PATHOLOGICAL PAIN: FROM MOLECULAR TO CLINICAL ASPECTS

Novartis Foundation

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Chair's introduction

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It is my great pleasure that we are holding this symposium on pathological pain here in Japan, as part of the Novartis Symposium series, a series that has had a brilliant history for more than half a century. As Chair of this symposium, I want to express my deep gratitude to all of the participants, who are joining here from all over the world, and to the Novartis Foundation for its generous support.

Before the 1970s, we learned much about pain from publications arising from two previous meetings on pain organized by the Foundation (then known as the Ciba Foundation): namely, *Pain and itch* in 1959 and *Touch, heat and pain* in 1966 (Ciba Foundation 1959, 1966). To my knowledge, except for these two meetings, there are no Novartis symposia focusing on the subject of pain. However, from the end of 1960s until now, pain research has undergone an explosive development. As all of you know, Dr Perl, one of the participants at this symposium, has played an important role in the development of the study of pain, from the pioneering early days until the present.

Over the last three decades, there have been two core phases in the development of pain research. The first was research on pain mechanisms in the normal state, from the late 1960s through the 1980s. The second, more recent focus has been on pain mechanisms in pathological states. Neurobiological research in the first phase of this explosive development of pain research uncovered detailed characteristics of the nociceptive system in normal states, from nociceptors to the cerebral cortex, and the existence of the endogenous analgesic system. The results obtained during this period were excellent, and we can now almost fully understand the mechanisms underlying nociceptive pain or so called 'acute pain', which warns of potential tissue damage. But the outcome of this research, on the other hand, has also shown that the information obtained in the normal state does not by itself explain mechanisms implicated in various mysterious pains of pathological states. The subsequent investigations have demonstrated that plastic changes take place in pain systems in chronic neuropathic states, and can result in structural changes of the nervous system. 'Plasticity' of the nervous system is becoming the most important key word in understanding pathological pain.

The terms 'acute pain' and 'chronic pain' remain very commonly used descriptors. But should we really be using the term 'chronic pain' which implies a chronological basis? Recent study on pain has revealed that acute pain has a physiological, nociceptive function. On the other hand, chronic pain may be caused by pathological, plastic changes of the neural system. This indicates that the difference between acute pain and chronic pain may be more than chronological: instead, it is mechanistic. The usage and definition of the terms acute and chronic pain therefore need reconsideration.

Why has 'plasticity' become a key word? This may reflect the fact that the pain system is primitive and not well differentiated. From the evolutionary point of view, the pain system was built up at the earliest stages of neural development, since alarm and defence systems are fundamental for survival. This evolutionary origin characterizes the nature of the system responsible for pain. First, the pain system has a high capacity for plastic changes, because its primitive nature provides a high degree of freedom for change. Second, the pain system is intimately related to instinctive functions and other fundamental bodily functions, such as autonomic or postural regulation. Third, humoral signalling is richly implicated, since these signalling means have roots in defence systems such as immune and inflammatory reactions.

Recent advances throw light on plasticity in humoral messenger systems as well as the organization of the neural systems. These neural plastic changes may underlie pathological pain. Reflecting these recent advances, in this symposium we will discuss mechanisms focusing on plastic changes in the pain system under various pathological states, at levels spanning from the molecular to clinical.

This symposium consists of five sessions. In the first two sessions, the roles of ion-channels, receptors and chemical messengers implicated in neuropathic pain will be discussed, mainly from a molecular perspective. Plasticity of the organization of the nervous system involved in pathological pain will be considered on the basis of molecular, electrophysiological and morphological analyses in the third session. Morphine tolerance is a notorious but important problem in pain management. The fourth session will consider the issue of opioid-induced plastic changes in the signalling pathways of anti-nociceptive and pro-nociceptive systems. In the last session, the mechanisms of pathological pain, such as bone cancer pain, complex regional pain syndrome (CRPS), and other chronic pain, will be discussed on the basis of experimental and clinical studies that aim to facilitate establishment of mechanism-based medicine.

I would now like to go back to this booklet published by the Ciba Foundation in 1959 (Ciba Foundation 1959). The title of this booklet, *Pain and itch* may tell us, at that period, that scientific knowledge on pain and itch were at similar levels of development. But at present, our knowledge of the pain system is far superior to that of itch, I think. In the chair's opening remarks of this book, Lord Adrian wrote

that 'Although pain is one of the central problems of medicine, it is disappointing that there is still so much to investigate.' I think that this remains very much so. He also wrote that, 'We may think that a discharge will not give pain unless it includes impulses in the non-medullated C fibres but the evidence is scarcely conclusive.' This point seems to have been the main interest of that conference. But at present, we know much about the receptor characteristics of C-fibre nociceptors and the whole nociceptive system. On the other hand, what we know now about itch is almost the same as it was at the beginning of the 1970s when neurophysiological studies on the nociceptive system began to flourish. As far as the pain system is concerned, the knowledge that we can obtain from this earlier symposium is quite limited at present. However, the interesting discussions included in this booklet are quite stimulating.

The Novartis Foundation Symposia have consistently attached importance to informal discussion. This is their distinctive feature and is testament to their importance. The present Symposium membership is made up of 14 speakers and nine discussants and the time scheduled for discussion is nearly 1.5 times longer than time for formal papers. To facilitate fruitful discussion, we have two excellent facilitators in each session whose role is to steer actively the process of discussion. I expect very stimulating discussions over the following three days. Thank you.

References

Ciba Foundation 1959 Pain and itch: nervous mechanism (Ciba Foundation Study Group Publication). Churchill, London

Ciba Foundation 1966 Touch, heat and pain. Churchill, London (Ciba Found Symp 91)

Regulation mechanisms of vanilloid receptors

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Abstract. The capsaicin receptor TRPV1 (also known as the vanilloid receptor VR1) is a non-selective cation channel and is activated not only by capsaicin but also by noxious heat or protons. Tissue damage associated with infection, inflammation or ischaemia, produces an array of chemical mediators that activate or sensitize nociceptor terminals. An important component of this pro-algeic response is ATP. In cells expressing TRPV1, ATP increased the currents evoked by capsaicin or protons through activation of P2Y metabotropic receptors in a PKC-dependent manner. In the presence of ATP, the temperature threshold for TRPV1 activation was reduced from 42 °C to 35 °C, such that normal body temperature could activate TRPV1. Functional interaction between P2Y receptors and TRPV1 was confirmed in a behavioural analysis using TRPV1-deficient mice. Direct phosphorylation of TRPV1 by PKC was confirmed biochemically and the two serine residues involved were identified. Extracellular Ca^{2+}-dependent desensitization of TRPV1 is thought to be one mechanism underlying the paradoxical effectiveness of capsaicin as an analgesic therapy. The Ca^{2+}-binding protein calmodulin binds to the C-terminus of TRPV1. We found that disruption of the calmodulin binding segment prevented TRPV1 desensitization even in the presence of extracellular Ca^{2+}.

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Pain is initiated when noxious thermal, mechanical or chemical stimuli excite the peripheral terminals of specialized primary afferent neurons called nociceptors (Wood & Perl 1999, Woolf & Salter 2000, Scholz & Woolf 2002). Many different kinds of ionotropic and metabotropic receptors are known to be involved in this process (McCleskey & Gold 1999, Caterina & Julius 1999, Julius & Basbaum 2001). Vanilloid receptors are nociceptor-specific cation channels that serve as the molecular target of capsaicin, the pungent ingredient in hot chilli peppers (Szallasi & Blumberg 1999). When expressed in heterologous systems, the cloned capsaicin receptor (TRPV1) can also be activated by noxious heat (with a thermal threshold > 43 °C) or protons (acidification), both of which cause pain *in vivo* (Caterina et al 1997, Tominaga et al 1998, Caterina & Julius 2001,

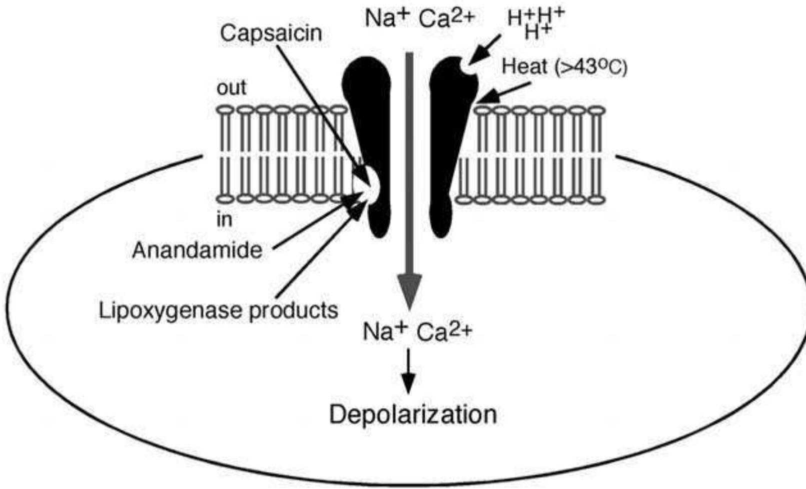

FIG. 1. Proposed model of TRPV1 function in a sensory neuron. TRPV1 can be activated not only by capsaicin but also by protons, heat, anandamide and lipoxygenase products. Cation influx leads to depolarization.

Tominaga 2000) (Fig. 1). Furthermore, analyses of mice lacking TRPV1 have shown that TRPV1 is essential for selective modalities of pain sensation and for tissue injury-induced thermal hyperalgesia (Caterina et al 2000, Davis et al 2000).

Tissue damage associated with infection, inflammation or ischaemia, produces an array of chemical mediators that activate or sensitize nociceptor terminals to elicit pain at the site of injury. An important component of this pro-algesic response is ATP released from different cell types (North & Barnard 1997, Burnstock & Williams 2000, Dun et al 2001). Extracellular ATP excites the nociceptive endings of nearby sensory nerves, evoking a sensation of pain. In these neurons, the most widely studied targets of extracellular ATP have been ionotropic ATP (P2X) receptors. Indeed, several P2X receptor subtypes have been identified in sensory neurons, including one ($P2X_3$) whose expression is largely confined to these cells (Dunn et al 2001). Our understanding of purinergic contributions to pain sensation may be incomplete, however, given that the potential involvement of widely distributed metabotropic ATP (P2Y) receptors has not yet been well investigated.

Results

To address whether metabotropic P2Y receptors are involved in TRPV1-mediated nociceptive responses, we examined the effects of extracellular ATP on TRPV1

expressed in human embryonic kidney-derived HEK293 cells and rat dorsal root ganglion (DRG) neurons (Tominaga et al 2001). In voltage-clamp experiments, low doses of capsaicin (10 or 20 nM) evoked small inward currents in the HEK293 cells expressing TRPV1. After a 2 min pretreatment with 100 μM extracellular ATP, the same doses of capsaicin produced much larger current responses (6.42 $\pm$ 1.01 [mean $\pm$ SEM]-fold, $n = 52$). A similar potentiating effect of extracellular ATP was observed on proton-evoked activation of TRPV1 (5.68 $\pm$ 0.92-fold, $n = 32$). To examine how ATP changes TRPV1 responsiveness, we measured TRPV1 currents in single cells by serially applying a range of concentrations of capsaicin or protons in the absence or presence of ATP. In both cases, maximal currents in the presence of ATP were almost the same as those obtained in the absence of ATP. The resultant dose–response curves clearly demonstrate that ATP enhances capsaicin and proton action on TRPV1 by lowering EC_{50} values without altering maximal responses. Potentiating effects of extracellular ATP were also examined on heat-evoked responses in HEK293 cells expressing TRPV1. When temperature ramps were applied to HEK293 cells expressing TRPV1 in the absence of ATP, heat-evoked currents developed at about 42 °C with an extremely steep temperature dependence. ATP treatment lowered the threshold temperature for TRPV1 activation significantly (41.7 $\pm$ 1.1 °C, $n = 7$ and 35.3 $\pm$ 0.7 °C, $n = 5$ without and with ATP treatment, respectively, $P < 0.01$). Thus, in the presence of ATP, normally non-painful thermal stimuli (even body temperature) are capable of activating TRPV1. These data clearly show that TRPV1 currents evoked by any of three different stimuli (capsaicin, proton or heat) are potentiated or sensitized by extracellular ATP.

In order to distinguish between the subtypes of P2Y receptors that might be involved in this process in HEK293 cells, we examined the effect of several ATP related reagents (each 100 μM) upon the TRPV1 response (Tominaga et al 2001). The resultant rank order of potency (ATP > ADP >> UTP > AMP) is most consistent with involvement of $P2Y_1$ receptors.

One major consequence of $P2Y_1$ receptor stimulation is activation of phospholipase C (PLC) through the G protein, $G_{q/11}$, leading to the production of inositol-1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG) (Burnstock & Williams 2000) (Fig. 2). Ca^{2+} mobilization by IP_3 is not a likely mechanism for the capsaicin-evoked current increase observed in our experiments because cytosolic free Ca^{2+} is tightly chelated with 5 mM EGTA included in the pipette solution. Therefore, activation of PKC by DAG remains a more likely mechanism for ATP-induced potentiation. To test this possibility, we examined the effect of a highly potent and selective PKC inhibitor, calphostin C (Tominaga et al 2001). When 1 μM calphostin C was added to the pipette solution, the ATP effect was almost completely abolished (1.13 $\pm$ 0.13-fold, $n = 7$, $P < 0.05$ vs. ATP alone). Furthermore, direct activation of PKC by 100 nM

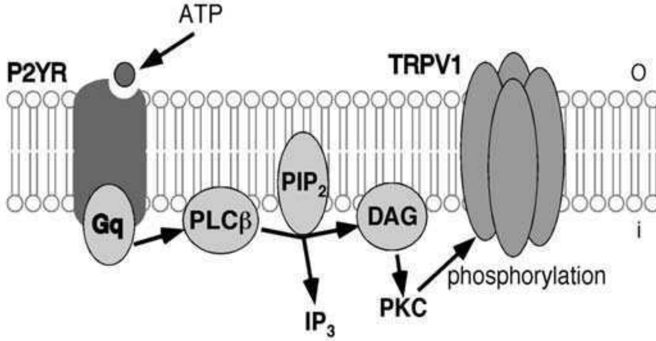

FIG. 2. PKC-dependent regulation of TRPV1. Gq-coupled P2Y receptor activation leads to production of IP_3 and DAG through PLCβ activation. PKC activation by DAG causes phosphorylation of TRPV1, leading to functional potentiation. PLCβ, phospholipase Cβ; DAG, diacylglycerol; PIP_2, phosphatidylinositol-4,5-bisphosphate; IP_3, inositol 1,4,5-trisphosphate; o and i, outside and inside of cell, respectively.

phorbol-12-myristate-13-acetate (PMA) caused a robust increase in the magnitude of capsaicin-evoked currents (8.09 ± 2.81-fold, $n = 10$, $P < 0.05$ vs. control). These data clearly indicate the involvement of a PKC-dependent pathway in TRPV1 potentiation by ATP.

In order to confirm the interaction between ATP and TRPV1 in the context of ATP-induced hyperalgesia *in vivo*, we performed a behavioural analysis using wild-type mice and TRPV1-deficient mice (Moriyama et al 2003). A significant reduction in paw withdrawal latency to radiant paw heating was observed for 5–30 min following ATP injection in wild-type mice. On the other hand, TRPV1-deficient mice developed no such thermal hypersensitivity in response to ATP injection, suggesting a functional interaction between ATP and TRPV1. A pharmacological analysis of ATP-induced potentiation of TRPV1 currents evoked by capsaicin in HEK293 cells expressing TRPV1 suggested the involvement of $P2Y_1$ receptors. Therefore, we extended our behavioural analyses to $P2Y_1$-deficient mice. Surprisingly, following ATP injection, mice lacking $P2Y_1$ exhibited a reduction in heat-evoked withdrawal latency similar to that observed in wild-type mice, indicating that $P2Y_1$ receptors are not involved in ATP-induced thermal hyperalgesia in mice.

To explore the identity of the P2Y subtypes responsible for ATP-induced thermal hyperalgesia in mice, we first examined the effects of ATP on the capsaicin-evoked response in isolated mouse DRG neurons (Moriyama et al 2003). In DRG neurons of wild-type mice, extracellular ATP caused significant increase of low dose capsaicin-evoked currents (1.07 ± 0.26-fold, $n = 3$; control vs. 4.01 ± 0.92-fold, $n = 8$; with ATP, $P < 0.05$). Similar potentiation of

capsaicin-evoked currents was observed in $P2Y_1$-deficient mice (4.37 ± 0.74-fold, $n=4$, $P<0.01$ vs. control), suggesting lack of involvement of $P2Y_1$ receptors in mouse DRG neurons, consistent with our behavioural analyses. We next examined the effect of another ATP-related molecule, UTP, because this molecule is thought to be a relatively selective agonist of $P2Y_2$ and $P2Y_4$ receptors. UTP potentiated the capsaicin-evoked current responses to a similar extent as ATP (6.24 ± 1.59-fold, $n=4$, $P<0.05$ vs. control), suggesting the involvement of $P2Y_2$ or $P2Y_4$ subtypes. Finally, the fact that suramin, which blocks $P2Y_2$ but not $P2Y_4$, abolished the potentiation by UTP (0.95 ± 0.57-fold, $n=3$, $P<0.05$ vs. UTP in wild-type) implicates $P2Y_2$ as the most likely P2Y subtype involved in the potentiation of capsaicin-evoked current responses in mouse DRG neurons.

To confirm this $P2Y_2$ receptor involvement *in vivo*, we examined the effect of UTP in mice (Moriyama et al 2003). UTP was found to cause thermal hyperalgesia with a time course similar to that observed in ATP injection, suggesting that $P2Y_2$ receptors are involved in the ATP-induced thermal hyperalgesia in mice.

The data described above suggest that direct phosphorylation of TRPV1 or a closely associated protein by PKC changes the agonist sensitivity of this ion channel (Fig. 2). We tried to confirm the *in vivo* phosphorylation of TRPV1 by PKC (Numazaki et al 2002). Following the treatment with [γ-^{32}P]ATP, the cells expressing TRPV1 were stimulated with PMA. TRPV1 protein immunoprecipitated with anti-rat TRPV1 antibody showed more ^{32}P incorporation into TRPV1 upon PMA stimulation compared to the TRPV1 without PMA stimulation, indicating the direct phosphorylation of TRPV1 by PKC. There are 16 putative Ser or Thr residues that are candidate substrates for PKC-dependent phosphorylation in the TRPV1 N-terminus, first intracellular loop and C-terminus. To distinguish among these possibilities, recombinant proteins carrying GST fused to the three segments of the cytoplasmic domains of TRPV1 were generated for use in an *in vitro* kinase assay. This assay demonstrated that the first intracellular loop and the C-terminus contained the substrates for PKCε. To identify the specific TRPV1 amino acids involved, eight Ser or Thr residues in the first intracellular loop and the C-terminal were individually replaced with Ala and the resulting mutant proteins were subjected to functional analysis using a whole-cell patch-clamp technique. After a 1 min pretreatment with 100 nM PMA, the same low dose of capsaicin produced a much larger current responses in the cells expressing TRPV1 (7.95 ± 2.72-fold, $n=8$). Among the mutants tested, S502A and S800A showed significantly smaller potentiation of capsaicin-evoked current responses by (2.13 ± 0.41-fold, $n=9$ for S502A; 2.76 ± 0.52-fold, $n=11$ for S800A) ($P<0.05$). Furthermore, double mutant, S502A/S800A exhibited almost no PMA potentiation effect (0.95 ± 0.04-fold,

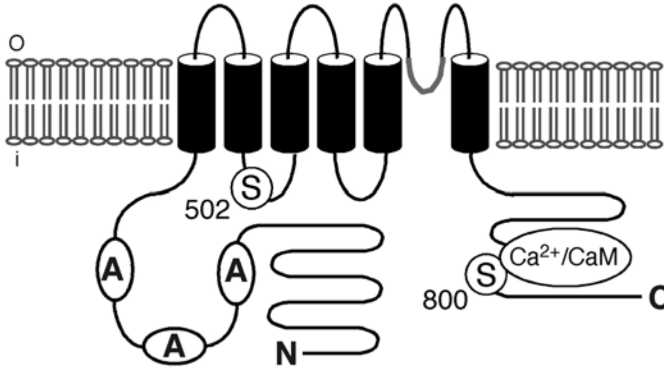

FIG. 3. Predicted membrane topology of TRPV1. Two serine residues for PKC-dependent phosphorylation and Ca^{2+}/calmodulin (CaM) binding site are shown. o and i, outside and inside of cell, respectively.

$n = 7$) ($P < 0.05$), suggesting that these two Ser residues were the major substrates for PKC-dependent phosphorylation (Fig. 3).

Of great physiological relevance is whether these mutants affect the response of TRPV1 to heat. Therefore, potentiating effects of PMA were examined on heat-evoked responses in HEK293 cells expressing wild-type TRPV1 or S502A/S800A mutant (Numazaki et al 2002). When temperature ramps were applied to HEK293 cells expressing wild-type TRPV1, heat-evoked currents developed at about 42 °C. PMA (100 nM) treatment lowered the temperature threshold for wild-type TRPV1 activation significantly (41.9 ± 0.9 °C, $n = 3$ and 31.8 ± 1.6 °C, $n = 4$ without and with PMA treatment, respectively, $P < 0.01$). On the other hand, no reduction of the threshold was observed in the mutant upon PMA treatment. These data further indicate the involvement of these two Ser residues in TRPV1 sensitization.

Extracellular Ca^{2+}-dependent desensitization of TRPV1 has been observed in patch-clamp experiments using both heterologous expression systems and native sensory ganglia (Docherty et al 1996, Caterina et al 1997, Szallasi & Blumberg 1999). The inactivation of nociceptive neurons by capsaicin has generated extensive research on the possible therapeutic effectiveness of capsaicin as a clinical analgesic tool (Campbell et al 1993, Szallasi & Blumberg 1999). Still, however, the underlying mechanism of this inactivation process is not known. There have been several studies reporting that calmodulin (CaM) mediates Ca^{2+}-dependent inhibition or inactivation of cyclic nucleotide-gated channels, NMDA receptor ion channels, L-type Ca^{2+} channels, P/Q type Ca^{2+} channels and small conductance Ca^{2+}-activated potassium channels many of which have high Ca^{2+} permeability (Molday 1996, Ehlers et al 1996, Xia et al 1998, Zuhlke et al 1999, Lee et al 1999, Levitan 1999). Furthermore, several members of the TRP ion

channel superfamily have been found to be regulated by CaM binding (Scott et al 1997, Chevesich et al 1997). Despite the fact that TRPV1 contains no obvious CaM binding sites, the fact that TRPV1 is a member of the TRP ion channel superfamily suggests the possibility that CaM inactivates TRPV1 in a Ca^{2+}-dependent manner.

We examined the direct interaction of TRPV1 with CaM (Numazaki et al 2003). In HEK293 cells expressing both TRPV1 and Myc-tagged CaM, CaM could be co-immunoprecipitated with TRPV1 in the presence of Ca^{2+}. Moreover, the amount of CaM co-immunoprecipitated with TRPV1 was increased upon capsaicin treatment. This finding suggests that an increased Ca^{2+} influx through TRPV1 results in making Ca^{2+}/CaM complex, leading to TRPV1 desensitization. To confirm that such an interaction occurs and to identify the domains of TRPV1 involved, recombinant proteins carrying GST fused to the four cytoplasmic domains of TRPV1 were generated for use in an *in vitro* binding assay with CaM. This assay demonstrated that the C-terminus of TRPV1 contains the segment necessary for interaction with CaM. In order to further narrow down the amino acids involved, we fused GST to the four segments of the C-terminus and the resultant fusion proteins were subjected to the *in vitro* binding assay. A 35 amino acid (AA) segment in the C-terminus was found to be sufficient for this interaction (Fig. 3). In addition, the C-terminus of TRPV1 lacking the 35 AA segment failed to bind CaM, further indicating that this 35 AA is essential for binding of TRPV1 with CaM.

The functional importance of this 35 AA segment was examined using the patch-clamp technique in HEK293 cells expressing either wild-type TRPV1 or a mutant TRPV1 lacking the 35 AA (Δ35AA) (Numazaki et al 2003). Interestingly, in the presence of extracellular Ca^{2+}, the desensitization induced by short capsaicin applications was almost completely abolished in the Δ35AA mutant (23.9 ± 7.5%, $n = 4$ for wild-type; 93.3 ± 5.5%, $n = 4$ for the mutant; $P < 0.01$), indicating that the 35 AA segment plays an important role in desensitization to such short repetitive stimuli.

Discussion

Inflammatory pain is initiated by tissue damage/inflammation and is characterized by hypersensitivity both at the site of damage and in adjacent tissue. One mechanism underlying these phenomena is the modulation (sensitization) of ion channels, such as TRPV1, that detect noxious stimuli at the nociceptor terminal. Sensitization is triggered by extracellular inflammatory mediators that are released *in vivo* from surrounding damaged or inflamed tissue and from nociceptive neurons themselves. Among the mediators, extracellular ATP potentiated or sensitized TRPV1 responses through phosphorylation of two serine residues of TRPV1 by PKC in the down stream of $P2Y_2$ receptor activation. This represents a novel

mechanism through which extracellular ATP might cause pain in a pathway distinct from the activation of P2X receptors. Most attention in the pain field has focused on the role of ionotropic ATP receptors in ATP-evoked nociception. Our findings suggest that $P2Y_2$ is also involved in this process and may represent a fruitful target for the development of drugs that blunt nociceptive signalling through capsaicin receptors. $P2Y_2$ receptors confer responsiveness to UTP and ATP to a similar extent, suggesting a possible role for UTP as an important component of pro-algesic response in the context of tissue injury. Therefore, UTP as well as ATP should be taken into account when purinergic contributions through P2Y receptors to pain sensation are examined.

We identified a structural determinant of TRPV1 which interacts with CaM. The interaction may underlie, in part, the paradoxical use of capsaicin as analgesic. Moreover, compounds acting on the 35 AA segment of TRPV1 could prove useful in the treatment of pain by interfering with Ca^{2+}/CaM function.

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References

Burnstock G, Williams M 2000 P2 purinergic receptors: modulation of cell function and therapeutic potential. J Pharmacol Exp Ther 295:862–869

Campbell E, Bevan S, Dray A 1993 Clinical applications of capsaicin and its analogues. In: Wood JN (ed) Capsaicin in the study of pain. Academic Press, San Diego, p 255–269

Caterina MJ, Julius D 1999 Sense and specificity: a molecular identity for nociceptors. Curr Opin Neurobiol 9:525–530

Caterina MJ, Julius D 2001 The vanilloid receptor: a molecular gateway to the pain pathway. Annu Rev Neurosci 24:487–517

Caterina MJ, Schumacher MA, Tominaga M, Rosen TA, Levine JD, Julius D 1997 The capsaicin receptor: a heat-activated ion channel in the pain pathway. Nature 389:816–824

Caterina MJ, Leffler A, Malmberg AB et al 2000 Impaired nociception and pain sensation in mice lacking the capsaicin receptor. Science 288:306–313

Chevesich J, Kreuz AJ, Montell C 1997 Requirement for the PDZ domain protein, INAD, for localization of the TRP store-operated channel to a signaling complex. Neuron 18:95–105

Davis JB, Gray J, Gunthorpe MJ et al 2000 Vanilloid receptor-1 is essential for inflammatory thermal hyperalgesia. Nature 405:183–187

Docherty RJ, Yeats JC, Bevan S, Boddeke HW 1996 Inhibition of calcineurin inhibits the desensitization of capsaicin-evoked currents in cultured dorsal root ganglion neurones from adult rats. Pflugers Arch 431:828–837

Dunn PM, Zhong Y, Burnstock G 2001 P2X receptors in peripheral neurons. Prog Neurobiol 65:107–134

Ehlers MD, Zhang S, Bernhardt JP, Huganir RL 1996 Inactivation of NMDA receptors by direct interaction of calmodulin with the NR1 subunit. Cell 84:745–755

Julius D, Basbaum AI 2001 Molecular mechanisms of nociception Nature 413:203–210
Lee A, Wong ST, Gallagher D et al 1999 Ca^{2+}/calmodulin binds to and modulates P/Q-type calcium channels. Nature 399:155–159
Levitan IB 1999 It is calmodulin after all! Mediator of the calcium modulation of multiple ion channels. Neuron 22:645–648
McCleskey EW, Gold MS 1999 Ion channels of nociception. Annu Rev Physiol 61:835–856
Molday RS 1996 Calmodulin regulation of cyclic-nucleotide-gated channels. Curr Opin Neurobiol 6:445–452
Moriyama T, Iida T, Kobayashi K et al 2003 Possible involvement of P2Y2 metabotripic receptors in ATP-induced transient receptor potential vanilloid receptor 1-mediated thermal hypersensitivity. J Neurosci 23:6058–6062
North AN, Barnard EA 1997 Nucleotide receptors. Curr Opin Neurobiol 7: 346–357
Numazaki M, Tominaga T, Toyooka H, Tominaga M 2002 Direct phosphorylation of capsaicin receptor VR1 by PKCε and identification of two target serine residues. J Biol Chem 277:13375–13378
Numazaki M, Tominaga T, Takeuchi K, Murayama N, Toyooka H, Tominaga M 2003 Structural determinant of TRPV1 desensitization interacts with calmodulin. Proc Natl Acad Sci USA 100:8002–8006
Scholz J, Woolf CJ 2002 Can we conquer pain? Nat Neurosci 5:S1062–S1067
Scott K, Sun Y, Beckingham K, Zuker CS 1997 Calmodulin regulation of Drosophila light-activated channels and receptor function mediates termination of the light response in vivo. Cell 91:375–383
Szallasi A, Blumberg PM 1999 Vanilloid (Capsaicin) receptors and mechanisms. Pharmacol Rev 51:159–211
Tominaga M 2000 Capsaicin receptor and its homologue in nociception. Pain Reviews 7:97–104
Tominaga M, Caterina MJ, Malmberg AB et al 1998 The cloned capsaicin receptor integrates multiple pain-producing stimuli. Neuron 21:531–543
Tominaga M, Wada M, Masu M 2001 Potentiation of capsaicin receptor activity by metabotropic ATP receptors as a possible mechanism for ATP-evoked pain and hyperalgesia. Proc Natl Acad Sci USA 98:6951–6956
Wood JN, Perl ER 1999 Pain. Curr Opin Genet Dev 9:328–332
Woolf CJ, Salter MW 2000 Neuronal plasticity: increasing the gain in pain. Science 288:1765–1768
Xia X-M, Fakler B, Rivard A et al 1998 Mechanism of calcium gating in small-conductance calcium-activated potassium channels. Nature 395:503–507
Zuhlke RD, Pitt GS, Deisseroth K, Tsien RW, Reuter H 1999 Calmodulin supports both inactivation and facilitation of L-type calcium channels. Nature 399:159–162

DISCUSSION

Belmonte: Is the reduction in temperature threshold due to the fact that all receptors become sensitive to a lower temperature, or because the recruitment of some receptor molecules — the pooled response — starts now at a cooler temperature? I think this is important. At a single channel level, do you also always get desensitization, or do you need the whole population of receptors in order to see this effect?

Tominaga: All the results shown here today were obtained from the whole cell recording. However, I did some single channel recordings. At the single channel

level the temperature threshold was also lowered, suggesting that the receptor has become sensitive to a lower temperature. It seems that even at below 30 °C some of the channels start to open at the single channel level.

Belmonte: So your idea, then, is that something changes in the structure of the channel that reduces the threshold.

Tominaga: Yes, it is.

McMahon: You reported that the ATP modulation of TRPV1 was not associated with any direct activation by ATP itself. But there are multiple reports in the literature that many DRG cells, including cells that are capsaicin sensitive, show P2X-mediated currents when exposed to ATP. Did you have some mechanism for avoiding these, or are you selecting cells that don't have these ATP-induced currents?

Tominaga: The reason why we did experiments on ATP sensitivity was because we wanted to simplify things. We did the experiment in DRG neurons. There are two kinds of cells: those expressing both TRPV1 and $P2X_3$, and those expressing either one or the other. In this experiment, I showed just the results from DRG neurons that express only TRPV1. But the results from the DRG neurons expressing TRPV1 and $P2X_3$ are not significantly different. This suggests that the $P2X_3$ receptor is not involved in this P2Y receptor-mediated potentiation of the capsaicin receptor.

McMahon: In reading the literature on modulation of TRPV1, one is struck by the wide range of intracellular mechanisms that are claimed to be effective. For some stimuli, such as nerve growth factor (NGF), there are at least four distinct intracellular cascades claimed to contribute, and which ones are apparent may depend on the circumstances under which one looks. For instance, one of the recent reports from Peter McNaughton's laboratory used the paradigm of repeated capsaicin challenge to get small but consistent capsaicin responses. I wonder if in those circumstances it may be possible to observe potentiation of a stable but desensitized response — that is, a 'de-desensitization'. In other circumstances, perhaps pertinent here, different mechanisms may operate.

Tominaga: I showed only two traces here. But in my experiment, when I tried the third and fourth application of capsaicin the potentiation was persistent. This suggests that some cytosolic mechanism is involved, like the mechanism I showed in my slides. As you said, there have been many mechanisms reported for regulation of TRPV1. I am not sure which are true. At least in my hands, PKCε or other PKC blockers can completely inhibit the potentiation by G protein-coupled receptor activation, which suggests that the PKC-dependent pathway could be the predominant one involved in the regulation of TRPV1.

Oh: Obviously, TRPV1 also has many different mechanisms of desensitization. PKC, PKA and CaM kinase II are known to be involved in desensitization. It is puzzling which kinase action is truly involved in this process.

Reeh: It may make a difference whether TRPV1 desensitization is prevented or antagonized, or whether there is primary sensitization or disinhibition of TRPV1. It appears that there are different molecular targets for each mechanism.

Dray: Returning to Carlos Belmonte's initial question about the sensitization process: I was trying to translate this into psychophysical terms. If the TRPV1 receptor is sensitized by inflammatory mediators and thus can be activated at normal body temperatures, how do you see that being translated into its involvement in inflammatory pain? Is it responsible for some persistent pain state?

Tominaga: This is a matter of the balance between sensitization and desensitization. As long as the PKC pathway is active in the cell, I think that reduction of the temperature threshold should persist for a long time. Does this answer your question?

Dray: Not really. Usually when I think of an inflammatory pain state, I think that the pain is not spontaneous: it is evoked by a heat or mechanical stimulus. I am trying to understand the impact of TRPV1 sensitization in psychophysical terms.

Reeh: This may not be a question of molecular principles in nociceptors. There are descending pain inhibition pathways, for example. On the other hand there is no acute inflammatory pain that could not be immediately relieved by cooling.

Dray: Related to this, early in your paper you mentioned that there is an up-regulation of TRPV1 following inflammation, with increased expression in Aδ fibres. What is the relationship between the increased expression of TRPV1 and the metabotropic-type ATP channels?

Tominaga: I have no idea about the connection. As shown in my slides, expression of TRPV1 is increased both in C and Aδ fibres, although the expression is increased more predominantly in Aδ fibres. Two days after the onset of inflammation, expression of TRPV1 is already increased. At this time point, both increase in receptor number and receptor sensitization might contribute to the development of inflammatory nociception. Perhaps this increase of TRPV1 receptor in Aδ fibres will help to facilitate the sensitization in these fibres.

Wood: Is it possible to do current clamp experiments at different temperatures? You could presumably see the very rapidly inactivating effects of ATP on channels such as $P2X_3$. Then you could look at the actual effect on excitability and excitation of sensory neurons in a more physiological situation than HEK cells.

Tominaga: Yes, it is. That would be a good experiment to do.

Reeh: Gordon Reid has done a similar experiment, blocking capsaicin-induced currents in DRGs by cooling (Babes et al 2002).

Devor: I have a question that will probably take us more to a systems level. What is the normal concentration of ATP in the extracellular fluid?

Tominaga: I'm probably not the best person to answer the question. Dr Inoue may be able to answer this question better. As far as I know, ATP is quite rapidly

metabolized to ADP, AMP and adenosine. But, after a short time period, it is out there at high concentrations, probably submillimolar.

Devor: What is the basal concentration?

Inoue: Usually the extracellular concentration of ATP is very low, at around picomolar concentrations. However, many cells contain ATP at levels of more than 6 mM in the cytosol. In vesicles it can be almost 500 mM. An exocytosis or leak from damaged cells leads to the existence of ATP at more than micromolar concentrations.

Devor: Putting aside emergencies, if you have a high millimolar concentration of ATP inside the cell, can't some of that leak out, either by a leak itself or by baseline exocytosis? The level of ATP right on the external surface of the membrane, just outside, where the receptors are, might be in a range that would sensitize. Therefore this whole phenomenon might be part of normal physiology.

McMahon: ATP antagonists have failed to reveal an endogenous 'ATP-ergic' tone. If this is generally true it would explain why P2X antagonists on their own do nothing.

Devor: This brings us back to the question on desensitization. Although we had a discussion, I am not sure that I understood the bottom line. If you apply 10 μM ATP for an hour, after the end of that hour do you still have sensitization?

Tominaga: ATP is easily broken down to ADP, AMP or adenosine. To do the experiment you propose, we would have to use continuous perfusion with newly made ATP. This is a hard experiment to do. I have tried this for up to 20 min, and we still see the huge potentiation that I have been talking about.

Gintzler: If I understood correctly, a very specific isoform of PKC is phosphorylating TRPV1, PKCε. Is that correct?

Tominaga: There is an old report claiming that PKCε is expressed a lot in sensory neurons and is involved in nociception. This arose from experiments in PKCε knockout mice. We made this conclusion from the results showing that PKCε translocation inhibitor almost completely blocks the phenomena we observe with ATP.

Gintzler: How certain are you that there is no contribution from other PKC isoforms?

Tominaga: There were several other reports indicating that PKCα and δ could also be involved, but we believe that PKCε is predominantly involved.

Belmonte: I want to play the devil's advocate. In my laboratory, we tested the effect of various ATP analogues on the impulse activity of a large number of single polymodal nociceptor fibres of the cornea. We never obtained a response or detected an increased responsiveness to physiological stimuli following topical application of purinergic agonists. In my view, a good, direct proof that under

normal conditions, release of ATP during injury is changing significantly the sensitivity of nerve endings is still lacking. I know that tissue-cultured primary sensory neurons become extremely sensitive to ATP but there are also data (Stebbing et al 1998) that neurons of the intact dorsal root ganglion are not activated by exogenous ATP while 6 h after culture the same neurons respond beautifully.

McMahon: We have evidence exactly opposite to that! First, we recorded from some 50–100 primary afferent C fibres in a skin nerve preparation, and about half of those did respond directly to exogenous ATP (Hamilton et al 2001). Thus, a substantial group of nociceptors are clearly responsive to ATP.

Second, we have shown that ATP iontophoresed into the skin of human volunteers, reliably induces a modest pain. (Hamilton et al 2000).

Dray: I think Carlos Belmonte's question is much more fundamental: whether effects seen in cultured systems relate to how the native receptor would behave? We have to be careful in extrapolating from different experimental conditions.

McMahon: Perhaps this is not the place to have a discussion about the physiological role of ATP as a peripheral mediator of pain. But while there are some negative data, there are also several reports showing altered pain related behaviours after antisense and knockout experiments targeting the $P2X_3$ receptor.

Tominaga: The cornea cells might not have sufficient metabotropic ATP receptors. We are looking at only the final output in terms of the TRPV1 channel as a result of P2Y receptor activation. To have a functional sensitization of TRPV1 in the downstream of P2Y receptors, all the players such as G protein, PLC and diacylglycerol (DAG) should be good there. Otherwise we cannot get potentiation of TRPV1 activity.

Reeh: Carlos Belmonte's comment has gone a bit further. He was doubting that cultured DRG neurons are a suitable model of their own nerve endings in every respect.

Mao: I would like to raise an issue that relates to the clinical perspective. Before the discovery of TRPV1 there were non-steroidal anti-inflammatory drugs (NSAIDs), and after the discovery of TRPV1 there were still NSAIDs, in terms of treatment of inflammatory pain. How would you put into your scheme the fact that in the clinical setting NSAIDs work pretty well in many situations of inflammatory pain?

Tominaga: That is a hard question to answer. I have submitted a paper recently in which we show that there are interactions between prostaglandins and TRPV1. This indicates that one of the final targets of the prostaglandin action could be TRPV1. In other words, NSAIDs seem to function to reduce the sensitization of TRPV1 through prostaglandin receptor activation.

Wood: There is also some evidence that NSAIDs directly block TRPV1.

Baron: You talked about the up-regulation of TRPV1 in C fibres and Aδ fibres. Is there any evidence of up-regulation in Aβ fibres in the inflammatory state or in neuropathic states?

Tominaga: We have looked at many sections, but we haven't seen this.

Zhang: How about central expression of TRPV1 after inflammation? What is the percentage of DRG neurons expressing this channel?

Tominaga: Usually less than 5% of Aδ neurons express TRPV1. This level increased up to 30% in inflammation. In terms of central expression of TRPV1, there seem to be no reports.

McMahon: I have a general question about species variability in TRPV1 expression. In the mouse some large myelinated fibres (which may or may not be nociceptors) normally respond to TRPV1. It is not clear whether this expression is seen in other species. There are other clear differences in the properties of TRPV1 in the rat — extensively used experimentally, of course — and in human. So, how many of the sensitizing phenomena that you described apply to human TRPV1 channels, and does this affect our interpretation of what might be the functions of these channels in pathological pain states?

Tominaga: We haven't done experiments with a human TRPV1, but we have lots of data from mouse and rat TRPV1. That's all I can say.

McMahon: I believe there is some preliminary evidence from the Novartis Institute in London that TRPV1 antagonists are effective in a guinea-pig neuropathic pain model, but not in an equivalent model in the rat. If true, this would be important.

Tominaga: It seems that rat TRPV1 is different from other TRPV1s that have been reported. When we used a mouse TRPV1 we found pretty similar results to those obtained with rat TRPV1, which suggests that this phenomenon is probably applicable to other species.

Wood: Have you extended your studies to other members of the TRPV family, TRPV1, 2, 3 and 4, which all seem to have some significance in sensory neuron function? Do you have any evidence that similar mechanisms apply to those channels?

Tominaga: No, we haven't. These are things I should look at.

Dray: My comment is related to the phosphorylation sites you described. You have identified two very specific serine residues. One hypothesis driving drug discovery is that inhibition of the TRPV1 channel through either antagonists of the TRPV1 receptor or TRPV1 channel block would be a way of producing analgesia. If the phosphorylation sites are ubiquitous for a number of converging control mechanisms, this would be another very effective target to regulate afferent excitability. Do you have any evidence for this? You mentioned some antibody work.

Tominaga: I think that in TRPV1, the two serine residues would be an effective target for the development of antinociceptive agents. We are now doing the experiment, using antibodies against phosphorylated TRPV1. If the antibody functions as a blocking antibody for the protein, this would be fascinating. We aligned the sequence of S800 and found that this is the only candidate specific for a PKC-dependent phosphorylation, because S502 is also phosphorylated by PKA. We also aligned human, mouse and rat TRPV1 sequences and realized that S800 is conserved in all three species. It indicates that this area could be important for TRPV1 function and could be a very promising target for development of antinociceptive agents.

References

Babes A, Amuzescu B, Krause U, Scholz A, Flonta ML, Reid G 2002 Cooling inhibits capsaicin-induced currents in cultured rat dorsal root ganglion neurones. Neurosci Lett 317:131–134

Hamilton SG, Warburton J, Bhattacharjee A, Ward J, McMahon SB 2000 ATP in human skin elicits a dose related pain response which is potentiated under conditions of hyperalgesia. Brain 123:1238–1246

Hamilton SG, McMahon SB, Lewin G 2001 Selective activation of nociceptors by P2X receptor agonists in normal and inflamed rat skin. J Physiol 534:437–445

Stebbing MJ, McLachlan EM, Sah P 1998 Are there functional P2X receptors on cell bodies in intact dorsal root ganglia of rats? Neuroscience 86:1235–1244

Sodium channels and neuropathic pain

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Abstract. Although it has long been known that sodium channels play an important role in the generation of abnormal neuronal activity and neuropathic pain, it is only recently that we have begun to understand the subtypes of sodium channels which are particularly important in neuropathic pain. Many of the identified subtypes of sodium channels are localized in dorsal root ganglion (DRG) neurons. Based on their sensitivity to tetrodotoxin (TTX), these sodium channels are classified as TTX-sensitive (TTXs) or TTX-resistant (TTXr) subtypes. In *in vitro* electrophysiological experiments, ectopic discharges arising from DRG neurons with injured axons are blocked by TTX at doses that are too low to block TTXr subtypes. Furthermore, the same low doses of TTX applied to the DRG of the injured segment in neuropathic rats significantly reduce pain behaviours. These data suggest that TTXs subtypes of sodium channels are playing an important role in the generation of both ectopic discharges and neuropathic pain. Analysis of mRNA of the TTXs subtypes of sodium channels in the DRG after spinal nerve ligation showed that $Na_v1.3$ (Type III) and Na_x (NaG) are the only two subtypes that are up-regulated, suggesting their potentially important role in ectopic discharge and neuropathic pain generation.

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An injury to a peripheral nerve leads to the development of abnormal afferent activity including ectopic discharges from axotomized afferents and spontaneous activity of sensitized intact nociceptors. These abnormal activities enter the spinal cord to set up and maintain central sensitization, which is an important underlying mechanism of chronic neuropathic pain. Since central sensitization appears to be initiated and maintained by ectopic discharge input, finding the triggering mechanism for ectopic discharges is an important step in the investigation of neuropathic pain mechanisms. One important factor that has long been recognized as contributing to the generation of abnormal afferent activity as well as neuropathic pain is the change in sodium channels after injury to the peripheral nerve (Chabal et al 1992, Devor et al 1989, 1992, 1993).

Various sodium channel subtypes have been cloned and characterized in recent years and many of these are localized in dorsal root ganglion (DRG) neurons,

suggesting their potential roles in sensory function (Goldin et al 2000). Therefore, it is important to identify which sodium channel subtype(s) is(are) involved in neuropathic pain not only for better understanding neuropathic pain mechanisms but also for developing more specific analgesic drugs with little side effects.

During the last several years, our laboratory has performed a series of experiments identifying the important sodium channel subtypes in neuropathic pain. Our efforts have concentrated more on the subtypes that may be up-regulated in axotomized afferents, but not so much on those in sensitized intact nociceptors. The results of these findings are summarized here.

Importance of ectopic discharges in neuropathic pain

Neuropathic pain is considered to be a state of abnormal central processing in that not only noxious peripheral input produces severe pain (hyperalgesia) but normally innocuous input also produces pain (allodynia) (Gracely et al 1992). This abnormal central processing state is initiated and maintained by abnormal peripheral inputs, and an important source of these inputs is ectopic discharge of axotomized afferent fibres. Blocking entrance of ectopic discharges to the spinal cord by dorsal rhizotomy reduces neuropathic pain behaviours significantly, suggesting that at least some of the pain behaviours are maintained by ectopic discharges (Chung & Chung 2002).

Importance of sodium channels in ectopic discharges and neuropathic pain

Sodium channels play an important role in the generation of ectopic discharges. Supporting evidence for this includes that: (1) sodium channels accumulate at the neuroma of a cut sensory nerve where ectopic discharges arise (Devor et al 1989, 1993), and (2) application of sodium channel blockers silences ectopic discharges (Abdi et al 1998, Devor et al 1992, Liu et al 2001, Matzner & Devor 1994, Omana-Zapata et al 1997). Furthermore, applications of sodium channel blockers reduce neuropathic pain in humans (Chabal et al 1992) or pain behaviours in animal models (Abdi et al 1998, Abram & Yaksh 1994, Chaplan et al 1995, Lyu et al 2000).

TTX sensitivity of ectopic discharges and neuropathic pain

Ten different sodium channel subtypes have been cloned and characterized up to now (for a review see Goldin et al 2000), and most of them are found in the nervous system. Since there are many subtypes, it is important to find out which one(s) of

them is(are) critically involved in neuropathic pain. As an initial step, it would be useful to find out whether those important subtypes belong to the tetrodotoxin (TTX) sensitive (TTXs) or resistant (TTXr) family since sodium channel subtypes can be grouped into these two, based on their TTX sensitivity (Hunter & Loughhead 1999). It has been known that ectopic discharges are readily suppressed by TTX either applied topically to the neuroma (Matzner & Devor 1994) or given intravenously (Omana-Zapata et al 1997). However, one cannot be sure of the effective dose of TTX when it is applied in *in-vivo* preparations, thus it is difficult to determine with certainty whether TTXs or TTXr subtypes are involved in this case.

To determine TTX sensitivity of ectopic discharges more accurately, we tested the sensitivity using an *in vitro* preparation (Liu et al 2001). At various times after L5 spinal nerve ligation in the rat, the L5 dorsal root ganglion (DRG) along with the dorsal root and the ligated spinal nerve were removed and placed on a recording chamber, which was perfused with artificial cerebrospinal fluid. Single unit ectopic discharges were recorded from the teased dorsal root filaments. Sustained ectopic discharges could be recorded from 13 hours after spinal nerve ligation and most of these ectopic discharges originated from the DRG (Liu et al 1999, 2001). These ectopic discharges are extremely sensitive to TTX applied to the DRG, the site where the discharges are originating, so that the average dose of TTX is 22 nM for significant reduction of the discharge rate (Fig. 1). This reduction is apparently not due to conduction block, since that would require a much higher (437 nM) dose of TTX. Since the dose required to reduce ectopic discharges is about two orders of magnitude lower than the TTX sensitivity of TTXr subtypes of sodium channels, TTXs subtypes must be the ones that play an important role in the generation of ectopic discharges. In conducting experiments such as this, confirming the origin of ectopic discharges from the DRG in each individual unit is important. This is to ensure that TTX is actually applied to the site of ectopic discharge generation and that the rate of ectopic discharges is reduced by acting on the discharge generator mechanisms. Therefore, our data collection was done only on units that were proven to be originating from the DRG.

If TTXs subtypes of sodium channels are critically important for ectopic discharge generation, are they important for neuropathic pain? One way to resolve this question is to test the sensitivity of TTX on neuropathic pain behaviours. In the L5 spinal nerve ligation model of neuropathic pain, TTX was applied topically onto the L5 DRG using a chronically implanted catheter (Lyu et al 2000). Neuropathic pain behaviours were significantly reduced by low doses of TTX (12.5–50 nM). Again, the fact that neuropathic pain is reduced by TTX doses that are too low to block TTXr subtypes suggests that TTXs subtypes of sodium channels are involved in neuropathic pain behaviours.

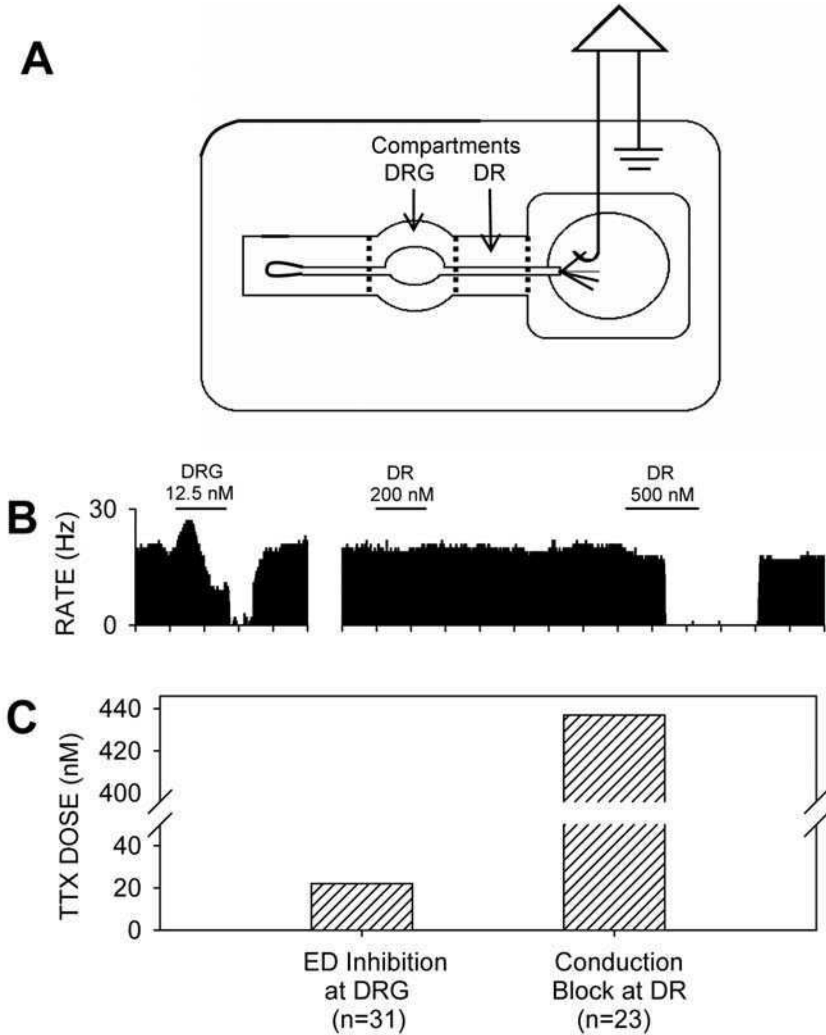

FIG. 1. (A) A schematic drawing of the experimental set-up. The L5 dorsal root ganglion (DRG) along with its dorsal root (DR) and previously ligated spinal nerve was removed from the rat and placed in a recording chamber. Single unit recordings were made from the proximal DR fascicles by teased preparation. The recording chamber was divided into four compartments, which were separated by Vaseline barriers (dotted lines). The DRG and the distal DR compartments were perfused independently with artificial cerebrospinal fluid (ACSF). A known concentration of TTX was added to the perfusion solution of each compartment for a few minutes. (B) The effects of TTX on ectopic discharges (ED). TTX was applied either to the DRG or to the distal DR with the specified doses during the periods indicated by horizontal bars. (C) Average doses required for inhibition of ectopic discharges in the DRG and for conduction block at the DR.

Up-regulation of TTXs sodium channel subtypes

Since TTXs subtypes of sodium channels seem to be important in neuropathic pain as well as in ectopic discharges, the next task is to find out which of the six TTXs subtypes that have been found in the DRG ($Na_v1.1$, $Na_v1.2$, $Na_v1.3$, $Na_v1.6$, $Na_v1.7$, and Na_x) is the important one. The approach we took for this task was to examine the expression of TTXs subtypes that were up-regulated in the DRG in synchrony with the development of ectopic discharges. The levels of mRNAs for the six TTXs subtypes in the DRG were measured with RNase protection assays (RPA) at various times after spinal nerve ligation (Kim et al 2001, 2002). The mRNAs of $Na_v1.1$, $Na_v1.2$, $Na_v1.6$, and $Na_v1.7$ declined after the nerve ligation, while those of $Na_v1.3$ and Na_x increased. The increase in $Na_v1.3$ mRNA was already evident 16 h after spinal nerve ligation and was maintained up to 7 days, whereas the increase in mRNA of Na_x was seen at 5 days but not at 1 day after the injury (Fig. 2). On the other hand, as mentioned above, ectopic discharges develop 13 h after spinal nerve ligation and continue to discharge for a long period of time. Therefore, $Na_v1.3$ is the only subtype of TTXs sodium channel whose mRNA

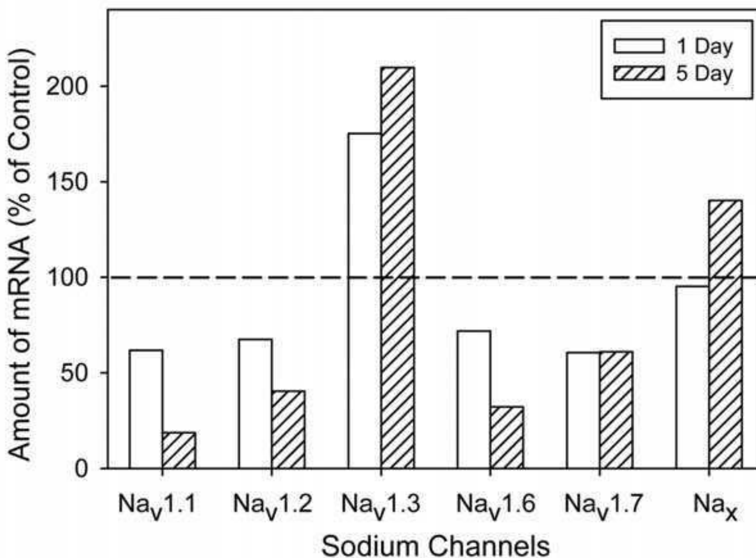

FIG. 2. Comparison of changes in mRNAs for TTX sensitive (TTXs) subtypes of sodium channels. The mRNAs of six TTXs subtypes ($Na_v1.1$, $Na_v1.2$, $Na_v1.3$, $Na_v1.6$, $Na_v1.7$ and Na_x) were measured in the DRG by RNase protection assay at 1 (1D) and 5 days (5D) after spinal nerve ligation. The amounts of mRNA are expressed as a percentage of control (normal) values. The mRNA of four subtypes are decreased after spinal nerve ligation, while two subtypes ($Na_v1.3$ and Na_x) increased. Up-regulation of Na_x was evident only at postoperative day 5, while that of $Na_v1.3$ was obvious both at day 1 and 5.

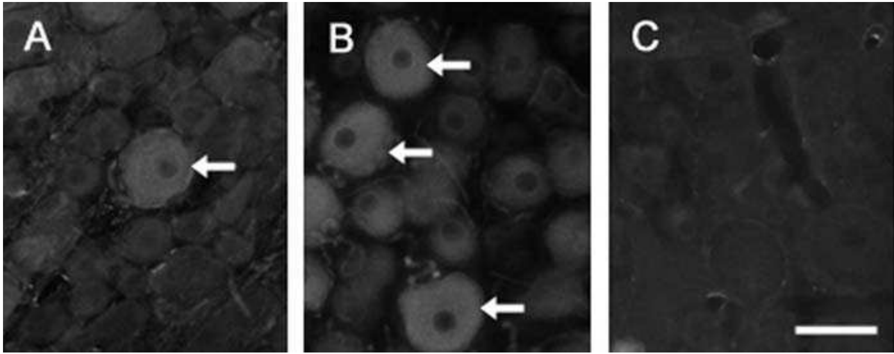

FIG. 3. Photomicrographs of immunostained L5 DRGs for $Na_v1.3$ subtype of sodium channels. A and B show DRGs taken from the contralateral and ipsilateral sides, respectively, 1 day after unilateral tight ligation of the L5 spinal nerve. Note the much higher number of immunostained cells (mainly large diameter neurons) in the ligated side (some labelled neurons are indicated by arrows). In C, preabsorption control tissue does not show any immunostaining for $Na_v1.3$. Calibration bar $= 50\,\mu m$. (Reprinted from Kim et al 2001 with permission from Elsevier.)

up-regulated in the DRG in synchrony with the development of ectopic discharges. In addition, immunohistochemical study of DRG neurons in spinal nerve ligation showed that the number of $Na_v1.3$ immunoreactive DRG neurons greatly increased, mostly in large-sized DRG neurons (Fig. 3) (Kim et al 2001). Assuming the up-regulation of sodium channels is a critically important factor for the generation of ectopic discharges and neuropathic pain, these data suggest that $Na_v1.3$ is the important sodium channel subtype, although the possible role of the Na_x subtype at a later postoperative period cannot be ruled out. The results of our studies are consistent with others in that up-regulation of $Na_v1.3$ sodium channel subtype has also been observed previously by others at a relatively long time after axotomy (7–9 days) (Boucher et al 2000, Waxman et al 1994).

Importance of TTXr sodium channel subtypes

While TTXs subtypes of sodium channels are likely involved in the generation of ectopic discharges, TTXr subtypes seem to be down-regulated in axotomized DRG. Evidence for this includes both the expression of TTXr subtype (Dib-Hajj et al 1998, Okuse et al 1997) and TTXr sodium currents (Cummins & Waxman 1997) that are reduced in axotomized DRG neurons. However, TTXr subtypes may play an important role in other aspects of neuropathic pain mechanisms, such as sensitization of intact nociceptive afferents. Supporting

evidence for such contention includes that $Na_v1.8$ protein level increases (Porreca et al 1999) in the DRG of the intact neighbouring segment of the spinal nerve ligation where nociceptors are observed to be sensitized (Wu et al 2001). Furthermore, inflammation of tissue causes up-regulation of TTXr subtypes as well as increased TTXr current (Tanaka et al 1998). Therefore, it is plausible that both TTXs and TTXr subtypes play important but somewhat different roles in neuropathic pain. An injured peripheral nerve usually contains a mixture of axotomized and neighbouring intact but inflamed afferent fibres. TTXr sodium channel subtypes may sensitize and generate spontaneous activity in intact nociceptors whereas TTXs subtypes are involved in the development of ectopic discharges in axotomized afferents.

Conclusions

It has long been suggested that changes in sodium channels play an important role in ectopic discharge generation in injured afferent nerves and the subsequent development of neuropathic pain. Recent cloning and characterization of multiple subtypes of sodium channels have made it feasible to identify the subtype that is critically important. Recent studies indicate that both ectopic discharges and neuropathic pain are extremely sensitive to TTX, suggesting that TTXs subtypes are critically involved in these phenomena. Systematic examination of the mRNA of TTXs sodium channel subtypes in the DRGs of injured segments revealed that $Na_v1.3$ and Na_x are the two subtypes that are up-regulated in the DRGs of axotomized segments, suggesting that they are potentially important subtypes in neuropathic conditions. Furthermore, up-regulation of $Na_v1.3$ was synchronous with the development of ectopic discharges, whereas that of Na_x was only apparent at a later time point. These data suggest that $Na_v1.3$ is the important sodium channel subtype for the generation of ectopic discharges and neuropathic pain after peripheral nerve injury. While TTXs subtypes are important for the generation of ectopic discharges in axotomized afferents, TTXr sodium channel subtypes may play an important role in sensitizing intact (non-axotomized) nociceptors, further contributing to neuropathic pain. Therefore, both TTXs and TTXr sodium channel subtypes seem to play somewhat different but important roles in neuropathic pain generation mechanisms.

References

Abdi S, Lee DH, Chung JM 1998 The anti-allodynic effects of amitriptyline, gabapentin, and lidocaine in a rat model of neuropathic pain. Anesth Analg 87:1360–1366

Abram SE, Yaksh TL 1994 Systemic lidocaine blocks nerve injury-induced hyperalgesia and nociceptor-driven spinal sensitization in the rat. Anesthesiology 80:383–391

Boucher TJ, Okuse K, Bennett DLH, Munson JB, Wood JN, McMahon SB 2000 Potent analgesic effects of GDNF in neuropathic pain states. Science 290:124–127
Chabal C, Jacobson L, Mariano A, Chaney E, Britell CW 1992 The use of oral mexiletine for the treatment of pain after peripheral nerve injury. Anesthesiology 76:513–517
Chaplan SR, Bach FW, Shafer SL, Yaksh TL 1995 Prolonged alleviation of tactile allodynia by intravenous lidocaine in neuropathic rats. Anesthesiology 83:775–785
Chung JM, Chung K 2002 Importance of hyperexcitability of DRG neurons in neuropathic pain. Pain Practice 2:87–97
Cummins TR, Waxman SG 1997 Downregulation of tetrodotoxin-resistant sodium currents and upregulation of a rapidly repriming tetrodotoxin-sensitive sodium current in small spinal sensory neurons after nerve injury. J Neurosci 17:3503–3514
Devor M, Keller CH, Deerinck TJ, Levinson SR, Ellisman MH 1989 Na^+ channel accumulation on axolemma of afferent endings in nerve end neuromas in Apteronotus. Neurosci Lett 102:149–154
Devor M, Wall PD, Catalan N 1992 Systemic lidocaine silences ectopic neuroma and DRG discharge without blocking nerve conduction. Pain 48:261–268
Devor M, Govrin-Lippmann R, Angelides K 1993 Na^+ channel immunolocalization in peripheral mammalian axons and changes following nerve injury and neuroma formation. J Neurosci 13:1976–1992
Dib-Hajj SD, Tyrrell L, Black JA, Waxman SG 1998 NaN, a novel voltage-gated Na channel, is expressed preferentially in peripheral sensory neurons and down-regulated after axotomy. Proc Natl Acad Sci USA 95:8963–8968
Goldin AL, Barchi RL, Caldwell JH et al 2000 Nomenclature of voltage-gated sodium channels. Neuron 28:365–368
Gracely RH, Lynch SA, Bennett GJ 1992 Painful neuropathy: altered central processing maintained dynamically by peripheral input. Pain 51:175–194
Hunter JC, Loughhead D 1999 Voltage-gated sodium channel blockers for the treatment of chronic pain. Curr Opin Investig Drugs 1:72–81
Kim CH, Oh Y, Chung JM, Chung K 2001 The changes in expression of three subtypes of TTX sensitive sodium channel in sensory neurons after spinal nerve ligation. Mol Brain Res 95:153–161
Kim CH, Oh Y, Chung JM, Chung K 2002 Changes in three subtypes of tetrodotoxin sensitive sodium channel expression in the axotomized dorsal root ganglion in the rat. Neurosci Lett 323:125–128
Liu X, Chung K, Chung JM 1999 Ectopic discharges and adrenergic sensitivity of sensory neurons after spinal nerve injury. Brain Res 849:244–247
Liu X, Zhou J-L, Chung K, Chung JM 2001 Ion channels associated with the ectopic discharges generated after segmental spinal nerve injury in the rat. Brain Res 900:119–127
Lyu YS, Park SK, Chung K, Chung JM 2000 Low dose of tetrodotoxin reduces neuropathic pain behaviors in an animal model. Brain Res 871:98–103
Matzner O, Devor M 1994 Hyperexcitability at sites of nerve injury depends on voltage-sensitive Na^+ channels. J Neurophysiol 72:349–359
Okuse K, Chaplan SR, McMahon SB et al 1997 Regulation of expression of the sensory neuron-specific sodium channel SNS in inflammatory and neuropathic pain. Mol Cell Neurosci 10:196–207
Omana-Zapata I, Khabbaz MA, Hunter JC, Clarke DE, Bley KR 1997 Tetrodotoxin inhibits neuropathic ectopic activity in neuromas, dorsal root ganglia and dorsal horn neurons. Pain 72:41–49
Porreca F, Lai J, Bian D et al 1999 A comparison of the potential role of the tetrodotoxin-insensitive sodium channels, PN3/SNS and NaN/SNS2, in rat models of chronic pain. Proc Natl Acad Sci USA 96:7640–7644

Tanaka M, Cummins TR, Ishikawa K, Dib-Hajj SD, Black JA, Waxman SG 1998 SNS Na^+ channel expression increases in dorsal root ganglion neurons in the carrageenan inflammatory pain model. Neuroreport 9:967–972

Waxman SG, Kocsis JD, Black JA 1994 Type III sodium channel mRNA is expressed in embryonic but not adult spinal sensory neurons, and is reexpressed following axotomy. J Neurophysiol 72:466–470

Wu G, Ringkamp M, Hartke TV et al 2001 Early onset of spontaneous activity in uninjured C-fiber nociceptors after injury to neighboring nerve fibers. J Neurosci 21:RC140(1–5)

DISCUSSION

Belmonte: I have two questions. The first relates to the point of origin of the ectopic activity. Do you think this is located in the soma or at the initial segment of the axon of dorsal root ganglion neurons? The second relates to the mechanism itself. Changes in Na^+ channel expression ($Na_v1.3$) may increase excitability. But you need some type of subthreshold mechanism to make them fire spontaneously. Do you think that other cationic conductances may be responsible for the observed hyperexcitability?

Chung: The first question is about the exact location of the ectopic activity. Obviously, we don't know this. This is why I said it was the cell body or the vicinity because all we know is that it is a part of the neuron located in the DRG. There is no way we can tell the exact origin. And with respect to the mechanism, I'm not saying that the Na^+ channel expression is the only one that is changing or is important. We and Marshall Devor have looked at this with a variety of channel blockers and their role in silencing ectopic discharges. It is hard to tell which is the most important.

Devor: I think it is a little naïve to say that a particular ion channel is the answer to everything. We have been talking about action potential generation, electrogenesis. But if the subject is pain, we are no longer talking about the generation of single action potentials; we are talking about repetitive firing. If a spike happens, or a 100 ms burst, that is not going to hurt you for a year. You need a process that is able to sustain rhythmic firing for very long periods. From our work, there is only one mechanism that does that in sensory neurons. I wish we could study it in sensory endings in the skin or cornea, but we can't. In the cell body we can. The phenomenon that drives sustained firing, that is necessary for it, is subthreshold oscillations. I refer to high frequency (around 100 Hz), spontaneous oscillation of the membrane potential. The fast rise time of the depolarizing limb of oscillations has the function of overcoming accommodation. When oscillations are present, any slow depolarization of the cell will give you sustained firing. If you don't have subthreshold oscillations then no sustained depolarization of the cell will give you repetitive firing. There are probably 100 different things that can

give you a slow sustained depolarization. Those are all the things that will excite a neuron if it is excitable. A key distinction needs to be made between exciting a sensory neuron and making the neuron excitable, making it capable of sustained firing. I think Jin Mo Chung makes an excellent point. The $Na_v1.3$ transcript looks like it is critically important not for excitation but for excitability. In fact, since these oscillations are so fast, $Na_v1.3$ is probably the only channel up-regulated in neuropathy that is fast enough to sustain them. I think it is a mistake for us to be focusing on agents and receptors that depolarize. We should be thinking about what changes the fundamental excitability of neurons: not what excites them, but what makes them excitable.

Dray: Marshall Devor, could you volunteer your mechanistic explanation of how the subthreshold oscillations would be set up in terms of conventional understanding of ion channel biology? Does it change from the focus on Na^+ channels that we have now?

Devor: We have actually worked quite a bit on this specific question. Oscillation is a resonance between an inward and outward current. To make a moderately long story short, the resonance here looks like it is between fast, transient openings of $Na_v1.3$, working against a voltage-insensitive passive K^+ leak conductance, probably one of the 2P channels (Amir et al 2002). In contrast, when you block the voltage-sensitive K^+ channels in the cell this strongly facilitates the oscillations, and enhances repetitive firing. Norepinephrine will do this, for example. We had always thought that norepinephrine activates injured sensory neurons by depolarization. Instead, its primary mode of action in causing ectopic firing may turn out to be that it makes the neurons more resonant. There are many different cells in the nervous system that have subthreshold oscillations, but this, as far as I know, is the simplest and most elegant example: a sustained oscillation with only a single voltage-sensitive conductance involved.

Belmonte: I think this view may also be too simplistic. You are assuming that the only thing that changes is threshold.

Devor: It is repetitive firing threshold that I am referring to, which is a very different thing from single spike threshold.

Belmonte: In my view, we should also look at other ion channels whose expression could be altered.

Devor: Which ones do you like?

Belmonte: There are many classes of ion channels in primary sensory neurons. I prefer not to speculate now!

Mao: It seems that there are different strategies at this point in terms of targeting Na^+ channels, such as $Na_v1.3$, that is, to find those agents that block the generation of ectopic discharges without interrupting normal or physiological nerve conduction. Having said this, is it possible that at the molecular level one can find lidocaine-type agents that would specifically block $Na_v1.3$ but not the

regular Na^+ channel. What we are looking at is the Na^+ channel that is responsible for the generation of ectopic or non-physiological discharges.

Chung: That is where we are now. The next critical step is to establish a way to selectively block $Na_v1.3$. Obviously, we don't have this yet. There are some problems facing us. One is that $Na_v1.1$, $Na_v1.2$ and $Na_v1.3$ are very similar. We need a molecular biological technique to design something very specific for $Na_v1.3$. We have tried very hard to use an antisense approach, with no success yet.

Oh: Frank Porecca insists that $Na_v1.8$ is very important in maintaining neuropathic pain.

Chung: Multiple components seem to be involved in neuropathic pain. One is the component of injured afferents and, as I talked about, $Na_v1.3$ seems to be important. Another is the component of uninjured afferents, which I did not talk much about. I believe that is where $Na_v1.8$ plays an important role. His conclusions are based on antisense experiments, which involved intrathecal injection of $Na_v1.8$ antisense. It is difficult to say which component is affected by intrathecal injection of antisense.

Tominaga: Have you checked the mRNA or protein expression of $Na_v1.3$ or $Na_v1.8$ in your model?

Chung: Both the expression of $Na_v1.3$ mRNA and protein go up in the DRG of the injured segment. Others have reported that mRNA of $Na_v1.8$ goes down in the injured segment whereas protein of $Na_v1.8$ goes up in the uninjured segment. Therefore, the expressions of $Na_v1.3$ and $Na_v1.8$ seem to go in opposite directions.

Wood: I was fascinated by your observation that the ectopic discharges arose from the somata, yet the neuroma presumably makes a contribution. What is the role of the neuroma?

Chung: We have looked at where the ectopic discharge originates. When we ligate the spinal nerve very close to the DRG, most of the activity comes from the DRG. But when we make a further injury distally, the situation seems to change. For example, we have compared spinal nerve ligation versus chronic constriction nerve injury (CCI), which is a distal injury. With CCI, more than half of the ectopic discharges come from the injury site, whereas practically all discharges originate from the DRG after spinal nerve injury. Perhaps the distance from the DRG is a factor here.

Baron: I'd like to come back to the issue of the uninjured fibres, which are very important for the clinicians because we can detect them relatively easily because they are still in the skin on the periphery. How many large myelinated fibres are present in the L4 level and how many of these are spontaneously active?

Chung: We haven't done a careful comparison of activity between the L4 and L5. Dick Meyer's group has recorded from the L4 spinal nerve after L5 ligation and they observed a low level of activity from a large number of C fibres, but they didn't

look at A fibres. However, Steve McMahon has looked at the L4, haven't you Steve?

McMahon: We have looked and reported on the findings (Boucher et al 2000). After spinal ligation of the L5 spinal nerve, one sees extensive ectopic activity in myelinated fibres in intact L4 spinal afferents. This is most apparent in the first week after the nerve ligation. If there is C-fibre activity in intact L4 spinal afferents, it must be at a very low rate.

Devor: I want to come back to Jianren Mao's question about the clinical relevance of this work. You said it would be nice if we had a selective $Na_v1.3$ blocker. Personally, I don't think this is necessary at all. We have very good $Na_v1.3$ blockers, albeit non-selective, from several different chemical families. These are the drugs that have been used for the last 20 years to control neuropathic pain. I'm referring to systemic local anaesthetics, anticonvulsants and tricyclics. They work great. I think the reason they work is that they stop ectopic firing very effectively in the dorsal root ganglion and also in the neuroma. Jin Mo pointed out in his recordings that the concentration of these drugs required to stop ectopic firing is between two and three orders of magnitude lower than what is required to stop nerve conduction. You are not going to kill anyone at these drug concentrations by stopping all of the nerve conduction. They are too low to stop nerve conduction. So what are the dose-limiting side effects in humans, in clinical applications? The answer is quite clear. The dose-limiting concentrations have nothing to do with muscles and only rarely the heart. The problem is that people get tired, dizzy and nauseous. These are central nervous system effects. But what we have just learned from Jin Mo is that the pain relieving effects that we are looking for are peripheral nervous system effects. I have been arguing for a long time that if we simply modified some of these tried and true drugs, which work in humans, so as to maintain their peripheral actions while preventing them from getting into the brain, then one might have marvellous analgesic drugs for neuropathic pain. This is not trivial, but it is doable.

Gintzler: Is there a net change in overall Na^+ channel expression? Some go up, some go down, but in the aggregate, is there any change?

Chung: Although it is easy to compare relative level of expression, it is harder to compare absolute amount of Na^+ channels. Therefore, it is hard to know the net change.

Gintzler: Different Na^+ channels have different biophysical properties. If overall the net amount of Na^+ channels is the same, but there is different prevalence of one type over another, is that of major functional significance?

Wood: The re-priming characteristics of $Na_v1.3$ would make it able to fire much more quickly, and thus increase excitability.

Zhang: What is the significance of Na^+ channel accumulation at the neuroma? Are they inserted in the membrane, or do they just accumulate in the cytoplasm?

Mao: I guess the question is whether the resolution of your technique is sufficient to identify the Na^+ channel in the plasma or approaching the membrane?

Devor: We have looked with EM immunocytochemistry. It is very highly concentrated in the membrane.

Dray: I have a question concerning the translation from the rat with respect to the redistribution of specific types of Na^+ channels, and human neuropathy. Is it possible to complement these findings by *in vitro* recording from human neuropathic DRGs? I agree very much with what Marshall Devor said with respect to the efficacy of local anaesthetics given intravenously at much lower concentrations than would be deleterious to other organs. Recent studies by Strichartz (Araujo et al 2003) showed that infusions of lidocaine reduced neuropathic pain behaviour by acute (hours) and sustained (days) mechanisms. Could you comment on this apparent discrepancy as the behaviours are not easily explained by a local channel block?

Chung: It is not only lidocaine, but TTX also. When you block ectopic discharge for a short period, behaviour lasts longer. The explanation I have is that when you block ectopic discharge you reduce central sensitization and it takes time for the reduced central sensitization to re-charge.

References

Amir R, Liu C-N, Kocsis JD, Devor M 2002 Oscillatory mechanism in primary sensory neurones. Brain 125:421–435

Araujo MC, Sinnott CJ, Strichartz GR 2003 Multiple phases of relief from experimental mechanical allodynia by systemic lidocaine: responses to early and late infusions. Pain 103:21–29

Boucher TJ, Okuse K, Bennett DLH, Munson JB, Wood JN, McMahon SB 2000 Potent analgesic effects of GDNF in neuropathic pain states. Science 290:124–127

Ion channel activities implicated in pathological pain

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Abstract. Altered expression of voltage-gated sodium, calcium and potassium channels has been associated with neuropathic pain conditions. In addition, roles for the ligand-gated $P2X_3$ and NMDA receptors, as well as pacemaker HCN channels have also been invoked in the pathogenesis of neuropathic pain. In this chapter, evidence of an important role for post-translational regulation of $Na_V1.9$ in setting pain thresholds is presented. Despite the importance of tactile allodynia and mechanical hyperalgesia in chronic pain, we remain ignorant of the molecular nature of mechanosensors present in sensory neurons. A number of candidate mechanosensor genes, identified because of their structural similarity with mechanosensors in *Caenorhabditis elegans* and *Drosophila melanogaster* have been identified. Acid-sensing ion channels (ASICs) are structurally related to putative mechanosensors in *C. elegans*, whilst transient receptor potential channels (TRPs) have been implicated in mechanosensation in the *Drosophila* acoustic system. Evidence against a role for ASICs as primary transducers of mechanosensation is provided here, and recent evidence implicating TRP channels is reviewed. Finally, the use of sensory neuron-specific gene deletion approaches to unravel the significance of individual ion channels in the regulation of sensory neuron excitability and the induction of pain will be described.

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Gene mis-regulation studies, knockouts and pharmacological insights have provided us with a range of new ion channel targets for analgesic drug development. Although a range of different mechanisms underlie neuropathic pain, common themes and interrelationships between many of the different deficits have been catalogued in animal models. Damage to sensory neurons or their axons can lead to alterations in responses to trophic factors and cytokines (e.g. nerve growth factor, tumour necrosis factor), leading to altered patterns of channel gene expression (e.g. the re-expression of genes normally expressed in early development such as the voltage-gated sodium channel $Na_V1.3$). Damage to

axons also leads to altered subcellular patterns of channel expression (e.g. voltage-gated sodium and potassium channels) that lead to hyperexcitability or spontaneous electrical activity. This in turn leads to altered patterns of input into the dorsal horn involving voltage-gated calcium channel subunits (e.g. $\alpha 2\delta 1$), and changes in second-order sensory neuron synaptic efficacy involving, amongst other signalling systems, NMDA receptors.

Table 1 contains a list of channels receptors and trophic factors that have been suggested to play a role in chronic pain pathogenesis.

One important and general clinical problem in pathological pain states is altered perception of mechanical stimuli. Both mechanical allodynia and mechanical hyperalgesia are often associated with chronic pain states. It might therefore be useful if specific drugs could be targeted at mechano-transducing molecules to treat such pain conditions. However, the detection of noxious or non-noxious mechanical stimuli is less well understood in molecular terms than the transduction processes involved in detecting thermal or chemical stimuli, where many of the receptors (e.g. TRPV1, CMH1, acid-sensing ion channels [ASICs]) have been identified. Genetic studies of mechanosensory mutants in *Caenorhabditis elegans* or *Drosophila melanogaster* have identified a number of candidate mechanosensors that have mammalian homologues, but the evidence for a role of any of these molecules in mechanosensation is indirect. At least three classes of receptors are candidate mechanosensors. Evidence for mechanical activation of $P2Y_1$ and $P2X_3$ receptors (ATP-gated receptors and cation-selective ion channels), transient receptor potential (TRP) channels (e.g. NompC and VR-OAC) and ASICs has been discussed (Ernstrom & Chalfie 2002).

ASICs comprise a family of two-pass transmembrane receptors that are encoded by four separate genes and exist as a variety of splice variants. They represent a subset of the epithelial sodium channel (ENaC) family of channels that are gated by a variety of stimuli, including peptides and low pH. Genetic screens for *C. elegans* mutants that are defective in mechanosensation have identified several loss-of-function genes, some of which encode ion channels; the two best characterized, MEC-4 and MEC-10, are homologues of the mammalian ASICs (amino acid identity between ASICs and MECs: 20–30%) (Tavernarakis & Driscoll 1997). All four ASIC genes as well as some related epithelial sodium channel subunits are expressed in DRG neurons (Waldmann & Lazdunski 1998, Akopian et al 2000). ASIC3 is expressed mainly in DRG neurons whilst ASIC1b is a splice-variant exclusively expressed by intermediate-sized DRG neurons (Chen et al 1998). ASIC2 and ASIC3 subunits are expressed in specialized mechanosensitive structures, as are some ENac subunits (Garcia-Anoveros et al 2001, Price et al 2000, 2001). Thus the distribution of expression of ASIC subunits is consistent with a role for these proteins in mechanosensation.

TABLE 1 Some of the ion channels, receptors, neurotrophins and cytokines that are known to influence neuropathic pain behaviour in rodent models of pathological pain

Channels and receptors	*Drug*	*Established role?*
Sodium channels		
$Na_v1.3$	Tetrodotoxin/lidocaine	correlative and pharmacological
$Na_v1.8$		antisense, knock-out
$Na_v1.9$		correlative
$\beta3$ subunits		correlative
Calcium Channels		
$Ca_v2.2$	N-type Calcium channel blockers	knock-out, pharmacological
$\alpha2\delta$	Gabapentin	pharmacological
Potassium channels		
$K_v1.4$		correlative
KCNQ	Retigabine	correlative and pharmacological
Pacemakers		
HCN	ZD7288	correlative and pharmacological
Ligand-gated		
TRPV1	Capsazepine	pharmacological
$P2X_3$	A317491	antisense and pharmacological
NMDA-NR2b	CP-101,606	pharmacological
mGluR1		antisense
mGluR2/3	LY379268	pharmacological — agonists
Galanin		transgenics
CB1	WIN55212-2	pharmacological
CB2		microglia?
Neurotrophins/Cytokines		
NGF	receptor-bodies	transgenics
BDNF	receptor-bodies	
GDNF	receptor-bodies	pharmacological
IL6		null mutant
TNF	thalidomide	pharmacological

Interestingly, many of the drugs that have reached clinical trials show activity, validating the animal models.

Heterologous expression of ASIC cDNAs has failed to demonstrate mechanically gated currents, but studies of null mutants of ASICs have demonstrated minor effects on mechanosensation. The sensitivity of low-threshold rapidly adapting mechanoreceptors is increased and the sensitivity of slowly conducting myelinated mechano-nociceptors is reduced in ASIC3

knockout mice as measured with the *in vitro* skin-nerve preparation (Price et al 2001), whilst the sensitivity of low-threshold rapidly adapting mechanoreceptors is reduced in ASIC2 knockout mice (Price et al 2000). The ionic basis of mechanosensitive currents is similar to those of proton-activated ASIC currents (see Waldmann & Lazdunski 1998). Immke & McCleskey (2001) have shown that ASIC-mediated currents in ischaemia-sensing neurons are modulated in a similar manner to mechanosensitive currents by changes in external Ca^{2+} (and Mg^{2+}) concentration. We found that the amplitude of mechanically activated-currents is not correlated with the amplitude of low pH-evoked responses in DRG and that these currents are not regulated by acidification of the external solution (Drew et al 2004). However, ASIC2a (Price et al 2000, Garcia-Anaveros et al 2001) and ASIC3 (Price et al 2001) are present on the endings of Aβ endings *in vivo* and these neurons are not activated by low pH. This observation underlies the suggestion that ASICs may be able to exist in a proton-insensitive state that is mechanosensitive (e.g. Welsh et al 2002).

McCarter et al (1999) first showed that cultured DRG neuron somata respond to mechanical stimulation with an inward cationic current. Such studies carried out in voltage-clamp configuration allow the primary mechanosensitive current to be isolated from other voltage-depended currents that may be subsequently activated. Thus mechanical stimulation of sensory neurons (as opposed to osmotic stimulation causing cell swelling coupled with calcium dye imaging) allows the characterization of mechanosensitive ion channels. These currents are specific to DRG neurons, suggesting that the current is related to mechanosensation at sensory neuron terminals. We extended these findings to show that there is diversity amongst the responses of DRG neurons to pressure *in vitro* that correlates with aspects of their *in vivo* properties (Drew et al 2002). Mechanical stimulation of the somata of cultured neonatal rat DRG neurons evoked inward cationic currents that displayed distinct properties between different subsets of cells. The presumptive nociceptor population, defined by capsaicin sensitivity, showed higher thresholds for the induction of an inward current and lower peak currents than other mechanosensitive neurons. A subset of capsaicin-sensitive IB4-positive sensory neurons was refractory to mechanical stimulation. All mechanically activated currents were blocked by gadolinium ($IC_{50} \approx 8\,\mu M$) and ruthenium red ($IC_{50} \approx 3\,\mu M$). Disruption of the actin cytoskeleton by acute application of $10\,\mu M$ cytochalasin B inhibited currents much more effectively in capsaicin-insensitive (61%) than sensitive neurons (20%). Extracellular calcium also attenuated mechanosensitive currents to a greater degree in capsaicin-insensitive neurons than capsaicin-sensitive neurons. Thus the somata of different types of cultured sensory neurons have distinct mechanosensitive phenotypes that may correspond to the receptor subtypes that they express *in vivo* (Drew et al 2002, 2004). Single channel analysis has suggested that distinct channel populations are

responsible for low and high threshold mechanically activated currents (Cho et al 2002) Inflammatory mediators that change sensitivity to mechanical stimulation *in vivo* have also been shown to regulate a subset of mechano-sensitive DRG patches *invitro* (Cho et al 2002). Recording from the somata of DRG neurons thus provides a route to identifying the receptor system involved in mechanosensation.

Having established a system to characterize mechanosensitive channels, we set out to test the role of ASIC subunits in mechanotransduction, using primary cultures of sensory neurons derived from adult mice with ASIC1, 2 or 3 gene deletions. Price et al (2000, 2001) had observed that mechanically evoked firing in Aβ fibres from ASIC2 nulls was reduced and that there was an increase in firing rates in rapidly activating mechanoreceptors from ASIC3 knockout mice. However, in our system the deletion of the genes for ASIC1, ASIC2 and ASIC3, either alone or for ASIC2/3 together, had no significant effect on either the sensitivity of large neurons to mechanical stimulation or on the kinetics of evoked responses. We therefore conclude that none of these ion channels contributes to the generation of mechanosensitive currents in isolated neurons. Mechanically evoked responses of small–medium neurons also showed no differences between wild-type neurons and those lacking ASIC2 and ASIC3.

The pharmacology of mechanosensitive channels is more reminiscent of TRP channels than ASICs (e.g. voltage-dependent block by ruthenium red), and recent evidence supports a role for TRP channels in mechanosensation. The TRPV4 channel is known to be activated by cell swelling, as well as lipoxygenase metabolites and thermal stimuli. A TRPV4 knockout mouse shows major deficits in response to noxious pressure applied with a Randall Selitto apparatus, although von Frey thresholds and thermal stimulation are normal (Suzuki et al 2003). Whether this channel acts directly as a mechanosensor remains uncertain, as heterologous expression of the channel does not confer mechanosensitivity on cell lines or sympathetic neurons.

Taken together these data suggest that both light touch and noxious mechanosensation are not transduced by ASIC channels. Although TRP channels are good candidates as primary mechanical transducers, the molecular nature of the mechanosensory complex remains to be determined.

Another important issue in pathological pain states concerns the alterations in sensory neuron activation thresholds that may underlie altered pain sensitivity. A role for sodium channels in this phenomenon has been suggested by many studies (Waxman & Wood 1999). Voltage-gated sodium channels comprise a family of 10 structurally related genes that are expressed in spatially and temporally distinct patterns in the mammalian nervous system. Two sodium channels, $Na_v1.8$ and $Na_v1.9$ are selectively expressed within the peripheral nervous system, predominantly in nociceptive sensory neurons, and these particular isoforms have attracted attention as analgesic drug targets (Waxman & Wood 1999). In

addition, an embryonic channel $Na_v1.3$ and a β subunit, $\beta3$, have been found to be up-regulated in dorsal root ganglion (DRG) neurons in neuropathic pain states (Cummins & Waxman 1997).

$Na_v1.9$ is also expressed in nociceptive neurons (Dib-Hajj et al 2002, Fang et al 2002) and underlies a persistent sodium current with substantial overlap between activation and steady-state inactivation (Cummins et al 1999). This channel may thus have a role in setting thresholds of activation (Dib-Hajj et al 2002, Baker et al 2003), suggesting that blockade of $Na_v1.9$ might be useful for the treatment of pain. Conversely, it has been suggested that $Na_v1.9$ activators might alleviate pain because $Na_v1.9$ is down-regulated after axotomy (Cummins et al 2000) and the resultant loss of the $Na_v1.9$ persistent current and its depolarizing influence on resting potential (Cummins et al 1999) might remove resting inactivation from other sodium channels. In the absence of a $Na_v1.9$ null mutants or selective blockers, there is inadequate information on the role of this channel in neuropathic pain, although normal level of expression seems to be dependent on the supply of NGF or glial cell-derived neurotrophic factor (GDNF) (Cummins et al 2000).

In vitro studies have demonstrated that $Na_v1.9$ current densities can be up-regulated in the presence of GTP or its non-hydrolysable analogue GTPγS. The functional consequences of up-regulation are dramatic. Over a period of several minutes, GTP may cause an increase in peak persisted tetrodotoxin (TTX)-resistant current of up to 10-fold. The threshold of activation of sensory neurons somata excursing $Na_v1.9$ falls by approximately 15 mV. At a holding potential around -60 mV this channel up-regulation results in spontaneous action potential propagation. Similar events occur if primary cultures of sensory neuron are bathed in a mixture of inflammatory mediators (Baker et al 2003), suggesting that at least in inflammatory conditions this channel may play a role in sensitizing pain thresholds. Interestingly, in diabetic neuropathy this channel is also up-regulated (Craner et al 2003) implying a potential role for $Na_v1.9$ in this important neuropathic pain condition.

Tissue-specific and inducible knockouts: the end of pharmacology?

Many broadly expressed genes encoding channels receptors, enzymes and regulatory molecules have a variety of different functions in distinct organ systems. This makes physiological function difficult to analyse in whole animal studies even with specific drugs (because of side effects), or by means of gene deletion experiments. However, by deleting the gene in specific subsets of cells associated with particular functions, the role of a particular transcript in a defined physiological system (e.g. pain pathways) may be analysed in an unambiguous fashion.

Sauer and collaborators (Le & Sauer 2002) exploited the recombinase activity of a bacteriophage enzyme Cre, to delete DNA sequences in mammalian cells that are flanked by loxP sites. Applying this technology to embryonic stem cells, it has proved possible to generate tissue-specific mouse null mutants. An analogous system exploits the Flp recombinase that recognizes Frt sites. By deleting genes only in a subset of cells, it is thus possible to examine the specialized role of a broadly expressed gene in a specific physiological system. Problems of developmental lethality may also be avoided using this approach.

In order to ablate genes in sensory ganglia, it is necessary to produce mice in which functional Cre recombinase is driven by sensory neuron-specific promoters. The effectiveness of expressed Cre in excising loxP-flanked genes can be measured with a reporter mouse using the β-galactosidase-expressing gene with a floxed (loxP flanked) stop signal. Where Cre removes the stop signal, β-galactosidase activity can be analysed histochemically. The $Na_v1.8$ gene is expressed predominantly in nociceptive sensory neurons, and is completely absent in tissue other than sensory neurons (Akopian et al 1999, Djouri et al 2003). Heterozygous null mutant $Na_v1.8$ mice are completely normal (Akopian et al 1999), suggesting that 'knocking-in' a Cre-recombinase into the $Na_v1.8$ locus is unlikely to have deleterious effects in heterozygous mice that express single alleles of $Na_v1.8$ and Cre. These mice were constructed and analysed, and showed no phenotypic deficits, whilst expressing Cre recombinase in a similar pattern to $Na_v1.8$ (Stirling et al 2004).

It would be even more useful to generate transgenic mice expressing drug-activatable Cre isoforms exclusively in subsets of sensory neurons. Such an approach would remove the problem of developmental compensatory mechanisms that may mask the phenotype caused by deletion of a particular gene. Recently a tamoxifen-activatable form of Cre recombinase has been developed. This form of Cre recombinase comprises a fusion protein between Cre and a human mutated oestrogen receptor. The addition of tamoxifen, but not endogenous steroids, releases the Cre recombinase from a cytoplasmic association with HSP90 and allows it to enter the nucleus (Metzger et al 2001). This allows the excision of genes at defined periods in adulthood.

This powerful technology is likely to be applied increasingly over the next few years, and together with siRNA promises to speed up target validation strategies in animal models of neuropathic pain. DRG-specific Cre-recombinase mice have been made (Stirling et al 2004, M. Nassar, in preparation), and an increasing number of floxed target genes (e.g. brain-derived neurotrophic factor [BDNF], Rios et al 2001) are also now available for this type of analysis.

Because a complex interplay between many types of molecules within damaged neurons, glia and cells of the immune system underlies the establishment of chronic neuropathic pain, a wealth of potential molecular targets which may be useful in

the treatment of pain have recently been described. A key to the development of useful analgesic drugs is to avoid unnecessary side effects. As yet there is not a single target that has been shown to be uniquely associated with the establishment of neuropathic pain, although sensory neuron-specific channels involved in pain pathways are attractive drug targets for obvious reasons. Unfortunately, specific pharmacological blockers or activators are still in development for these targets. However, the intense focus of academia and of the pharmaceutical industry on the problem of neuropathic pain suggests that new drugs directed at novel targets such as sodium channels should be available in the future. Mechanism-based medicine depends upon both the understanding of pathological mechanisms and the development of drugs that target the disease state. The financial and legal constraints on drug development cause enormous delays and frustration in translating research insights into therapies. Nevertheless, the wait will unquestionably be worthwhile.

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References

Akopian AN, Souslova V, England S et al 1999 The tetrodotoxin-resistant sodium channel SNS has a specialized function in pain pathways Nat Neurosci 2:541–548

Akopian AN, Chen CC, Ding Y, Cesare P, Wood JN 2000 A new member of the acid-sensing ion channel family. Neuroreport 11:2217–2222

Baker MD, Chandra SY, Ding Y, Waxman SG, Wood JN 2003 GTP-induced tetrodotoxin-resistant Na+ current regulates excitability in mouse and rat small diameter sensory neurones. J Physiol 548:373–382

Chen CC, England S, Akopian AN, Wood JN 1998 A sensory neuron-specific, proton-gated ion channel. Proc Natl Acad Sci USA 95:10240–10245

Cho H, Shin J, Shin CY, Lee SY, Oh U 2002 Mechanosensitive ion channels in cultured sensory neurons of neonatal rats. J Neurosci 22:1238–1247

Craner MJ, Klein JP, Renganathan M, Black JA, Waxman SG 2002 Changes of sodium channel expression in experimental painful diabetic neuropathy. Ann Neurol 52:786–792

Cummins TR, Waxman SG 1997 Down-regulation of tetrodotoxin-resistant sodium currents and up-regulation of a rapidly repriming tetrodotoxin-sensitive sodium current in small spinal sensory neurons following nerve injury. J Neurosci 17:3503–3514

Cummins TR, Dib-Hajj SD, Black JA et al 1999 A novel persistent tetrodotoxin-resistant sodium current in SNS-null and wild-type small primary sensory neurons. J Neurosci 19:RC43(1–6)

Cummins TR, Black JA, Dib-Hajj SD, Waxman SG 2000 GDNF up-regulates expression of functional SNS and NaN sodium channels and their currents in axotomized DRG neurons. J Neurosci 20:8754–8761

Cummins TR, Aglieco F, Renganathan M et al 2001 $Na_v1.3$ sodium channels: rapid repriming and slow closed-state inactivation display quantitative differences following expression in a mammalian cell line and in spinal sensory neurons. J Neurosci 21:5952–5961

Dib-Hajj S, Black JA, Cummins TR, Waxman SG 2002 NaN/Nav1.9: a sodium channel with unique properties. Trends Neurosci 25:253–259
Djouhri L, Fang X, Okuse K, Wood JN, Berry CM, Lawson SN 2003 The TTX-resistant sodium channel Nav1.8 (SNS/PN3): expression and correlation with membrane properties in rat nociceptive primary afferent neurons. J Physiol 550:739–752
Drew LJ, Wood JN, Cesare P 2002 Distinct mechanosensitive properties of capsaicin-sensitive and -insensitive sensory neurons. J Neurosci 22:RC228
Drew LJ, Rohrer DK, Price MP et al 2004 ASIC2 and ASIC3 do not contribute to mechanically activated currents in mammalian sensory neurons. J Physiol, in press
Ernstrom GG, Chalfie M 2002 Genetics of sensory mechanotransduction. Annu Rev Genet 36:411–453
Fang X, Djouhri L, Black JA et al 2002 The presence and role of the TTX resistant sodium channel $Na_v1.9$ (NaN) in nociceptive primary afferent neurons. J Neurosci 22:7425–7433
Garcia-Anoveros J, Samad TA, Zuvela-Jelaska L, Woolf CJ, Corey DP 2001 Transport and localization of the DEG/ENaC ion channel BNaC1alpha to peripheral mechanosensory terminals of dorsal root ganglia neurons. J Neurosci 21:2678–2686
Immke DC, McCleskey EW 2001 ASIC3: a lactic acid sensor for cardiac pain. ScientificWorldJournal 1:510–512
Le Y, Sauer B 2002 Conditional gene knockout using Cre recombinase. Mol Biotechnol 17:269–275
McCarter GC, Reichling DB, Levine JD 1999 Mechanical transduction by rat dorsal root ganglion neurons in vitro. Neurosci Lett 273:179–182
Metzger D, Chambon P 2001 Site- and time-specific gene targeting in the mouse. Methods 24:71–80
Price MP, Lewin GR, McIlwrath SL et al 2000 The mammalian sodium channel BNC1 is required for normal touch sensation. Nature 407:1007–1011
Price MP, McIlwrath SL, Xie J et al 2001 The DRASIC cation channel contributes to the detection of cutaneous touch and acid stimuli in mice. Neuron 32:1071–1083
Rios M, Fan G, Fekete C et al 2001 Conditional deletion of brain-derived neurotrophic factor in the postnatal brain leads to obesity and hyperactivity. Mol Endocrinol 15:1748–1457
Stirling C, Nassar M, Forlani G, Baker MD, Wood JN 2004 Nociceptor-specific gene deletion using heterozygous $Na_V1.8$-Cre recombinase mice. J Physiol (abstr) 555P C53
Suzuki M, Mizuno A, Kodaira K, Imai M 2003 Impaired pressure sensation in mice lacking TRPV4. J Biol Chem 278:22664–22668
Tavernarakis N, Driscoll M 1997 Molecular modeling of mechanotransduction in the nematode Caenorhabditis elegans. Annu Rev Physiol 59:659–689
Waldmann R, Lazdunski M 1998 H^+-gated cation channels: neuronal acid sensors in the NaC/DEG family of ion channels. Curr Opin Neurobiol 8:418–424
Waxman SG, Wood JN 1999 Sodium channels: from mechanisms to medicines? Brain Res Bull 50:309–310
Welsh MJ, Price MP, Xie J 2002 Biochemical basis of touch perception: mechanosensory function of degenerin/epithelial Na^+ channels. J Biol Chem 277:2369–2372

DISCUSSION

McMahon: Regarding mechanocurrents, is it correct that only about 50% of the DRG neurons respond with the stimuli you use?

Wood: It is a question of how hard you look. I would say more than that, in the range of 70–80%.

McMahon: That's what you'd expect.

Dray: Am I right in thinking that the $Na_V1.7$ knockout made in the conventional way didn't survive, but the conditional knockout did?

Wood: The $Na_V1.7$ null mouse, with $Na_V1.7$ deleted in sensory and sympathetic neurons died, but the nociceptor-specific knockout survived.

Dray: So the death is presumably due to some sympathetic dystrophy.

Wood: That is what we believe.

Dray: You said that you thought $Na_V1.7$ has some function in inflammatory pain. Can you elaborate on this?

Wood: We seem to lose the second phase of the formalin response.

Mantyh: How well do you think your probing of these mechanosensing channels reflects what happens in the sensory neurons? For example, if we are going to take a mechanosensitive neuron in a DRG and probe it in culture, how well would this cell reflect what occurs *in vivo* in the animal?

Wood: I think it is a poor reflection on the normal expressed complex at the nerve terminal, but is the only thing that we can do. As a test system for assessing knockouts, it is a useful system.

Mantyh: Do you think it really is a useful system? With the mechanosensitive fibres, do you think that the mechanonsensitive channels are actually inserted into the fibre, where they would normally be responding to a stimuli *in vivo* as opposed to the cell body?

Wood: When we acutely dissociate them we probably cause all kinds of aberrant trafficking. I accept your criticism, but this is the only system we have.

Reeh: Have you tried the hypotonic stimuli that Carlos Belmonte is interested in?

Belmonte: A couple of years ago we did exactly the same experiment using hypotonic solutions as stimulus (Viana et al 2001), and we obtained similar results: a population of neurons showing fast, short-lasting spikes that responded to moderately hypotonic solutions with a rapid rise in $[Ca^{2+}]_i$ and a second population of neurons with broader spikes and hump in the falling phase of the spike, which gave a smaller and slower $[Ca^{2+}]_i$ in response to hypotonic solutions. Calcium imaging could perhaps be a simpler technique to explore mechanical responses.

Wood: One of the good reasons for carrying on doing that is that some of the Ca^{2+} signalling is downstream of the initial target. By actually looking at current flow in voltage clamp we get around the problem of voltage-gated channel activity, and can look at the primary mechanosensitive channels.

Reeh: Have you actually compared hypotonic stimuli with your mechanical stimulus?

Wood: No.

Reeh: I would guess that more cells respond to hypotonic than to mechanical stimuli.

Wood: They probably do because we can't prod many of the very small cells.

Oh: I have a comment on the TRPV channel. There are differences between mechanical pressure and hypo-osmotic shock. The TRPV4 channel responds well to hypo-osmotic shock but never responds to mechanical pressure. I don't know why.

Belmonte: I agree. Hypotonic stimuli also stimulate stretch-activated channels because of the change of volume.

Reeh: Do you actually see an osmotically induced change in volume in every single cell? After all it should depend on water permeability of the plasma membrane, shouldn't it?

Belmonte: In those cells where we measured volume, we observed a change with the hypo-osmotic stimulus. The question I wanted to ask you concerns the 'mechanosensory apparatus'. There, proteins are apparently linked to an ion channel; but, is this channel the same in low- and high-threshold mechanosensory neurons? This is a critical question.

Wood: Dr Oh has a lot of single channel data on this.

Oh: We have recorded from DRG neurons. We found that there are two different types of mechanically stimulated ion channels: one type is low threshold, the other is high-threshold. What we found most frequently are both types of mechanosensitive channels. They are rarely in the same cell. But, this type of experiment is not that accurate, so I should be cautious.

Devor: Almost all cells have mechanosensitive channels. They keep cells from swelling too much. So one would have to know if we are talking about that process, cell size regulation, or true mechanosensation. It could be that evolutionarily, channels designed to maintain cell size, which are ubiquitous, have been adapted by sensory neurons for mechanosensation. Whatever the mechanosensing channel might be, you have the blocker for it, ruthenium red. Does ruthenium red have analgesic properties?

Wood: It blocks all TRPs. It is very non-specific.

Devor: Is it a good anti-nociceptive drug?

Reeh: We have applied it to the nerve endings, and it didn't block mechanosensitivity. Nor did gadolinium.

Wood: How did you stimulate the terminus?

Reeh: With von Frey hairs or with an electromagnetic probe. When we apply gadolinium up to millimolar concentration there is no effect. So it is not the gold standard for mechanosensitivity.

Belmonte: We did the same type of experiment, testing gadolinium in nociceptor fibres of the cornea and of the knee joint. We injected up to 5 mM gadolinium into the knee joint and it did not eliminate mechanically evoked impulse responses. In

contrast, in cultured trigeminal ganglion neurons, a very low dose of gadolinium blocked the Ca^{2+} rises evoked by mechanical (hypo-osmotic) stimuli. Thus, gadolinium does not seem to be a good blocker of mechanosensory responses of nociceptor endings.

Reeh: It isn't a good blocker of the native mechanosensory protein complex in the nerve ending. This complex may well contain the monomeric protein that is blocked by gadolinium as long as it is on its own and installed in the DRG cell membrane, but this is not the functional protein we are searching for.

McMahon: It may have actions in the cell soma that are distinct from and not reflected in its effects on transduction processes in peripheral terminals.

Wood: Another possibility is that there are several different mechanisms of mechanosensation. Things like ATP and $P2X_3$ could also play a partial role.

Reeh: We also see these discrepancies in the TRPV1 pharmacology. Ruthenium red is effective in HEK cells, is less effective in DRGs and is completely ineffective in the nerve endings against heat and proton responses. Still, TRPV1 plays an essential role in the nerve ending, but not by itself. Instead, it is combined with other TRPs in a heteromultimeric assembly.

Zhang: It is possible that the different subtypes of receptors and subtypes of the Na^+ channels have different trafficking. Perhaps some channels are inserted in the plasma membrane of the soma, while other channels are transported in the axons and inserted in the plasma membrane of the terminals.

Wood: We discussed earlier how it is likely that there is aberrant expression of the channels in the soma of DRG neurons on acute dissociation in culture. It is quite likely that the distribution of Na^+ channels *in vivo* is quite different.

Belmonte: First, nerve endings and soma cannot automatically be taken as identical functionally. They are probably quite different. Second, we cannot consider cultured neurons that are obtained from newborn animals and kept in very particular growing conditions as the ideal experimental model to study transduction mechanisms present in the intact nerve endings. They are useful for getting a broad idea of what is going on, but compared with intact, adult neurons they are probably expressing different ion channels or the same channels in different proportions, and this may change everything. Also, very often, when we record intracellularly from cultured neurons, we assume that we are seeing activity originating in the soma but actually it may originate at the growing branches. Thus, we need to analyse the information obtained from cultured neurons with great care.

Oh: I have a question about your Cre-lox system. What if channels have multiple genes, not just on one chromosome. Will the Cre-lox system still work then?

Wood: I think the distance between the loxP sites does have a bearing, but we can put them within 2–3 kb and this seems to work very efficiently. Most genes are expressed in single copies. I suppose there is always the process of gene

duplication as part of evolution. This is a danger here that can easily be controlled for.

Dray: You mentioned the use of cytochalasin B as a disruptor of the cytoskeleton. How useful would that be in the kind of experiment that Peter Reeh described?

Wood: The problem with cytochalasin is that it is extremely toxic. You can't do whole animal experiments with it.

Dray: I was thinking of using afferents *in situ*, rather than DRG neurons.

Wood: People have done that using very high doses for short periods.

Dray: Is it a useful tool to try and dissociate different types of mechanical channel?

Wood: Not in a mechanical context. I think John Levine has done some experiments on this.

McMahon: He disrupted the cytoskeleton in sensory neurons and showed that mechanical hyperalgesia was blocked *in vivo* and enhancement of Na^+ currents was blocked *in vitro*.

Ueda: What is the mechanism to activate Na^+ channels *in vivo*? Are G protein-coupled receptors involved, for example?

Wood: We have some evidence that protein kinase inhibitors block this. The difficulty with working with $Na_v1.9$ is that it doesn't seem to be expressed anywhere other than in DRG sensory neurons. If you transfect it, you can't get it to form functional channels. In fact, even if you inject it into sympathetic neurons it still doesn't work. There is clearly some additional co-factor that we don't have yet. Until we do, we won't be able to answer that question.

McMahon: About a year or so ago there was a suggestion that $Na_v1.9$ might be gated by ligands such as BDNF. Do you have any evidence in this regard?

Wood: There was a certain amount of scepticism about this idea, particularly in terms of the kinetics of the response to BDNF, and also the size of the currents and their properties. There is accumulating evidence that wherever persistent TTX-resistant Na^+ channels are seen, $Na_v1.9$ transcripts are present, particularly in the enteric nervous system (e.g. Rugiero et al 2003). So the correlation between persistent current and $Na_v1.9$ is strong, and only one group has reported BDNF gating of this channel (Blum et al 2002).

Reeh: And BDNF receptors are not expressed in primary sensory neurons.

Wood: Some, but not many.

Devor: I would like to broaden the discussion. Your focus has been strictly on nociceptors, including the technology of conditional knockouts of transcripts in nociceptors. Earlier on we heard Jin Mo Chung saying that at least for neuropathic pain — and there are many reasons to extend this also to inflammatory pain — the $A\beta$ cells may be the ones that are important for tactile allodynia. Spontaneous pain, amplified by central sensitization, is also due to $A\beta$ fibres. In chronic pain patients,

the skin becomes abnormally sensitive to heat, but the real clinical problem is the ongoing pain and tactile allodynia. So why invest this tremendous scientific power in nociceptors which may be important for pain when someone steps on your toe, but which for pathological pain might not be very important at all?

Wood: We do have Cre expressing mouse lines in large diameter sensory neurons as well as in nociceptors. There is a lot of hand waving about the role of different subsets of sensory neurons in neuropathic pain, and with these tools we will be able to answer these questions in a very precise way, by deleting genes only in subsets of sensory neurons. I agree with the point that you have made but I think that both nociceptor-specific and large diameter DRG neuron specific Cre mice as well as the adult inducible Cre-recombinase expressing mice will be extremely useful both in basic research and target validation for new analgesic drug targets. These tools will tell you if Aβ fibres are crucial in inflammatory pain or not.

Devor: Is there pain without nociceptors?

Perl: I'm sceptical about proposals that one can eliminate nociceptors and still have pain from peripheral stimulation. The evidence is that nociceptors and non-nociceptive neurons can be altered. The combination of such changes has a relationship to abnormal pain. The evidence for pain being evoked by low threshold mechanoreceptors alone is circumstantial and open to question.

McMahon: It is interesting that it is the mechanically insensitive C fibres that in human seem to be particularly responsible for inducing central sensitization. In the case of central sensitization it is clear that innocuous mechanoreceptors can produce pain, and here the important question may not relate to the particular type of C fibre activated, but to the consequences of C fibre activation in general.

Perl: Much of the evidence hangs on the fact that people have implicated a particular set of neurons in a situation based on conduction velocity or size considerations, and these are imperfect separations. There are smaller afferent fibres that are mechanically sensitive to the same degree as large afferent fibres.

Devor: What do you do with the Torebjork et al (1992) experiment where an intraneural microelectrode in humans stimulates very low threshold, and evokes a tingling buzz appropriate for Aβ fibres. Then, after causing a little bit of inflammation in the skin, the same electrical stimulation in the nerve now causes pain.

Perl: We did a relevant experiment during an early microneurographic study of human single afferent C fibre stimulation (Konietzny et al 1981). It proved necessary to adjust the intensity of stimulation carefully to avoid evoking activity in fibres other than the one under study. An observation, particularly relevant to your question, involved comparing afferent properties of a C fibre to evoked perception. A neural unit noted in the microneurographic recording was excited by innocuous mechanical skin stimulation. When the recording electrode was used to stimulate, the referred skin region for the lowest threshold sensory experience

was totally different than the receptive field established by recording responses to innocuous peripheral stimuli. The referred sensation was painful. It turned out that there were two different fibres involved with different recorded action potential amplitudes. The larger amplitude action potential conducted at C velocity and had distinct nociceptive features. Its peripheral receptive field was identical to that of the referred pain-like sensation. That experience makes me suspicious of experiments that do not carefully investigate the possibility that more than one fibre or class are activated by the stimulus used.

Zhang: In the monkey we have done an experiment that shows that the majority of the sensory neurons in the monkey DRG are small neurons. There are some large DRG neurons. If we label and trace these fibres, almost all the afferent fibres terminate in laminae I and II, only a few afferents are seen in laminae III. In the rat $A\beta$ afferents project to laminae III and IV. Is it possible that in humans and monkeys most of the afferent fibres terminate in laminae I and II?

McMahon: I don't think the evidence supports this. There have been some postmortem studies which have examined CGRP immunoreactivity and it shows the expected concentration in laminae I and II, not III. I don't know of any other data suggesting that human C fibres have central terminals concentrated in lamina III.

Mantyh: Humans look very similar to monkey, rats and mice concerning the termination patterns of primary afferent neurons in lamina I and II of the spinal cord.

References

Blum R, Kafitz KW, Konnerth A 2002 Neurotrophin-evoked depolarization requires the sodium channel $Na_V1.9$. Nature 419:687–693

Konietzny F, Perl ER, Trevino D, Light A, Hensel H 1981 Sensory experiences in man evoked by intraneural electrical stimulation of intact cutaneous afferent fibers. Exp Brain Res 42:219–222

Rugiero F, Mistry M, Sage D et al 2003 Selective expression of a persistent tetrodotoxin-resistant Na^+ current and $Na_V1.9$ subunit in myenteric sensory neurons. J Neurosci 23:2715–2725

Torebjork H, Lundberg L, LaMotte R 1992 Central changes in processing of mechanoreceptive input in capsaicin-induced secondary hyperalgesia in humans. J Physiol 448:765–780

Viana F, de la Pena E, Pecson B, Schmidt RF, Belmonte C 2001 Swelling-activated calcium signalling in cultured mouse primary sensory neurons. Eur J Neurosci 13:722–734

General discussion I

Dray: Dr Tominaga, one of the pieces of data you showed earlier was bradykinin sensitizing the TRPV1 response. Do you have any further characterization of the pharmacology of this response? One assumes that at low concentrations it was a bradykinin B2 receptor. But in conditions of chronic inflammation or neuropathy, there is significant expression of bradykinin B1 receptors. What would be the most important interaction for a sensitization mechanism with respect to TRPV1?

Tominaga: So far we have data about the interaction between TRPV1 and bradykinin B2 receptors. But we have the B1 receptor clone, too. So we should do the experiment, but using the 293 expression of both TRPV1 and the bradykinin B1 receptors.

Mantyh: The group led by Janet Winter recently published a paper on the expression of bradykinin 1 receptors in sensory neurons where she demonstrated that a high level of constitutive expression was further up-regulated in an inflammatory pain state (Fox et al 2003).

Dray: I think that was post-nerve lesion or post-inflammation.

Mantyh: I thought her paper showed that there was expression even before the injury. I always thought that it couldn't be found, but she had shown it was present.

Belmonte: I would like to raise a more general question. During inflammation, to what degree does nerve ending damage lead to axotomy, and thus to what degree can we extrapolate data from neuromas obtained by cutting the nerve to what occurs in the nerve endings of inflamed tissues? In many cases of chronic pain there is sustained inflammation. How similar are the changes in ion channel expression occurring at nerve endings during chronic inflammation to those observed in axotomized neurons?

Perl: Is it not possible that axotomized neurons create conditions for inflammation at the locus of nerve injury and thereby alter the environment of intact nerve fibres?

Belmonte: That was my question.

Perl: Whenever there is injury, the possibility of inflammation exists. Injury activates potent cytokines that influence neural tissue. Pure nerve injury and pure inflammation probably do not exist.

Dray: Should you redefine the question? That is, following an injury there are several sequelae related to the initiation and then the maintenance of chronic pain. There are a number of different mechanisms acting in parallel. There are those

which cause further injury, such as inflammatory mediators and those which are involved in repair and restoration. The important question is what is maintaining chronic pain? Are the mechanisms the same in the earlier phases, e.g. one day to a few days, as those several weeks or months later? It is a critical question if we want to address meaningful therapy. Perhaps we have been misguided because it's easier to study the earlier events.

Mao: I am not that old, but I have seen the pattern that pain research has taken. Some people have focused on the peripheral mechanisms, and others have focused on the central. One might argue that in terms of maintenance on pathological pain, if the central mechanism is somehow established after the initiation process, then the peripheral mechanism could theoretically be irrelevant if the central mechanism can be self-sustained. Obviously, this may not be the case. We know that there is a dynamic interaction between the peripheral and central mechanisms. The key question is, where is this interaction taking place? This is very important.

Devor: You have touched on what I think is a very important principle. To my way of thinking there is an error that has become embedded in the pain literature. That is, that if you have a painful input sustained for a long time, the process becomes 'centralized': something changes in the central nervous system, and now the pain becomes independent of the peripheral sustaining volleys, whether they are from Aβ fibres or from nociceptors. This idea has been around for a long while. I think it began with phantom limb pain — the fact that you can block neuromas in the stump and the phantom pain sometimes doesn't go away. People presumed the pain must have centralized, not realizing that it is necessary also to block ectopic firing in the ganglion before coming to this conclusion. The idea of centralization has also become part of the lore concerning reflex sympathetic dystrophy, with the claim that if you have this condition for a long time then it is almost impossible to get rid of it. This, of course, is a circular argument. It may have been a difficult case from the very beginning, and that is why it did not resolve and became chronic. If I think of other cases of chronic pain such as arthritic hip-joint pain, or rotten teeth, you can have quite severe pain for 20 years but when the peripheral source is removed by joint replacement, or tooth extraction, the pain goes away. I don't know of any cases where the pain has become centralized and the patient feels it for ever. If the peripheral source can be found and is blocked, I think the pain will go away. There are two quite different concepts here: centralization (for which I don't see any solid supporting data) and central sensitization, which is driven in a dynamic manner by a peripheral source. If we put aside the idea of centralization, then this should refocus our interest on the peripheral nervous system. If we can understand and stop the peripheral abnormality, then we will have stopped both the painful peripheral afferent drive and the central amplification process which it triggers and maintains. On the other hand, if we only stop the central process we still have the peripheral process which may remain problematic.

Mao: On this very issue, so far we have been talking about nociception and not pain. In terms of pain, the clinical experience is that we are really talking about a three-dimensional experience. It is not just a sensory discriminative aspect of pain, which is nociception in a sense. Clinical experience of pain also involves a cognitive aspect, an affect aspect and other factors including socio-economic aspects. The whole issue of pain experience is much more complicated than just modulating certain channels.

Perl: Marshall Devor has raised a point that I'd like to support. He brought up the question of whether a symptom should be called chronic pain, when in fact it is really a pathological pain. There are situations in which pain is associated with normal nociception when tissue is damaged and where selective afferent signalling occurs, which could last for one minute or years. That is what may be labelled physiological pain. There is another kind of process in which pain is generated by neural pathology. The ligation of nerves is one example. Central pain is another. In my view, there is little in the form of convincing evidence that the whole process is centralized after one exposure. We need to consider whether the label 'chronic' pain should be substituted by 'pathological' pain.

Dray: I think it is important that we agree on the terminology we intend to uses. To a clinician, 'chronic' pain has a specific meaning. If we are to advocate a common terminology, as you suggest, we need to engage in an aggressive cross-disciplinary dialogue.

Apkarian: Marshall Devor, are you saying that central sensitization does not happen? I assume that this is a phenomenon that has been around for a long time, and results from clinical approaches where fibres have been cut and the pain does not disappear. There are such reports for spinal cord lesions, thalamic lesions, as well as complete amputation of a painful leg, where the pain is unabated. The other part of central sensitization is increasing the gain of the afferent input through central synapses, both in the spinal cord and more cephalad. Also, it seems that there are ample data for potentiation of glutamatergic transmission in the spinal cord following either peripheral inflammation or peripheral neuropathic injury, at least in rodents.

Devor: Let's first put aside central pain: pain that is associated with CNS lesions. This is a whole other bag. I was contrasting the concept of central sensitization, which is rather new, with the concept of centralization, which is older and different. The idea of centralization is that as the pain persists, it changes its properties, its mechanism moves from the peripheral nervous system to the CNS and it becomes intractable. I don't see signs of centralization happening as a result of noxious input—pain itself. I do believe that you might get centralization following nerve injury, which is quite a different matter. Here too, however, I don't see much convincing evidence. No, let's talk about central sensitization. The way we think of central sensitization these days is as a dynamic process which is turned on

by afferent input and can be rapidly turned off again when the afferent input is gone. From my understanding of the central sensitization literature, if one blocks peripheral inputs, for example by an epidural administration of local anaesthetic, the pain goes away at least for the duration of the block.

Dray: How confident are we that this is really so?

Apkarian: I take the position of defining chronic pain by the criteria put forward by International Association for the Study of Pain (IASP) (Merskey & Bogduk 1994). This terminology is rather clear. Chronic pain is defined as pain persistent past the normal healing process. If one talks about chronic inflammatory versus neuropathic pain, then the question is what are the differences between them? Do these chronic pain conditions correspond to differential central sensitization? Our own studies certainly hint in this direction?

Devor: The clinicians can help us here. Are there examples of pains associated with either peripheral inflammation or peripheral nerve injury that cannot be blocked with a good epidural local anaesthetic block?

McMahon: Is this a fair test? If the epidural treatment blocks cord activity as well as sensory activity, and it can, one cannot definitively answer the question.

Devor: OK, now you're saying that centralization may reach the spinal cord but not the rest of the nervous system. So let me rephrase my question and ask whether pain, excluding pain due to direct CNS injury, ever persists despite complete foraminal block. There we probably don't have the clinical data, but they could be obtained. But with epidural blocks we probably do have the data. What is the answer?

Mao: That's true. For most acute pain cases such blocks work well, unless the catheter is not in the right place or the side effects of such a block prevents the medication from taking full effect. If you have an appropriate level of block you should block the pain, or nociceptive transmission. Chronic pain cases are much different. In some cases epidural is used as a treatment method to reduce chronic pain. But there are some cases where even with solid peripheral block the patient may not have complete pain relief. Then again, back to your point, it is not clear whether there is central pain or the pain is initially produced by central mechanisms independent of peripheral mechanisms that could be interrupted with blocks.

Zhou: Based on my experience in Washington University, this is not what we are talking about. I think clinicians also need to learn more about basic mechanisms of pain from basic scientists. It is quite easy to argue that anything seen in mice and rats is not relevant to humans. When we really understand each component, we can forge a new definition. But if we don't open the black box we can spend time guessing what is inside the box.

Apkarian: I do have some concrete examples on this issue: behavioural experiments done in Lebanon by Saade et al (2002) examining neuropathic pain behaviour changes with spinal cord lesions. The results are very interesting.

When they do bilateral dorsal column lesions in chronic constriction injury animals, for a two week period the animals lose their neuropathic behaviour. After two weeks they revert back to the same behaviour. It is a transient recovery. If lesions are performed to interrupt the anterolateral tracts, a similar behavioural time course is seen: namely, an initial recovery from neuropathic pain and a later resumption of pain. This is evidence for centralization in the sense that the specificity of information transmission across different pathways has been lost, coupled with transient recovery.

Devor: I don't think so. You don't have to use Lebanese rats to see that. There is a very long clinical literature on the subject. It makes great sense to do anterolateral column lesions in patients, and it works for a couple of weeks.

Perl: Actually, usually it is reported to be several months.

Devor: The pain almost always comes back. So this is another question: what alternative polysynaptic pathways might open up? But it still doesn't touch the question of where the problematic impulses are coming from. It just says that a new conduction pathway can open up, but the problematic impulses are still probably coming from the periphery or the ganglion.

Mao: Or, if you look at it this way — you have peripheral nerve injury, which ends up with pathological pain, and then you cut the nerve at a different site, causing a new injury. Obviously, one will see the pain coming back because one has produced new neuropathic pain.

Devor: So the solution is not to cut. You use a pharmacological lesion that blocks the impulse traffic but doesn't destroy the nerve.

Perl: Regarding the question of a spinal cord lesion, a few years ago Willis and colleagues pointed out that the spinal dorsal column of several species contains a pathway transmitting noxious afferent activity from the viscera. This is in addition to the well known ventrolateral pathway. I don't think one can partially lesion the spinal cord without leaving open at least one route signalling noxious events. Multiple paths could explain the apparent reappearance of the capacity to recognize pain-causing stimuli after traditional chordotomy.

Oh: I have a question regarding neuropathic pain and sodium channels. John Wood, you said that $Na_V1.3$ is important and is up-regulated once you cut the nerve. There are many ion channels, especially Na^+ channels, that inactivate rapidly. Doesn't $Na_V1.3$ inactivate?

Wood: Yes, but they reprime rapidly and are ready to open again very quickly in response to depolarization. It is fast compared to other Na^+ channels.

Mao: The other way to ask this question is that normal Na^+ channels have a cycle of activation and inactivation and inactivation is necessary for the re-activation, so does your Na^+ channel have the same properties?

Wood: Yes, it is just the temporal nature of this transition. Owing to the structure of the channel it is intrinsically faster to reactivate.

Dray: We heard at least three different possibilities for how nerve excitability can be changed by Na^+ channel regulation. One was increased expression of $Na_v1.3$ in the damaged afferents, increase expression of $Na_v1.8$ in the adjacent undamaged fine afferents, and thirdly a Na^+ channel-driven subthreshold membrane oscillation. Are these mechanisms independently regulated and is regulation condition dependent, i.e. related to a specific type of neuropathy?

Devor: I'd like to say something on this issue. $Na_v1.3$, this very fast activating/inactivating/repriming Na^+ channel, is the basis of the subthreshold oscillations in Aβ neurons. $Na_v1.8$ is not part of the story concerning Aβ fibres: it concerns C nociceptors. I don't think there is any evidence for an appearance of $Na_v1.8$ in Aβ neurons after injury or inflammation. A distinction must be made between Aβ fibres on the one hand, and C fibres/nociceptors on the other hand. This is why I raised earlier the subject of Aβ fibres, and whether they might be very important in pain. Another minor confusion relates to timing. Jin Mo reported that virtually all of the activity in the injured afferents arising from the neuroma or the dorsal root ganglion (DRG) during the early phase is Aβ fibres, the large myelinated fibres, perhaps with some contamination with Aδ fibres. Many labs agree with this. There is very little sign of ongoing C fibre activity during the first days after nerve injury. However, if you now wait a couple of weeks, then you start seeing a lot more ectopic activity generated in C fibres. There is a shift from Aβ fibres being the prominent source of ectopic firing to C fibres becoming more and more prominent. This is a very important transition that has to be kept in mind. The later emergence of ectopic hyperexcitability in C fibres might be related to $Na_v1.8$.

Wood: In $Na_v1.8$ knockouts, ectopic action potentials are lost at 3 weeks in neuromas (Roza et al 2003). This channel is expressed in 60% of Aα/β nociceptors (Djouri et al 2003).

Oh: I have a question about the role of TRPV1 in mediating inflammatory pain or other peripheral pain. To what extent does TRPV1 contribute to inflammatory or other peripheral pain?

Tominaga: I am currently doing an experiment looking at what percentage of pain is mediated by TRPV1. I gave a talk about the involvement of TRPV1 in acute inflammation. As you know, Caterina et al (2000) reported that TRPV1 is not involved in neuropathic pain, but I have to emphasize that after the paper there were several reports indicating a possible involvement of TRPV1 in neuropathic pain. I think we should realize that TRPV1 is involved not only in acute nociception and acute inflammation, but also in neuropathic pain.

Oh: Related to this question about TRPV1 being only for heat, acid sensation, or inflammatory pain, we developed a TRPV1 antagonist and then applied it to animals. Interestingly, it blocks mechanical hyperalgesia induced by inflammation. This clearly suggests that TRPV1 antagonists also reduce

mechanical hyperalgesia. We were surprised to see this. Not only does TRPV1 mediate heat, acid or inflammation-induced pain, it may also be related to mechanical hyperalgesia.

Dray: Another very important symptom in some neuropathic pain states is an abnormal sensitivity to cold stimuli. We have heard very little about cold transduction. I wondered whether there is any evidence linking abnormal functioning of cold receptors such as TRPM8 or ANKTM1 in neuropathic pain.

Belmonte: Our impression at this point is that TRPM8 is probably involved in the transduction of strong cold stimuli, but it may not be so important for the detection of small temperature changes around 32 °C. We think that cold neurons are highly excitable and are depolarized by cooling because temperature reductions decrease a resting, outward potassium current (Viana et al 2002). In cold-insensitive neurons a current called I_{Kd} antagonizes this effect, acting as a break against depolarization by cold. Such current is poorly expressed in cold neurons. We don't know how cold excites polymodal nociceptors. We distinguished two populations of neurons based on their threshold to cold, but we did not find a clear correspondence of the membrane properties of the neurons that had a high threshold to cold with those of polymodal nociceptive neurons. What may happen in polymodal nociceptor endings that respond to cold is that they express a particular mixture of different conductances that are affected by temperature and the net effect is depolarization.

Oh: When we have a pain, such as inflammatory pain, we have a hot sensation. Then we apply menthol, which activates a cold receptor, blocks the hot sensation.

Belmonte: Menthol doesn't produce cold by itself but makes cold neurons more sensitive to cooling. In many cases, menthol does not activate directly a cold neuron; in cold nerve endings, it shifts the stimulus-response curve towards the right, i.e. a given value of nerve impulse frequency is now obtained at a higher temperature value.

Reeh: I have a question directed to the clinicians. Does cold allodynia really exist as a clinical problem in neuropathic pain? The literature is not clear.

Baron: If you look at the most common neuropathic pain states, polyneuropathies and post-herpetic neuralgia, some recent data suggest that up to 10% have cold allodynia. It is a very small number. It might be different in chronic and mechanical nerve lesions. We have data coming out of our network in neuropathic pain indicating that 40% have cold allodynia, but patients with mechanical nerve lesions are not very common, so it is not a big clinical problem, although it is interesting.

Belmonte: Is it a possibility that cold allodynia is mediated by mechanosensory fibres? About 10% of low threshold mechanosensory fibres also respond to cold. This was already observed in some classical electrophysiological studies, where the sensitivity to cold of low threshold mechanoreceptors was first described. This is

the explanation offered by psychophysics to explain why the same object feels heavier if it is cold. Is this because they stimulate mechanosensory fibres?

Baron: But 90% of the patients with post herpetic neuralgia have dynamic mechanic allodynia and only 10% have cold allodynia.

Belmonte: My question is whether in this 10% of patients, cold activates mechanosensory fibres sufficiently to evoke allodynia. It would represent an activation of the same population of low threshold mechanosensory fibres with another modality of stimulus.

References

Caterina MJ, Leffler A, Malmberg AB et al 2000 Impaired nociception and pain sensation in mice lacking the capsaicin receptor. Science 288:306–313

Djouhri L, Fang X, Okuse K, Wood JN, Berry CM, Lawson SN 2003 The TTX-resistant sodium channel $Na_v1.8$ (SNS/PN3): expression and correlation with membrane properties in rat nociceptive primary afferent neurons J Physiol 550:739–752

Fox A, Wotherspoon G, McNair K et al 2003 Regulation and function of spinal and peripheral neuronal B1 bradykinin receptors in inflammatory mechanical hyperalgesia. Pain 104:683–691

Merskey HM, Bogduk N (eds) 1994 Classification of chronic pain, 2nd edn. IASP Press, Seattle

Roza C, Laird JM, Souslova V, Wood JN, Cervero F 2003 The tetrodotoxin-resistant Na^+ channel $Na_v1.8$ is essential for the expression of spontaneous activity in damaged sensory axons of mice. J Physiol 550:921–926

Saade NE, Baliki M, El-Khoury C et al 2002 The role of the dorsal columns in neuropathic behavior: evidence for plasticity and non-specificity. Neuroscience 115:403–413

Viana F, de la Pena E, Belmonte C 2002 Specificity of cold thermotransduction is determined by differential ionic channel expression. Nat Neurosci 5:254–260

Chronic pain and microglia: the role of ATP

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Abstract. Pain following nerve damage is an expression of pathological operation of the nervous system, one hallmark of which is tactile allodynia. We have been studying the role of ATP receptors in pain, and have already reported that activation of the $P2X_{2/3}$ heteromeric channel/receptor in primary sensory neurons causes acutely tactile allodynia. We report here that tactile allodynia under chronic pain states requires an activation of the $P2X_4$ ionotropic ATP receptor and p38 mitogen-activated protein kinase (MAPK) in spinal cord microglia. Two weeks after L5 spinal nerve injury, rats displayed a marked mechanical allodynia. In the rats, activated microglia were detected in the injury side of the dorsal horn where the level of the dually phosphorylated active form of p38MAPK (phospho-p38MAPK) was increased. We performed the double-immunostaining analysis using cell-type specific markers and found that phospho-p38MAPK-positive cells were microglia. Moreover, intraspinal administration of p38MAPK inhibitor, SB203580, suppressed the allodynia. We also found that the expression level of $P2X_4$ was increased strikingly in spinal cord microgila after nerve injury and that pharmacological blockade of $P2X_4$ reversed the allodynia. Intraspinal administration of $P2X_4$ antisense oligodeoxynucleotide (ODN) reduced induction of $P2X_4$ and suppressed tactile allodynia. Taken together our results demonstrate that activation of $P2X_4$ or p38 MAPK in spinal cord microglia is necessary for tactile allodynia following nerve injury.

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Injury of primary sensory neurons produces long-lasting abnormal hypersensitivity to normally innocuous stimuli, a phenomenon known as tactile allodynia (Woolf & Mannion 1999, Scholz & Woolf 2002). Tactile allodynia is the most troublesome of the neuropathic pain syndromes in humans and is nearly always resistant to known treatments such as non-steroidal anti-inflammatory drugs (NSAIDs) or even narcotics (Woolf & Mannion 1999, Scholz & Woolf 2002). The mechanisms by which nerve injury develops tactile allodynia have remained largely unknown.

Several lines of evidence have proposed that induction of tactile allodynia is attributed to central hyperactive states resulting from multiple plastic alterations in dorsal horn neurons as well as spinal glia following nerve injury (Woolf & Mannion 1999, Woolf & Salter 2000, Watkins et al 2001, Scholz & Woolf 2002). The present article introduces our recent study (Tsuda et al 2003, 2004) revealing crucial roles of two molecules — expression and activation of which are highly restricted in microglia in the spinal cord — in transmitting neuropathic pain: p38 mitogen-activated protein kinase (p38MAPK) which is one of four subgroups of the MAPK family, and the $P2X_4$ receptor which is a subtype of ionotropic ATP receptors. These findings suggest the importance of spinal cord microglia in chronic pain.

Experimental procedures

Animals

Male Wistar rats were used in all experiments in accordance with the guidelines of the National Institute of Health Sciences.

Neuropathic pain model

We used the spinal nerve injury model (Kim & Chung 1992) with some modifications: a unilateral L5 spinal nerve of rats was tightly ligated and cut just distal to the ligature. To assess the tactile allodynia, we applied calibrated von Frey filaments (0.4–15.1 g; Stoelting) to the plantar surface of the hindpaw from below the mesh floor. The 50% paw withdrawal threshold (PWT) was determined using the up–down method (Dixon 1980, Chaplan et al 1994).

Immunohistochemistry

The rats were perfused transcardially with phosphate-buffered saline (PBS) followed by 4% paraformaldehyde. Transverse L5 spinal cord sections (30 μm) were incubated in a blocking solution and then incubated in the primary antibody, anti-phospho-p38MAPK (Cell Signaling) or anti-$P2X_4R$ antibody (Alomone). Markers of microglia, OX42 (anti-OX42, Chemicon); astrocytes, glial fibrillary acidic protein (GFAP, anti-GFAP, Boehringer Mannheim); and neurons, NeuN (anti-NeuN, Chemicon) were used to identify the type of cells. Following incubation, tissue sections were washed and incubated in the secondary antibody solution (anti-rabbit IgG-conjugated Alexa FluorTM 488 or anti-mouse IgG-conjugated Alexa FluorTM 546, Molecular Probes).

Western blotting

The spinal cord segments L4–L6 ipsilateral to the nerve injury were homogenized in ice-cold PBS containing a mixture of phosphatase and protease inhibitors. The resulting homogenate (20 μg) was subjected to SDS-PAGE, and the proteins were transferred electrophoretically to nitrocellulose membranes. After blocking, the membranes were incubated with each antibody and then were incubated with HRP-conjugated secondary antibody. The blots were detected using a chemiluminescence method (LumiGLO; Cell Signaling).

Spinal administration of drugs

Rats were implanted with catheters for intrathecal injection according to the method described previously (Yaksh et al 1980). Compounds were injected intrathecally using a 25 μl Hamilton syringe with a 28 gauge needle.

Statistical analysis

Statistical analyses of the results were evaluated using the Student's t-test, the Student's paired t-test or the Mann–Whitney U test.

Results and discussion

p38MAPK is activated in spinal hyperactive microglia after nerve injury

Animals with spinal nerve injury displayed tactile allodynia. PWT (ipsilateral side) to mechanical stimulation significantly decreased at 7 and 14 days (Fig. 1A bottom panel). At day 7 and 14, the OX42 labelling was greater in the dorsal horn ipsilateral to the nerve injury (Fig. 1A top panel). OX42-positive cells were more numerous and displayed hypertrophic morphology in the dorsal horn on the side of the nerve injury as compared with the contralateral side (Tsuda et al 2003). To examine whether p38MAPK is activated in the spinal cord in rats that have developed tactile allodynia, we performed Western blot analysis using an antibody targeting the phosphorylated p38MAPK (phospho-p38MAPK). The band intensity of phospho-p38MAPK protein in the ipsilateral spinal cord dramatically increased 7 and 14 days after nerve injury compared with that in naïve rat (Fig. 1B). Furthermore, we observed strong phospho-p38MAPK immunofluorescence in the injury side of L5 dorsal spinal cord sections at 7 and 14 days after nerve injury (Tsuda et al 2004). The bilateral difference in phospho-p38MAPK levels parallel with the emergence of the tactile allodynia (Fig. 1A bottom panel). These results indicate that the p38MAPK is activated in the dorsal horn ipsilateral to the nerve injury, which may correlate with the nerve injury-induced tactile allodynia. To identify the type of cells in which p38MAPK was phosphorylated after nerve

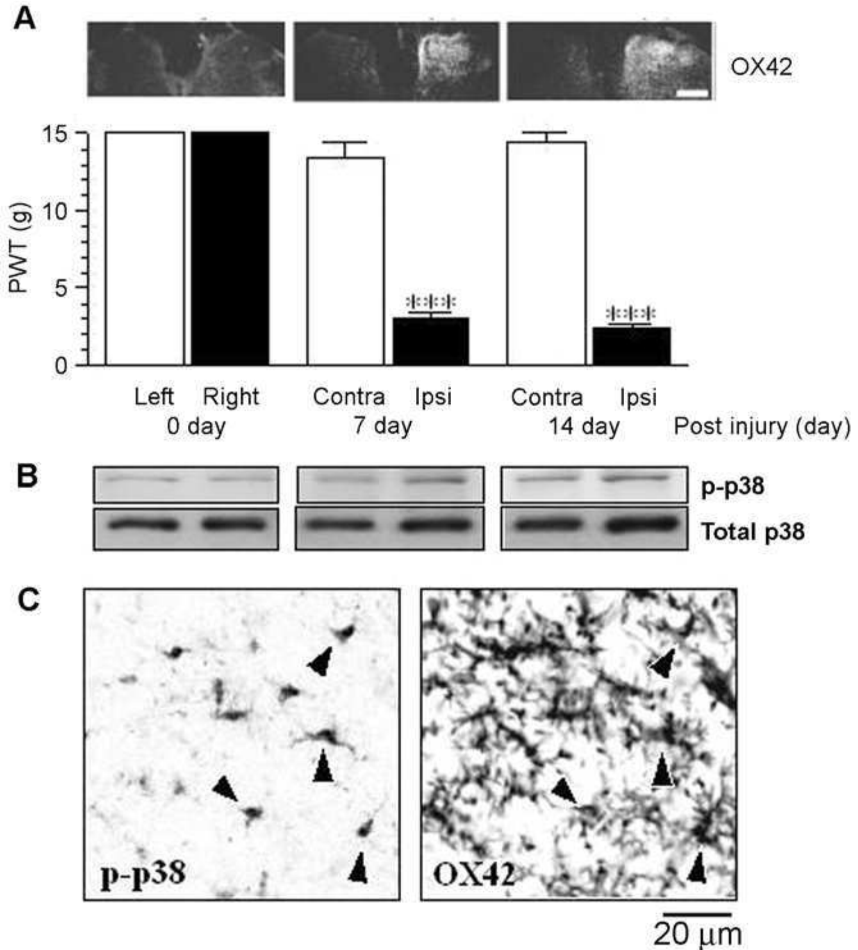

FIG. 1. Activation of p38MAPK in spinal hyperactive microglia after nerve injury. (A) (top panel) Microglia were activated following L5 spinal nerve injury 7 and 14 days after nerve injury. The change of the level of OX42 immunofluorescence following nerve injury was examined in transverse section of L5 dorsal horn. Scale bar, 200 μm. (Bottom panel) The change of PWT (mean $\pm$ SEM) in injury side after nerve injury (***$P < 0.001$ by the Student's paired t-test, compared with the threshold on day 0). Ipsi, ipsilateral; Contra, contralateral. (B) Immunoreactivity of phospho-p38MAPK (p-p38) detected by an antibody for dual-phosphorylated p38MAPK in L4–L6 dorsal spinal cord 7 and 14 days after nerve injury. The total protein from the spinal cord ipsilateral to the nerve injury on day 0 (naïve), 7 and 14 was subjected to Western blot analysis. The proteins of total p38MAPK were detected by an antibody for non-phosphorylated p38MAPK. (C) Double immunofluorescent labels of phospho-p38MAPK (p-p38, arrowheads, left panel) with OX42 (arrowheads, right panel), a marker of microglia was analysed. Scale bars $= 20\ \mu$m.

injury, we carried out double immunolabelling for phospho-p38MAPK and for cell type-specific markers. We found that cells showing phospho-p38MAPK immunofluorescence (Fig. 1C left panel) were double labelled with OX42 (Fig. 1C right panel) but not with NeuN or GFAP (Tsuda et al 2004), indicating that activation of p38MAPK in the dorsal horn is highly restricted to microglia (Tsuda et al 2004). OX42 recognizes the complement receptor type 3 (CR3), expression of which is greatly increased in hyperactive versus resting microglia after nerve injury (Aldskogius & Kozlova 1998, Tsuda et al 2003, 2004). These results indicate that nerve injury induced a switch from the resting to the hyperactive phenotype in the population of microglia in the dorsal horn. We observed a marked phosphorylation of p38MAPK in individual microglia in the ipsilateral dorsal horn (3.7-fold as compared with the contralateral side), particularly in hyperactive microglia that dramatically expressed OX42 (Tsuda et al 2003). Therefore, we conclude that in the dorsal horn following nerve injury hyperactive microglia are the cells that activate p38MAPK, and that the level of p38MAPK phosphorylation is dramatically increased in individual microglia.

p38MAPK activation in the spinal cord is required for development and maintenance of tactile allodynia following peripheral nerve injury

We examined whether intrathecal treatment with a potent inhibitor of p38MAPK, SB203580 alters the maintenance and development of tactile allodynia following nerve injury. Catheterized rats were treated with SB203580 (3 nmol/10 μl, $n=13$) once at day 7 of the nerve injury. SB203580-treated rats displayed a marked increase in PWT following nerve injury (Fig. 2A). When the rats were treated with SB203580 (30 nmol/10 μl, $n=9$) once a day during the 14 days following the nerve injury, SB203580-treated rats showed only a slight decrease in PWT (Fig. 2B). These results suggest that inhibiting spinal p38MAPK activation in microglia by intrathecal treatment with inhibitor for p38MAPK suppresses the maintenance and development of tactile allodynia following spinal nerve injury.

Involvement of the $P2X_4$ receptor in the tactile allodynia

We tested for involvement of P2X receptors in the tactile allodynia by using trinitrophenol (TNP)-ATP, an antagonist of P2X subtypes $P2X_{1-4}$. We found that following intrathecal injection of TNP-ATP (30 nmol) PWT increased gradually, peaked about 45 min after the injection and then returned to the pre-injection level over the subsequent 45 min. PWT at the peak of the effect of TNP-ATP was not different from that prior to nerve injury, and thus, tactile allodynia was reversed by TNP-ATP (30 nmol) on day 7. Intrathecal

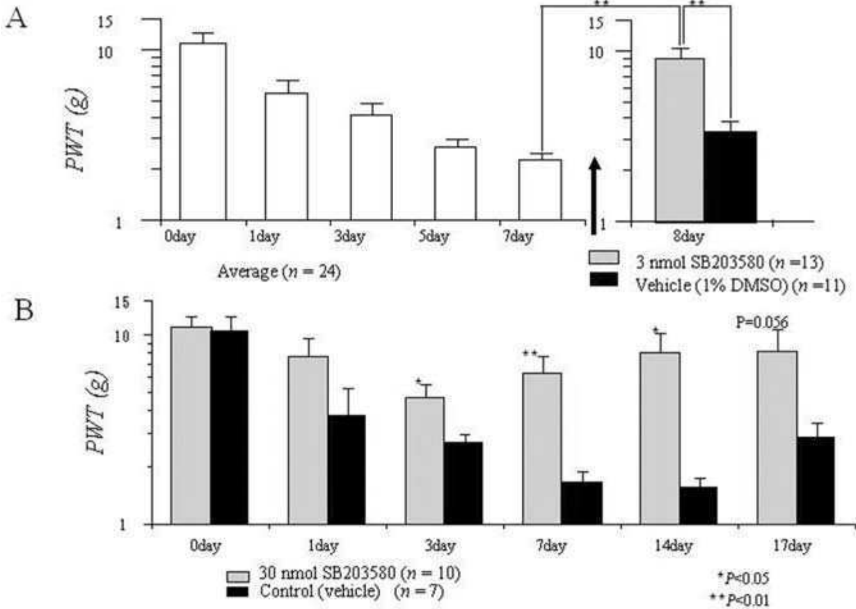

FIG. 2. Intrathecal administration of a potent inhibitor for p38MAPK, SB203580, suppresses tactile allodynia caused by L5 spinal nerve injury. (A) Rats were injected intrathecally once with SB203580 (3 nmol/10 μl, $n=9$) or vehicle (1% DMSO/10 μl, $n=7$) at the 7th day. SB203580 suppressed tactile allodynia. (B) Rats were injected intrathecally with SB203580 (30 nmol/10 μl, $n=9$) or vehicle (2% DMSO/10 μl, $n=7$) once a day for 14 days. PWT of tactile stimulation to the ipsilateral was examined on day 0 (before nerve injury), 1, 3, 7 and 14 at 12–14 h after intrathecal injection. Each data point represents the mean ± SEM of PWT ($^*P<0.05$, $^{**}P<0.01$ by the Mann–Whitney U test, compared with the threshold of vehicle-treated group). SB203580 suppressed the development of nerve injury-induced tactile allodynia.

administration of the vehicle or PPADS (pyridoxal-phosphate-6-azophenyl-2′,4′-disulfonic acid), an antagonist of P2X subtypes $P2X_{1,2,3,5,7}$ but not of $P2X_4$, had no effect on either testing day (Fig. 3A). The increase in PWT by TNP-ATP was dose dependent with the dose producing half-maximal effect calculated as 8.1 nmol on day 7 (Fig. 3B). We observed no alteration in motor behaviour following TNP-ATP administration (data not shown). These results together indicate that TNP-ATP caused a dose-dependent, reversible recovery of PWT on the nerve-injured side without a non-specific effect on motor or sensory functioning. At these intrathecal doses, PPADS is known to suppress nociceptive behaviours caused by intrathecal injection of the $P2X_{1,3}$ agonist α,β-methylene ATP. The lack of effect of PPADS on PWT together with the increase by TNP-ATP indicates that tactile allodynia caused by L5 nerve injury depends upon spinal P2X receptors that are sensitive to TNP-ATP and insensitive to PPADS. The pharmacological profile

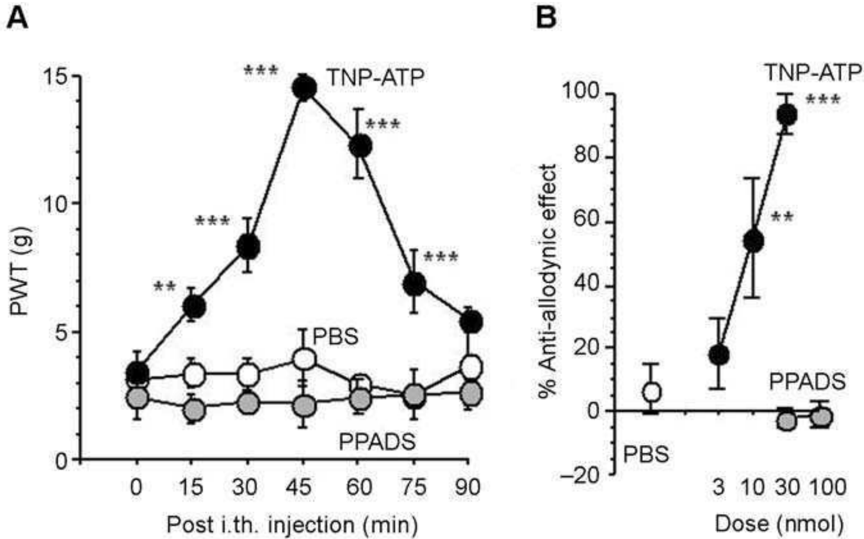

FIG. 3. Intrathecal administration of TNP-ATP but not PPADS reverses tactile allodynia caused by L5 spinal nerve injury. (A) The line graphs show the effects of intrathecal administration of TNP-ATP (30 nmol; black circles) and PPADS (30 nmol; grey circles) on the decrease in PWT 7 days after nerve injury (**$P < 0.01$ and ***$P < 0.001$ vs. PBS-treated group; open circles). (B) Anti-allodynic effect (mean ± SEM) of TNP-ATP 7 days after nerve injury (**$P < 0.01$ and ***$P < 0.001$ vs. PBS-treated group). Anti-allodynic effect (%) = 100× (test value − pre-injection value)/(15.1 g − pre-injection value).

of these P2Xs is consistent with that of the $P2X_4$ subtype and therefore, we further explored the role of $P2X_4$ in tactile allodynia following nerve injury.

$P2X_4$ in spinal microglia is responsible for tactile allodynia following nerve injury

We found that $P2X_4$ protein in the ipsilateral spinal cord increased dramatically after L5 nerve injury (Fig. 4A). The increase in $P2X_4$ was detected as early as day 1 and the highest level was observed on day 14. In contrast, the level of $P2X_4$ protein in the contralateral spinal cord was not different on either day 7 or day 14 as compared with naïve rats. The time-course of the change in $P2X_4$ level in the spinal cord and the bilateral difference in $P2X_4$ levels matched the emergence of the tactile allodynia (Fig. 1A). In order to examine the distribution of $P2X_4$, we performed immunofluorescence on sections of the L5 spinal dorsal horn (Fig. 4B). In the spinal cord ipsilateral to the nerve injury, we observed strong, punctate $P2X_4$ immunofluorescence in the dorsal horn on day 14 (arrowheads, Fig.4Ba–c). To identify the type of cell expressing $P2X_4$ after nerve injury, on

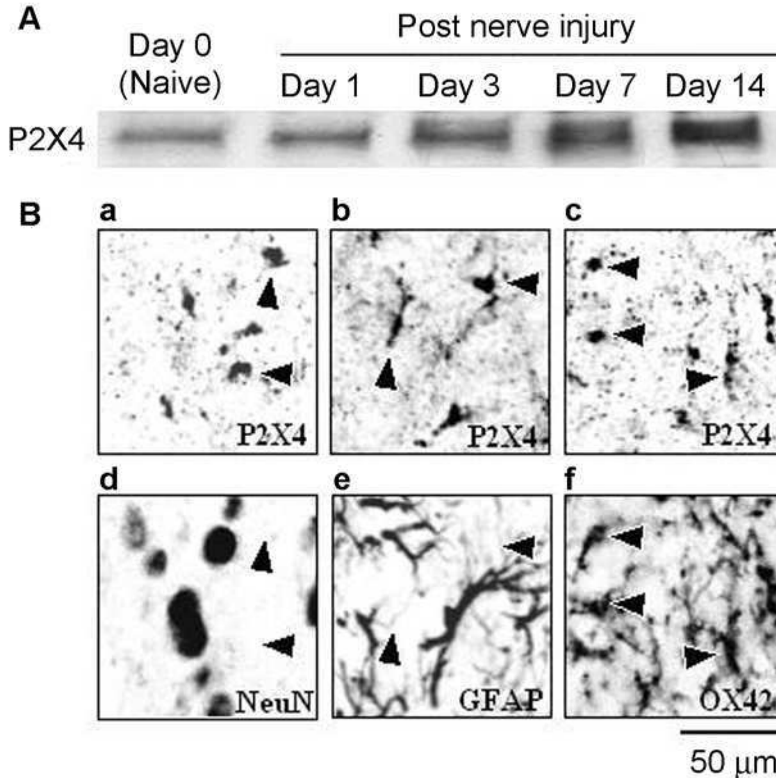

FIG. 4. Up-regulation of $P2X_4$ level in the spinal microglia after L5 nerve injury. (A) Western blot analysis of $P2X_4$ protein detected by $P2X_4$ antibody in the membrane fraction from the spinal cord ipsilateral to the nerve injury at different day. (B) $P2X_4$ was induced in hyperactive microglia but not in neurons or astrocytes. All experiments were done using the spinal cord sections 14 days after nerve injury. Double immunofluorescent labels of $P2X_4$ (a, b, c) with NeuN (d), a marker of neurons, GFAP (e), a marker of astrocytes, and OX42 (f), a marker of microglia. Most $P2X_4$-positive cells (c, arrowheads) are double-labelled with OX42 (f, arrowheads). Scale bars = 50 μm.

day 14 we carried out double immunofluorescence labelling for $P2X_4$ and for cell type-specific markers. We found that cells showing $P2X_4$ immunofluorescence (Fig. 4Ba,b) were not double-labelled for NeuN (Fig. 4Bd) or GFAP (Fig. 4Be). Rather, almost all of $P2X_4$-positive cells (Fig. 4Bc) were double-labelled with OX42 (Fig. 4Bf), indicating that $P2X_4$s were expressed in microglia, but not in neurons or astrocytes. Therefore, we concluded that in the dorsal horn following nerve injury hyperactive microglia are the cell types which express $P2X_4$ and that the level of $P2X_4$ expression is dramatically increased in individual hyperactive microglia.

Next we examined whether tactile allodynia following nerve injury is critically dependent upon functional $P2X_4$ in hyperactive microglia in the dorsal horn. We tested this by means of intrathecal treatment with an antisense ODN targeting $P2X_4$. The nerve injury-induced decrease in PWT was significantly inhibited in animals treated with $P2X_4$ antisense ODN as compared with that in animals treated with mismatch ODN (Tsuda et al 2003). Furthermore, we found that the level of $P2X_4$ protein in homogenates from the spinal cord of antisense ODN-treated rats was $32.0 \pm 4.8\%$ less than that of mismatch ODN-treated rats (Tsuda et al 2003). These results indicate that intrathecal treatment with $P2X_4$ antisense ODN suppressed both the tactile allodynia and the increase in $P2X_4$ expression following nerve injury.

Summary

In the present article we demonstrate that activation of p38MAPK and $P2X_4$ in spinal cord microglia are essential for tactile allodynia following peripheral nerve injury. Tactile allodynia was reversed rapidly by pharmacological blockade of p38MAPK activation or $P2X_4$ receptors implying that nerve injury-induced pain hypersensitivity depends upon ongoing signalling via $P2X_4$ and/or p38MAPK, likely activated by ATP which may be released from primary sensory terminals (Sawynok et al 1993, Li et al 1998, Nakatsuka & Gu 2001), dorsal horn neurons (Sawynok et al 1993, Bardoni et al 1997, Jo & Schlichter 1999) or dorsal horn astrocytes (Fam et al 2000). As a consequence of peripheral nerve injury microglia in the spinal dorsal horn are converted to the hyperactive phenotype and have dramatically expressed $P2X_4$ and activated p38MAPK. Thus, preventing the up-regulation of $P2X_4$ expression and/or inhibiting these receptors or p38MAPK in spinal microglia can be novel therapeutic approaches for treating pain hypersensitivity caused by nerve damage, for which there is currently no effective therapy.

References

Aldskogius H, Kozlova EN 1998 Central neuron-glial and glial-glial interactions following axon injury. Prog Neurobiol 55:1–26

Bardoni R, Goldstein PA, Lee CJ, Gu JG, MacDermott AB 1997 ATP P2X receptors mediate fast synaptic transmission in the dorsal horn of the rat spinal cord. J Neurosci 17:5297–5304

Chaplan SR, Bach FW, Pogrel JW, Chung JM, Yaksh TL 1994 Quantitative assessment of tactile allodynia in the rat paw. J Neurosci Methods 53:55–63

Dixon WJ 1980 Efficient analysis of experimental observations. Annu Rev Pharmacol Toxicol 20:441–462

Fam SR, Gallagher CJ, Salter MW 2000 $P2Y_1$ purinoceptor-mediated Ca^{2+} signaling and Ca^{2+} wave propagation in dorsal spinal cord astrocytes. J Neurosci 20:2800–2808

Jo YH, Schlichter R 1999 Synaptic corelease of ATP and GABA in cultured spinal neurons. Nat Neurosci 2:241–245
Kim SH, Chung JM 1992 An experimental model for peripheral neuropathy produced by segmental spinal nerve ligation in the rat. Pain 50:355–363
Li P, Calejesan AA, Zhuo M 1998 ATP P2X receptors and sensory synaptic transmission between primary afferent fibers and spinal dorsal horn neurons in rats. J Neurophysiol 80:3356–3360
Nakatsuka T, Gu JG 2001 ATP P2X receptor-mediated enhancement of glutamate release and evoked EPSCs in dorsal horn neurons of the rat spinal cord. J Neurosci 21:6522–6531
Sawynok J, Downie JW, Reid AR, Cahill CM, White TD 1993 ATP release from dorsal spinal cord synaptosomes: characterization and neuronal origin. Brain Res 610:32–38
Scholz J, Woolf CJ 2002 Can we conquer pain? Nat Neurosci 5:S1062–S1067
Tsuda M, Shigemoto-Mogami Y, Koizumi S et al 2003 $P2X_4$ receptors induced in spinal microglia gate tactile allodynia after nerve injury. Nature 424:778–783
Tsuda M, Mizokoshi A, Shigemoto-Mogami Y, Koizumi S, Inoue K 2004 Activation of p38 mitogen-activated protein kinase in spinal hyperactive microglia contributes to neuropathic pain hypersensitivity following peripheral nerve injury. Glia 45:89–95
Watkins LR, Milligan ED, Maier SF 2001 Spinal cord glia: new players in pain. Pain 93:201–205
Woolf CJ, Mannion RJ 1999 Neuropathic pain: aetiology, symptoms, mechanisms, and management. Lancet 353:1959–1964
Woolf CJ, Salter MW 2000 Neuronal plasticity: increasing the gain in pain. Science 288:1765–1769
Yaksh TL, Jessell TM, Gamse R, Mudge AW, Leeman SE 1980 Intrathecal morphine inhibits substance P release from mammalian spinal cord *in vivo*. Nature 286:155–157

DISCUSSION

Mantyh: What causes the up-regulation of the $P2X_4$ receptor?

Inoue: We have no evidence yet. We are examining this using a DNA chip.

Mantyh: Do the macrophages which invade the peripheral nerve after injury also express $P2X_4$ receptors?

Inoue: We don't have any data on this.

Baron: Are you suggesting that ATP is leaking from the damaged C fibres in the spinal cord if the C fibres are damaged in the periphery? Do you have any direct evidence for this leakage?

Inoue: We don't have direct evidence. We are trying to find this.

Malmberg: I have a question about the time course of this phenomenon. Your studies look up to 2 weeks after the injury. Have you looked further out, after 5–7 weeks?

Inoue: Microglia activation was weaker at 4 weeks and had vanished 8 weeks after nerve injury.

Malmberg: My question is prompted by the observation that after sham surgery we often see increased sensitivity to thermal and mechanical stimuli one to two weeks after the injury followed by return to normal thresholds. We believe these initial behavioural changes mainly reflect tissue injury or the inflammation

processes. In addition, the pharmacology of mechanical allodynia one to two weeks after the injury appears to be different compared to nerve injured animals showing allodynia at later time points, that is, four or five or more weeks after nerve injury.

Devor: My question has to do with cause and effect. We have heard from Jin Mo that when the spinal nerve is injured, many cells in the ganglion are active. One doesn't even have to talk about the leak of ATP, because there is probably a lot of active release due to spike activity. Is it possible that the actual spike activity, lasting 24 h, is enough to activate microglia? Has anyone tried electrically stimulating a nerve without any injury, to see whether this would activate the microglia? Has anyone tried injuring the nerve and then using TTX or local anaesthetic to block the ectopic activity, to see whether that would prevent the activation of the glia? Perhaps more importantly, all the drugs you are injecting intrathecally and believe are acting directly on the microglia also have access to the ganglion. I wonder whether they might not be simply turning off the abnormal activity generated from the ganglia.

Inoue: After intrathecal administration, does the drug act on the DRG?

Devor: Yes.

Dray: But I thought the expression of $P2X_4$ receptors was only in microglia, not in astrocytes and not in the satellite cells, and that the antagonist does not affect microglial activation.

Inoue: $P2X_4$ up-regulation was only seen in spinal microglia. We think that the main target of the blocker injected (intrathecally) is the spinal macroglia.

Dray: In the experiment where you exposed the spinal cord cultures to microglia plus ATP, the result was a behavioural pain hypersensitivity which was blocked with TNP-ATP but not PPADS. You interpreted this as ATP-induced activation of microglial $P2X_4$ receptors and the release of excitatory mediators. Normally ATP induces spinal hypersensitivity that can be blocked by PPADS. Can you explain how the exogenous microglia were treated to express a $P2X_4$-predominant pharmacology?

Oh: Did you ever check whether satellite cells or some other types of cells in DRG increased the expression of $P2X_4$ receptors?

Inoue: We did not check whether the satellite cells or DRG neurons up-regulated $P2X_4$ receptors after nerve injury.

Perl: When I am confronted by phenomena which manifest one week after peripheral nerve injury, reach a peak in two weeks and then decline, it brings to mind denervation supersensitivity. You have produced an injury to a nerve. Is it possible that an up-regulation of receptors or some similar change in signalling properties that peaked after two weeks has taken place?

Inoue: Our findings suggest the importance of $P2X_4$ in the spinal microglia for the allodynia after nerve injury. There might be so many factors causing

and maintaining the allodynia. We, however, have no evidence to discuss the relationship between these factors. We are now examining other factors and mechanisms before and after $P2X_4$-activation of microglia. $P2X_4$ is a new player in neuropathic pain, and I think many interesting findings will come soon.

Wood: It was very striking that this compound A317491, which is $P2X_{2/3}$ specific, blocks mechanical allodynia. Does it have any effect on $P2X_4$? Also, you showed that aconitide, which blocks $P2X_3$, also produces an effect? Does this affect $P2X_4$?

Inoue: Since PPADS blockade of $P2X_{2/3}$ did not affect $P2X_4$-dependent allodynia after intrathecal injection, I think that A317491 administered the same way wouldn't have effects on the allodynia in this neuropathic pain model.

Wood: There are interleukin (IL)6 knockout mice and there are neutralizing monoclonal antibodies to tumour necrosis factor (TNF). Do you have any idea what is happening downstream of the activating microglia? Is this mediated through these kinds of cytokines?

Inoue: We found recently that ATP stimulation causes IL6 and TNFα release, and that TNFα stimulation of neurons causes IL6 receptor expression after 4 h. These data suggest that a part of the downstream effect of the stimulation of $P2X_4$ in spinal microglia may be mediated through cytokines.

McMahon: I want to comment on Dr Devor's question about whether you need spike activity to activate microglia. The early literature on microglial activation clearly showed spinal activation in motoneuron pools after peripheral nerve injury, starting in less than a day. In these circumstances, it seems extremely unlikely that one would need nerve activity. Of course, whether or not activity is sufficient is an open question.

Devor: Is that the same phenomenon as the dorsal horn picture that we just saw?

McMahon: I think so. In your data, the strongest activation is around motor neurons first and then in the dorsal horn.

Dray: I am just trying to understand these important experiments again. Peripheral nerve lesions cause activation of spinal microglia and the expression of $P2X_4$ receptors. If you block the receptor expression with antisense, the microglia stay activated, but allodynia is attenuated. A number of other microglial receptors have been shown to be up-regulated (e.g. chemokine receptors, cannabinoid receptors) and can respond to their ligands in the spinal milieu. I find it difficult to understand why blocking only $P2X_4$ receptors can affect behaviour in such a profound way and yet still leave the microglia in a state of activation.

Inoue: I think that activated microglia are not enough to cause allodynia.

Dray: But you administered the antisense, tested for behaviour, and according to the OX43 expression the microglia were still in a state of activation.

Inoue: I meant that active substances released from the microglia by $P2X_4$ stimulation will cause the allodynia. Our data suggest that the microglia of the activated form need $P2X_4$ stimulation to cause allodynia through the release of factors such as cytokines. It may cause synaptic transmission enhancement in pain sensation.

Zhou: Do we know that cytokines actually cause long-term enhancement of synaptic transmission in spinal dorsal horn sensory synapses?

Inoue: Not yet. This is just an assumption.

Gintzler: Andy Dray, you would like to block some of these other receptors and show the absence of an effect.

Dray: I guess what I am struggling with is whether the microglia rather than other neuroglial cell types have some kind of pivotal role in neuropathic pain. Your experiment shows the critical importance of microglia, ATP and $P2X_4$ receptors in neuropathic pain behaviour.

Gintzler: Why is that troubling?

Dray: The fact that the microglia are still in an activated state and presumably susceptible to non-ATP influences when pain behaviour is absent.

Inoue: Our data don't exclude the involvement of other factors. As you mention, activated microglia express many different receptors, including $P2X_4$. TNP-ATP blocks the allodynia causes by nerve injury in this model. We don't have any direct evidence to explain why $P2X_4$ blocker totally inhibited the allodynia though microglia are staying in activated form. If many factors do not act independently (in parallel) to cause the pain but cause the pain in a series of reactions, or if the main actions of these factors are related to $P2X_4$ function, we can accept the data that $P2X_4$ blockers completely inhibit the allodynia.

Wood: There is a chemokine receptor 2 knockout mouse which has lost all mechanical allodynia. This strongly supports the involvement of microglia.

Mantyh: I believe they also saw that the macrophages in the peripheral nerve expressed the chemokine receptor 2 too.

Inoue: If any of you have good tools or models concerning the effects of TNP-ATP, please let me know.

Neurotrophic influences on neuropathic pain

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Abstract. Damage to peripheral nerves following trauma or disease has a number of consequences including the emergence of neuropathic pain. Commonly, neuropathic pain sufferers experience spontaneous burning pain in and radiating from the area innervated by the damaged nerves, and an exquisite sensitivity to light touch stimuli, which are now perceived as painful. These neuropathic pains are often refractory to conventional analgesic therapy, with most patients obtaining at best only partial relief. Unfortunately, neuropathic pains are frequently also very persistent and do not resolve with time. Thus, neuropathic pain is often an extremely debilitating condition with a bleak outlook. In this paper we review the pathophysiological mechanisms that underlie these neuropathic pain states with particular emphasis on the therapeutic role of neurotrophic factors.

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The International Association for the Study of Pain defines Neuropathic pain as 'Pain initiated or caused by a primary lesion or dysfunction in the nervous system' (Merskey & Bogduk 1994). Clearly, this is a very broad definition and gives very little insight into the neurobiological mechanisms of such pains. Commonly, neuropathic pain sufferers experience spontaneous burning pain within and radiating from the area innervated by the damaged nerves, and many report an exquisite sensitivity to light touch stimuli, which are now perceived as painful — a condition known as allodynia. These neuropathic pains are often refractory to conventional analgesic therapy, with most patients obtaining only partial relief. Unfortunately, neuropathic pains are commonly very persistent and do not resolve with time. Thus, neuropathic pain is often an extremely debilitating condition with a bleak outlook.

The diverse causes of neuropathic pain also offer only limited mechanistic understanding. Perhaps the most common form of neuropathic pain is that associated with metabolic abnormality, notably diabetes. Many diabetics, especially those with poor blood sugar control, ultimately develop a distal

symmetrical and painful neuropathy that initially affects the longest peripheral axons, but with time spreads proximally. Another large and growing group of neuropathic patients have pain secondary to infection. Many AIDS sufferers, perhaps up to 50%, develop painful neuropathy similar to those seen in diabetics. The incidence of HIV-induced neuropathy is increasing with improvements in antiretroviral drug therapy (Moyle & Sadler 1998) partly because patients are living longer with the disease. The pain experienced in the wake of an attack of Herpes Zoster is another example of neuropathic pain and the intensity and persistence of post-herpetic neuralgia increase with age. A third important cause of neuropathic pain is iatrogenic, as a side-effect of several drug treatments, including some anticancer drugs (where neuropathy may be the dose-limiting factor) and some of the drugs currently used to treat HIV infection. A further cause of neuropathic pain is that associated with traumatic nerve injuries. While such injuries are not very common in peacetime, the advent of high velocity firearms means gunshot wounds have added significantly to the burden of neuropathic pain around the world. All of the above causes are associated with damage or disease to the peripheral nervous system. However, neuropathic pain can be associated with damage to central structures. The clearest examples are pains associated with spinal cord injury and pains associated with vascular lesions of the thalamus. It seems unlikely that neuropathic pain of central and peripheral origin has a common underlying mechanism, but it is also unclear whether there are multiple contributing mechanisms of neuropathic pain of peripheral origin. This is of considerable practical as well as academic interest, since, as we will review below, most of our understanding of mechanisms arises from the study of a very limited type of animal model.

In total, neuropathic pain is likely to affect some 1.8 million people in the USA and for only a few of these (e.g. those with pain associated with carpal tunnel syndrome) is there a straightforward and effective treatment.

Animal models of neuropathic pain

Many of the disease states causing neuropathic pain can be modelled in animals. For instance, there are reasonably well-characterized models of diabetic neuropathy and several animal models of neuropathies associated with anticancer drug treatments. There have also been attempts to induce HIV neuropathy in rats (Milligan et al 2001), and several reports of abnormal pain sensitivity after experimental spinal cord injury in rats (e.g. see Yezierski & Burchiel 2002). However, for reasons of reproducibility and simplicity, most studies of neuropathic pain use traumatic nerve injury, usually in rodents. One important caveat, as previously mentioned, is that it is not clear whether understanding derived from the study of such models will be applicable to neuropathic pain

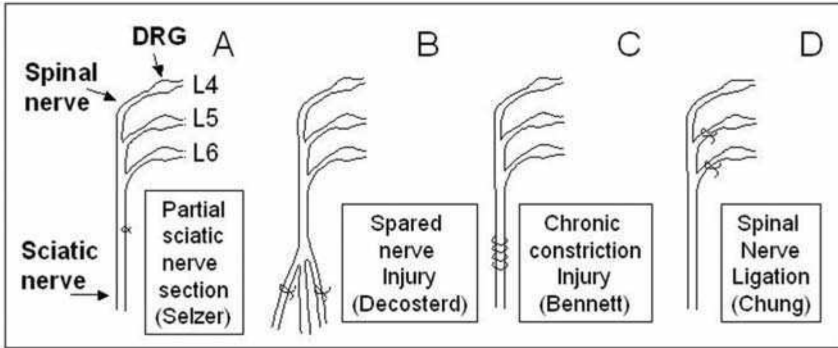

FIG. 1. Schematic illustration of different experimental models of neuropathic pain. In each case the sciatic nerve and its projection through dorsal root ganglia are shown. (A) A portion, typically about 50% of the sciatic nerve is tightly ligated (from Seltzer et al 1990). (B) Ligatures are loosely tied around the sciatic nerve (from Bennett et al 1988). (C) One or more branches of the sciatic nerve are tightly ligated and cut (from Deosterd & Woolf 2000). (D) One or more spinal nerves are ligated and cut (from Kim & Chung 1992). See text for more details.

associated with other causes. There are several such models in fairly common use, and the nature of the nerve lesions is illustrated in Fig. 1. The different models share the common feature of degeneration of some but not all sensory fibres in a major peripheral nerve, so that a peripheral target is partially denervated and conversely, partially innervated. One model introduced by Selzter et al (1990) involves partial nerve ligation (Fig. 1A). Typically one-third to one-half of the sciatic nerve is tightly ligated with a silk suture. Since sensory fibres in the sciatic nerve exhibit considerable mixing as they travel distally, this procedure does not result in total denervation of a confined area, but a partial denervation throughout much of the sciatic innervation territory. A more recent derivation of this approach is to tie off one or more of the branches of the sciatic nerve (Doesterd & Woolf 2000, Fig. 1B). The damaged sensory fibres do innervate a more restricted area in this case, but because of the overlap of nerve territories, there are broader zones of partial innervation which exhibit neuropathic signs (i.e. altered sensibility to sensory stimuli). A third extensively used model is that of chronic nerve constriction (Fig. 1C, Bennett & Xie 1988). Here several chromic ligatures are loosely tied around the sciatic nerve at mid-thigh level. The sutures are only tight enough to partially restrict blood flow in superficial vessels in the nerve. But the nerve swells and a marked constriction results. Anatomical studies (Coggeshall et al 1993) show that a substantial fraction but not all fibres undergo Wallerian degeneration distal to the ligation site. This model appears to have a greater inflammatory component than the others. The presence particularly of chromic suture material may exacerbate the inflammatory response (Maves et al 1993). As we review below, the liberation of cytokines or other factors (e.g. nerve growth factor) from

immunocytes at the site of constriction is also likely to contribute to neuropathic symptoms.

The most commonly used model today involves the ligation of one or two spinal nerves (usually L5 or L5 and L6), just distal to the dorsal root ganglion (DRG) (Fig. 1D, Kim & Chung 1992). Since the sciatic nerve carries large numbers of sensory fibres from the L4 and L5 spinal nerves (and smaller numbers from the L6 nerve), this lesion results in degeneration of about 50% of the fibres in the sciatic nerve, and these project throughout the normal sciatic innervation territory. One advantage of this model for mechanistic studies is that in a particular DRG virtually all sensory neuron cell bodies are either axotomized or intact. This contrasts with other models, where the cell bodies of injured and uninjured neurons are mixed together in one or more DRGs.

In all of these animal models pronounced sensory changes are seen, similar to those observed in many neuropathic pain patients. Thus, neuropathic animals will guard and avoid weight-bearing on the affected paw, consistent with the existence of some ongoing pain. Animals also show escape behaviour to very light tactile stimulation of the paw, indicating allodynia. Cold stimuli also trigger greatly exaggerated responses, and this state is frequently referred to as cold allodynia. In response to tests of noxious heating, these models also show increased sensitivity, but this thermal hyperalgesia is usually quite modest. An example of the degree and time-course of these sensory changes seen in one group of animals subjected to spinal nerve ligation lesions (as in Fig. 1D) is shown in Fig. 2. Note that mechanical allodynia emerges very rapidly, and is fully developed within one or two days. It seems quite possible that the mechanical allodynia and thermal hyperalgesia may have different underlying causes. For instance, treating neuropathic animals with the C-fibre neurotoxin resiniferatoxin reportedly abolishes the thermal hyperalgesia whilst leaving mechanical allodynia (Ossipov et al 1999).

Traumatic injury models of neuropathic pain have been used in many different studies aimed at elucidating the factor or factors that might contribute to the emergence of neuropathic symptoms. These studies have demonstrated a series of pathophysiological reactions to the injuries that sweep forward from the site of the injury, involving considerable changes in gene expression in the cell bodies of sensory neurons in DRG and also marked alterations in the central processing of sensory information, particularly within the dorsal horn of the spinal cord. These changes are reviewed below.

Which sensory neurons are responsible for neuropathic pain behaviour?

In the traumatic nerve injury models described above, one common feature is that some but not all of the sensory axons running in a major peripheral nerve are

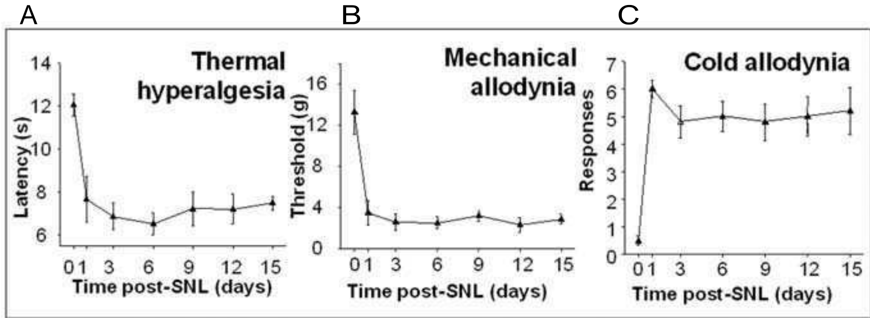

FIG. 2. Typical sensory changes seen in neuropathic pain models. Here the responses of rats were studied before and after an L5 spinal nerve ligation (SNL). (A) and (B) show changes in the mechanical and thermal threshold, respectively, necessary to elicit withdrawal reflexes. (C) Shows the number of paw flinches on exposure to cold stimulus of about 1 °C for 30 seconds (T. J. Boucher, S. B. McMahon, unpublished data).

axotomized. The distal portions of those damaged axons begin very rapidly to undergo Wallerian degeneration. The axotomized proximal stumps cannot regenerate in these models, because they are trapped at the site of a nerve ligation. These fibres form a distinct functional group that have lost contact with the peripheral targets that they normally innervate, and consequently any target-derived trophic factors that are normally provided to them (for instance, NGF, see below).

However, there is a second functionally distinct group of sensory neurons. These are intact neurons, 'spared' from injury, but running in the same peripheral nerves. These 'spared' sensory neurons have axons running through an area of Wallerian degeneration and may be subject to the altered chemical environment of the degenerating nerve and alterations in target-derived factors (see below).

The existence of two such groups of sensory neurons can plausibly be suggested for all neuropathic pain states associated with peripheral nerve injuries. In diabetic neuropathy (and in several other causes of neuropathic pain) the nerve damage may be very distal and some axons may only die back relatively modest distances from peripheral targets. Nonetheless, some neurons may be injured and disconnected from their normal innervation fields, while others are intact but present amongst degenerating fibres.

In one traumatic model — that produced by spinal nerve ligation (Fig. 1D) — there is considerable anatomical separation of these two subgroups, because all neurons of one dorsal root ganglion and dorsal root, will be either injured or 'spared'. The anatomical advantage of this model has permitted a number of experiments aimed at identifying the contribution of intact versus 'spared' neurons in the evolution of neuropathic pain. Surprisingly, perhaps, because the

experiment would seem rather straightforward, there is a great deal of controversy about the result. Cutting the L5 dorsal root (after L5 spinal nerve ligation) prevents activity from reaching the CNS from the damaged neurons in the L5 dorsal root ganglion (DRG), and is reported to abolish signs of neuropathic pain by some (Sheen & Chung 1993, Yoon et al 1996, Sukhotinsky et al 2004), but not all (Li et al 2000) groups.

These studies are further complicated by reports that dorsal rhizotomy (i.e. a section of dorsal roots) may itself produce behavioural hyperalgesia (Colburn et al 1999, Li et al 2000), and so it might be that this lesion abolishes one form of neuropathic pain behaviour only to be replaced with another. The infusion of local anaesthetic onto the L5 DRG (after L5 spinal nerve ligation) blocks sensory nerve activity and is reported to reverse neuropathic pain behaviour (Sukhotinsky et al 2004).

Li et al (2000) reported on a number of apparently well-controlled behavioural studies of neuropathic pain generated by spinal nerve ligation. They found that signs of neuropathic pain were selectively abolished when sensory activity in 'spared' afferents was prevented from reaching the cord. They also reported that interrupting signalling from injured axons was without effect. These authors have suggested that influences arising from Wallerian degeneration are crucial in altering responsiveness of 'spared' neurons. In support, the same group have reported that ventral rhizotomy (which damages motoneurons but also causes Wallerian degeneration in the sciatic nerve) also leads to neuropathic pain behaviour.

The reasons for the discrepancies in these reports are not obvious. They do not seem related to animal strain, time points studied or other simple methodological variables. One interpretation of these data is that it would be prudent to consider both these groups of sensory neurons as potentially important contributors to neuropathic pain.

Changes in peripheral sensory neurons in neuropathy models

A logical question to ask is 'What aspect or consequence of nerve injury is important for neuropathic pain?' There is now good evidence to suggest that post-injury sequelae are dictated by at least two principal processes. The first is an alteration in the availability of target derived neurotrophic factors, and the second is the generation of injury-induced factors, such as cytokines and chemokines. The former constitutes a 'negative' signal to some sensory neurons. That is, a factor normally supplied to some sensory neurons is lost or diminished. But both the former and the latter can be 'positive' signals to some neurons — becoming available *de novo* or at increased levels. Both these groups of factors are responsible for inducing very marked changes in gene expression in sensory

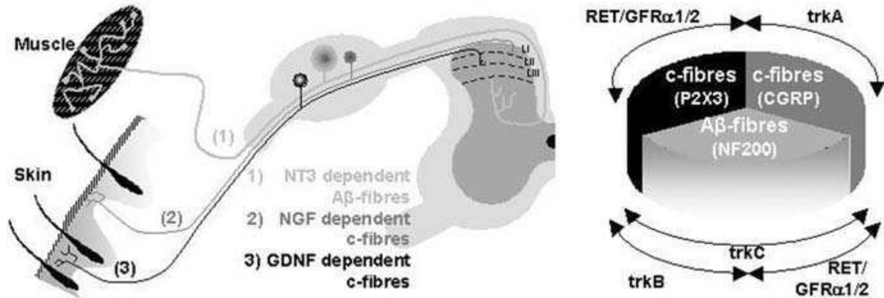

FIG. 3. Neurotrophic factor dependency of sensory neurons. The left hand side of the figure illustrates three principal subgroups of primary sensory neurons. These are: (1) large diameter, mainly innocuous mechanoreceptors innervating skin and muscle, nearly all of which are sensitive to NT3; (2) about one-half of the small diameter, mainly nociceptive, neurons are sensitive to NGF; (3) the other half of the small diameter population, also nearly all nociceptors, are sensitive to GDNF. The right hand side shows the relative proportion of these subgroups and other phenotypic properties of the neurons.

neurons, which in turn lead to the emergence of abnormal electrical activity, known to be essential for the manifestation of neuropathic pain behaviour. We will consider each of these changes in turn.

Trophic factor availability

Sensory neurons depend upon limited amounts of neurotrophic factors produced by target tissues during development to maintain an appropriate peripheral innervation. Expression of high affinity neurotrophic factor receptors by functionally distinct sub-populations of sensory neurons ensures physiological connectivity (see Fig. 3). While expression levels of neurotrophic factors are maintained into adulthood, albeit at a low level, these factors are not necessary to maintain the survival of sensory neurons. Nonetheless, they can exert very profound effects on sensory systems. For instance, there is now substantial evidence that highlights the pro-nociceptive role of the proto-typical neurotrophic factor, nerve growth factor (NGF) (see McMahon & Bennett 1999).

Damaged sensory fibres. CX Disconnection of damaged sensory axons from peripheral targets interrupts the retrograde trophic support these neurons normally receive from peripheral targets (Heumann 1987, Raivich 1991). There is now good evidence for a greatly reduced retrograde transport of at least three important trophic factors in damaged sensory axons. These are NGF, which normally supports about one-half of the small diameter (very largely nociceptive) sensory neurons; NT3, which supports most large diameter (mostly

mechanosensitive) sensory neurons; and glial cell-derived neurotrophic factor (GDNF), which supports the other half of small sensory neurons as well as a subgroup of some large neurons (see Fig. 3). The loss of retrograde supply of these factors to the cell bodies of sensory neurons causes dramatic alterations in the expression of neuropeptides, ion channels and receptors (see below). Many studies have shown that exogenous delivery of appropriate neurotrophic factors rescues or ameliorates many of these changes.

After nerve injury, there is increased expression of NGF and GDNF by Schwann cells distal to the injury site in areas undergoing Wallerian degeneration (Heumann 1987, Herzberg et al 1997, Naveilhan et al 1997). However, these factors do not appear to be available to the proximally damaged axons, or at least not available in sufficient amounts to compensate for the lost target-derived supply. After peripheral axotomy, there is also increased expression of NGF and NT3 in satellite cells surrounding sensory neuron cell bodies in dorsal root ganglia (DRG) (Zhou et al 2000). While it remains an open and intriguing question of what signal is responsible for this change, it too does not appear to be sufficient to substitute for lost target-derived supplies. (It may, however, be sufficient to trigger the sprouting of sympathetic fibres within the DRG after peripheral nerve injury and in this way contribute to neuropathic pain — see Ramer et al 1999.) Direct measurement of NGF protein levels in DRG after nerve injury confirms the net reduction in bioavailability of this factor.

Other members of the neurotrophin family have been shown to be key modulators in the maintenance of neuropathic pain and therefore remain intriguing therapeutic targets. Brain-derived neurotrophic factor (BDNF) appears an important target-derived factor for many placode-derived sensory neurons, such as vagal afferents innervating visceral structures. However, for the neural crest derived sensory neurons of the DRG, this role seems less important. Unlike NGF, BDNF is synthesized by sensory neurons themselves (Ernfors et al 1990, 1993) and its expression is subject to alteration after nerve injury (Michael et al 1997). Both L5 spinal nerve ligation (Fukuoka et al 2001) and chronic constriction nerve injury (CCI) (Obata et al 2003) precipitate a net loss in BDNF expression levels in small diameter TrkA-expressing neurons, presumably due to a loss of target-derived NGF. BDNF is thought to modulate sensory processing via its accumulation (Michael et al 1997) and subsequent release with activity from primary afferent terminals in the dorsal horn (Lever et al 2001). Several studies have illustrated that sequestering centrally released BDNF can attenuate behavioural signs of neuropathic pain (Theodosiou et al 1999, Yajima et al 2002). After partial nerve injury, intact or 'spared' neurons face less competition for target-derived factors owing to partial target denervation. Expression of BDNF is up-regulated in 'spared' DRG neurons in L4 after L5 spinal nerve ligation and after chronic constriction injury of the sciatic nerve (Fig. 1). Not all of these

'spared' neurons express TrkA and therefore alteration in BDNF expression cannot be due entirely to increased availability of NGF. This suggests a role for a currently unknown injury induced factor.

Neurotrophin 3 (NT3), another target-derived neurotrophic factor, maintains the adult phenotype of large diameter myelinated mechanoreceptors. These cells express the high affinity TrkC receptor (Fig. 3) and are subject to modification after peripheral nerve injury owing in part to their loss of target-derived support. Treatment of damaged nerves with exogenous NT3 has been shown to ameliorate some of these changes (Ohara et al 1995, Munson et al 1997). Despite the observation that nerve injury induces the expression of NT3 mRNA within satellite cells in the DRG (Zhou et al 1999), application of NT3 antisera and or TrkC fusion proteins failed to elicit a profound alteration in pain thresholds after L5 spinal nerve ligation (Zhou et al 2000, Deng et al 2000), thereby highlighting a more subtle role of NT3 as a possible therapy for neuropathic pain.

Another neurotrophic factor, structurally unrelated to the neurotrophins, is GDNF (see Fig. 3). This supports the survival of the non-peptidergic small diameter nociceptive C-fibres (Naveilhan et al 1997). As is the case with NGF and NT3, nerve injury-induced interruption of target derived GDNF alters the expression of neuromodulators and receptors within the cell bodies of damaged sensory neurons, alterations that can be reversed by the exogenous delivery of GDNF (Bennett et al 1998, Cummins et al 2000, Boucher et al 2000). Together these data clearly show that interruption of target-derived trophic support precipitates many of the phenotypic changes seen in sensory neurons after nerve injury. However, experimental approaches that aim to replenish neurotrophic support to neurons disconnected from their target may fail to address additional maladaptive consequences that neurotrophic support can have on intact neurons.

Intact sensory fibres. Few experimental models of nerve injury fully transect an entire nerve fascicule; therefore, many intact fibres are closely opposed to injured fibres and consequently share the same environmental consequences of nerve injury. There are two ways in which these 'spared' sensory neurons may be exposed to increased levels of neurotrophic factors. First, the peripheral targets they innervate are partially denervated. Since the expression of target-derived factors does not seem to depend on innervation density, 'spared' fibres will have fewer others to compete with for these factors. Second, Schwann cells reacting to Wallerian degeneration and other cells invading the nerve as a part of the process start to express several of the factors normally expressed by peripheral targets. The best examples are NGF and GDNF. Spared axons running through this environment are exposed to these factors and we have direct and indirect evidence that the net result is an increased retrograde supply of some factors to the cell bodies of spared axons. Fukuoka et al (2001) measured NGF protein

levels in the L4 DRG after a spinal nerve ligation of L5. They found a progressive increase in NGF protein in this ganglion, but not in the (axotomised) L5 DRG. There have also been several studies on the expression of specific genes known to be regulated by NGF and GDNF. These studies (see below) report changes consistent with increased NGF and GDNF levels in these spared neurons.

It is important to consider the likely consequences of increased trophic factor supply to 'spared' afferents. NGF has a potent algogenic effect on intact TrkA-expressing sensory neurons, causing robust thermal and mechanical hyperalgesia within hours of systemic administration (Lewin et al 1993). Local administration of NGF sensitizes cutaneous nociceptors to thermal and mechanical stimuli via direct action on the afferent fibres, but also via an indirect action on resident non-neuronal cellular elements, such as mast cells. Mast cells also express TrkA (Horigome et al 1993) and in response to NGF proliferate, degranulate and release inflammatory mediators such as interleukin (IL)10, serotonin (5HT) and tumour necrosis factor (TNF)α (Woolf et al 1995). Furthermore, the delivery of function blocking molecules has demonstrated that NGF contributes to abnormal pain sensitivity in several animal models (e.g. McMahon et al 1995).

As previously mentioned, NGF stimulates expression of BDNF in small-diameter peptidergic C-fibres and the intrathecal administration of BDNF has been shown to cause mechanical and thermal hyperalgesia (Zhou et al 2000). It is known that BDNF levels increase in 'spared' sensory neurons after CCI (Obata et al 2003) and in intact L4 DRG after L5 spinal nerve ligation (SNL) (Fukuoka et al 2001), in an NGF-dependent manner, and this may contribute to neuropathic pain behaviour. One would predict that 'spared' neurons that express TrkC may experience less competition for target derived NT3. If this is indeed the case there is no evidence to suggest that NT3 is directly algogenic.

Injury-induced factors

Peripheral nerves have an immune privilege maintained by the blood–nerve barrier, which allows for minimum immune surveillance, mainly by activated T lymphocytes. Nerve injury dissolves this privilege and the nerve is subject to invasion from dedifferentiating and proliferating fibroblasts, macrophages and Schwann cells. Broadly speaking, injury-induced cytokines initiate a loop of self promoting activity; by increasing vascular permeability at the site of trauma and concomitant up-regulation of endothelial adhesion molecules, thereby enhancing leukocyte adhesion and extravasation. While the key action of recruited cells is to remove cellular debris and facilitate axonal regeneration, it is clear that these cells produce a variety of pro-inflammatory cytokines and chemokines (summarized in Fig. 4) which have been implicated in the generation of neuropathic pain either via direct sensitizing actions on nociceptors, or indirectly by stimulating the release of

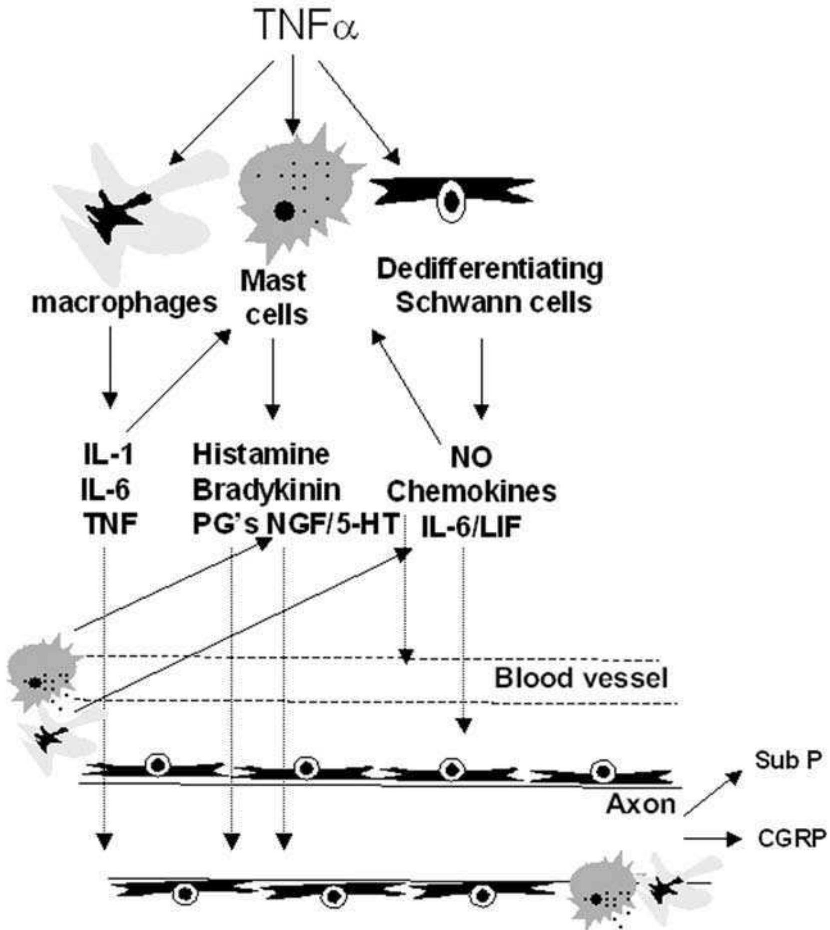

FIG. 4. Neural inflammatory response. Summary of injury-induced neural mediators that initiate and maintain the inflammatory response. TNFα released locally stimulates the release of cytokines IL1, IL6 and LIF (arrows) from resident macrophages and Schwann cells. Subsequent release of chemokines (CCL2) from activated macrophages and Schwann cells initiates the recruitment of further phagocytic cells, which infiltrate and continue the release of cytokines. Resident Mast cells degranulate in response to injury-induced stimuli and release prostaglandins, NGF and histamine. The locations of action of these mediators are indicated by broken arrows. Cytokines (such as TNFα) directly influence the axon via interactions with sodium and calcium channels. Prostaglandins (PGs), bradykinin and NGF released from mast cells sensitize axons directly. Injury-induced chemokines (CCL2) directly increase vascular permeability thereby enhancing leukocyte extravasation.

agents that act on neurons (reviewed by Watkins & Maier 2002). The expression of injury-induced factors is not limited to the distal stump of transected axons or areas undergoing Wallerian degeneration. Therefore both injured and intact neurons are subject to their influence. Overall, the cytokine response to nerve injury is highly complex, involving the up-regulation of pro- and anti-inflammatory factors that act and interact on a broad number of neuronal and non-neuronal cells producing transcription-dependent and -independent alterations in sensory processing.

Nerve trauma initiates a potent immune response typified by the early release of TNFα from infiltrating and resident macrophages (George et al 1999) and Schwann cells. Within 5 hours of nerve injury, TNFα levels are elevated within resident Schwann cells, which owing to their intimate proximity can directly sensitize nearby neurons (Shamash et al 2002). Subsequently TNFα stimulates the sequential production and release of IL1 and IL6 from infiltrating macrophages and dedifferentiating Schwann cells (Wagner & Myers 1996, Bolin et al 1995, Sommer 1999) along the entire length of the degenerating nerve. Simply delaying the infiltration of macrophages after nerve injury delays the development of neuropathic pain (Myers et al 1996), while delivering neutralizing antibodies to TNFα and IL1 reduces behavioural signs of experimental neuropathic pain (Shafers et al 2001, Sommer et al 1999). Furthermore $IL6^{-/-}$ mice fail to exhibit neuropathic pain after nerve injury (Ramer et al 1998, Murphy et al 1999). Much of the evidence to suggest a role for cytokines and chemokines in the initiation and maintenance of neuropathic pain come from studies such as these that have utilized tools that block cytokine function after experimental injury, or have been conducted in mice that experience delayed Wallerian degeneration (Ramer et al 1997). These mice fail to show signs of mechanical and thermal hypersensitivity after chronic constriction injury, highlighting a crucial role of degeneration-induced factors, such as cytokines and chemokines.

Intact and injured sensory neurons are known to express receptor components capable of transducing extracellular TNFα (Pollock et al 2002), IL1 and IL6 (Gardiner et al 2002). Indeed intraneuronal (Wagner & Myers 1996) and intraplantar injection of TNFα induces mechanical (Cunha et al 1992) and thermal hyperalgesia (Perkins et al 1994), via the TNFα1 receptor (Sommer et al 1998). While sensory neurons are a substrate for direct sensitization by TNFα, the underlying mechanism remains to be fully determined. Evidence from non-neuronal cells indicates an interaction with endogenous sodium and calcium channels (Wilkinson et al 1996). Intriguingly, trimers of TNFα have been reported to insert into membranes and form functional voltage-dependent sodium channels (Kagan et al 1992), which may allow for a generalized sensitization of sensory neurons in the absence of functional TNFα receptors.

It is clear that TNFα initiates a cascade of nerve injury-induced cytokine production (Woolf et al 1997, Shamash et al 2002), a self-promoting loop that also recruits production and release of IL1 and IL6. Intradermal injection of IL1 causes both mechanical and thermal hyperalgesia within minutes (Fukuoka et al 1994), suggesting a direct role on nociceptors. However, a dependence of IL1-induced hyperalgesia on bradykinin receptors 1 and 2 (Davis & Perkins 1994), prostaglandins (Schweizer et al 1988) and production of NGF (Lewin et al 1994) has also been observed.

The chemokine CCL2 (formerly known as monocyte chemoattractant protein 1) is another injury-induced product that accumulates within sensory neurons and contributes to macrophage recruitment. Recent data from our laboratory have implicated CCL2 in the maintenance of neuropathic pain: exogenous application of CCL2 to the sciatic nerve results in transient mechanical and thermal hyperalgesia (M. Thacker, B. J. Cafferty, S. Thompson, S. B. McMahon, unpublished observations). IL6, the prototypical member of the gp130 cytokines is absent from the adult peripheral nervous system, but is rapidly up-regulated by neurons (Murphy et al 1999) and Schwann cells at the site of nerve injury (Bolin et al 1995, Kurek et al 1996) probably via injury-induced TNFα release. Along with its related cytokine, LIF (leukaemia inhibitory factor; Thompson et al 1996), IL6 has been shown to promote touch-evoked allodynia after exogenous application (DeLeo et al 1996). The precise role of gp130 cytokines is complicated by the observation that some studies have highlighted an anti-inflammatory role for LIF and IL6 in models of cutaneous inflammation (reviewed by Gadient & Patterson 1999). However, their roles in nerve injury are better defined, having been shown to be crucial in the up-regulation of key modulators of sensory processing such as BDNF (Murphy et al 2000), galanin and substance P (Sun & Zigmond 1996) after peripheral nerve injury.

Electrophysiological changes

There is considerable evidence that activity in sensory neurons after injury is necessary for the elaboration of neuropathic symptoms. For instance, blocking sensory inflow by cutting dorsal roots, or applying local anaesthetics or the sodium channel blocker TTX, reportedly prevents the emergence of neuropathic pain in some circumstances in animal models (Lyu et al 2000, Liu CN et al 2000, Liu X et al 2000, Sheen & Chung 1993, Sukhotinsky et al 2004, Yoon et al 1996). Clinical observations also support the idea that abnormal sensory inputs trigger neuropathic pain (Price et al 1989, Campbell et al 1988). Electrophysiological recordings of more than a quarter of a century ago showed that damaged peripheral nerves became the source of abnormal activity (Wall & Gutnick 1974). Some of this activity appears to arise from the damaged sensory nerve

terminals (particularly those trapped in the neuroma that forms at the site of peripheral nerve injury). Some activity also clearly arises at the level of the cell body in the dorsal root ganglion (Wall & Devor 1983). However, it is only in the last few years that a clear picture has emerged as to which particular type of fibre becomes abnormally active.

Primary sensory neurons can be divided crudely into two functional subgroups. First, a group of small diameter cells with slowly conducting axons (so called Aδ and C axons). More than 90% of these cells are nociceptors. The second group are large diameter neurons with rapidly conducting (Aβ) axons, most of which are innocuous mechanoreceptors. One can easily see how ectopic or abnormal activity arising in nociceptors would provide a ready explanation for the ongoing pain seen in many neuropathic states. But there is also considerable evidence that activity in Aβ fibres can elicit pain in the presence of central sensitization — that is an enhanced excitability of central neurons. It is generally accepted that most of the mechanical hyperalgesia seen following peripheral nerve injury arises because of this reason. For instance, in human neuropathic pain states, activation of these Aβ afferents is capable of inducing pain (Campbell et al 1988). A matter of considerable debate, however, is the event(s) responsible for inducing the central nervous system (CNS) sensitization that allows Aβ afferent activity to produce pain. One clear possibility is that C-fibre activity initiates central sensitization and Aβ activity plays on this to maintain neuropathic touch-evoked pain. A second issue that now appears to be of central importance is between those fibres that are damaged in neuropathic conditions, and those that are intact but run alongside the damaged ones.

Damaged sensory fibres. Following nerve injury, some axotomised afferent neurons begin to discharge spontaneously (see Devor & Seltzer 1999). This afferent barrage provides constant input to the CNS, and thus may induce central sensitization. In many circumstances it is clear that only nociceptor activity is capable of inducing central sensitization (see Coderre et al 1993). Following L5 spinal nerve ligation, however, spontaneous activity arises almost exclusively in myelinated fibres (at least during the first week or two after injury, when neuropathic pain behaviour starts and becomes well established) (Boucher et al 2000, Li et al 2000, Liu X et al 2000). This is perhaps surprising but has been repeatedly determined by independent groups. There are conflicting reports on the importance of these ectopic discharges in damaged nerves to neuropathic pain behaviours (see above).

'Spared' sensory fibres. Damage to some afferents in a peripheral nerve leaves the remaining, intact, neighbouring fibres facing less competition for target-derived factors and subject to putative degeneration factors in the peripheral nerve. Recent work has shown that these intact 'spared' afferents (such as those running through

L4 after L5 SNL) show remarkable plastic changes, including the development of spontaneous activity. Myelinated fibres show very similar changes to those seen in damaged afferents, albeit slightly less well developed. That is, many Aβ afferents begin to generate relatively high frequency bursts or trains of action potentials that bombard the spinal cord (Boucher et al 2000, Michaelis et al 2000). It is interesting that myelinated afferents innervating muscle rather than skin seem to show a much greater propensity to generate these ectopic discharges (Proske et al 1995, Michaelis et al 2000). It is not clear what the functional significance of this observation might be, but one might imagine that specialized length and tension detectors in muscle would be the least likely group of afferents to generate or maintain neuropathic pain. We have observed that these ectopic discharges are reduced by GDNF treatment (Boucher et al 2000).

In 'spared' afferents (and not damaged ones) there are also reports of spontaneous activity arising in unmyelinated, nociceptive afferents (Koltzenburg et al 1994, Ali et al 1999). This activity has not been seen by all workers (e.g. Boucher et al 2000), but this may be because it occurs at very low rates, typically in the order of fewer than 0.1 Hz (Ali et al 1999). Indeed it is not clear what the consequences are of such low rates of C-fibre activity. However it has been reported that low level nociceptor activation (not eliciting pain) is sufficient to produce manifestations of central sensitization (Cervero et al 1993). Thus, it is possible that the key precipitating event in the development of neuropathic sensory abnormalities is the emergence of these C-fibre ectopic discharges in fibres spared by the injury, but running in the same peripheral nerves. The discharges in myelinated fibres (overwhelmingly innocuous mechanoreceptors originally) may only produce pain because they impinge on a CNS sensitized by the nociceptor inputs. In support, there are some behavioural data suggesting that blocking the spared afferent input can block the development of mechanical allodynia (Li et al 2000).

Altered gene expression in sensory neurons

In addition to the electrophysiological changes described above, models of experimental neuropathy lead to striking changes in gene expression in primary sensory neurons. Again it is important to distinguish between damaged and spared sensory neurons and to address which factors are responsible for these alterations.

Damaged sensory fibres. As discussed above, damaged neurons lose target-derived support. The damaged sensory neurons show changes in gene expression that affect virtually all aspects of the neurons' function, as summarized in Fig. 4. From the perspective of neuropathic pain, there are two types of change in gene

expression that may be particularly important. One is in the type and level of the neurotransmitters/neuromodulators that are produced by the damaged afferents, and released in the spinal cord with activity. Since, among the damaged afferents, it is myelinated fibres that become spontaneously active, changes here may be of particular importance. Some damaged A fibres (i.e. those with myelinated axons) appear to undergo a phenotypic shift, and begin to express transmitters normally associated with nociceptors, that is, substance P and BDNF. These factors are now released with A fibre activity (Malcangio et al 2000). Since there is good evidence that these factors are important contributors to central sensitization (see Woolf & Slater 2000), one can easily envisage that this contributes to neuropathic pain states. Further, many damaged fibres, including a large number of myelinated ones, begin to express the neuropeptide galanin. Traditionally, galanin has been thought of as an inhibitory neuropeptide in the dorsal horn of the spinal cord. However, it now emerges that different galanin receptors may be coupled to excitatory and inhibitory mechanisms and, using mice with null mutations in the galanin gene, we have directly observed reduced neuropathic pain behaviour in the absence of galanin expression (Kerr et al 1999).

A second observation relates to alterations in the expression of ion channels in damaged nerves. Clearly, there has to be a molecular correlate of the emergence of ectopic activity in damaged myelinated fibres. The most ready explanation is an altered expression of ion channels. Most studies have focused on changes in expression of sodium channels, the overexpression of which could alone lead to ectopic activity. One particular transcript, that encoding the Brain III sodium channel (now known as $Na_V1.3$) is up-regulated in damaged sensory neurons. Other known subtypes are all down-regulated. $Na_V1.3$ channels have rapidly repriming characteristics appropriate to maintain high frequency spontaneous activity. We have further correlative data in that GDNF treatment (that prevents neuropathic pain behaviour) largely reverses the up-regulation of $Na_V1.3$ channels in damaged afferents (Boucher et al 2000). The potential role of other channels is considered in the following section.

Spared sensory neurons. Sensory neurons running alongside injured fibres in neuropathic models also show marked changes in gene expression (Fig. 4). Increased bioavailability of target-derived neurotrophic factors and the abnormal expression of several chemokines and cytokines arising in damaged nerves (as described above) are likely triggers for transcriptional regulation. Spared afferent fibres frequently show the opposite pattern of gene changes to that seen in damaged axons and many of the examples of altered gene expression in this group can most parsimoniously be explained by increased availability of NGF (see Fukuoka et al 2000). Thus, substance P and TRPV1 (both increased in spared C-fibres) are known to be strongly regulated by NGF. And these changes are likely

to increase the sensitivity of C fibres or increase their central effectiveness. There are also increases of $P2X_3$ expression in spared neurons in some but not all neuropathy models (Tsuzuki et al 2001, Fukuoka et al 2002) most readily explained by increased availability of GDNF.

The molecular basis for the increased ectopic activity in spared afferents is not well understood. On the one hand, there is no or only minor up-regulation of $Na_v1.3$ (Boucher et al 2000) (the altered expression of which is well correlated with ectopic activity in damaged axons — see above). There has been some interest in the notion that the TTX resistant channel $Na_v1.8$ (formally SNS), might play a critical role in the generation of neuropathic pain behaviour. Antisense treatment targeting this protein reportedly reduces neuropathic pain (Lai et al 2002). This protein is normally confined to nociceptive neurons, and it is known to be down-regulated after injury. Thus, it is unlikely to play a role in damaged afferents. However, it is up-regulated in spared afferents (Boucher et al 2000), presumably C-fibres (although this is not formally established). It would be expected to increase the excitability of these neurons and could therefore account for the low levels of spontaneous activity seen in the spared nociceptors. However, a further confounding factor is the observation that $Na_v1.8$ knockout mice do not show any appreciable loss of neuropathic pain behaviour (Kerr et al 2001). In short, the molecular basis of this increased excitability of spared myelinated and unmyelinated afferents is currently unknown.

The relative contribution of ectopic inputs from damaged and spared afferents remains a contentious issue presently. However, the shift in focus to the undamaged afferents in neuropathic pain states provides new (and testable) hypotheses about the mechanisms underlying neuropathic pain.

Neurotrophic factor treatment for neuropathic pain

From the foregoing discussion, it is clear that several or many of the pathophysiological features associated with neuropathic pain appear to be secondary to altered neurotrophic factor availability. While the precise role (if any) of each of these observed changes to neuropathic pain itself is not established, a testable hypothesis is that normalizing neurotrophic factor availability will be of some use in the treatment of neuropathic pain.

The administration of NGF can induce strong neuroprotective effects on damaged neurons (reviewed in McMahon & Bennett 1999). However, the equally strong algogenic actions of NGF (McMahon & Bennett 1999) are likely to compromise the usefulness of this approach. There is one report of a beneficial effect of NGF in HIV neuropathy in human (Schifitto et al 2001). It is not clear if all subjects remained blinded on this trial and other attempts to use NGF clinically for the treatment of neuropathic pain states have been unsuccessful (see Apfel 2002).

The need to keep doses of NGF below pain-producing levels clearly limits its usefulness. Of course, if 'spared' rather than 'damaged' sensory neurons are more important for the evolution of neuropathic pain, then strategies aimed at limiting NGF availability to these neurons might be therapeutically useful, a suggestion for which there is supporting evidence (Theodosiou et al 1999).

We have assessed the effects of GDNF in several animal models. GDNF maintains the development of the non-peptidergic C fibres, and its exogenous delivery is able to reverse many of the alterations in gene expression induced by nerve injury that are crucial for the manifestation of neuropathic pain (Boucher et al 2000). Although many intact neurons express receptor components for GDNF and its related members (Bennett et al 1998), delivery of GDNF to intact animals failed to cause hyperalgesia or alter sensory processing when delivered to normal animals (Boucher et al 2000). However, GDNF does affect experimental neuropathic pain. We observed that intrathecal treatment with GDNF relieved neuropathic symptoms, and dramatically reduced the afferent barrage arising from damaged myelinated sensory neurons (Boucher et al 2000). The up-regulation of $Na_v1.3$ in damaged sensory neurons was also reversed by GDNF treatment, further supporting a pivotal role for this channel in the generation of ectopic activity and subsequently neuropathic pain.

Artemin is a neurotrophic factor structurally related to GDNF. Artemin and GDNF bind to different receptors. The binding protein for artemin (so called GFRα3) is expressed in many small diameter sensory neurons (but not large diameter neurons, unlike GDNF). Recently, it has been reported that this factor can also reverse many of the changes in gene expression that occur in damaged sensory neurons with appropriate receptors — that is, small but not large diameter ones (Gardell et al 2003). Artemin was also reported to prevent and reverse neuropathic pain behaviour in animals models. Since the distribution of artemin receptors is very restricted (with almost none in the CNS), side effects of treatment might be limited. Artemin, unlike NGF, does not appear to be algogenic.

Changes in spinal sensory processing in neuropathy models

In this chapter, we have focused primarily on changes that occur in peripheral sensory neurons. But the fact that pain can be evoked by activation of innocuous mechanosensitive fibres with Aβ axons in many neuropathic patients, clearly indicates an altered state of sensory processing in these subjects. The animal models described above all exhibit mechanical allodynia — pain-related behaviour to very gentle tactile stimuli. Touch-evoked pain can emerge very quickly in patients, and in the models too allodynia is seen within one or two days. The animal models also show a marked increase in the sensitivity to cold

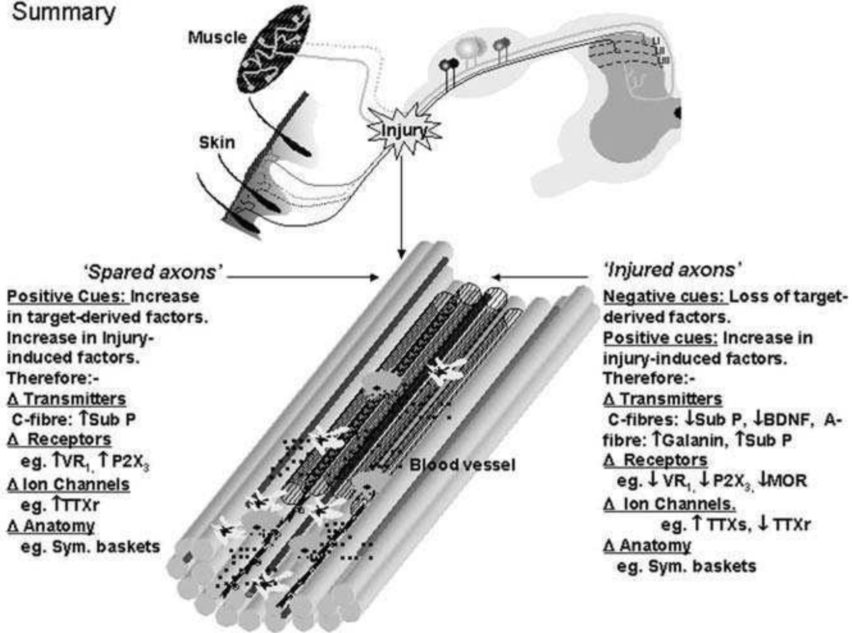

FIG. 5. Some of the changes in gene expression in sensory neurons in models of neuropathic pain. Changes occurring in neurons damaged in the model are listed separately from those occurring in intact neurons spared by the lesion but running in the same peripheral nerve. Damaged (outlined axons) and spared (filled axons) fibres are shown juxtaposed to one another after a partial injury to a peripheral nerve. The site of injury is typified by the recruitment and proliferation of non-neuronal elements (Schwann cells, Mast cells, macrophages) which release TNFα, NGF, IL6 and CCL2 (indicated by small dark grey squares). Abbreviations: Sub P, substance P; BDNF, brain-derived neurotrophic factor; MOR, μ opiate receptor.

stimuli, again a common finding in neuropathic patients, and again an indicator of altered central processing of sensory information (since we have no evidence for an increased sensitivity of cold-sensitive primary sensory neurons in these conditions). Together, these observations suggest that the animal models do indeed accurately reflect at least some of the symptoms typically seen in patients, and it is reasonable to assume that mechanisms identified in experimental work will have some relevance to those occurring in humans. In fact, a great number of abnormalities have been identified in the central processing of sensory information in these models and one problem is in identifying those that might contribute significantly to neuropathic pain. A discussion of these central factors is beyond the scope of this chapter, but is dealt with in several other contributions to this volume.

Conclusions

Our understanding of the neuronal mechanisms contributing to neuropathic pain has advanced significantly during the past few years. Myriad changes occur following nerve injury as summarized in Fig. 5. Several independent groups have reported that among damaged sensory neurons, ectopic activity initially appears only in myelinated afferents, most of which, of course, are erstwhile innocuous mechanosensitive afferents. There is also new recognition that a critical role may be played not only by damaged afferents, but also by their spared neighbours. Rather remarkably, there are major changes in gene expression in these afferents, and consequential changes in anatomy and physiological function. The signal for change in these intact neighbours has not been revealed. A partial denervation of target tissue will lead to increased availability of target-derived factors, such as NGF, for the remaining afferents. These factors are known to powerfully regulate sensory neuron phenotype (McMahon et al 1995), and may be involved in the ectopic activity generation seen in spared unmyelinated afferents (Ali et al 1999). An alternative source of signal may arise from the process of Wallerian degeneration of damaged axons. This is associated with a rapid and massive invasion of degenerating nerves by macrophages, a ready source of neuroactive molecules such as cytokines. Schwann cells around degenerating axons also up-regulate their expression of trophic factors. The increased understanding of the roles of these target-derived and injury-induced factors offers the opportunity to develop novel therapeutic strategies for treatment of neuropathic pain states.

References

Ali Z, Ringkamp M, Hartke T et al 1999 Uninjured C-fiber nociceptors develop spontaneous activity and alpha-adrenergic sensitivity following L6 spinal nerve ligation in monkey. J Neurophysiol 81:455–466

Apfel SC 2002 Nerve growth factor for the treatment of diabetic neuropathy: what went wrong, what went right, and what does the future hold? Int Rev Neurobiol 50:393–413

Bennett GJ, Xie YK 1988 A peripheral mononeuropathy in rat that produces disorders of pain sensation like those seen in man. Pain 33:87–107

Bennett DLH, Michael GJ, Ramachandran N et al 1998 A distinct subgroup of small DRG cells express GDNF receptor components and GDNF is a protective for these neurons following nerve injury. J Neurosci 18:3059–3072

Bolin LM, Verity AN, Silver JE, Shooter EM, Abrams JS 1995 Interleukin-6 production by Schwann cells and induction in sciatic nerve injury. J Neurochem 64:850–858

Boucher TJ, Okuse K, Bennett DL, Munson JB, Wood JN, McMahon SB 2000 Potent analgesic effects of GDNF in neuropathic pain states. Science 290:124–127

Campbell JN, Raja SN, Meyer RA, Mackinnon SE 1988 Myelinated afferents signal the hyperalgesia associated with nerve injury. Pain 32:89–94

Castro-Lopes JM, Tavares I, Coimbra A 1993 GABA decreases in the spinal cord dorsal horn after peripheral neurectomy. Brain Res 620:287–291

Cervero F, Gilbert R, Hammond RGE, Tanner J 1993 Development of secondary hyperalgesia following non-painful thermal stimulation of the skin: a psychological study in man. Pain 54:181–189

Coderre TJ, Katz J, Vaccarino AL, Melzack R 1993 Contribution of central neuroplasticity to pathological pain: review of clinical and experimental evidence. Pain 52:259–285

Coggeshall RE, Dougherty PM, Pover CM, Carlton SM 1993 Is large myelinated fiber loss associated with hyperalgesia in a model of experimental peripheral neuropathy in the rat? Pain 52:233–242

Colburn RW, Rickman AJ, DeLeo JA 1999 The effect of site and type of nerve injury on spinal glial activation and neuropathic pain behavior. Exp Neurol 157:289–304

Cunha FQ, Poole S, Lorenzetti BB, Ferreira SH 1992 The pivotal role of tumour necrosis factor alpha in the development of inflammatory hyperalgesia. Br J Pharmacol 107: 660–664

Cummins TR, Black JA, Dib-Hajj SD, Waxman SG 2000 Glial-derived neurotrophic factor upregulates expression of functional SNS and NaN sodium channels and their currents in axotomized dorsal root ganglion neurons. J Neurosci 20:8754–8761

Davis AJ, Perkins MN 1994 The involvement of bradykinin B1 and B2 receptor mechanisms in cytokine-induced mechanical hyperalgesia in the rat. Br J Pharmacol 113:63–68

DeLeo JA, Colburn RW, Nichols M, Malhotra A 1996 Interleukin-6-mediated hyperalgesia/ allodynia and increased spinal IL-6 expression in a rat mononeuropathy model. J Interferon Cytokine Res 16:695–700

Deng YS, Zhong JH, Zhou XF 2000 Effects of endogenous neurotrophins on sympathetic sprouting in the dorsal root ganglia and allodynia following spinal nerve injury. Exp Neurol 164:344–350

Devor M, Seltzer Z 1999 Pathophysiology of damaged nerves in relation to chronic pain. In: Wall PD, Melzack R (eds) Textbook of pain, edn 4. Churchill Livingstone, London, p 129–164

Doesterd I, Woolf CJ 2000 Spared nerve injury: an animal model of persistent peripheral neuropathic pain. Pain 87:149–158

Ernfors P, Wetmore C, Olson L, Persson H 1990 Identification of cells in rat brain and peripheral tissues expressing mRNA for members of the nerve growth factor family. Neuron 5:511–526

Ernfors P, Rosario CM, Merlio JP, Grant G, Aldskogius H, Persson H 1993 Expression of mRNAs for neurotrophin receptors in the dorsal root ganglion and spinal cord during development and following peripheral or central axotomy. Brain Res Mol Brain Res 17:217–226

Fukuoka H, Kawatani M, Hisamitsu T, Takeshige C 1994 Cutaneous hyperalgesia induced by peripheral injection of interleukin-1 beta in the rat. Brain Res 657:133–140

Fukuoka T, Kondo E, Dai Y, Noguchi K 2000 The role of neighboring intact DRG neurons in a rat neuropathic pain model. In: Devor M, Rowbotham M, Weisenfeld-Hallin Z (eds) Progress in pain research and management. IASP press, Seattle, p 137–146

Fukuoka T, Kondo E, Dai Y, Hashimoto N, Noguchi K 2001 Brain-derived neurotrophic factor increases in the uninjured dorsal root ganglion neurons in selective spinal nerve ligation model. J Neurosci 21:4891–4900

Fukuoka T, Tokunaga A, Tachibana T, Dai Y, Yamanaka H, Noguchi K 2002 VR1, but not P2X(3), increases in the spared L4 DRG in rats with L5 spinal nerve ligation. Pain 99:111–120

Gadient RA, Patterson PH 1999 Leukemia inhibitory factor, Interleukin 6, and other cytokines using the GP130 transducing receptor: roles in inflammation and injury. Stem Cells 17: 127–137

Gardell LR, Wang R, Ehrenfels C et al 2003 Multiple actions of systemic artemin in experimental neuropathy. Nat Med 9:1383–1389

Gardiner NJ, Cafferty WB, Slack SE, Thompson SW 2002 Expression of gp130 and leukaemia inhibitory factor receptor subunits in adult rat sensory neurones: regulation by nerve injury. J Neurochem 83:100–109
George A, Schmidt C, Weishaupt A, Toyka KV, Sommer C 1999 Serial determination of tumor necrosis factor-alpha content in rat sciatic nerve after chronic constriction injury. Exp Neurol 160:124–132
Herzberg U, Eliav E, Dorsey JM, Gracely RH, Kopin IJ 1997 NGF involvement in pain induced by chronic constriction injury of the rat sciatic nerve. Neuroreport 6:1613–1618
Heumann R 1987 Regulation of the synthesis of nerve growth factor. J Exp Biol 132:133–150
Horigome K, Pryor JC, Bullock ED, Johnson EM Jr 1993 Mediator release from mast cells by nerve growth factor. Neurotrophin specificity and receptor mediation. J Biol Chem 268:14881–14887
Kagan BL, Baldwin RL, Munoz D, Wisnieski BJ 1992 Formation of ion-permeable channels by tumor necrosis factor-alpha. Science 255:1427–1230
Kerr BJ, Cafferty WBJ, Gupta YK et al 1999 Galanin knockout mice reveal nociceptive deficits following peripheral nerve injury. Eur J Neurosci 12:793–802
Kerr BJ, Souslova V, McMahon SB, Wood JN 2001 A role for the TTX-resistant sodium channel Nav 1.8 in NGF-induced hyperalgesia, but not neuropathic pain. Neuroreport 12:3077–3080
Kim SH, Chung JM 1992 An experimental model for peripheral neuropathy produced by segmental spinal nerve ligation in the rat. Pain 50:355–363
Koltzenburg M, Kees S, Budweiser S, Ochs G, Toyka KV 1994 The properties of unmyelinated nociceptive afferents change in a painful chronic constriction neuropathy. In: Gebhart GF, Hammond DL, Jensen TS (eds) Proceedings of the 7th World Congress on Pain: progress in pain research and management. IASP Press, Seattle, p 511–522
Kurek JB, Austin L, Cheema SS, Bartlett PF, Murphy M 1996 Up-regulation of leukaemia inhibitory factor and interleukin-6 in transected sciatic nerve and muscle following denervation. Neuromuscul Disord 6:105–114
Lai J, Gold MS, Kim CS et al 2002 Inhibition of neuropathic pain by decreased expression of the tetrodotoxin-resistant sodium channel, NaV1.8. Pain 95:143–152
Lever IJ, Bradbury EJ, Cunningham JR et al 2001 Brain-derived neurotrophic factor is released in the dorsal horn by distinctive patterns of afferent fiber stimulation. J Neurosci 21:4469–4477
Lewin GR, Ritter AM, Mendell LM 1993 Nerve growth factor-induced hyperalgesia in the neonatal and adult rat. J Neurosci 13:2136–2148
Lewin GR, Rueff A, Mendell LM 1994 Peripheral and central mechanisms of NGF-induced hyperalgesia. Eur J Neurosci 6:1903–1912
Li Y, Dorsi MJ, Meyer RA, Belzberg AJ 2000 Mechanical hyperalgesia after an L5 spinal nerve lesion in the rat is not dependent on input from injured nerve fibers. Pain 85:493–502
Liu CN, Wall PD, Ben-Dor E, Michaelis M, Amir R, Devor M 2000 Tactile allodynia in the absence of C-fiber activation: altered firing properties of DRG neurons following spinal nerve injury. Pain 85:503–521
Liu X, Eschenfelder S, Blenk K-H, Janig W, Habler H-J 2000 Spontaneous activity of axotomized afferent neurons after L5 spinal nerve injury in rats. Pain 84:309–318
Lyu YS, Park SK, Chung K, Chung JM 2000 Low dose of TTX reduces neuropathic pain behaviours in an animal model. Brain Res 871:98–103
Malcangio M, Ramer MS, Jones MG, McMahon SB 2000 Abnormal substance P release from the spinal cord following injury to primary sensory neurons. Eur J Neurosci 12:397–399
Maves TJ, Pechman PS, Gebhart GF, Meller ST 1993 Possible chemical contribution from chromic gut sutures produces disorders of pain sensation like those seen in man. Pain 54:57–69

McMahon SB, Bennett DLH 1999 Trophic factors and pain. In: Wall PD, Melzack R (eds) Textbook of pain, 4th edn. Churchill-Livingstone, London, p 105–128

McMahon SB, Bennett DL, Priestley JV, Shelton DL 1995 The biological effects of endogenous nerve growth factor on adult sensory neurons revealed by a trkA-IgG fusion molecule. Nat Med 1:774–780

Merskey H, Bogduk N (eds) 1994 Classification of chronic pain. Descriptions of chronic pain syndromes and definitions of pain terms, 2nd edn. IASP Press, Seattle

Michaelis M, Liu X, Janig W 2000 Axotomized and intact muscle afferents but no skin afferents develop ongoing discharges of dorsal root ganglion origin after peripheral nerve lesion. J Neurosci 20:2742–2748

Michael GJ, Averill S, Nitkunan A et al 1997 Nerve growth factor treatment increases brain-derived neurotrophic factor selectively in TrkA-expressing dorsal root ganglion cells and in their central terminations within the spinal cord. J Neurosci 17:8476–8490

Milligan ED, O'Connor KA, Nguyen KT et al 2001 Intrathecal HIV-1 envelope glycoprotein gp120 induces enhanced pain states mediated by spinal cord proinflammatory cytokines. J Neurosci 21:2808–2819

Moyle GJ, Sadler M 1998 Peripheral neuropathy with nucleoside antiretrovirals: risk factors, incidence and management. Drug Saf 19:481–494

Munson JB, Shelton DL, McMahon SB 1997 Adult mammalian sensory and motor neurons: roles of endogenous neurotrophins and rescue by exogenous neurotrophins after axotomy. J Neurosci 17:470–466

Murphy PG, Ramer MS, Borthwick L, Gauldie J, Richardson PM, Bisby MA 1999 Endogenous interleukin-6 contributes to hypersensitivity to cutaneous stimuli and changes in neuropeptides associated with chronic nerve constriction in mice. Eur J Neurosci 11: 2243–2253

Murphy PG, Borthwick LA, Altares M, Gauldie J, Kaplan D, Richardson PM 2000 Reciprocal actions of interleukin-6 and brain-derived neurotrophic factor on rat and mouse primary sensory neurons. Eur J Neurosci 12:1891–1899

Myers RR, Heckman HM, Rodriguez M 1996 Reduced hyperalgesia in nerve-injured WLD mice: relationship to nerve fiber phagocytosis, axonal degeneration, and regeneration in normal mice. Exp Neurol 141:94–101

Naveilhan P, ElShamy WM, Ernfors P 1997 Differential regulation of mRNAs for GDNF and its receptors Ret and GDNFR alpha after sciatic nerve lesion in the mouse. Eur J Neurosci 9:1450–1460

Obata K, Yamanaka H, Fukuoka T et al 2003 Contribution of injured and uninjured dorsal root ganglion neurons to pain behavior and the changes in gene expression following chronic constriction injury of the sciatic nerve in rats. Pain 101:65–77

Ohara S, Tantuwaya V, DiStefano PS, Schmidt RE 1995 Exogenous NT-3 mitigates the transganglionic neuropeptide Y response to sciatic nerve injury. Brain Res 699: 143–148

Ossipov M, Bian D, Malan TJ, Lai J, Porreca F 1999 Lack of involvement of capsaicin-sensitive primary afferents in nerve-ligation injury induced tactile allodynia in rats. Pain 79:127–133

Perkins MN, Kelly D 1994 Interleukin-1 beta induced-desArg9bradykinin-mediated thermal hyperalgesia in the rat. Neuropharmacology 33:657–660

Pollock J, McFarlane SM, Connell MC et al 2002 TNF-alpha receptors simultaneously activate Ca^{2+} mobilisation and stress kinases in cultured sensory neurones. Neuropharmacology 42:93–106

Price DD, Bennett GJ, Rafii A 1989 Psychophysical observations on patients with neuropathic pain relieved by a sympathetic block. Pain 36:273–288

Proske U, Iggo A, Luff AR 1995 Mechanical sensitivity of regenerating myelinated skin and muscle afferents in the cat. Exp Brain Res 104:89–98

Raivich G, Hellweg R, Kreutzberg GW 1991 NGF receptor-mediated reduction in axonal NGF uptake and retrograde transport following sciatic nerve injury and during regeneration. Neuron 7:151–164
Ramer MS, French GD, Bisby MA 1997 Wallerian degeneration is required for both neuropathic pain and sympathetic sprouting into the DRG. Pain 72:71–78
Ramer MS, Murphy PG, Richardson PM, Bisby MA 1998 Spinal nerve lesion-induced mechanoallodynia and adrenergic sprouting in sensory ganglia are attenuated in interleukin-6 knockout mice. Pain 78:115–121
Ramer MS, Thompson SW, McMahon SB 1999 Causes and consequences of sympathetic basket formation in dorsal root ganglia. Pain (suppl 6):S111–S120
Schafers M, Brinkhoff J, Neukirchen S, Marziniak M, Sommer C 2001 Combined epineurial therapy with neutralizing antibodies to tumor necrosis factor-alpha and interleukin-1 receptor has an additive effect in reducing neuropathic pain in mice. Neurosci Lett 310: 113–116
Schifitto G, Yiannoutsos C, Simpson DM et al 2001 Long-term treatment with recombinant nerve growth factor for HIV-associated sensory neuropathy. Neurology 57:1313–1316
Schweizer A, Feige U, Fontana A, Muller K, Dinarello CA 1988 Interleukin-1 enhances pain reflexes. Mediation through increased prostaglandin E2 levels. Agents Actions 25: 246–251
Seltzer Z, Dubner R, Shir Y 1990 A novel behavioral model of neuropathic pain disorders produced in rats by partial sciatic nerve injury. Pain 43:205–218
Shamash S, Reichert F, Rotshenker S 2002 The cytokine network of Wallerian degeneration: tumor necrosis factor-alpha, interleukin-1alpha, and interleukin-1beta. J Neurosci 22: 3052–3060
Sheen K, Chung JM 1993 Signs of neuropathic pain depend on signals from injured nerve fibers in a rat model. Brain Res 610:62–68
Sommer C 1999 Animal studies on neuropathic pain: the role of cytokines and cytokine receptors in pathogenesis and therapy (German). Schmerz 13:315–323
Sommer C, Schmidt C, George A 1998 Hyperalgesia in experimental neuropathy is dependent on the TNF receptor 1. Exp Neurol 151:138–142
Sommer C, Petrausch S, Lindenlaub T, Toyka KV 1999 Neutralizing antibodies to interleukin 1-receptor reduce pain associated behavior in mice with experimental neuropathy. Neurosci Lett 270:25–28
Sukhotinsky I, Ben-Dor E, Raber P, Devor M 2004 Key role of the dorsal root ganglion in neuropathic tactile hypersensibility. Eur J Pain 8:135–143
Sun Y, Zigmond RE 1996 Leukaemia inhibitory factor induced in the sciatic nerve after axotomy is involved in the induction of galanin in sensory neurons. Eur J Neurosci 8:2213–2220
Theodosiou M, Rush RA, Zhou XF, Hu D, Walker JS, Tracey DJ 1999 Hyperalgesia due to nerve damage: role of nerve growth factor. Pain 81:245–255
Thompson SW, Dray A, Urban L 1996 Leukemia inhibitory factor induces mechanical allodynia but not thermal hyperalgesia in the juvenile rat. Neuroscience 71:1091–1094
Tsuzuki K, Kondo E, Fukuoka T et al 2001 Differential regulation of P2X(3) mRNA expression by peripheral nerve injury in intact and injured neurons in the rat sensory ganglia. Pain 91:351–360
Wagner R, Myers RR 1996 Schwann cells produce tumor necrosis factor alpha: expression in injured and non-injured nerves. Neuroscience 73:625–629
Wall PD, Gutnick M 1974 Ongoing activity in peripheral nerves: the physiology and pharmacology of impulses arising from a neuroma. Exp Neurol 45:576–589
Wall PD, Devor M 1983 Sensory afferent impulses originate from dorsal root ganglia as well as from the periphery in normal and nerve-injured rats. Pain 17:321–339

Watkins LR, Maier SF 2002 Beyond neurons: evidence that immune and glial cells contribute to pathological pain states. Physiol Rev 82:981–1011

Wilkinson MF, Earle ML, Triggle CR, Barnes S 1996 Interleukin-1β, tumor necrosis factor-alpha, and LPS enhance calcium channel current in isolated vascular smooth muscle cells of rat tail artery. FASEB J 10:785–791

Woolf CJ, Slater M 2000 Neuronal plasticity: increasing the gain in pain. Science 288:1765–1768

Woolf CJ, Shortland P, Coggeshall RE 1992 Peripheral nerve injury triggers central sprouting of myelinated afferents. Nature 355:75–78

Woolf CJ, Shortland P, Reynolds M, Ridings J, Doubell T, Coggeshall RE 1995 Reorganization of central terminals of myelinated primary afferents in the rat dorsal horn following peripheral axotomy. J Comp Neurol 360:121–134

Woolf CJ, Allchorne A, Safieh-Garabedian B, Poole S 1997 Cytokines, nerve growth factor and inflammatory hyperalgesia: the contribution of tumour necrosis factor alpha. Br J Pharmacol 121:417–424

Yajima Y, Narita M, Narita M, Matsumoto N, Suzuki T 2002 Involvement of a spinal brain-derived neurotrophic factor/full-length TrkB pathway in the development of nerve injury-induced thermal hyperalgesia in mice. Brain Res 958:338–346

Yezierski RP, Burchiel KJ 2002 Spinal cord injury pain: assessment, mechanisms, management progress in pain research and management. Vol. 23, IASP Press, Seattle

Yoon YW, Na HS, Chung JM 1996 Contributions of injured and intact afferents to neuropathic pain in an experimental rat model. Pain 64:27–36

Zhou XF, Deng YS, Chie E et al 1999 Satellite-cell-derived nerve growth factor and neurotrophin-3 are involved in noradrenergic sprouting in the dorsal root ganglia following peripheral nerve injury in the rat. Eur J Neurosci 11:1711–1722

Zhou XF, Deng YS, Xian CJ, Zhong JH 2000 Neurotrophins from dorsal root ganglia trigger allodynia after spinal nerve injury in rats. Eur J Neurosci 12:100–105

DISCUSSION

Baron: Have you shown that application of GDNF prevents fibre death in the dorsal horn of IB4-positive neurons?

McMahon: We don't think these cells are dying after the peripheral nerve injury. We think they are just down-regulating the sugars that bind the lectin IB4.

Baron: Did you correlate these findings with the behavioural findings?

McMahon: The data I showed you were from preparations with sciatic nerve axotomy, and we are unable to do meaningful behavioural analysis in these cases. But we have looked at IB4 binding in neuropathic models — spinal nerve ligation — and we see the same changes in damaged sensory neurons. There is a general correlation in that IB4 binding is reduced at one and two weeks after injury, when neuropathic behaviour is present. And GDNF treatment prevents both the immunohistochemical and behavioural changes in this time frame. We have not examined the correlation on an animal-by-animal basis.

Belmonte: We have an interesting observation in the cornea. When we damaged corneal nerve endings located in the superficial corneal epithelium where they do not have anymore Schwann cells, we don't see *c*-jun expression in the cell bodies. Whereas if the corneal lesion is deeper, affecting the stroma where the fibres are

covered by Schwann cells, there is a marked *c*-jun expression in the soma of corneal neurons. What is your speculation about the role played by Schwann cells in the effects of growth factors seen after axotomy?

McMahon: Well, it's a bit more than speculation because there are some data that quiescent Schwann cells don't make a lot of NGF. However, Schwann cells in the presence of degenerating axons start churning it out. There is evidence that this occurs secondary to IL1β stimulation of the Schwann cells. But I think you are asking 'what are the consequences of these changes?' If a peripheral nerve is crushed, fibres start regenerating into the degenerating distal nerve almost immediately (within a day or two). Under those circumstances many of the candidate genes that are regulated by NGF don't change. The simple explanation is that NGF is replenished in the crushed fibres as they regrow in an NGF-rich environment. But if a nerve is cut and prevented from regenerating, while the Schwann cells at the site of nerve damage start making excess NGF, it is not enough to compensate for what the sensory axon would normally get from its peripheral terminals. The corollaries of your question are also very interesting. That is, in neuropathies that are associated with dying back of axons, how far do they have to die back before they lose target-derived trophic support? I don't know the answer to this. There are indications from intradermal and topical capsaicin studies. Just the terminals are lost with these treatments and there does not seen to be extensive retrograde degeneration of axons. Under these circumstances, C-fibres look as if they have lost their peripheral neurotrophic support.

Devor: When you have this dying back into the nerve trunk, are these dying back axon ends like a distributed microneuroma? Do you have a Tinel sign along the nerve in diabetic neuropathy? Is this something that has been tested?

McMahon: I think the dying back occurs without any Tinel sign. Studies using quantitative evaluation of C fibre epidermal innervation in humans show reduced regenerative capacity of C fibres challenged with capsaicin in diabetic patients or HIV patients when the patient is completely asymptomatic and before they start to lose innervation from the epidermis.

Devor: But their symptoms might begin when these dying back axon ends start to become hyperexcitable, one of the important symptoms being an ongoing burning pain, for example.

Wood: Have you looked in behavioural experiments at neurturin and artemin, and do all GDNF family members have the same effects?

McMahon: Yes, we have repeated them with both neurturin and artemin, two other members of the GDNF family. Neurturin has been difficult to use, because of its limited solubility and we have no convincing data. We have studied neuropathic behaviour in several experiments using artemin. In about a half of these we have seen a good neuroprotective action, but in other, apparently identical experiments we have seen nothing. The experiments are done blind and

we don't understand the lack of reproducibility. The effects we have seen emerge later than with GDNF treatment. Subsequently a large study by Frank Porecca and others found that artemin did produce a strong but delayed behavioural recovery of neuropathic behaviour. So, several members of the GDNF family may be effective. But this in itself is quite perplexing, since different groups of sensory neurons have receptors for artemin and GDNF. This doesn't help us understand the mechanisms.

Noguchi: What is the mechanism underlying the effect of GDNF on the L4 ganglia following L5 spinal nerve injury?

McMahon: In animals with an L5 spinal nerve ligation, we don't think there is any deficit in GDNF or NGF in the L4 dorsal root ganglion. If you look at markers that are induced or supported by either of these factors, they don't decrease dramatically. So there is no need to propose that tropic factors work by offering neuroprotection to these intact afferents. But there is a problem: I told you that intact myelinated afferents become spontaneously active, as do axotomised ones, and GDNF treatment dampens down activity in both these groups. But when we looked for ionic changes that might contribute to the spontaneous activity, we could only see an up-regulation of $Na_V1.3$ in the damaged afferents. We don't see a change in $Na_V1.3$ by PCR in the spared afferents. So the relationship between ectopic activity and channel expression in those two sets of neurons is unclear. We don't have a simple explanation.

Apkarian: Could you explain what the roots of your scepticism are about the lack of central anatomical reorganization?

McMahon: It is not just my view: four separate groups now have data suggesting that the anatomical reorganization (sprouting) that has been reported after peripheral axotomy is based on a methodology that isn't sound. The classic method used to identify this sprouting is the bulk transport of CTB (the β subunit of cholera toxin). Several groups have now shown that after peripheral nerve injury, C fibres start to transport CTB. Therefore the change previously interpreted as sprouting may not be sprouting at all, but rather *de novo* transport. That suggests that the anatomical data are more difficult to interpret than we would like. If one asks whether there is positive evidence against sprouting, then there is. One line of evidence comes from studies where a peripheral nerve is labelled with CTB and subsequently damaged peripherally. *De novo* transport by damaged C fibres is not possible here, and one does not see any signs of 'sprouting'. In a recent study Hughes et al (2003) labelled Aβ fibres with very small injections of CTB into dorsal columns. This labelled small numbers of A fibres that could be studied anatomically, again without significant contamination by *de novo* transport in C fibres. They too found no evidence of sprouting.

Baron: I thought there was electrophysiological evidence showing an activation of nociceptive neurons in the dorsal horn by Aβ fibres.

McMahon: There clearly are functional changes that take place in the spinal cord, but the explanation for these is uncertain. I should say that in addition to the bulk labelling experiments, there are data from anatomical reconstructions of single A fibres, some of which do and some of which do not suggest sprouting. Both the bulk labelling and the single fibre fills throw some doubt on the simple conclusion that second order cells in the spinal cord are beginning to receive *de novo* monosynaptic connections from A*β* fibres. There are other possible explanations, such as the unmasking of existing A*β* connections, or perhaps the strengthening of such connections.

Yoshimura: We have tested a change of synaptic connections in the spinal dorsal horn following inflammation in my present talk, but we have also reported the reorganization of the synaptic transmission in the spinal cord in sciatic nerve transected rats. What we found is that in the early stage of inflammation, A*β* afferent fibres made synaptic connections with interneurons which had already established synaptic contacts with substantia gelatinosa neurones. Therefore, there were many polysynaptic inputs from A*β* afferents to substantia gelatinosa neurons. After 7–10 days of inflammation, the A*β* fibres then made a direct synaptic contact with substantia gelatinosa neurons. Similar to the peripheral inflammation, the sprouting is also observed in sciatic nerve transected rats originally reported by Woolf's group (Woolf et al 1992, Okamoto et al 2001). Although A*β* fibres make synaptic contact with superficial dorsal horn neurons, only a few neurons have a direct input from A*β* fibres, and many of the inputs from A*β* are polysynaptic. Thus, the sprouting patterns of the A*β* afferent fibres are distinct in different pain models.

Devor: I also wonder whether this could happen within the 24 h time frame that Jin Mo has set for us. Also, if there is a hardwiring change of that sort, how can you turn the allodynia on and off by stopping the ectopic activity in the ganglion and neuroma?

Malmberg: I would like to hear your comments on your other questions, and the relationship between two of your comments, namely does NGF promote neuropathic pain and is there a need for ectopic activity in C fibres? Given that NGF-positive neurons are C fibres, is it possible that NGF is promoting ectopic activity in C fibres?

McMahon: These are related questions. One hypothesis is that the critical peripheral event is C fibre activity. The only candidate appears to be intact C fibres. And there does seem to be an up-regulation of genes in these afferents that are likely to be controlled by NGF. A consistent hypothesis would be that C fibre activity is important and arises because of increased bioavailability of NGF to those intact afferents. This is testable, but the necessary reagents (anti-NGF) are not freely available.

Malmberg: The groups that have performed these studies have found that anti-NGF has some effect, particularly in inflammatory models, but the effect on neuropathic pain is less convincing.

Devor: On a similar issue, is there any sign that these altered L4 C afferents begin to respond to very light stimuli? I'm thinking of the sort of stimuli that evoke the tactile allodynia — the response to weak von Frey hairs. I don't think that this happens. It is a misconception that many people have had. It would be easy to interpret tactile allodynia if that happened: if C fibres became sensitive in the skin. If anything, the role of the very low rate of abnormal C fibre activity would be to contribute to the central sensitization, which raises an alternative question: Is it possible that injured A fibres, which now have changed expression of many peptides and other molecules, could have acquired the ability to turn on and maintain central sensitization, an ability which normally they don't have.

Dray: There is an important role for neurotrophins in the regulation of ion channels and recent reports of enhanced dorsal root reflexes suggest neurotrophins regulate redistribution of ion channels and ionic transport mechanisms in neuropathic pain. Could you comment on the relevance of neurotrophins in this respect?

McMahon: I don't think there is any direct evidence that differential trafficking is controlled by trophic factor availability. The claim is that after injury there may be translocation of channels, but the causative agents are unknown. My own prejudice is that such translocation is an unlikely explanation for neuropathic pain, because of the continuing neuropathic behaviour in $Na_V1.8$ knockout mice.

Dray: There has been another discussion about neurotrophin regulation of chloride channels. Is there a relationship between GDNF and chloride channels?

McMahon: Most of the interest that I am aware of relates to regulation by other trophic factors, most notably BDNF, which has been suggested to regulate a chloride transporter in dorsal horn neurons.

Chung: I have a question regarding the activity of the spared intact nerve after spinal nerve ligation. You showed a picture of the Remak bundle with a reduced number of unmyelinated fibres. This tells me that there is plenty of opportunity for interaction between the degenerating and the intact fibres. What I don't see is clear evidence that there is strong interaction. If there is a strong interaction, I would expect to see a whole bunch of intact C fibres firing like crazy, which I do not see.

McMahon: What one sees probably depends on what is being produced. Several putative factors won't necessarily induce high rates of C fibre active. NGF itself, if given in large measures, can activate some deep afferents, but mainly is associated with sensitizing the peripheral terminals of intact C fibres. Secondly, if one asks whether there are other signs of increased trophic factor bioavailability, there is indeed considerable evidence that this is the case, as seen by changes in gene

expression in spared sensory axons. Some of these will be reviewed in other papers at this meeting. The fact that there are such changes in gene expression itself is highly suggestive of altered availability of neurotrophic factors or cytokines that have neurotrophic effects.

Chung: The activity that you report is different from that of Dick Meyer's group. Do you know whether your activity is coming from damaged or undamaged afferents?

McMahon: I don't know what causes that activity. One issue is whether spared afferents are really intact. But simply doing the surgery to make a spinal nerve ligation of L5 threatens to damage L4, which sits alongside. We recently undertook a study in which we used the marker ATF-3 (a transcription factor that marks cells that have been axotomised). We found that in some preparations there was very little ATF-3 in L4 after L5 spinal ligation, but in other preparations, up to 30% of L4 DRG neurons appeared to have been axotomised. Interestingly, there was no correlation between ATF3 expression in L4 after L5 ligation and L4 ectopic activity in the same animals. We still don't know what causes the damage, but it appears to be different from what causes the ectopic activity in these spared afferents.

Devor: The activity reported by the Baltimore group in residual C fibres in L4 is something like 3–5 spikes every five minutes. We are talking about exceedingly low rates of firing. Many of us who have done recording would have thought of artefacts, that maybe this has to do with the refrigerator turning on in the next door lab! I should add, though, that the claim is that quite a high percentage of fibres show this very low level activity.

Apkarian: I had a similar question related to the issue of anatomic reorganization. The other issue that comes up repeatedly is central cell death. Where does this stand?

McMahon: Recent evidence from the Woolf lab suggests that dorsal horn cell death is a very active phenomenon that may explain some of the disinhibition phenomena seen in neuropathic models. The difference from the original claim is that cell death only arises in models of partial nerve injury, those associated with neuropathic pain.

Apkarian: If that is believable how could anatomical reorganization not happen, if you also have apoptosis happening at the same time?

McMahon: You could have anatomical reorganization which does not affect or involve sprouting of Aβ afferents.

Devor: The loss of inhibitory neurons in spinal cord also has the problem (along with Aβ sprouting) of explaining the reversal of tactile allodynia and of spontaneous pain with peripheral nerve block, which is an almost universal report from the clinicians. If you find the source of the ectopic firing in the peripheral nerve and you block it with local anaesthetic, the pain goes away until

the block vanishes. If the key change was happening in the spinal cord and the pain signal originated there, this wouldn't be possible.

McMahon: You may have a disinhibited spinal cord that allows weak peripheral inputs to generate abnormal pain sensations.

Devor: But would the residual peripheral inputs remain if you blocked the injured area? Again, we go back to the question of whether we need the ectopic firing coming from the injured nerve, or whether the pain has become centralized. As I said earlier, I don't see the evidence of frank centralization.

Apkarian: It can still be driven from the periphery but magnified centrally.

Devor: Tactile allodynia means you are driving tactile receptors in the skin. Their signal is amplified in the spinal cord. But if the amplification is due to a change in the spinal cord that doesn't require ectopic input from the periphery, then blocking the ectopic input shouldn't stop the allodynia. But it does (Gracely et al 1992).

Mantyh: In light of your data, would you say that IB4 neurons are uniquely involved in neuropathic pain? What are they normally doing?

McMahon: There are some data from selective ablations using the saporin conjugates, although we have had no luck with this approach. Since it is always easier to believe one's own data, I am not clear the approach provides any compelling insight into the selective role of IB4 fibres. You could turn the question round and say what do we know about those fibres from their normal physiology? There we end up with a clear answer. In rodents, half of the IB4-binding cells are capsaicin sensitive and half are not, and half are heat sensitive and half are not. In our hands, quite a few appear to be ATP sensitive. They don't seem to have unusual properties compared to non-IB4-binding C fibres. The simple conclusion would be that they are just a bunch of nociceptors, but that they have a unique central connectivity. But what this means to the animal, I don't know.

Dray: I concur with what you said with respect to the selective ablation. However, an important question is raised from human microneurography studies showing the existence of a specific population of mechano-insensitive C fibres called 'silent nociceptors'. Amongst other characteristics these fibres respond more dramatically to capsaicin exposure and make an extraordinary contribution to the initiation of spinal sensitization. Little or nothing is known of their phenotype.

Devor: What do we know about how activity in C fibres turns on central sensitization?

McMahon: Those fibres produce flare responses. They are presumably peptidergic in nature, and we predict that they would be NGF sensitive, and not GDNF sensitive. But this is indirect evidence and somewhat speculative.

Devor: Do we know which peptide it is, if it is a peptide that is turning on the central effect? These C fibres, whether they are originally silent or not, never become responsive to these very light stimuli that drive tactile allodynia. Until someone can find C fibres that are really sensitized, we have to talk about Aβ activity as being misinterpreted by a sensitized spinal cord. Now the question becomes how do these C fibre inputs sensitize the cord? One popular idea is that a peptide that is released — perhaps substance P — produces a tonic depolarization in post-synaptic neurons and therefore enables NMDA receptors, which now become responsive to the Aβ input. However, from the work of Dr Noguchi and others, we know that after a nerve injury substance P is down-regulated and there is much less of it in the C fibres, while there is much more of it in the A fibres. This happens quite soon after axotomy. Is it possible that the injured Aβ fibres, due to this phenotypic switch, are now able to induce central sensitization? If so, the injured A fibres not only fire and produce an input, but also maintain the central sensitization that amplifies this input.

Reeh: Shouldn't we ask the author the first of your series of questions? That is, whether low threshold C or Aδ mechanoreceptors contain neuropeptides that eventually could be released. He is the only one who could comment on that.

Perl: The evidence is incomplete for the low threshold C afferent fibres. We labelled few, and never studied them at the electron microscope level. Low threshold myelinated mechanoreceptive fibres in general do not have dense core vesicles in their synaptic terminals. The issue is half-answered. We do know that there are many peripheral C fibres in mammals that act as low threshold mechanoreceptors. They are remarkably sensitive; if they were involved, their input could easily explain tactile allodynia.

Devor: Yes, if they were nociceptors. But they have a low tactile threshold, so they are not nociceptors.

Perl: C-mechanoreceptors are not nociceptors. They have peculiar characteristics and they are reported to have important functions in human beings. They have been shown to be involved in a peculiar emotional experience by a patient with no functioning myelinated fibres below the neck (Olausson et al 2002).

Devor: If low threshold C fibres were capable of turning on central sensitization, just brushing the skin lightly for a minute or two would turn on central sensitization, and it doesn't.

Reeh: That is not the sort of stimulus that would evoke any flare reaction or CGRP release in the periphery. We could assume that those fibres are not peptidergic.

Devor: There is an interesting observation from Molander et al (1994) in Stockholm. Normally *c*-fos is turned on in dorsal horn neurons only by C fibre stimulation of the peripheral nerve. However, if there has been a prior nerve

injury, now Aβ fibre stimulation will activate *c*-fos in the spinal cord. This is another piece of evidence suggesting that Aβ fibres may acquire the ability to trigger central sensitization after injury, a capability that they didn't have before.

Mao: I'd like to ask a different type of question. If we imagine that whatever the mechanisms so far we have proposed for neuropathic pain, whether central or peripheral mechanisms, the end point is a common pathway, for example, activating the spinal projection pathway to the brain. If this were the case, in terms of peripheral mechanisms we would have a new generator, and in terms of central mechanisms we would have an increased gain of input. But why then do patients with neuropathic pain often describe the pain as having a different quality from physiological pain. They don't use the same words as those used to describe physiological pain. Whatever the mechanisms involved, if it is simply to turn on the common pathway or to enhance the gain of this common pathway, why do they choose different pain descriptors? Why has the quality of the pain changed? With similar nerve injury, some patients will have allodynia and others have hyperalgesia, and this can change dynamically over time.

Devor: This might be a good point to mention recent results by Frank Porreca and his group (Porreca et al 2002). Central sensitization refers to a gain in amplification in the spinal cord. This amplifier is controlled by many different things. Afferent input, and in particular afferent C fibre input, is clearly one of them. I have raised the possibility that Aβ input along injured Aβ fibres might be another. Porreca points out the possibility that this spinal amplifier might also be controlled by descending pathways from the brainstem. He shows that cutting some of the descending pathways can eliminate tactile allodynia. This is one more thing that can control this amplifier. When we talk about individual variability in neuropathic sensation, or changes in the quality of the sensation from time to time in a given patient, this could be dependent on the diversity of inputs to the amplifier, including descending control from the head.

Belmonte: We have done experiments in the human cornea using a gas aesthesiometer that allows us to stimulate polymodal nociceptors alone or in combination with mechano-nocicceptors (Acosta et al 2001). The quality of the pain sensation is completely different from one case to another. In my view, the final quality of the sensation depends on the degree of activation of the various classes of sensory receptors. In the above mentioned experiment where two subpopulations of nociceptors are activated to a variable degree, different qualities of pain were experienced. In neuropathic pain, the sensation felt by patients may be particular because many types of fibres are being simultaneously and abnormally activated.

McMahon: Why assume there is only one single pathway leading to one unique sensation?

Apkarian: The other issue here is that the discussion keeps centring on allodynia. Chronic pain can clearly lead to spontaneous pain, which is the most common form of chronic pain but it is difficult to measure and design experiments to study. Perhaps the slow acting C fibres are critical for the perception of spontaneous pain.

Devor: Perhaps, but if there is central sensitization for whatever reason, because of these abnormal L4 C fibres, or because injured Aβ fibres now are able to turn on central sensitization, or because the central sensitization is induced by descending control from the head, the spontaneous activity that many of us have been pointing out will also be amplified. This is an obvious potential source of spontaneous painful sensation. The neuroma and the DRG activity are now amplified by central sensitization which, parenthetically, may be maintained by that same ectopic input. I wanted to raise the topic of microarray experiments. Steve McMahon showed a slide from Dr Zhang's work (Xiao et al 2002) on mRNA expression from axotomized DRG. Of the 8000 odd transcripts on the array, 167 were significantly up- or down-regulated after axotomy. This experiment has now been done by several other groups. It is safe to say that at least 1000 transcripts in the DRG are significantly regulated after nerve axotomy. This is only in the DRG. If we were to do the same arrays in spinal cord, we may find another couple of thousand mRNAs changed, and who knows what happens in the brain and skin. I think we are facing a crisis in our understanding: we began with having no theories of neuropathic pain and now we have many thousands. We will need to come up with strategies to figure out which are central to pain and which aren't.

Zhou: I think that in the future there will be a requirement for researchers using more integrated approaches to address pain mechanisms. Personally, I think in the future more collaborations will help to solve this problem.

Devor: The problem is that we are talking about thousands of transcripts — it's not a regular collaboration.

Mantyh: There have been a couple of very nice studies in ovarian cancer by the group led by Dr Bagnato in Italy. What she showed was that if she blocked the endothelin A receptor in precancerous cells, it could block most of the downstream events. She used microarrays to show this. In arrays, the change between precancerous and cancerous cells involved thousands of genes. I am wondering if you ran microarrays and looked at the effect of GDNF, how many of these changes in gene expression would you see?

McMahon: The London Pain Consortium is undertaking studies on genes regulated in sensory neurons by trophic factors. These are ongoing and we hope to post data on a publically available website.

Gintzler: Thousands of transcripts changing doesn't mean thousands of theories. More importantly, it needs to be pointed out that a change in transcript level doesn't mean there is a change in protein level. Until one knows the protein

that is encoded by all of these transcripts, we can't validate that there are meaningful changes in protein level.

Zhou: There are lots of proteins which Western blots are not sensitive enough to detect. In most cases, biochemical analysis uses samples with mixed populations of cells from brain regions.

Wood: The subcellular localization of proteins is also important.

Devor: An important feature is threshold discontinuity in a response function. This is very characteristic of the abnormal firing that Jin Mo was talking about. If you are operating just above repetitive firing threshold, a small inhibitory stimulus will kick you from a substantial rate of firing to zero. Since you are at threshold it could be that all of these various things turn off allodynia despite the fact that each makes only a small contribution overall.

Wood: Just to turn that argument on its head, if there is cooperativity between large numbers of different mediators which summate to change thresholds, this can explain why so many different drugs, antisense and knockouts all have dramatic effects. If we can form a reasonable theory based on temporal analysis of how these things change then we may be able to home in on the kind of bottleneck which could be a globally interesting target.

References

Acosta MC, Belmonte C, Gallar J 2001 Sensory experiences in humans and single-unit activity in cats evoked by polymodal stimulation of the cornea. J Physiol 534:511–525

Gracely R, Lynch S, Bennett G 1992 Painful neuropathy: altered central processing, maintained dynamically by peripheral input. Pain 51:175–194 (Erratum in Pain 52:251–253)

Hughes DI, Scott DT, Todd AJ, Riddell JS 2003 Lack of evidence for sprouting of Aβ afferents into the superficial laminas of the spinal cord dorsal horn after nerve section. J Neurosci 23:9491–9499

Molander C, Hongpaisan J, Persson JK 1994 Distribution of c-fos expressing dorsal horn neurons after electrical stimulation of low threshold sensory fibers in the chronically injured sciatic nerve. Brain Res 644:74–82

Okamoto M, Baba H, Goldstein PA, Higashi H, Shimoji K, Yoshimura M 2001 Functional reorganization of sensory pathways in the rat spinal dorsal horn following peripheral nerve injury. J Physiol (Lond) 532:241–250

Olausson H, Lamarre Y, Backlund H et al 2002 Unmyelinated tactile afferents signal touch and project to insular cortex. Nat Neurosci 5:900–904

Porreca F, Ossipov MH, Gebhart GF 2002 Chronic pain and medullary descending facilitation. Trends Neurosci 25:319–325

Woolf CJ, Shortland P, Coggeshall RE 1992 Peripheral nerve injury triggers central sprouting of myelinated afferents. Nature 355:75–78

Xiao H-S, Huang Q-H, Zhang F-X et al 2002 Identification of gene expression profile of dorsal root ganglion in the rat peripheral axotomy model of neuropathic pain. Proc Natl Acad Sci USA 99:8360–8365

Changes in DRG neurons and spinal excitability in neuropathy

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Abstract. An intracellular signalling pathway in the dorsal root ganglion (DRG) and spinal neurons is a popular target in pain research that is relevant to the neuroplastic changes that occur during chronic pain conditions. First, we examined the phosphorylation of ERK in DRG neurons after peripheral inflammation and sciatic nerve transection without any stimulation to the receptive field. We found an activation of ERK in different populations of DRG neurons after peripheral inflammation and axotomy, which developed from alterations in target-derived nerve growth factor (NGF). We observed that the ERK signalling regulates the brain-derived neurotrophic factor (BDNF) expression in DRG neurons in both conditions. We also demonstrated that very rapid phosphorylation of ERK occurred in DRG neurons that were involved in the transmission of various noxious signals under normal conditions. Further, we examined the pERK labelling after the mechanical stimuli into the inflamed tissue and found that the pERK labelling occurred through the $P2X_3$ receptors in the terminals. This activity-dependent activation of the ERK signal pathway may be useful for identifying which DRG neurons are involved in transmission of noxious stimuli under normal and pathological conditions.

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Primary sensory neurons are highly specialized for transducing and transmitting sensory information from the periphery to the CNS and are selectively equipped to detect different kinds of stimuli (Snider & McMahon 1998). The initial step in pain perception is that noxious thermal, mechanical, or chemical stimuli excite specialized nociceptive transducer receptor/ion channel complexes in peripheral terminals of nociceptors. Action potentials that are transmitted from the periphery may activate the intracellular signalling pathway and regulate gene expression in dorsal root ganglion (DRG) neurons (Fields et al 1997). The alteration in gene expression and the resultant changes in the excitability in DRG neurons may be involved in peripheral and central sensitization in acute and chronic pain conditions (Dubner & Ruda 1992, Woolf & Salter 2000).

Recent reports have shown that inflammatory mediators, such as prostaglandin E2, serotonin, epinephrine and nerve growth factor (NGF), produce hyperalgesia

through activation of protein kinase A, protein kinase C or extracellular signal-regulated kinases (ERKs) in the primary afferent neurons (Gold et al 1998, Aley et al 2001). ERK is a mitogen-activated protein kinase (MAPK) that mediates several cellular responses to mitogenic and differentiation signals (Lewis et al 1998). ERKs are activated by an upstream kinase, MEK (Chang & Karin 2001), and are known as one of the intracellular signalling pathways involved in neuronal plasticity (Fields et al 1997, Martin et al 1997, Impey et al 1999). Physiological and pathological activity-dependent activation of ERK occurs in the CNS (Obrietan et al 1998, Atkins et al 1998). Several groups have recently employed immunohistochemistry and phospho-specific antisera to analyse the distribution and level of activation of signalling components in the DRG neurons *in vivo* (Aley et al 2001, Averill et al 2001, Ma et al 2001). Therefore, the purpose of this study was to examine the specific activation pattern of ERK in primary afferent neurons in normal and pathological pain conditions and assess its functional significance in the pain transmission cascade.

Materials and methods

The methods of animal surgery, pain stimulation, immunohistochemistry, *in situ* hybridization, pain behaviour and Western blotting have been described in detail in previous reports (Dai et al 2002, Obata et al 2003).

Results and discussion

Phosphorylation of pERK in primary afferent neurons following peripheral inflammation (Obata et al 2003)

pERK-IR was located in neurons, as well as in surrounding satellite cells, as reported before (Averill et al 2001). The pERK-IR neurons in the ipsilateral DRG 1 day after the complete freund's adjuvant (CFA) injection increased in terms of the number of labelled neurons and the intensity of labelling compared with those in the contralateral DRG. Most of these pERK-IR neurons were small-to-medium diameter DRG neurons. pERK-IR neurons of naïve rats in the L4/5 DRG were $2.7 \pm 0.4\%$ of the total neurons, and inflammation induced a significant increase in the percentage of pERK-IR neurons in the ipsilateral DRG at 1 day ($8.1 \pm 0.7\%$). The levels gradually declined and returned to normal by 3 days.

Intrathecal administration of U0126 significantly reduced both the inflammation-induced heat and mechanical hypersensitivity measured at 1 day. To examine whether brain-derived neurotrophic factor (BDNF) expression in the DRG is regulated by ERK activation , we compared the immunoreactivity for BDNF in DRG neurons in the vehicle and U0126 groups. The MEK1/2

inhibitor, U0126, significantly inhibited the inflammation-induced increase in BDNF-IR, which was seen mainly in small- and medium-sized neurons. BDNF mRNA as revealed by ISHH in the ipsilateral DRG neurons in the U0126 group at 3 days after CFA injection decreased compared with those of rats in the vehicle group. Next, to determine whether the pERK-IR neurons and BDNF-expressing neurons belonged to the same subset of DRG neurons, we performed double immunofluorescence for pERK and BDNF. In the vehicle group at 3 days after the CFA injection, pERK was detected in a subpopulation of BDNF-labelled neurons (67.0 ± 11.4%) and BDNF was detected in approximately 80% of pERK-labelled cells.

To elucidate whether alterations of endogenous NGF can trigger changes in both the phosphorylation of ERK and BDNF expression similar to those seen after peripheral inflammation, we performed intrathecal injections of rat recombinant βNGF. The DRG neurons in the NGF group had clear increases in the number of pERK-IR, BDNF-IR, and BDNF mRNA-positive neurons compared to the saline group at 3 days after surgery. These pERK- and BDNF-labelled neurons were primarily of small or medium diameter.

The present study demonstrated that peripheral inflammation produced heat and mechanical hypersensitivity on the ipsilateral hind paw and an increased number of BDNF-IR neurons and an increased expression of BDNF mRNA, suggesting an increased BDNF synthesis in the DRG. This increase was suppressed by intrathecal delivery of the MEK1/2 inhibitor, U0126. The contribution of NGF to the phenotypic change of DRG neurons has been investigated in several experimental peripheral inflammation models. We found that NGF injection produced an increase in the phosphorylation of ERK in the DRG, and further, an increase in the percentage of BDNF-IR neurons in the DRG. Taken together, these observations suggest that the activation of the ERK pathway is a key intracellular signalling event in the NGF-induced increase in the expression of BDNF in the peripheral inflammation model.

Phosphorylation of ERK in primary afferent neurons following peripheral axotomy (Obata et al 2003)

The number of pERK-IR neurons in the ipsilateral DRG markedly increased at 7 days after peripheral nerve injury and the increase in pERK-IR was seen mainly in medium- and large-sized neurons. In the ipsilateral DRG, the number of pERK-IR satellite cells was also greatly increased, particularly around large diameter neurons. The increase in the percentage of pERK-IR neurons in the ipsilateral DRG was first evident at 3 days after sciatic nerve lesion (6.8 ± 1.7%) and remained significant at 14 days after surgery (8.4 ± 2.1%), compared to those of naïve control rats. The size of neurons labelled for pERK-IR following sciatic

nerve transection was much larger than that of neurons labelled for pERK-IR following peripheral nerve injury.

We examined whether ERK activation is involved in BDNF up-regulation after the nerve lesion. The MEK inhibitor, U0126, suppressed the axotomy-induced elevation of BDNF-IR in the ipsilateral DRG. The neurons labelled for BDNF-IR in the vehicle group at 7 days after the lesion were clearly increased (26.2±1.1%), but the increase in the percentage of BDNF-IR neurons was blocked significantly by this MEK inhibitor (17.6±2.4%). Furthermore, to determine whether these pERK-IR neurons also expressed BDNF, we performed double immunostaining and found a significant number of neurons showing colocalization of pERK and BDNF in DRG neurons.

We also found that anti-NGF induced the increase in the number of pERK-IR, BDNF-IR and BDNF mRNA-positive neurons at 3 days after the intrathecal injection, but these pERK- and BDNF-labelled neurons were medium-to-large diameter sensory neurons. The pERK was located not only in large neuronal cells but also in some surrounding satellite glial cells. These data suggested that NGF antiserum could induce axotomy-like changes in pERK and BDNF expression in intact DRG.

The increased pERK expression that occurred after axotomy appeared mainly in medium- and large-sized neurons. The changes after axotomy thus contrast starkly with the massive pERK increase in TrkA-containing small neurons that occurs with NGF treatment (Averill et al 2001) and also peripheral inflammation in this study. Furthermore, pERK expression was also up-regulated in satellite glial cells that surrounded, in particular, the larger diameter neuronal somata. In the present study, sciatic nerve transection produced autotomy behaviour. Considering that U0126 blocked axotomy-produced ERK activation and autotomy behaviour, it is suggested that the ERK pathway in the DRG might be involved in the pathophysiology of neuropathic pain, as well as painful inflammation. The pathophysiological mechanisms of the phosphorylation of ERK and the increase in BDNF that occur in the axotomized medium-to-large diameter DRG neurons are not clear at this point. The reduction in endogenous NGF may be important for triggering a variety of changes in neuropeptides after axotomy, since exogenous NGF can reverse these changes (Verge et al 1995), and further, an antiserum against NGF can trigger changes in peptide expression similar to those seen after axotomy (Shadiack et al 2001).

Increase in the phosphorylation of ERK in DRG neurons following noxious stimulation to the normal tissue (Dai et al 2002)

We examined the pERK labelling after natural stimulation. We first examined the relationship between thermal stimuli at different temperatures and the induction of

pERK in DRG neurons. We applied thermal stimuli by immersion of the hind paw into warm to hot water (42–60 °C). The thermal stimulus of 42 °C only induced pERK in very small cells. Noxious heat stimulation at higher temperature was found to induce pERK in more and larger neurons. The double-labelling experiment revealed that some labelled neurons at 60 °C showed colocalization with NF200, indicating the activation of neurons with A fibres at higher temperatures.

We also examined pERK labelling after mechanical stimulation of the peripheral tissue. We applied high and low intensities of pinch stimulation to the plantar surface of the hind paw. The high-intensity pinch, in contrast with the low-intensity pinch, produced a greater number of DRG neurons that were labelled for pERK and these neurons tended to be larger. These data suggest that the increase in thermal and mechanical stimulus intensity was highly correlated with the number and size of DRG neurons in which the ERK cascade was activated.

An interesting finding in the present study is that the natural stimulation of the receptive field with different intensities resulted in changes in a subpopulation of pERK-labelled neurons. Most pERK-labelled neurons are presumably C polymodal nociceptors. Our data suggest that smaller neurons have lower thresholds in terms of pERK activation in C polymodal receptors. The intense stimuli induced pERK in NF200-containing neurons, probably in Aδ mechano-heat nociceptors. The threshold of the Aδ mechano-heat nociceptor is higher than that of C polymodal nociceptors (LaMotte et al 1983), which is consistent with our data that higher temperatures induce double labelling with pERK and NF200.

The characteristics of pERK labelling in DRG neurons after noxious stimuli clearly indicated that the pERK labelling is correlated with the activation state of primary afferent neurons. Therefore, we believe that examination of pERK is very useful as an indicator of the activated DRG neurons after noxious stimuli *in vivo*. Different noxious stimuli, such as capsaicin injection, mechanical stimuli and thermal stimuli, induced a variable number of DRG neurons labelled for pERK, suggesting that each noxious stimulus may have a different threshold for activation of the ERK pathway.

Mechanical stimuli to the inflamed tissue induce pERK in DRG neurons

We also examined the mechanical stimulation-evoked phosphorylation of ERK in DRG neurons in CFA-inflamed rats and normal rats. Mechanical stimulation to the hind paw of normal rats caused pERK labelling in a few small-sized DRG neurons. In contrast, we found many pERK-labelled small- and some medium-sized DRG neurons in CFA-inflamed rats following mechanical stimulation. To determine whether this phosphorylation of ERK is mediated by P2X receptors, we examined the effect of P2X receptor antagonists on the mechanical

stimulation-induced pERK labelling. Both PPADS (pyridoxal-phosphate-6-azophenyl-2′,4′-disulfonic acid) and trinitrophenyl(TNP)-ATP partly but significantly reversed the mechanical stimulation-induced phosphorylation of ERK in DRG neurons of the CFA rats. In order to identify these mechanical stimulation-evoked pERK-labelled neurons, we double labelled with $P2X_3$ and pERK. We found only a few double-labelled neurons in normal rats with mechanical stimulation. However, in CFA-inflamed rats that received mechanical stimulation, many DRG neurons labelled for pERK also expressed $P2X_3$.

In normal rats, we found that mechanical stimulation-induced pERK does not localize in neurons expressing $P2X_3$. Both PPADS and TNP-ATP failed to block the ERK phosphorylation induced by mechanical stimulation in DRG neurons, suggesting that the P2X receptors might not be involved in the mechanical response of DRG neurons in the normal condition. Phosphorylation of ERK induction by mechanical stimulation in normal rats might occur through the activation of channels other than P2X receptors. Alternatively, the amount of leaked extracellular ATP may be too small to activate the non-sensitized $P2X_3$ receptors under normal conditions. This finding agrees with a behavioural study using the von Frey test on $P2X_3$ knockout mice (Souslova et al 2000).

Phosphorylation of ERK in dorsal horn neurons in chronic pain model

We examined noxious stimuli-induced ERK phosphorylation in spinal dorsal horn neurons in a rat model of neuritis of the nerve root (Kominato et al 2003). Male Sprague–Dawley rats received the implantation of disc tissue that was obtained from coccygeal intervertebral discs. The number of phospho-ERK-LI neurons in L4/5 DRG in the neuritis group after the noxious mechanical stimulation significantly increased compared to the sham-treated group at 3 and 7 days after surgery. The increase in ERK phosphorylation in the spinal cord dorsal horn neurons indicates that responses/activation by the noxious stimulation applied to the periphery were elevated in spinal cord neurons in this neuritis model of the lumbar nerve root.

Summary

We have examined the detailed expression pattern of phosphorylated ERK in primary afferent neurons and also in dorsal horn neurons. We found that pERK showed distinctive expression in DRG neurons after peripheral inflammation, peripheral nerve transection or noxious stimuli to the normal tissue. The activation of an intracellular signal cascade in DRG neurons may have important roles in functional alteration of primary afferent neurons after pathological conditions or noxious stimulation. We also examined the pERK in post-synaptic

neurons after noxious stimuli in different chronic models and found several novel findings related to the neuroplastic changes in dorsal horn neurons.

References

Aley KO, Martin A, McMahon T, Mok J, Levine JD, Messing RO 2001 Nociceptor sensitization by extracellular signal-regulated kinases. J Neurosci 21:6933–6939
Atkins CM, Selcher JC, Petraitis JJ, Trzaskos JM, Sweatt JD 1998 The MAPK cascade is required for mammalian associative learning. Nat Neurosci 1:602–609
Averill S, Delcroix JD, Michael GJ, Tomlinson DR, Fernyhough P, Priestley JV 2001 Nerve growth factor modulates the activation status and fast axonal transport of ERK 1/2 in adult nociceptive neurones. Mol Cell Neurosci 18:183–196
Chang L, Karin M 2001 Mammalian MAP kinase signalling cascades. Nature 410:37–40
Dai Y, Iwata K, Fukuoka T et al 2002 Phosphorylation of extracellular signal-regulated kinase in primary afferent neurons by noxious stimuli and its involvement in peripheral sensitization. J Neurosci 22:7737–7745
Dubner R, Ruda MA 1992 Activity-dependent neuronal plasticity following tissue injury and inflammation. Trends Neurosci 15:96–103
Fields RD, Eshete F, Stevens B, Itoh K 1997 Action potential-dependent regulation of gene expression: temporal specificity in Ca^{2+}, cAMP-responsive element binding proteins, and mitogen-activated protein kinase signalling. J Neurosci 17:7252–7266
Gold MS, Levine J, Correa AM 1998 Modulation of TTX-R Ina by PKC and PKA and their role in PGE2-induced sensitization of rat sensory neurons in vitro. J Neurosci 18:10345–10355
Impey S, Obrietan K, Storm DR 1999 Making new connections: role of ERK/MAP kinase signaling in neuronal plasticity. Neuron 23:11–14
Kominato Y, Tachibana T, Dai Y et al 2003 Changes in phosphorylation of ERK and Fos expression in dorsal horn neurons following noxious stimulation in a rat model of neuritis of the nerve root. Brain Res 967:89–97
LaMotte RM, Thalhamme JG, Robinson CJ 1983 Peripheral neural correlates of magnitude of cutaneous pain and hyperalgesia: a comparison of neural events in monkey with sensory judgments in human. J Neurophysiol 50:1–26
Lewis TS, Shapiro PS, Ahn NG 1998 Signal transduction through MAP kinase cascades. Adv Cancer Res 74:49–139
Ma W, Zheng WH, Powell K, Jhamandas K, Quirion R 2001 Chronic morphine exposure increases the phosphorylation of MAP kinases and the transcription factor CREB in dorsal root ganglion neurons: an in vitro and in vivo study. Eur J Neurosci 14:1091–1104
Martin KC, Michael D, Rose JC et al 1997 MAP kinase translocates into the nucleus of the presynaptic cell and is required for long-term facilitation in Aplysia. Neuron 18:899–912
Obata K, Yamanaka H, Dai Y et al 2003 Differential activation of extracellular signal-regulated protein kinase in primary afferent neurons regulates brain-derived neurotrophic factor expression after peripheral inflammation and nerve injury. J Neurosci 23:4117–4126
Obrietan K, Impey S, Storm DR 1998 Light and circadian rhythmicity regulate MAP kinase activation in the suprachiasmatic nuclei. Nat Neurosci 1:693–700
Shadiack AM, Sun Y, Zigmond RE 2001 Nerve growth factor antiserum induces axotomy-like changes in neuropeptide expression in intact sympathetic and sensory neurons. J Neurosci 21:363–371
Snider WD, McMahon SB 1998 Tackling pain at the source: new ideas about nociceptors. Neuron 20:629–632
Souslova V, Cesare P, Ding Y et al 2000 Warm-coding deficits and aberrant inflammatory pain in mice lacking P2X3 receptors. Nature 407:1015–1017

Verge VM, Richardson PM, Wiesenfeld Hallin Z, Hokfelt T 1995 Differential influence of nerve growth factor on neuropeptide expression *in vivo*: a novel role in peptide suppression in adult sensory neurons. J Neurosci 15:2081–2096

Woolf CJ, Salter MW 2000 Neuronal plasticity: increasing the gain in pain. Science 288: 1765–1769

DISCUSSION

Mantyh: In the spinal cord and the dorsal column nuclei, is it neurons or glia that express pERK?

Noguchi: Both. There were definitely neurons, but other people have published that glial cells express pERK in this kind of experiment. We found that both glial cells and neurons express pERK.

Mantyh: So can you see this pERK expression increase in spinal cord dorsal column nuclei, thalamus and cortex?

Noguchi: As I have shown here, pERK expression is increased in spinal cord and dorsal column nuclei. However, there is no change in pERK expression in thalamus and cortex. For several years I have looked for something that shows a change of expression in the thalamus, but so far I haven't been successful.

Mantyh: How many days after treatment did you look?

Noguchi: I looked 3 days, 7 days and 2 weeks after nerve injury, but no longer.

Devor: How long after the electrical stimulation did you look in the dorsal horn?

Noguchi: I looked at pERK 2–3 minutes after electrical stimulation. It is very difficult to look at pERK sooner than 2 minutes because of methodological limitations. It is a very rapid response. This time course is similar to the phosphorylation of ERK in the dorsal horn reported by Ji and Woolf.

Dray: I have a question relating to the spared nerve model. Were the features that you measured correlated with the time course of the behaviour 7–14 days after lesion? It seemed that some markers were still expressed at a high level after 14 d. I wonder whether after 6 weeks or several months the expression remains high and correlates with behaviour, or whether there is a mismatch between neuropathic behaviour and gene expression.

Noguchi: At first, I have to say that the time course of changes in L4 and L5 DRG are different in Chung's model. The change in L4 DRG does not last so long. After several days we can detect a significant increase that continues for at least one or two weeks. But after several weeks there is almost no change. In contrast, L5 DRG after axotomy showed a very consistent and prolonged change in their expression. I think the L4 changing pattern is like that after peripheral inflammation. Two or three days after CFA injection into the hindpaw, we can detect thermal and mechanical hyperalgesia, and elevated expression of several molecules, so there is some correlation with behaviour and gene expression. In fact, I agree that there are

some mismatches of time course between neuropathic pain behaviour and gene expression in L4 DRG in Chung's model.

Dray: I would like to understand whether it is continued nerve activation as well as some injury related factor which causes the increased expression of the P kinase and ERK. Have you done the experiment where you block the input with a local anaesthetic or give an opioid? How does this affect the pattern of distribution you see after a nerve injury?

Noguchi: I have explained that pERK induction after noxious stimuli to normal tissue is a rapid response. These responses are completely abolished by application of lidocaine to the sciatic nerve. Clearly, this response is due to the transmission of action potentials, and not other factors. Of course I also examined CFA-induced pERK induction in L5 DRG and found the significant increase one day after CFA injection. In the experiment of nerve injury, we did not do any blocking experiments. I do not have any data as to whether the nerve activity affects the pERK expression pattern in injured primary afferents.

Dray: There has been much discussion about reducing the need for analgesics with pre-emptive block on the afferent input. If you do this, does this modify what occurs subsequently in the spinal cord? How long must block be maintained to prevent spinal changes?

Noguchi: I understand what you are saying. However, I have never done such experiments to examine the pre-emptive effects of local anaesthetics on the spinal cord so far. I have no idea whether very long local anaesthetic application affects the changes in dorsal horn described here.

I showed the pERK activation in the dorsal horn neurons. I have used several pain models that are clinically related to see the activity of dorsal horn neurons. For example, we made a lumbar canal stenosis model and examined the change of pERK in dorsal horn neurons to see the effect of a drug. We found a decrease of pERK after a drug was administered but this was not pre-emptive.

Belmonte: I was puzzled by the response of the large DRG neurons when you reached 60 °C. Do you think this is a specific response to heat, of a native ion channel present in these cells, or that at 60 °C you start to produce non-specific damage to the axon? How do you explain the ERK response in these large neurons when you reach these high temperatures?

Noguchi: I don't have an answer. I found 42 °C induced pERK in very small neurons. But high temperature showed larger neurons, but still C fibres are mainly labelled for pERK. I have looked for many physiological papers to see the relationship between cell sizes in the C fibre population and temperature threshold or any other functions, but I couldn't find these kinds of data.

Perl: Carlos Belmonte has asked an important question. That is, why do you see the up-regulation of pERK in large neurons at high temperature? We need to keep in mind that the correlation between fibre diameter and soma size in the DRG is not

perfect. Small DRG somata are not necessarily only the cell bodies of the smallest diameter fibres in the periphery. Large somata may in fact be associated with medium or small/medium diameter fibres. A subset of cutaneous nociceptors with myelinated fibres have relatively large cell bodies. Some of these are excited by high temperatures after prior exposure to noxious heat or sometimes after prolonged exposure to noxious heat. These are usually classified as high threshold mechanoreceptors. So just because it's a large DRG cell body, does not necessarily specify a large fibre. Conversely, a small cell body does not specify a small diameter peripheral fibre.

McMahon: To follow that up, if you stimulate a peripheral nerve electrically, do you get induction of ERK in all of the cells that are activated? This would be the implication of your findings, but have you tested it explicitly?

Noguchi: I don't know whether all excited cells express pERK. I don't know how to check this.

McMahon: You could simply electrically stimulate the sciatic nerve.

Noguchi: Yes. I have stimulated the sciatic nerve, and found many but not all neurons are positive for pERK. We found very few neurons are positive at 0.1 mA, but at 0.3 mA some neurons were. But the C-fibre-level stimulation induced pERK in many neurons. Whether all excited neurons express pERK is a tough question to answer, but maybe not all neurons express pERK.

McMahon: You could stimulate a spinal nerve, where you would be stimulating all of the axons coming from one dorsal root ganglion.

Noguchi: I don't think all C fibres express pERK. This means that each neuron has a different threshold for expressing pERK. This is my supposition.

McMahon: What is your explanation for the effects of anti-NGF? This struck me as an unexpected finding. You are giving an antibody intrathecally and this changes gene expression very rapidly.

Noguchi: We checked it three days after injection.

McMahon: Even so, how is that working? It presumably is not getting into the DRG cells? How is it changing the availability of NGF in DRG cells? Or is it simply an effect of giving a large amount of foreign protein? Is there a control with some other antibody?

Noguchi: I agree, it is an unexpected finding. One explanation is that many medium-sized neurons express pERK, and TrkA is expressed in small neurons and some medium sized neurons. So these medium sized neurons may also be regulated by NGF. These neurons express BDNF and neuropeptide Y after injection of NGF antibody, suggesting that these neurons are negatively regulated by NGF. The NGF antibody injected intrathecally may come to DRG cell bodies and result in the decrease of available NGF in the DRG, and induce pERK, and finally increase the expression of BDNF.

Oh: You saw a great increase in ERK or MAP kinase in the DRG cells for a long time. Does this lead to cell death or cell toxicity?

Noguchi: Usually MAP kinase is known to regulate transcription. There may be some effects on apoptosis, but I don't have any data on this.

Mao: It seems to me that the MAP kinase family is unlikely to be a specific marker of pathological pain for several reasons. First, it can be activated by transient stimulation, although it can also be activated under conditions such as nerve injury and inflammation. Second, the activation of MAP kinases is rather far down in the intracellular pathway, well downstream. It almost serves as a converging point for many other kinases. It could lose specificity because of this. Third, in my limited experience with MAP kinases, the outcome of regulating MAP kinases depends on what sort of target gene MAP kinase is trying to regulate. It has been shown that MAP kinases are involved in regulating cannabinoid receptors, but they also regulate the expression of glutamate transporters. The outcome or the interpretation of the involvement of MAP kinase in neuropathic pain is rather complex. If it regulates genes involved in the generation of neuropathic pain, then MAP kinases are probably contributing to the mechanisms of neuropathic pain. On the other hand, MAP kinases could regulate genes that prevent or reduce neuropathic pain.

Noguchi: I understand your comments. MAP kinase is widely distributed in all tissues. Of course, these kinds of intracellular cascades involving PKA and PKC are also widespread. I'm not saying that this event is specific for neuropathic pain. So far I don't have any intention to target MAP kinases for treatment of neuropathic pain. At least I can see the excitability changes in some specific neurons in the DRG, to show the very dynamic changes in neuropathic pain conditions. So we can get important information this way.

Mao: That is precisely what I am trying to get at. If it were not a specific mechanism, I don't think it would be appropriate to target MAP kinases as a treatment tool. If MAP kinases regulate genes encoding different outcome proteins, then if you shut down MAP kinases you shut down all the downstream pathways, which could probably create a situation that is difficult to interpret.

Perl: Could you go over the experimental circumstances that led you to the observation that the deeply located dorsal horn neurons showed pERK activation? There was nerve injury, and electrical stimulation.

Noguchi: Yes, there was nerve injury and we stimulated the injured nerve electrically.

Perl: Were there lesions of L4 and L5 roots or spinal nerves?

Noguchi: I cut the sciatic nerve 7 d previously, and I stimulated it electrically just proximal to the neuron. We didn't have a spinal nerve injury in that preparation. We saw the changes I described.

Gintzler: The implication is that whenever you see these neurochemical changes, they are causative, mediating hypersensitivity or neuropathic pain. But they could also be the body's attempt to compensate for the increased sensitivity or hyperexcitability. I'd be interested to see how people dissect this. How do you distinguish between something that is causative and mediates the hyperalgesia, versus neurochemical changes that represent the body's attempt to compensate and to ameliorate this hyperexcitability?

McMahon: The c-fos knockout is an example in point. It doesn't show any deficit in pain studies, so it may be that *c*-fos is a compensatory mechanism. So, I agree with you.

Noguchi: I also agree. The crucial question is what target protein of the transcription factor there is or what is downstream of it. It is a very difficult question to answer: whether a response is causative or a compensating mechanism.

Mantyh: You showed several changes in the neurons present in DRG. Do you also see these changes in the Schwann cells, non-myelinating and myelinating?

Noguchi: I haven't studied this. I found a report in the literature suggesting that Schwann cells express pERK after nerve injury (Reynolds et al 2001).

Mantyh: Is that in both the myelinating and the non-myelinating Schwann cells?

Noguchi: I'm not sure.

McMahon: We have looked in damaged nerve and see a clear up-regulation in Schwann cells.

Mantyh: Are there differences between the myelinating and non-myelinating?

McMahon: We haven't looked.

Dray: With respect to the expression of p38 and ERK, there have been a number of studies using selective p38 inhibitors as well as ERK inhibitors. These clearly show that they do affect neuropathic pain behaviour. The suggestion is that the expression is related to the hyperexcitability state and relates to the behavioural manifestations, such as mechanical allodynia. Earlier, I was pressing the point about the importance of the time in terms of the expression pattern. This was because the studies that I have seen suggesting that only early interventions, up to a couple of weeks after the nerve injury, change pain behaviour. Later intervention with an inhibitor does not change behaviour. This suggests that p38 involvement is a transient event and that it may not be related to the chronicity of the neuropathic pain. This bears some further re-examination.

Wood: What is the relationship between activated neurons and the support cells that are showing this increased kinase activity? Can you speculate on the significance of these changes of activity in the support cells?

Noguchi: Dr Inoue talked about the importance of glial cells in pain transmission. Recently, many pain researchers have suggested that glial cells are important in modulating pain. In addition to astrocytes and microglia in the spinal cord, we also need to examine the role of satellite cells, because the DRG

neurons are surrounded by them. If these satellite cells release neuroactive molecules, such as cytokines or neurotrophic factors, perhaps the neurons may change their excitability. The change in MAP kinases in satellite cells after peripheral nerve injury may be very important to regulate the excitability of DRG neurons. For instance, in a lumbar canal herniation model we reported, the inflammatory responses occur in DRG neuron cell bodies, and inflammatory cells invade the DRG tissue and release NGF and proinflammatory cytokines. These molecules might change glial cells or satellite cells.

Reference

Reynolds AJ, Hendry IA, Bartlett SE 2001 Anterograde and retrograde transport of active extracellular signal-related kinase 1 (ERK1) in the ligated rat sciatic nerve. Neuroscience 105:761–771

Functional reorganization of the spinal pain pathways in developmental and pathological conditions

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Abstract. Following inflammation, a subpopulation of Aβ afferents that terminates preferentially in deeper laminae have been shown to extend their axons to the superficial dorsal horn, particularly substantia gelatinosa (SG). Similarly, SG neurons in immature spinal cord receive mainly Aβ afferent inputs. To clarify whether the reorganized sensory pathway in the inflamed rats has a functional similarity with that in the developmental state, we compared synaptic inputs from primary afferents using *in vitro* and *in vivo* patch-clamp recordings from SG neurons. SG neurons in the mature state had monosynaptic inputs from Aδ and C afferents, while only a few neurons received inputs from Aβ afferents. Following inflammation, the Aβ afferents extended their axons to SG and established functional monosynaptic transmission. Meanwhile, SG neurons in the immature state received preferentially Aβ as well as Aδ afferent inputs, and the majority of Aβ afferent inputs were monosynaptic. These observations support the idea that the sprouting of the large afferent fibres observed in inflamed rats is, at least in part, a regeneration process. However, the process, maybe distinct at some point from the process during development, therefore, produces pathological pain. Though the idea that the regeneration mimics the developmental process has been widely accepted, other possibilities cannot be excluded.

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Hyperalgesia or allodynia has been considered as either an increased sensation of pain following noxious stimuli (hyperalgesia) or as the sensation of pain in response to normally innocuous stimuli (allodynia), and these changes are commonly observed in peripheral inflammation. A change in excitability of neurons in the dorsal horn may also participate in the generation of hyperalgesia. This change in excitability manifests as either an increase in firing rates in response to electrical stimulation or a novel occurrence of responses to low-intensity stimuli (Simone et al 1991, Woolf et al 1994); they appear to be mediated by various substances, such as glutamate (Ma & Woolf 1995a), substance P (Ma & Woolf

1995b) and ATP (Moriyama et al 2003). These modifications collectively constitute the phenomenon of central sensitization. Alternatively, the allodynia may be produced by a change in circuitry in the dorsal horn, in such a way that afferents innervating low threshold mechanoreceptors begin to induce pain. Evidence for this idea has come from neuroanatomical studies (Woolf et al 1992, Koeber et al 1994), although it is unknown whether this reorganization is functional. An electrophysiological study has provided evidence suggesting that interneurons within the substantia gelatinosa (SG) establish a novel synaptic connection with the sprouting Aβ afferents following the inflammation (Nakatsuka et al 1999). Considering that the SG plays a critical role in the processing of the sensation of pain and that Aβ afferents convey innocuous information, the transfer of innocuous information to SG neurons is conceivably involved in the development of allodynia. Analogously, neurons in SG at the early developmental state are innervated predominantly by Aβ rather than C afferents (Park et al 1999). This physiological innervation of SG neurons by Aβ afferents in the immature state is interpreted by anatomical evidence as demonstrating that the development of C afferents is delayed and C afferents enter the superficial dorsal horn at the 2–3 week postnatal state (Fitzgerald 1987). This developmental delay in transmission of certain sensory information may be compensated for by the innervation of Aβ afferents. These observations imply that the sprouting of Aβ afferents following inflammation is in part a regenerative process. This notion is further supported by several lines of evidence. For instance, each muscle fibre is innervated by multiple motor nerves in the immature state; following maturation each muscle fibre becomes singly innervated. Whereas after nerve cutting, motor units mimic the immature state, the single muscle fibre is again innervated by multiple motor nerves (Brown et al 1981).

The present study was designed to address the consequential changes in sensory pathways following peripheral inflammation and distinction in the pathways of the spinal dorsal horn between inflamed and immature states, by comparing glutamatergic excitatory synaptic responses elicited in SG neurons by the stimulation of primary afferents both in spinal cord slices and *in vivo* preparations.

Methods

In vitro slice patch-clamp recording

Male Sprague–Dawley rats (7–10 weeks old) were anaesthetized with urethane and then a lumbosacral laminectomy was performed (Ito et al 2000, Okamoto et al 2001). About 2 cm of lumbar spinal cord was removed and placed in a cold Krebs solution. After this, we cut all the ventral and dorsal roots near the root entry zone, except for the L4 or L5 dorsal root on one side. Following removal of the

pia-arachnoid membrane except around the preserved L4 or L5 dorsal root, we cut a 600–650 μm thickness of a transverse slice with the dorsal root attached using a Vibratome. The slice was placed on a nylon mesh in the recording chamber and perfused with warmed preoxygenated Krebs solution. We then recorded miniature excitatory postsynaptic currents (EPSCs) in the presence of tetrodotoxin (TTX) and evoked responses by the dorsal root stimulation were recorded by the whole cell patch-clamp recording. The dorsal root was stimulated with a suction electrode. Similar slice preparations were also made from immature rats (3 weeks old).

In vivo patch-clamp recording

Under artificial ventilation, and monitoring body temperature and blood pressure, we performed a lumbosacral laminectomy at the level of L4 or L5 and the rat was then placed in a stereotaxic apparatus. The surface of the spinal cord was perfused continuously with warmed preoxygenated Krebs solution. A patch electrode was inserted into the dorsal horn at an angle of 30° through a window opened at the pia-arachnoid membrane. Identification of neurons as SG was made by their depth from the surface of the spinal cord and also by morphological features through injection of neurobiotin through the electrodes. The noxious and innocuous mechanical stimuli used were pinching of skin folds with toothed forceps and puffing air onto the skin, respectively. To confirm that the responses evoked by pinch were really mediated by the selective activation of nociceptors, the forceps were fixed on a rod and various sizes of weights were put on the forceps. The response was increased in frequency with increasing weight and accommodation was hardly observed, indicating that the responses were mediated by the selective activation of nociceptors (Furue et al 1999, Narikawa et al 2000).

Inflamed rat preparation

Injection of complete Freund's adjuvant (CFA) into the hindpaw of rats caused significant hypersensitivity to mechanical stimulation tested with von Frey hairs. The hypersensitivity lasted for at least 2 weeks. The spinal cord slice preparations with the dorsal root attached were made from the inflamed rats at 10 days after inflammation.

Intracellular recordings from DRG neurons

To determine thresholds and conduction velocities of Aβ, Aδ and C afferents, intracellular recordings were made from dorsal root ganglion (DRG) neurons isolated from normal, inflamed and immature rats (Nakatsuka et al 1999, Park

et al 1999). The proximal end of the dorsal root was stimulated with the same suction electrode that was used to activate the afferents in the slice experiments. Based on conduction velocities, threshold stimulus intensities and dv/dt of the antidromic action potentials, the afferents were divided into three subclasses, Aβ, Aδ and C afferents. The conduction velocities and stimulus intensities were not significantly different in normal and inflamed rat DRG. The parameters tested in immature rats were significantly different from those in mature rats. The conduction velocities were slower and the stimulus thresholds increased significantly, but the afferents could still be divided into three subgroups according to the criteria used for mature rat DRG. These conduction velocities and stimulus intensities were applied to identify the afferents in slice examinations.

Results and discussion

Synaptic inputs to SG neurons from afferents in normal rat spinal cord

Stimulation of the dorsal root with the suction electrode elicited Aδ and/or C afferent EPSCs in SG neurons of normal rats. 69% of Aδ afferent-evoked EPSCs were monosynaptic and 30% of C afferent-evoked EPSCs were also monosynaptic. A few neurons received Aβ afferent monosynaptic EPSCs (2%). The remaining 29% and 70% of Aβ/Aδ and C afferents, respectively, were mediated through interneurons (Table 1). All together, these observations indicate that SG neurons receive inputs from primary afferents, preferentially Aδ and C. These observations

TABLE 1 Synaptic inputs to substantia gelatinosa neurons in normal and inflamed rats

A-fibre evoked EPSCs		Nomal ($n=41$)	Inflamed ($n=30$)
Aβ evoked	Mono	1 (2%)	10 (33%)
	Poly	3 (7%)	2 (7%)
Aδ evoked	Mono	28 (69%)	6 (20%)
	Poly	9 (22%)	12 (40%)
C-fibre evoked EPSCs		Normal ($n=36$)	Inflamed ($n=33$)
	Mono	11 (30%)	10 (30%)
	Poly	25 (70%)	23 (70%)
A and C responses		Normal ($n=43$)	Inflamed ($n=43$)
		35 (81%)	20 (47%)

Note that Aβ afferent-evoked EPSCs are observed in only 2% of neurons, while about 70% of neurons receive monosynaptic inputs from Aδ afferents in normal condition. In contrast, Aβ afferent-evoked monosynaptic responses are increased significantly to 33% in inflamed rats.

are consistent with previously reported anatomical examinations, in which the Aβ afferents show a flame-like arborization at the border of lamina III and SG, and a few Aβ afferents penetrate the border and enter the SG.

Synaptic inputs to SG neurons from afferents in inflamed rat spinal cord

Next we tested the synaptic inputs to SG neurons from primary afferents in inflamed rats. In contrast to the normal rat spinal dorsal horn, SG neurons in slices received a large number of monosynaptic Aβ-afferent inputs. Single stimuli with strength sufficient to activate Aβ afferents elicited a monosynaptic EPSC in 30% ($n = 36$) of neurons tested, since the latency of EPSC was short (less than 0.8 ms) and no failures were observed when the dorsal root was stimulated repetitively at 20 Hz. In addition, SG neurons receiving monosynaptic Aδ afferent inputs decreased in number (from 69% to 20%). Coincidently, polysynaptic Aδ afferent inputs increased from 22% to 40%.

In vivo patch-clamp recordings from SG neurons in inflamed rat spinal cord

Observed Aβ afferents sprouting into SG are presumably induced by a change in synaptic transmission at the early stage of inflammation. To address this possibility, the *in vivo* patch-clamp recordings were made from SG neurons in rats 2 days after inflammation (unpublished observation). In normal rats, all SG neurons recorded exhibited spontaneous EPSCs; these EPSCs were still observed in the presence of TTX, indicating that these were miniature EPSCs (mEPSCs). Mechanical noxious and innocuous stimuli applied to the ipsilateral hindpaw elicited a barrage of large amplitude EPSCs. The EPSCs evoked by noxious stimuli showed no accommodation, while the innocuous stimuli elicited a response at the beginning and end of the stimuli. Thus the innocuous response showed significant accommodation. In contrast, the majority of neurons in inflamed rat spinal cord exhibited large amplitude EPSCs as well as mEPSCs. The large amplitude EPSCs were blocked by TTX applied at the dorsal root, indicating that the responses are mediated by spontaneous firings initiated at the inflamed region. The innocuous stimuli in inflamed rats elicited a continuous barrage of EPSCs without accommodation (Fig. 1). The noxious stimuli produced EPSCs with much higher frequency than that in normal rats. These observations suggest that a large amount of sensory inputs to SG neurons are, at least in part, responsible for triggering the sprouting observed in SG 10 days after inflammation.

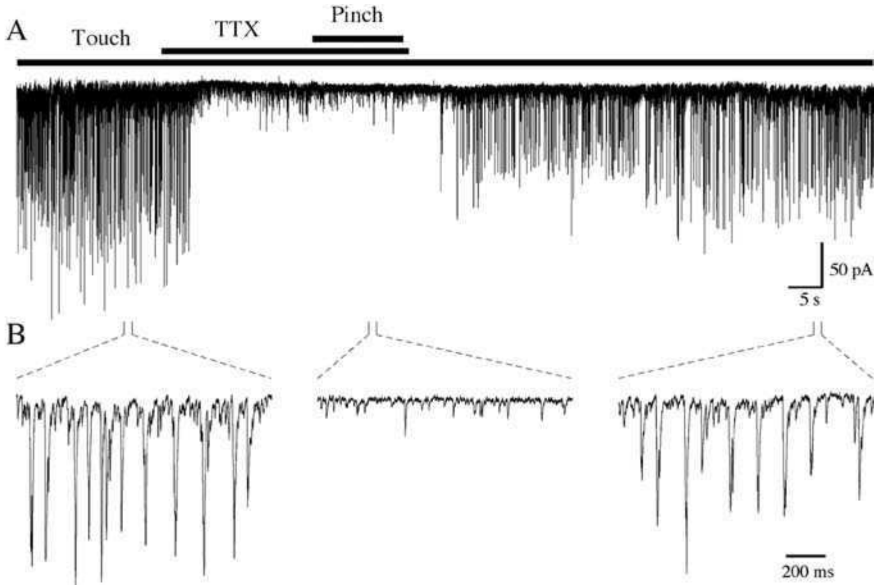

FIG. 1. (A) Synaptic responses of substantia gelatinosa (SG) neurons recorded *in vivo* in response to touch and pinch stimuli applied to the hindlimb. The touch stimulation produces a barrage of large amplitude EPSCs without showing accommodation. TTX applied to the dorsal root eliminates the large amplitude EPSCs, leaving small EPSCs that seem to be miniature EPSCs. Pinch stimuli evoke no response in the presence of TTX. (B) shows synaptic responses with fast time scale.

Synaptic responses evoked in immature SG neurons in response to primary afferent stimulation

In immature rat SG neurons, 10 out of 24 neurons exhibited monosynaptic EPSCs evoked by low intensity stimulation sufficient to activate Aβ afferents that had short and constant latencies even when the dorsal root was stimulated repetitively at the frequency of 50 Hz. The remaining EPSCs in 14 neurons had a long and variable latency, indicating these were elicited through the polysynaptic pathway. Thus, these results indicate that the EPSCs in immature SG neurons receive predominantly Aβ afferent inputs (Nakatsuka et al 1999).

The results obtained from inflamed and immature rats have some similarities. In normal rat, a few Aβ afferents penetrate the border between lamina III and SG, while many of Aβ afferents in inflamed rats sprout into SG neurons and establish a functional synaptic contact (Fig. 2). This plastic change is analogous to the circuit in the immature state. The innervation of SG neurons by Aβ afferents is presumably due to a slow development of unmyelinated C afferent fibres (Fitzgerald 1987). Using a selective labelling technique with horseradish

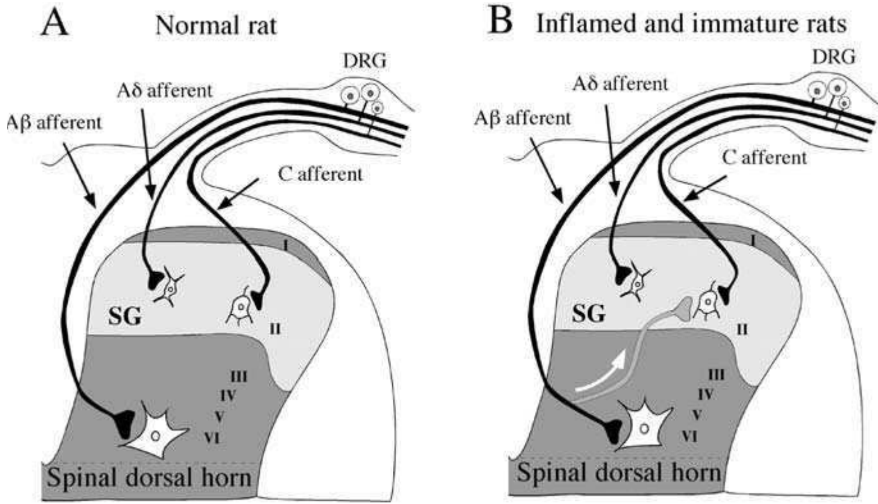

FIG. 2. Schematic diagram of the termination of primary afferents and possible plastic change of Aβ afferents induced by inflammation. Note that in immature condition, a small number of C afferents makes synaptic contact with substantia gelatinosa neurons.

peroxidase conjugated to choleragenoid, she has detected dense Aβ afferent termination in SG until postnatal day 21. Thereafter, the Aβ afferents retract and the terminal fields are restricted to laminae III to V. As mentioned earlier, the anatomical evidence demonstrates that the development of C afferents has a delay and enters the superficial dorsal horn at 1–2 weeks postnatally (Fitzgerald 1987). This time course of C afferent development is somewhat earlier than that we observed in an electrophysiological study. This difference may be due to a delay in establishing a functional synaptic contact of C afferents with SG neurons at the early state. The sprouting of Aβ afferents into SG neurons and formation of synaptic contact with SG neurons following inflammation are similar to those occurring in the immature state. As has been reported in various regions, particularly in the periphery, the regeneration process of damaged peripheral nerves mimics the early developmental state. For example, if motor nerves are cut in adults, they then sprout and innervate muscle fibres. Different from normal conditions, a single muscle fibre is innervated by multiple nerves (Brown et al 1981). Additionally a nicotinic receptor subtype that is expressed at the neuromuscular junctions is also substituted by the other subtype that is prevalent in the immature state (Mishina et al 1986). This transient change in innervation of muscle fibres and receptor expression again returns to the mature state with time. Therefore, the sprouting of Aβ afferent into SG is assumed to be a process of

regeneration. However, the regeneration process could not mimic entirely the process of development: in other words, the plastic changes observed in inflammation are somewhat distinct from those of development. These differences may be responsible for hypersensitivity in sensory transmission. Therefore, it will be important to clarify these differences in order to prevent the production of pathological pain.

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References

Brown MC, Holland RL, Hopkins WG 1981 Motor nerve sprouting. Annu Rev Neurosci 4:17–42

Fitzgerald M 1987 Prenatal growth of fine-diameter primary afferents into the rat spinal cord: a transganglionic tracer study. J Comp Neurol 261:98–104

Furue H, Narikawa K, Kumamoto E, Yoshimura M 1999 Responsiveness of rat substantia gelatinosa neurones to mechanical but not thermal stimuli revealed by in vivo patch-clamp recording. J Physiol 521:529–535

Ito A, Kumamoto E, Takeda M et al 2000 Mechanisms for ovariectomy-induced hyperalgesia and its relief by calcitonin: participation of 5-HT1A-like receptor on C-afferent terminals in substantia gelatinosa of the rat spinal cord. J Neurosci 20:6302–6308

Koeber HR, Mirnics K, Brown PB, Mendell LM 1994 Central sprouting and functional plasticity of regenerated primary afferents. J Neurosci 14:3655–3671

Ma Q-P, Woolf CJ 1995a Noxious stimuli induce an N-methyl-D-aspartate receptor-dependent hypersensitivity of the flexion withdrawal reflex to touch: implications for the treatment of mechanical allodynia. Pain 61:383–390

Ma Q-P, Woolf CJ 1995b Involvement of neurokinin receptors in the induction but not the maintenance of mechanical allodynia in rat flexor motoneurones. J Physiol 486:769–777

Mishina M, Takai T, Imoto K et al 1986 Molecular distinction between fetal and adult forms of muscle acetylcholine receptor. Nature 321:406–411

Moriyama T, Iida T, Kobayashi K et al 2003 Possible involvement of P2Y2 metabotropic receptors in ATP-induced transient receptor potential vanilloid receptor 1-mediated thermal hypersensitivity. J Neurosci 23:6058–6062

Nakatsuka T, Park J-S, Kumamoto E, Tamaki T, Yoshimura M 1999 Plastic changes in sensory inputs to rat substantia gelatinosa neurons following peripheral inflammation. Pain 82:39–47

Narikawa K, Furue H, Kumamoto E, Yoshimura M 2000 In vivo patch-clamp analysis of IPSCs evoked in rat substantia gelatinosa neurons by cutaneous mechanical stimulation. J Neurophysiol 84:2171–2174

Okamoto M, Baba H, Goldstein PA, Higashi H, Shimoji K, Yoshimura M 2001 Functional reorganization of sensory pathways in the rat spinal dorsal horn following peripheral nerve injury. J Physiol 532:241–250

Park J-S, Nakatsuka T, Nagata K, Higashi H, Yoshimura M 1999 Reorganization of the primary afferent termination in the rat spinal dorsal horn during post-natal development. Brain Res Dev Brain Res 113:29–36

Simmone DA, Sorkin LS, Oh U et al 1991 Neurogenic hyperalgesia: central neural correlates in responses of spinothalamic tract neurons. J Neurophysiol 66:228–246
Woolf CJ, Shortland P, Coggeshall RE 1992 Peripheral nerve injury triggers central sprouting of myelinated afferents. Nature 355:75–78
Woolf CJ, Sortland P, Sivilotti LG 1994 Sensitization of high mechanothreshold superficial dorsal horn and flexor motor neurones following chemosensitive primary afferent activation. Pain 58:141–155

DISCUSSION

Zhuo: Those were beautiful data. I want to offer one possible interpretation of increased Aβ mediating the monosynaptic response. This possibility could be that the post-synaptic trafficking of the AMPA receptor is altered after injury. Previously you couldn't detect a response but now you can.

Yoshimura: We haven't looked at this yet. You are considering a silent synapse (Li & Zhuo 1998), but we didn't check the synaptic responses at the different holding potentials. It is possible that inflammation transforms a silent synapse into a functional one. In the early stages of inflammation, we could see that there was some sprouting of the Aβ fibres to the interneurons located at the deeper laminae. In that condition we saw only polysynaptic inputs from Aβ fibres to substantia gelatinosa neurons. Following the gradual change of Aβ sprouting during the early stage of inflammation, we re-examined the monosynaptic connection of Aβ afferents with substantia gelatinosa neurons 7–10 d later. These gradual changes in synaptic connection in the spinal dorsal horn following inflammation may not be consistent with the notion you mentioned. However, we cannot exclude your possibility with certainty.

Zhuo: I have another question relating to the effect of BDNF on sensory synaptic transmission. Have you followed this for a long time? People have reported BDNF producing an effect lasting for a few hours.

Yoshimura: We applied BDNF on slice preparations for just one or two minutes. The effects of BDNF lasted for about 4–5 minutes, which is not long lasting in our conditions.

Tominaga: You seem to explain the phenomenon by the failure of the regeneration process, for example, found in the motoneurons. In the case of the motoneurons, the polysynaptic connection will go back to the monosynaptic one at the end of the process. But in the case of the neuropathic pain, will the behavioural abnormality last a long time?

Yoshimura: One of the behavioural studies shows that the inflamed rats exhibit a gradual increase in threshold to mechanical stimulation and no significant difference in mechanical threshold is observed 4 weeks after inflammation. In addition, the disappearance of hyperalgesia induced by chronic constriction injury to the sciatic nerve is also reported by the same authors (Goff et al 1998).

Therefore, hyperalgesia or allodynia induced by nerve injury or inflammation may not be long lasting, at least in certain pain models. These observations seem to be analogous to that observed in the periphery. In the case of motoneurons, the multi-motor fibre innervation was shown to have reverted to the mature condition, in which the single muscles were innervated by single motoneurons, a long time after nerve transection.

Tominaga: Have you checked this in your system? If that is the case in the neuropathic pain model, the phenomenon which you observed should become normal after 4 weeks.

Yoshimura: No, we haven't done this using slice preparations. The behavioural study suggests that the plastic changes in the sensory circuitry in the spinal cord induced by nerve injury or inflammation are restored to the normal pathways. We should test this possibility in the slice preparations obtained from rats 1–2 months after inflammation.

Perl: I have no problem with the observations, but I am concerned about some conclusions. First, you suggest that there is sprouting of the Aβ fibres based on the presence of Aβ fibre-related responses after inflammation. There is evidence that Aβ fibre nociceptors terminate in the superficial dorsal horn under normal circumstances. What you may have seen could be explained by an increase of background excitability which would allow subthreshold responses to manifest themselves. There is good evidence that there are substantially different synaptic connections to different groups of SG neurons. These differences are related to neuronal morphology. You are aware of the evidence that we have presented in this direction (Grudt & Perl 2002). Neurons in the substantia gelatinosa in slice preparations from normal rodents have substantial TTX-insensitive background activity. Other neurons in the same preparations exhibit TTX-sensitive spontaneous EPSPs. In the slices from normal animals did you have examples of SG neurons that had TTX insensitive spontaneous activity?

Yoshimura: As I mentioned in my talk, many neurons exhibited spontaneous large amplitude EPSCs which were mediated by action potentials initiated at the periphery, because those were blocked by TTX and disappeared in slice preparations in which the peripheral inflamed site was cut. When you look at the amplitude of miniature EPSCs in the presence of TTX or in the slice preparations, the amplitude of mEPSCs was not significantly different from those observed in normal preparations, *in vivo* or *in vitro*. These results indicate that the sensitivity of postsynaptic receptors is not significantly affected by inflammation. Thus, the increase in the incidence of inputs from Aβ afferents may not be due to the increase in excitability of SG neurons. As I mentioned in my talk and demonstrated in the previous papers, I believe that the small number of SG neurons receive direct inputs from Aβ afferents even in the normal condition and this ratio is increased when records are made from slices obtained from neonatal or

young adult rats (1–4 weeks). The decrease in the number of neurons receiving Aβ afferents in the mature state is not likely to be due to the development of silent synapses, since the silent synapses decrease in number with maturation (Baba et al 2000). In response to your last question, what I said is that we didn't see any neurons with spontaneous EPSCs in the slice preparations. 'Spontaneous' used here means EPSCs mediated by action potentials but not quantal release of glutamate. Thus, all neurons we tested showed only miniature EPSCs but not spontaneous EPSCs in the slice preparations.

Perl: Did you not note spontaneous activity after TTX?

Yoshimura: I am saying that SG neurons exhibited only miniature but not spontaneous EPSCs after TTX.

Perl: I agree. The large amplitude spontaneous EPSPs are mostly TTX-sensitive. But there are some neurons that show such activity and others which do not in tissue from normal animals. Thus, you often find mixed spontaneous activity with both TTX sensitive and TTX insensitive EPSPs present. Therefore one cannot use the presence of TTX sensitive spontaneous EPSPs as an absolute judge of the difference between normal and experimental preparations.

Yoshimura: Under our recording conditions in slices, we didn't see any spontaneous EPSCs even without TTX. Similarly, any neurons in slices obtained from inflamed rats also showed no spontaneous EPSCs. The spontaneous EPSCs were detected in *in vivo* preparations obtained from inflamed rats, after the sensitization of peripheral sensory receptors.

Perl: My main point is that there are different kinds of SG neurons. Therefore, one cannot compare unknown populations of SG neurons unless you develop a spectrum of characteristics that allow you to define and specify which neurons are involved. Anatomical evidence shows projections from myelinated fibres of medium size to the superficial dorsal horn. The changes in responses from normal to inflamed rat are not necessarily indicative of sprouting. It may simply reflect alterations in background level of excitability so that subthreshold responses become superthreshold.

Yoshimura: We always check the morphological features of the recorded neuron. We are making a correlation between the morphology and the synaptic input to SG neurons. So far we haven't found any significant difference in synaptic input in different types of SG neurons.

Perl: Eventually you will find these differences!

Yoshimura: It was quite difficult to find any correlation between the morphology and synaptic input from the primary afferents. I can't say anything about the correlation between synaptic inputs and cell types.

Devor: I also have problems with the interpretation. In the sketches you showed you only depicted the cell soma, but these cells have quite long dendrites and it is hard to know what sort of selection bias an *in vivo* patch electrode might have.

When you record from cells with your neurobiotin labelling do you see some cells with dendrites that go down across the border into L3, where they would probably normally be able to pick up lots of Aβ inputs? Then the interpretation would not be sprouting but just a change in the space constant of those dendrites.

Yoshimura: Yes, I think some of the neurons have dendrites near L3 and in L3. Perhaps they receive Aβ inputs there. As I showed you in Table 1 (p 119), we could record from some neurons which showed the synaptic inputs from Aβ fibres. However, the population of these neurons was quite small. It was about 7–8%.

Devor: That is by electrophysiological criteria. But what I am saying is that perhaps 30% have those inputs, but you are not able to see the EPSPs because the synapses are too far out on the dendrite. A change in the conductance of the dendrite could mean that you are now able to see inputs that you couldn't see previously, without any structural change at all.

Yoshimura: That's possible. But we tested the membrane properties of neurones from normal and inflamed rats. Any significant changes in input resistance, membrane time constant and membrane potential were not found. In addition, the amplitudes of EPSCs evoked by presumed Aβ afferents had large (not small) amplitudes and the time course was similar to those of Aδ and C afferent-evoked EPSCs, suggesting that the Aβ afferent-evoked EPSCs are likely elicited by synapses located at the similar distance to those of Aδ and C afferents. These observations suggest that the appearance of Aβ afferent-evoked EPSCs in inflamed rats is not due to a change in length constant of the dendrites of SG neurons. However, our electrophysiological evidence may not be enough to prove our notion. Morphological data, such as obtained by single fibre injection of dye into Aβ afferents, are further required.

Devor: I can accept the observation, but I don't think this proves sprouting.

Perl: There is no question about the validity of the observations, rather the issue is whether the results are a sound basis for the conclusions. For example, are there silent synapses from Aβ fibres which become functional in the presence of an increase in background excitability? There is solid evidence for a myelinated fibre input to the SG. It is limited, selective and organized in a particular way. Will differences in the population emerge when specifically examined in neurons that exhibit these phenomena? If so, are the novel responses possibly related to existing connections that are uncovered by the inflammatory process?

Yoshimura: As you know, Baba et al (2000) have reported that the silent synapses are evident, in particular in the immature condition, while these are found in only a few dorsal horn neurons in the mature stage. Therefore, it is not likely that the Aβ afferent inputs to the SG neurons are due to the activation of the silent synapses. However, these data are not conclusive. It might be good to inject some dye into single Aβ afferent fibres and look at the termination of the fibres.

Zhuo: Or can you use an inhibitor to inhibit the sprouting process?

Yoshimura: We are trying to check whether anti-BDNF antibody blocks the sprouting. There is evidence suggesting that synthesis and expression of TrkB may be involved in the plastic changes occurring in the spinal cord. We don't have enough data yet.

Zhang: Have you considered tracing methods to see what the projection is?

Yoshimura: The tracing of Aβ afferents is one of the reliable methods for improving our electrophysiological observations. Dr Woolf's group has published a paper showing that there is some sprouting of Aβ fibres to the SG. However, there are many arguments saying that the phenotypic changes occur following nerve damage; the tracer is taken up into C afferent fibres. We cannot exclude this possibility. We are considering injecting some dye into a single Aβ afferent before and after inflammation. Combining electrophysiological and morphological evidence, then we will be able to say the sprouting of Aβ afferents occurs with certainty.

McMahon: Did you find in your *in vivo* patching experiments that two days after inflammation you found no cells that were heat responsive? If so, why might this be? Also, the A fibre touch responses went from being transient to sustained. Did you interpret this as a peripheral change? If so, why not consider the possibility of a post-synaptic increase in excitability that may facilitate those touch inputs?

Yoshimura: I didn't say that no cells responded to heat after inflammation. We haven't tested an effect of heat stimulation of SG neurons in inflamed rats. What I said is that no SG neurons respond to heat under normal conditions. In response to the second question, we conclude that the elimination of the adaptation following inflammation is preferentially due to a peripheral change, because it has been reported that the adaptation is a property of sensory receptors, and the barrage of EPSCs ceased immediately after cessation of the stimulation and no after-discharge was observed. If the change were due to a post-synaptic increase in excitability, we could expect a long lasting response even after cessation of the touch stimulation. In addition to this, we couldn't observe any significant increase in amplitude of mEPSCs in inflamed rats in comparison with that in normal rats.

McMahon: Is there any evidence for a change in peripheral sensitivity of touch fibres in these inflammatory models? You are suggesting that the input is changed because the tactile afferents become sensitized in the inflammatory state.

Yoshimura: To my knowledge, I don't have any supporting evidence for a change in peripheral sensitivity of touch fibres following inflammation, although it is well known that nociceptors are sensitized by chemical mediators released during inflammation.

McMahon: What is the cause of the lack of heat responses in your prep?

Yoshimura: Our results obtained by the *in vivo* patch-clamp recordings indicate that SG neurons don't make synaptic contact with the primary afferent fibres

conveying noxious heat sensation. I know many heat-sensitive afferent fibres terminate in SG.

McMahon: Presumably this is a technical problem. We know that lots of cells respond with *c*-fos to acute noxious heat stimuli. It is therefore surprising that you can't see the postsynaptic effects of these stimuli.

Yoshimura: Although I didn't tell you, the heat sensation seemed to be transferred to deep dorsal horn neurons since those neurons exhibited an increase in the barrage of EPSCs with large amplitude in response to heat stimulation. Interestingly, the response was mediated by a fast EPSC but not by a slow current, indicating no involvement of peptides, such as substance P or CGRP. This observation may be supported by the anatomical evidence demonstrating that some deep dorsal horn neurons extend their dendrites to lamina II and receive synaptic inputs from primary afferents.

McMahon: In anaesthetized animals, paws dipped in hot water for two minutes show evidence of a lot of post-synaptic activation in L1 and L2, using markers such as pERK. This could imply that there is some rapid and some direct activation of L1, 2 with heat stimuli. I wonder why you are not seeing this.

Yoshimura: As mentioned before, the heat sensation seems to be transferred to the deeper laminae but not to SG, in spite of the dense termination of C afferent fibres in there and the pERK expression in SG neurons. One possible explanation for these discrepancies is that activated afferent fibres by heat may release unknown chemicals onto SG neurons which trigger the expression of pERK or *c*-fos without transmission of electrical activity. However, to my knowledge, there is no such report supporting this possibility.

Ueda: How long did you wait after surgery?

Yoshimura: We did our whole cell patch recording at least 1 h after operation.

McMahon: But the *c*-fos data would say that the cells with the nucleus in L2 are somehow activated, presumably via synaptic activation.

Yoshimura: The expression of *c*-fos or pERK doesn't necessary mean synaptic activation.

Perl: There may be a simple explanation for the lack of heat evoked responses, possibly related to the way that we often go about recording from SG neurons in spinal cord slices. The recording electrode is aimed at the translucent region in the dorsal horn. This area is mostly lamina II inner. The major projection of heat-sensitive nociceptors, mainly unmyelinated fibres, is to lamina II outer, a very thin lamina in the rat. It is difficult to record from these neurons. The problem may be that in patch electrode experiments we don't sample the areas containing the major heat nociceptive input.

Yoshimura: In *in vivo* experiments we injected neurobiotin, and after the experiment we checked the morphology and location of the neurons. We found

that we recorded not only from the inner lamina II but also from the dorsal part of lamina II and those neurons exhibited variety in shape as you reported, suggesting that we were recording from the different types of SG neurons, but not from small classes of neurons.

Oh: Your exposed spinal cord is too cold, because it is at room temperature. You recorded a neuron in the SG, which is quite close to the surface.

Yoshimura: The surface of the spinal cord was continuously perfused with a solution at 38 °C. The spinal cord was maintained at nearly body temperature. Therefore, we can exclude that possibility.

Baron: I would like to come back to the possible retraction of these sprouts. This is potentially very important for clinical neuropathy. Has anyone looked at a longer time period in neuropathic rats? You mentioned that you didn't look at longer time periods after inflammation.

Yoshimura: Although we didn't look at the synaptic changes a long time after inflammation, a behavioural study has shown that the mechanical hyperalgesia observed in inflamed and also chronic sciatic nerve constriction models is gradually reduced and this decrease in threshold becomes insignificant at day 28 (Goff et al 1998). These observations have some similarity with those reported in the neuromuscular junction occurring after nerve cutting.

McMahon: Have you considered the role of endogenous BDNF? At relatively early time points after inflammation, endogenous levels of sensory neuron BDNF are going up. You have done a number of experiments in which you have added BDNF. Have you tried to ask whether BDNF released with primary afferent activity was important in the changes you observed? Is it possible to do this experiment using sequestering molecules or receptor antagonists?

Yoshimura: We didn't study the effect of BNDF in the presence of antagonist. We have only tested the effect of BDNF on the synaptic transmission in SG neurons in slice preparations obtained from normal and inflamed rats. BDNF had no significant effects on the synaptic transmission in normal rats and 10 days after inflammation.

McMahon: Have you any evidence that endogenous BDNF is activating second-order cells? We published a paper a year or so ago (Pezet et al 2002) showing that C fibre activation, especially in inflamed conditions, led to TrkB phosphorylation, presumably mostly on postsynaptic cells. And this will have a number of downstream consequences. Do you have any direct evidence of electrophysiological consequences?

Yoshimura: That is interesting information for us, but we haven't done this. We will be able to look at the effect of BDNF on the synaptic responses evoked by primary afferent fibres. This experiment tells us whether BDNF acts on presynaptic or postsynaptic cells.

References

Baba H, Doubell TP, Moore KA, Woolf CJ 2000 Silent NMDA receptor-mediated synapses are developmentally regulated in the dorsal horn of the rat spinal cord. J Neurosci 83:955–962

Goff JR, Burkey AR, Goff DJ, Jasmin L 1998 Reorganization of the spinal dorsal horn in models of chronic pain: correlation with behaviour. Neuroscience 82:559–574

Grudt TJ, Perl ER 2002 Correlations between neuronal morphology and electrophysiological features in the rodent superficial dorsal horn. J Physiol 540:189–207

Li P, Zhuo M 1998 Silent glutamatergic synapses and nociception in mammalian spinal cord. Nature 393:695–698

Pezet S, Malcangio M, Lever IJ et al 2002 Noxious stimulation induces Trk receptor and downstream ERK phosphorylation in the spinal dorsal horn. Mol Cell Neurosci 21:684–695

Central plasticity in pathological pain

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Abstract. Neurons and synapses in the central nervous systems are very dynamic and plastic, and can undergo changes throughout life. Studies of molecular and cellular mechanisms of such changes not only provide important insight into how we learn and store new knowledge in our brains, but also reveal the mechanisms of pathological changes occurring following an injury. Here, we propose that while neuronal mechanisms underlying physiological functions such as learning and memory may share some common signalling molecules with abnormal or injury-related changes in the brain, distinct synaptic mechanisms are involved in pathological pain as compared with that of cognitive learning and memory. Using genetically altered mice and classic physiological approaches, we showed that N-methyl-D-aspartate (NMDA) receptor-dependent, calcium–calmodulin-activated adenylyl cyclases (AC1 and AC8) in the anterior cingulate cortex (ACC) play important roles in the induction and expression of persistent inflammatory and neuropathic pain. In contrast, acute pain was not significantly affected. Calcium–calmodulin-dependent protein kinase IV, which is widely expressed in central areas related to pain and memory, primarily contributes to injury-related fearful memory and emotional responses. Our studies suggest distinct signalling pathways are responsible for physiological responses to the injury, including behavioural, emotional and memory.

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Tissue or nerve injury often leads to pain which lasts for an extended period of time after the injury. Hyperalgesia and allodynia are often associated with persistent pain. Both peripheral and central sensitization contributes to persistent pain. Peripheral sensitization reflects increased sensitivity of primary afferent nociceptors, and includes lowered thresholds and an increased responsiveness of the skin. Furthermore, during as well as after injury, synaptic transmission in the central nervous system undergoes long-lasting changes. Some of these central changes are permanent, altering the brain's perception of future sensory stimuli. Despite recent progress in dissecting the pathophysiological mechanisms of persistent pain, cellular and molecular mechanisms for chronic pain remain unclear. Understanding these mechanisms is essential to the development of clinical strategies aimed at alleviating chronic pain. Here I present our recent

findings using integrative approaches to investigate central plasticity, in particular the anterior cingulate cortex (ACC) involvement in persistent pain caused by inflammation, nerve injury or amputation.

Spinal cord: first central synapses undergo long-term potentiation

Primary afferent fibres form synapses with dorsal horn sensory neurons in the spinal cord. Some of these dorsal horn neurons send ascending projecting fibres and make synapses with neurons located at supraspinal sites, such as the thalamic nuclei. These ascending pathways are important for conveying sensory information from the periphery to the brain. Glutamate is a major neurotransmitter between primary afferent fibres and dorsal horn neurons (Levine et al 1993) and postsynaptic responses are mainly mediated by glutamate α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) and kainate receptors (Yoshimura & Jessell 1990, Hori et al 1996, Bardoni et al 1998, Li et al 1998, 1999a,b). Glutamatergic synapses are heterogeneous in the spinal dorsal horn (Bardoni et al 1998, Li et al 1998, 1999a, Wang & Zhuo 2002). At least three different types of glutamatergic synapses are found:

- In some synapses, only functional NMDA receptors are found. Neither AMPA nor kainate receptors are present or functional in these synapses (so-called 'silent' synapses).
- At sensory synapses that are receiving low-threshold inputs, only AMPA receptors are found. No functional kainate receptors exist.
- Finally, at synapses receiving high-threshold inputs, both AMPA and kainate receptors are found. In both of the cases above, NMDA receptors are always detected.

Besides being reliable and fast, glutamate synaptic transmission in the spinal cord is dynamic and plastic. Not only can the release of glutamate be regulated by different agents, including opioids, but also postsynaptic glutamate receptors can be regulated. Postsynaptic glutamate receptors are put into the place through a family of proteins containing PDZ domains (see Zhuo 2000 for review). Furthermore, these postsynaptic protein–protein interactions are very dynamic and can be involved in the clustering, removal or insertion of postsynaptic receptors (Li et al 1999b), providing a novel and efficient way to regulate synaptic strength. Protein–protein interactions contribute to the switch in the phenotype of dorsal horn sensory synapses, i.e. changing silent synapses into functional synapses (Li et al 1999b). In addition to postsynaptic regulation of glutamate receptors, presynaptic regulation of the release of glutamate may play important roles in dorsal horn plasticity. Retrograde messenger generators,

including haem oxygenases and nitric oxide synthases are reported in the spinal dorsal horn neurons (Fig. 1). They may contribute to long-term potentiation (LTP) as do those reported in the hippocampus (see Zhuo et al 1993, 1994).

The forebrain in pain perception and higher brain functions

Forebrain neurons are important for pain-related perception (see Fig. 2). Recent studies from both human and animals consistently suggest that the ACC and its related areas are important for processing pain perception. Lesions of the medial frontal cortex including the ACC significantly increase acute nociceptive responses as well as injury-related aversive memory behaviours (Lee et al 1999, Johansen et al 2002). In patients with frontal lobotomies or cingulotomies, the unpleasantness of pain is abolished (see Zhuo 2002 for review). Electrophysiological recordings from both animals and humans demonstrate that neurons within the ACC respond to noxious stimuli, including nociceptive-specific neurons (Sikes & Vogt 1992, Hutchison et al 1999). Neuroimaging studies further confirm these observations and show that the ACC, together with other cortical structures, are activated by acute noxious stimuli (Rainville et al 1997, 2001, Talbot et al 1991, Casey 1999). Thus, understanding of synaptic mechanisms within the ACC will greatly help us to gain insights into plastic changes in the brain related to central pain.

LTP and LTD in the ACC

Similar to the spinal dorsal horn, glutamate is the major fast excitatory transmitter in the ACC (Wei et al 1999). Different types of glutamate receptors, including AMPA, kainate, NMDA and metabotropic receptors (mGluRs) are distributed in the ACC. Fast synaptic responses induced by local stimulation or stimulation of thalamocortical projection pathways are mediated by AMPA/kainate receptors. Glutamatergic synapses in the ACC can undergo long-lasting plastic changes such as long-term potentiation (LTP) and long-term depression (LTD). We recently found that theta-burst stimulation, a paradigm more closely related to the activity of ACC neurons, induced LTP, which lasted for at least 40–120 min (Wei et al 2002a). cAMP signalling pathways known to be important for LTP in the other central synapses such as the hippocampus are required for the induction of ACC LTP. Preliminary studies using gene knockout mice and pharmacological activators/inhibitors found that calcium-stimulated adenylyl cyclases subtype 1 (AC1) and 8 (AC8) contribute to the induction of LTP in the ACC. In addition, calmodulin and CaMKIV, another protein kinase responding to calcium–calmodulin, are also required for the induction of LTP (Wei et al 2002a, 2003).

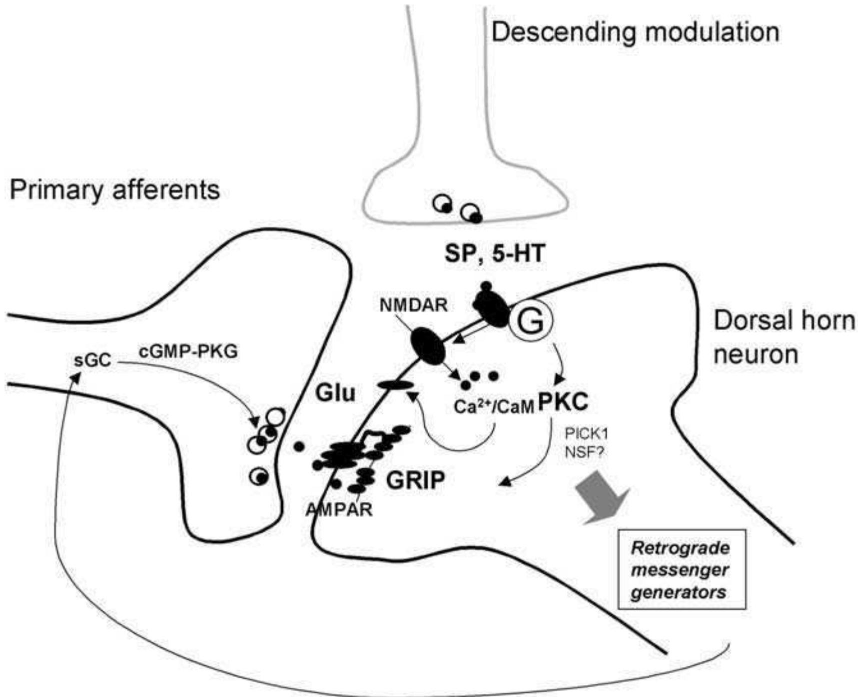

FIG. 1. A model of LTP in spinal dorsal horn neurons. Peripheral tissue injury activates nociceptors and causes the release of glutamate (filled circles) as well as substance P, CGRP and other putative transmitters (not shown) from the central terminals in the spinal dorsal horn. Activation of glutamate NMDA receptors leads to an increase in postsynaptic Ca^{2+} in dendritic spines. Ca^{2+} serves as an important intracellular signal for triggering a series of biochemical events that contribute to the expression of spinal LTP. Ca^{2+} binds to CaM and leads to activation of various Ca^{2+}-dependent enzymes and release of potential diffusible retrograde messengers, such as nitric oxide, carbon monoxide, arachidonic acid, platelet-activating factor and neurotrophins. Retrograde messengers will enhance transmitter release from presynaptic terminals by regulating presynaptic ion channels and/or synaptic vesicle release and/or recycling pathways. Activation of protein kinases and protein phosphatases regulate phosphorylation and dephosphorylation of different target proteins to enhance postsynaptic excitability by (1) regulation of glutamate AMPA and NMDA receptors and/or (2) gene regulation and protein synthesis. In addition, activation of postsynaptic silent AMPA receptors may contribute to the expression of spinal LTP. Several G protein coupled receptors can also contribute to the regulation of spinal synaptic transmission. Activation of G protein coupled receptors, such as 5-HT (released from descending modulatory systems; see Basbaum & Fields 1984, Urban & Gebhart 1999, Zhuo 2000) or SP receptors, activate PKC and other related protein kinases, and alter synaptic responses through similar mechanisms. Glu, glutamate; mGluRs, metabotropic glutamate receptors; AMPARs, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate receptors; NMDARs, N-methyl-D-aspartate receptors; SP, substance P; 5-HT, serotonin; GRIP, glutamate receptor interacting protein; sGC, souble guanylyl cyclase; PKG, cGMP-dependent protein kinase; PICK1, protein interacting with C kinase.

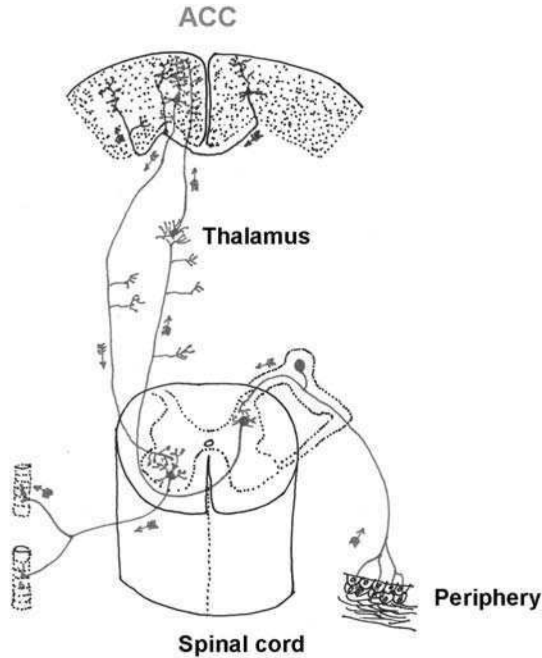

FIG. 2. Model of ascending sensory including pain pathways. Sensory inputs from the periphery enter the brain through three major synaptic relays, including the spinal dorsal horn, thalamus and anterior cingulate cortex. At each synaptic relay, glutamate is the major fast excitatory transmitter. While AMPA and kainate receptors mediate most of the synaptic response at resting conditions, NMDA receptors serve as a coincidence detector to enhance synaptic responses in an activity-dependent manner. Long-lasting potentiation is likely to occur at each sensory synapse, including the spinal cord, thalamus and the ACC.

What goes up needs to come down. LTD has been thought to be a reversed form of plasticity for LTP. In the ACC slices of adult rats and mice, LTD can be induced by repetitive stimulation for a long period of time (15 min) (Wei et al 1999 and preliminary data). Prolonged, low frequency stimulation (1 Hz for 15 min) produced long-lasting depression of synaptic responses. Depression is input-specific, and unstimulated pathways remain unchanged. There are several properties of LTD in the ACC that differ from the hippocampus. 5 Hz stimulation (3 min) induced LTD in the ACC but not in hippocampal slices. Unlike hippocampal LTD, which required activation of NMDA receptors, LTD induction required activation of mGluRs and L-type voltage-gated calcium channels (LVGCCs). Furthermore, LTD in adult ACC slices is easily detected (Wei et al 1999).

Therefore, sensory synapses in pain-related forebrain areas are plastic, regulated in a biphasic manner and can undergo LTP and LTD.

Loss of long-term depression in the ACC after amputation

What makes ACC more interesting is that neuronal activity and plasticity show plastic changes after tissue injury or amputation. Activity-dependent immediate early genes, such as *c*-fos, Egr1 and adenosine 3′,5′-monophosphate response element binding protein (CREB) are activated in the ACC neurons after tissue inflammation or amputation (Wei et al 1999, 2001). Furthermore, these plastic changes persist for a long period of time. Studies using AC1 and AC8 double knockout or NR2B-overexpressing mice show that NMDA receptors, AC1 and AC8 contribute to activation of immediate early genes by injury (Wei et al 2001, 2002b). In parallel with these dramatic changes in gene expression, synaptic plasticity recorded from *in vitro* ACC slices is altered. In ACC slices of animals with amputation, the same repetitive stimulation produced less or no LTD. The loss of LTD is regionally selective, and no change was found in other cortical areas (Wei et al 1999). One possible physiological mechanism for LTD in the ACC is to serve as an autoregulatory mechanism. LTD induced during low-frequency repetitive stimulation may help to maintain appropriate neuronal activity within the ACC by reducing synaptic transmission. In amputated or injured animals, the loss of autoregulation of synaptic tone may lead to overexcitation in the ACC neurons and contribute to enhancement of pain or unpleasantness related to the injury.

Long-term enhancement of synaptic responses in the ACC after amputation

In order to demonstrate that synaptic changes occur in the ACC after amputation, it is important to show that some physiological changes also happen in whole animals. First, to measure synaptic responses to peripheral electrical shocks, we placed a recording electrode in the ACC of anaesthetized rats (Wei & Zhuo 2001). At high intensities of stimulation, sufficient to activate Aδ and C fibres, evoked field EPSPs were found in the ACC. The field EPSPs recorded from the ACC were obviously polysynaptic in nature, likely involving at least primary afferent fibres and spinothalamic and thalamocortical tracts (the estimated latency for the onset of field EPSPs was 12.0 ± 0.1 ms). Because amputation caused damage to local skin as well as nerves innervating the digit, we performed amputation at the hindpaw contralateral to the one to which stimulation was delivered. Interestingly, after amputation of a central digit of the hindpaw, we observed a rapid enhancement of sensory responses to peripheral electrical shocks delivered to the normal hindpaw. The potentiation was long-lasting; evoked responses remained enhanced for at least 120 min (Wei & Zhuo 2001).

In order to address whether synaptic changes may occur locally within the ACC, we measured field EPSPs to focal ACC electrical stimulation. Consistently, we

observed a long-lasting potentiation of field EPSPs after amputation that lasted for at least 90 min (Wei & Zhuo 2001). The amount of potentiation is not significantly different from that in field recordings evoked by hindpaw stimulation. We hypothesize LTP within the ACC is likely due to abnormal activity during and after amputation. One important question is whether potentiated sensory responses required persistent activity from the injured hindpaw. To test this, we locally injected a local anaesthetic, QX-314, into the hindpaw (5%, 50 μl) at 120 min after amputation. We found that QX-314 injection did not significantly affect the synaptic potentiation induced by amputation (Wei & Zhuo 2001).

Genetic enhancement of persistent pain by forebrain NR2B overexpression

In order to investigate molecular and cellular mechanisms for pain-related plasticity in the ACC, we decided to use genetic approaches together with integrative neuroscience techniques to investigate synaptic mechanisms in the ACC. First, we want to test if persistent pain may be enhanced by genetically enhanced NMDA receptor functions, a key mechanism for triggering central plasticity in the brain (Zhuo 2002). Functional NMDA receptors contain heteromeric combinations of the NR1 subunit plus one or more of NR2A-D. While NR1 shows a widespread distribution in the brain, NR2 subunits exhibit regional distribution. In humans and rodents, NR2A and NR2B subunits predominate in forebrain structures. NR2A and NR2B subunits confer distinct properties to NMDA receptors; heteromers containing NR1 plus NR2B mediate a current that decays three to four times more slowly than receptors composed of NR1 plus NR2A. Unlike other ionotropic channels, NMDA receptors are 5–10 times more permeable to calcium, a critical intracellular signalling cation, than to Na^+ or K^+. NMDA receptor mediated currents are long-lasting compared with the rapidly desensitizing kinetics of AMPA and kainate receptor channels. In transgenic mice with forebrain-targeted NR2B overexpression, the normal developmental change in NMDA receptor kinetics was reversed (Tang et al 1999). NR2B subunit expression was observed extensively throughout the cerebral cortex, striatum, amygdala and hippocampus, but not in the thalamus, brainstem or cerebellum. In both the ACC and insular cortex, NR2B expression was significantly increased, and NMDA receptor mediated responses were enhanced (Wei et al 2001). NMDA receptor-mediated responses in the spinal cord, however, were not affected. NR2B transgenic and wild-type mice were indistinguishable in tests of acute nociception, NR2B transgenic mice exhibited enhanced behavioural responses after peripheral injection of formalin. Late-phase nociceptive responses but not early responses were enhanced. Furthermore, mechanical allodynia measured in the complete Freund's adjuvant (CFA) model

were significantly enhanced in NR2B transgenic mice. These findings provide the first genetic evidence that forebrain NMDA receptors play a critical role in chronic pain.

Selective blockade of persistent pain by double knockout of AC1 and AC8

Next, we want to know if inhibition of NMDA receptor-dependent, calcium-stimulated signalling pathways in the ACC may help to reduce chronic pain while keeping acute pain sensation intact (this is critical for animal or human self-protection). AC1 and AC8, the two major calmodulin-stimulated ACs in the brain, couple NMDA receptor activation to cAMP signalling pathways. In the ACC, strong and homogeneous patterns of AC1 and AC8 expression were observed in all cell layers (Wei et al 2002b). Behavioural studies found that wild-type, AC1, AC8 or AC1/AC8 double-knockout mice were indistinguishable in tests of acute pain including the tail-flick test, hot-plate test, and the mechanical withdrawal responses. However, behavioural responses to peripheral injection of two inflammatory stimuli, formalin and CFA, were reduced in AC1 or AC8 single knockout mice. Deletion of both AC1 and AC8 in AC1/AC8 double knockout mice produced greater reduction in persistent pain (Wei et al 2002a). More importantly, microinjection of an AC activator, forskolin, can rescue defects in chronic pain in AC1/AC8 double knockout mice. Consistently, pharmacological blocking of NMDA receptors as well as cAMP signalling pathways within the ACC also produced inhibitory effects on persistent pain in normal or wild-type animals, supporting the roles of ACC in persistent pain. Microinjection of NMDA receptor antagonists or cAMP-dependent protein kinase (PKA) inhibitors reduced or blocked mechanical allodynia related to inflammation (Wei et al 2002b).

CaMKIV: a molecule for fear but not pain

One major physiological effect of pain is to trigger emotional fear and form environment-related fear memory (LeDoux 2000, Zhuo 2003). Emotional learning and its expression in mammals, such as fear, require the involvement of higher brain structures including the amygdala, hippocampus and related cortical areas. Cumulative evidence consistently shows that within these areas, long-term changes in synaptic transmission and structure are important for the establishment and consolidation of such memory. CREB is a key transcription factor for synaptic plasticity and memory consolidation. Two major pathways responsible for CREB activation are the cAMP signalling pathway and calcium/calmodulin-dependent protein kinase pathway. While the inhibition of CREB impaired behavioural

performance in various memory tests across different species, the overexpression of CREB is reported to facilitate long-term fear memory. To investigate possible roles of CaMKIV-dependent signalling pathways, we performed experiments using CaMKIV knockout mice. Two forms of associative emotional memory in wild-type and CaMKIV knockout mice were studied: contextual and auditory fear conditioning. No significant difference in contextual freezing was found immediately following training, nor at 1 hour post-training. In contrast, when tested at 1 and 7 days, contextual freezing was significantly reduced in CaMKIV knockout mice. Furthermore, when testing auditory fear conditioning at 1 day or 7 days after training, freezing in response to the tone was also significantly reduced in CaMKIV knockout mice compared to that in wild-type mice (Wei et al 2002a).

Next, we asked whether the lack of CaMKIV affected acute nociceptive transmission or persistent pain. No significant difference in tail-flick latency was observed. Likewise, no differences were found in latencies to a hot-plate test. Furthermore, behavioural responses to peripheral injection of two inflammatory stimuli, formalin and CFA, were similar in wild-type and CaMKIV knockout mice (Wei et al 2002a). Together, these results indicate that CaMKIV is preferentially involved in fear memory induced by a noxious shock but not in the behavioural responses to acute noxious stimuli or tissue inflammation. This finding confirms that behavioural responses to peripheral tissue inflammation commonly used in pain research do not reflect fear memory (Kerchner et al 2001).

Cellular model for plastic changes in the ACC

In summary, we believe that molecular and cellular mechanisms for central plasticity in the ACC are starting to be revealed. Figure 3 is a model proposed based on current studies. Neural activity triggered by injuries releases the

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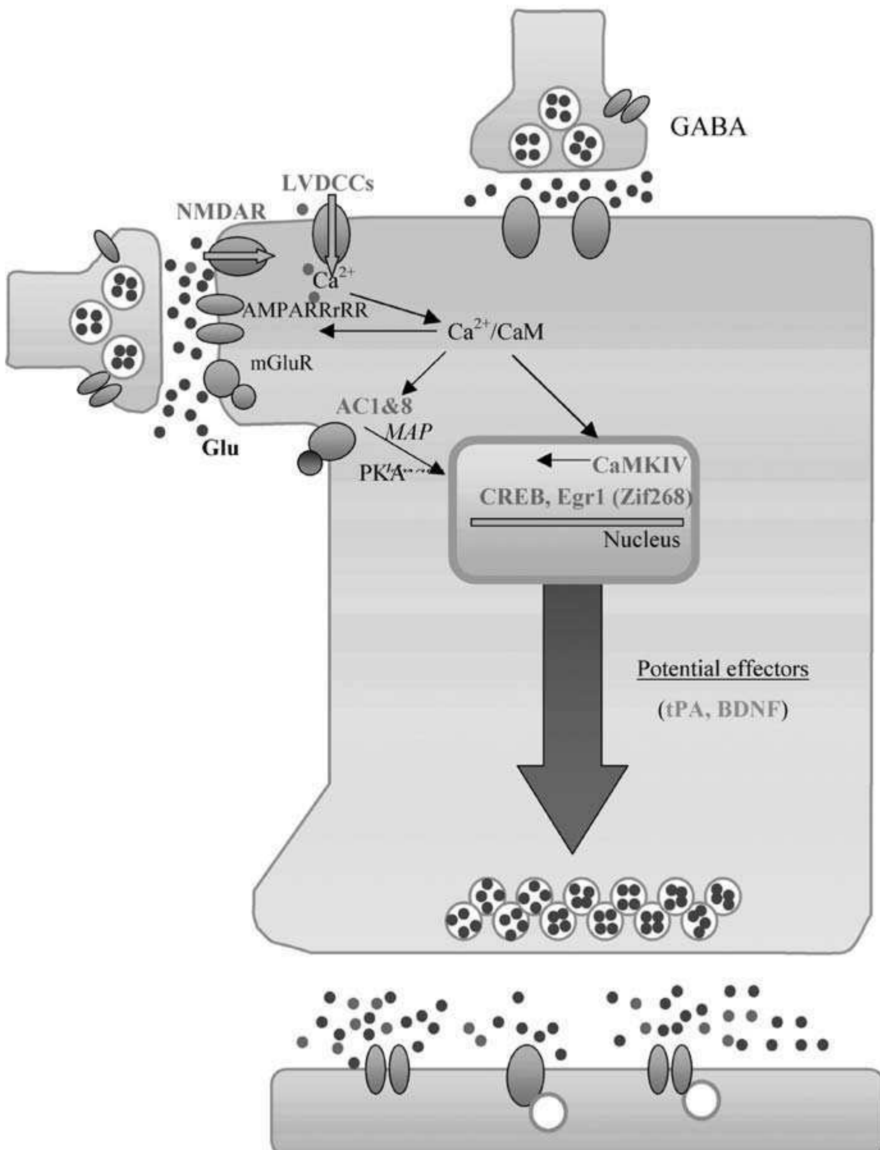
FIG. 3. Model of signalling pathways in the ACC for plastic changes related to injuries. Neural activity triggered by injuries releases excitatory neurotransmitter glutamate (Glu, filled circles) in the ACC synapses. Activation of glutamate NMDA receptors leads to an increase in postsynaptic Ca^{2+} in dendritic spines. Ca^{2+} serves as an important intracellular signal for triggering a series of biochemical events that contribute to the expression of LTP. Ca^{2+} binds to CaM and leads to activation of calcium-stimulated ACs, including AC1 and AC8 and Ca^{2+}/CaM-dependent protein kinases (PKC, CaMKII and CaMKIV). The Ca/CaM-dependent protein kinases phosphorylate glutamate AMPA receptors, increasing their sensitivity to glutamate. Activation of CaMKIV, a kinase predominantly expressed in the nuclei, will trigger CaMKIV-dependent CREB. In addition, activation of AC1 and AC8 leads to activation of PKA, and subsequently CREB as well. CREB as well as other immediate early genes (e.g. Egr1) in turn activates targets that are thought to lead to more profound structural changes.


GABA
LVDCCs
NMDAR
Ca2+
AMPARRrRR
Ca2+/CaM
mGluR
AC1&8
MAP
Glu
PKA
CaMKIV
CREB, Egr1 (Zif268)
Nucleus
Potential effectors
(tPA, BDNF)

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Positive feedback control: a key enhancer for persistent pain

As described in the beginning, we are far from understanding the physiological mechanisms of chronic pain if we only focus on what is happening at individual synapses. I believe that changes in individual synapses can lead to alterations of neuronal network functions related to pain transmission and modulation. Here, a *positive feedback control* is proposed to serve as the key pathological mechanism for chronic pain. Positive enhancement occurs not only at single synapses, but also between multiple neuronal synapses in different parts of the brain (Fig. 4). Several mechanisms may contribute to synaptic enhancement:

- Postsynaptic regulation of glutamate receptors, including phosphorylation and dephosphorylation.
- Recruitment of functional glutamate receptors (for example, in spinal dorsal horn neurons, recruitment of postsynaptic functional AMPA receptors).
- Presynaptic enhancement of glutamate release.
- Structural changes in synapses. At network levels, heterosynaptic facilitation or dis-inhibition can lead to enhancement as well.

It is well documented that dorsal horn neurons receive descending facilitatory modulation from the brainstem neurons. Recent study further suggests that the activation of supraspinal structures including ACC neurons can also facilitate spinal responses (Zhuo & Gebhart 1997, Calejesan et al 2000) and trigger long-term fear memory (J. Tang, S. Ko, H. K. Ding, C-S Qiu, M. Zhuo, unpublished data). The consequence of this *positive feedback control* will lead central neurons to a much enhanced and overexcited status; a weak input will lead to significantly greater neuronal action potentials. Such a mechanism most likely contributes to several chronic pain related states, such as allodynia and central pain.

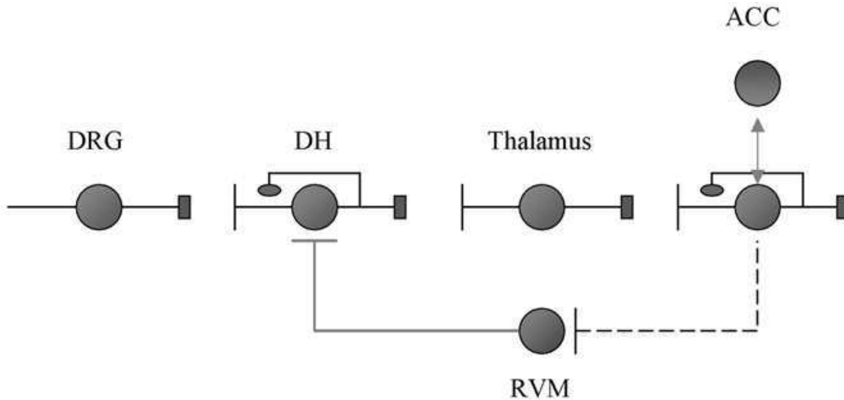

FIG. 4. Positive feedback control as a key central mechanism for persistent pain or central pain. Sensory inputs inducing painful stimuli enter the brain through three major synaptic relays, including the dorsal horn (DH), thalamus and anterior cingulate cortex (ACC). At each synaptic relay, glutamate is the major fast excitatory transmitter. While AMPA and kainate receptors mediate most of the synaptic response at resting conditions, NMDA receptors serve as a coincidence detector to enhance synaptic responses in an activity-dependent manner. Long-lasting potentiation is likely to occur at each sensory synapse. Within the ACC, cortical connections may also undergo plastic changes, and may serve as highest central loci to store unpleasantness or pain. In addition, dorsal horn sensory synapses receive heterosynaptic facilitatory modulation from supraspinal structures, including the forebrain and brainstem. The RVM in the brainstem is likely to serve as a final relay for this descending facilitatory or excitatory modulation. Both homosynaptic and heterosynaptic enhancement will lead central sensory neurons to an enhanced excitatory status, so that a gentle trigger or stimulation can cause massive firing of action potentials and thus cause pain. In the case of central pain, spontaneous activity of neurons in the network itself can also lead to action potential firing and pain.

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References

Bardoni R, Magherini PC, MacDermott AB 1998 NMDA EPSCs at glutamatergic synapses in the spinal cord dorsal horn of the postnatal rat. J Neurosci 18:6558–6567

Basbaum AI, Fields HL 1984 Endogenous pain control system: brainstem spinal pathways and endorphin circuitry. Annu Rev Neurosci 7:309–338

Calejesan AA, Kim SJ, Zhuo M 2000 Descending facilitatory modulation of a behavioral nociceptive response by stimulation in the adult rat anterior cingulate cortex. Eur J Pain 4:83–96

Casey KL 1999 Forebrain mechanisms of nociception and pain: analysis through imaging. Proc Natl Acad Sci USA 96:7668–7674

Hori Y, Endo K, Takahashi T 1996 Long-lasting synaptic facilitation induced by serotonin in superficial dorsal horn neurones of the rat spinal cord. J Physiol 492:867–876

Hutchison WD, Davis KD, Lozano AM, Tasker RR, Dostrovsky JO 1999 Pain-related neurons in the human cingulate cortex. Nat Neurosci 2:403–405

Johansen JP, Fields HL, Manning BH 2002 The affective component of pain in rodents: direct evidence for a contribution of the anterior cingulated cortex. Proc Natl Acad Sci USA 98:8077–8082

Kerchner GA, Wei F, Wang GD et al 2001 Do 'smart' mice feel more pain, or are they just better learners? Nat Neurosci 4:453–454

LeDoux JE 2000 Emotion circuits in the brain. Annu Rev Neurosci 23:155–184

Lee DE, Kim SJ, Zhuo M 1999 Comparison of behavioral responses to noxious cold and heat in mice. Brain Res 845:117–121

Levine JD, Fields HL, Basbaum AI 1993 Peptides and the primary afferent nociceptor. J Neurosci 13:2273–2286

Li P, Zhuo M 1998 Silent glutamatergic synapses and nociception in mammalian spinal cord. Nature 393:695–698

Li P, Calejesan AA, Zhuo M 1998 ATP P_{2X} receptors and sensory transmission between primary afferent fibers and spinal dorsal horn neurons in rats. J Neurophysiol 80:3356–3360

Li P, Wilding TJ, Kim SJ, Calejesan AA, Huettner JE, Zhuo M 1999a Kainate-receptor-mediated sensory synaptic transmission in mammalian spinal cord. Nature 397:161–164

Li P, Kerchner GA, Sala C et al 1999b AMPA receptor-PDZ protein interaction mediate synaptic plasticity in spinal cord. Nat Neurosci 2:972–977

Rainville P, Duncan GH, Price DD, Carrier B, Bushnell MC 1997 Pain affect encoded in human anterior cingulate but not somatosensory cortex. Science 277:968–971

Rainville P, Bushnell MC, Duncan GH 2001 Representation of acute and persistent pain in the human CNS: potential implications for chemical intolerance. Ann NY Acad Sci 933:130–141

Sikes RW, Vogt BA 1992 Nociceptive neurons in area 24 of rabbit cingulate cortex. J Neurophysiol 68:1720–1732

Talbot JD, Marrett S, Evans AC, Meyer E, Bushnell MC, Duncan GH 1991 Multiple representations of pain in human cerebral cortex. Science 251:1355–1358

Tang YP, Xhimizu E, Dube GR et al 1999 Genetic enhancement of learning and memory in mice. Nature 401:63–69

Urban MO, Gebhart GF 1999 Supraspinal contributions to hyperalgesia. Proc Natl Acad Sci USA 96:7687–7692

Wang GD, Zhuo M 2002 Synergistic enhancement of glutamate-mediated responses by serotonin and forskolin in adult mouse spinal dorsal horn neurons. J Neurophysiol 87:732–739

Wei F, Zhuo M 2001 Potentiation of synaptic responses in the anterior cingulate cortex following digital amputation in rat. J Physiol 532:823–833

Wei F, Li P, Zhuo M 1999 Loss of synaptic depression in mammalian anterior cingulate cortex after amputation. J Neurosci 19:9346–9354

Wei F, Wang GD, Kerchner GA et al 2001 Genetic enhancement of inflammatory pain by forebrain NR2B overexpression. Nat Neurosci 4:164–169

Wei F, Qiu CS, Liauw J, Robinson DA, Ho N, Chatila T, Zhuo M 2002a Calcium calmodulin-dependent protein kinase IV is required for fear memory. Nat Neurosci 6:573–579

Wei F, Qiu CS, Kim SJ, et al 2002b Genetic elimination of behavioral sensitization in mice lacking calmodulin-stimulated adenylyl cyclases. Neuron 36:713–726

Wei F, Xia XM, Tang J et al 2003 Calmodulin regulates synaptic plasticity in the anterior cingulate cortex and behavioral responses: a microelectroporation study in adult rodents. J Neurosci 23:8402–8409

Yoshimura M, Jessell TM 1990 Amino acid-mediated EPSPs at primary afferent synapses with substantia gelatinosa neurones in the rat spinal cord. J Physiol 430:315–335
Zhuo M 2000 Silent glutamatergic synapses and long-term facilitation in spinal dorsal horn neurons. Prog Brain Res 129:101–113
Zhuo M 2002 Glutamate receptors and persistent pain: targeting forebrain NR2B subunits. Drug Discov Today 7:259–267
Zhuo M 2003 Synaptic and molecular mechanisms for pain and memory. Acta Physiologica Sinica 55:1–8
Zhuo M, Gebhart GF 1997 Biphasic modulation of spinal nociceptive transmission from the medullary raphe nuclei in the rat. J Neurophysiol 78:746–758
Zhuo M, Small SA, Kandel ER, Hawkins RD 1993 Nitric oxide and carbon monoxide produce activity-dependent long-term synaptic enhancement in hippocampus. Science 260:1946–1950
Zhuo M, Hu Y, Schultz C, Kandel ER, Hawkins RD 1994 Role of guanylyl cyclase and cGMP-dependent protein kinase in long-term potentiation in hippocampus. Nature 368:635–639

DISCUSSION

Wood: I'd like to play devil's advocate. With your AC knockouts, there is very strong evidence that things like peripheral nociceptors are regulated in terms of their channel expression by cAMP. How can you distinguish the behavioural consequences of deleting AC in the primary nociceptors and what is taking place in the cingulate cortex?

Zhuo: That's a wonderful question. There is evidence that AC1/AC8 are also expressed in the DRG and dorsal horn neurons at a much lower level. Less expression doesn't mean that they are not important. In the future we need to address this using a forebrain knockout of AC1/AC8. My collaborator in Washington University (Dr Muglia) is working to develop this mouse. We have also published data to show that in the spinal cord slices AC1 and AC8 are required for synaptic potentiation induced by co-application of serotonin and forskolin (Wang & Zhuo 2002). The strong evidence to indicate the involvement of ACC is the 'rescue' experiment using forskolin. We introduced forskolin in the double knockout into the ACC and we get allodynia back. This finding strongly suggests that loss of chronic pain is not simply due to a developmental defect.

Dray: Perhaps you could clarify whether the effects in ACC were specifically localized? Or is this a generalized phenomenon if you look at a number of different forebrain sites?

Zhuo: Most glutamatergic synapses can undergo LTP in the central nervous system. In our previous studies using *in vitro* ACC slices, we have shown that loss of long-term depression after amputation is limited to the ACC (Wei et al 1999). Data using immediate early genes as activity markers also showed distinct patterns of activation in the cortical areas. It is quite likely that pain-related cortical areas may also undergo plastic changes, as responses for other pain-related cognitive functions in the brain. Future studies are needed to address these potential changes.

Dray: There appears to be a relationship between expression of the NR2B receptor and the amplification of the pain signal. Thus NR2B receptors have been a favoured analgesia target because they are highly expressed in dorsal horn. However block of this receptor in the CNS may also impair memory or other performance. Would you comment on this?

Zhuo: That is an important question. We have an ongoing project to address this. With regards to NR2B in the spinal cord versus the cingulate cortex, I have read a recent report in which a NR2B antagonist was used, and it was suggested that the action of the NR2B antagonist is unlikely in the spinal cord dorsal horn (Chizh et al 2001). In the cingulate cortex we have unpublished data showing that microinjection of NR2B antagonists can block mechanical allodynia. How does this happen? If we apply what we learn from the mechanism for NMDA receptors in learning and memory, we expect that NMDA receptors will not contribute to the expression of chronic pain. Why can we still inhibit behavioural allodynia? I think this is the key question, and makes the pain field more attractive than learning and memory. In learning memory as we open a book, the brain forms the memory. We stop learning when we close the book. In case of chronic pain, we open a book and we are forced to read it day and night, because the abnormal sensory inputs keep coming into the central nervous system. This could be why we are still able to rescue the behavioural allodynia by using forskolin, providing strong evidence that chronic pain is really different from learning memory. In both brain slices and freely moving mice we have shown that there is a big slow response mediated by the NMDA receptor in the cingulate cortex (J. Tang, L-J. Wu, M. Zhuo, unpublished data; Liauw et al 2003). This has been reported previously by Roger Nicoll's group in 1991 (Sah & Nicoll 1991). They see a slow component there. NMDA receptor mediates normal transmission in the cingulate cortex. We found that in a chronic pain condition this NMDA that mediates normal synaptic transmission is enhanced (J. Tang, L-J. Wu, M. Zhuo, unpublished data). For NR2B, what happens with memory defects? My take home message is that pain is so important that it takes your whole brain to deal with it. You can't just have a magic bullet to cure the pain: you have to pay a price to get rid of chronic pain. No pain, no gain! There is a study from Joe LeDoux's lab (Rodrigues et al 2001) which shows that if you block NR2B before the induction of fear memory this will interfere with the formation of memory in mice. But if you let the animal form fear memory and then inject NR2B, there is no effect. This is different from chronic pain, because when we inject NR2B it could be 3 d after induction. In fear memory, 3 d after induction NR2B has no effect on established fear memory.

Mao: I am impressed because you showed there is such an early response in the cingulate cortex after a peripheral insult, which could potentially lead to a long-term pathological change. This is a significant finding, but I have some difficulty understanding that the cingulate cortex is necessary for the expression of allodynia.

If you do spinalization in your model, do you think that allodynia will be abolished?

Zhuo: Withdrawal will be blocked when you spinalize animals.

Mao: No, withdrawal will most likely be intact if you do a high spinalization.

Zhuo: My argument for not doing this is that spinalization will cause spinal cord injury, and may trigger even greater changes in the brain. It is hard to use this approach to address the contribution of spinal cord vs. supraspinal structures.

McMahon: This is seen in chronically decerebated animals. Astonishingly, those animals show many complex behaviours to noxious and inflammatory stimuli. They clearly don't need any forebrain for these.

Zhuo: Obviously, the animal under test is in an intact conscious condition. We previously showed that stimulation of the cingulate cortex could only produce descending facilitation, and not descending inhibition of spinal tail flick reflex (Calejesan et al 2001). Our data show that cingulate excitability is quite critical in normal intact animals.

Mao: Ironically, in human subjects perhaps the cingulate cortex is important even in the appreciation or expression of an allodynic response. However, in animal models, because the behaviour test we use is a reflex, I suspect that the cingulate cortex may not be necessary.

Zhuo: We have tried to address this. Instead of using behavioural withdrawals, we use the classic fear memory to study the role of the ACC in pain-related fear responses. So what we do is give a free-moving animal an electric shock in the cingulate cortex. If mice don't like it, they should develop fear memory. What happens is that we give an electric shock, and three days later we put the mice back and they show a nice freezing response. The freezing response depends on the amygdala, feeding into the classic fear memory pathway. Therefore we argue that you need to have input into the amygdala in order for the rat to establish this fear memory. This is why pain is fearful. Hopefully in the future this will help us avoid the withdrawal reflex models.

Wood: You said that L-type Ca^{2+} channel blockers blocked LTP in the cingulate cortex. Do they also block wind-up in the spinal cord?

Zhuo: The N-type Ca^{2+} channel is important for synaptic transmission, so it may not be a good target. I think L-type Ca^{2+} channels may be a better candidate.

Malmberg: While there are a few studies on L-type Ca^{2+} channels and pain, there are significantly more data in favour of N-type Ca^{2+} channel involvement in pain modulation.

Zhuo: My argument is let's not try to use one single model to interpret behaviour. Wind-up and LTP may not directly correlate to behavioural withdrawal in freely moving animals at all. We do the animal work to nail down molecular candidates and then we go on to humans. Mice are not the final answer.

Devor: You were able to 'rescue' allodynia in the double knockouts by putting forskolin into the anterior cingulate. From my understanding of the discussion, this is the only thing that connects this report to pain. Have you tried rescuing by injecting forskolin into other parts of the cortex or other parts of the brain?

Zhuo: That is a good suggestion but we haven't done this yet. I still think the best rescue experiment will be using double-knockout mice with AC1/AC8 expressed back to the ACC.

Devor: So it could be that injecting forskolin anywhere rescues allodynia.

Wood: It is not rescue.

Zhuo: I think that more experiments are needed to test these possibilities.

References

Calejesan AA, Kim SJ, Zhuo M 2000 Descending facilitatory modulation of a behavioral nociceptive response by stimulation in the adult rat anterior cingulate cortex. Eur J Pain 4:83–96

Chizh BA, Reissmuller E, Schlutz H, Scheede M, Haase G, Englberger W 2001 Supraspinal vs spinal sites of the antinociceptive action of the subtype-selective NMDA antagonist ifenprodil. Neuropharmacology 40:212–220

Liauw J, Wang GD, Zhuo M 2003 NMDA receptors contribute to synaptic transmission in anterior cingulate cortex of adult mice. Sheng Li Xue Bao 55:373–380

Rodrigues SM, Schafe GE, LeDoux JE 2001 Intra-amygdala blockade of the NR2B subunit of the NMDA receptor disrupts the acquisition but not the expression of fear conditioning. J Neurosci 21:6889–6896 (Erratum in J Neurosci 22:1A)

Sah P, Nicoll RA 1991 Mechanisms underlying potentiation of synaptic transmission in rat anterior cingulate cortex in vitro. J Physiol 433:615–630

Wang GD, Zhuo M 2002 Synergistic enhancement of glutamate-mediated responses by serotonin and forskolin in adult mouse spinal dorsal horn neurons. J Neurophysiol 87: 732–739

Wei F, Li P, Zhuo M 1999 Loss of synaptic depression in mammalian anterior cingulate cortex after amputation. J Neurosci 19:9346–9354

General discussion II

Tominaga: I have a comment on a question raised by Dr Noguchi's presentation about the different cell size distribution in rats in response to different heat stimuli. This can be explained by expression of TRPV2, another type of TRP thermosensitive receptor expressed in larger cells. It is expressed not in C fibres but in Aδ fibres.

Dray: With respect to the TRPV1 distribution, I have heard that this is also found in non-neuronal cells. What impact does this have on our interpretation of TRPV1 biology? In particular, if the observations are correct that TRPV1 is expressed in the urothelial cells of the urinary bladder, inhibition of TRPV1 activity might have side effects related to bladder dysfunction.

Oh: TRPV1 knockout mice behave normally, except for a reduction in inflammatory hyperalgesia. Even though there are many TRPV channels in the brain, the function of these may not be measurable. Also, TRPV1 is found in epithelial cells, and some say that these channels are present in skin cells. I don't know what they are doing. If we inject TRPV1 antagonists into the animals they behave normally.

Wood: Our discussion has very much focused on TRPV1 as a thermosensor. In fact, thermosensation is normal in the TRPV1 null mutant animals. Proton activation and the effects of eicosanoids may be just as important in these tissues.

Reeh: As far as I know keratinocytes express a low level of TRPV1, 20 times lower than DRGs, for example. The protein can just be detected using immunocytochemistry. If we apply capsaicin to the keratinocytes we don't see an influx of Ca^{2+}. We know how animals look that have been treated neonatally with capsaicin. They have no major deficits in cutaneous function or keratinocyte growth. They have certain sensory deficits, but these are explainable in terms of sensory neuronal distribution of TRPV1.

Belmonte: To add more confusion to the picture, 30% of cold neurons express TRPV1 and respond to capsaicin. This should cause a cold sensation.

Tominaga: There are three places expressing TRPV1 in the body: epithelial cells such as the skin, peripheral sensory neurons and the CNS. We did a lot of immunostaining in the CNS. TRPV1 mRNA and protein are present, especially in the substantia nigra. It might be doing something, but not a lot in the brain. A nice paper by Birder et al (2002) raises the possibility that it might be activated by pressure in the bladder. But I don't think this is likely. I did lots of experiments

applying mechanical stress to sensory nerves and HEK cells expressing TRPV1 and no cells responded at all to the pressure. In the keratinocytes, as Peter Reeh points out, there is very low expression of TRPV1 protein and it is not functional there.

Dray: There appear to be several secondary consequences in the spinal cord following a peripheral nerve injury including activation of neuroglia and a number of kinases. There is also evidence of dysfunction of interneurons leading to disinhibition in spinal cord. In some cases this can result from cell death suggesting that some peripheral neuropathies cause central degeneration similar to other neurological diseases (Moore et al 2002). There is some dispute about such cell losses and the identity of the interneurons involved; believed to be GABA-ergic (see Polgár et al 2003). Although Marshall Devor told us yesterday that the peripheral drive is very important to maintain chronic pain situations, these secondary effects in the spinal cord also set up profound hyperexcitability states, that may also maintain the chronic pain state.

Devor: What makes you so sure that these secondary effects have anything to do with spinal hyperexcitability? They may be just a few more of the hundreds of things that change after nerve injury.

Dray: The literature suggests that there is a relationship between the degree of dysfunction of interneurons or loss of interneurons, and cold allodynia in particular in a specific pathological pain model.

Devor: Of course. The greater the degree of injury in the periphery, the more prominent the central changes, changes causally related to pain processing and changes that are not related. At least give me some evidence that the pain doesn't begin in the periphery and doesn't require the periphery to be sustained.

Dray: It probably does begin in the periphery quite often, but we are talking about central plasticity. Central plasticity does exist in several different manifestations one of which involves an irreversible loss of spinal cells.

Devor: There isn't an irreversible pain. The physicians here told us yesterday that if we block peripheral input from the injured nerve the pain goes away until the block fades and then the pain comes back (Gracely et al 1992).

Apkarian: There are patients who have pain for 35 years.

Devor: Sure, because they have a peripheral lesion for 35 years, or a CNS lesion. If you have a rare example of someone who started with a peripheral nerve lesion and now has pain for 35 years that persists despite a really good spinal block, then we can study this phenomenon on him or her.

Perl: I favour the concept that often the periphery is important in pathological pain. However, numerous clinical situations suggest existence of persisting central phenomena. Cases of long-term peripheral inputs related to particular pains are reported which can be kindled or rekindled by CNS stimulation. We need to leave open the possibility that CNS mechanisms are involved wherein a pattern

of activity occurring as a consequence of persisting peripheral input leads to a central plasticity that sets into place a neural pattern capable of driving expression of the reported pain even after the peripheral initiating circumstances have disappeared.

Devor: I am not denying that things change centrally after peripheral nerve lesion. I am sure that things change, and I accept that the things that change might modulate the peripheral input. I am just looking for an example where you can prove that the pain persisted without peripheral input.

Mantyh: There are a couple of papers by Dr Banati where he uses PET with the peripheral benzodiazepine receptor ligand PK11195 which binds with high affinity to the acute phase reactant α1-acid glycoprotein and can label activated microglia in the CNS (Gerhard et al 2003, Cagnin et al 2001). He has been able to look in nerve injury. I agree with Marshall in that the question is really how long do the central changes persist once the peripheral drive has gone? He has been able to show changes in the cord, thalamus and cortex.

Devor: There is a long list of things that change, but this doesn't mean that they are important for pain.

McMahon: But some of them are important. Coming back to touch-evoked pain, this says there is a central component, whatever peripheral manipulations do. And your criteria seem to me to be unreasonably strict. Spinal cord block will stop some of these central changes. Logically, you have to ask what the evidence is that purely peripheral blocks can eliminate all forms of persistent pain. Here there is less evidence, because it can be technically very difficult to ensure that all possible peripheral inputs have been blocked.

Devor: Agreed, but are they independent of the peripheral input?

McMahon: Whether or not they are independent doesn't change the fact that there are central changes and they contribute to these abnormal pain states.

Devor: The possibility is wide open. The clinicians have to bring us the evidence. What we need are reversible blocks of peripheral input that don't involve blocking the spinal cord. Your reply to the evidence of pain relief from epidural block is effectively that despite lots of central changes above the spinal cord, these are not important. The only central changes of importance are in the spinal cord. So what you do is foraminal block, blocking the root and the dorsal root ganglia, which will block the periphery without blocking the spinal cord. We need the evidence. We have been talking around this issue for a long time. The case of phantoms is probably the best example. The methods are available and easy to apply: anaesthesiologists use foraminal blocks all the time.

Mantyh: In the bone cancer model you can get rid of peripheral sensitization so you can see what I think is central sensitization. This persists much longer. Do they persist forever? I don't know.

Devor: How much longer?

Mantyh: We have been looking 20 days later. By then there is no peripheral drive and we can still see the glial changes. These are much more difficult to reverse than the others.

Devor: In your model, is there activity coming out of the dorsal root ganglia (DRG)?

Mantyh: No. We kill the tumour and then we still see the glial changes. I am not saying that they won't ever go back, but once they have occurred they take longer to return.

Devor: So you have killed the tumour, but this doesn't mean that the DRG cells aren't continuing to fire. Their endings, which were in the bone marrow and which were destroyed by the tumour, have effectively been axotomized. My presumption is that the DRG cells are firing away like crazy, and this might be maintaining your glial changes and tactile allodynia for 20 days or 20 months.

Mantyh: I don't think they are firing away because we don't see any release of substance P or any internalization. It is just that if the glial changes do come back, they take longer than markers of neural activity.

Devor: There is no doubt about this, but is it the glial changes that are causing the tactile allodynia that you see 20 days after, or is it persistent firing of A and C fibres that were cut? This is why we need foraminal block experiments. We need to block the peripheral ectopic input from the injured nerve and see that allodynia in fields served by neighbouring intact nerves remains.

Baron: The problem is that we don't have the answers because the right experiments haven't been done. I believe that there are a few chronic pain states which are centralized, but I can't prove this because we don't have the data.

Zhuo: Obviously, persistent changes occur in the brain after amputation. In monkeys with amputations, if we look over years we see that there is structural change in the somatosensory cortex. There is no doubt that these central changes occur. If pain triggering is due to central mechanisms, peripheral block would obviously not be a good idea.

Mao: This brings us back to the issue of nociception versus pain. This is a fundamental issue in this discussion. In terms of nociception, without nociceptive stimulation you wouldn't have nociception. Whether nociceptive stimulation occurs at the peripheral site or the central site, it probably doesn't matter. However, if we are talking about pain, this becomes a completely different issue. Are we agreed that one could have psychological (psychogenic) pain? In other words, pain being expressed in someone's mind without peripheral or central nociceptive stimulation. This is a key issue. We may have made interchanges between these two issues (nociception vs. pain) in our discussion, and this is why, I guess, we could not agree.

Gintzler: What is psychological pain?

Mao: I would say it is 'pain' without an identifiable pain generator. This is a difficult issue to discuss.

Reeh: That is what anaesthesiologists in pain clinics do every day. They apply an epidural block and ask whether the pain has gone or not. Tell me, what pains are resisting this block and which pains are gone? To my knowledge even 50% of the phantom limb pain is gone after epidural block.

Dray: There is a very strong focus on blocking the drive from the periphery, because this is a logical way of approaching pain therapy. What I am trying to understand is the extent to which the plasticity in the nervous system offers alternative approaches. The peripheral input creates conditions of abnormal pain sensitivity. If these conditions can be reversed then the peripheral input would be processed as if it were a physiological pain.

Devor: How can you say that? A normal healthy person doesn't have a hypersensitive spinal cord. Yet if I step on their toe it hurts them.

Dray: It only hurts them for a little while.

Devor: If I repeatedly step on their toe it will hurt them for a long time. You don't need a sensitized spinal cord to feel pain.

McMahon: I agree that it would be good to eliminate some changes as not being relevant to pain if possible. But it doesn't follow that all changes have to be eliminated. You can feel pain without them but that doesn't mean they are not important.

Baron: I want to give some more examples. In very severe post-herpetic neuralgia patients it has been shown that nearly all the DRG cells have died in these patients due to degeneration and virus infection. They have a complete loss of afferent sensation. I can't imagine where you could block this nerve to produce any reduction in pain.

Devor: This is deafferentation pain, a result of the degeneration of central afferent terminals, and arguably an example of central pain. I agree that it strongly implies a central mechanism.

Dray: There is a huge literature showing that blockade of spinal sensitized mechanisms, will reduce allodynia. To me that is the evidence that you have reduced the relevance of the abnormality of the peripheral input.

Devor: You need a conscious brain to feel pain. It could be that epileptic seizure-like activity in the spinal cord forms a focus of activity which is felt by a conscious brain as pain. I am just looking for a neuropathic condition where that happens. You can inject convulsants like bicucullin or penicillin into the spinal cord and there is no doubt that this causes pain, and that the pain signal originates at the intraspinal injection site. But while this experimental intervention may be a model of central pain, I don't believe that it models peripheral neuropathy.

Dray: Another mechanism that increases spinal excitability is descending facilitation. This can be reduced by lesions of the rostroventral medulla. This is

further evidence of central plasticity as it takes some time for this mechanism to manifest itself following a peripheral nerve injury.

Devor: The example I gave was 20 years of hip pain; when the hip is replaced the pain is gone. Other examples? Impacted teeth, kidney stones, childbirth. Where is the centralization?

References

Birder LA, Nakamura Y, Kiss S et al 2002 Altered urinary bladder function in mice lacking the vanilloid receptor TRPV1. Nat Neurosci 5:856–860

Cagnin A, Myers R, Gunn RN et al 2001 In vivo visualization of activated glia by [^{11}C] (R)-PK11195-PET following herpes encephalitis reveals projected neuronal damage beyond the primary focal lesion. Brain 124:2014–2027

Gerhard A, Banati RB, Goerres GB et al 2003 [^{11}C](R)-PK11195 PET imaging of microglial activation in multiple system atrophy. Neurology 61:686–689

Gracely RH, Lynch SA, Bennett GJ 1992 Painful neuropathy: altered central processing, maintained dynamically by peripheral input. Pain 51:175–194 (Erratum in Pain 52:251–253)

Moore KA, Kohno T, Karchewski LA, Scholz J, Baba H, Woolf CJ 2002 Partial peripheral nerve injury promotes a selective loss of GABAergic inhibition in the superficial dorsal horn of the spinal cord. J Neurosci 22:6724–6731

Polgár E, Hughes DI, Riddell JS, Maxwell DJ, Puskar Z, Todd AJ 2003 Selective loss of spinal GABAergic or glycinergic neurons is not necessary for development of thermal hyperalgesia in the chronic constriction injury model of neuropathic pain. Pain 104:229–239

Anti-opioid systems in morphine tolerance and addiction — locus-specific involvement of nociceptin and the NMDA receptor

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Abstract. Mechanisms for opioid tolerance and addiction are divided into two types of plasticity — cellular level and those occurring through multiple neuronal networks. Receptor desensitization through phosphorylation and endocytosis are currently well discussed using cell lines expressing opioid receptors in relation to acute tolerance mechanisms, while altered gene expression is mainly discussed in relation to the model mechanisms of chronic tolerance and dependence. However, little is known of mechanisms operating through plasticity of neuronal networks. In our approach, we began with the assumption that some non-opioid neurons with anti-opioid activity may cause neuronal plasticity, showing opioid adaptation and dependence. In mice lacking nociceptin/orphanin FQ receptor (NOP), or the NMDA receptor ε1 subunit, both of which mediate anti-opioid activities, analgesic tolerance and dependence following chronic morphine treatments were markedly attenuated. Chronic morphine-treatments increased NOP gene or ε1 subunit protein expression in the spinal cord or specific brain loci, respectively. Furthermore the rescue of the ε1 subunit gene in the specific brain locus of knockout mice recovers the tolerance and dependence. All these results suggest that the enhanced anti-opioid system may contribute to the development of morphine tolerance and dependence, and their contribution could be brain locus specific.

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Recent clinical evidence claimed that morphine treatments for cancer patients with pain do not cause clinically problematic tolerance and addiction, as long as it is used appropriately. However, we have to consider at the same time the fact that terminal cancer patients with severe pain need to take increasing doses during longer survival. As higher doses of morphine are more likely to cause sub-sensitivity to morphine and to worsen quality of life (QOL) by exerting side effects, we need to study how we can prevent the adaptation to morphine. Here I summarize the

current status of studies of morphine tolerance and addiction, and propose some potential molecular targets for developing analgesic adjuvants for new cancer pain.

Cellular mechanisms for morphine tolerance and addiction

The cAMP hypothesis

In animal studies, the adaptation caused by prolonged exposure to large amounts of morphine causes analgesic tolerance and leads to an addiction, such as psychological and physical dependence. The physical dependence is manifested by withdrawal symptoms when morphine is withdrawn or opioid antagonist is given. As the withdrawal symptoms such as hyperalgesia, hyperpnea and diarrhoea are quite opposite to the analgesia, respiratory depression and constipation observed by acute morphine treatment, the mechanisms underlying addiction have been presumed to be derived from cellular adaptation. In the cell culture system, on the other hand, opioid-mediated inhibition of cAMP production reverses to the control level after long exposure to opioid, while challenge by opioid antagonist causes excess production of cAMP. Thus, it has been accepted that cAMP may play a key role in morphine tolerance and addiction. This so-called cAMP hypothesis (Sharma et al 1975) is accepted in a different form, in which chronic morphine treatments mediate gene expression of several molecules to increase cellular cAMP levels (Monteggia & Nestler 2003). Maldonado et al (1996) demonstrated that the development of morphine dependence is significantly inhibited in mutant mice with genetic deletion of cAMP-response element binding protein (CREB), which is not only stimulated by cAMP-dependent protein kinase, but also promotes the gene expression of adenylyl cyclase (Lane-Ladd et al 1997). Although details of morphine-induced CREB activation remain to be determined, some novel mechanisms through activations of the Ras–MAP kinase pathway (Xing et al 1996) through G protein $\beta\gamma$ subunits have recently been claimed.

The PKC and RAVE hypotheses

Current studies have also claimed that desensitization mechanisms through opioid receptor signalling and trafficking play important roles in the development of acute morphine tolerance. Molecular events underlying the reduction of opioid receptor function following morphine pretreatments have been correlated with receptor trafficking, through

- phosphorylation
- internalization/endocytosis and
- sequestration/recycling or
- down-regulation/breakdown (Law et al 2000).

According to our current understanding, longer exposure to higher concentrations of opioids desensitizes the opioid receptor through a phosphorylation process in the C-terminus (Pak et al 1997, Afify et al 1998) and/or third intracellular loop. On the other hand, receptor endocytosis is also influenced by receptor phosphorylation, leading to resensitization or down-regulation through endosomal trafficking (Miller & Lefkowitz 2001). Recent studies revealed that opioid receptors are phosphorylated by many kinds of kinases, such as cAMP-dependent protein kinase (PKA) (Harada et al 1990), protein kinase C (PKC) (Gucker & Bidlack 1992, Ueda et al 1995, Zhang et al 1996), Ca^{2+}/calmodulin-dependent protein kinases (Koch et al 1997), G protein-coupled receptor kinases (GRKs) (Pei et al 1995, Zhang et al 1998), and mitogen-activated protein kinase (Belcheva et al 2001, Polakiewicz et al 1998). For opioid receptor internalization, the GRK mechanism is known to play the most important role (Zhang et al 1998).

In 1989 we first discovered that G_i-coupled receptor functionally mediates phospholipase C (PLC) activation in brain membranes in addition to known inhibition of adenylyl cyclase (Ueda et al 1989). This finding extends the view that the opioid receptor might also mediate an activation of PKC as well as an inhibition of PKA. In the *Xenopus* oocyte expressing δ-opioid receptor (DOP), repeated application of the agonist caused a rapid desensitization of evoked Ca^{2+}-dependent chloride currents through a PKC mechanism (Ueda et al 1995). In this study we first demonstrated that DOP and muscarinic M2 receptor are both functionally coupled to G_{i1}, PLC and downstream Ca^{2+}-activated chloride channel activation by selective co-expression of $G\alpha_{i1}$ subunit and by use of pharmacological tools. This desensitization could be characterized to be quasi-homologous, since DOP desensitization was evoked by repeated DOP stimulations, but not by the stimulation of M2, which shares common post-receptor signalling. As the DOP desensitization rapidly recovers after the application of PKC inhibitor, the desensitization is unlikely to be due to receptor down-regulation, but to the temporal inhibition of receptor function.

We have further demonstrated that PKC mediates opioid tolerance in *in vivo* studies in association with *in vitro* receptor endocytosis mechanisms (Inoue & Ueda 2000, Ueda et al 2001). This study shows the inverse relationship between tolerance liability and μ-opioid receptor (MOP) endocytosis. DAMGO causes a rapid endocytosis of MOP, but morphine does not (Whistler et al 1999). DAMGO (the μ-opioid selective agonist) does not induce acute analgesic tolerance, while morphine does. PKC inhibitors abolish not only acute morphine tolerance, but also the DAMGO tolerance observed when MOP endocytosis is prohibited by *in vivo* treatment with dynamin-negative mutant adenovirus (Ueda et al 2001). He et al (2002) proposed that relative activity versus endocytosis (RAVE) is correlated with opioid tolerance liability, and demonstrated that the

combination of agonist of low RAVE value with morphine alleviates the tolerance. So acute tolerance through receptor trafficking could be explained by so-called PKC and RAVE hypotheses.

Plasticity in neuronal networks involved in morphine tolerance and dependence

Lack of peripheral analgesic tolerance

A clear difference between chronic and acute morphine tolerance has not been demonstrated. In algogenic-induced paw flexion (APF) tests in mice (Inoue et al 1998), peripheral morphine analgesia develops acute tolerance after 4 h pretreatment with 3 nmol (i.pl.) of morphine (Ueda et al 2001). However, the peripheral morphine analgesia has completely recovered 24 h after the initial morphine treatment. Daily administration of 10 mg/kg s.c. of morphine for 5 days showed a marked chronic tolerance in the tail pinch test, which involves higher central nervous mechanisms. However, peripheral morphine analgesia was not affected in such mice showing so-called 'central tolerance'. Thus, it is evident that acute morphine tolerance is mediated by mechanisms distinct from those mediating chronic tolerance, and chronic tolerance is likely mediated through the plasticity in neuronal networks present in the CNS.

Anti-opioid hypothesis

We propose the hypothesis that anti-opioid neuronal systems are involved in the plasticity of neuronal networks during chronic morphine treatments. From the literature, anti-opioid candidates include cholecystokinin (Mitchell et al 2000, Pommier et al 2002), neuropeptide FF (Malin et al 1990), nociceptin (N/OFQ) (Mogil & Pasternak 2001, Ueda et al 1997) and glutamate stimulating the NMDA receptor (Trujillo & Akil 1991). In our approaches we have attempted to examine the validity of this hypothesis using nociceptin (N/OFQ) and NMDA receptor mechanisms.

Nociceptin receptor (NOP) knockout mice showed a partial loss of morphine tolerance (Ueda et al 1997, 2000). In our morphine tolerance experiments, morphine was given daily 10 mg/kg s.c. for 5 days, and the attenuation of morphine analgesia was evaluated on the 6th day. The loss of morphine analgesic tolerance in NOP knock out mice was more evident in the nociception test based on the spinal reflex (tail flick test) than in the systemic biting behaviour (tail pinch test). This was supported by the experiments using the NOP antagonist, J-113397. The intrathecal injection of J-113397 (3 nmol) abolished the morphine analgesic tolerance in the tail flick test, but intracerebroventricular injection (10 nmol) did not. Naloxone-precipitated morphine withdrawal abstinence was also markedly

attenuated in NOP knockout mice and by systemic injection of J-113397. In the paradigm of morphine dependence, morphine was given every 8 h from doses of 20 to 100 mg/kg s.c. for 3 days. The naloxone (1 mg/kg i.p.) injection was given 2 h after the last morphine (100 mg/kg s.c.) injection. J-113397 (10 or 30 mg/kg s.c.), on the other hand, was given 1 h before the naloxone injection.

NOP gene expression measured by RT-PCR was enhanced specifically in the spinal cord by daily morphine administration, according to the tolerance paradigm. On day 5, the NOP level in the spinal cord was 50% higher than the control level through chronic morphine treatment. A similar increase in the NOP level (by 60% of the control level) was observed in the spinal cord from mice pretreated with morphine, according to dependence paradigm. Thus, the plasticity underlying the morphine tolerance and dependence may be attributed at least to the enhanced spinal NOP expression. As NOP knockout mice did not show any change in acute morphine analgesia, the N/OFQ system is unlikely to be downstream of opioid neurons.

The NMDA receptor has long been supposed to play important roles in the development of morphine tolerance and dependence, since several compounds possessing the antagonistic activity inhibit morphine tolerance and dependence (Trujillo & Akil 1991, Trujillo 2000, Mao 2002). As general NMDA antagonists have serious side effects, the development of antagonists specific for the blockade of morphine tolerance has been explored. One approach to find specific antagonists began with identification of the subunit of the NMDA receptor involved in morphine tolerance and addiction. As mice lacking GluRε1 (NR2A) are now available, we have investigated involvement of this subunit in the development of morphine tolerance and dependence using knockout mice. In our experiments (Inoue et al 2003), GluRε1 knockout mice showed an enhancement in acute morphine analgesia in the tail pinch test, which uses more supraspinal mechanisms for nociceptive responses. This unique finding suggests that glutamatergic neurons to stimulate the GluRε1 subunit could be located downstream of opioid neurons, and inhibit endogenous and exogenous opioid actions. This contrasts with the results from the NOP knockout mice. Chronic pretreatment with morphine (10 mg/kg s.c. for 5 days) markedly attenuated the morphine analgesia on the 6th day in wild-type mice in the tail pinch test. However, such analgesic tolerance was not observed in GluRε1 knockout mice. As the protein expression of GluRε1 significantly increased by 100–200% of control levels only in the periaqueductal grey matter (PAG), ventral tegmental area (VTA) and nucleus accumbens (Nacc) throughout the brain, it was speculated that enhanced expression of the anti-opioid NMDA receptor system counteracts morphine analgesia during chronic treatments. In this report we firstly attempted to rescue the GluRε1 gene in specific brain regions of knockout mice to examine the locus-specificity by use of an electroporation technique. The

rescue of this gene into PAG or VTA, but not Nacc successfully recovers the morphine analgesic tolerance. The rescued protein level of GluRε1 was confirmed by western blot analysis. The protein level in PAG was almost the same as that in wild-type mice, and lasted at least 9 days after electroporation. This electroporation technique also has the advantage that the PAG did not cause any morphological damage.

Similar approaches were used to study the mechanisms for morphine dependence. Morphine was given to mice subcutaneously in increasing doses from 20–100 mg/kg for 3 days, and naloxone 1 mg/kg (i.p.) was administered to precipitate withdrawal behaviours 2 h after the last morphine (100 mg/kg s.c.) injection on the 4th day. The withdrawal behaviours such as jumping, withdrawal locomotion, sniffing and defaecation, which were observed in wild-type mice, were markedly inhibited in GluRε1 knock-out mice. The significant increase (by 100% of the control level) in protein expression of GluRε1 by chronic morphine was observed in Nacc of wild-type mice. Indeed, the locus-specific recovery of withdrawal behaviours was observed when the GluRε1 gene was rescued in the Nacc of knockout mice.

All these findings strongly suggest that enhanced anti-opioid systems may counteract the actions of morphine and show an adaptation, and this reversal of adaptation may lead to withdrawal symptoms. One of the most interesting conclusions here is that the plasticity in neuronal networks through such anti-opioid systems during chronic morphine treatments occurs at least to some extent in locus-specific brain regions.

References

Afify EA, Law PY, Riedl M, Elde R, Loh HH 1998 Role of carboxyl terminus of μ- and δ-opioid receptor in agonist-induced downregulation. Mol Brain Res 54:24–34

Belcheva MM, Szucs M, Wang D, Sadee W, Coscia CJ 2001 μ-Opioid receptor-mediated ERK activation involves calmodulin-dependent epidermal growth factor receptor transactivation. J Biol Chem 276:33847–33853

Gucker S, Bidlack JM 1992 Protein kinase C activation increases the rate and magnitude of agonist-induced δ-opioid receptor downregulation in NG108-15 cells. Mol Pharmacol 42:656–665

Harada H, Ueda H, Katada T, Ui M, Satoh M 1990 Phosphorylated μ-opioid receptor purified from rat brains lacks functional coupling with Gi1, a GTP-binding protein in reconstituted lipid vesicles. Neurosci Lett 113:47–49

He L, Fong J, von, Zastrow M, Whistler JL 2002 Regulation of opioid receptor trafficking and morphine tolerance by receptor oligomerization. Cell 25:271–282

Inoue M, Ueda H 2000 Protein kinase C-mediated acute tolerance to peripheral μ-opioid analgesia in the bradykinin-nociception test in mice. J Pharmacol Exp Ther 293:662–669

Inoue M, Kobayashi M, Kozaki S, Zimmer A, Ueda H 1998 Nociceptin/orphanin FQ-induced nociceptive responses through substance P release from peripheral nerve endings in mice. Proc Natl Acad Sci USA 95:10949–10953

Inoue M, Mishina M, Ueda H 2003 Locus-specific rescue of GluRe1 NMDA receptors in mutant mice identifies the brain regions important for morphine tolerance and dependence. J Neurosci 23:6529–6536

Koch T, Kroslak T, Mayer P, Raulf E, Hollt V 1997 Site mutation in the rat μ-opioid receptor demonstrates the involvement of calcium/calmodulin-dependent protein kinase II in agonist-mediated desensitization. J Neurochem 69:1767–1770

Lane-Ladd SB, Pineda J, Boudy VA et al 1997 CREB (cAMP response element-binding protein) in the locus coeruleus: biochemical, physiological, and behavioral evidence for a role in opiate dependence. J Neurosci 17:7890–7901

Law PY, Wong YH, Loh HH 2000 Molecular mechanisms and regulation of opioid receptor signaling. Annu Rev Pharmacol Toxicol 40:389–430

Maldonado R, Blendy JA, Tzavara E et al 1996 Reduction of morphine abstinence in mice with a mutation in the gene encoding CREB. Science 273:657–659

Malin DH, Lake JR, Hammond MV et al 1990 FMRF-NH2-like mammalian octapeptide: possible role in opiate dependence and abstinence. Peptides 11:969–972

Mao J 2002 Opioid-induced abnormal pain sensitivity: implication in clinical opioid therapy. Pain 100:213–217

Miller WE, Lefkowitz RJ 2001 Expanding roles for beta-arrestins as scaffolds and adapters in GPCR signaling and trafficking. Curr Opin Cell Biol 13:139–145

Mitchell JM, Basbaum AI Fields HL 2000 A locus and mechanism of action for associative morphine tolerance. Nat Neurosci 3:47–53

Mogil JS, Pasternak GW 2001 The molecular and behavioral pharmacology of the orphanin FQ/nociceptin peptide and receptor family. Pharmacol Rev 53:381–415

Monteggia LM, Nestler EJ 2003 Opiate addiction: role of the cAMP pathway and CREB. In: Maldonado R (ed) Molecular biology of drug addiction. Humana Press Inc, Totowa, NJ, p 37–44

Pak Y, O'Dowd BF, George SR 1997 Agonist-induced desensitization of the μ opioid receptor is determined by threonine 394 preceded by acidic amino acids in the COOH-terminal tail. J Biol Chem 272:24961–24965

Pei G, Kieffer BL, Lefkowitz RJ, Freedman NJ 1995 Agonist-dependent phosphorylation of the mouse delta-opioid receptor: involvement of G protein-coupled receptor kinases but not protein kinase C. Mol Pharmacol 48:173–177

Polakiewicz RD, Schieferl SM, Dorner LF, Kansra V, Comb MJ 1998 A mitogen-activated protein kinase pathway is required for μ-opioid receptor desensitization. J Biol Chem 273:12402–12406

Pommier B, Beslor F, Simon A et al 2002 Deletion of CCK2 receptor in mice results in an upregulation of the endogenous opioid system. J Neurosci 22:2005–2011

Sharma SK, Klee WA, Nirenberg M 1975 Dual regulation of adenylate cyclase accounts for narcotic dependence and tolerance. Proc Natl Acad Sci USA 72:3092–3096

Trujillo KA 2000 Are NMDA receptors involved in opiate-induced neural and behavioral plasticity? A review of preclinical studies. Psychopharmacology (Berl) 151:121–141

Trujillo KA, Akil H 1991 Inhibition of morphine tolerance and dependence by the NMDA receptor antagonist MK-801. Science 251:85–87

Ueda H, Yoshihara Y, Misawa H et al 1989 The kyotorphin (tyrosine-arginine) receptor and a selective reconstitution with purified Gi, measured with GTPase and phospholipase C assays. J Biol Chem 264:3732–3741

Ueda H, Miyamae T, Hayashi C et al 1995 Protein kinase C involvement in homologous desensitization of δ opioid receptor coupled to Gi1-phospholipase C activation in *Xenopus* oocytes. J Neurosci 15:7485–7499

Ueda H, Yamaguchi T, Tokuyama S, Inoue M, Nishi M, Takeshima H 1997 Partial loss of tolerance liability to morphine analgesia in mice lacking the nociceptin receptor gene. Neurosci Lett 237:136–138
Ueda H, Inoue M, Takeshima H, Iwasawa Y 2000 Enhanced spinal nociceptin receptor expression develops morphine tolerance and dependence. J Neurosci 20:7640–7647
Ueda H, Inoue M, Matsumoto T 2001 Protein kinase C-mediated inhibition of mu-opioid receptor internalization and its involvement in the development of acute tolerance to peripheral mu-agonist analgesia. J Neurosci 21:2967–2973
Whistler JL, Chuang HH, Chu P, Jan LY, von Zastrow M 1999 Functional dissociation of μ opioid receptor signaling and endocytosis: implications for the biology of opiate tolerance and addiction. Neuron 23:737–746
Xing J, Ginty DD, Greenberg ME 1996 Coupling of the RAS-MAPK pathway to gene activation by RSK2, a growth factor-regulated CREB kinase. Science 273:959–963
Zhang L, Yu Y, Mackin S, Weight FF, Uhl GR, Wang JB 1996 Differential μ opioid receptor phosphorylation and desensitization induced by agonists and phorbol esters. J Biol Chem 271:11449–11454
Zhang J, Ferguson SSG, Barak LS et al 1998 Role for G protein-coupled receptor kinase in agonist-specific regulation of μ-opioid receptor responsiveness. Proc Natl Acad Sci USA 95:7157–7162

DISCUSSION

Zhuo: What happened with the NMDA receptor-mediated current in the NR2A knockout mice?

Ueda: We didn't measure this.

Zhuo: There are a lot of areas in the brain where NR2A is quite highly expressed. Is that where you see the current disappear?

Ueda: The hippocampal CA1 area shows a significant decrease in the NMDA receptor-mediated excitatory postsynaptic currents in NR2A knockout mice. But I have no idea about the brain loci we see.

Dray: Can you say whether the dependence or tolerance that you proposed with respect to nociceptin was regionally specific? Could you elaborate on this? You made some inference that there was some regional specificity about nociceptin.

Ueda: As far as the tolerance study is concerned, nociceptin in the spinal cord appears to make the best contribution as an anti-opioid system or the development of morphine tolerance. In the case of dependence, however, we could not obtain clear regional specificity in the brain and spinal cord. But we obtained some changes in the nociceptin receptor expression in the spinal cord in the paradigm of dependence. We tried to look for changes of nociceptin receptor expression in the brain, but the data fluctuate highly. We are now doing some rescue experiments to test which part of the brain is most important for the nociception regulation.

Dray: You focused on nociceptin and the glutamate system. Are there other systems that have been proposed to be involved in morphine tolerance?

Ueda: Nociceptin was discovered in 1995 and then immediately it was proposed that this was an anti-opiate system. We then used knockout mice to show that anti-opioid activity is involved, in 1997. Our proposal using knockout mice would be the first. NPFF and cholecystokinin systems have also been proposed as anti-opioid systems in terms of morphine tolerance. There are some other reports suggesting that opioid κ receptor and the met-enkephalin system are also related to tolerance development, on the basis of mouse knockouts.

Belmonte: I am surprised that there is so much distance between our scientific knowledge about tolerance mechanism for morphine action and the availability of new drugs to prevent the appearance of tolerance in patients. Why haven't we got these drugs, considering how much we know about the nociceptin system and others?

Ueda: It's difficult to answer this. If we use different kinds of knockout mice, such as the enkephalin mice, they have a similar tolerance mechanism. The NMDA system is the most important, and the next is nociceptin. There are also some other reports showing changes in knockout mice. Some reports show the loss of tolerance in knockout mice lacking a certain gene. However, the experiments have been done using the 129-base knockout mouse. As these mice are very stupid animals, possibly possessing some deficiency in the memory and learning process, they cannot develop morphine tolerance. So we have to be careful to evaluate which gene knockout mice truly display a real defect of tolerance liability. As far as we know, the spinal-level response is very important for tolerance liability, and nociceptin might be more important here. Experiments using nociceptin antagonists strongly supported our findings from knockout mice.

Belmonte: But still I don't know why you can't apply this knowledge to humans. There should be drugs which would mimic some of the effects that you have shown, but my impression is that there is still a big gap between basic knowledge and clinical usefulness of this knowledge. Has nociceptin or its analogues been used as a drug?

Ueda: We will have to wait a long time for clinical trials of drugs related to this basic research. Although a certain company I have collaborated with is now not so enthusiastic about developing the nociceptin antagonist for such purposes, I am convinced that it would be a good candidate.

Devor: I want to ask Carlos Belmonte's question in a slightly different way. You get your full effect of tolerance after 5–6 injections. I have some experience in rats where after 2 or 3 injections tolerance is present. My understanding is that in humans you don't get tolerance after 5 injections. You do get some, ultimately, but you reach a plateau. Then you can continue giving morphine for a year or two and you don't get a loss of the effect with the steady dose. Are we talking about the same things here?

Mao: It's an argument that has existed for some years. I think it depends on what kind of pain condition and patient population you investigate. In some patient populations tolerance isn't an issue at all. In others, tolerance does develop.

Gintzler: In animal models, we can easily demonstrate profound tolerance of one or even two orders of magnitude. I am curious as to why this wouldn't be the case in the human population. Why is there any debate about whether or not tolerance develops in humans, when I don't know of a single animal model where tolerance can't be demonstrated?

Mao: It comes down to the difference between the clinical situation and animal models. In humans the only answer is to the question, 'Do you have pain relief?' or you ask them to rate their pain on a scale of 0–10. Is morphine actually treating pain? You are not getting a direct answer about whether tolerance is developing.

Devor: I am willing to take the word of a person who reports their pain over that of a rat that flicks its tail.

Gintzler: It is more complicated: the question is whether morphine doesn't interfere somewhat with the perception of pain but influences the affective component of pain. The patient might say 'Yes, it hurts, but I don't care'.

Devor: Is that what the patients say?

Mao: A lot of times, yes.

Mantyh: What's the situation with neuropathic pain?

Mao: That's another matter of debate.

Devor: So are you saying that morphine doesn't relieve pain in humans, it only makes them say that they don't care anymore?

Mao: In animal studies we think morphine works through several mechanisms. It may block the Ca^{2+} channels or reduce the release of neurotransmitter. Postsynaptically it may have a direct inhibitory effect on cells. On the other hand, it has other side effects. It could change mental status or somehow make patients feel calm and care less. One of my colleagues is a pain physician and one time he had a renal colic. This is a very painful condition, and he was taken to the emergency department and given a dose of morphine. As a pain physician, he said that he could still feel pain, but he didn't care anymore. Morphine is not as clean as the animal models would suggest. It is very difficult to test the clinical utility of these mechanisms we are proposing in animal studies in terms of opioid tolerance because we can't get a clean model of this.

Dray: You can get other objective measures of μ opioid receptor effects by measuring morphine side effects, such as respiratory depression and constipation. Could you comment on the fact that often you can get very rapid tolerance developing to the respiratory depressant effects of morphine, but you get little tolerance to the constipative effects of morphine? I know your focus has been on μ opioid-mediated analgesia, but why are these opioid effector systems regulated in different ways?

Ueda: Before I answer I have to add something. If the animal or human has pain, their morphine tolerance is reduced in the experimental situations using the animals, so the pain state can inhibit tolerance. Respiratory inhibition by morphine is also said to easily develop tolerance, while constipation does not develop tolerance. As I mentioned in my talk, the lack of tolerance of constipation may be related to the sparse neuronal networks in myenteric plexus. With regard to the analgesic tolerance, when we use an excessive amount of morphine (10 mg/kg) in animal experiments, this causes tolerance.

Devor: Are you saying that in animals you are using vastly higher concentrations than the clinicians will use? Why?

Ueda: That's correct. In humans, however, we start with as small a dose as 10 mg a day and go gradually upwards. So it is not hard to understand that morphine in clinical doses does not cause tolerance.

Devor: Why not use clinically relevant doses?

Perl: Rodents are known to need much higher doses of morphine than human beings and other primates to produce demonstrable changes.

Devor: The question returns: are we talking about the same thing?

Mao: That is an issue. For the sake of a testable model, animals serve a useful purpose.

Dray: We have a fundamental issue about translating from the rat to human.

Ueda: Problems might be observed in the case of terminal cancer patients. They will take as much as several hundred milligrams a day, a dose equivalent to that used for the study of tolerance in experimental animals.

Dray: That is testable. Is there any evidence that MK801, even in small amounts, combined with morphine in tolerant patients improves opioid efficacy?

Mao: Yes, but not with MK801. MK801 cannot be used in human subjects.

Ueda: Yes, I agree. I believe we need some adjuvant drugs to reduce the morphine tolerance liability, such as NMDA or nociceptin receptor antagonists.

Gintzler: The implication is that you have selective tolerance: you have tolerance for the analgesic effects but you don't develop tolerance to the activation of the anti-opioid system. I want to get to a point that Andy Dray made. In response to the question as to whether or not you get clinical tolerance in cancer patients, who could be getting a gram of morphine a day, this is a dose that would kill them by respiratory depression normally. Yet they are not dead. Clearly there is profound tolerance to the respiratory depressive effects. This is indisputable. With regard to whether you get equal tolerance to the analgesic effect, one of the confounds is that morphine does lots of things. It may well be that the mechanism for tolerance development is different for each of the various effects that the opioids produce.

Perl: That is true. I remember an addict patient who had main-lined (intravenous) 1 g of morphine. He arrived in the emergency room respiring once

a minute, turning cyanotic between breaths. Yet this man complained of pain when pricked with a needle during the physical examination.

Dray: One of the mechanisms you suggested involved microglia and the release of cytokines. Do you have any evidence to support this as a mechanism of receptor regulation?

Ueda: These microglial studies suggest that morphine acts and causes some morphological and functional changes on the cell. Activated microglial cells might release various kinds of neurotrophic factors and cytokines, which in turn cause plasticity of neuronal circuits and neural anti-opioid gene expression.

Dray: Can you block the mechanism with anti-TNF, for example?

Ueda: I haven't tried this yet.

Dray: There is some recent evidence that high doses of morphine can be cytotoxic.

Ueda: Long-term morphine exposure might cause some apoptosis in the spinal cord neurons through the action of microglia. The chronic state is different from the acute setting.

Oh: I don't understand why DAMGO and morphine act on the same opioid receptor, but one goes to tolerance and the other does not. Is it a matter of potency?

Ueda: Potency or kinetics.

Gintzler: There is also another important difference, which is that DAMGO is highly selective for the μ opioid receptor whereas morphine is not. It interacts with all of the major classes of opioid receptor (μ, δ and κ), particularly at the doses used for tolerance experiments.

Ueda: The other thing is that GPCR receptors are present as dimers. So the dimerization of μ and δ opioid receptor might change such ligand interactions. Dimerization is another key point.

Chronic morphine-induced plasticity among signalling molecules

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Abstract. Most formulations of the consequences of the persistent activation of opioid receptors have centred on the diminution or loss of opioid receptor-coupled signalling mechanisms. Activation of opposing compensatory circuits remains another of the adaptations proposed to underlie the extreme loss of the antinociceptive potency of narcotics following their chronic administration. Recent research has revealed that adaptations to chronic morphine involve not only the impairment of opioid receptor functionality but also the altered consequences of its G protein coupling. Pre-eminent among the biochemical perturbations that underlie the chronic morphine-induced emergence of new signalling strategies are enhanced phosphorylation and altered expression of key signalling molecules. These molecular changes include the up-regulation and augmented phosphorylation of adenylyl cyclase type II isoforms, which underlies the ability of morphine to shift opioid receptor G protein signalling from predominantly $G_{i\alpha}$ inhibitory to $G_{\beta\gamma}$ stimulatory. Persistent morphine exposure also enhances the concomitant phosphorylation of G protein receptor kinase, β arrestin and the G protein G_{β} subunit, one consequence of which is to further enhance G protein receptor signalling via the $G_{\beta\gamma}$ subunit. This review will focus on our increasing understanding of the importance of qualitative changes among components of opioid receptor-coupled signalling pathways, as opposed to the interruption of such signalling, as the predominant mode of adapting to the presence of opioids.

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Multiple adaptations are elicited in response to the persistent presence of morphine. These are thought to underlie the reestablishment of homeostasis in the central nervous system (CNS) and mediate the development of pharmacological tolerance. Despite enormous scientific efforts, tolerance to the antinociceptive effects of opioids remains a predominant impediment to their medicinal use for pain relief. Research in this area continues to be inspired by the unique perspective it provides on signal transduction plasticity in the CNS. Additionally, such research is inspired by the conviction that elucidation of the adaptations that are causally associated with opioid tolerance will translate into

the development of pharmacotherapies for its amelioration. Either, individually and collectively, would have a substantial impact on the effectiveness of medicinal treatments for acute and chronic pain.

A remaining major conundrum that confounds investigations of chronic morphine-induced adaptations that are causally related to tolerance formation is parsing those that are contributory to opioid tolerance versus parallel or epiphenomenona. An additional complicating factor is that tolerance is always defined relative to specific opioid actions such as analgesia, sedation and respiratory depression, which frequently manifest differential tolerance development. This suggests that different opioid functions are mediated via different signalling strategies that could elicit varied adaptational mechanisms. Thus, the general applicability and exclusivity of any model of opioid tolerance should be rigorously assessed prior to acceptance. These caveats notwithstanding, the plethora of adaptations to chronic morphine that have thus far been demonstrated are testimony to the profound and perturbing effects of opioids on CNS equilibria.

Adaptations to the persistent presence of morphine generally fall into two main formulations, those that result in the actual loss of specific opioid receptor-mediated signalling and those that result in the *apparent* loss of this activity via its masking. Examples of adaptations resulting in the loss of opioid action would include the diminution of spare opioid receptors (Chavkin & Goldstein 1984), altered opioid receptor density/internalization (Bohn et al 2000, Chakrabarti et al 1995) and altered G protein content (Ammer & Schultz 1995). Additionally, studies utilizing GTPγS^{35} binding, which reflects the exchange of GTP for GDP on the heterotrimeric G protein and thus its activation, have demonstrated decreased opioid receptor G protein coupling following chronic systemic morphine (Sim et al 1996).

The best characterized exemplar of adaptations that result in masking opioid receptor functionality is adenylyl cyclase (AC) superactivation or overshoot (Duman et al 1988, Sharma et al 1975a). This refers to the robust up-regulation of AC activity that is manifest upon the acute withdrawal of an opioid after chronic opioid treatment. Its demonstration is often interpreted to indicate the presence of enhanced AC activity in chronic morphine-treated cells prior to agonist withdrawal (although this inference has never been unequivocally demonstrated). This is the basis for postulating that the reestablishment of 'normal' levels of AC activity in chronic morphine-treated cells results from the up-regulation of this signalling enzyme, which counter balances the inhibitory influence of the continued presence of morphine.

It is important to note that the marked increase in AC activity that occurs following the acute removal of morphine from chronically treated tissue also reveals that opioid receptor functionality (signalling) persists in these

preparations. In other words, it indicates that even after chronic morphine treatment and the manifestation of profound opioid tolerance, opioids can still negatively modulate AC activity (Sharma et al 1975b). This consideration underscores that interference with opioid receptor functionality e.g. opioid receptor desensitization, regardless of the underlying mechanism(s) cannot be the exclusive basis for opioid tolerance.

The AC superactivation theory of tolerance also has significant limitations. AC superactivation is not uniformly observed in all brain regions. For example, in rats, chronic *in vivo* administration of morphine increased basal, G protein and forskolin-stimulated AC activity in the locus coeruleus but not in the dorsal raphe, frontal cortex or neostriatum (Duman et al 1988). Furthermore, the CNS contains a multiplicity of AC isoforms, several of which do not manifest superactivation. In fact, those of the type II AC family (AC II, IV, VII) manifest *reduced* activity following chronic morphine exposure and its abrupt withdrawal (Avidor-Reiss et al 1997).

More recent observations indicate that in addition to the quantitative adaptations noted above, chronic morphine also elicits qualitative changes in opioid receptor-coupled signalling. Specifically, recent research has revealed that adaptations to chronic morphine involve not only the impairment of opioid receptor functionality but also the altered consequences of its G protein coupling. Underlying the chronic morphine-induced emergence of new signalling strategies are fundamental changes in the nature of effectors that are coupled to opioid receptor-G protein-signalling pathways. These adaptations reflect the availability of multiple opioid receptor-coupled signalling strategies, the relative predominance of which can be altered in response chronic morphine (Fig. 1).

Several observations were the predicate for this formulation. In some tissues such as the guinea pig longitudinal muscle myenteric plexus (LMMP) preparation, opioid responsiveness is clearly bimodal. Low concentrations of opioids enhance whereas higher concentrations inhibit transmitter release (methionine-enkephalin) and cAMP formation (Wang & Gintzler 1994, Xu et al 1989). Pharmacological observations indicate that G_s- and G_i/G_o-like G proteins mediate the positive and negative opioid modulation, respectively (Gintzler & Xu 1991, Wang & Gintzler 1997). Chronic morphine treatment results in a shift in opioid functionality from predominantly inhibitory to excitatory (Gintzler et al 1987, Wang & Gintzler 1995). Notably, however, although chronic morphine augments the G_s-mediated low dose μ opioid facilitative pathway (Wang & Gintzler 1997), this adaptation does not underlie the concomitant reversal of high dose sufentanil inhibition to enhancement since the former is abolished by cholera toxin while the latter is not (Wang & Gintzler 1997). Additionally, the chronic morphine-induced modality shift in opioid functionality does not result

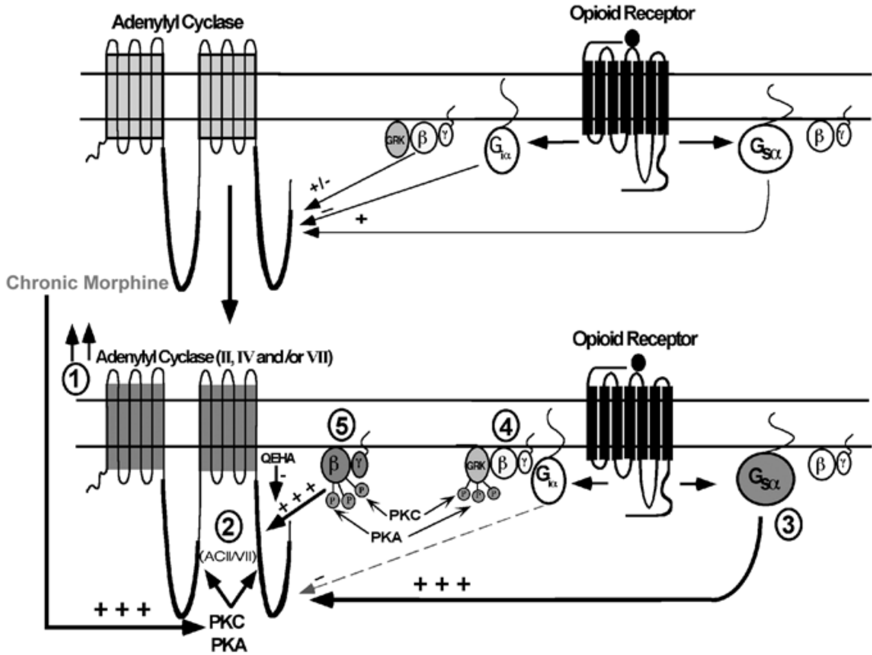

FIG. 1. Schematic representation of adaptations among signalling molecules induced by chronic morphine. Conclusions are drawn from experiments utilizing longitudinal muscle myenteric preparations obtained from opioid naive and chronic morphine-treated guinea pigs. # 1 depicts the AC isoform specific upregulation of AC IV and VII; #2 depicts the substantial enhancement of AC phosphorylation (most likely AC II and VII) via PKC; #3 depicts the augmented stimulatory response of AC to $G_{s\alpha}$ that results from AC phosphorylation #4 and #5 depicts the concomitant augmentation of GRK2/3 and G_{β} phosphorylation, respectively, both predominantly via PKC and PKA. Adaptations act in concert to shift opioid receptor-coupled signalling from predominantly $G_{i\alpha}$ inhibitory to $G_{\beta\gamma}$ AC stimulatory. Up-regulation of ACs of the type 2 family ($G_{s\alpha}$-dependent $G_{\beta\gamma}$ stimulated) and their phosphorylation (which augments their $G_{\beta\gamma}$ stimulatory responsiveness) would greatly augment the $G_{\beta\gamma}$ stimulatory arm of G_i-coupled signal transduction pathways. Furthermore, phosphorylation of G_{β} decreases interaction of $G_{\beta\gamma}$ with GRK making more available for interaction with effectors such as AC. These consequences would be further exacerbated by the augmented phosphorylation of the G_{β} subunit, which approximately doubles the magnitude of $G_{s\alpha}$-dependent $G_{\beta\gamma}$ stimulation of AC. Thus, the chronic morphine-induced augmented prominence of $G_{\beta\gamma}$ stimulatory AC signalling is determined by a confluence of factors, which in the aggregate are multiplicative (modified from Gintzler & Chakrabarti 2000).

from the loss of inhibitory G_i-mediated responsiveness (such as that which occurs following treatment with pertussis toxin). In fact, in chronic morphine-treated LMMP tissue, despite the shift from inhibitory to excitatory opioid modulation, there appears to be a paradoxical augmentation of μ opioid receptor coupling to the inhibitory (G_i-mediated) opioid pathway. This finding is consistent with (a) the

subsequent demonstration (Ingram et al 1998) of enhanced efficacy of μ opioid receptors to inhibit GABAergic evoked inhibitory postsynaptic potentials in periaquiductal gray neurons following *in vivo* chronic morphine and (b) the seemingly contradictory observation (Lang & Schulz 1989) that the LMMP content of $G_{i\alpha}$ is elevated following chronic *in vivo* morphine exposure, despite the loss of opioid receptor-coupled inhibitory signalling.

Thus, despite the numerous demonstrations of an opioid stimulatory (presumably G_s-mediated) signalling pathway, it seems unlikely that its augmentation along with the impairment of the inhibitory (G_i) pathway contributes to the tolerant-associated predominance of sufentanil facilitative effects. In other words, in tolerant/dependent tissue, the predominance of opioid excitatory responses is, most likely, not mediated via an alteration of the balance between facilitative and inhibitory mechanisms that are operative in opioid naïve tissue. Instead, in the aggregate, these observations point to a greater subtlety of adaptations that are elicited by chronic morphine. Recently obtained data reveal that isoform-specific adaptation of key signalling proteins, e.g. enhancement of their phosphorylation, could play a pivotal role in adapting to the continued presence of morphine.

Adenylyl Cyclase

AC isoform-specific synthesis

It is very well established that multiple isoforms of AC exist, many of which are differentially regulated by the $G_{\beta\gamma}$ subunit of G proteins (Tang & Gilman 1991, 1992). For example, $G_{\beta\gamma}$ inhibits ACI but stimulates AC isoforms that comprise the type two family (ACII, IV and VII) (Tang & Gilman 1991 1992). Thus, the relative predominance of AC isoforms and alterations thereof can have enormous physiological consequences. Chronic morphine-induced AC isoform-specific synthesis is, in fact, one adaptation to chronic morphine that is a basis for the diminution of inhibitory opioid receptor AC signalling and the emergence of excitatory opioid receptor-coupled sequelae.

Following chronic systemic morphine administration, mRNA encoding AC IV (Rivera & Gintzler 1998) and AC VII (Gintzler & Chakrabarti 2000) are significantly elevated in LMMP tissue. Additionally, in these preparations AC protein levels, most likely comprised predominantly of AC isoforms IV and/or VII, are also significantly augmented (56%) (Chakrabarti et al 1998a). This is of particular functional relevance to adaptations that are elicited by chronic morphine since, in contrast to ACI, which is inhibited by $G_{\beta\gamma}$, ACIV and ACVII are both stimulated by $G_{\beta\gamma}$ (Gao & Gilman 1991, Tang & Gilman 1991, Watson et al 1994).

AC phosphorylation

Persistent activation of opioid receptors with morphine also has profound effects on the phosphorylation state of AC (ACII, IV, VII), which is dramatically increased (Chakrabarti et al 1998b). Moreover, the magnitude of the chronic morphine-induced augmented AC phosphorylation is substantially attenuated by chelerythrine, a protein kinase C (PKC)-selective inhibitor. Chelerythrine pretreatment also blocks the chronic morphine-induced shift in opioid receptor AC signalling from predominantly inhibitory to stimulatory (Wang et al 1996) underscoring the potential relevance of increased (PKC-mediated) phosphorylation, presumably of AC albeit not exclusively, to adaptations elicited by chronic morphine.

Functional consequences of AC isoform-specific synthesis and phosphorylation

Up-regulation of AC isoforms IV and VII and augmented phosphorylation of AC II and/or VII would have convergent physiological consequences. Both, in combination, would be expected to result in a shift in receptor/G protein signalling from predominantly $G_{i\alpha}$ inhibitory to $G_{\beta\gamma}$ stimulatory AC signalling. This laboratory has in fact demonstrated such a shift (Chakrabarti et al 1998a), the evidence for which consists of two critical observations: (1) chronic *in vivo* treatment with morphine significantly enhances the magnitude of AC stimulation produced by activated recombinant $G_{s\alpha}$ and (2) sub-optimal concentrations of the $G_{\beta\gamma}$ blocking peptide QEHA (Chen et al 1995) abolish this chronic morphine-induced increment in AC stimulation by recombinant $G_{s\alpha}$. The latter observation indicates that the enhanced stimulation of AC produced by $G_{s\alpha}$ is most likely mediated via augmented ($G_{s\alpha}$-dependent) $G_{\beta\gamma}$ stimulatory AC responsiveness in these preparations.

These biochemical changes could underlie the previously demonstrated shift from high dose opioid inhibition to stimulation of AC activity (Wang & Gintzler 1995, 1997) and transmitter release (Gintzler et al 1987). Increased AC stimulation by $G_{s\alpha}$, which is also known to result from augmented, PKC-mediated, ACII/ACVII phosphorylation (Jacobowitz & Iyengar 1994, Watson et al 1994, Zimmermann & Taussig 1996), could also underlie the previously reported (Wang & Gintzler 1997) enhancement of low-dose sufentanil facilitation of stimulatory AC responsiveness that is elicited by chronic morphine treatment.

Concomitant phosphorylation of GRK2/3, β-arrestin and the G_β subunit of G proteins

In addition to eliciting increased AC isoform-specific synthesis and their phosphorylation, chronic morphine also induces the concomitant

phosphorylation of several other signalling proteins resulting in their altered association. Specifically, in LMMP tissue, the phosphorylation state of GRK2/3, β arrestin and G_β are concomitantly augmented $\approx$twofold. Augmented phosphorylation of all three proteins is evident in immunoprecipitate obtained using either anti-GRK2/3 or G_β antibodies, but not their pre-adsorbed controls, suggesting that they exist, at least in part, as a multi-molecular complex.

Of particular relevance, phosphorylation of GRK2/3, β arrestin and G_β has opposing consequences on their ability to associate. Phosphorylation of G_β attenuates its association with GRK2/3 and promotes its dissociation from the complex containing GRK2/3 and β arrestin. This is evidenced by two observations: (1) the total amount of GRK that co-immunoprecipitates with G_β is significantly reduced in chronic morphine-treated tissue and (2) *in vitro* phosphorylation of purified bovine $G_{\beta\gamma}$ via PKC and PKA, both of which contribute to its chronic morphine-induced augmented phosphorylation *in vivo*, attenuates its interaction with recombinant GRK2 protein (Chakrabarti et al 2001).

In contrast, phosphorylation of GRK2/3 (and β arrestin) increases its kinase activity and promotes their association with non-phosphorylated G_β (Chakrabarti et al 2001, Chuang et al 1995, Sarnago et al 1999). This is of particular functional importance since it could explain the inability of chronic morphine to attenuate membrane GRK2/3 content. This should be expected, based on the chronic morphine-induced augmentation of G_β phosphorylation and the consequent reduction in the size of the pool of nonphosphorylated G_β, which recruits GRK to the membrane (Inglese et al 1993). The most plausible explanation for this paradox is that although phosphorylation of G_β effectively reduces the amount of this protein that would be available for interaction with GRK, this effect is partially compensated for by the enhanced ability of phosphorylated GRK (levels of which are augmented following chronic morphine) to interact with non-phosphorylated G_β. Following chronic morphine, non-phosphorylated $G_{\beta\gamma}$ would continue to recruit GRK2/3 to the membrane that is then stored in association with charged membrane phospholipids, via a pleckstrin homology domain (DebBurman et al 1995).

The consequences of the chronic morphine-induced altered interaction between GRK2/3 and G_β, the net effect of which is the increased availability of (phosphorylated) $G_{\beta\gamma}$ has substantial functional implications. These directly pertain to the observed alteration in opioid receptor-coupled signalling that has been documented to occur in response to persistent opioid receptor activation. Decreased association of $G_{\beta\gamma}$ with GRK2/3 would increase its availability for interaction with biological effectors such as AC. This could contribute to the augmented $G_{\beta\gamma}$ stimulatory AC signalling demonstrated in chronic morphine-treated LMMP tissue (Chakrabarti et al 1998a). Additionally, the phosphorylated G_β subunit could also participate in an enzymatic transfer of a phosphate to GDP at

G protein α subunits (Wieland et al 1993) and thereby prevent reformation of heterotrimeric G proteins. This would maintain the pool of free $G_{\beta\gamma}$ subunits available for signalling.

Most recently, we have investigated the effect of *in vitro* phosphorylation of G_{β} on its ability to stimulate AC. *In vitro* phosphorylation of the G_{β} subunit via the catalytic subunit of either PKA or PKC increases ≈twofold the increment in $G_{s\alpha}$-dependent ACII activity produced by $G_{\beta\gamma}$ (Chakrabarti & Gintzler 2003). Moreover, of particular relevance to altered functionality induced by chronic morphine is the current observation that (threonine) phosphorylated G_{β} not only occurs naturally in the spinal cord but its levels are augmented ≈60% following chronic morphine (Chakrabarti & Gintzler 2003). Therefore, multiple biochemical adaptations, chemical modification of the G_{β} subunit itself as well as modification of its effector (AC), underlie the increased signalling via $G_{\beta\gamma}$ subunits that is elicited in response to chronic morphine.

In conclusion, there is abundant evidence that opioid receptor–effector coupling remains substantially intact following chronic morphine, the most obvious being the enormously robust magnitude of naloxone precipitated withdrawal that can be elicited from chronic morphine-treated animals as well as isolated cells and tissues. Despite some evidence of the loss of opioid receptor functionality, it is clear that this cannot be the exclusive underpinning of opioid tolerance. Adaptations to chronic morphine involving the emergence of new consequences of opioid receptor-coupled signal transduction processes, which may actually be the antithesis of the consequences predominant in the naïve state, should be considered to be major potential contributors to the tolerant condition. The most prevalent biochemical mechanism underlying such changes thus far appears to be the concomitant phosphorylation of key signalling molecules. Further elucidation of the molecular components of opioid tolerance-producing mechanisms holds out the promise of developing adjunct pharmacotherapies that would selectively minimize their contribution and thus enhance the pharmacological control of pain.

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References

Ammer H and Schultz R 1995 Chronic activation of inhibitory δ-opioid receptors cross-regulates the stimulatory adenylate cyclase-coupled prostaglandin E1 receptor system in neuroblastoma X glioma (NG108-15) hybrid cells. J Neurochem 64:2449–2457

Avidor-Reiss T, Nevo I, Saya D, Bayewitch M, Vogel Z 1997 Opiate-induced adenylyl cyclase superactivation is isozyme-specific. J Biol Chem 272:5040–5047

Bohn LM, Gainetdinov RR, Lin FT, Lefkowitz RJ, Caron MG 2000 Mu-opioid receptor desensitization by beta-arrestin-2 determines morphine tolerance but not dependence. Nature 408:720–723

Chakrabarti S, Gintzler AR 2003 Phosphorylation of Gβ is augmented by chronic morphine and enhances G$\beta\gamma$ stimulation of adenylyl cyclase activity. Mol Brain Res 119: 144–151

Chakrabarti S, Law P-Y and Loh HH 1995 Neuroblastoma neuro2A cells stably expressing a cloned μ-opioid receptor: a specific cellular model to study acute and chronic effects of morphine. Mol Brain Res 30:269–278

Chakrabarti S, Rivera M, Yan S-Z, Tang W-J, Gintzler AR 1998a Chronic morphine augments G$\beta\gamma$/Gsα stimulation of adenylyl cyclase: relevance to opioid tolerance. Mol Pharmacol 54:655–662

Chakrabarti S, Wang L, Tang W-J, Gintzler AR 1998b Chronic morphine augments adenylyl cyclase phosphorylation: relevance to altered signalling during tolerance/dependence. Mol Pharmacol 54:949–953

Chakrabarti S, Oppermann M, Gintzler AR 2001 Chronic morphine induces the concomitant phosphorylation and altered association of multiple signaling proteins: a novel mechanism for modulating cell signaling. Proc Natl Acad Sci USA 98:4209–4214

Chavkin C, Goldstein A 1984 Opioid receptor reserve in normal and morphine-tolerant guinea pig ileum myenteric plexus. Proc Natl Acad Sci USA 81:7253–7257

Chen J, DeVivo M, Dingus J et al 1995 A region of adenylyl cyclase 2 critical for regulation by G protein $\beta\gamma$ subunits. Science 268:1166–1169

Chuang TT, LeVine H and De Blasi A 1995 Phosphorylation and activation of β-adrenergic receptor kinase by protein kinase C. J Biol Chem 270:18660–18665

DebBurman SK, Ptasienski J, Boetticher J, Lomasney JW, Benovic JL, Hosey MM 1995 Lipid-mediated regulation of G protein-coupled receptor kinases 2 and 3. J Biol Chem 270:5742–5747

Duman RS, Tallman JF, Nestler EJ 1988 Acute and chronic opiate-receptor regulation of adenylate cyclase in brain: specific effects in locus coerulus. J Pharmacol Exp Ther 246:1033–1039

Gao B, Gilman AG 1991 Cloning and expression of a widely distributed (type IV) adenylyl cyclase. Proc Natl Acad Sci USA 88:10178–10182

Gintzler AR, Xu H 1991 Different G proteins mediate the opioid inhibition or enhancement of evoked [5-methionine] enkephalin release. Proc Natl Acad Sci USA 88:4741–4745

Gintzler AR, Chakrabarti S 2000 Opioid tolerance and the emergence of new-opioid receptor-coupled signalling. Mol Neurobiol 21:21–33

Gintzler AR, Chan WC, Glass J 1987 Evoked release of methionine-enkephalin from tolerant/dependent enteric ganglia: paradoxical dependence on morphine. Proc Natl Acad Sci USA 84:2537–2539

Inglese J, Freedman NJ, Koch WJ, Lefkowitz RJ 1993 Structure and mechanism of the G protein-coupled receptor kinases. J Biol Chem 268:23735–23738

Ingram SL, Vaughan CW, Bagley EE, Connor M, Christie MJ 1998 Enhanced opioid efficacy in opioid dependence is caused by an altered signal transduction pathway. J Neurosci 18:10269–10276

Jacobowitz O, Iyengar R 1994 Phorbol ester-induced stimulation and phosphorylation of adenylyl cyclase 2. Proc Natl Acad Sci USA 91:10630–10634

Lang J, Schulz R 1989 Chronic opiate receptor activation in vivo alters the level of G-protein subunits in guinea pig myenteric plexus. Neuroscience 32:503–510

Rivera M, Gintzler AR 1998 Differential effect of chronic morphine on mRNA encoding adenylyl cyclase isoforms: relevance to physiological sequela of tolerance/dependence. Mol Brain Res 54:165–169
Sarnago S, Elorza A, Mayor F 1999 Agonist-dependent phosphorylation of the G protein-coupled receptor kinase 2 (GRK2) by Src tyrosine kinase. J Biol Chem 274:34411–34416
Sharma SK, Nirenberg M, Klee W A 1975a Morphine receptors as regulators of adenylate cyclase activity. Proc Natl Acad Sci USA 72:590–594
Sharma SK, Klee WA, Nirenberg M 1975b Dual regulation of adenylate cyclase accounts for narcotic dependence and tolerance. Proc Natl Acad Sci USA 72:3092–3096
Sim LJ, Selley DE, Dworkin SI, Childers SR 1996 Effects of chronic morphine administration on mu opioid receptor-stimulated [^{35}S]GTPγS autoradiography in rat brain. J Neurosci 16:2684–2692
Tang W-J, Gilman AG 1991 Type-specific regulation of adenylyl cyclase by G protein $\beta\gamma$ subunits. Science 254:1500–1503
Tang W-J, Gilman AG 1992 Adenylyl cyclases. Cell 70:869–872
Wang L, Gintzler AR 1994 Bimodal opioid regulation of cAMP formation: implications for positive and negative coupling of opiate receptors to adenylyl cyclase. J Neurochem 63:1726–1730
Wang L, Gintzler AR 1995 Morphine tolerance and physical dependence: reversal of opioid inhibition to enhancement of cAMP formation. J Neurochem 64:1102–1106
Wang L, Gintzler AR 1997 Altered μ-opiate receptor-G-protein signal transduction following chronic morphine exposure. J Neurochem 68:248–254
Wang L, Medina VM, Rivera M, Gintzler AR 1996 Relevance of phosphorylation state to opioid responsiveness in opiate naive and tolerant/dependent tissue. Brain Res 723:61–69
Watson PA, Krupinski J, Kempinski AM, Frankenfiedl CD 1994 Molecular cloning and characterization of the type VII isoform of mammalian adenylyl cyclase expressed widely in mouse tissues and in S49 mouse lymphoma cells. J Biol Chem 269:28893–28898
Wieland T, Nurnberg B, Ulibarri I, Kaldenberg-Stasch S, Schulz G, Jakobs KH 1993 Guanine nucleotide-specific phosphate transfer by guanine nucleotide-binding regulatory protein beta-subunits. Characterization of the phosphorylated amino acid. J Biol Chem 268:18111–18118
Xu H, Smolens I, Gintzler AR 1989 Opioids can enhance and inhibit the electrically evoked release of methionine-enkephalin. Brain Res 504:36–42
Zimmermann G, Taussig R 1996 Protein kinase C alters the responsiveness of adenylyl cyclase to G protein α and $\beta\gamma$ subunits. J Biol Chem 271:27161–27166

DISCUSSION

Dray: If your conclusions can be related to the effects of morphine on the brain *in vivo*, then morphine tolerance could be explained entirely in terms of the fine regulation of G protein signalling.

Gintzler: While it is tempting for me to say that, of course, I won't. It would be superficial to draw that conclusion at the moment. As Jianren Mao pointed out, given the activation of the systems that Hiroshi Ueda spoke about, if morphine is doing it, then the question is, what enables morphine to turn those systems on? While I don't know experimentally whether there is a direct connection, one could certainly formulate a milieu in which the NMDA system and the nociceptin system are downstream effectors of the $G_{\beta\gamma}$ story. It is important to

note that we use adenylate cyclase, which has been the predominant effector used to monitor opioid effects, but it certainly is by no means the only effector for $G_{\beta\gamma}$. There are a plethora of other effectors whose activity is modulated by $G_{\beta\gamma}$. I am quite sure that the story wouldn't just end here.

Ueda: Did you show direct coupling of the receptor to the G_s receptor?

Gintzler: It all depends on what you consider as direct coupling. We have demonstrated in the myenteric plexus a cholera toxin-sensitive effect of opiates which is not blocked by pertussis toxin. If you are asking about whether we have ever been able to show that G_s co-immunoprecipitates (IPs) with the μ receptor, the answer is no. At the present, the evidence for G_s coupling to opioid receptors is indirect and pharmacological.

Oh: If you have a tissue that is chronically treated with morphine, and instead of probing with an opioid, you probe it with an M2 agonist, would you see a tolerance response?

Gintzler: That's a good question. We haven't looked. In the AC system, the effects of $G_{\beta\gamma}$ are determined by the particular isoform(s) of AC that is (are) present as well as its state of phosphorylation, both of which are modulated by chronic morphine. So there is a great deal of morphine-induced plasticity. I don't know what $\beta\gamma$ does to the channels modulated by M2 agonists. If channel responsiveness to $G_{\beta\gamma}$ is phosphorylation-state dependent, which is modulated by chronic morphine, and if the α subunit inhibits while the $\beta\gamma$ subunit facilitates channel activity, then possibly changes analogous to adaptations observed in the AC system would also occur.

Oh: The reason I am asking is I am anticipating a similar thing in another system. Normally, $G_{i\alpha}$ inhibits AC. But you said that on repeated application $G_{\beta\gamma}$ kicks in which stimulates AC. Do you see a similar pathway in other systems which use $G_{i\alpha}$ to down-regulate AC?

Ueda: I might be able to answer this question. In 1995 I published a paper using *Xenopus* oocytes expressing the opioid δ receptor and the M2 receptor in this experiment (Ueda et al 1995). When we reconstitute the classical G_α which is sensitive to pertussis toxin, to see the Ca^{2+} current. Repeated challenge with δ agonists causes desensitization but this is blocked by PKC inhibitors. In the same system repeated challenge with acetylcholine did not affect the δ opioid receptor sensitivity, though acetylcholine also causes Ca^{2+} current through the same pertussis toxin-sensitive G_α subunit. So δ opioid receptor, but not downstream G protein, is desensitized by PKC. As far as we know the M2 receptor has no site for PKC and cannot be desensitized by PKC.

Oh: There is not much tolerance or desensitization through M2 receptors.

Ueda: In oocyte experiments, repeated acetylcholine stimulation did not show homologous desensitization.

Oh: What happens in the AC knockout mouse? Does it develop morphine tolerance?

Gintzler: I don't know whether there is an AC II knockout. There have been knockouts of AC I and AC VIII, but they are not relevant because these isoforms are not stimulated by $\beta\gamma$.

Mantyh: Have you looked at the different cell populations that would be involved in tolerance? Does it then follow that this more or less predicts which ones are going to show tolerance and which ones don't, in a rank order?

Gintzler: I do not have any specific data pertaining to various cell types. However, I would certainly infer that cells unable to express AC isoforms of the type II family (AC II, IV and VII) and/or modulate their levels of phosphorylation would be limited in the spectrum of adaptations to chronic morphine that are available to them.

Mantyh: This is relevant to an earlier question by Andy Dray about why certain lines show tolerance more than others, and why certain side effects are more prevalent than others.

Gintzler: We haven't addressed this, but certainly addressing such issues could provide critical insight into tolerance mechanisms. I think that it has a lot to do with subtleties of signal transduction that are not often addressed. These would include relative predominance of AC, G_β and PKC isoforms, composition of macro-molecular signalling complexes, etc.

Mantyh: Can one apply what you have found therapeutically?

Gintzler: Potentially, yes. Since $G_{\beta\gamma}$ is one of the mediators of tolerance, interfering with its opioid receptor-coupled signalling could have medicinal value. Currently the only way of chelating $\beta\gamma$ is to overexpress chelating proteins. This isn't to say that we couldn't develop a non-peptide small molecule that could inhibit $G_{\beta\gamma}$. This would probably have substantial side effects, given the universality of signalling through $G_{\beta\gamma}$. However, if one succeeded in identifying relevant populations of cells and in targeting a $G_{\beta\gamma}$ blocker to those cells, it might be possible to achieve sufficient specificity for such a blocker to have medicinal value.

Dray: Can you generalize your conclusions to other μ opioid ligands? You have used morphine exclusively.

Gintzler: We are thinking of doing those experiments. We have continued to use morphine because of its clinical importance. I have no reason to suspect that we wouldn't see it with more selective ligands.

Dray: There have been a number of subtypes of μ receptor suggested (Snyder & Pasternak 2003). Is there anything that can be generalized to these subtypes in terms of the way that they would be regulated?

Gintzler: One of the problems in the tolerance field is that putative mechanisms are often not parsed with respect to specific opioid functions. Also, it is important

to note that the adaptations that I have discussed involve regulating opioid receptor-coupled signalling downstream from the receptor. So, they would be pertinent regardless of the opioid receptor subtype that is involved in mediating opioid effects. For example, as far as I know, there is little evidence indicating that the large number of proposed subtypes of μ opioid receptors each have distinct signalling pathways.

Zhang: I am curious to know if there is any kind of dimerization of opioid receptors in the DRG neurons. μ opioid receptor, δ opioid receptor and κ receptor are expressed in small DRG neurons and usually they colocalize. What do you think the possibility is that the signal pathways integrate the functions of different types of opioid receptor?

Gintzler: Are you asking me how dimerization would fit into this mechanism? I don't know how dimerization would fit into this. Most of the evidence of μ/δ or κ/δ dimerization has been in cell experiments where these receptors are over expressed. I'm not sure that anyone has demonstrated dimerization convincingly in native receptors. This may simply be due to methodological reasons. What I have proposed doesn't require any dimerization: it addresses adaptations and plasticity of the signalling downstream from the opioid receptor. On the other hand, the formulations I have been discussing do not exclude contributions from altered signalling that might result from opioid receptor hetero- and/or homodimerization.

Dray: There is a recent publication about the involvement of μ opioid receptor regulation of δ opioid receptor trafficking (Cahill et al 2001). In these experiments cortical cells that were exposed to morphine trafficked δ opioid receptors, which are normally internalized on peptide-containing vesicles, to the cell surface.

Gintzler: This mechanism fits in perfectly with my formulations as previously published (Chakrabarti et al 2001). Lefkowitz had shown some years ago that in order for the β arrestin to react with and internalize receptors, it had to be dephosphorylated (Lin et al 1997). Morphine promotes the phosphorylation state of β arrestin, possibly explaining why morphine does not internalize opioid receptors. It is 'locking' the β arrestin out and thus interfering with the internalization pathway. It was based on these data, which I showed you: Eisinger et al (2002) predicted that exposure to morphine followed by challenge with a δ receptor agonist should interfere with δ receptor internalization; precisely what was reported.

Zhang: Could you comment on the role of the κ opioid receptors in the dorsal horn?

Gintzler: This is not directly related to what I have just presented, but we have data that under certain conditions, activation of spinal κ opioid receptors produces antinociception. κ opioid receptors in the spinal cord are in a minority. The most abundant type of opioid receptor in the spinal cord is μ. Much less is δ and an even

lesser amount is counted for by κ. Clinically, spinal fentanil or sufentanil, both of which are μ-preferring, are the opioids most often utilized to induce spinal analgesia. We have data that at least under certain conditions — physiological pregnancy and its hormonal simulation — spinal κ opioid receptors (in combination with δ opioid receptors) are also analgesic.

References

Cahill CM, Morinville A, Lee MC, Vincent JP, Collier B, Beaudet A 2001 Prolonged morphine treatment targets delta opioid receptors to neuronal plasma membranes and enhances delta-mediated antinociception. J Neurosci 21:7598–7607

Chakrabarti S, Oppermann M, Gintzler AR 2001 Chronic morphine induces the concomitant phosphorylation and altered association of multiple signaling proteins: a novel mechanism for modulating cell signaling. Proc Natl Acad Sci USA 98:4209–4214

Eisinger DA, Ammer H, Schulz R 2002 Chronic morphine treatment inhibits opioid receptor desensitization and internalization. J Neurosci 22:10192–10200

Lin F-T, Krueger M, Kendall HE et al 1997 Clathrin-mediated endocytosis of the β-adrenergic receptor is regulated by phosphorylation/dephosphorylation of β-arrestin 1. J Biol Chem 272:31051–31057

Snyder SH, Pasternak GW 2003 Historical review: opioid receptors. Trends Pharmacol Sci 24:198–205

Ueda H, Miyamae T, Hayashi C et al 1995 Protein kinase C involvement in homologous desensitization of δ-opioid receptor coupled to G_{i1}-phospholipase C activation in *Xenopus* oocytes. J Neurosci 15:7485–7499

Opioid tolerance and neuroplasticity

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Abstract. Opioid analgesics are highly effective for treating many forms of acute and chronic pain. The development of opioid analgesic tolerance is a pharmacological phenomenon indicative of the cellular and system adaptation that could affect the clinical use of opioid analgesics. Activation of N-methyl-D-aspartate receptors and protein kinase C as well as regulation of glutamate transporters has been implicated in the mechanisms of opioid tolerance, suggesting a possible link between neural plasticity and the mechanisms of opioid tolerance. More recent studies have shown that neural plasticity associated with the development of opioid tolerance may activate a pronociceptive mechanism within the central nervous system that could counteract the analgesic effects of opioids. Thus, exposure to opioids could lead to two seemingly unrelated cellular processes, i.e. (1) the development of opioid tolerance — a negative sign of cellular adaptation, and (2) the development of opioid-induced pain sensitivity — a positive sign of cellular adaptation. The converging effects of these cellular mechanisms would significantly reduce the opioid analgesic efficacy. The current evidence also suggests new approaches for improving the clinical use of opioid analgesics.

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Opioid analgesics are highly effective for treating many forms of acute and chronic pain. The development of opioid analgesic tolerance is a pharmacological phenomenon indicative of the cellular and system adaptation that could affect the clinical use of opioid analgesics. Activation of N-methyl-D-aspartate receptors (NMDAR) and protein kinase C as well as regulation of glutamate transporters (GTs) has been implicated in the mechanisms of opioid tolerance, suggesting a possible link between neural plasticity and the mechanisms of opioid tolerance. More recent studies have shown that neuroplasticity associated with the development of opioid tolerance may activate a pronociceptive mechanism within the central nervous system (CNS) that could counteract the analgesic effects of opioids. Thus, exposure to opioids could lead to two seemingly unrelated cellular processes, i.e.

- the development of opioid tolerance — a negative sign of cellular adaptation, and
- the development of opioid-induced pain sensitivity — a positive sign of cellular adaptation.

The converging effects of these cellular mechanisms would significantly reduce the opioid analgesic efficacy. The current evidence also suggests new approaches to improving the clinical use of opioid analgesics. In this chapter, I will specifically examine the evidence regarding the role of glutamate homeostasis in neuroplasticity associated with the development of opioid tolerance.

Regional glutamate homeostasis regulated by glutamate transporters

Glutamate is a major excitatory amino acid neurotransmitter in the CNS participating in the maintenance of important physiological functions such as synaptic plasticity and cognitive awareness. Maintaining a low extracellular glutamate concentration is key to preventing glutamate over-excitation and neurotoxicity that could occur under many pathological conditions. An efficient, high-capacity GT system within the CNS regulates the extracellular glutamate concentration, because clearance of extracellular glutamate via glutamate metabolism or diffusion is virtually negligible. Thus far, at least five cell membrane GT proteins have been identified and cloned (Robinson & Dowd 1997, Danbolt 2001). Among these cell membrane GTs, EAAT1 (GLAST), EAAT2 (GLT-1), and EAAT3 (EAAC1) are particularly relevant to the regulation of glutamate uptake in broad CNS regions. In addition, there have been increasing reports of vesicular GTs and their functional role and regulations remain to be determined (Takamori et al 2000, Bellocchio et al 2000).

EAAC1 is generally considered as a neuronal GT whereas GLAST and GLT-1 are primarily astroglial GTs (Robinson & Dowd 1997, Danbolt 2001). GTs are primarily located in the CNS with a sporadic extra-CNS presence in the heart, kidney, and gastrointestinal system. Subcellularly, GTs are located in plasma membranes, mitochondria and synaptic vesicles with the vast majority of GTs being associated with plasma membranes of both neuronal and glial cells (Robinson & Dowd 1997, Danbolt 2001). GTs participate in regulating the uptake of L-glutamate as well as L- or D-aspartate in a Na^+- and K^+-dependent manner. The exact stoichiometry of glutamate uptake by GTs in relation to Na^+ and K^+ ions remains unclear. In general, GTs transport glutamate from the low-concentration extracellular compartment to the high-concentration intracellular compartment at the cost of both Na^+ and K^+ ion gradients. Under certain circumstances such as global CNS ischaemia, reversed uptake (from intracellular to extracellular compartments) could take place secondary to a weakened driving force from decreased transmembrane electrochemical gradients (Robinson & Dowd 1997, Danbolt 2001).

Role of GTs in neuroplasticity associated with neurological disorders and neuropathic pain

Glutamate plays a dual role both as a major excitatory neurotransmitter essential for physiological functions and a neurotoxic mediator contributory to pathological processes. Of significance is that, although inhibition of GT activity may not significantly prolong a single stimulus-induced excitatory postsynaptic glutamate current, it does so if the stimulus is repetitive and excessive (Overstreet et al 1999), a condition that can be encountered under many pathological circumstances. Reduced GT function leads to the accumulation of extracellular glutamate, causing excessive activation of glutamate receptors and initiating processes of glutamate-mediated neuronal over-excitation and excitotoxicity. To date, a large number of studies have shown the role of GTs in neuroplasticity associated with a variety of neurological disorders including brain ischaemia, epilepsy, spinal cord injury, amyotrophic lateral sclerosis, AIDS neuropathy, and Alzheimer's disease (Rothstein et al 1996, Mennerick et al 1999, Lievens et al 2000, Vorwerk et al 2000, Trotti et al 2001, Bigini et al 2001, Rao et al 2001, Vera-Portocarrero et al 2002).

GTs have also been shown to be involved in the spinal nociceptive processing in response to the hindpaw formalin injection or exogenous NMDA or prostaglandins (Minami et al 2001, Niederberger et al 2003). A series of recent experiments have demonstrated that both expression and uptake activity of spinal GTs changed following peripheral nerve injury and contributed to neuropathic pain behaviours in rats (Sung et al 2003). Intrathecal administration of the tyrosine kinase receptor inhibitor K252a and the mitogen-activated protein kinase inhibitor PD98059 reduced and nearly abolished the increase in GT expression, respectively. Moreover, peripheral nerve injury significantly reduced spinal GT uptake activity, which was prevented by riluzole (a positive GT activity regulator). Riluzole also effectively attenuated and gradually reversed neuropathic pain behaviours. These results indicate that spinal GTs may play a critical role in both induction and maintenance of neuropathic pain following nerve injury via regulating regional glutamate homeostasis. The involvement of GTs in the mechanism of neuropathic pain is of particular interest because compelling evidence has indicated that neuropathic pain and opioid tolerance may have much in common in terms of their neural mechanisms (Mao et al 1995a).

Role of GTs in neuroplasticity associated with opioid tolerance and dependence

In animal models of morphine tolerance, subcutaneous injection of a proposed GT activator MS-135 diminished the development of morphine tolerance (Nakagawa

et al 2001). More recently, chronic morphine administered through either intrathecal boluses or continuous infusion has been shown to induce a dose-dependent down-regulation of GTs (EAAC1 and GLAST) in the rat's superficial spinal cord dorsal horn (Mao et al 2002a). This GT down-regulation was mediated through opioid receptors because naloxone blocked such GT changes. Morphine-induced GT down-regulation reduced the ability to maintain *in vivo* glutamate homeostasis at the spinal level, since the hyperalgesic response to exogenous glutamate was enhanced, including an increased magnitude and a prolonged time course, in morphine-treated rats with reduced spinal GTs. Moreover, the down-regulation of spinal GTs exhibited a temporal correlation with the development of morphine tolerance. Consistently, the GT inhibitor PDC potentiated, whereas the positive GT regulator riluzole reduced, the development of morphine tolerance. The effects from regulating spinal GT activity by PDC were at least in part mediated through activation of NMDAR, since the non-competitive NMDAR antagonist MK-801 blocked morphine tolerance that was potentiated by PDC. These results indicate that spinal GTs may contribute to the neural mechanisms of morphine tolerance by means of regulating regional glutamate homeostasis.

Recent evidence suggests that GTs may play a role in opioid dependence as well. First, changes in the GLT-1 mRNA level occurred following naloxone-precipitated morphine withdrawal (Ozawa et al 2001). Second, during the withdrawal period from a sustained morphine treatment, glutamate uptake activity at hippocampal synapses was substantially increased accompanied by an increase in the expression of GLT-1 (Xu et al 2003). These results indicate that there may be a compensatory change in GT activity and expression, a response that is likely to serve as a buffer system to minimize the impact of glutamate surges accompanying opioid withdrawal.

Opioid-induced neuronal apoptosis and pain sensitivity

Both preclinical and clinical studies have indicated that the development of morphine tolerance could be associated with an increased pain sensitivity, which may contribute to the manifestation of opioid tolerance (Mao et al 1995b, Ossipov et al 1995, Celerier et al 2001). In addition, neurotoxic events in the form of neuronal *apoptosis* have been demonstrated in association with the development of morphine tolerance (Mao et al 2002b). These apoptotic cells were predominantly located in the superficial spinal cord dorsal horn and most apoptotic cells also expressed GAD, a key enzyme for the synthesis of the inhibitory neurotransmitter GABA. In addition, increased nociceptive sensitivity to heat stimulation was observed in these same rats and modulation of GT activity regulated the occurrence of both opioid-induced neuronal apoptosis and increased pain sensitivity (Mao et al 2002b). These results are consistent with the role of the

spinal glutamatergic system in both opioid tolerance and neuropathic pain and provide new insights into interactions between the cellular mechanisms underlying both opioid tolerance and pain hypersensitivity.

Clinical implications

The importance of neuroplasticity in the development of opioid tolerance suggests a new strategy for preventing opioid tolerance, opioid-induced neuronal apoptosis and pain sensitivity. We now recognize that the development of opioid tolerance is not merely a process of negative cellular adaptation that leads to the diminished pharmacological effects of opioids. This process is accompanied by the activation of a pronociceptive system, a positive sign of cellular adaptation. The endpoint of both processes would be the reduction of opioid analgesic effects and the restoration of nociceptive signalling. An active role of GTs in both processes is the regulation of regional glutamate homeostasis. Thus, regulation of regional glutamate homeostasis using a GT regulator such as riluzole may modulate neuroplasticity associated with the development of opioid tolerance. Investigation is under way to explore such possibilities. Furthermore, studies on regulation of glutamate homeostasis, neuroplasticity and opioid tolerance may also provide new insights into the neural mechanisms of substance abuse.

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References

Bellocchio EE, Reimer RJ, Fremeau RT Jr, Edwards RH 2000 Uptake of glutamate into synaptic vesicles by an inorganic phosphate transporter. Science 289:957–960
Bigini P, Bastone A, Mennini T 2001 Glutamate transporters in the spinal cord of the wobbler mouse. Neuroreport 12:1815–1820
Celerier E, Laulin JP, Corcuff JB, Le Moal M, Simonnet G 2001 Progressive enhancement of delayed hyperalgesia induced by repeated heroin administration: a sensitization process. J Neurosci 21:4074–4080
Danbolt NC 2001 Glutamate uptake. Prog Neurobiol 65:1–105
Lievens JC, Bernal F, Forni C, Mahy N, Kerkerian-Le Goff L 2000 Characterization of striatal lesions produced by glutamate uptake alteration: cell death, reactive gliosis, and changes in GLT-1 and GADD45 mRNA expression. Glia 29:222–232
Mao J, Price DD, Mayer DJ 1995a Mechanisms of hyperalgesia and opiate tolerance: a current view of their possible interactions. Pain 62:259–274
Mao J, Price DD, Mayer DJ 1995b Experimental mononeuropathy reduces the antinociceptive effects of morphine: implications for common intracellular mechanisms involved in morphine tolerance and neuropathic pain. Pain 61:353–364

Mao J, Sung B, Ji RR, Lim G 2002a Chronic morphine induces downregulation of spinal glutamate transporters: implications in morphine tolerance and abnormal pain sensitivity. J Neurosci 22:8312–8323

Mao J, Sung B, Ji RR, Lim G 2002b Neuronal apoptosis associated with morphine tolerance: evidence for an opioid-induced neurotoxic mechanism. J Neurosci 22:7650–7661

Mennerick S, Shen W, Xu W et al 1999 Substrate turnover by transporters curtails synaptic glutamate transients. J Neurosci 19:9242–9251

Minami T, Matsumura S, Okuda-Ashitaka E et al 2001 Characterization of the glutamatergic system for induction and maintenance of allodynia. Brain Res 895:178–185

Nakagawa T, Ozawa T, Shige K, Yamamoto R, Minami M, Satoh M 2001 Inhibition of morphine tolerance and dependence by MS-153, a glutamate transporter activator. Eur J Pharmacol 419:39–45

Niederberger E, Schmidtko A, Rothstein JD, Geisslinger G, Tegeder I 2003 Modulation of spinal nociceptive processing through the glutamate transporter GLT-1. Neuroscience 116:81–87

Ozawa T, Nakagawa T, Shige K, Minami M, Satoh M 2001 Changes in the expression of glial glutamate transporters in the rat brain accompanied with morphine dependence and naloxone-precipitated withdrawal. Brain Res 905:254–258

Ossipov MH, Lopez Y, Nichols ML, Bian D, Porreca F 1995 The loss of antinociceptive efficacy of spinal morphine in rats with nerve ligation injury is prevented by reducing spinal afferent drive. Neurosci Lett 199:87–90

Overstreet LS, Kinney GA, Liu YB, Billups D, Slater NT 1999 Glutamate transporters contribute to the time course of synaptic transmission in cerebellar granule cells. J Neurosci 19:9663–9673

Rao VL, Dogan A, Todd KG et al 2001 Antisense knockdown of the glial glutamate transporter GLT-1, but not the neuronal glutamate transporter EAAC1, exacerbates transient focal cerebral ischemia-induced neuronal damage in rat brain. J Neurosci 21:1876–1883

Robinson MB, Dowd LA 1997 Heterogeneity and functional properties of subtypes of sodium-dependent glutamate transporters in the mammalian central nervous system. Adv Pharmacol 37:69–115

Rothstein JD, Dykes-Hoberg M, Pardo CA et al 1996 Knockout of glutamate transporters reveals a major role for astro-glial transport in excitotoxicity and clearance of glutamate. Neuron 16:675–686

Sung B, Lim G, Mao J 2003 Altered expression and uptake activity of spinal glutamate transporters after nerve injury contributes to the pathogenesis of neuropathic pain in rats. J Neurosci 23:2899–2910

Takamori S, Rhee JS, Rosenmund C, Jahn R 2000 Identification of a vesicular glutamate transporter that define the glutamatergic phenotype in neurons. Nature 407: 189–194

Trotti D, Aoki M, Passinelli P, Berger UV et al 2001 Amyotrophic lateral sclerosis-linked glutamate transporter mutation has impaired glutamate clearance capacity. J Biol Chem 276:576–582

Vera-Portocarrero LP, Mills CD, Ye Z et al 2002 Rapid change in expression of glutamate transporters after spinal cord injury. Brain Res 927:104–110

Vorwerk CK, Naskar R, Schuettauf F et al 2000 Depression of retinal glutamate transporter function leads to elevated intravitreal glutamate levels and ganglion cell death. Invest Ophthalmol Vis Sci 41:3615–3621

Xu NJ, Bao L, Fan HP et al 2003 Morphine withdrawal increases glutamate uptake and surface expression of glutamate transporter GLT1 at hippocampal synapses. J Neurosci 23:4775–4784

DISCUSSION

Mantyh: So the idea is that opiates down-regulate the glutamate/aspartate transporter, which is also known as GLAST or EAAC1 (excitatory amino acid transporter 1)? You indicated that they expressed a new opiate receptor.

Mao: Yes, we did some co-immunostaining and the μ opioid receptor does co-localize with GLAST.

Mantyh: Is it the microglia or the astrocyte?

Mao: We don't know yet.

Mantyh: We have looked for astrocytes expressing the opiate receptors from cord and haven't been able to see that.

Devor: A short while ago we heard the *in vitro* and mouse people asking themselves whether their results are relevant to clinical tolerance. If the way you have told the story is right, the clinical people have a similar problem. You are describing results from populations of street addicts with and without methadone, who have all sorts of other problems, and data on tolerance based on clinical populations with cancer, mostly, where there is disease progression and we don't know how the underlying stimulus is changing over time. Is it not possible to take normal healthy volunteers and give them a series of morphine injections, to see whether tolerance develops?

Mao: This experiment is certainly doable. I don't know in normal subjects how long it would take to induce 'tolerance', which I think would be both dose- and drug-related, but is testable.

Devor: If it has anything to do with the mice, it should just take a couple of days, or weeks at the outside. It sounds like an experiment that ought to be done.

Mao: The problem is that it doesn't necessarily answer the question of pathological pain either. The responsiveness to opioids in patients with pathological pain may have a different pattern: this has been shown in animal models. This experiment would certainly show whether tolerance will or will not be induced in those subjects. But this doesn't necessarily answer the question in a general patient population.

Belmonte: Is the hyperalgesia that you see limited to nociceptors? Is the sensitivity to other non-nociceptive stimuli, such as cold, normal? You say that these people have higher cold pain sensitivity, but what about their response to innocuous cooling, is cold threshold normal?

Mao: I don't know at this point. Generally speaking, some physicians are quite sceptical about the possibility of the existence of this phenomenon. Physicians are trained to increase the dose of a medication if the dose doesn't solve the problem. This may be true with most drugs, but I guess opioid treatment may be an exception.

Zhang: What is the dose of morphine being used to induce cell apoptosis?

Mao: It is 10 μg given intrathecally. This also is related to Dr Devor's earlier question, which I didn't answer fully. Interestingly, the systemic dose of opioids used in animal models is probably 50–100 times higher than in humans, but intrathecal doses are comparable. Maybe pharmacokinetic issues are involved in the difference in systemic delivery between rats and humans.

Devor: Is the serum level in rats given these large doses 100 times higher than the level in humans, or is that just the dose that is injected?

Mao: I don't know — that's a good question, and there must be data out there on this issue.

Zhuo: In your model you are suggesting that opiate treatment causes a change in glutamate transporter activity. One of the predicted results would be that glutamatergic receptor functions may be enhanced. Recently we have shown in the spinal cord there are a lot of presynaptic glutamate receptors that could inhibit glutamate release, which might lead to the opposite in the model (Kerchner et al 2002); or they can regulate the GABA release which might support your model (see Kerchner et al 2001). Second, you mention the PKC regulation of the sensitivity of NMDA receptors to magnesium. Do you have your own evidence for this?

Mao: I know this is controversial, but for the convenience of the model we are accepting this. It could be direct or indirect in terms of interactions between μ opioid and NMDA receptor functions.

Zhuo: I have heard that this phenomenon may not be common in the central nervous system.

Mao: Sure.

Apkarian: What is the magnitude of apoptosis that you see?

Mao: In these rats it is about 8–12 per spinal section. I don't know how this translates to the human setting. One thing about this apoptotic change is that it is difficult to confirm this in human subjects, because we need to collect and fix the tissue, and no one is willing to donate a piece of their spinal cord.

Apkarian: In rats, is it localized to the point of injection?

Mao: It is mainly in the lumbar area.

Mantyh: With respect to the apoptosis issue, there are a lot of studies that have been done on cancer patients. I would imagine that cord is relatively obtainable. There have been some recent studies, which although they haven't done TUNEL staining, have produced cytological stains suggestive of the pathological apoptosis at the tip of the catheter which is related to the dose of opioid agonist given intrathecally (Horais et al 2003, Coffey & Burchiel 2002).

Mao: That is one of the many confounding factors. Apoptosis can be induced by many factors including metabolic imbalance or simply taking the tissue out.

Inoue: I would like to ask you about the hypersensitivity to morphine treatment. I think this phenomenon is irreversible. What happens to morphine-evoked

hypersensitivity after morphine treatment is finished? Does the sensitivity recover to normal?

Mao: That's a good question. We haven't looked at this. I was careful not to link apoptosis directly to tolerance in terms of its contribution to pain sensitivity. I am not quite sure whether apoptosis is the end product of this process or is in some way contributory in terms of increased pain sensitivity or tolerance.

Baron: You mentioned that addicts who receive methadone have a higher pain sensitivity. Is this correct?

Mao: Yes.

Baron: But methadone has a considerable NMDA-blocking effect. Why isn't there a reversible effect on this sensitivity?

Mao: First of all, methadone has been regarded as a NMDA receptor antagonist in certain circumstances. There is a problem with methadone, though. If you think about an agent that has actions on two opposing systems, meaning that if methadone serves both as a μ opioid receptor agonist and a NMDA receptor antagonist, the outcome on either system would be self-limited. If you increase a methadone dose to counteract the NMDA receptor-mediated development of tolerance, you would also increase the μ activity, which would facilitate the development of tolerance. That is, the outcome of this agent would be determined by the intrinsic ratio of this agent on the μ and NMDA receptor activity. One cannot preferentially choose one pharmacological property of methadone without affecting the other.

Dray: I have a question related to the expression of apoptotic markers. Does this necessarily mean cells will degenerate, or does it indicate cells in distress but which have the potential for recovery?

Mao: I am not in this field, as apoptosis itself is an independent field that is ever evolving. I am using these markers for *in situ* identification of DNA fragmentation. I know there is debate about whether the TUNEL staining indicates that the cell is undergoing an irreversible process.

Dray: Is there any evidence that people who are tolerant to opioids (i.e. consume large quantities) have characteristic neurodegenerative signs?

Mao: I don't think there is much evidence at this point. If you consider patients who are on opioids for months or years, and if a transient morphine administration to rats induces a detectable apoptotic process, you would expect that the spinal cord of these patients with long-term opioid therapy would be severely affected. This has not been looked at in the clinical setting.

Dray: If the MK801 mechanism prevents opioid tolerance, I would have expected that drug abusers would have figured this out by now and it would be traded on the street to reduce the expense of opioid drugs! Perhaps you don't get the desirable effect if you mix say heroin with MK801.

Oh: The tolerance of receptor/ligand is kind of a protection mechanism for the cell. If you provoke tolerance, why does that cell undergo apoptosis? Tolerance should be protecting the cell.

Mao: As I said, I think both sensitization and desensitization processes are mechanisms for a biological system to resist certain changes in response to narcotic analgesics. These changes would potentially have detrimental outcomes, because one cannot survive without the ability to feel pain. If the nociceptive system is somehow compromised, either by the integrity of the nociceptor pathway being damaged or the activity of this system being reduced by a pain medication such as opioid, the system tries to counteract such changes beyond adaptation. That is why I think sensitization and desensitization processes are not mutually exclusive, but actually work in concert to counteract such changes.

Gintzler: I think his question was because tolerance is adaptive, why is apoptosis occurring which is maladaptive?

Mao: I guess it depends on how you look at the total outcome from these two processes. The process of adaptation (desensitization) may not be able to protect this system fully during the process.

Inoue: I have a slightly unusual suggestion. Suramin is believed to block the ATP receptor, but we have reported that it also blocks the NMDA receptor and AMPA. Then instead of MK801 why don't you use suramin as a blocker of the glutamate receptor function? Suramin is a drug that we have used for more than 80 years for the treatment of trypanosomiasis. It is relatively safe and cheap.

Mao: I probably wouldn't choose that agent. I would use a more selective NMDA antagonist. Unfortunately, as you know, most NMDA receptor antagonists are not usable in human subjects, MK801 being an example. There are a few clinically available NMDA receptors, but they are not highly selective.

References

Coffey RJ, Burchiel K 2002 Inflammatory mass lesions associated with intrathecal drug infusion catheters: report and observations on 41 patients. Neurosurgery 50:78–87

Horais K, Hruby V, Rossi S et al 2003 Effects of chronic intrathecal infusion of a partial differential opioid agonist in dogs. Toxicol Sci 71:263–275

Kerchner GA, Wang GD, Qiu CS, Huettner JE, Zhuo M 2001 Direct presynaptic regulation of GABA/glycine release by kainate receptors in the dorsal horn: an ionotropic mechanism. Neuron 32:477–488

Kerchner GA, Wilding TJ, Huettner JE, Zhuo M 2002 Kainate receptor subunits underlying presynaptic regulation of transmitter release in the dorsal horn. J Neurosci 22:8010–8017

General discussion III

Zhang: Dr Gintzler, if the μ opioid receptors stay on the surface and keep functional, what do you think about the process of the μ opioid receptor being desensitized and then resensitized after chronic exposure to morphine?

Gintzler: The classical pattern is for it to be internalized, dephosphorylated and then reinserted into the membrane. This is the classical way in which the receptors become resensitized. You are asking what happens when the receptors stay on the surface and are not allowed to be internalized, why don't they remain desensitized? Perhaps this is contributory to some extent to the tolerance. We don't get internalization, but over time perhaps there is some desensitization. There is a delicate balance between the receptors that become internalized and which ones become reinserted into the membrane or continue on the lysosomal degradation pathway. If I am reading your question correctly, you are asking how one can explain continued functionality of the μ opioid receptor, which I am proposing albeit in a qualitatively different way, if they are not internalized and thus cannot be resensitized.

Zhang: Does the receptor stay on the cell surface and always couple with the signal transduction pathways? I am trying to understand how these receptors staying on the surface still interact with signal transduction molecules and what is the mechanism of desensitization and resensitization under this condition?

Gintzler: Perhaps they never desensitize to begin with.

Oh: DAMGO doesn't undergo tolerance, so why isn't it used clinically?

Ueda: Several trials attempted to use this peptide a long time ago, but pharmaceutical companies aren't very interested in peptides. In the clinic tolerance is not such a big deal in the beginning of cancer patient treatment. We don't have to think about tolerance initially. The point is that later on we need several hundred milligrams of morphine a day to treat patients with, and morphine is much more economical than an expensive peptide-based drug. We need to use morphine in the clinic for economic and safety reasons. If we use morphine in terminal care there is some tolerance. For this purpose we need an adjuvant to inhibit that tolerance as much as possible.

Oh: Perhaps chemically we can synthesize a ligand that only stimulates the μ receptor but which does not undergo tolerance.

Gintzler: They would if they were able to but they can't find such a drug.

Dray: Tolerance is not the problem. μ agonists have an upper limit of efficacy because there are serious side effects which are dose limiting. These are mainly respiratory depression, constipation and dysphoria. In fact there has been an attempt to restrict the CNS effects of opioids by developing peripherally restricted μ agonists that can provide analgesia without CNS side effects.

Oh: Perhaps chemically we can synthesize a ligand that only stimulates the μ receptor but which does not undergo tolerance.

Gintzler: It may be, however, that the formation of tolerance is inseparable from the activation of the opiate receptor. If you activate it for long enough, you will get tolerance.

Zhou: Is there evidence that receptor internalization is required for tolerance?

Gintzler: No. In fact the evidence is that it is not important.

Dray: Even if you didn't have tolerance you would still have other undesirable receptor related effects.

Devor: If the dose-limiting effects are in gut, then why not use a peripherally restricted μ receptor antagonist together with morphine, to protect against the gut effects?

Dray: There is a lot of commercial interest in pursuing such possibilities, but thus far this concept has not had sufficient clinical validation.

Devor: I meant that the blocker should be peripherally restricted, not the morphine.

Dray: I think this possibility is being explored.

Gintzler: Some of the gut effects are central, as well.

Dray: Another concept that is being explored stems from the observations that there are two types of μ receptor, one inhibitory and the other facilitatory. Extremely low doses of naloxone favour one receptor, the excitatory one, over the other. There is some clinical support for this being developed as combinations of morphine and very low dose naloxone actually give better clinical analgesic efficacy.

Mao: This is in phase II trial with a drug company based in California. There is an opioid receptor antagonist with peripheral effects, but I have forgotten the name. The bioavailability of naloxone is not that high and it is variable. In extreme cases of constipation induced by high doses of opioids, naloxolone can be used as a treatment, at the same time trying to avoid the central antagonistic effect (i.e. reversing the analgesic effect), with some success. This is to take advantage of the poor bioavailability (or absorption) of oral naloxolone.

Dray: Another commercial initiative has been to develop κ opioid receptor analgesics. This has not been successful because of high incidence of side effects. Yet another possibility is to target δ opioid receptors. δ receptors lack the constipative effects of μ agonists, and lack the dysphoric effects of κ agonists. They also don't produce respiratory depression. It has also been advocated that

mixing μ and δ features in one molecule will provide a more favourable clinical profile. There have been attempts to manipulate the endogenous opioid systems in some advantageous way e.g. with enkephalinase inhibitors, hitherto without marked clinical success.

Baron: Are there opioid compounds available that have κ receptor effects?

Dray: For clinical use? I don't think so. For irritable bowel syndrome, κ agonists with a peripherally restricted effect have been advocated, but they were unsuccessful in clinical trials. The idea behind this was evidence for peripheral expression of a novel κ receptor.

Reeh: The Danish company Ferring has two κ opioid compounds in clinical trials for anti-nociceptive efficacy.

Zhang: Are there any δ acting compounds?

Dray: Apart from the $\mu\delta$ mixes there are some δ compounds that have been advocated for irritable bowel. Again, these are peripherally restricted. There are a few companies developing δ-selective agonists for chronic pain but to my knowledge these compounds have not yet been clinically evaluated.

A mechanism-based understanding of bone cancer pain[1]

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Abstract. Although bone cancer pain can be severe and is relatively common, as it frequently arises from metastases from breast, prostate and lung tumours, relatively little is known about the basic mechanisms that generate and maintain this chronic pain. To begin to define the mechanisms that give rise to bone cancer pain, we developed a mouse model using the intramedullary injection and containment of osteolytic sarcoma cells into the mouse femur. These tumour cells induced bone destruction as well as ongoing and movement evoked pain behaviours similar to that found in patients with bone cancer pain. In addition, there was a significant neurochemical reorganization of sensory neurons that innervate the tumour bearing bone as well as in the spinal cord segments that received sensory input from the cancerous bone. This reorganization generated a neurochemical signature of bone cancer pain that was different from that observed in mouse models of chronic neuropathic or inflammatory pain. These data suggest that there is an inflammatory, neuropathic and tumorigenic component to bone cancer pain. Therefore defining when and how these different components drive bone cancer pain may allow the development of more selective analgesic agents to treat this chronic pain state.

2004 Pathological pain: from molecular to clinical aspects. Wiley, Chichester (Novartis Foundation Symposium 261) p 194–219

Recently, the first animal models of bone cancer pain have been developed. In the mouse femur model, bone cancer pain is induced by injecting murine osteolytic sarcoma cells into the intramedullary space of the femur (Fig. 1) (Schwei et al 1999). Critical to this model is ensuring that the tumour cells are confined within the marrow space of the injected femur and that they do not invade adjacent soft tissues, which would directly affect the joints of the muscle, making behavioral

[1]This review includes material already published by the author and Stephen P. Hunt in the context of *Osteoarthritic joint pain* in the preceding Novartis Foundation Symposium volume (Mantyh PW, Hunt SP 2004 Mechanisms that generate and maintain bone cancer pain. In: Osteoarthritic joint pain. Wiley, Chichester (Novartis Found Symp 260), p 221–238).

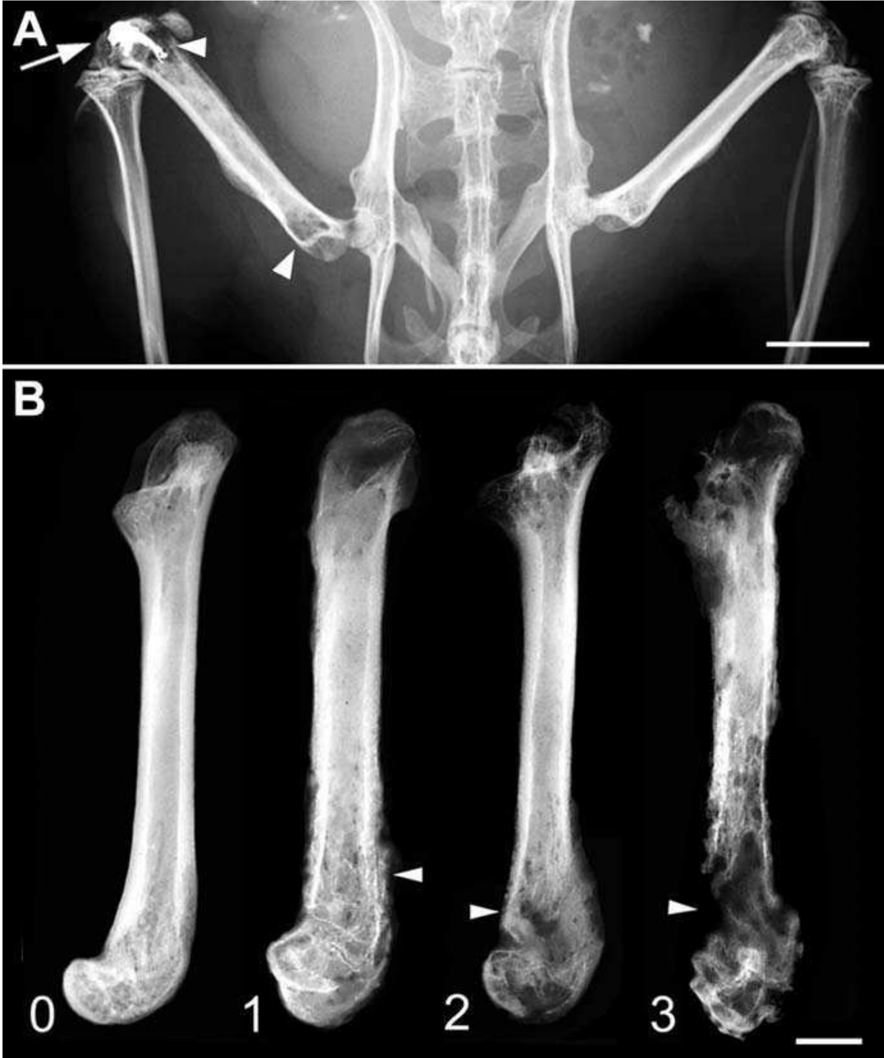
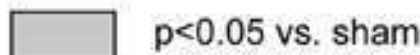


FIG. 1. Progressive destruction of mineralized bone in mice with bone cancer. (A) Low-power anterior–posterior radiograph of mouse pelvis and hindlimbs after a unilateral injection of sarcoma cells into the distal part of the femur and closure of the injection site with an amalgam plug (arrow), which prevents the tumour cells from growing outside the bone (Honore et al 2000a). Radiographs of murine femora (B) show the progressive loss of mineralized bone caused by tumour growth. These images are representative of the stages of bone destruction in the murine femur. At week 1 there is a minor loss of bone near the distal head (arrow); at week 2, substantial loss of mineralized bone at both the proximal and distal (arrow) heads; and at week 3, loss of mineralized bone throughout the entire femur and fracture of distal head (arrow). Scale bar = 2 mm. Modified from Schwei et al (1999).

TABLE 1 Changes in pain behaviour with time in the mouse femur model of bone cancer pain

PAIN BEHAVIOR	NAIVE	SHAM	SARCOMA day 6	SARCOMA day 10	SARCOMA day 14
I. Ongoing Pain					
- guarding (sec) over 2-min. observation period	0.4 ± 0.2	1.4 ± 0.5	2.1 ± 0.5	4.3 ± 0.8	15.2 ± 3.3
- flinches (count) over 2-min. observation period	1.7 ± 0.7	3.1 ± 0.7	7.7 ± 1.6	13.0 ± 2.0	24.5 ± 3.8
II. Movement-evoked Pain					
A. AMBULATORY PAIN					
- forced ambulation on rotarod (score) 5 (normal) to 0 (impaired)	4.7 ± 0.3	4.4 ± 0.3	3.8 ± 0.4	2.5 ± 0.3	2.3 ± 0.3
- limb use during normal ambulation (score) 4 (normal) to 0 (impaired)	4.0 ± 0.0	3.9 ± 0.1	3.7 ± 0.2	3.5 ± 0.3	2.7 ± 0.3
B. PALPATION-EVOKED PAIN (over 2-min. period)					
- guarding (sec) following non-painful palpation	0.4 ± 0.4	1.4 ± 0.5	1.9 ± 0.6	7.1 ± 0.6	18.1 ± 4.0
- flinches (count) following non-painful palpation	2.0 ± 1.2	3.1 ± 0.7	7.0 ± 2.1	19.0 ± 1.1	30.5 ± 5.1



analysis problematic (Schwei et al 1999, Honore et al 2000a, Luger et al 2001). Following injection the tumour cells proliferate, and ongoing, movement-evoked and mechanically evoked pain-related behaviours develop that increase in severity with time (Table 1). These pain behaviours correlate with the progressive tumour-induced bone destruction that ensues, which appears to mimic the condition in patients with primary or metastatic bone cancer. These models have allowed us to gain mechanistic insights into how cancer pain is generated and how the sensory information it initiates is processed as it moves from sense organ to the cerebral cortex under a constantly changing molecular architecture. As detailed below, these insights promise to fundamentally change the way cancer pain is controlled.

Primary afferent sensory neurons

Primary afferent sensory neurons are the gateway by which sensory information from peripheral tissues is transmitted to the spinal cord and brain (Fig. 2), and these neurons innervate the skin and every internal organ of the body, including mineralized bone, marrow and periosteum. The cell bodies of sensory fibres that

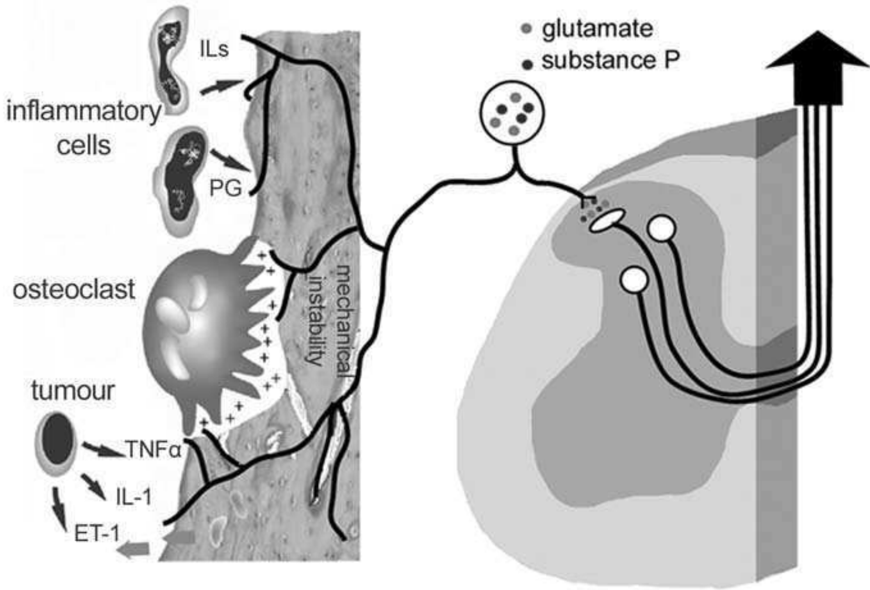


FIG. 2. Sensory neurons and detection of noxious stimuli due to tumour cells. Nociceptors use a diversity of signal-transduction mechanisms to detect noxious physiological stimuli, and many of these mechanisms may be involved in driving cancer pain. Thus, when nociceptors are exposed to products of tumour cells, tissue injury, or inflammation, their excitability is altered and this nociceptive information is relayed to the spinal cord and then to higher centres of the brain. Some of the mechanisms that appear to be involved in generating and maintaining cancer pain include activation of nociceptors by factors such as extracellular protons (+), endothelin 1 (ET-1), interleukins (ILs), prostaglandins (PG), and tumour necrosis factor (TNF).

innervate the head and body are housed in the trigeminal and dorsal root ganglia (DRG), respectively, and can be divided into two major categories: myelinated A fibres and smaller-diameter unmyelinated C fibres. Nearly all large-diameter myelinated Aβ fibres normally conduct non-noxious stimuli applied to the skin, joints and muscles, and thus these large sensory neurons usually do not conduct noxious stimuli (Djouhri et al 1998). In contrast, most small-diameter sensory fibres — unmyelinated C fibres and finely myelinated A fibres — are specialized sensory neurons known as nociceptors, whose major function is to detect environmental stimuli that are perceived as harmful and convert them into electrochemical signals that are then transmitted to the central nervous system (CNS). Unlike primary sensory neurons involved in vision or olfaction, which are required to detect only one type of sensory stimulus (light or chemical odorants, respectively), individual primary sensory neurons of the pain pathway have the remarkable ability to detect a wide range of stimulus modalities, including those of physical and chemical nature (Basbaum & Jessel 2000, Julius & Basbaum

2001). To accomplish this, nociceptors express an extremely diverse repertoire of transduction molecules that can sense forms of noxious stimulation (thermal, mechanical and chemical), albeit with varying degrees of sensitivity.

The past few years have seen remarkable progress toward understanding the signalling mechanisms and specific molecules that nociceptors use to detect noxious stimuli. For example, the vanilloid receptor TRPV1 (formerly known as VR1), which is expressed by most nociceptors, detects heat (Kirschstein et al 1999), and also appears to detect extracellular protons (Bevan & Geppetti 1994, Caterina et al 2000, Welch et al 2000) and lipid metabolites (Tominaga et al 1998, Nagy & Rang 1999). In order to detect noxious mechanical stimuli, nociceptors express mechanically gated channels that initiate a signalling cascade upon excessive stretch (Price et al 2001). The cells also express several purinergic receptors capable of sensing adenosine triphosphate (ATP), which may be released from cells upon excessive mechanical stimulation (Krishtal et al 1988, Xu & Huang 2002).

To sense noxious chemical stimuli, nociceptors express a complex array of receptors capable of detecting inflammation-associated factors released from damaged tissue. These factors include protons (Bevan & Geppetti 1994, Caterina et al 2000), endothelins (Nelson & Carducci 2000), prostaglandins (Alvarez & Fyffe 2000), bradykinin (Alvarez & Fyffe 2000), and nerve growth factor (McMahon 1996). Aside from providing promising targets for the development of more selective analgesics, identification of receptors expressed on the nociceptor surface has increased our understanding of how different tumours generate cancer pain in the peripheral tissues they invade and destroy.

In addition to expressing channels and receptors that detect tissue injury, sensory neurons are highly 'plastic', in that they can change their phenotype in the face of a sustained peripheral injury. Following tissue injury, sensory neuron subpopulations alter patterns of signalling peptide and growth factor expression (Woolf & Salter 2000). This change in phenotype of the sensory neuron in part underlies peripheral sensitization, whereby the activation threshold of nociceptors is lowered so that a stimulus that would normally be mildly noxious is perceived as highly noxious (hyperalgesia). Damage to a peripheral tissue also activates previously 'silent' or 'sleeping' nociceptors, which then become highly responsive both to normally non-noxious stimuli (allodynia) and to noxious stimuli (hyperalgesia).

There are several examples of nociceptors that undergo peripheral sensitization in experimental cancer models (Schwei et al 1999, Honore et al 2000b, Luger et al 2001). In normal mice, the neurotransmitter substance P is synthesized by nociceptors and released in the spinal cord in response to a noxious, but not to a non-noxious, palpation of the femur. In mice with bone cancer, normally non-painful palpation of the affected femur induces the release of substance P from

primary afferent fibres that terminate in the spinal cord. Substance P in turn binds to and activates the neurokinin 1 receptor that is expressed by a subset of spinal cord neurons (Mantyh et al 1995a,b, Hunt & Mantyh 2001). Similarly, normally non-noxious palpation of tumour-bearing limbs of mice with bone cancer also induces the expression of *c*-fos protein in spinal cord neurons. In normal animals that do not have cancer, only noxious stimuli will induce the expression of *c*-fos in the spinal cord (Hunt et al 1987). Thus, peripheral sensitization of nociceptors appears to be involved in the generation and maintenance of bone cancer pain.

Properties of tumours that excite nociceptors

Tumour cells and tumour-associated cells that include macrophages, neutrophils and T lymphocytes secrete a wide variety of factors that sensitize or directly excite primary afferent neurons (Fig. 2). These include prostaglandins (Nielsen et al 1991, Galasko 1995), endothelins (Nelson & Carducci 2000, Davar 2001), interleukins 1 and 6 (Watkins et al 1995, Leskovar et al 2000, Opree & Kress 2000), epidermal growth factor (Stoscheck & King 1986), transforming growth factor (Poon et al 2001, Roman et al 2001), and platelet-derived growth factor (Daughaday & Deuel 1991, Radinsky 1991, Silver 1992). Receptors for many of these factors are expressed by primary afferent neurons. Each of these factors may play an important role in generating pain in particular forms of cancer, and therapies that block two of these factors, prostaglandins and endothelins, are currently approved for use in patients with other (non-cancer) indications.

Prostaglandins are pro-inflammatory lipids that are formed from arachidonic acid by the action of cyclooxygenase (COX) and other downstream synthetases. There are two distinct forms of the COX enzyme, COX-1 and COX-2. Prostaglandins are involved in the sensitization or direct excitation of nociceptors by binding to several prostanoid receptors (Vasko 1995). Several tumour cells and tumour-associated macrophages express high levels of COX-2 and produce large amounts of prostaglandins (Dubois et al 1996, Molina et al 1999, Kundu et al 2001, Ohno et al 2001, Shappell et al 2001).

The COX enzymes are a major target of current medications, and COX inhibitors are commonly administered for reducing both inflammation and pain. A major problem with using COX inhibitors such as aspirin or ibuprofen to block cancer pain is that these compounds inhibit both COX-1 and COX-2, and inhibition of the constitutively expressed COX-1 can cause bleeding and ulcers. In contrast, the new COX-2 inhibitors or coxibs preferentially inhibit COX-2 and avoid many of the side effects of COX-1 inhibition, which may allow their use in treating cancer pain. Other experiments have suggested that COX-2 is involved in angiogenesis and tumour growth (Masferrer et al 2000, Moore & Simmons 2000), so in cancer patients, in addition to blocking cancer pain,

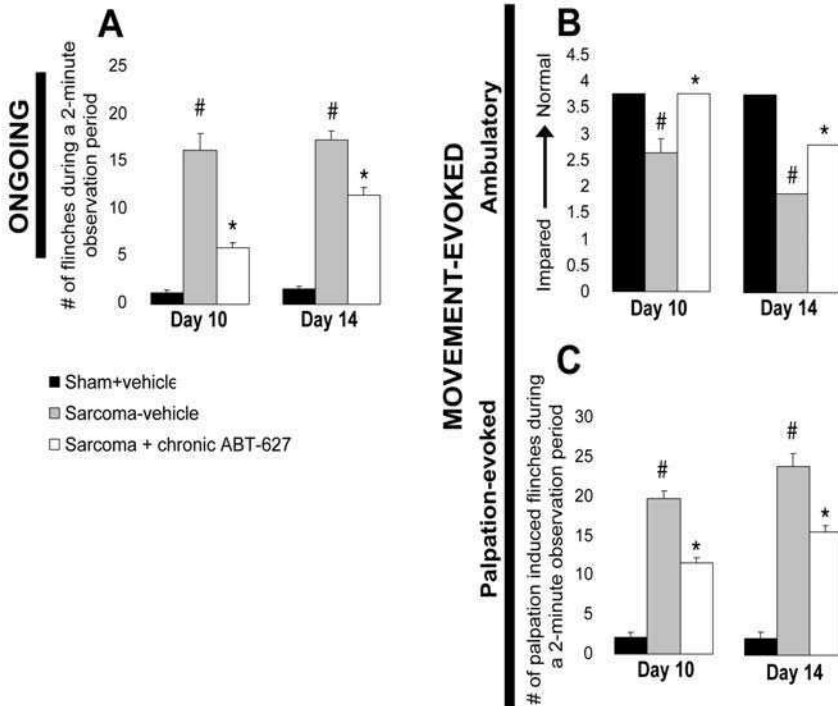


FIG. 3. Selective ET_AR inhibition attenuates ongoing and movement-evoked bone cancer pain behaviours. (A) The number of spontaneous flinches of the cancerous limb over a 2 min observation period was used as a measure of ongoing pain. (B) Parameters of movement-evoked pain include assessment of the sarcoma-bearing limb during normal ambulation in an open field. (C) Quantification of the number of flinches evoked by normally non-noxious palpation of the sarcoma bearing limb over a 2 min observation period following palpation was used as a measure of palpation-evoked pain. All pain behaviours were significantly reduced 10 and 14 days after sarcoma injection with chronic administration of ABT-627 beginning at 6 days after sarcoma injection: bars, $\pm$SEM. # $P < 0.05$ versus sham; *$P < 0.05$ versus sarcoma + vehicle group. Note that the ability of chronic ET_AR inhibition to attenuate ongoing pain was significantly reduced from day 10 to day 14 post sarcoma injection. Modified from Peters et al (2003).

COX-2 inhibitors may have the added advantage of reducing the growth and metastasis of the tumour. COX-2 antagonists show significant promise for alleviating at least some aspects of cancer pain, although clearly more research is required to fully define the actions of COX-2 in different types of cancer.

A second pharmacological target for treating cancer pain is the peptide endothelin 1 (Fig. 3). Several tumours, including prostate cancer, express high levels of endothelins (Shankar et al 1998, Kurbel et al 1999, Nelson & Carducci 2000), and clinical studies have reported a correlation between the severity of the

pain in patients with prostate cancer and plasma levels of endothelins (Nelson et al 1995). Endothelins could contribute to cancer pain by directly sensitizing or exciting nociceptors, given that a subset of small unmyelinated primary afferent neurons express receptors for endothelin (Pomonis et al 2001). Direct application of endothelin to peripheral nerves activates primary afferent fibres and induces pain behaviours (Davar et al 1998). Like prostaglandins, endothelins that are released from tumour cells are also thought to be involved in regulating angiogenesis (Dawas et al 1999) and tumour growth (Asham et al 1998), suggesting again that endothelin antagonists may be useful not only in inhibiting cancer pain but in reducing the growth and metastasis of the tumour.

Tumour-induced release of protons and acidosis

Tumour cells become ischaemic and undergo apoptosis as the tumour burden exceeds its vascular supply (Helmlinger et al 2002). Local acidosis, a state where an accumulation of acid metabolites is present, is a hallmark of tissue injury (Reeh & Steen 1996, Julius & Basbaum 2001). In the past few years the concept that sensory neurons can be directly excited by protons or acidosis has generated intense research and clinical interest. Studies have shown that subsets of sensory neurons express different acid-sensing ion channels (Olson et al 1998, Julius & Basbaum 2001). The two major classes of acid-sensing ion channels expressed by nociceptors are TRPV1 (Caterina et al 1997, Tominaga et al 1998) and the acid-sensing ion channel 3 (ASIC-3) (Bassilana et al 1997, Olson et al 1998, Sutherland et al 2000). Both of these channels are sensitized and excited by a decrease in pH. More specifically, TRPV1 is activated when the pH falls below 6.0, while the pH that activates ASIC-3 appears to be highly dependent on the coexpression of other ASIC channels in the same nociceptor (Lingueglia et al 1997).

There are several mechanisms by which a decrease in pH could be involved in generating and maintaining cancer pain. As tumours grow, tumour-associated inflammatory cells invade the neoplastic tissue and release protons that generate local acidosis (Helmlinger et al 2002). A second mechanism by which acidosis may occur is apoptosis of the tumour cells. Release of intracellular ions may generate an acidic environment that activates signalling by acid-sensing channels expressed by nociceptors.

Tumour-induced release of protons and acidosis may be particularly important in the generation of bone cancer pain. In both osteolytic (bone-destroying) and osteoblastic (bone-forming) cancers there is a significant proliferation and hypertrophy of osteoclasts (Clohisy et al 2000). Osteoclasts are terminally differentiated, multinucleated cells of the monocyte lineage that are uniquely designed to resorb bone by maintaining an extracellular microenvironment of acidic pH (4.0–5.0) at the interface between osteoclast and mineralized bone

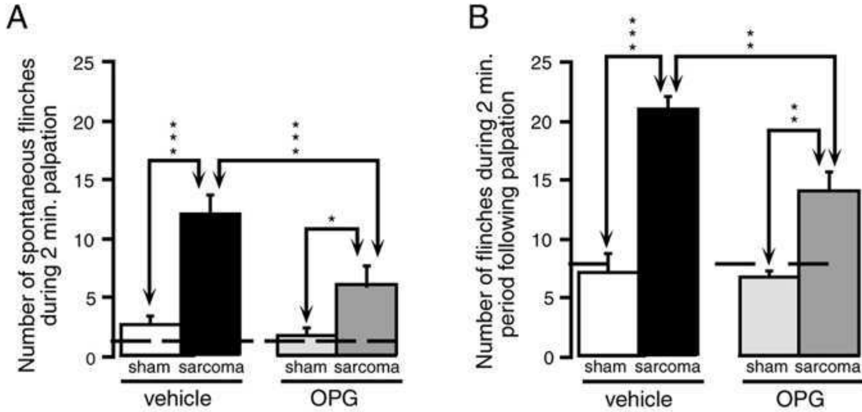


FIG. 4. Attenuation of bone cancer pain by osteoprotegerin (OPG). Histograms show that administration of OPG beginning 6 days after tumour implantation attenuated both (A) spontaneous and (B) palpation-evoked pain in mice at day 17 following tumour implantation (modified from Honore et al 2000a). OPG is a naturally occurring protein that is a secreted decoy receptor that inhibits osteoclast differentiation, proliferation, and hypertrophy, resulting in reduced osteoclast activity and bone resorption.

(Delaisse & Vaes 1992). Studies have shown significant expression of ASIC (Olson et al 1998) and TRPV1 (Tominaga et al 1998, Guo et al 1999) in peptidergic afferent fibres, and we have localized peptidergic fibres in bone marrow and cortical bone (Mach et al 2002). This evidence suggests that exposure of these sensory fibres to the osteoclast's acidic extracellular microenvironment could activate resident proton-sensitive ion channels, stimulating pain sensation. Recent experiments in a murine model of bone cancer pain reported that osteoclasts play an essential role in cancer-induced bone loss, and that osteoclasts contribute to the aetiology of bone cancer pain (Honore et al 2000a, Luger et al 2001). Recent work has shown that osteoprotegerin (Honore et al 2000a) and a bisphosphonate (Fulfaro et al 1998, Mannix et al 2000), both of which are known to induce osteoclast apoptosis, are effective in decreasing osteoclast-induced bone cancer pain (Fig. 4). Similarly, TRPV1 or ASIC antagonists may be used to reduce pain in patients with soft tumours or bone cancer by blocking excitation of the acid-sensitive channels on sensory neurons.

Release of growth factors by tumour cells

One of the most important discoveries in the past decade has been the demonstration that the biochemical and physiological status of sensory neurons is maintained and modified by factors derived from the innervated tissue.

Changes in the periphery associated with inflammation, nerve injury, or tissue injury are mirrored by changes in the phenotype of sensory neurons (Honore et al 2000b,c,d). After peripheral nerve injury, expression of a subset of neurotransmitters and receptors by damaged sensory neurons is altered in a highly predictable fashion. These changes are caused, in part, by a change in the tissue level of several growth factors released from the environment local to the injury site, including nerve growth factors (NGF) (Fu & Gordon 1997, Koltzenburg 1999, Fukuoka et al 2001) and glial-derived neurotrophic factor (GDNF) (Boucher & McMahon 2001, Hoke et al 2002). These neurochemical changes can be reversed in a receptor-specific fashion by intrathecal or peripheral application of NGF or GDNF (Bennett et al 1996, 1998, Boucher et al 2000, Ramer et al 2000).

While the level of NGF expression reportedly correlates with the extent of pain in pancreatic cancer (Zhu et al 1999, Schneider et al 2001), relatively little is known about how other tumours affect the synthesis and release of growth factors. However, one certainty is that the repertoire of growth factors to which the sensory neuron is exposed will change as the developing tumour invades the peripheral tissue that the neuron innervates. Thus, in addition to a disruption of the growth factors normally released by the intact peripheral tissue, one can expect release of a variety of additional growth factors by tumour cells as well as by tumour-infiltrating leukocytes, which can comprise up to 80% of the total tumour mass (Zhang et al 2002). Activated leukocytes synthesize and release high levels of several growth factors (Stoscheck & King 1986, Daughaday & Deuel 1991, Radinsky 1991, Silver 1992, Leon et al 1994, Caroleo et al 2001, Poon et al 2001, Roman et al 2001), and thus one would expect a significant change in the phenotype and response characteristics of the sensory neurons following tumour invasion of a peripheral organ.

While tumour growth alters the invaded tissue, it is also clear that the affected tissue also influences the phenotype of the invading tumour cell (Mundy 2002). Because the local environment can influence the molecules that tumour cells express and release, it follows that the same tumour in the same individual may be painful at one site of metastasis but not at another. Clinical observations reveal that pain from cancer can be quite perplexing because the size, location, or type of cancer tumour does not necessarily predict symptoms. Different patients with the same cancer may have vastly different symptoms. Kidney cancer may be painful in one person and asymptomatic in another. Metastases to bone in the same individual may cause pain at the site of a rib lesion, but not at that of a humeral lesion. Small cancer deposits in bone may be very painful, while large soft-tissue cancers may be painless (Mantyh et al 2002). Important areas for future research include identification of tissue-specific mechanisms of cancer pain, comparing soft tissue with bone, as well as site-specific mechanisms, comparing flat bones (ribs) with

tubular bones (femurs). It will also be of interest to determine patient-specific factors that influence disease progression and its relationship to pain perception.

Tumour-induced distension and destruction of sensory fibres

In general, previous reports have suggested that tumours are not highly innervated by sensory or sympathetic neurons (O'Connell et al 1998, Seifert & Spitznas 2001, Terada & Matsunaga 2001). However, in many cancers, rapid tumour growth frequently entraps and injures nerves, causing mechanical injury, compression, or ischaemia or direct proteolysis (Mercadante 1997). Proteolytic enzymes produced by the tumour can also injure sensory and sympathetic fibres, causing neuropathic pain.

The capacity of a tumour to injure and destroy peripheral nerve fibres has been directly observed in an experimental model of bone cancer. Following injection and containment of lytic murine sarcoma cells intramedullary to the mouse femur, tumour cells grow in the marrow space and disrupt innervating sensory fibres (Figs 5,6). As the tumour cells grow they first compress and then destroy both the haematopoietic cells of the marrow and the sensory fibres that normally innervate the marrow, mineralized bone and periosteum (Schwei et al 1999).

While the mechanisms by which any neuropathic pain is generated and maintained are still not well understood, several therapies that have proven useful in the control of other types of neuropathic pain may also be useful in treating tumour-induced neuropathic pain. For example, gabapentin, which was originally developed as an anticonvulsant but whose mechanism of action remains unknown, is effective in treating several forms of neuropathic pain and may also be useful in treating cancer-induced neuropathic pain (Ripamonti & Dickerson 2001).

Central sensitization in cancer pain

A critical question is whether the spinal cord and forebrain also undergo significant neurochemical changes as a chronic cancer pain state develops. The murine cancer pain model revealed extensive neurochemical reorganization within spinal cord segments that receive input from primary afferent neurons innervating the cancerous bone (Honore et al 2000b,c,d, Luger et al 2001). These changes included astrocyte hypertrophy (Fig. 7) and up-regulation of the prohyperalgesic peptide dynorphin. Spinal cord neurons that normally would only be activated by noxious stimuli were activated by non-noxious stimuli. These spinal cord changes were attenuated by blocking the tumour-induced tissue destruction and pain (Honore et al 2000a, Luger et al 2001). Together, these neurochemical changes suggest that cancer pain induces, and is at least partially maintained by, a state of

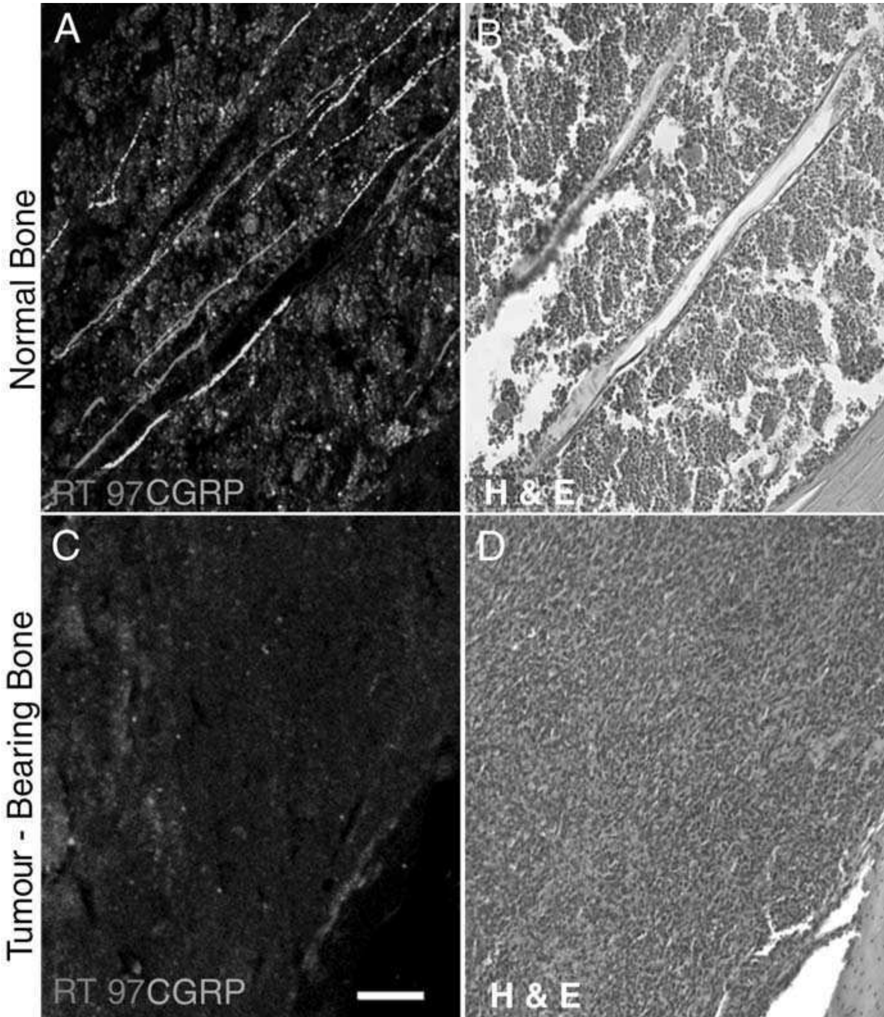


FIG. 5. Sensory nerve fibres in the marrow of the mouse femur are destroyed by invading sarcoma tumour cells. Confocal (A,C) images show calcitonin gene related peptide (CGRP) and neurofilament-200 (RT-97) and serially adjacent sections (B,D) stained with Haematoxylin and Eosin (H&E; B,D) in the normal (A,B) and tumour-bearing (C,D) marrow. In the normal marrow CGRP and RT-97-expressing sensory fibres are generally associated with the vasculature (A,B) whereas 14 days following injection and confinement of the tumour cells to the marrow space (C,D) few if any CGRP or RT-97 expressing sensory fibres can be detected. Scale bar = 150 μm.

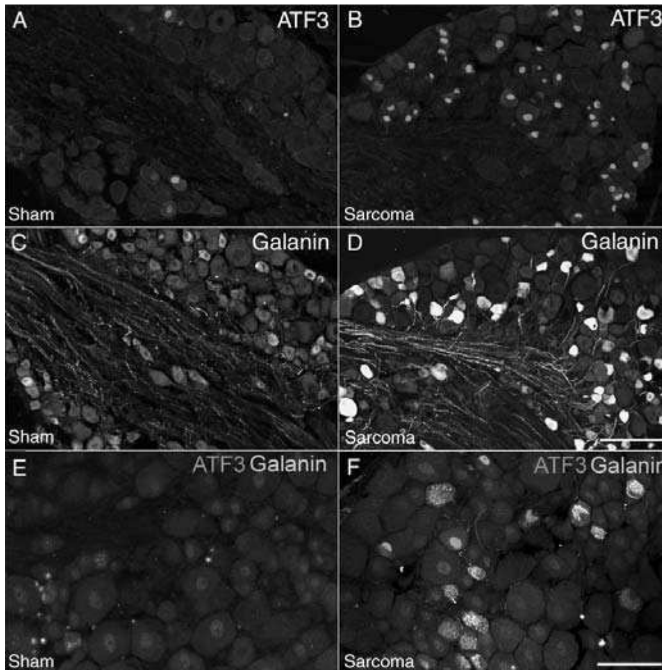


FIG. 6. Tumour-induced destruction of sensory nerve fibres in the tumour-bearing bone results in the up-regulation of activated transcription factor-3 and galanin in the cell body of sensory neurons that innervate the tumour-bearing femur. Neurons in the normal L2 dorsal root ganglia express low levels of both transcription factor-3 (ATF-3) (A) or the neuropeptide galanin (C) whereas 14 days following injection and confinement of sarcoma cells to the marrow space there is a marked up-regulation of both ATF-3 and galanin in sensory neurons in the L2 dorsal root ganglia ipsilateral to the tumour-bearing bone. Many sensory neurons which show an up-regulation of galanin in response to tumour-induced destruction of sensory fibres in the bone also show up-regulation of ATF-3 in their nucleus (compare E vs. F). These data suggest that as tumour cells invade the bone, sensory nerve fibres that normally innervate the bone are destroyed with a resulting generation of the neurochemical signature of neuropathy in sensory neurons that innervate the tumour-bearing bone. Scale bar = 100 μm. Modified from Mantyh et al (2004).

central sensitization, in which an increased transmission of nociceptive information allows normally non-noxious input to be amplified and perceived as noxious stimuli.

Once nociceptive information has been transmitted to the spinal cord by primary afferent neurons, it can travel via multiple ascending 'pain' pathways that project from the spinal cord to higher centres of the brain. Classically, the main emphasis in examining the ascending conduction of pain has been placed on spinothalamic tract neurons. However, data from recent clinical studies have

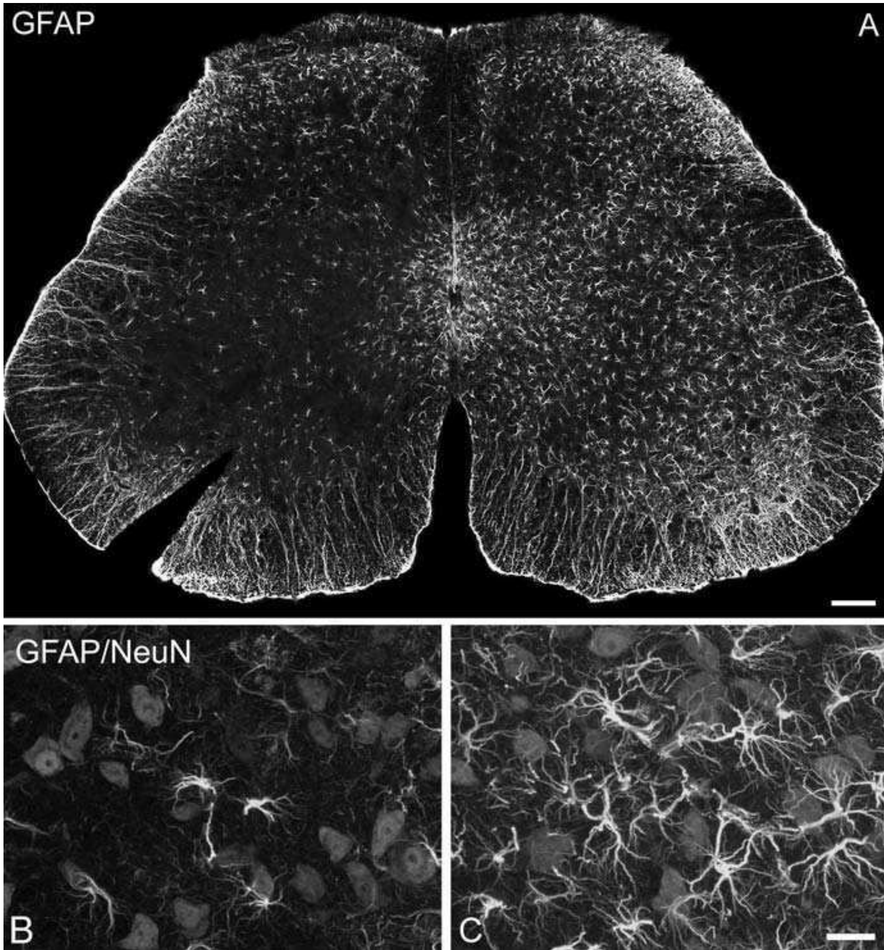


FIG. 7. Cancer-induced reorganization of the CNS. Chronic cancer pain not only sensitizes peripheral nociceptors, but also can induce significant neurochemical reorganization of the spinal cord. This reorganization may participate in the phenomenon of central sensitization, i.e. an increased responsiveness of spinal cord neurons involved in transmission of pain. (A) Confocal image of a coronal section of the mouse L4 spinal cord showing glial fibrillary acidic protein (GFAP) positive astrocytes (white) which have undergone hypertrophy on the side ipsilateral to the tumour-bearing bone. Panels B and C show higher magnification of the ipsilateral and contralateral dorsal horn seen in panel A, with colocalization of the neuron-specific antibody NeuN. Note that while the astrocytes (spindle-shaped cells) have undergone a massive hypertrophy, there does not appear to be any significant loss of NeuN positive neurons. Scale bars: A, 200 μm; B and C, 30 μm. Modified from Schwei et al (1999).

necessitated a reassessment of this position by showing significant attenuation of some forms of difficult-to-control visceral cancer pain following lesion of the axons of non-spinothalamic tract neurons (Willis et al 1999, Nauta et al 2000). Together these data suggest that one reason that cancer pain is frequently perceived as such an intense and disturbing pain is that it ascends to higher centres of the brain via multiple parallel neuronal pathways. Importantly for cancer patients, many of whom frequently experience anxiety or depression, it is clear that higher centres of the brain can modulate the ascending conduction of pain. Descending pathways that modulate the ascending conduction of cancer pain may play an important role in either enhancing or inhibiting the patient's perception of pain. The general mood and attention of the patient thus may be significant factors in determining the pain's intensity and degree of unpleasantness.

A changing set of factors may drive cancer pain with disease progression

Cancer pain frequently becomes more severe as the disease progresses, and adequate control of cancer pain becomes more difficult to achieve without encountering significant unwanted side effects (Payne 1998, Foley 1999, Portenoy & Lesage 1999). While tolerance may contribute to the escalation of the dose of analgesics required to control cancer pain, a compatible possibility is that with the progression of the disease, different factors assume a greater importance in driving cancer pain. For example, in the mouse model of bone cancer, as tumour cells first begin to proliferate, pain-related behaviours start to occur long before any significant bone destruction is evident. This pain may be due to prohyperalgesic factors such as prostaglandins and endothelin that are released by the growing tumour cells and subsequently activate nociceptors in the marrow. Pain at this stage might be attenuated by COX-2 inhibitors and endothelin antagonists. As the tumour continues to grow, sensory neurons innervating the marrow are compressed and destroyed, causing a neuropathic pain to develop that may best respond to treatment with drugs such as gabapentin that are known to attenuate non-cancer-induced neuropathic pain. When the tumour begins to induce proliferation and hypertrophy of osteoclasts, the pain due to excessive osteoclast activity might be largely blocked by anti-osteoclastogenic drugs such as bisphosphonates or osteoprotegerin (Fig. 4). As the tumour cells completely fill the intramedullary space, tumour cells begin to die, generating an acidic environment; antagonists to TRPV1 or ASICs may attenuate the pain induced by this acidosis. Finally, as bone destruction compromises the mechanical strength of the bone, antagonists that block the mechanically gated channels and/or ATP receptors in the richly innervated periosteum may attenuate movement-evoked pain.

While the above pattern of tumour-induced tissue destruction and nociceptor activation may be unique to bone cancer, an evolving set of nociceptive events probably occurs in other cancers. This complex pattern may in part explain why cancer pain is frequently difficult to treat and why it is so heterogeneous in nature and severity. Changes in tumour-induced tissue injury, in nociceptor activation, and in the brain areas involved in transmitting these nociceptive signals as the disease progresses suggest that different therapies will be efficacious at particular stages of the disease. Understanding how tumour cells differentially excite nociceptors at different stages of the disease, and how the phenotype of nociceptors and CNS neurons involved in nociceptive transmission change as the disease progresses, should allow a mechanistic approach to designing more effective therapies to treat cancer pain.

Future directions

For the first time, animal models of cancer pain are now available that mirror the clinical picture of patients with cancer pain. Information generated from these models should elucidate the mechanisms that generate and maintain different types of cancer pain. Many of these cancer models have been developed in mice and rats, but implantation of human tumours in immunocompromised rodent strains should allow examination of the pain that different human tumours generate. These animal models may also offer insight into one of the major conundrums of cancer pain: that the severity of cancer pain is so variable from patient to patient, from tumour to tumour, and even from site to site. Newer molecular techniques using microarrays and proteomics should reveal which specific features of different tumours are important in inducing cancer pain. Once we have determined the mechanisms by which the different types of cancer induce pain, we can identify molecular targets and develop mechanism-based therapies. Ultimately, the key will be to integrate information about tumour biology and the host's response to neoplasia with our understanding of how chronic pain is generated and maintained. These studies should improve the quality of life of all those who suffer from cancer pain.

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References

Alvarez FJ, Fyffe RE 2000 Nociceptors for the 21st century. Curr Rev Pain 4:451–458
Asham EH, Loizidou M, Taylor I 1998 Endothelin-1 and tumour development. Eur J Surg Oncol 24:57–60
Basbaum AI, Jessel TM 2000 The perception of pain. In: Kandel ER, Schwartz JH, Jessell TM (eds) Principles of neural science. McGraw-Hill, New York, p 472–490
Bassilana F, Champigny G, Waldmann R et al 1997 The acid-sensitive ionic channel subunit ASIC and the mammalian degenerin MDEG form a heteromultimeric H^+-gated Na^+ channel with novel properties. J Biol Chem 272:28819–28822
Bennett DL, French J, Priestley JV, McMahon SB 1996 NGF but not NT-3 or BDNF prevents the A fiber sprouting into lamina II of the spinal cord that occurs following axotomy. Mol Cell Neurosci 8:211–220
Bennett DL, Michael GJ, Ramachandran N et al 1998 A distinct subgroup of small DRG cells express GDNF receptor components and GDNF is protective for these neurons after nerve injury. J Neurosci 18:3059–3072
Bevan S, Geppetti P 1994 Protons: small stimulants of capsaicin-sensitive sensory nerves. Trends Neurosci 17:509–512
Boucher TJ, McMahon SB 2001 Neurotrophic factors and neuropathic pain. Curr Opin Pharmacol 1:66–72
Boucher TJ, Okuse K, Bennett DL et al 2000 Potent analgesic effects of GDNF in neuropathic pain states. Science 290:124–127
Caroleo MC, Costa N, Bracci-Laudiero L, Aloe L 2001 Human monocyte/macrophages activate by exposure to LPS overexpress NGF and NGF receptors. J Neuroimmunol 113:193–201
Caterina MJ, Schumacher MA, Tominaga M et al 1997 The capsaicin receptor: a heat-activated ion channel in the pain pathway. Nature 389:816–824
Caterina MJ, Leffler A, Malmberg AB et al 2000 Impaired nociception and pain sensation in mice lacking the capsaicin receptor. Science 288:306–313
Clohisy DR, Perkins SL, Ramnaraine ML 2000a Review of cellular mechanisms of tumor osteolysis. Clin Orthop Res 373:104–114
Clohisy DR, Ramnaraine ML, Scully S et al 2000b Osteoprotegerin inhibits tumor-induced osteoclastogenesis and bone growth in osteopetrotic mice. J Orthop Res 18: 967–976
Clohisy DR, O'Keefe PF, Ramnaraine ML 2001 Pamidronate decreases tumor-induced osteoclastogenesis in mice. J Orthop Res 19:554–558
Coyle NJ, Adelhardt KM, Foley KM, Portenoy RK 1990 Character of terminal illness in the advanced cancer patient: pain and other symptoms during the last four weeks of life. J Pain Symptom Manage 5:83–93
Daughaday WH, Deuel TF 1991 Tumor secretion of growth factors. Endocrinol Metab Clin North Am 20:539–563
Davar G 2001 Endothelin-1 and metastatic cancer pain. Pain Med 2:24–27
Davar G, Hans G, Fareed MU et al 1998 Behavioral signs of acute pain produced by application of endothelin-1 to rat sciatic nerve. Neuroreport 9:2279–2283
Dawas K, Laizidou M, Shankar A et al 1999 Angiogenesis in cancer: the role of endothelin-1. Ann R Coll Surg Engl 81:306–310
Delaisse J-M, Vaes G 1992 Mechanism of mineral solubilization and matrix degradation in osteoclastic bone resorption. In: Rifkin BR, Gay CV (eds) Biology and physiology of the osteoclast. CRC, Ann Arbor, p 289–314
de Wit R, van Dam F, Loonstra S et al 2001 The Amsterdam Pain Management Index compared to eight frequently used outcome measures to evaluate the adequacy of pain treatment in cancer patients with chronic pain. Pain 91:339–349

Djouhri L, Bleazard L, Lawson SN 1998 Association of somatic action potential shape with sensory receptive properties in guinea-pig dorsal root ganglion neurones. J Physiol 513:857–872
Dubois RN, Radhika A, Reddy BS, Entingh AJ 1996 Increased cyclooxygenase-2 levels in carcinogen-induced rat colonic tumors. Gastroenterology 110:1259–1262
Foley KM 1999 Advances in cancer pain. Arch Neurol 56:413–417
Fu SY, Gordon T 1997 The cellular and molecular basis of peripheral nerve regeneration. Mol Neurobiol 14:67–116
Fukuoka T, Kondo E, Dai Y et al 2001 Brain-derived neurotrophic factor increases in the uninjured dorsal root ganglion neurons in selective spinal nerve ligation model. J Neurosci 21:4891–4900
Fulfaro F, Casuccio A, Ticozzi C, Ripamonti C 1998 The role of bisphosphonates in the treatment of painful metastatic bone disease: a review of phase III trials. Pain 78:157–169
Galasko CS 1995 Diagnosis of skeletal metastases and assessment of response to treatment. Clin Orthop 312:64–75
Guo A, Vulchanova L, Wang J et al 1999 Immunocytochemical localization of the vanilloid receptor 1 (VR1): relationship to neuropeptides, the P2X3 purinoceptor and IB4 binding sites. Eur J Neurosci 11:946–958
Helmlinger G, Sckell A, Dellian M et al 2002 Acid production in glycolysis-impaired tumors provides new insights into tumor metabolism. Clin Cancer Res 8:1284–1291
Hoke A, Gordon T, Zochodne DW, Sulaiman OA 2002 A decline in glial cell-line-derived neurotrophic factor expression is associated with impaired regeneration after long-term Schwann cell denervation. Exp Neurol 173:77–85
Honore P, Luger NM, Sabino MA et al 2000a Osteoprotegerin blocks bone cancer-induced skeletal destruction, skeletal pain and pain-related neurochemical reorganization of the spinal cord. Nat Med 6:521–528
Honore P, Rogers SD, Schwei MJ et al 2000b Murine models of inflammatory, neuropathic and cancer pain each generates a unique set of neurochemical changes in the spinal cord and sensory neurons. Neuroscience 98:585–598
Honore P, Menning PM, Rogers SD et al 2000c Neurochemical plasticity in persistent inflammatory pain. Prog Brain Res 129:357–363
Honore P, Schwei J, Rogers SD et al 2000d Cellular and neurochemical remodeling of the spinal cord in bone cancer pain. Prog Brain Res 129:389–397
Hoskin P 2000 Tumor-induced bone remodelling In: Body J-J (ed) Radiotherapy, tumor bone diseases and osteoporosis in cancer patients. Marcel Dekker, New York, p 263–286
Hunt SP, Mantyh PW 2001 The molecular dynamics of pain control. Nat Rev Neurosci 2:83–91
Hunt SP, Pini A, Evan G 1987 Induction of c-fos-like protein in spinal cord neurons following sensory stimulation. Nature 328:632–634
Julius D, Basbaum AI 2001 Molecular mechanisms of nociception. Nature 413:203–210
Kirschstein T, Greffrath W, Busselberg D, Treede RD 1999 Inhibition of rapid heat responses in nociceptive primary sensory neurons of rats by vanilloid receptor antagonists. J Neurophysiol 82:2853–2860
Koltzenburg M 1999 The changing sensitivity in the life of the nociceptor. Pain(suppl 6):S93–102
Krishtal OA, Marchenko SM, Obukhov AG 1988 Cationic channels activated by extracellular ATP in rat sensory neurons. Neuroscience 27:995–1000
Kundu N, Yang QY, Dorsey R, Fulton AM 2001 Increased cyclooxygenase-2 (COX-2) expression and activity in a murine model of metastatic breast cancer. Int J Cancer 93: 681–686
Kurbel S, Kurbel B, Kovacic D et al 1999 Endothelin-secreting tumors and the idea of the pseudoectopic hormone secretion in tumors. Med Hypotheses 52:329–333

Leon A, Buriani A, Dal Toso R et al 1994 Mast cells synthesize, store, and release nerve growth factor. Proc Natl Acad Sci USA 91:3739–3743

Leskovar A, Moriarty LJ, Turek JJ et al 2000 The macrophage in acute neural injury: changes in cell numbers over time and levels of cytokine production in mammalian central and peripheral nervous systems. J Exp Biol 203:1783–1795

Linguеglia E, Weille JR, Bassilana F et al 1997 A modulatory subunit of acid sensing ion channels in brain and dorsal root ganglion cells. J Biol Chem 272:29778–29783

Luger NM, Honore P, Sabino MAC et al 2001 Osteoprotegerin diminishes advanced bone cancer pain. Cancer Res 61:4038–4047

Mach DB, Rogers SD, Sabino MC et al 2002 Origins of skeletal pain: sensory and sympathetic innervation of the mouse femur. J Neurosci 113:155–166

Mannix K, Ahmedazai SH, Anderson H et al 2000 Using bisphosphonates to control the pain of bone metastases: evidence based guidelines for palliative care. Palliat Med 14:455–461

Mantyh PW, DeMaster E, Malhotra A et al 1995a Receptor endocytosis and dendrite reshaping in spinal neurons after somatosensory stimulation. Science 268:1629–1632

Mantyh PW, Allen CJ, Ghilardi JR et al 1995b Rapid endocytosis of a G protein-coupled receptor: substance P evoked internalization of its receptor in the rat striatum in vivo. Proc Natl Acad Sci USA 92:2622–2626

Mantyh PW, Clohisy DR, Koltzenburg M, Hunt SP 2002 Molecular mechanisms of cancer pain. Nat Rev Cancer 2:201–209

Mantyh PW, Sabino MC, Schwei MJ et al 2004 Tumor-induced injury of primary afferent sensory nerve fibers in bone cancer pain. Neuroscience. Submitted

Masferrer JL, Leahy KM, Koki AT et al 2000 Antiangiogenic and antitumor activities of cyclooxygenase-2 inhibitors. Cancer Res 60:1306–1311

McMahon SB 1996 NGF as a mediator of inflammatory pain. Phil Trans R Soc Lond B Biol Sci 351:431–440

Mercadante S 1997 Malignant bone pain: pathophysiology and treatment. Pain 69:1–18

Mercadante S, Arcuri E 1998 Breakthrough pain in cancer patients: pathophysiology and treatment. Cancer Treat Rev 24:425–432

Meuser T, Pietruck C, Radbruch L et al 2001 Symptoms during cancer pain treatment following WHO guidelines: a longitudinal follow-up study of symptom prevalence, severity and etiology. Pain 93:247–257

Molina MA, Sitja-Arnau M, Lemoine MG et al 1999 Increased cyclooxygenase-2 expression in human pancreatic carcinomas and cell lines: growth inhibition by nonsteroidal anti-inflammatory drugs. Cancer Res 59:4356–4362

Moore BC, Simmons DL 2000 COX-2 inhibition, apoptosis, and chemoprevention by nonsteroidal anti-inflammatory drugs. Curr Med Chem 7:1131–1144

Mundy GR 2002 Metastases to bone: causes, consequences, and therapeutic opportunities. Nat Rev Cancer 2:584–593

Nagy I, Rang H 1999 Noxious heat activates all capsaicin-sensitive and also a sub-population of capsaicin-insensitive dorsal root ganglion neurons. Neuroscience 88:995–997

Nauta HJW, Soukup VM, Fabian RH et al 2000 Punctate midline myelotomy for the relief of visceral cancer pain. J Neurosurg 92(suppl 2):125–130

Nelson JB, Carducci MA 2000 The role of endothelin-1 and endothelin receptor antagonists in prostate cancer. BJU Int 85(suppl 2):45–48

Nelson JB, Hedican SP, George DJ et al 1995 Identification of endothelin-1 in the pathophysiology of metastatic adenocarcinoma of the prostate. Nat Med 1:944–999

Nielsen OS, Munro AJ, Tannock IF 1991 Bone metastases: pathophysiology and management policy. J Clin Oncol 9:509–524

O'Connell JX, Nanthakumar SS, Nielsen GP, Rosenberg AE 1998 Osteoid osteoma: the uniquely innervated bone tumor. Mod Pathol 11:175–180

Ohno R, Yoshinaga K, Fujita T et al 2001 Depth of invasion parallels increased cyclooxygenase-2 levels in patients with gastric carcinoma. Cancer 91:1876–1881
Olson TH, Riedl MS, Vulchanova L et al 1998 An acid sensing ion channel (ASIC) localizes to small primary afferent neurons in rats. Neuroreport 9:1109–1113
Opree A, Kress M 2000 Involvement of the proinflammatory cytokines tumor necrosis factor-alpha, IL-1 beta, and IL-6 but not IL-8 in the development of heat hyperalgesia: effects on heat-evoked calcitonin gene-related peptide release from rat skin. J Neurosci 20:6289–6293
Payne R 1997 Mechanisms and management of bone pain. Cancer 80(suppl 8):1608–1613
Payne R 1998 Practice guidelines for cancer pain therapy. Issues pertinent to the revision of national guidelines. Oncology 12:169–175
Payne R, Mathias SD, Pasta DJ et al 1998 Quality of life and cancer pain: satisfaction and side effects with transdermal fentanyl versus oral morphine. J Clin Oncol 16:1588–1593
Peters CM, Lyndsay TH, Pomonis JD et al 2004 Endothelin and the tumorigenic component of bone cancer pain. Neuroscience, in press
Pomonis JD, Rogers SD, Peters CM et al 2001 Expression and localization of endothelin receptors: implication for the involvement of peripheral glia in nociception. J Neurosci 21:999–1006
Poon RT, Fan ST, Wong J 2001 Clinical implications of circulating angiogenic factors in cancer patients. J Clin Oncol 19:1207–1225
Portenoy RK, Lesage P 1999 Management of cancer pain. Lancet 353:1695–1700
Portenoy RKD, Payne D, Jacobsen P 1999 Breakthrough pain: characteristics and impact in patients with cancer pain. Pain 81:129–134
Price MP, McIlwrath SL, Xie JH et al 2001 The DRASIC cation channel contributes to the detection of cutaneous touch and acid stimuli in mice. Neuron 32:1071–1083
Radinsky R 1991 Growth factors and their receptors in metastasis. Semin Cancer Biol 2:169–177
Ramer MS, Priestley JV, McMahon SB 2000 Functional regeneration of sensory axons into the adult spinal cord. Nature 403:312–316
Reeh PW, Steen KH 1996 Tissue acidosis in nociception and pain. Prog Brain Res 113:143–151
Ripamonti C, Dickerson ED 2001 Strategies for the treatment of cancer pain in the new millennium. Drugs 61:955–977
Roman C, Saha D, Beauchamp R 2001 TGF-beta and colorectal carcinogenesis. Microsc Res Tech 52:450–457
Schneider MB, Standop J, Ulrich A et al 2001 Expression of nerve growth factors in pancreatic neural tissue and pancreatic cancer. J Histochem Cytochem 49:1205–1210
Schwei MJ, Honore P, Rogers SD et al 1999 Neurochemical and cellular reorganization of the spinal cord in a murine model of bone cancer pain. J Neurosci 19:10886–10897
Seifert P, Spitznas M 2001 Tumours may be innervated. Virchows Archiv 438:228–231
Shankar A, Loizidou M, Aliev G et al 1998 Raised endothelin 1 levels in patients with colorectal liver metastases. Br J Surg 85:502–506
Shappell SB, Manning S, Boeglin WE et al 2001 Alterations in lipoxygenase and cyclooxygenase-2 catalytic activity and mRNA expression in prostate carcinoma. Neoplasia 3:287–303
Silver BJ 1992 Platelet-derived growth factor in human malignancy. Biofactors 3:217–227
Stoscheck CM, King Jr LE 1986 Role of epidermal growth factor in carcinogenesis. Cancer Res 46:1030–1037
Sutherland S, Cook S, Ew M 2000 Chemical mediators of pain due to tissue damage and ischemia. Prog Brain Res 129:21–38
Terada T, Matsunaga Y 2001 S-100-positive nerve fibers in hepatocellular carcinoma and intrahepatic cholangiocarcinoma: an immunohistochemical study. Pathol Int 51:89–93
Tominaga M, Caterina MJ, Malmberg AB et al 1998 The cloned capsaicin receptor integrates multiple pain-producing stimuli. Neuron 21:531–543

Vasko MR 1995 Prostaglandin-induced neuropeptide release from spinal cord. Prog Brain Res 104:367–380

Watkins LR, Goehler LE, Relton J et al 1995 Mechanisms of tumor necrosis factor-alpha (TNF-alpha) hyperalgesia. Brain Res 692:244–250

Welch JM, Simon SA, Reinhart PH 2000 The activation mechanism of rat vanilloid receptor 1 by capsaicin involves the pore domain and differs from the activation by either acid or heat. Proc Natl Acad Sci USA 97:13889–13894

Willis WD, Al-Chaer ED, Quast MJ, Westlund KN 1999 A visceral pain pathway in the dorsal column of the spinal cord. Proc Natl Acad Sci USA 96:7675–7679

Woolf CJ, Salter MW 288 Neuronal plasticity: increasing the gain in pain. Science 288:1765–1769

Xu GY, Huang LYM 2002 Peripheral inflammation sensitizes P2X receptor-mediated responses in dorsal root ganglion neurons. J Neurosci 22:93–102

Zhang F, Lu W, Dong Z 2002 Tumor-infiltrating macrophages are involved in suppressing growth and metastasis of human prostate cancer cells by INF-beta gene therapy in nude mice. PG-2942-51. Clin Cancer Res 8:2942–2951

Zhu ZW, Friess H, diMola FF et al 1999 Nerve growth factor expression correlates with perineural invasion and pain in human pancreatic cancer. J Clin Oncol 17:2419–2428

DISCUSSION

Dray: When tumours metastasize and go to several different bone locations, which ones are the most painful?

Mantyh: That is a good question, because it is the conundrum of bone cancer. Let's say a person has 10 metastases. Three of them could be painful. Why are three painful and seven not? The vertebral metastases tend to be very painful. There can be significant pain in the femur. We have begun to look at the tumour cells with microarrays, as have others. Originally we were thinking about the pro-hyperalgesic compounds that tumour cells could be making. But in some of the cases we see there is an up-regulation of enkephalin. The idea is that perhaps there are a variety of compounds made by tumours that are anti-nociceptive in some cases. Clearly, the environment that the tumour cell is in will dictate what it expresses. This is what characterizes tumour cells: they are genetically unstable. They will begin to make different things depending on what clone they are now at. For example, the Dunning model of prostate cancer is a wildly unstable tumour cell. Depending on what you put it in, it will either make one thing or the other. I think it can be any bone, but in general the vertebrae, ribs and long bones tend to be extremely painful.

Dray: Have the results with OPG been translated into any therapy?

Mantyh: I think it is now in clinical trials at Sloan-Kettering. It was originally developed for osteoporosis. The problem was that they were going to have to go head to head with bisphosphonate, and the time of the experiment was going to be significantly longer. With an experiment like this, however, within a year you would have an answer.

Dray: What about the endothelin A antagonist?

Mantyh: Abbot was originally using this. Its primary end-point was going to be reduced tumour burden in patients with prostate cancer. They said that they halted clinical trials in prostate cancer because it didn't reduce tumour burden although they left open the possibility that it could be used for the control of pain. It reduced pain but it did not reduce tumour burden. You must have been in this situation occasionally in a pharmaceutical company where you don't get the answer you want and the question is what you then do with it. Prostate and ovarian cancer express extremely high levels of endothelin 1.

Belmonte: What is the density of the innervation in a bone like the femur? Are there few neurons branching extensively or many sensory neurons? In other words, bone pain is very strong: is this because the bone is richly innervated by many nociceptive neurons or just a few neurons produce a lot of pain? Anatomically, is this innervation part of that of the soft tissues of the joints? And has anyone recorded from these fibres?

Mantyh: I think the skeleton is richly innervated, not from the standpoint that it has more fibres per millimetre cubed than skin, but when you think about how large a volume the skeleton makes up there are plenty of fibres to bump into. The areas that receive the greatest amount of stress are also the ones with the greatest number of Haversian canals per unit area. These canals tend to always have a sensory fibre in them.

Belmonte: Let me put this in another way. How many neurons do you label in the DRG when you fill up the bone medulla with a dye like fluorogold?

Mantyh: If we just look at ATF-3-expressing cells, we see them in L1, L2 and L3, and in L2 about 25% of the neurons will show ATF-3 if we confine it to the femur. It drops down to about 5% in L1 and it is about 5% in L3. The innervation is precisely where you don't want it. Or maybe you do want it here: it is the area you are most likely to fracture. If the sensory fibres are there to alert you to when you injure your bone they are in the right place. They also happen to be in the place where the tumour cells like to home in to.

Devor: You made the point that when you reduce the pain, you also get a substantial reduction in the astrogliosis. Does this not indicate that the pain is driving the gliosis, rather than the pain being a result of the gliosis?

Mantyh: I don't know which one it is. People have suggested that when there is this hypertrophy of astrocytes they down-regulate their glutamate transporters. Is the pain driving that? I don't know.

Devor: Is there any reason to believe that OPG might be affecting gliosis directly? If it is acting in the bone by the mechanism you describe, and reducing drive on nociceptors, then I would imagine that the gliosis goes down secondarily in the OPG animals. The implication is that the spikes are making the glial cells light up and not the other way round.

Reeh: CGRP is known to increase proliferation of glial cells.

Devor: There is probably something released from the neurons that have spikes. I am not saying that the action potentials themselves are responsible. Secondly, on the question of treatment, you draw the bone with one or two nerve branches going in. Would it make sense, rather than developing new non-steroidal anti-inflammatory drugs (NSAIDs) or things that work on the sensory terminals, just to denervate the bone?

Mantyh: I don't think this schematic was anatomically correct. You see the fibres coming in the nutrient foramen but if you look at the sites of the periosteum, periosteal fibres are piercing the Haversian and Wollman canals. To denervate the bone would be close to impossible because you would have to cut all the periosteal fibres entering that way.

Devor: In myeloma, it is quite common that at the beginning of the first cycle of adriamycin treatment the pain is powerfully reduced. Do you know why?

Mantyh: No.

Tominaga: Between the bone and osteoclast the pH is very low. It is much lower than the threshold for activation of either TRPV1 or ASIC. Many cancers have relatively small vascularity, leading to hypoxia, and are therefore in acidic conditions. Which is more predominantly involved in nociception in bone, TRPV1 or ASIC?

Mantyh: I would imagine that both are there. We have been able to show that TRPV1 is present. This nicely corresponds with the innervation of bone.

Tominaga: If my understanding is correct, a cancer must originate from the epithelial cells. Sarcoma is not epithelial. Have you used other kinds of cancer cells in your system?

Mantyh: Yes, we have used breast, colon and prostate cancer cells. We get the same results. From the standpoint of osteoclast involvement we don't see any real difference between these different types.

Baron: I would like to come back to the neuropathic component. If other tumours infiltrate nerve tissue there is a distinct change in the characteristics of the pain. I am not aware of such things in tumour patients. If they are so richly innervated I would assume that there must be some changes in these characteristics.

Mantyh: One key question is whether every tumour cell will avidly destroy the sensory fibres as well as the sarcoma, as well as the prostate and breast. There could be tumour cells which are not as aggressive and don't destroy either as quickly or don't have the same enzymatic potential that these particular cells do. I am not suggesting that all bone cancer pain is the same: I don't think it is. I think different tumour cells will do different things to the fibres depending on what they are releasing and their enzymatic potential. By getting a signature of what different tumour cells do and what they release at what stage, one can begin to make sense of the conundrum of bone cancer pain.

Devor: I'd like to raise the question of neuropathy from destruction of intramedullary innervation. It comes back to the example of hip replacement therapy. In severe osteoarthritis of the hip the standard treatment is to cut through the femur, remove the whole end of the bone and put a metal prosthesis in its place. There you would be cutting through a lot of the intramedullary innervation. I don't know of descriptions of neuropathic pain associated with this.

Apkarian: Eventually the end fibres are being eaten away by the tumour. What is the role of afferents relative to behaviour at that stage?

Mantyh: At least a component of the behaviour is responsive to gabapentin. Pfizer is stating the gabapentin is not just for neuropathic pain anymore but that it may also be useful in treating inflammatory pain. All we know is that ATF-3 is expressed, gabapentin is there, and we can see macrophages and supporting cells, similar to what we see if we damage the nerve. Do we have a neuropathic pain state? I don't know. I think we have nerve injury, we have markers of injury, we see macrophages in the nerve and DRG, and we see supporting cells also.

Apkarian: Is the behaviour of the animal different before nerve destruction versus after nerve destruction?

Mantyh: I think that the fibres are being destroyed as the tumour cells invade. I think it is a continuum. That's a very good point: if I said I know that there is neuropathic pain here, I certainly mis-stated. We don't know that. I was surprised to see ATF-3 there. We have done the same thing in the paw, injecting complete Freund's adjuvant (CFA) and looking at 3, 7 and 14 days, but we don't see ATF-3 come up there. We really have to injure the nerve fibres in some way, which is suggestive to us that when we see this expression and the changes in the glial cells, there is actually injury to the nerve fibres.

Wood: I wanted to go back to the topic of lots of different tumour cell types all recruiting activated osteoclasts. Is the low pH that might be caused by rapid cell division playing a role in activating mast cells?

Mantyh: It is known that tumour cells have lower pH than normal cells. Tumours typically grow rapidly at the leading edge and then you get necrosis, which could contribute to the pH. It is usually at the leading edge, though, that we see the greatest number of osteoclasts plastered up against the mineralized bone. It is staggering when you see the number of osteoclasts against the bone and the bone resorption.

Wood: Do you have an idea of other specific recruitment mechanisms of the tumour cells for the osteoclasts?

Mantyh: There is a whole debate in tumour biology about what is driving the osteoclasts. This is why they thought that endothelin 1 was going to reduce tumour burden. The idea was that tumour cells were releasing endothelin 1, stimulating the osteoblasts which then up-regulated the OPGL and drove RANK. But tumour burden wasn't reduced.

Dray: I have a question about the results that you have seen with MF tricyclics, second-generation COX-2 inhibitors. Did you say that they reduce sarcoma growth?

Mantyh: Yes.

Dray: Does this reduce the potential invasion of bone from other tumour sources?

Mantyh: If you are asking whether giving the MF tricyclic actually reduces the number of metastases to bone, then we haven't done this.

Dray: If the sarcoma can be reduced through a COX-2 inhibitory mechanism, can second generation of COX-2 inhibitors cause pain relief in bone cancer?

Mantyh: We have given NS398 acutely and we see a reduction of pain. I think that there it is probably acting on the sensory fibres and cord, as well as elsewhere. It can acutely reduce pain, although I don't think this has anything to do with tumour burden at that point. But if you chronically give the COX-2 inhibitor, COX-2 reduces tumour growth. I think it is then reducing the pain in two ways, by directly acting on sensory fibres to inhibit the prostaglandins and it is reducing tumour growth.

Dray: I am coming back to the fact that people with bone cancer pain are taking huge amounts of morphine. Your data would predict that COX-2 inhibitors would be good therapies for bone cancer pain.

Mantyh: I would think they would be.

Dray: Do you have any idea of what kind of prostanoid receptors are involved in sarcoma growth and bone pain?

Mantyh: No. I think there is a reason most people haven't worked with bone before. It is a horrible organ to work with. It is mineralized, so you have to try to demineralize it just to the point where you haven't destroyed your antigen, and then you have to try to cut a partially mineralized section. Some one asked earlier about recording from fibres: can you imagine trying to stick a microelectrode through mineralized bone to find a fibre?

McMahon: At several points you suggested that acid might be a mediator. I wonder if there is any direct evidence for this. This is particularly interesting in view of the fact that you may be destroying many nerve terminals, then the acid might be acting on the axons. And receptors such as TRPV1 are known to be strongly down-regulated after axotomy. In your DRG material, where you are using ATF-3 as a putative marker of innervation of the bone, have you looked at histochemistry there to see whether TRPV1 or ASICs are still expressed in those particular cells? Or do you have any other evidence that acid may be an important peripheral mediator?

Mantyh: That is a good point. These tumour cells, like many others, express high levels of NGF. In the ATF-3-expressing neurons there does not appear to be a significant alteration in the expression of TRPV1, substance P or CGRP. I think

we have nerve injury with NGF continually pumped out, and there is no lack of data suggesting that at least in some tumours that NGF may be involved in tumour proliferation.

McMahon: That is interesting. NGF will suppress ATF-3 expression, so if there is a lot of NGF reaching the fibres that are in the bone, ATF might be a difficult marker to use in these conditions.

Mantyh: ATF is not all or nothing. The more injury there is, the more ATF there is.

Mao: You mentioned two major mechanisms. One is the massive denervation with loss of pain fibres. The other is an acidic condition. These two mechanisms seem to be mutually exclusive. If you have a massive denervation, TRPV1 would not be adequately expressed.

Mantyh: It is a difficult pain to control, and there must be a reason for this. My hypothesis would be that it is because there are multiple mechanisms that are simultaneously driving it. There is a tumorigenic mechanism kicking out NGF and a variety of other pro-hyperalgesic factors. There is injury to nerve fibres and there are inflammatory cells there also. Nothing is going well for trying to reduce the pain in these individuals. Finally, there is the instability of the bone itself. Once the osteoclast has bored through into the proximal distal head and the bone is breaking, that is a significant pain. Now you have the mechanosensitive effectors which we know are in the periosteum which are beginning to move. This is why if you rub a sheet over a person with advanced bone cancer this is extremely disturbing to them.

Reeh: You don't need intact nerve endings to sense acid or heat. All along the length of the unmyelinated axon it is fully equipped with functionally effective TRPV1 molecules, and the axons are sensitive to heat as well as to protons in the same way as the endings. What are needed are just axons or even stumps of axons to create the same amount of pain as with full blown arborized nerve endings.

Mantyh: Another person in our lab has looked at these GFP-transfected cells which show what the tumour does. It doesn't just invade the marrow space but creeps through the Haversian canals. Then it recruits osteoclasts in the Haversian canals. So when you ask about the total amount of nerve–tumour interface, it becomes huge. The interface is not just in the marrow space.

Mao: For a long time the prevailing teaching point has been that tumour expansion is what is causing the pain. What you have presented changes the way we should be explaining bone pain.

Mantyh: I don't think there is any significant pressure issue. Mineralized bone is pretty tough, and the pain begins long before there is any significant bone erosion.

Belmonte: After listening to your presentation it is quite obvious to me that the distinction between neuropathic pain originated at the peripheral nerve endings and inflammatory pain is very difficult to make. In chronic inflammation a certain degree of damage to the nerve fibres is likely to occur.

Mechanistic and clinical aspects of complex regional pain syndrome (CRPS)

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Abstract. Complex regional pain syndromes (CRPS, reflex sympathetic dystrophy, causalgia) are painful disorders that develop after trauma affecting a limb with (I) or without (II) nerve injury. Clinical features are pain, impairment of motor function, swelling and autonomic abnormalities (changes in sweating and blood flow). *Autonomic abnormalities.* The maximal skin temperature difference between the affected and unaffected extremity that occurs during a controlled thermoregulation can be used as a diagnostic tool. *SMP.* Sympathetic outflow to the painful extremity was experimentally activated. The intensity as well as area of spontaneous pain and mechanical hyperalgesia increased considerably in patients that had been classified as having SMP by positive sympathetic blocks. A pathological interaction between sympathetic vasoconstrictor and afferent neurons within the affected skin is the likely explanation for SMP in CRPS patients. *Motor abnormalities.* Kinematic analysis of target reaching as well as grip force analysis showed a pathological sensorimotor integration located in the parietal cortex. Furthermore, MEG studies demonstrated a continuous inhibition of the primary motor cortex. *Neurogenic inflammation.* Some features of acute CRPS (vasodilatation, swelling, pain) indicate a localized inflammatory process. Transcutaneous electrical stimulation of nociceptive C-fibre provoked protein extravasation into the interstitial fluid (microdialysis) only in CRPS patients and not in controls.

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Definition of CRPS

The IASP (International Association for the Study of Pain) classification of chronic pain redefined pain syndromes formerly known as reflex sympathetic dystrophy and causalgia. The term 'complex regional pain syndrome' (CRPS) describes 'a variety of painful conditions following injury which appears regionally having a distal predominance of abnormal findings, exceeding in both magnitude and duration the expected clinical course of the inciting event often resulting in significant impairment of motor function, and showing variable progression

over time'. These chronic pain syndromes comprise different additional clinical features including spontaneous pain, allodynia, hyperalgesia, oedema, autonomic abnormalities and trophic signs. In CRPS type I (reflex sympathetic dystrophy) minor injuries or fractures to a limb precede the onset of symptoms. CRPS type II (causalgia) develops after injury to a major peripheral nerve (Harden et al 2001, Janig & Baron 2003).

Clinical characteristics

CRPS Type I (reflex sympathetic dystrophy)

The most common precipitating event is a trauma affecting the distal part of an extremity (65%), especially fractures, postsurgical conditions, contusions and strain or sprain. In rare occasions central nervous system lesions such as spinal cord injuries and cerebrovascular accidents are described as well as cardiac ischaemia.

CRPS I patients develop asymmetrical distal extremity pain and swelling without presenting an overt nerve lesion. These patients often report a burning spontaneous pain felt in the distal part of the affected extremity. Characteristically, the pain is disproportionate in intensity to the inciting event. The pain usually increases when the extremity is in a dependent position. Stimulus-evoked pains are a striking clinical feature; they include mechanical and thermal allodynia and/or hyperalgesia. These sensory abnormalities often appear early, are most pronounced distally, and have no consistent spatial relationship to individual nerve territories or to the site of the inciting lesion. Typically pain can be elicited by movements and pressure at the joints (deep somatic allodynia), even if these are not directly affected by the inciting lesion.

Autonomic abnormalities include swelling and changes of sweating and skin blood flow. In the acute stages of CRPS I the affected limb is commonly warmer than the contralateral limb. Sweating abnormalities, frequently hyperhidrosis are present in most patients. The acute distal swelling of the affected limb depends very critically on aggravating stimuli.

Trophic changes such as abnormal nail and hair growth, fibrosis, thin glossy skin and osteoporosis may be present, particularly in chronic stages. Restrictions of passive movement are often present in long-standing cases and may be related to both functional motor disturbances and trophic changes of joints and tendons.

Weakness of all muscles of the affected distal extremity is often present. Small accurate movements are characteristically impaired. Nerve conduction and electromyography studies are normal, except in patients in very chronic and advanced stages. About half of the patients have a postural or action tremor that

represents an increased physiological tremor. In about 10% of cases dystonia of the affected hand or foot develops.

CRPS Type II (causalgia)

The symptoms of CRPS II are similar to those of CRPS I. The only exception is that a lesion of peripheral nerve structures and subsequently focal deficits are mandatory for the diagnosis. The symptoms and signs spread beyond the innervation territory of the injured peripheral nerve and often occur *remote* from the site of injury, but a restriction to the territory is not in conflict with the current definition.

Pathophysiological mechanisms

Sensory abnormalities and pain

On the basis of numerous animal experiments, spontaneous pain and various forms of hyperalgesia at the distal extremity are thought to be generated by processes of peripheral and central sensitization.

These changed somatosensory perceptions are likely due to changes in the central representation of somatosensory sensations in the thalamus and cortex. Accordingly, positron emission tomography (PET) studies have demonstrated adaptive changes in the thalamus during the course of the disease. Furthermore, recent magnetoencephalography (MEG) and functional magnetic resonance imaging (fMRI) studies demonstrated a shortened distance between little finger and thumb representations in the sensorimotor (SI) cortex on the painful side. The MEG SI responses were increased on the affected side, indicating processes of central sensitization (Fig. 1).

Functional MRI studies indicate that prefrontal cortical networks are involved in SMP in CRPS patients. Furthermore, in one CRPS patient a traumatic cerebral contusion of the left temporal lobe resolved the symptoms.

We do not know the extent to which these central changes depend on continuous nociceptive input from the affected extremity or whether these generalized sensory changes disappear after successful treatment of the pain.

Autonomic abnormalities

A partial nerve lesion is the important preceding event in *CRPS II*. Therefore, it has generally been assumed that abnormalities in skin blood flow *within the territory of the lesioned nerve* are due to peripheral impairment of sympathetic function and sympathetic denervation. During the first weeks after transection of vasoconstrictor fibres, vasodilatation is present within the denervated area. Later

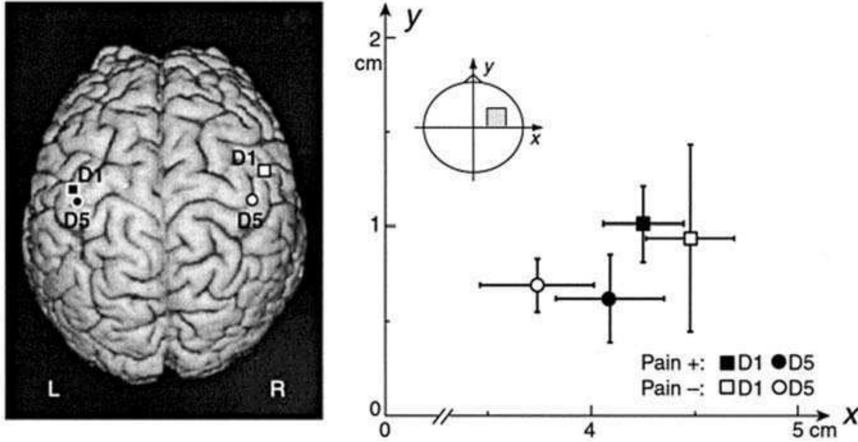


FIG. 1. MEG in CRPS. (Left) Sources of cortical responses to tactile stimuli of the healthy (open symbols) and painful (filled symbols) sides applied to digits 1 (squares) and 5 (circles) in a patient with CRPS. (Right) The mean (±SEM) source locations in the SI region. The head insert illustrates the coordinate system where the *x* axis goes from the left to the right preauricular point and *y* axis from inion to nasion.

the vasculature may develop increased sensitivity to circulating catecholamines, probably due to up-regulation of adrenoceptors.

Sympathetic denervation and denervation hypersensitivity cannot completely account for vasomotor and sudomotor abnormalities in CRPS. First, in CRPS I there is *no overt nerve lesion* and second, in CRPS II the autonomic symptoms spread *beyond the territory of the lesioned nerve*. In fact, there is direct evidence for a reorganization of central autonomic control in these syndromes.

In patients with hyperhidrosis, resting sweat output, as well as thermoregulatory and axon reflex sweating are increased in CRPS I patients. Increased sweat production cannot be explained by a peripheral mechanism since, unlike blood vessels, sweat glands do not develop denervation supersensitivity.

In CRPS I central sympathetic vasoconstrictor reflexes induced by thermoregulatory (whole-body warming, cooling) and respiratory stimuli were analysed. Sympathetic effector organ function, i.e. skin temperature and skin blood flow, was measured bilaterally by infrared thermometry and laser Doppler flowmetry. Under normal conditions these reflexes do not show inter-side differences. In CRPS patients three distinct vascular regulation patterns were identified related to the duration of the disorder. In the warm regulation type (acute stage, <6 months) the affected limb was warmer and skin perfusion values were higher than contralaterally during the entire spectrum of sympathetic activity. Even massive body cooling failed to activate sympathetic vasoconstrictor neurons.

Consistently, direct measurements of noradrenaline levels from the venous effluent above the area of pain show a *reduction* in the affected extremity. In the intermediate type, temperature and perfusion were either warmer or colder depending on the degree of sympathetic activity. In the cold type (chronic stage), temperature and perfusion were lower on the affected side during the entire thermoregulatory cycle. Noradrenaline levels, however, were still lower on the affected side. These data support the idea that CRPS I is associated with a pathological unilateral inhibition of cutaneous sympathetic vasoconstrictor neurons leading to a warmer affected limb in the acute stage. The locus of pathophysiological changes underlying such disturbed reflex activity must be in the central nervous system. Secondary changes in the neurovascular transmission may induce severe vasoconstriction and cold skin in chronic CRPS. Accordingly, α-adrenoceptor density is increased in skin biopsies of CRPS I patients.

Abnormalities in central autonomic control are consistent with experimental findings in animals, which show that the reflex pattern in single cutaneous vasoconstrictor neurons may change after peripheral nerve injury. The few microneurographic studies of small sympathetic nerve fascicles that have been performed so far in patients with CRPS, however, have not confirmed the presence of reflex abnormalities; the average skin sympathetic activity (i.e. a combination of vasoconstrictor and sudomotor activity) was not different on the two sides.

Neurogenic inflammation

Some of the clinical features of CRPS particularly in its early phase could be explained by an inflammatory process. Consistent with this idea, corticosteroids are often successfully used in acute CRPS.

There is increasing evidence that a localized *neurogenic inflammation* might be involved in the generation of acute oedema, vasodilatation and increased sweating. Scintigraphic investigations with radiolabelled immunoglobulins show extensive plasma extravasation in patients with acute CRPS I. Furthermore, synovial effusion is enhanced in affected joints as measured with MRI which seems to be a valuable additional diagnostic tool. In acute untreated CRPS I patients neurogenic inflammation was elicited by transcutaneous electrical stimulation via intradermal microdialysis capillaries. Protein extravasation that was simultaneously assessed by the microdialysis system was only provoked on the affected extremity as compared with the normal side. Furthermore, axon reflex vasodilatation was significantly increased. In the fluid of artificially produced skin blisters significantly higher levels of interleukin (IL)6 and tumour necrosis factor (TNF)α were observed in the involved extremity as compared with the uninvolved extremity.

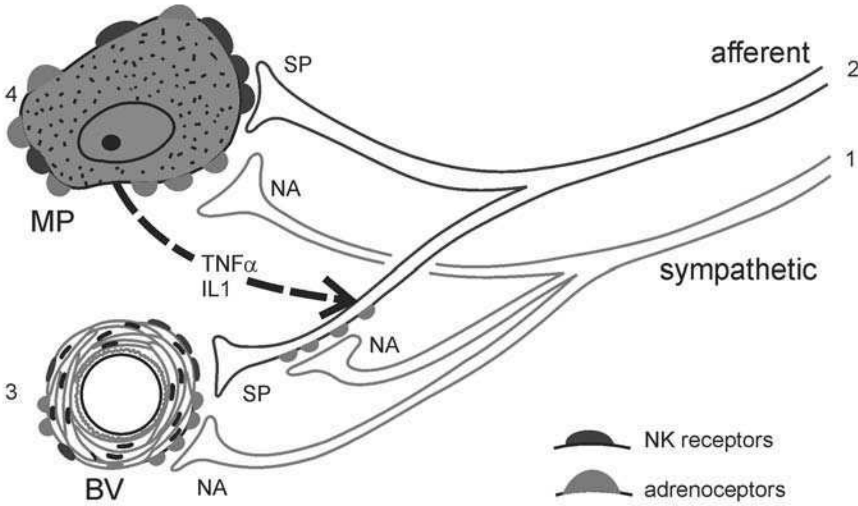


FIG. 2. Hypothetical relation between sympathetic noradrenergic nerve fibres (1), peptidergic afferent nerve fibres (2), blood vessels (3) and macrophages (4). The activated and sensitized afferent nerve fibres activate macrophages (via substance P release). The immune cells start to release cytokines, such as tumour necrosis factor α (TNFα) and interleukin 1 (IL1) which further activate afferent fibres by enhancing sodium influx into the cells. Substance P (and CGRP) released from the afferent nerve fibres reacts with neurokinin 1 (NK1) receptors in the blood vessels (arteriolar vasodilation, venular plasma extravasation; neurogenic inflammation). The sympathetic nerve fibres interact with this system on three levels: (i) via adrenoceptors (mainly α) on the blood vessels (vasoconstriction); (ii) via adrenoceptors (mainly β) on macrophages (further release of cytokines), and (iii) via adrenoceptors (mainly α) on afferents (further sensitization of these fibres). (From Janig & Baron 2003, with permission).

The exact mechanisms of the initiation and maintenance of these inflammatory reactions in early CRPS are unclear. One central issue is whether the sympathetic nervous system may contribute to the inflammatory state. *De novo* expression of adrenoreceptors on macrophages after experimental nerve lesion supports this idea. Figure 2 illustrates the possible interactions between sympathetic fibres, afferent fibres, blood vessels and non-neural cells related to the immune system (e.g. macrophages) leading theoretically to the inflammatory changes observed in CRPS patients.

Motor abnormalities

About 50% of the patients with CRPS show motor abnormalities. It is unlikely that these motor changes are related to a peripheral process (e.g. influence of the sympathetic nervous system on neuromuscular transmission and/or contractility of skeletal muscle). These somatomotor changes are more likely generated by

central changes of activity in the motoneurons. With kinematic analysis of target reaching, as well as grip-force analysis to quantitatively assess motor deficits in CRPS patients, abnormalities in the cerebral motor processing were revealed. A pathological sensorimotor integration located in the parietal cortex may induce an abnormal central programming and processing of motor tasks. Interestingly, the motor performance is also slightly impaired on the contralateral unaffected side. According to this view, a neglect-like syndrome was clinically described as responsible for the disuse of the extremity. A recent controlled study also supports an incongruence between central motor output and sensory input as underlying mechanism in CRPS. Using the method of mirror visual feedback the visual input from a moving unaffected limb to the brain was able to re-establish the pain-free relationship between sensory feedback and motor execution. After six weeks of therapy pain and function were improved.

Sympathetically maintained pain (SMP)

Neuropathic pain patients presenting with similar clinical signs and symptoms can clearly be divided into two groups by the negative or positive effect of selective sympathetic blockade or antagonism of α-adrenoceptor mechanisms. The pain component that is relieved by specific sympatholytic procedures is considered 'sympathetically maintained pain' (SMP). Thus, *SMP* is now defined to be a *symptom* or the underlying *mechanism* in a subset of patients with neuropathic disorders and *not a clinical entity*. The positive effect of a sympathetic blockade is *not essential* for the diagnosis.

After nerve lesion in animal experiments afferent nociceptive and non-nociceptive neurons undergo dramatic functional and anatomical changes including up-regulation of α-adrenoceptors. Noradrenaline released by the sympathetic nerve fibres may activate and/or sensitize the afferent neurons. This sympathetic-afferent coupling forms the theoretical basis for the clinical phenomenon of SMP. The interaction may occur at the lesion site, along the lesioned nerve or even in the dorsal root ganglion.

Clinical studies in humans support the idea that nociceptors develop catecholamine sensitivity after nerve lesions. In CRPS II, intradermal noradrenaline, in physiologically relevant doses, was demonstrated to evoke greater pain in the affected regions of patients with SMP than in the contralateral unaffected limb, and in control subjects.

In patients with CRPS I cutaneous sympathetic vasocontrictor outflow to the painful extremity was experimentally activated to the highest possible physiological degree by whole body cooling. During the thermal challenge the affected extremity was clamped to 35 °C in order to avoid thermal effects at the nociceptor level. The intensity as well as area of spontaneous pain and

mechanical hyperalgesia (dynamic and punctate) increased significantly in patients that had been classified as having SMP by positive sympathetic blocks but not in SIP patients (Fig. 3). The experimental setup used in the latter study selectively alters sympathetic cutaneous vasoconstrictor activity without influencing other sympathetic systems innervating the extremities, i.e. pilo arrector, sudomotor and muscle vasoconstrictor neurons. Therefore, the interaction of sympathetic and afferent neurons measured here is likely to be located within the skin. Interestingly, the relief of spontaneous pain after sympathetic blockade was more pronounced than changes in spontaneous pain that could be induced experimentally by sympathetic activation. One explanation for this discrepancy might be that a complete sympathetic block affects all sympathetic outflow channels projecting to the affected extremity. It is very likely that in addition to a coupling in the skin, a sympathetic-afferent interaction may also occur in other tissues, in particular in the deep somatic domain such as bone, muscle or joints. Furthermore, patients may exist who are characterized by a selective or predominant sympathetic-afferent interaction in deep somatic tissues sparing the skin.

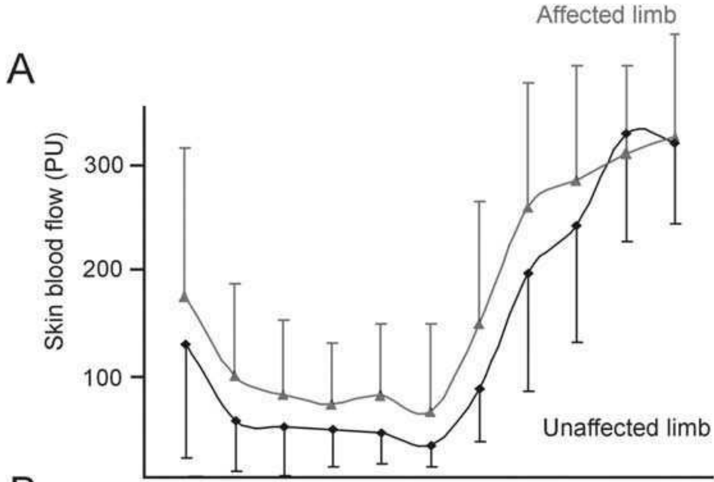
Genetics of CRPS

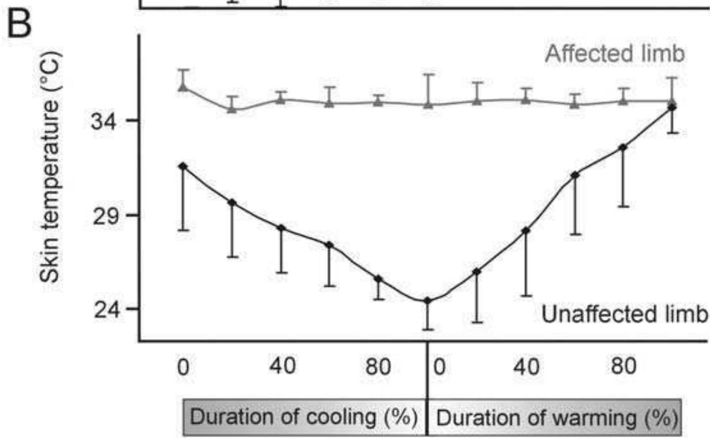
One of the unsolved features in human pain diseases is the fact that only a minority of patients develop chronic pain after identical inciting events. Similarly, in certain nerve-lesion animal models, differences in pain susceptibility were found to be due to genetic factors. Gene technology has revealed some genetic patterns of patients at risk of developing CRPS. In CRPS patients class I and II major histocompatibility antigens were typed. The frequency of HLA-DQ1 was found to be significantly increased compared with control frequencies. In patients with CRPS who progressed towards multifocal or generalised tonic dystonia an association with HLA-DR13 was reported. Furthermore, a different locus, centromeric in HLA-class I, was detected to be associated with spontaneous development of CRPS, suggesting an interaction between trauma severity and genetic factors that describe CRPS susceptibility.

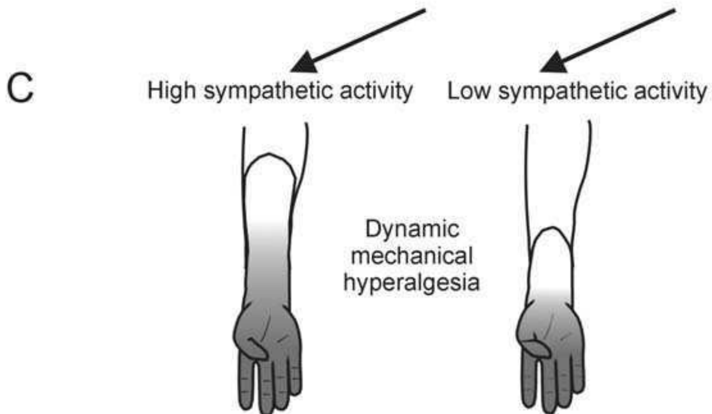
Diagnostic tests which support the diagnosis of CRPS

In the mineralization phase of the bone scintigraphy a pathological uptake in the metacarpophalangeal or metacarpal bones is sensitive and specific for CRPS. It only shows significant changes during the subacute period (up to 1 year).

In plain radiographs subperiostal and trabecular bone resorption, spotty and localized bone demineralization or osteoporosis are specific but only positive in


A
Affected limb
Skin blood flow (PU)
300
200
100
Unaffected limb


B
Affected limb
Skin temperature (°C)
34
29
24
Unaffected limb
0
40
80
0
40
80
Duration of cooling (%)
Duration of warming (%)


C
High sympathetic activity
Low sympathetic activity
Dynamic
mechanical
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chronic stages. MRI scans are proposed to be more reliable than radiographic examination and scintigraphy but have to prove their value in further studies.

Autonomic testing with the QSART (quantitative sudomotor axon reflex test) can provide information about the function of sudomotor reflex loops. Swelling can be quantified by measuring water displacement.

Skin temperature measurements are an easy measure of vascular function and may be particularly helpful for diagnosis of CRPS. We performed a study using controlled thermoregulation (whole-body warming, cooling) to change cutaneous sympathetic vasoconstrictor activity. Skin temperature at the affected and unaffected limbs (infra-red thermometry) was measured under resting conditions (before temperature challenge in the office at room temperature) and continuously monitored during controlled modulation of sympathetic activity. Only minor skin temperature asymmetries were present between both limbs under resting conditions in most patients. However, during controlled thermoregulation temperature differences between both sides increased dynamically and were most prominent at a high to medium level of vasoconstrictor activity. In patients suffering from painful limbs of other origin and in healthy volunteers (control groups), there were only minor side differences in temperature both in rest and during thermoregulatory changes of sympathetic activity. When comparing the diagnostic value of skin temperature asymmetries in CRPS, sensitivity was only 32% under resting conditions, but increased up to 76% during controlled alteration of sympathetic activity. Specificity was 100% at rest and 93% at controlled thermoregulation (Fig. 4).

In conclusion, the degree of unilateral vascular disturbances in CRPS and the temperature side differences depend critically on environmental temperature and spontaneous sympathetic activity. However, the maximal skin temperature

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FIG. 3. (A,B) Influence of cutaneous sympathetic vasoconstrictor activity on pain in a chronic CRPS I patient with SMP. Thermal stimuli were applied that induce changes of activity in sympathetic vasoconstrictor neurons innervating skin and of noradrenaline release. The patient was lying in a thermal suit (see above) in order to cool or warm the whole body. Cooling induces a massive tonic activation of cutaneous vasoconstrictor neurons, warming leads to a decrease of their activity. In this way sympathetic activity was switched on and off in a controlled manner during simultaneous measurement of pain sensation (measurement of area of mechanical allodynia to phasic light touch exerted by gently moving a cotton swab over the skin). (C) High sympathetic activity during cooling induces a decrease of skin temperature due to vasoconstriction (unaffected side; monitor for the effect of whole-body cooling and warming). During the experiment the forearm temperature on the affected side was clamped to 35 °C by a feedback-controlled heat lamp to exclude temperature effects on the sensory receptors (upper record). Activation of cutaneous vasoconstrictor neurons leads to an increase in the area of allodynia in this CRPS patient indicating that in CRPS with SMP a pathologic coupling between sympathetic and nociceptive neurons does exist. This functional coupling is absent in CRPS patients without SMP.

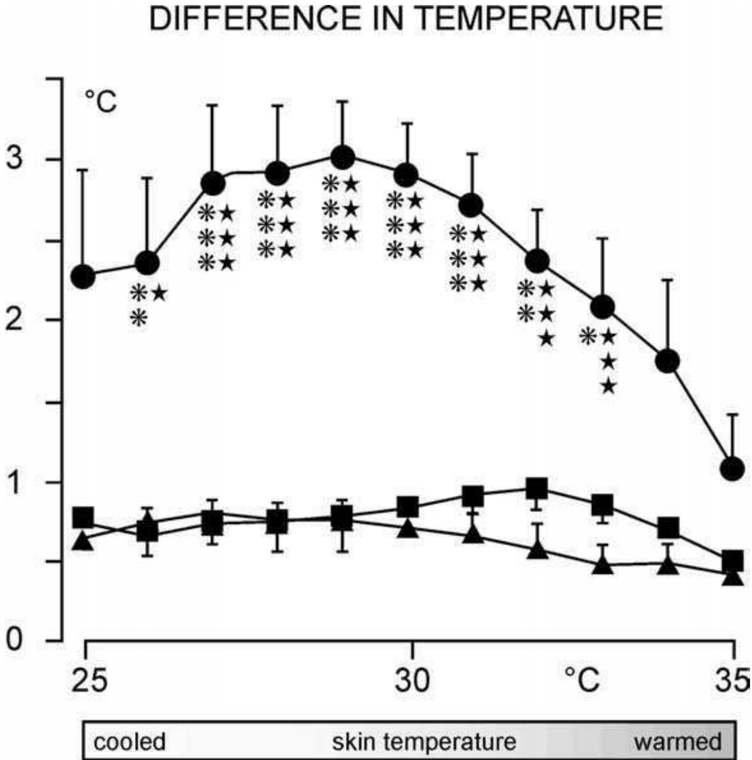

FIG. 4. Average absolute side differences in skin temperature of the fingers (toes) of both hands (feet) in 25 patients with CRPS (●) in 20 healthy controls (■) and in 15 control patients with extremity pain of other origin (▲) during a controlled thermoregulatory cycle (controlled alteration in cutaneous sympathetic activity). The level of the overall cutaneous sympathetic vasoconstrictor activity was estimated indirectly by using the skin temperature on the unaffected side (or right side in healthy controls) as reference value. A skin temperature on the healthy side of 25 °C indicates a high level, a temperature of 30 °C an intermediate level and a temperature of 35 °C a complete inhibition of sympathetic vasoconstrictor activity to the skin. Mean ± SEM. CRPS compared with healthy controls, CRPS compared with control patients with extremity pain of other origin. Asterisks show CRPS compared with healthy controls, stars show CRPS compared with control patients with extremity pain of other origin. One symbol, $P < 0.05$; two symbols, $P < 0.01$; three symbols, $P < 0.001$.

difference that occurs during the thermoregulatory cycle distinguishes CRPS from other extremity pain syndromes with high sensitivity and specificity.

Therapeutic strategies in CRPS

Lack of understanding of the underlying pathophysiological abnormalities and lack of objective diagnostic criteria make it difficult to conduct clinical trials for

CRPS. Therefore, only a few evidence-based treatment regimens for CRPS are available so far and outcome studies find little consistent information regarding the pharmacological agents and methods for treatment of CRPS. Treatment of CRPS should be immediate, pain-free and directed toward restoration of the full function of the extremity within a multidisciplinary setting.

Conclusions

The sensory, sympathetic, somatomotor and trophic changes (including swelling), observed in variable combinations in patients with CRPS are the results of changes and distorted processing of information in the central nervous system involving the somatosensory non-nociceptive and nociceptive systems, the endogenous neuronal systems controlling nociceptive impulse transmission, the sympathetic system and also the somatomotor system. Various levels of integration are probably involved, such as spinal cord, brain stem, diencephalon (hypothalamus, thalamus) and forebrain (cortex and limbic system). A key player in generation and maintenance of CRPS is most likely the nociceptive system. However, this system must not be seen to *cause* CRPS in the sense that CRPS can be reduced to the malfunctioning of the nociceptive system. By the same token, although the sympathetic nervous system is important, CRPS cannot be reduced to a malfunctioning of this system or components of it. Thus, CRPS is a real, complex disorder.

Acknowledgements

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References

Ali Z, Raja SN, Wesselmann U et al 2000 Intradermal injection of norepinephrine evokes pain in patients with sympathetically maintained pain. Pain 88:161–168

Apkarian AV, Thomas PS, Krauss BR, Szeverenyi NM 2001 Prefrontal cortical hyperactivity in patients with sympathetically mediated chronic pain. Neurosci Lett 311:193–197

Baron R, Levine JD, Fields HL 1999 Causalgia and reflex sympathetic dystrophy: does the sympathetic nervous system contribute to the generation of pain? Muscle Nerve 22:678–695

Baron R, Schattschneider J, Binder A, Siebrecht D, Wasner G 2002 Relation between sympathetic vasoconstrictor activity and pain and hyperalgesia in complex regional pain syndromes: a case-control study. Lancet 359:1655–1660

Birklein F, Sittle R, Spitzer A et al 1997 Sudomotor function in sympathetic reflex dystrophy. Pain 69:49–54

Casale R, Elam M 1992 Normal sympathetic nerve activity in a reflex sympathetic dystrophy with marked skin vasoconstriction. J Auton Nerv Syst 41:215–219

Christensen K, Jensen EM, Noer I 1982 The reflex dystrophy syndrome response to treatment with systemic corticosteroids. Acta Chir Scand 148:653–635

Deuschl G, Blumberg H, Lücking CH 1991 Tremor in reflex sympathetic dystrophy. Arch Neurol 48:1247–1452

Drummond PD, Finch PM, Smythe GA 1991 Reflex sympathetic dystrophy: the significance of differing plasma catecholamine concentrations in affected and unaffected limbs. Brain 114:2025–2036

Drummond PD, Skipworth S, Finch PM 1996 Alpha 1-adrenoceptors in normal and hyperalgesic human skin. Clin Sci (Lond) 91:73–77

Forouzanfar T, Koke AJ, van Kleef M, Weber WE 2002 Treatment of complex regional pain syndrome type I. Eur J Pain 6:105–122

Fukumoto M, Ushida T, Zinchuk VS, Yamamoto H, Yoshida S 1999 Contralateral thalamic perfusion in patients with reflex sympathetic dystrophy syndrome. Lancet 354:1790–1791

Galer BS, Butler S, Jensen MP 1995 Case reports and hypothesis: a neglect-like syndrome may be responsible for the motor disturbance in reflex sympathetic dystrophy (Complex Regional Pain Syndrome-1). J Pain Symptom Manage 10:385–391

Graif M, Schweitzer ME, Marks B, Matteucci T, Mandel S 1998 Synovial effusion in reflex sympathetic dystrophy: an additional sign for diagnosis and staging. Skeletal Radiol 27:262–295

Harden RN, Baron R, Jänig W (eds) 2001 Complex regional pain syndrome. Progress in pain research and management, Vol. 22. IASP Press, Seattle

Huygen FJ, De Bruijn AG, De Bruin MT et al 2002 Evidence for local inflammation in complex regional pain syndrome type 1. Mediators Inflamm 11:47–51

Janig W, Baron R 2002 Complex regional pain syndrome is a disease of the central nervous system. Clin Auton Res 12:150–164

Juottonen K, Gockel M, Silen T et al 2002 Altered central sensorimotor processing in patients with complex regional pain syndrome. Pain 98:315–323

Kemler MA, van de Vusse AC, van den Berg-Loonen EM et al 1999 HLA-DQ1 associated with reflex sympathetic dystrophy. Neurology 53:1350–1351

Maleki J, LeBel AA, Bennett GJ, Schwartzman RJ 2000 Patterns of spread in complex regional pain syndrome, type I (reflex sympathetic dystrophy). Pain 88:259–266

McCabe CS, Haigh RC, Ring EF et al 2003 A controlled pilot study of the utility of mirror visual feedback in the treatment of complex regional pain syndrome (type 1). Rheumatology (Oxford) 42:97–101

Merskey H, Bogduk N 1995 Classification of chronic pain: descriptions of chronic pain syndromes and definition of terms. IASP press, Seattle

Oyen WJ, Arntz IE, Claessens RM et al 1993 Reflex sympathetic dystrophy of the hand: an excessive inflammatory response? Pain 55:151–157

Ribbers GM, Mulder, T, Geurts, AC, den Otter, RA 2002 Reflex sympathetic dystrophy of the left hand and motor impairments of the unaffected right hand: impaired central motor processing? Arch Phys Med Rehabil 83:81–85

Sintzoff S, Sintzoff S Jr, Stallenberg B, Matos C 1997 Imaging in reflex sympathetic dystrophy. Hand Clin 13:431–442

van de Beek WJ, Roep BO, van der Slik AR, Giphart MJ, van Hilten BJ 2003 Susceptibility loci for complex regional pain syndrome. Pain 103:93–97

van der Laan L, Goris RJ 1997 Reflex sympathetic dystrophy. An exaggerated regional inflammatory response? Hand Clin 13:373–385

van Hilten JJ, van de Beek WJ, Roep BO 2000 Multifocal or generalized tonic dystonia of complex regional pain syndrome: a distinct clinical entity associated with HLA-DR13. Ann Neurol 48:113–116

Wasner G, Heckmann K, Maier C, Baron R 1999 Vascular abnormalities in acute reflex sympathetic dystrophy (CRPS I) — complete inhibition of sympathetic nerve activity with recovery. Arch Neurol 56:613–620

Wasner G, Schattschneider J, Heckmann K, Maier C, Baron R 2001 Vascular abnormalities in reflex sympathetic dystrophy (CRPS I): mechanisms and diagnostic value. Brain 124:587–599
Wasner G, Schattschneider J, Baron R 2002 Skin temperature side differences — a diagnostic tool for CRPS? Pain 98:19–26
Wasner G, Schattschneider J, Binder A, Baron R 2003 Complex regional pain syndrome — diagnostic, mechanisms, CNS involvement and therapy. Spinal Cord 41:61–75
Weber M, Birklein F, Neundorfer B, Schmelz M 2001 Facilitated neurogenic inflammation in complex regional pain syndrome. Pain 91:251–257
Zyluk A 1999 The usefulness of quantitative evaluation of three-phase scintigraphy in the diagnosis of post-traumatic reflex sympathetic dystrophy. J Hand Surg [Br] 24:16–21

DISCUSSION

McMahon: What is the incidence of sympathetically maintained pain in your CRPS patients? I get the impression that its significance has been dwindling over the last decade. Is that true?

Baron: It is dwindling in the chronic phase of the disease. It is much higher in the acute phase of CRPS. There is a change in the cause of the disease, which is very important to realize. If we want to treat them early to facilitate physiotherapy we have to apply sympathetic blocks as early as possible.

McMahon: Whole body temperature warming and cooling has a very dramatic effect on cutaneous vasoconstrictor outflow. Is it known that you are modulating deep sympathetic outflow to the same degree?

Baron: No.

McMahon: So it is possible that the body warming is only revealing a fraction of the total sympathetic contribution that is participating.

Baron: That is correct. We just modulate the skin sympathetic output, because the sympathetic system is organized in distinct output channels, which we can influence separately. We checked for muscle vasoconstrictor neurons by measuring blood pressure during whole body warming, and there is no significant change. This indicates that there is no change in muscle vasoconstrictor tone. This is the reason why we think that the deep sympathetic component is perhaps much more important. If we compare the amount of pain which we can modulate with the cutaneous changing with sympathetic blocks where we block the entire sympathetic outflow, the latter is much higher. The deep coupling might therefore be more important than the cutaneous coupling.

Dray: I have a question relating to the stability of the plastic changes in the sympathetic system. If you lesion the sympathetic system, often the pain goes away and then returns. Why?

Baron: With surgical sympathectomy, most people with SMP will have pain relief for some weeks or months. This is not a long-lasting effect. The idea is that with time there is up-regulation of denervation supersensitivity and severe vasoconstriction. Also, if you do surgical sympathectomy on animals there is an

up-regulation of these receptors on nociceptive fibres without any nerve lesion. This will come in addition to the receptors which are already there.

Dray: Does the appearance of the new α2A receptor subtype translate into humans?

Baron: I have no idea. We have some indication that it might be α receptors, but no subtypes so far.

Perl: The literature suggests strongly it is an α adrenoreceptor. The subtype isn't clear.

Dray: Is that because it hasn't been done in human material?

Perl: As far as I am aware the subtype in human subjects is not definitively established.

Baron: It has been looked at.

Malmberg: Some clinical and primate studies implicate α1 adrenergic receptors, while the majority of rodent work implicates α2 adrenergic receptors. What do you think about studying these mechanisms in animals? Is it worthwhile?

Baron: I think it is worthwhile.

Malmberg: But if the receptor subtype is different in humans versus rodents?

Baron: It hasn't been established which adrenoreceptor type is present in humans.

Dray: You have related sympathetic pain to changes in afferent excitability. What about changes in the local vasculature which might then indirectly affect excitability?

Baron: We are not able to rule this out. If we get rid of the temperature effect we can rule out things like chronic allodynia due to cooling of the skin. But we see the severe vasoconstriction due to activity of vasoconstrictor neurons. It might be that this is indirect due to vasoconstriction and changes in skin blood flow. It is hard to test in the human situation. You could apply vasoconstrictors which are not adrenergic to get rid of this bias, but we haven't done this.

Perl: There is another question that needs to be addressed relative to these considerations. Drummond et al (1991) reported that there was evidence of decreased sympathetic activity in an affected limb of RSD patients compared to the normal contralateral limb. This fits with some earlier microneurographic data. Denervation supersensitivity has been evoked as a possible explanation.

Baron: It seems to be contradictory that we can demonstrate a newly lateral inhibition of sympathetic efferents in these patients. We also have shown decreased levels of noradrenaline in the affected extremity. This seems to be true. The old story of hyperactivity of the sympathetics is not true. It is inhibited all the time. But you have to keep in mind that if present, this remaining activity will be able to drive afferent fibres if there is a coupling. And we have evidence for a decrease in activity (an inhibition) in the cutaneous outflow. It might well be that if we look at the different channels, we have inhibition in cutaneous areas but

hyperactivity in deep areas, and that most of the interaction will take place in deep areas. This is open because we are not able to measure sympathetic activity in deep tissues.

Apkarian: I had a couple of simple questions. One concerns the motor abnormalities. How common is this?

Baron: In acute patients they are very common. In particular they have weakness. The coordination deficits are obvious. There is some nice work using precision grip analysis by Schattschneider et al (2001) who have evidence that this is a central, cerebral problem in the integration of visual, afferent and motor interaction. Deficits in this precision grip are very similar to patients with parietal stroke.

Apkarian: The other question concerns the data you showed us about andrenergic sensitivity in carpal tunnel syndrome. Are the two populations behaviourally distinct? Do the patients complain of different types of pain?

Schattschneider: From a clinical point of view all the patients show typical signs of a carpal tunnel syndrome, which means that they are complaining about the same type of pain, especially at night time. We can't distinguish clinically between patients with sensitized C-fibres and normal C-fibres. To identify the two populations we have performed a quantitative thermotest in the area innervated by the medial nerve and found that some patients showed a decrease in heat pain thresholds indicating sensitization of C-fibres.

Devor: In the old nomenclature and the new there is a distinction between situations where there is clear nerve injury (CRPS II) and ones where there isn't. By definition, CRPS I doesn't have a demonstrable nerve lesion. This raises the question, is this properly characterized as neuropathic pain? I'd like your comments on this. The second issue is an idea that has been around a lot which deserves discussing. You have paid most attention in your presentation to sensory abnormalities: hyperalgesia and ongoing pain, and the subject of motor disturbances, which really is an open question, has come up. There are also the trophic changes which you mentioned. This is where the sympathetic sensory coupling in the ganglion might be of particular importance. If in fact there is a long-term sustained sympathetic drive on sensory fibres from the ganglion, then impulses would run centrally and contribute to sensory abnormalities. But in addition they would run peripherally, leaking various peptides out into the skin over long periods of time. This could be an underlying cause of trophic changes.

Baron: I agree. We don't have any idea about trophic changes so far. But the afferent system might be involved. We do not need the DRG to explain retrograde afferent activity.

Devor: You do, in a way—axon reflex isn't enough. If it were, then any condition with tactile allodynia would show these same trophic changes. The

axon reflex would be common to anything that hurts in the skin, which I don't think is the case.

Perl: One must consider the time course of the blood flow changes. Part of the mechanism of this syndrome is related to changes in peripheral tissue. That was Weir Mitchell's concept over 100 years ago.

Baron: It might be the efferent sympathetic outflow that causes skin changes.

Devor: What is your feeling about the neuropathic issue in CRPS I?

Baron: Every tissue damage or fracture will involve some nerve effects, for sure. But by definition it is not really a neuropathic pain syndrome, although the clinical features are very similar. I think it is wise to differentiate, because if there is a large nerve involved there are other things going on in addition to the features we see in classical CRPS. Interestingly, there is now evidence that in acute CRPS I there is an additional inflammatory reaction. One nice study involved skin suction blisters, looking at the interstitial fluid in the blister. They found that some cytokines are increased in the affected side. There is also recent evidence from Linda Watkins showing that macrophages are able to express α receptors on their membrane in the inflammatory environment.

Mao: I have a housekeeping question about your experimental paradigm. Even though the whole body cooling was meant to change the sympathetic tone, you maintained the limb at 35 °C. This means that the limb would not be affected by the change of sympathetic tone.

Baron: The whole idea behind this experimental set-up is that you are able to change the sympathetic tone to the whole body, including the affected limbs. The vasoconstrictor neurons are firing like hell, although you keep the affected extremities at a certain temperature.

Mao: Through what mechanism?

Baron: The body is cooled, and this induces sympathetic activity everywhere in the body. We warm just the affected extremities because we would like to avoid the cooling at the receptor level.

Mao: But the limb is maintained at 35 °C, which would minimize the change in the sympathetic tone.

Baron: No, it is the heating lamp outside which we use just to warm this area.

Mao: If you warm that area you change the sympathetic tone.

Baron: The body is still cooled.

Mao: The body is cooled but the limb is not. The limb's sympathetic tone would be unchanged.

Devor: The question is how topographic the autonomic outflow is.

Baron: We checked the effect of this high vasoconstrictor tone by using laser doppler. We can see a huge vasoconstriction in the affected limb, indicating massive vasoconstrictor activity to the limb, although the local temperature is warm.

Perl: The kinds of stimuli being considered are stressful and may reflexly involve the adrenal medulla and release of epinephrine, which in part would explain some of the distant effects.

Baron: If you bring in the adrenal medulla, it is true that we can't rule out its effects.

Mao: Another housekeeping question is the local injection of noradrenaline. I thought this could cause extreme pain.

Baron: With the quantities we use it is not painful in normal subjects.

Chung: I would remind the group that we need to distinguish between adrenergically maintained pain and sympathetically maintained pain. With adrenergically maintained pain we are talking about a condition with up-regulation of adrenergic receptors. On the other hand, with sympathetically maintained pain we are talking about a condition that has excessive sympathetic ligands. These two phenomena may frequently occur concurrently but may also occur independently. That is to say, we can have pain showing increased adrenergic sensitivity yet sympathetic block may have no effect or vice versa. Therefore, it is dangerous to think that increased adrenergic sensitivity is the basis of sympathetically maintained pain. A related question is: when we talk about sympathetically maintained pain in patients, how much is maintained? Is it totally maintained or just partially? I ask this because Doo Lee observed a variable effect of sympathectomy in the spinal nerve ligation model, depending on the test spots — a very sensitive spot and a less sensitive spot. The end result was that in the less sensitive spot the initial threshold was high, but after sympathectomy there was no change. In the more sensitive spot, sensitivity was high to begin with and then went to a less sensitive level after the sympathectomy. So is there any possibility that sensitive allodynia is sympathetically maintained whereas less sensitive hyperalgesia is not?

Baron: In every patient where there is this phenomenon there are pain mechanisms besides SMP. The SMP component comes in addition to these. If you block the system entirely you will, if you are lucky, achieve 80% in pain reduction — the SMP component. There are other patients who have a 20% SMP component. We always have spontaneous pain and allodynia in parallel. We think that we first influence spontaneous pain activity in the nociceptors, and then we influence allodynia by reducing central sensitization.

Ueda: I have a question concerning the sympathetic sprouting into the DRG neurons. To which DRG neurons do sympathetic neurons send sproutings?

Baron: A fibres. It is mainly large neurons.

Devor: That is the general rule, although it is not absolute. I'd like to remind everyone that in the presence of central sensitization or nerve injury, the behaviour and phenotype of the large neurons changes. Sympathetically driven

activity in these large neurons could now be giving a sensation of pain. In particular, if we are talking about axotomized neurons, they may be behaving in ways which are characteristic of nociceptors in the sense that they can release substance P which is up-regulated in A fibres. They may also be able to trigger and maintain central sensitization. Remember Aβ pain.

Ueda: Which type of α receptor is involved in your experiment? Is it α2? This is G_i-coupled.

Baron: We haven't done the animal work.

Devor: I'd like to add a bit of light concerning α1 and α2 receptor pharmacology in experiments on sympathetic–sensory coupling in injured nerve. In rats, at least, 65% of afferents show α2 receptor selectivity, but something like 25% show α1, or a mix of α1 plus α2 pharmacology (Chen et al 1996). I should add that firing in some neurons is suppressed, especially in long-surviving preparations.

Perl: The situation is more complicated than Professor Devor implied. There are at least three major subtypes of α2 receptors, each with different functional characteristics.

Devor: Most investigators presume that an adrenergic stimulus depolarizes or hyperpolarizes a neuron, and in that way changes its firing. We have published evidence recently suggesting quite a different process (Amir et al 2002). We recorded from DRG neurons and monitored the subthreshold oscillations which, as I mentioned previously, appear to be critical for ectopic firing. At least in some cells, you can apply noradrenaline and get no change at all in the membrane potential, and yet the resonance of the cell is enhanced. Cells become capable of firing repetitively without membrane depolarization. Things may be more complicated than the simple dichotomy of depolarization or hyperpolarization.

References

Amir R, Michaelis M, Devor M 2002 Burst discharge in primary sensory neurons: triggered by subthreshold oscillations, maintained by depolarizing afterpotentials. J Neurosci 22:1187–1198

Chen Y, Michaelis M, Janig W, Devor M 1996 Adrenoreceptor sub-type mediating sympathetic-sensory coupling in injured sensory neurons, J Neurophysiol 76:3721–3730

Drummond PD, Finch PM, Smythe GA 1991 Reflex sympathetic dystrophy: the significance of differing plasma catecholamine concentrations in affected and unaffected limbs. Brain 114:2025–2036

Schattschneider J, Wenzelburger R, Deuschl G, Baron R 2001 Kinematic analysis of the upper extremity in CRPS. In: Harden RN, Baron R, Jänig W (eds) Complex Regional Pain Syndrome, Vol 22. IASP Press, Seattle, p 119–128

Cortical pathophysiology of chronic pain

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Abstract. Studies in my laboratory have been employing multiple non-invasive brain imaging techniques to study the characteristics of patients with chronic pain. Some of these results are briefly outlined in this communication. Our studies regarding brain activity in chronic pain are summarized, emphasizing the unique role of the prefrontal cortex in chronic, especially neuropathic pain states. I also review our work examining brain chemistry abnormalities in chronic pain. Given these results, we have examined chronic pain patients in a cognitive task, designed to probe brain regions that we think are specifically abnormal in chronic pain, these results are also summarized. An overview of the mechanisms that may be pertinent to the observed results is included.

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The symposium that took place in Tsukuba, Japan, was organized to examine peripheral and central processes underlying chronic pain states. This chapter examines central pathophysiology of such conditions from the viewpoint of the human condition, and by taking into consideration cortical processes. A brief perusal of other chapters in this book should demonstrate that the largest portion of this symposium was dedicated to examining peripheral or spinal cord processes, which have been looked at in various animal models of chronic pain. Implicit in these studies is the notion that understanding and then reversing some of these processes should give rise to decreased pain-like behaviour in these animals. Thus, they are hinting at therapeutical approaches that one can then test in humans in clinical settings. This approach has been extensively tested over the last at least 15 years, i.e. since the advent of well-defined chronic pain-like behavioural models in animals (Bennett 1993). Unfortunately, outside of the example of cancer pain (see Mantyh 2004), we have yet to see a single new medication or therapeutic approach, developed specifically from data gathered from such animal models, that has had clinical impact.

One obvious question is the extent of correspondence between animal models and the human clinical chronic pain condition. This, although an important issue, will not be addressed here. A second, important assumption is the localizability of factors involved in chronic pain: meaning that the majority of the animal studies assume that mechanisms of chronic pain impact local processes be it in the periphery or the spinal cord. Many previous chapters deal with notions of peripheral vs. central sensitization, where the 'central' is localized, at least implicitly, to the spinal cord.

The studies outlined here are based on a different set of hypotheses:

- Since the extent of correspondence between human chronic pain conditions and animal models remains to be established, examining the human condition simply eliminates such ambiguities.
- Given that the brain is a highly interconnected dynamical network, and that chronic pain conditions are states that are maintained over years and even a whole lifetime, injury-induced reorganization in a given portion of the pain system must propagate throughout the network and impact the overall system.

Thus, we assume that chronic pain involves central sensitization that engages and restructures connectivity between the periphery, spinal cord, thalamus, and cortex. In fact, the data presented in this chapter point to a central reorganization at the highest level of the brain, i.e. the prefrontal cortex. The pain clinician is amply aware of the fact that chronic pain is more than increased excitability of peripheral afferents and spinal cord neurons, because chronic pain patients' behaviour is a combination of many interacting dimensions, including emotional, social, and environmental factors. In the presented studies we show that some of these factors can be objectively demonstrated.

A diversion to Tokyo

A second part of this meeting was held in Tokyo, where many Japanese pain clinicians attended the talks. For me this presented the opportunity to begin to learn how life goes on in a mega-metropolis, i.e. Tokyo. I cannot help myself but make the analogy between this city and the brain, and to compare the properties of this magnificent city to the brain in normal conditions and following acquisition of chronic pain-like behaviour.

Tokyo with its suburbs is home to more than 47 million people. A staggering size for a city and yet this would correspond to a few millimetres of cortical tissue, if we make the analogy of people to neurons. This analogy is not original and a number of physicists have suggested that understanding the self-organizational properties of a city should give profound insight to the dynamical properties of

the brain. I only spent a few days in Tokyo, so my impressions are limited and relatively superficial. Still, one cannot escape the organizational success of a city where people are bustling by — thousands in every major street intersection — and at the same time, there is limited traffic congestion. Major train stations like at Shinjuku, Ginza and Tokyo are a marvel to watch, and they are whole cities within themselves. Hundreds of trains, running on multiple platforms at the precision of seconds, transport millions of people all across the city. This enormous connectivity at a very high temporal resolution undoubtedly is a major contributor to the social and economic success of Tokyo. One can imagine the extent of havoc that failure in one of the stations, or even one of the lines, would generate locally, quickly propagating it throughout the whole city within a few hours. Alternatively, if the influx of people from a particular suburb suddenly increases by 10-fold, again the balance of the city would be immediately disrupted. If we assume, moreover, that this influx is composed of a specific minority group not welcomed by the society in general, then the reverberations would be more severe, especially if it is sustained over weeks, months, or years. The human history of treating unwanted minorities is very ugly, and yet it seems like a reasonable analogy for the brain in chronic pain. I do not want to belabour this line of thought; perhaps the reader can follow the thread between the analogy and our observations of the brain in chronic pain.

Examining the brain in chronic pain

Non-invasive brain imaging technologies provide the opportunity to directly study human clinical conditions. We have spent considerable time and effort in devising methodologies that would be applicable in studying clinical chronic pain conditions and used them to examine and compare between different chronic pain conditions. Functional magnetic resonance imaging (fMRI) and positron emission tomography (PET) have become standardized techniques with which human brain neuronal activity can be studied indirectly. A large number of studies have examined human brain activity, using fMRI or PET, to identify cortical circuitry for acute pain perception in normal subjects, see reviews by (Treede et al 2000, Bushnell et al 1999, Apkarian et al 2004a). There is now ample evidence that a well-defined cortical network participates in human perception of acute pain. The areas most consistently observed include: primary and secondary somatosensory cortices (SI and SII), anterior cingulate (ACC), insula (IC), prefrontal cortex (PFC), thalamus (Th), and cerebellum (CB). Our own studies of fMRI activity for acute pain is consistent with this pattern of brain activity (Apkarian et al 1999, Gelnar et al 1999, Apkarian et al 2000). When brain activity to acute thermal painful stimuli are examined in chronic pain patients, the resultant pattern is very similar to that seen for acute pain in normal subjects, independent of

the type of chronic pain, as seen in chronic back pain patients (Derbyshire et al 2002), and in complex regional pain syndrome (CRPS) patients (Apkarian et al 2001a). Thus, although chronic pain patients may have various cutaneous sensory abnormalities (e.g. Gracely et al 1992, Petzke et al 2003), mapping brain responses to acute pain does not distinguish them from normal subjects. On the other hand, when brain activity specifically related to the chronic pain is isolated then activity seems to be preferentially involving prefrontal cortical regions (Apkarian et al 2001a). We have preliminary data to indicate that this is also true in chronic back pain (CBP) patients (Baliki et al 2002), when brain activity in CBP patients is examined specifically in relation to the fluctuations of the subjective report of pain by the patients (Apkarian et al 2001b). To further examine the hypothesis that chronic pain preferentially involves PFC activity, in a large review article where brain imaging studies of pain were summarized over the last 15 years, a meta-analysis was performed examining the incidence of significant reports of brain activity in PFC as compared to SI, SII, IC, Th, and ACC (Apkarian et al 2004a). The results indicate that across almost 100 studies, incidence of PFC activity is significantly higher in chronic pain conditions as compared to acute pain states; the opposite is true for the other regions, namely their incidence is significantly decreased in chronic pain conditions in contrast to acute pain in normal subjects. Therefore, it seems there is now solid evidence for the idea that chronic pain conditions preferentially involve PFC. The PFC is a complicated large structure with many sub-specializations (Fuster 1997). The specific portions involved in chronic pain remains somewhat ambiguous, and thus the specific functional significance of this activity requires further studies.

The significance of preferential PFC activity needs to be commented on: the shift of activity from parietal and cingulate cortices to prefrontal regions implies that there is also a shift in perceptual characteristics in the experience of pain. The simplest explanation would be that perception has become dominated by cognitive evaluations of the condition, with decreased emphasis on its sensory properties. This interpretation is consistent with notions advanced recently regarding regional specialization of different brain regions in acute pain (Price 2000). Price (2000) subdivides pain unpleasantness to primary and secondary affective conditions, with the secondary component localized to PFC. Our fMRI data indicate that different subregions of PFC are involved in distinct chronic pain conditions, implying that they may be differentiated along the extent to which they are dominated by affective or cognitive-evaluative burdens. We think that the extent to which a given chronic pain condition may be dominantly affective or cognitive will depend on the type of chronic pain, and even on the individual patient's history of chronic pain.

The shift in brain activity pattern with chronic pain also implies reorganization of information flow in nociceptive pathways. To examine substrates of this

reorganization we turned to investigating brain biochemistry, using magnetic resonance spectroscopy (MRS). The methodology has the advantage that it can document long-term effects since instantaneous cognitive or perceptual states do not affect the measurements. In a series of MRS studies (Melzack 1987, Grachev et al 2000, 2001, 2002, Grachev 2001), we have now examined changes in brain regional chemistry in CBP patients, as compared to age- and gender-matched healthy control subjects. We observe decreased brain chemical concentrations for multiple chemicals in both dorsolateral prefrontal cortex (DLPFC) and orbital prefrontal cortex (OFC), with no detectable changes in SI-motor cortex, ACC, IC or Th. Moreover, we could demonstrate that across-region relationships between brain chemicals are disrupted in CBP, in a unique pattern in relation to pain, as compared to anxiety. We could also demonstrate that the specific dimensions of pain, as measured by the short-form McGill Pain Questionnaire (Melzack 1987), could be correlated with brain-regional chemistry changes. Thus, this directly links perceptual states of pain to brain chemistry.

N-acetyl-aspartate (NAA) was the main chemical that we observed to decrease regionally in CBP. NAA is localized mainly in neuronal cell bodies and has been observed to decrease in many neurodegenerative conditions; as a result, it is thought to be a marker for neuronal density. Therefore, we interpret the decrease in NAA as evidence for brain atrophy in these patients. Recently, we embarked on a study to compare brain grey-matter overall volume and regional grey-matter density in CBP patients as compared to age- and gender-matched normal subjects. Preliminary analysis of the data shows both global grey-matter volume decreases in CBP patients, beyond normal aging; as well as regional grey-matter density changes (Apkarian et al 2002), thus confirming the notion that the brain in chronic pain undergoes atrophy. The specific pattern of this atrophy remains to be determined.

Given the observed decreases in brain chemistry and brain grey matter and the fMRI evidence for preferential involvement of PFC in chronic pain conditions, we reasoned that cognitive tasks, especially ones that are emotionally driven, may be impaired in chronic pain patients. To this end we tested performance of CBP and CRPS patients, as compared to age-, gender-, and education-matched normal control volunteers, on an emotional decision making task (Apkarian et al 2004b). Both CBP and CRPS patients performed worse than controls on the gambling task. Moreover, gambling task outcomes in CBP were correlated with the intensity of back pain; while in CRPS patients when the pain was manipulated with sympathetic blocks (all CRPS were of sympathetically maintained type) performance could not be modulated. This cognitive deficit seems specific since CBP and CRPS patients tested normal for attention, language and short-term memory tasks. Moreover, when the gambling task was tested, in normal volunteers when one hand was immersed in painful hot water, and compared to a

second group where the hand was in warm non-painful water, there was no difference in performance between them. Thus, the deficit in gambling task performance seems unique to the chronic pain group and does not generalize to acute pain conditions.

In summary, our results indicate that cortical circuitry underlying chronic pain is distinct from that observed in acute pain, and preferentially involves OFC. In chronic back-pain patients brain chemistry is abnormal and there is a decreased cortical grey matter size, as well as decreased prefrontal cortical grey matter density. Moreover, chronic pain patients show a specific cognitive deficit which is consistent with the brain activity observed in such patients and with the observed chemical and morphological abnormalities as well. We, therefore, conclude that chronic pain is reflected at the cortical level, and is associated with cortical reorganization and perhaps even neurodegeneration.

References

Apkarian AV, Darbar A, Krauss BR, Gelnar PA, Szeverenyi NM 1999 Differentiating cortical areas related to pain perception from stimulus identification: temporal analysis of fMRI activity. J Neurophysiol 81:2956–2963

Apkarian AV, Gelnar PA, Krauss BR, Szeverenyi NM 2000 Cortical responses to thermal pain depend on stimulus size: a functional MRI study. J Neurophysiol 83:3113–3122

Apkarian AV, Thomas PS, Krauss BR, Szeverenyi NM 2001a Prefrontal cortical hyperactivity in patients with sympathetically mediated chronic pain. Neurosci Lett 311:193–197

Apkarian AV, Krauss BR, Fredrickson BE, Szeverenyi NM 2001b Imaging the pain of low back pain: functional magnetic resonance imaging in combination with monitoring subjective pain perception allows the study of clinical pain states. Neurosci Lett 299:57–60

Apkarian AV, Sosa Y, Krauss BR et al 2002 Cognitive deficits in chronic pain patients: impaired ability in decision making. Society for Neuroscience, Washington (abstr on *www.sfn.org*)

Apkarian AV, Bushnell MC, Treede RD, Zubieta JK 2004a Human brain mechanisms of pain perception and regulation in health and disease. Eur J Pain. Submitted

Apkarian AV, Sosa Y, Krauss BR et al 2004b Chronic pain patients are impaired on an emotional decision-making task. Pain 108:129–136

Baliki M, Sosa Y, Parrish TB, Harden RN et al 2002 Chronic back pain (CBP) is an orbitofrontal condition: an fMRI study of ongoing chronic pain. Society for Neuroscience, Washington (abstr on *www.sfn.org*)

Bennett GJ 1993 An animal model of neuropathic pain: a review. Muscle Nerve 16:1040–1048

Bushnell MC, Duncan GH, Hofbauer RK, Ha B, Chen JI, Carrier B 1999 Pain perception: is there a role for primary somatosensory cortex? Proc Natl Acad Sci USA 96:7705–7709

Derbyshire SW, Jones AK, Creed F et al 2002 Cerebral responses to noxious thermal stimulation in chronic low back pain patients and normal controls. Neuroimage 16:158–168

Fuster JM 1997 The prefrontal cortex. Anatomy, physiology, and neuropsychology of the frontal lobe. Lippincott-Raven, Philadelphia

Gelnar PA, Krauss BR, Sheehe PR, Szeverenyi NM, Apkarian AV 1999 A comparative fMRI study of cortical representations for thermal painful, vibrotactile, and motor performance tasks. Neuroimage 10:460–482

Gracely RH, Lynch SA, Bennett GJ 1992 Painful neuropathy: altered central processing maintained dynamically by peripheral input. Pain 51:175–194

Grachev ID 2001 Spectroscopic brain mapping the N-acetyl aspartate to cognitive-perceptual states in chronic pain. Mol Psychiatry 6:124
Grachev ID, Fredrickson BE, Apkarian AV 2000 Abnormal brain chemistry in chronic back pain: an in vivo proton magnetic resonance spectroscopy study. Pain 89:7–18
Grachev ID, Fredickson BE, Apkarian AV 2001 Dissociating anxiety from pain: mapping the neuronal marker N-acetyl aspartate to perception distinguishes closely interrelated characteristics of chronic pain. Mol Psychiatry 6:256–258
Grachev ID, Fredrickson BE, Apkarian AV 2002 Brain chemistry reflects dual states of pain and anxiety in chronic low back pain. J Neural Transm 109:1309–1334
Mantyh P 2004 A mechanism based understanding of bone cancer pain. In: Pathological pain: from molecular to clinical aspects (Novartis Found Symp 261). Wiley, Chichester, p 194–219
Melzack R 1987 The short-form McGill Pain Questionnaire. Pain 30:191–197
Petzke F, Clauw DJ, Ambrose K, Khine A, Gracely RH 2003 Increased pain sensitivity in fibromyalgia: effects of stimulus type and mode of presentation. Pain 105:403–413
Price DD 2000 Psychological and neural mechanisms of the affective dimension of pain. Science 288:1769–1772
Treede RD, Apkarian AV, Bromm B, Greenspan JD, Lenz FA 2000 Cortical representation of pain: functional characterization of nociceptive areas near the lateral sulcus. Pain 87: 113–119

DISCUSSION

Mao: Can you tell for sure the area which lights up by fMRI actually represents neuronal excitation or inhibition?

Apkarian: This is a tough question that has been debated for a long time but I think the data nowadays are quite solid. A series of studies have shown that excitability of neurons is highly correlated with blood flow changes. Still, this is an indirect method. In the cortex at least, there is very little question that when there is decreased blood flow this represents inhibition and when there is increased flow this represents activation.

Mao: One of the issues that have been debated is whether neural inhibition also involves an energy requirement. It does.

Apkarian: But the overall net energy is the crucial factor, and at the cortical level the data are solid in that for the most part excitatory synaptic activity is the main drive for fMRI signal. Having said that, it should be added that proportionality between increases and decreases in neuronal activity and fMRI signal tend to be complicated and not linear except for a small range of stimulus intensities and durations.

Dray: I have a question relating to measuring the activity of areas that can induce neural inhibition. Have you ever asked a patient to imagine that they have less pain, so that areas that inhibit pain perception are highlighted? Would they be the same or different areas that light up when intense pain is felt?

Apkarian: In a sense this method includes these data. One could simply look at the opposite. I showed the map for activity when the pain is high versus when the

pain is low. If we do the opposite subtraction then we should observe brain regions that are inhibited when the pain is high. We have earlier reported on such regions in acute pain experiments (Apkarian et al 2000), where we report many regional decreases in fMRI signal. In the current study of chronic back pain we do not observe any significant decreased regional activity for the high-low comparison. I should add that the statistical analyses of such data are complicated and we constantly change, and hopefully improve, these methods. Thus I cannot rule out that the differences between our earlier results and the current are not based on technical differences. I prefer to think of these differences as reflecting distinct circuitry for chronic vs. acute pain. This data set is in patients with quite bad pain. If we do reverse the vectors we don't see anything activated. If we ask the question when the pain is low and compare this with when the pain is high, we don't see anything in the brain, whatever this means. On the other hand if we ask the question as to which brain areas are active when the pain perception transitions from a high to a low level, we get a complicated result that approximates the negative of the regions involved with high vs. low pain. Essentially implying that decreases in pain perception are probably involving similar but not exactly the same brain activity as for increases in pain perception.

Dray: Can such fMRI studies be done in animal models?

Apkarian: Of course.

Dray: You have highlighted an important role of prefrontal cortical areas: is there an equivalent in rodents?

Apkarian: Yes, I think so. In fact, we have an ongoing rodent study examining the role of the prefrontal cortex. We are showing similar effects in neuropathic pain models. When we do lesions in the prefrontal cortex in rodents we see changes in the neuropathic behaviour. By and large we are ignorant about the prefrontal cortex in rodents, but this doesn't mean that it does not have a functional equivalence to that observed in humans.

Perl: What is the resolution of the fMRI technique?

Apkarian: In human fMRI studies the usual voxel size that we currently use is around 3 mm^3. The actual spatial resolution would be three-to-four times larger than this value.

Perl: This doesn't give much detail over a rodent brain.

Apkarian: For rodents voxel size can be decreased to about 0.2 mm^3, which in turn results in a spatial resolution of three-to-four times this value. In rodents one can gain resolution by increasing scan time, especially since such experiments will most likely require scanning under anaesthesia.

McMahon: In your chronic pain patients, how certain can you be that a difference in brain activity when they are in a lot of pain versus less pain is really a measure of brain areas associated with pain processing? Might it be confounded by other

concurrent changes? Have you any ways of controlling for the non-painful things that co-vary with pain?

Apkarian: This is hard to do. Perhaps what we are seeing is not so much the pain, but the suffering of the pain. The regions that we are looking at may have to do with emotional evaluation, and specifically negative emotional evaluation. All this is saying is that this is the significant component of the pain that the patients have, as opposed to what the pain is. It is more like the 'flavour' of the pain that they have continuously. The fMRI scans that we do routinely include a variety of control scans that we use to subtract from the brain activity motoric responses, cognitive evaluative responses, as well as non-specific spurious correlations. Only after correcting for all of these can we make the claim that the remaining activity has a good chance of being related to the ongoing pain.

McMahon: The findings would perhaps be less interesting if patients were simply trying to move their leg to minimize their pain, for instance.

Apkarian: Fortunately those areas of the brain are not activated.

Devor: I think it is important to pursue the theme that Steve McMahon has just raised. You began with the statement, or at least the implication, that the cortex is the organ of pain. To challenge this would be like going to the Vatican to say that God doesn't exist. And yet there are difficulties. An implication of what you are saying is that the neuroactivity you showed us is directly related to the percept of pain. This seems logical and the correlates all fit. Who would challenge this conclusion? Yet, since the 1950s we have had the extensive work of Penfield and colleagues (Penfield & Rasmussen 1955) who electrically activated surface areas of cortex and only extremely rarely found any area where stimulation produced a percept of pain, including areas like S1 that routinely show 'activation' in modern imaging studies. This is not true of other senses: vision, smell and somatosensory percepts are readily evoked by cortical stimulation. Pain is not evoked, or rarely evoked. You could say that these authors only had access to the cortical surface and not deeper areas such as the medial temporal lobe and the insula. However, seizures happen there often, and it is extremely rare for seizures to be painful. I am thinking in the same general direction as Steve McMahon. Is it possible that what you are looking at is the cortex monitoring pain percepts that are happening in the brainstem or the cerebellum; and that the cortex is using this information to decide what to do next, but the actual activity you are seeing in the cortex is not the neural substrate of a pain percept?

Apkarian: If we go back to the early clinical studies that dispute the presence of pain in the cortex, there are also a number of opposite data sets. Whether they balance each other, I am not sure, but one could argue both ways. On the other hand, we now have fairly decent anatomical and physiological data from the cortex in animals, which demonstrate the presence of nociceptive neurons.

Devor: I don't deny that these cells are responding or changing their activity in response to a painful stimulus. This doesn't mean that their activity is generating a pain sensation.

Apkarian: Now you want me to discuss where the perception is happening; whether it is in or below the cortex. I don't know. The cortex has something to do with it. If there was something downstream that was the primary region of these events we should see it. Why isn't it there? Unfortunately, all current electrophysiological and brain imaging methods remain correlative in nature and there is no understanding of how perception comes about for any sensory modality, including pain.

Devor: Many subcortical modules show nice correlates to pain.

Apkarian: So do all of these cortical units.

Devor: But you prefer cortex to the cerebellum.

Apkarian: The data are clear. One can show intensity-dependent changes and unpleasantness-dependent changes. We don't have a way of knowing where consciousness is happening: we can only correlate simple parameters of perception to brain activity. With these tools it seems to work.

Devor: I've given you an alternative way of thinking about the correlation: it has nothing to do with perception, it only has to do with future planning, while the percept is happening in the brainstem or elsewhere. I am not disputing that the cortex is 'involved' in pain. I am just saying that the nature of its involvement might be quite different from what you are implying.

Perl: One must consider the human data on discrete cerebral cortical lesions. Certain cortical lesions are reported to abolish the ability to recognize pain selectively in parts of the body. Those who doubt the influence of the cerebral cortex on pain perception should carefully read John Marshall's 1951 article (Marshall 1951) reporting an analysis of missile wound injuries to the cortex that occurred in World War II. The cerebral cortex has something very particular to do with the recognition of painful stimuli and distinguishing them from other somatosensory events.

Devor: Cortical lesions have not been very useful in terms of clinical treatment for chronic pain.

Apkarian: Let me turn that upside down. We all agree that pain has many dimensions to it, and it has strong social, psychological and environmental influences. All of these are an integration of multiple functions. One assumes that they have to be integrated at a high level. The cortex has to be involved.

Devor: One could argue the same for the cerebellum.

Apkarian: There is no evidence anywhere for any perception at the level of the cerebellum.

Devor: There is plenty of evidence for the upper brainstem.

Apkarian: Now there are reports of deep insula cortex stimulation giving rise to pain perception.

Dray: I didn't think you were saying that the cortex can be singled out for being involved in pain signalling. I thought you were saying that there were several simultaneous regions that are either inhibited or activated, and the cortex is just one of them.

Devor: All the areas presented were in the cortex.

Apkarian: No. Sorry, I believe in the integration of information throughout the CNS. Saying perception happens in the cortex versus the cerebellum tends to diminish the network as a system within which perception happens. I am not going to deny the role of nociceptive neurons in the spinal cord. At the same time, the cortex has to be involved. The simple fact that the spinal cord neurons project, through a couple of synapses, to the cortex implies to me that changes in excitability of spinal cord cells has to be reflected also at the cortical level, and as such the perception of pain has to be the coordinated activity across the whole network, including spinal cord, brainstem, thalamus, and cortex. I have not seen any evidence that refutes this position.

Devor: Stimulation of the spinal cord does evoke pain, as does stimulation of the thalamus and many places in the brain stem. In the cortex it doesn't, basically.

Perl: That's not true. Stimulation of the cortex has been reported to cause pain. The areas involved are mainly hidden in sulci or folds.

Apkarian: The insular cortex does this beautifully.

Devor: There are very few cases reported. This is an area that is prominently involved in seizures. Seizures are common and very rarely painful.

Apkarian: Some of them are painful and the ones that are painful are in the right regions in the cortex.

Devor: I'm not sure we should base a whole theory of pain on these rare instances when most activity in the cortex does not evoke pain.

Apkarian: I think the new technology gives us a handle to move on, rather than being stuck with methodologies that were inadequate for the most part.

Baron: I was confused about your data that different chronic pain states have completely different networks in the brain.

Apkarian: It is not so much that they are very different but that they seem to be distinct subsets. The neuropathic patients seem to have very similar brain regions activated but the specific pattern seems different for complex regional pain syndrome patients as compared to chronic back pain patients, implying that the affective and cognitive perceptual properties of the pain may be unique for different chronic pain conditions.

Gintzler: How do you subtract out the element of distraction? When you are in chronic pain you are obviously going to be distracted: how do you eliminate this?

Apkarian: I can't. The only control for this is to test the attentional abilities of such patients, which we do. When we actually test for attentional abilities, these patients don't show any abnormality. On the other hand these patients are distracted specifically with their ongoing pain, and in our fMRI studies we ask them to ignore everything else and only evaluate the fluctuations of this pain. Thus, we are taking advantage of their distraction with their pain.

Gintzler: How do you assess attention?

Apkarian: It is a separate standard cognitive test looking for attentional abnormalities.

Dray: Do you have a hypothesis to account for the apparent loss of grey matter that you have measured?

Apkarian: Yes. There is a beautiful paper recently published from Dr Casey's lab (Lorenz et al 2003) which tried to subdivide the prefrontal cortical regions in a capsaicin thermal hyperalgesia test. It shows that there is antagonistic activity between the dorsolateral prefrontal cortex (DLPFC) and the orbitofrontal cortex (OFC). It seems like the DLPFC is trying to control OFC activity, in essence trying to limit the amount of suffering. This would be exactly the kind of brain area that would be most stressed in a chronic pain patient where pain could not be controlled. In a sense the hypothesis would be that these are overstressed, over-active neurons that are slowly dying because of over-activity, given their functional role in pain perception control. Alternatively, if such patients already have a smaller DLPFC then they would be predisposed to develop chronic pain because of their inability to control OFC activity.

Perl: Another possibility is fluid accumulation in association with an interaction between active neurons and glial cells.

Apkarian: I haven't said what the specific mechanism for the atrophy is. There are many candidates. Is there a functional reason for it? This is my explanation for the specific site.

Mantyh: A similar argument has been made for depression. The idea was that a selective serotonin reuptake inhibitor (SSRI) or another agent that could alleviate the depression would reduce the atrophy. Have you looked at this also? In pain patients in which the pain is reduced, do you see a reduction in atrophy?

Apkarian: No, but we will. This is the first study that we have done in the subject. In a sense we would like to do a longitudinal study on this, with two populations: one with proper pain management and another without. These patients I have shown essentially have no management of their chronic pain.

Mantyh: One thing about depression studies is that they have been focusing on specific areas of the brain. If they didn't focus on these, they would lose all signal. Do you think that there are specific areas of the brain that are showing greater atrophy than others?

Apkarian: Yes, the main area is DLPFC, which is very consistent with our MR spectroscopy data, which shows decreased N-acetyl-aspartate (NAA) concentration in chronic back pain patients (Grachev et al 2000). Decreased NAA has been reported in all neurodegenerative conditions, as a result it is assumed to be a marker for neuronal atrophy. This initial finding prompted us to perform the morphometric study. Our starting hypothesis for the morphometric study was that the DLPFC would be one of the main areas that shows atrophy in chronic pain. The regional atrophy that we observe in these patients is distinct from that of depression or anxiety, for example.

Mao: I have a couple of comments. First is that you used a new pain rating scale?

Apkarian: That is actually an old pain scale, initially devised by S. S. Stevens back in the 1950s. In fact, he has shown that finger-span is a natural linear scale for magnitude estimation.

Mao: My other comment is that I am concerned that the population of chronic pain patients you have used may be selective because of the monetary compensation, which is rather common in clinical studies.

Apkarian: They may be motivated to get the money, but they have no clue what we are doing to them, especially when they are strapped into a scanner. They have no idea what to do to satisfy us.

Mao: I am just saying that they are collected in this systematic manner which might bias the outcome.

Wood: Have there been any fMRI studies of patients with phantom limb pain?

Apkarian: There is a whole series of studies examining brain responses and reorganization in subjects with phantom sensations as compared to subjects with painful phantom sensations. Herta Flor and colleagues have pioneered the work in this field (Flor et al 1995). Their reported results are quite relevant. Primarily these are magnetoencephalography studies where they show primary somatosensory cortex region reorganization. They show a correlation of the extent of the reorganization with the amount of pain that these patients have. Of course, they are correlating tactile reorganization with the amount of pain, so it is a little unclear how this correlation can have a causal relationship with pain perception. Still the data clearly indicates that phantom pain is very strongly related to changes that occur in the cortex.

Zhou: The idea of top-down descending facilitation is now getting more support. In addition to descending inhibitory or analgesic systems, descending facilitation systems are believed to be important, particularly in disease conditions. We have shown that this top-down system can facilitate responses of spinal dorsal horn neurons to various sensory stimuli (Zhuo & Gebhart 1992, 1997, 2002a,b, Zhuo et al 2002). From my reading of the literature on injuries and the cingulate cortex, there are two reports of particular relevance here. One recently showed that using placebo analgesic in humans also caused aberrations

in the cortex. They had previously shown that pain activates the cingulate cortex. My interpretation would be that imaging can show activation of neurons but cannot tell whether it is a pyramidal cell or an inhibitory interneuron in the cortex.

Apkarian: If placebo is a real effect and if the placebo perception is a decrease in activity in the cortical network for pain, one would expect that placebo would show the same sort of effect.

Zhou: No, pain increased the activity in imaging, and placebo which reduced the pain also showed the same increase.

Mantyh: If you compare results on your pain stimulation with your colleagues who are doing visual work, for example, are many more areas of the cortex activated in pain versus visual stimuli?

Apkarian: No, it is comparable. The only difference tends to be that the visual areas are much more contiguous with each other. In the field of visual experimentation, the science is much more sophisticated: one can look at colour discrimination versus motion detection versus face identification, and for these one can identify subsystems. This is what we have not yet been able to do in pain. What is the role of S1, S2, anterior cingulate, insular cortex or prefrontal cortex in the overall dimensions of the pain perception? As to the question of the number of areas, I think a strict comparison between sensory modalities is not very helpful. If for example one performs a brain imaging task where rest state is contrasted to a rich visual stimulus, like watching a silent colour movie, then a very large number of cortical regions will be activated. On the other hand, if the task is a contrast between objects and faces then most likely only the face region within the temporal cortex would be observed. The more specific the task the more localized and specific brain activity should be. So far, in the pain field the only comparable studies are those that have attempted to map intensity of pain with different brain regions (e.g. Coghill et al 1999).

Mantyh: When you look at the changes of blood flow for visual stimuli versus pain, are they similar?

Apkarian: Yes.

Mantyh: When I've looked at the data it has always struck me how with vision there are intense areas lit up, but with pain there are many more areas that light up and the areas are less precise. Do you think that pain is just activating many more areas of cortex?

Apkarian: It seems to be more distributed in the sense that there are more separate areas. Unfortunately, unlike vision where there are a lot of nice animal data on physiological functions to look for, we have essentially no animal data to guide us. As a result the experimental paradigms tend to be less specific in the kinds of questions we ask, which inevitably will give rise to a more distributed activity.

Dray: Why are there cognitive deficits in your patient population? Have you ever looked at the association between cognitive deficits and the intractability to pain therapy?

Apkarian: I showed you all the data I have. The only striking difference here is that between CRPS and back pain, there is a significant difference between the two populations in their cognitive performance. CRPS patients are much worse. I am not sure what this is due to. It is also important to keep in mind that the orbitofrontal cortex is an area that is tightly involved with autonomic regulation. If the task is specifically looking at orbitofrontal performance, then it is consistent with the CRPS patients doing worse on it.

Dray: Can you exclude things like previous drug histories that could have affected cognition?

Apkarian: I cannot exclude previous drug therapies in that study. I can exclude this factor in our morphometric study, where we actually quantified drug use but did not observe any significant relationship between drug use and brain atrophy. I should mention that we routinely exclude patients who are highly depressed, highly anxious, and who are on polypharmacy for their pain control. Thus, the general population of chronic pain patients that we are studying tend to be first highly chronic (which in the majority cases are people who have discovered that drugs are not helping and use them only occasionally), and second they are mostly engaged in normal lifestyle (that is, most of them work and lead a fairly routine life).

Oh: I was told that the somatosensory cortex has a column organizaton such that one column is fast adapting mechanosensory and the next column is slowly adapting mechanosensory. Is there any pain-specific column in the primary somatosensory cortex?

Apkarian: I think that Dan Kenshalo (Kenshalo et al 2000) would say that there is. When you find one nociceptive neuron in the cortex the chances of finding more are much higher.

Reeh: In the depth of the same sulcus. There are no pain-specific columns to my knowledge.

Apkarian: But once you find one neuron then finding more is likely. Dr Perl should comment on this.

Perl: The evidence is not striking. The problem, at least in part, seems to be that the nociceptive neurons in the primary cortex, somatosensory 1, are in the central sulcus. Therefore they are rarely approached by electrodes introduced from the surface. The central sulcus is deep and the nociceptive neuronal activity reported is from cells between the sensory and motor zones, not readily accessible by electrodes introduced from the surface. This is at least one explanation for the limited information about somatosensory neurons that specifically respond to noxious stimuli. Observations by Kenshalo on monkeys fit with the human

lesion data by Marshall (1951) which show that a loss of capacity to recognize painful stimuli is associated with limited lesions of the central sulcus region. Unfortunately, definitive studies require well focused studies on primates. Apparently cortical organization involves nests of cells. Whether they are columns or not is uncertain.

Devor: Returning to the cognitive matter, there is a substantial literature on cognitive deficits, and emotional deficits, in patients with chronic pain. Here, once again, the evidence suggests that if the peripheral cause of pain can be addressed and definitively removed, then the cognitive and emotional problems resolve themselves. It is not as if your pain burns out your cortex, so that you can't come back again. The one control that you offered is of normal people being submitted to an acute pain of equal intensity. But this has a very different meaning to your life than knowing that you are going to have pain for the next 20 years. I don't think that control really answers the question at all.

Apkarian: That control was only to show that this is not simply a distraction of the presence of pain. There is no way that I can come up with a control that equates the chronic pain with an acute one.

Devor: My point is that the change you are observing in the cortex isn't necessarily evidence of centralization. It may just be a reaction to a terrible life state. If we could make the pain go away, people might jump for joy.

Apkarian: That should show up as a more generalized cognitive deficit. It is not: it is a very specific deficit. The specificity gives us a clue that it is not a degenerate condition and nicely corresponds to the brain areas where we see the activity. Moreover, the evidence for brain atrophy taken together with the cognitive deficit, in fact is indicative of the likelihood that the chronic pain has burned out the brain and therapy may reinstate a happier personality but may not necessarily reinstate the ability of proper decision making.

References

Apkarian AV, Gelnar PA, Krauss BR, Szeverenyi NM 2000 Cortical responses to thermal pain depend on stimulus size: a functional MRI study. J Neurophysiol 83:3113–3122

Coghill RC, Sang CN, Maisog JM, Iadarola MJ 1999 Pain intensity processing within the human brain: a bilateral, distributed mechanism. J Neurophysiol 82:1934–1943

Flor H, Elbert T, Knecht S et al 1995 Phantom-limb pain as a perceptual correlate of cortical reorganization following arm amputation. Nature 375:482–484

Grachev ID, Fredrickson BE, Apkarian AV 2000 Abnormal brain chemistry in chronic back pain: an in vivo proton magnetic resonance spectroscopy study. Pain 89:7–18

Kenshalo DR, Iwata K, Sholas M, Thomas DA 2000 Response properties and organization of nociceptive neurons in area 1 of monkey primary somatosensory cortex. J Neurophysiol 84:719–729

Lorenz J, Minoshima S, Casey KL 2003 Keeping pain out of mind: the role of the dorsolateral prefrontal cortex in pain modulation. Brain 126:1079–1091

Marshall J 1951 Sensory disturbances in cortical wounds with special reference to pain. J Neurochem 14:187–204

Penfield W, Rasmussen T 1955 The cerebral cortex of man. Macmillan, New York

Zhuo M, Gebhart GF 1992 Characterization of descending facilitation and inhibition of spinal nociceptive transmission from the nuclei reticularis gigantocellularis and gigantocellularis pars alpha in the rat. J Neurophysiol 67:1599–1614

Zhuo M, Gebhart GF 1997 Biphasic modulation of spinal nociceptive transmission from the medullary raphe nuclei in the rat. J Neurophysiol 78:746–758

Zhuo M, Gebhart GF 2002a Modulation of noxious and non-noxious spinal mechanical transmission from the rostral medial medulla in the rat. J Neurophysiol 88:2928–2941

Zhuo M, Gebhart GF 2002b Facilitation and attenuation of a visceral nociceptive reflex from the rostroventral medulla in the rat. Gastroenterology 122:1007–1019

Zhuo M, Sengupta JN, Gebhart GF 2002 Biphasic modulation of spinal visceral nociceptive transmission from the rostroventral medial medulla in the rat. J Neurophysiol 87:2225–2236

Final discussion

Translating basic research to the clinic

Kumazawa: The discussions over the last few days extended over a very wide range of topics, so it's hard to bring everything together in a summary. However, I'd like to suggest four questions that we might focus on for our final discussion. The first is, how do we connect basic science with clinical work, and translate our research findings to the clinic? The second is what are the differences between acute pain and chronic pain? Thirdly, is there direct coupling between sympathetic and sensory systems? Finally, do Aβ fibres really sprout in the spinal cord in the pathological pain states, and if so, is this functional or structural?

Perl: I think we should focus on the first question. How do we connect basic science concepts with the clinical reality? This has been brought up as an issue but we haven't discussed it thoroughly.

McMahon: One of the things that strikes me is that we use different tests for animals and people. In our animal models, we rarely apply the sensory tests that are used in patients. Accordingly, we don't have very strong correlations between assessment of our models and assessment of the clinical cases. It seems to me there is a strong case for trying to define a common series of tests that you would apply to neuropathic pain patients and to animals.

Mao: First let me say that I really appreciate being invited to this meeting and I have learned a lot. There is a recent movie called 'Lost in Translation'. I haven't seen it, but the thought related to the title is interesting. The translation should be bi-directional in our field: ideas are lost in translation from the bench to the clinic and also the other way. By raising this issue we are recognizing that there is a need to address this issue. A meeting like this, which brings the basic scientists and clinicians together to discuss this issue, is a good beginning. The NMDA receptor mechanism is a perfect example. It has been well studied in many laboratories using quite a few animal models, yet in the clinical setting there is nothing that works effectively. There are many reasons for this. One is that we could not produce an animal model that actually represents clinical pain, because pain is a subjective experience. So we have to find a way to make the interpretation of the laboratory data closer to the clinical situation.

Devor: I'd like to make three points. First, a lot of clinical pain is spontaneous pain and it is very difficult to know about this in an animal. You can look at electrophysiological correlates, but behavioural correlates, like autotomy, always

involve some uncertainty. Second, tactile allodynia did not become obvious until people started testing rat feet using von Frey filaments. The first (classic) report on Gary Bennett's CCI (chronic constriction injury) model, for example, notes thermal but not tactile allodynia. It was the effectiveness of the von Frey hair stimulus which led to the current profusion of neuropathic pain models. The nature of the lesion is much less important. I made partial lesions of the sciatic nerve years before the current alphabet soup of models emerged, but didn't see striking tactile hypersensitivity. What I did wrong was to stimulate the way Ralf Baron does: I was touching the paw and brushing it with a paint brush. Rats don't show obvious pain to these stimuli. But they do show striking withdrawal responses to probing with a fine von Frey hair. I think we may be fooling ourselves with the belief that the animal models are really the same as the clinical states. My third point is that patients self select. Ralf Baron has a clinical centre that is expert on CRPS (chronic regional pain syndrome), and the centre is collecting from a population of probably several million people. Trigeminal neuralgia, which is one of my favourites, is rare, with a few cases per 100 000 of the population. How could we ever find a spontaneous trigeminal neuralgia in a rat? We'd have to sample a few million rats before we came up with enough cases for a research study. CRPS and trigeminal neuralgia may happen spontaneously in rats, but we may have to screen a very large number of animals before we find them. The truth is that we don't know how to make proper models of these sorts of conditions.

Baron: I think we have to improve the interaction between the clinicians and preclinical workers. We have to carefully detect symptoms and signs in our patients and communicate these problems to the scientific community. Researchers should come to the clinic sometimes and look for the assessment of stimuli in patients. I go to many conferences, and sometimes I show my videos of assessment to the researchers, so they see it first hand. Perhaps this is a first step. Then the preclinical workers have to establish models which mimic the clinical situations as far as possible to find hypotheses. Then these have to come back into the clinic so we can test whether they are relevant. Sometimes they are not. Most of the animal models we know so far have SMP (sympathetically maintained pain), but only very few patients have SMP. There is something different, and we have to find out what it is. Then we have to improve our techniques in the human setting. We have methods like microneurography available so we can record from afferent fibres. It is problematic sticking needles into damaged nerves in humans, but we could do this to test these ideas. We have to improve imaging and all these techniques.

Perl: I submit that one factor that needs to be improved is communication between the clinicians and basic scientists. As a person who has had contact with the clinic and has concentrated in basic science, I know that the difference in mindset between these areas is substantial. It is difficult for a clinical neurologist

to spend four hours examining a single patient when he has 20 patients waiting to be seen. When you talk to the clinicians about using basic science techniques their eyes roll back and they say there is no way they can do that. We need more people like Dr Baron and Dr Mao to communicate to clinicians what has been learned from basic science and can be usefully applied in the clinic.

Baron: I spend half my time doing this.

Wood: There are both positive and negative examples of animal models. For example, gabapentin works in both humans and animal models, and yet the NK1 antagonists which were so interesting in rodents have turned out not to be useful in human. Given the fact that the drug development process takes several years and costs hundreds and millions of dollars, the most important thing we can do is for the clinicians and the basic scientists to prioritize what they think the most exciting targets are at the moment, and to form a consensus view. We have heard of around 25 interesting targets over the last few days, and it is not practical for the drug companies to address all of them. If we can prioritize a list that would be helpful.

Dray: There has always been a problem with the translation of animal to human data and vice versa. Predicting the efficacy of new analgesic therapies is an enormous challenge. It may be of benefit to identify the best clinical situation to make a rapid proof of principle evaluation to guide further development and investment? There is a lobby that advocates early drug characterization in humans without reference to animal models. But I don't believe this is how we should build our new analgesia approaches. Animal models are essential for mechanistic exploration and for testing a specific hypothesis related to chronic pain. We are not at a stage where we can do this in human since our knowledge of clinical pain is extremely poor and we have a very limited toolbox. I am very impressed by the conviction of scientists like Marshall Devor, who have proposed a strong hypothesis about peripheral pain generation that can be tested with the right kind of chemical and biological tools. Molecular studies have yielded a very large but confusing array of molecular targets. Clearly we have underestimated the huge complexities of chronic pain mechanisms and we have overestimated our ability to provide timely solutions. I would submit that we need more information from specific clinical pain conditions in order to model those conditions better. We should be moving away from the idea that we can create generic models for nociceptive or neuropathic pain.

Perl: You have just defined the second problem. The one we started with was how do we get basic science information to the clinic in a way that is useful and practical. But now you are defining the question of how we get basic science information to developers of pharmaceutical agents. The same people may not or cannot carry the message to both.

Apkarian: In a sense we are in a position to be able to test the questions of animal models versus humans. We have an array of animal models and an array of clinical

conditions. We also have the technology for testing how closely they correspond to each other. To my knowledge this effort has not been seriously addressed yet.

Malmberg: An important issue that Marshall Devor brought up is that the way that we assess the pain is different. There is a big disconnect here. In humans we use pain scores, whereas in animals we look at thresholds. These are very different.

Devor: The trouble is that the animals simply do not withdraw from a firm brushstroke stimulus on the foot. So we look where the light is: the animals do respond to a von Frey hair so that is what we do. They ought to respond to a brush if this were truly a model of what Ralf is studying.

Chung: That isn't the case. They do respond to a brush.

Devor: Not my rats! Everyone in the literature uses von Frey hairs.

McMahon: We too have opted for von Frey hairs, for practical reasons. We are now trying jets of air, which seem to elicit pain in animal models.

Devor: It ought to be so obvious. If you touch these patients with a bed sheet they scream. It ought to be that obvious in the rats.

Apkarian: It is not that obvious in the human, either.

Dray: Many plastic changes have been highlighted in chronic pain models: each one represents an opportunity for drug discovery. Unfortunately, a major stumbling block has been an inability to evaluate the significance of all these molecular changes with respect to human pain and the potential for therapy.

Mao: There is a phenomenon, I think, in our pain field, if you look at what is going on in each laboratory that examines the mechanisms of central sensitization, each laboratory appears to have their own molecule of interest. Each demonstrates that if they block this molecule pain would be abolished or reduced. How could that be? Back in the 1970s, each lab had a nucleus in the brain and claimed that if you lesion or stimulate this nucleus, you can modulate pain (nociceptive transmission) and it is the important one, if not the most important one, in the world. This is why, I would guess, drug companies have had problems translating lab information into the clinical setting.

Gintzler: Everyone would agree that we have to enhance communication from basic science to the clinicians, but no one seems to have any suggestions how we go about doing this. In the USA it is becoming increasingly more burdensome for clinicians to do research. There are enormous clinical pressures. How should one go about trying to improve the communication.

Perl: I offer one person's opinion. In my view, the effective transfer of information to clinicians will be by other clinicians who have at least a foot in the laboratory, even if they don't have their whole soul there. Such individuals need to speak to their clinician colleagues. Clinicians find credible information from someone who speaks their language, knows their situation, and suffers the same problems in patient care and time. I believe this to be an effective way to bridge the gap.

Mantyh: I think what is needed is specific carved-out time, and compensating the clinicians for their efforts. For example, the National Cancer Institute has a group at Mayo called the North Central Cancer Control Group and this group is remarkably effective. If you interact with them on a regular basis you can hone your animal models so that they look more and more like the patient. Without this one is working in the dark. NCI has umbrella grants that allows basic scientists to interact with clinicians.

Devor: The specific model development that you represented is very different from the problem of neuropathic pain. All patients who have metastases growing in many places in their bones have severe pain. On the other hand, peripheral nerve injuries are very variable in terms of whether they produce pain or not. We have to face this problem of variability. It is not just a question of me sitting with Ralf and seeing some of his patients, and then being able to do the same thing in rats. Even when the same injuries occur in humans, most people don't develop pain problems.

Mantyh: Steve McMahon pointed out that there are different types of neuropathic pain, but we concentrate on one segment of it and models haven't been developed for the other types. But I think models can be developed. It takes a long time to develop the model, validate it and have others accept it. And you have to have a clinician involved in seeing those patients to say whether he thinks it works.

Devor: I don't think these models are fundamentally different. If you look at the drug treatments, all of the neuropathic pain conditions respond to the same classes of drugs, and the animal models do too.

McMahon: If they all responded brilliantly to the drugs we have, then we wouldn't be in this room. The fact that there is a problem means that we don't have a complete answer.

Devor: I am saying that post-herpetic neuralgia and amputation stump pain, which are very different clinically, would be treated with the *same* drugs.

McMahon: The point that John Wood made is that a plethora of targets have come into focus over the last decade. This is true. It probably means that there are many mechanisms that might contribute to abnormal pain states. The bad news is that people in pain may have different mechanisms participating. One approach is to generate models that closely resemble the actual disease state. Professor Mantyh has shown that elegantly, and by studying mechanisms in that model, one may come up with novel targets or solutions. Presumably it would be less easy to identify those same targets using surrogate models. This demonstration therefore is an important one for us all and suggests that we could all benefit by creating better models.

Baron: Most animal models rely on mechanical nerve lesions, which are relatively rare in the clinic. We all look in the clinic for post-herpes neuralgia and diabetic neuropathy, which is different from mechanical nerve lesions.

McMahon: There are many models of neuropathy that have been generated. Not all have been systematically analysed mechanistically for their pain contributions. But half the effort has already been done. There are models of diabetic, HIV and drug-induced neuropathy in animals.

Kumazawa: I'd like to switch the discussion to address the issue of acute versus chronic pain.

Mao: The first question we should ask about acute versus chronic pain is how we define these. Second, mechanistically we need to answer the question of whether chronic pain is simply a continuum of acute pain. This is an important question. In basic science research, we often assume that if we block the early response induced by a condition causing pain or nociception, we would be able to block the development of chronic pain. Is that really true? A lot of lab studies are based on this assumption.

Dray: Can we get a very clear definition of what people who treat pain patients would regard as acute pain? We often think of it as being mainly post-operative pain. Chronic pain is pain of six months duration or longer, and which may or may not respond to therapy.

Mao: From the clinician's perspective if the pain doesn't go away that becomes chronic pain. It appears mainly to be a chronological issue. This does not necessarily mean one should only use a defined time period to determine whether pain is acute or chronic.

Apkarian: I thought the functional definition of chronic pain was pain after the time of healing.

Mantyh: I think that is a better definition. I think the one based exclusively on chronology excludes too many individuals with chronic pain. For example, if someone has pancreatic cancer, they will have that pain until they die, which might be sooner than six months. But this is still chronic pain.

Perl: Needed is a definition that makes it clear that chronic pain involves many different syndromes and circumstances, and by using that term you are not defining mechanisms. 'Chronic' describes only the temporal course. Professor Kumazawa has suggested using 'pathological' pain as opposed to 'chronic' pain. In place of acute pain we might consider using the terms 'physiological' pain or 'normal' pain.

Baron: Whatever definition we use, the animal models are never chronic.

Zhuo: There is one thing I wanted to add about pain-related synaptic plasticity. I know there is recent progress using invertebrate models looking at nociceptive-related proteins. In the future it could be helpful to look at genes and proteins related to pain.

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Non-participating co-authors are indicated by asterisks. Entries in bold indicate papers; other entries refer to discussion contributions.

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