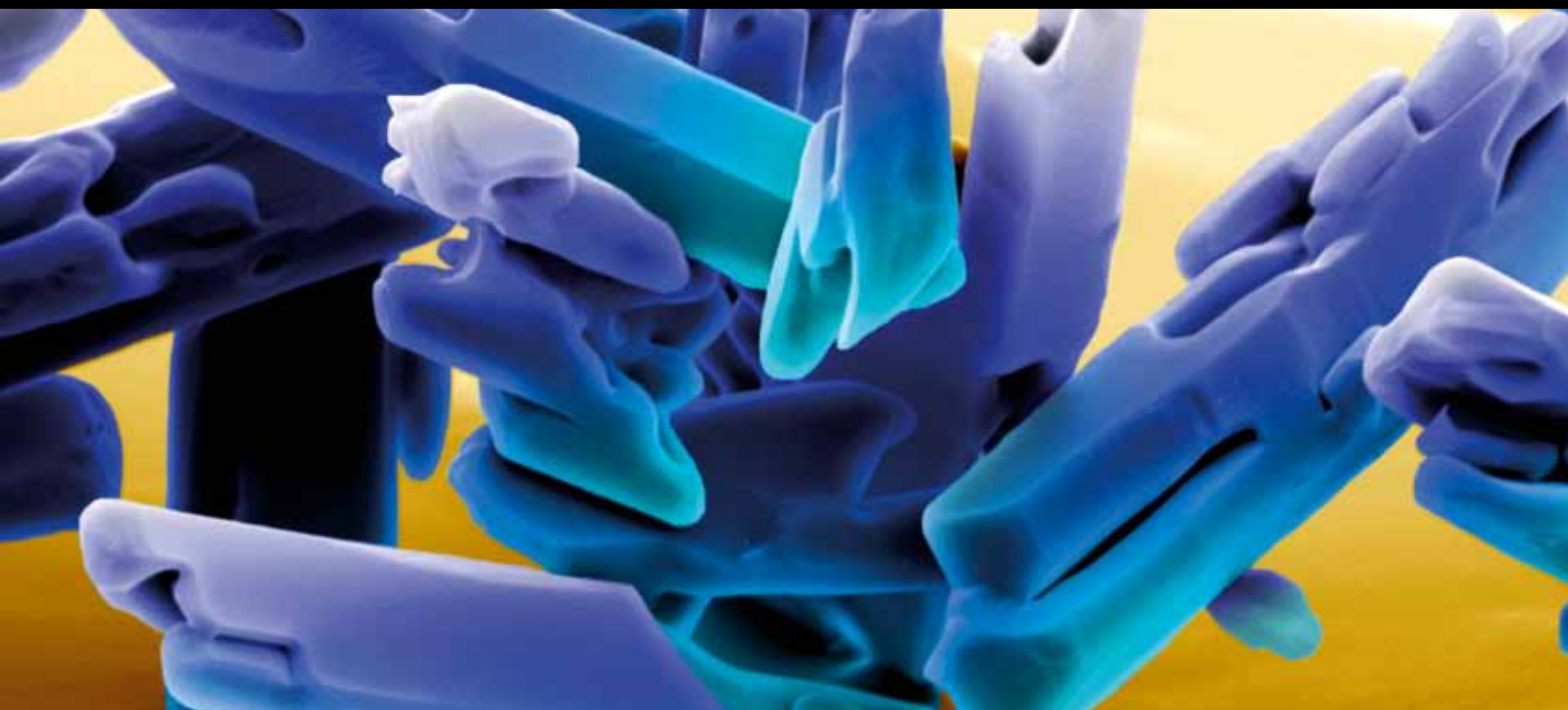# Primary Afferent Nociceptor as a Target for the Relief of Pain

Guest Editors: Carlos A. Parada, Caudia H. Tambeli, Paul G. Green, and Brian E. Cairns

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Editorial Board

Contents

Editorial

Primary Afferent Nociceptor as a Target for the Relief of Pain

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Primary afferent nociceptors (A-delta and C fibres) are generally the first structures to be involved in the perception of pain. These specialized primary afferent sensory neurons have also been described as important to the development of inflammatory and neuropathic pain. Primary afferent nociceptors are particularly interesting as a target for the development of new drugs to control pain given the fact that they are located outside the central nervous system and separated from it by the blood brain barrier. Although significant advances have been made to better understand the molecular mechanisms involved in activation of primary afferent nociceptors, important aspects of this process remain unclear. In this special issue are papers that address some of these aspects.

Dr. R. Hulse and colleagues discuss the importance of galanin and the galanin receptor 2 on primary afferent neurons as a potential target to control neuropathic pain. As described by the authors, despite the dramatic increase of galanin expression in the peripheral nervous system following nerve injury, few studies have investigated the role that this neuropeptide plays in modulating nociception at the level of the primary afferent nociceptor. It is important to highlight that the most widely used medicines to control neuropathic pain include anticonvulsants (antiseizure medications) and antidepressants and that these agents are usually used for central nervous system disorders. As a result, these therapies for neuropathic pain are associated with significant central nervous system side effects, such as drowsiness, dizziness, and cognitive deficits. Therefore, the finding of increased expression of galanin in primary afferent nociceptors could emerge as an interesting peripheral target to control neuropathic pain that might be devoid of problematic central nervous system side effects.

Dr. V. Chaban summarizes the role that estrogen plays in visceral nociception at the level of primary sensory neurons. This review paper reports that the incidence of persistent, episodic, or chronic "functional" visceral pain associated with disorders such as irritable bowel syndrome, fibromyalgia, painful bladder syndrome, chronic pelvic pain, and others is much more prevalent in women than in men. Based on this observation, the author hypothesizes that viscerovisceral cross-sensitization is mediated by estrogen modulation of the response of dorsal root ganglion neurons to adenosine triphosphate (ATP). This estrogen-mediated modulation of visceral afferent nociceptors may also explain the observed clinical and animal sex-related differences in visceral hypersensitivity.

Dr. Y. Tousignant-Laflamme and Dr. S. Marchand hypothesize that aging, independent of the hormonal status, changes pain responses in young postmenopausal women. They tested this hypothesis by examining pain responses to different experimental nociceptive stimuli in a group of young postmenopausal women and compared the results to nonmenopausal women. They undertook these experiments while the nonmenopausal women were menstruating, since sex hormone levels are at their lowest during menses. Blood sampling enabled them to assure that both groups had comparable sex hormones levels for progesterone and estrogen, the main female sex hormones. The authors concluded that age seems to be the main factor influencing changes in tonic pain perception in the group of midlife postmenopausal women. They also suggested that a reduction of the peak pain intensity in the postmenopausal women was probably due to a reduction of function in myelinated Aδ fibers that occurs naturally with age.

Dr. Y.-H. Jin and colleagues hypothesize that glutamate is released in peripheral tissues following activation of the TPRV1 receptors. This increase in peripheral glutamate then acts on glutamate receptors on primary afferent nociceptor terminals to activate nociceptors. They tested this hypothesis by measuring c-Fos expression in the spinal cord following injection of capsaicin alone (to activate TRPV1 receptors) and capsaicin with various glutamate receptor antagonists. Neuronal c-Fos expression was significantly increased by capsaicin, and this effect was antagonized when capsaicin was coadministered with glutamate receptor antagonists. Based on the effectiveness of the different glutamate antagonists used, the authors conclude that glutamate receptors are present on peripheral terminals of primary afferent nociceptors and that activation of these ionotropic glutamate receptors mediate hyperalgesia produced by activation of TRPV1.

Dr. K. E. Miller and colleagues describe findings of an increase in the enzyme glutaminase in primary afferent sensory neurons during inflammation. They hypothesize that elevated glutaminase action in primary afferent nociceptor cell bodies could produce increased glutamate synthesis in the peripheral and spinal terminal endings of these neurons. This increase in glutaminase activity may contribute to the mechanisms that underlie central sensitization and may also provide a potential target for novel therapies for the treatment of chronic pain.

The paper by M. Gautam and colleagues examines the underlying mechanism of pain in a model of long-lasting mechanical hyperalgesia that employs repeated injections of a low-pH solution into the gastrocnemius muscle at 5-day intervals. One unanswered question about this model is whether the hyperalgesia is mediated by a long-term alteration in the expression and/or response properties of acid-sensing ion channels (ASICs) on muscle nociceptors. Their paper combined whole cell patch clamp and behavioral testing to determine whether long-term changes in the properties of peripheral ASICs contribute to the mechanical hyperalgesia seen in this model. The authors conclude that ASICs are not involved in long-term maintenance of hyperalgesia in this model.

Carlos A. Parada
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Review Article

Peripheral Galanin Receptor 2 as a Target for the Modulation of Pain

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The neuropeptide galanin is widely expressed in the nervous system and has an important role in nociception. It has been shown that galanin can facilitate and inhibit nociception in a dose-dependent manner, principally through the central nervous system, with enhanced antinociceptive actions after nerve injury. However, following nerve injury, expression of galanin within the peripheral nervous system is dramatically increased up to 120-fold. Despite this striking increase in the peripheral nervous system, few studies have investigated the role that galanin plays in modulating nociception at the primary afferent nociceptor. Here, we summarise the recent work supporting the role of peripherally expressed galanin with particular reference to the dual actions of the galanin receptor 2 in neuropathic pain highlighting this as a potential target analgesic.

1. Introduction

The 29-amino-acid neuropeptide galanin was first identified in porcine intestine [1] and later in the rat central nervous system and intestine [2]. Since then galanin has been shown to play important roles in a number of physiological processes including cognition [3], feeding [4], and nociception [5]. This paper will consider activation of galanin receptors on primary afferent nociceptors as a possible target for pain treatment.

2. Galanin-Historical Perspectives and Spinal Nociceptive Processing

Galanin is expressed in many areas of the nervous system involved in somatosensation including the dorsal root ganglia (DRG) and spinal cord [6, 7], and also in other CNS regions such as the arcuate nucleus and periaqueductal grey [8, 9]. In the peripheral nervous system, low levels of galanin expression is present in the DRG of intact adult rodents, with the peptide expressed in fewer than 5% of DRG sensory neurons [10]. These galanin-expressing neurons belong to

a group of small diameter sensory afferents that respond to capsaicin [7, 11], which are characteristically C fibre nociceptors [12]. Galanin is now considered to be an injury-response peptide, as it is dramatically upregulated in DRG neurons in sciatic [6, 10] and saphenous nerve injury models [13–17]. The original observations of galanin upregulation after peripheral nerve injury strongly suggested a functional role for galanin in nociception and that these actions were through modulation of spinal nociceptive processing.

Prior to the identification and characterization of galanin receptors in the central nervous system, functional studies demonstrated that galanin could modulate spinal nociceptive reflexes. Behaviourally, intrathecal galanin administration was initially reported to have differential effects on thermal and mechanical nociception in the normal animal; thermal responses were inhibited whereas mechanical responses were enhanced [18, 19]. Numerous further studies resulted in the recognition that galanin has differential actions on spinal nociceptive processing, in that low galanin concentrations exert pronociceptive [20–23] and higher concentrations lead to antinociceptive effects [24, 25]. In contrast, in nerve-injured rodents, intrathecal galanin has

predominantly antinociceptive actions at the spinal level, acting on a greater number of neurons, and these actions are more pronounced than those seen in naïve animals [26–32].

These apparently conflicting spinal actions of galanin at different concentrations are thought to be due to the differential distributions, and/or activation of the galanin receptor subtypes. To date, three galanin G-protein-coupled receptor subtypes have been identified, galanin receptor (GalR) 1 [33], GalR2 [34], and GalR3 [35]. Galanin binding is abundant in the superficial laminae of the dorsal horn, being localized to GABA and glycine containing inhibitory interneurons [36]. The lack of specific antibodies against the galanin receptors [37] has hampered localisation attempts, but GalR1 mRNA is abundant in the superficial laminae of the dorsal horn [38, 39], in glutamatergic neurons [40]. GalR2 and GalR3 mRNA are found in a very small number of superficial dorsal horn neurones and in lamina X, and both cell numbers and intensity of expression are very low in comparison to GalR1 in the same areas [38, 41], and the type(s) of neurones on which these receptors are expressed are unknown.

Galanin has actions at both pre- and postsynaptic sites in the dorsal horn [27] (Figure 1). Galanin exerts presynaptic inhibition of neurotransmitter release, through activation of presynaptic GalR2, reducing primary afferent input into the dorsal horn [25, 42]. Activation of postsynaptic GalR1 in superficial dorsal horn laminae [38] leads to a reduction in postsynaptic neuronal excitability through activation of inward rectifying potassium currents [42]. Central sensitisation is key to the observed behavioural changes consequent to peripheral nerve injury [43], and galanin reduces central sensitisation of spinal neuronal circuits [44], particularly after nerve injury [44, 45]. There are also reports of entirely excitatory (pronociceptive) effects of galanin on wide dynamic range spinal neurons [20], possibly mediated through GalR2 [23]. Data derived from studies on intrathecal administration of the galanin fragment, Gal2-11, which is an agonist for GalR2/3, and as there is very little or no GalR3 expressed, indicates that many of the concentration-dependent effects of intrathecal galanin are exerted through GalR2, despite the relatively small numbers of spinal neurons expressing GalR2 mRNA [23].

The contributions of different GalR subtypes to spinal nociceptive processing are still unclear, and, unfortunately, the development of GalR knockout (KO) animals has not greatly clarified this area [53]. GalR1 knockout animals have only subtle differences from wild types [54], possibly as a result of altered expression of GalR2 and GalR3 in these animals [54, 55]. Intact GalR2 KO animals have no observable nociceptive phenotype, and the nociceptive responses to exogenous galanin have not been investigated in these GalR2 KO animals. Pharmacological investigation had originally suggested that GalR2 may mediate the pronociceptive effects of spinal galanin and that, after nerve, injury GalR1 may underpin the antinociceptive actions [23].

An additional layer of complexity arises from the intracellular signalling of the galanin receptors, as the second messenger cascades activated give rise to different functional outcomes. All the reported galanin receptors are known to couple to $G_{i/o}$ and inhibit adenylyl cyclase activity. GalR1 and GalR3 activation then results in neuronal hyperpolarization, as a result of increased potassium conductance. GalR2, however, can couple to $G_{i/o}$ and G_q [56]; activation of the latter G protein results in activation of the phospholipase C-protein kinase C pathway [57], which appears to be essential to the GalR2 mediated pronociceptive effect [58]. Activation of G_i and G_q proteins is fundamental to nociceptive processing, resulting in anti- and pronociceptive effects, respectively, when activated through different GPCRs, in the P2Y family [59]. This suggests that different G protein activation by GalR2 may result in pro- or antinociceptive downstream effects. In other galanin receptor systems, opposing effects can be evoked by activation of these G-proteins by the same receptor, for example, in a model of epilepsy, GalR2-G_i activation is antiepileptic and GalR2-G_q is proepileptic [60]. In addition, different agonist concentrations have also been reported to exert opposing effects in different receptor systems, for example, low concentrations of angiotensin II lead to an inhibition of vesicular neurotransmitter release whereas high concentrations potentiate exocytosis [61]. It is hypothesised that when galanin expression rises to high levels within the peripheral nervous system, such as after peripheral nerve injury, and is released into the dorsal horn [46, 62], GalR2 activation switches from a G_q- (low galanin concentration) to a $G_{i/o}$- dependent pathway (high galanin concentration), that is from a pro- to antinociceptive signalling pathway [59].

Thus, the biphasic concentration-dependent actions of galanin on spinal nociception may occur through a combination of activation of different galanin receptors (GalR1 and GalR2), expressed on different dorsal horn neurons (inhibitory or excitatory), or acting at different sites (pre- and post-synaptic), and/or through activation of different signal transduction pathways (which are concentration-dependent GalR2 activation of either $G_{i/o}$ or G_q second messenger pathways (Figure 1).

3. Direct Actions of Galanin on Primary Afferent Nociceptors

Binding and expression studies have shown that GalR1 and GalR2 are found in DRG neurons [63–65], and these receptors are functional on the central terminals of primary afferents [25, 42]. Little or no GalR3 mRNA expression is found in either the DRG or spinal cord [66, 67]. Given that proteins synthesised by DRG neurons are usually transported peripherally in addition to centrally, these observations raise the possibility that galanin may modulate the function of nociceptors by actions on their peripheral, in addition to their central terminals. Galanin expression is not limited to the nervous system but has also been identified in nonneuronal peripheral tissues such as keratinocytes, sweat glands, macrophages, and blood vessels [68]. Galanin released from such peripheral sites could therefore modulate peripheral nociceptive function, for example, keratinocytes in the skin have been demonstrated to alter primary sensory neuronal function through release of various mediators [69].
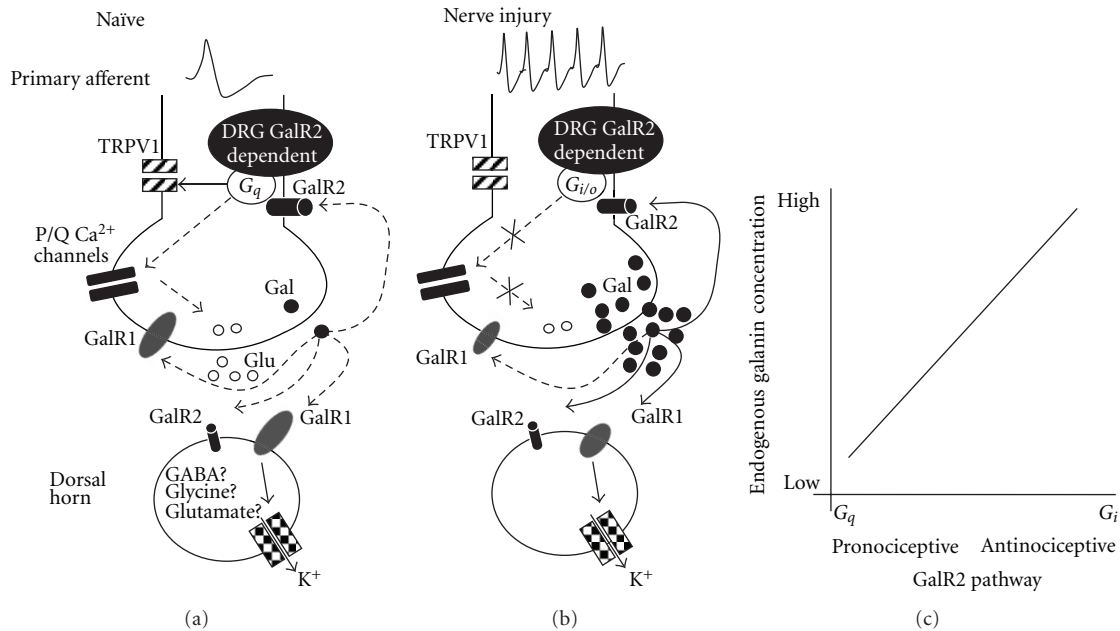
FIGURE 1: Putative galanin-mediated neuronal pro- and antinociceptive mechanisms in the dorsal horn of the spinal cord. (a) In the naïve animal, GalR1 and GalR2 are expressed on the central terminals of a large proportion of small diameter TRPV1 expressing C fibres. Galanin itself is expressed at very low levels in a small number of neurons. Peripheral activation of nociceptive C fibre afferents leads to neurotransmitter release (e.g., glutamate) at the first synapse in the superficial dorsal horn in the spinal cord, including galanin release (filled circles) [46]. In the uninjured state, galanin release is low at this synapse. Evoked galanin release or exogenous galanin is postulated to activate presynaptic GalR2 (solid arrow). This stimulates signalling through G_q in the central terminals, which then regulates both the sensitisation and expression of TRPV1 and hence afferent sensitivity [11, 47]. In addition, G_q acts on P/Q type calcium channels [11], which would serve to enhance neurotransmitter release (e.g., glutamate, open circles), enhancing excitation of postsynaptic neurons. Postsynaptic neurons express both GalR1 and GalR2. GalR1 is expressed on both excitatory (glutamatergic) and inhibitory (GABA- and glycinergic) postsynaptic neurons, and activation reduces excitability of these neurons through actions on potassium channels (checkered boxes). Postsynaptic GalR2 activation is postulated to result in the low concentration pronociceptive effects of galanin [23]. The net effect of the activation of GalR1 and GalR2 on spinal nociceptive processing will differ depending on the degree of presynaptic activation and whether excitatory or inhibitory postsynaptic neurons are affected. GalR1 is also expressed on DRG neurons, but whether presynaptic effects of galanin are also mediated through GalR1 is not yet known as there is no evidence that these receptors are functional. Dashed lines indicate minimal effects on the pathways shown. (b) After nerve injury, galanin levels are massively upregulated in DRG neurons. Up to 50% of neurons now express galanin and to a much higher level, resulting in a 120-fold increase in DRG galanin expression. There is also a small increase in galanin expression in the dorsal horn, [13] where galanin is largely found in inhibitory neurons [48, 49]. Spontaneous firing increases in primary afferents, and galanin release into the dorsal horn is increased after both nerve injury [46] and nociceptor stimulation [46]. Spinal GalR levels are only minimally altered under these conditions. Increased galanin release into the dorsal horn would increase basal activation of presynaptic GalR2, which under high galanin concentrations couples to $G_{i/o}$. TRPV1 sensitisation is therefore reduced. $G_{i/o}$ coupling also stops the activation of calcium channels thereby greatly reducing glutamate release and hence nociceptive input to the dorsal horn [25]. In addition, galanin exerts greater postsynaptic effects, effectively reducing central sensitisation [27, 44, 45]. In nerve injury, therefore, increased endogenous or exogenous galanin enhances these actions and results in antinociception. (c) (Inset) A schematic representation of the galanin concentration-dependent system. Pronociceptive actions are exerted by low-concentration galanin when GalR2 couples to G_q. This then activates the protein kinase C-phospholipase C pathway to lead to enhanced nociceptor excitability and behavioural hypersensitivity. When galanin concentrations are higher, for example, after nerve injury, GalR2 couples to G_i, reducing nociceptor excitability through inhibition of peripheral sensitisation.

Galanin enhances the excitability of TRPV1-expressing nociceptive DRG neurones both *in vitro* [11] and *in vivo* [58]. Exogenous galanin, delivered in the periphery, has been shown to modulate the properties of primary afferent nociceptors *in vivo*, in a manner similar to that seen in the spinal cord, that is, galanin exerts both facilitatory and inhibitory effects on primary afferent nociceptors [27, 51, 70]. We have shown that the opposing actions of galanin on primary afferent nociceptors are, as in the spinal cord, concentration dependent. Low concentrations of galanin sensitise primary afferent nociceptors in naïve rodents, resulting in decreased mechanical activation thresholds and increased mechanically evoked activity, whereas higher concentrations inhibit nociceptor responses. These actions are of similar magnitude in nerve-injured animals [51]. The concentration-dependent effects of galanin on primary afferents are mediated through peripheral GalR2, as both the reduction in threshold and increase in evoked activity are seen only in afferents expressing functional GalR2 [51]. Similar concentration-dependent actions of galanin have been reported in DRG neurons *in vitro*, for example, inhibition of DRG P/Q calcium channel activity through
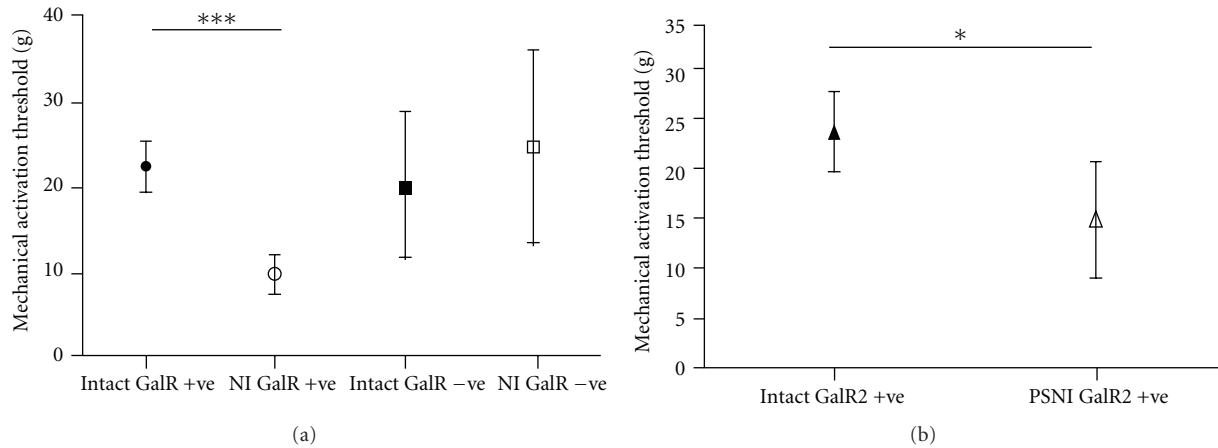
(a)

(b)

FIGURE 2: Mechanical responses of a characterised subset of C fibre nociceptor afferents expressing functional galanin receptors, in intact and PSNI-injured rats. Nociceptors were defined as those afferents with a von Frey mechanical threshold greater than 1 g [50]. Functional galanin receptors were identified in individual afferents by their response to close arterial injection of galanin and Gal2-11. Functional receptors were deemed to be present if the mechanically evoked response was increased in the afferent after galanin or Gal2-11, as at the concentrations using (close intra-arterial injection 0.1 mM) afferent responses were facilitated in both naïve and nerve injured animals [51]. (a) High threshold mechanoreceptive (nociceptive) afferents in rats with a peripheral nerve injury [14, 52] with functional galanin receptors (NI GalR+ve) had lower mechanical activation thresholds than those in uninjured rats (Intact GalR+ve). The thresholds were also lower in NI GalR+ve afferents compared to nociceptive afferents that did not express functional galanin receptors (NI and Intact GalR−ve) irrespective of whether the animals had a peripheral nerve injury or not (***$P < 0.001$, Kruskal Wallis test with Dunn's multiple comparison test, afferent number intact $n = 50$, PSNI $n = 43$). (b) Nociceptive afferents from animals with nerve injury with functional GalR2 (NI GalR2+ve) also had lower mechanical thresholds compared to those from naïve animals (Intact GalR2+ve). (*$P < 0.05$, Mann-Whitney test afferent number intact = 23, PSNI = 13).

activation of GalR2 shows similar concentration dependence [11, 71]. These findings therefore suggest that galanin receptors, specifically GalR2, in primary afferent nociceptors are possible analgesic targets *in vivo*, as nociceptor properties can be directly modulated by activation of peripheral GalR2.

4. Galanin, Galanin Receptors, and Actions on Primary Afferent Nociceptors Following Peripheral Nerve Injury

After a nerve injury, galanin expression is upregulated in the peripheral nervous system and galanin release is enhanced in the dorsal horn [46, 62]. The advent of knockout and transgenic animals has allowed further examination of the nociceptive role played by endogenous galanin after nerve injury. This includes using galanin-promoter-driven nerve-injury-induced galanin overexpression [72] and doxycycline-induced suppression of galanin overexpression [73]. Overexpression of galanin prevented the development of mechanical allodynia after nerve injury [51, 72], and allodynia was reversed on doxycycline administration and, importantly, reestablished on doxycycline withdrawal [73]. Study of galanin knockout mice surprisingly revealed a neurotrophic effect of galanin acting through GalR2, as both galanin and GalR2 knockout animals lost a specific subset of sensory neurones [66, 74]; therefore, those nociceptive phenotypes in these transgenic models cannot be interpreted. These findings indicate a substantial contribution of albeit overexpressed, endogenous galanin to spinal nociceptive processing

in nerve injury, in that increased spinal galanin release can alleviate nerve-injury-induced allodynia. Taken together, the effects of galanin overexpression indicate that physiologically, endogenous galanin exerts antinociceptive actions under conditions in which nociceptive processing is enhanced, such as peripheral nerve injury [72, 75].

Despite the evidence that galanin can affect the peripheral terminals of primary afferent nociceptors, most work has concentrated on the actions of galanin in the spinal cord, on presynaptic nociceptor terminals and on postsynaptic dorsal horn neurones. This is attributable to the key role of central sensitisation in altered pain behaviours consequent to nerve injury, and also because peripheral sensitisation has long been thought to contribute little to neuropathic pain. Recently, however, peripheral sensitisation has been described in nerve injury models, including reduction in primary afferent activation threshold and the onset of ongoing activity [50, 52, 76–78] and, importantly, in patients with neuropathic pain [79, 80]. When identified subsets of nociceptors are studied in inflammatory models, clear reductions in mechanical activation thresholds, that is, peripheral mechanical sensitisation, can be seen [50]. We have shown that functional GalR2 expression is a marker for those primary afferents that become sensitised to mechanical stimulation after nerve injury, that is, the afferents that express GalR2 after nerve injury are those that exhibit peripheral mechanical sensitisation (Figure 2). This is not to say that the action of galanin on primary afferents results in sensitisation and more that GalR2 is a possible target for identification and possible reversal of peripheral mechanical

sensitisation. Using galanin overexpressing (GalOE) mice, we tested the hypothesis that increased endogenous galanin expression after nerve injury might directly affect the properties of the peripheral nociceptors, rather than the central processing of nociceptive inputs. In GalOE animals with peripheral nerve injury, nociceptive behaviours did not change, reduction in primary afferent nociceptor threshold was not seen, and nociceptor ongoing activity did not develop, although all of these changes were seen in wild type controls [51]. Our findings, therefore, indicate that GalR expressing primary afferent nociceptors represent at least a proportion of the population of peripheral neurons that show peripheral sensitisation after nerve injury and that increased endogenous galanin can prevent the development of peripheral sensitisation.

In order for GalR2 in primary afferent nociceptors to represent an effective analgesic target, activation of the receptor must also be effective in chronic pain states, which is suggested by the results described above. The data in the naïve animal show that galanin can have facilitatory, in addition to inhibitory actions on primary afferents, depending on the concentration [27, 51, 70]. Facilitation would, of course, be detrimental in a chronic pain state. As described, the galanin system is highly plastic following peripheral nerve injury [26, 29], and, under these conditions, the inhibitory actions of both spinal [31] and peripheral galanin appear enhanced [27]. Determination of the contributions of specific GalR to peripheral sensitisation in neuropathy will be fundamental to the development of a potential peripheral GalR-targeted analgesic. Little, however, is known about GalR regulation in peripheral nociceptor terminals after nerve injury, due to the lack of specific GalR antibodies. GalR1 and GalR2 mRNAs are downregulated in DRG sensory neurons after peripheral nerve axotomy [66, 81], although the decrease in GalR2 is less profound [82].

Functionally, behavioural studies in GalR knockout mice do not give specific information on receptor function in peripheral nociceptors. Using methods that specifically study peripheral afferents, we have shown that peripheral activation of GalR2 modulates C fibre nociceptor function in nerve injured rats and shows a concentration dependence that is similar to that seen in naïve animals [51]. This is in contrast to the previously reported enhancement of the antinociceptive effect of galanin at the spinal level in neuropathic pain [27, 29]. While these findings might suggest that peripheral GalR may not represent good analgesic targets, we suggest that in pathological conditions when endogenous galanin levels would be dramatically increased, further GalR activation would be hypothesised to be more likely to drive GalR-$G_{i/o}$- mediated signalling and would therefore result in anti-nociceptive actions.

This paper highlights peripheral GalR2 as a potential peripheral analgesic target. GalR2 activation by high concentration galanin inhibits primary afferent nociceptor activity and thereby reduces nociceptive input to the spinal cord. Once specific GalR2 pharmacological tools with favourable characteristics such as long *in vivo* half-lives are developed, peripheral antinociceptive GalR2-mediated mechanisms can be fully characterized. GalR2 may represent a therapeutic target that may be effective for the alleviation of neuropathic pain.

Authors' Contribution

R. P. Hulse, L. F. Donaldson, and D. Wynick wrote the paper.

Acknowledgment

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Research Article

Glutaminase Immunoreactivity and Enzyme Activity Is Increased in the Rat Dorsal Root Ganglion Following Peripheral Inflammation

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Following inflammation, primary sensory neurons in the dorsal root ganglion (DRG) alter the production of several proteins. Most DRG neurons are glutamatergic, using glutaminase as the enzyme for glutamate production, but little is known about glutaminase following inflammation. In the present study, adjuvant-induced arthritis (AIA) was produced in rats with complete Freund's adjuvant into the hindpaw. At 7 days of AIA, DRG were examined with glutaminase immunohistochemistry, Western blot immunoreactivity, and enzyme activity. Image analysis revealed that glutaminase was elevated most in small-sized neurons (21%) ($P < 0.05$). Western blot analysis revealed a 19% increase ($P < 0.05$) in total glutaminase and 21% in mitochondrial glutaminase ($P < 0.05$). Glutaminase enzyme activity was elevated 29% ($P < 0.001$) from 2.20 to 2.83 moles/kg/hr. Elevated glutaminase in primary sensory neurons could lead to increased glutamate production in spinal primary afferent terminals contributing to central sensitization or in the peripheral process contributing to peripheral sensitization.

1. Introduction

Several animal models of tonic pain, for example, subcutaneous and intraarticular injections of inflammatory agents such as complete Freund's adjuvant (CFA), are used to mimic human chronic pain [1]. During the acute phase of inflammation, bradykinin, serotonin, prostaglandins, ATP, H^+, and glutamate activate and/or sensitize the afferent limb of primary sensory neurons by increasing spontaneous activity, lowering activation threshold, and increasing or prolonging firing to stimuli [2, 3]. Sensory neurons respond chronically to inflammation by increasing neurotransmitter/neuromodulator, for example, tachykinin (substance P (SP)) and calcitonin gene-related peptide (CGRP), expression and content in dorsal root ganglia (DRG) [4–6], and

enhanced immunoreactivity in the spinal dorsal horn [7], skin, and joints [8, 9]. These peptidergic neurons also are glutamatergic [10, 11], using glutaminase (GLS) as the synthetic enzyme for neurotransmitter glutamate production [3, 12]. Despite data regarding functional, morphological, and neuropeptide alterations in sensory neurons, little is known about long-term regulation of glutamate production in tonic pain models.

Acutely, glutamate is released from central primary afferent terminals following noxious stimulation [13–16]. Acute glutamate release, along with SP and CGRP, is responsible for sensitization of spinal neurons leading to persistent or chronic changes [2]. After the induction of knee joint inflammation in monkeys, glutamate-immunoreactive fibers in the spinal cord increase 30% at 4 hr and nearly 40% at

8 hr [17]. At 24 hrs, extracellular levels of spinal glutamate in rats are 150% above controls [15] indicating a possible prolonged, activity-dependent recruitment of glutamate release from central primary afferents. These studies suggest that glutamate production and release is modified during painful conditions.

Alteration in glutamate production at these acute and intermediate time points most likely represents modification in flux control and/or local modifications of glutamine cycle enzymes, such as GLS [18, 19]. Longer-term evaluations of glutamate metabolism have not been performed in tonic pain models as for neuropeptides in DRG neurons. Based on previous glutamate studies and evaluations of neuropeptide production, we hypothesized that persistent inflammation would cause DRG neurons to increase glutaminase production. The present study, therefore, was to examine glutaminase immunoreactivity and enzyme activity in the rat DRG at seven days after adjuvant-induced arthritis (AIA).

2. Materials and Methods

2.1. Adjuvant-Induced Arthritis. Adult Sprague Dawley male rats, 250–350 g, were used in this study ($n = 36$). Adjuvant-induced arthritis was caused in the right hindpaw ($n = 20$) by the intraplantar injection of $150\,\mu$L of complete Freund's adjuvant (CFA; Mycobacterium butyricum, Sigma) emulsified in saline ($1:1$) [20]. Controls ($n = 16$) were naïve rats that received no injection. Procedures in this study were conducted according to guidelines from the International Association for the Study of Pain [21] and the National Institutes of Health publication number 80-23 and were approved by the University of Oklahoma Health Sciences Center and Oklahoma State University Center for Health Science Institutional Animal Care and Use Committees. Efforts were made to minimize the number of animals used for this study.

The L_4 DRG was examined for the following reason. The tibial nerve innervates the majority of the plantar surface of the rat hindpaw [22, 23]. Approximately 99% of tibial DRG neuronal perikarya of rats are located in the L_4-L_5 DRG and the L_4 DRG contains more than twice the number than L_5 [22, 24].

2.2. Behavioral Testing. Two days prior to and for the days following AIA, rats were tested for pressure sensitivity with von Frey hairs (Semmes-Weinstein monofilaments; Stoelting, Inc.). Rats acclimated for five minutes in a plastic box ($25 \times 25 \times 25$ cm) with 6 mm holes spaced every 6 mm [25]. Monofilaments calibrated for specific forces were inserted through the holes underneath to probe the plantar surface of the hindpaw, 5 times in 3-4-seconds intervals in different places on the plantar surface. Filaments with light force were used first, followed by filaments of increasing force. A filament slowly was applied perpendicularly to the plantar surface until bending of the filament occurred. If the paw did not retract three out of five times, the next larger filament was used. The threshold force was defined as the filament (force) that caused the foot retraction without bending the monofilament three out of five times. Using

a conversion table for the filaments (Stoelting), thresholds were reported as gram force.

Thermal latencies for the footpaw plantar surface were determined with Plantar Test apparatus (Ugo Basile, Comerio, Italy) at an intensity of 55 mW/cm^2. Rats were placed on an elevated glass plate (3 mm) in clear plastic boxes with air holes in the lids and acclimated for 10 min. Radiant heat was applied to the plantar surface of the hindpaw and the withdrawal latency recorded. A second test was followed after 5 min. All behavioral testing occurred at 21-22°C with indirect lighting in the testing room.

2.3. Glutaminase Immunohistochemistry. For immunohistochemical localization of GLS, rats ($n = 6$ AIA 7-day; $n = 4$ control) were anesthetized with sodium pentobarbital (90 gm/kg) and transcardially perfused with fixative: 0.2% paraformaldehyde (PFA), 70% (v/v) picric acid (PA) in 0.1 M phosphate buffer, pH 7.4 [26]. Initial immunohistochemical studies had indicated that only small-diameter DRG neurons were GLS immunoreactive (IR) [27], but subsequent studies have determined that high concentrations of paraformaldehyde mask antigenic sites on GLS and decrease GLS immunoreactivity [11, 12, 26]. The fixative used in the current study provides a more accurate immunohistochemical staining pattern with all DRG neurons exhibiting GLS immunoreactivity [26]. Right L_4 DRG were removed and placed overnight in fixative at 4°C; the PFA concentration was increased to 2% for postfixation. DRGs were transferred to 20% sucrose in 0.1 M Sorenson's phosphate buffer, pH 7.4 for 24–96 hr at 4°C. The tissue was frozen, sectioned at 20 μm in a cryostat, thaw mounted onto gelatin-coated slides, and dried for 1 hr at 37°C. Every fifth section was used to reduce the possibility of evaluating a neuron twice. Sections were washed three times for 10 min in phosphate buffered saline (PBS) and incubated in 10% normal goat serum, 10% normal horse serum, 10% fetal bovine serum, 2% BSA, and 1% polyvinylpyrrolidone in PBS with 0.3% Triton (PBS-T).

Sections were incubated for 48 hrs at 4°C in rabbit antiglutaminase ($1:6000$; gift from Dr. N. Curthoys, Colorado State University, Fort Collins, Colo) in PBS-T. The tissue was washed three times in PBS and incubated in biotinylated goat anti-rabbit IgG secondary antibody (3 μg/mL; Vector) in PBS-T for 1 hr at room temperature. Sections were washed two times in PBS following secondary antibody incubation, washed in sodium carbonate buffered saline (SCBS), pH 8.5, incubated in fluorescein-avidin (1.5 μg/mL; Vector) in SCBS for 1 hr, and washed three times in PBS. Coverslips were apposed with Vectashield mounting media (Vector) to retard fading of immunofluorescence. Glutaminase purified from rat brain was incubated with rabbit antiglutaminase for an absorption control. Tissue sections incubated with absorbed primary antibody were processed as described above. Other controls included exclusion of primary and secondary antisera.

Immunofluorescent sections of 7 day AIA and control DRG were observed with an Olympus Provis AX70 microscope with a 20x objective and digital images were obtained with a SPOT CCD camera (Diagnostic Instruments). The entire section of DRG was photographed in a series of images

and images were saved as uncompressed TIFF files. The exposure time for all images was the same for all tissue sections from all animals. The exposure time was determined empirically so that weakly stained neurons could be distinguished for tracing, but that intensely stained neurons were not oversaturated [26, 28]. This approach allowed images to be evaluated along the linear aspect of immunofluorescence intensity [28]. The glutaminase-immunoreactive DRG images were analyzed using the SCION Image program (Scion Co., Frederick, Md). Only DRG neurons with a nucleus were evaluated. Individual DRG neurons were circumscribed and the area, pixel number, and intensity were recorded. Neuronal cell bodies in the DRG were distributed into the following three sizes for analysis: 100–600 μm^2 (small), 600–1200 μm^2 (medium), and >1200 μm^2 (large) [29].

2.4. Glutaminase Enzyme Assay. For GLS enzyme assays, rats ($n = 6$ AIA; $n = 4$ control) were anesthetized (sodium pentobarbital, 90 mg/kg) and decapitated. Right L$_4$ DRG were removed quickly, placed in embedding molds with M-1 mounting media (Lipshaw), and frozen on dry ice. Individual DRGs were sectioned at $-20°$C on a cryostat at 30 μm, sections were placed in aluminum racks for lyophilization, and samples were stored under vacuum at $-20°$C. The embedding media was removed from around the lyophilized DRG sections using a Wild Heerbrugg 181300 dissecting microscope and DRG sections were weighed using quartz-fiber balances [30–32].

GLS enzyme assay was performed according to Curthoys and Lowry [33]. Five to six randomly selected DRG sections from rats with AIA and from control rats were placed individually in a 40 μL volume of reaction mixture containing 20 mM glutamine, 100 mM K$_2$HPO$_4$, 0.6 mM EDTA, 0.01% Triton-X 100, 0.01% BSA in 50 mM TRIS, pH 8.65, for 45 minutes at 37°C. The reaction was stopped by adding 20 μL of 0.7 N HCl and placing the samples at 4°C. Indicator buffer (1 mL) containing 300 μM ADP, 360 μM NAD, 50 μg/mL glutamate dehydrogenase (GDH, rat liver, Boehringer Mannheim, Indianapolis, Ind) in 50 mM TRIS, pH 8.5 was added for 20 minutes, r.t. In this reaction, glutamate produced by GLS is converted to 2-oxoglutarate via GDH with the formation of NADH. Reduction of NAD$^+$ was measured using a fluorometer (Farrand Inc.) with an excitation wavelength of 365 nm and emission at 340 nm. Quantitation was accomplished by reacting increasing concentrations of glutamate standards in the indication reaction. The GLS activity from each DRG section was determined and a mean activity for each DRG was calculated.

2.5. Western Blot for Total Glutaminase. For immunoblotting, rats ($n = 5$, AIA, control) were killed with CO$_2$ and decapitated. Right L$_4$ DRG were removed rapidly and homogenized [34, 35]. Whole brain, spinal cord and kidney also were obtained for evaluation. DRG were homogenized individually with lysis buffer (50 mM Tris pH 7.4, 2 mM EDTA, 0.05% Triton-X 100) with phosphatase inhibitor cocktail I and II and protease inhibitor (Sigma). Homogenates were

centrifuged (70,000 rpm, 20 minutes) and the protein concentration of the supernatant was measured (BCA Protein Assay Kit, Pierce, Rockford, Ill) to normalize the samples. Rabbit anti-GLS antibody (gift from Dr. N. Curthoys) was bound to M-280 Dynabeads (Invitrogen) conjugated with sheep anti-rabbit antibody [34]. Equal amounts of total protein (75 mg/mL) were exposed to rabbit anti-GLS antibody beads (16 hr, 4°C) for GLS purification [35]. Samples were exposed to a magnet to collect the bead antibody-protein complex. The purified protein was eluted using Laemmli buffer (10 mM Tris, 1 mM EDTA, 2.5% SDS, 5% β-mercaptoethanol, 5% bromophenol blue, pH 8.0) and by heating the samples at 100°C for three minutes.

GLS electrophoresis was performed on a 12.5% homogenous polyacrylamide gel (Phast-System, Promega) [34, 35] along with molecular weight standards (Novagen). Proteins were transferred to a nitrocellulose membrane in a buffer of 25 mM Tris, pH 8.0, 192 mM glycine and 20% methanol at 25 mA for 20 minutes. Immunoblotting was performed using the Protoblot II AP System (Promega) [34, 35]. Membranes were dried at 37°C, rinsed in 20 mM Tris-HCL, 150 mM NaCl, and 0.05% Tween 20, pH 7.5 (TBST), washed in 1% bovine serum albumin (BSA) in TBST, and incubated in rabbit anti-GLS antibody (1/1000, TBST) for 1 hour at room temperature. Samples were washed in TBST followed by incubation in alkaline phosphatase conjugated goat anti-rabbit antibody for 30 minutes. Samples were washed twice in TBST and TBS. Membranes were incubated in Western Blue stabilized substrate for alkaline phosphatase (Promega; 5-bromo-4-chloro-3-indolyl-phosphate, nitro blue tetrazolium).

2.6. Mitochondria Isolation for Glutaminase Western Blots. For mitochondrial isolation, rats ($n = 3$, AIA, control) were killed with CO$_2$ and decapitated. Right L$_4$ DRG were removed rapidly, manually homogenized in a buffer containing 10 mM Tris-HCL pH 7.4, 0.32 M sucrose, 1 mM EDTA. The supernatant was centrifuged and supernatant (P1) use for mitochondria isolation. P1 was exposed to a rabbit ant-porin antibody (Millipore) overnight at 4°C. Samples were exposed to a goat anti-rabbit antibody conjugated to M-500 magnetic beads (Dynal) 30 minutes [36], exposed to a magnet and reconstituted in a Lysis buffer of 50 mM Tris-HCL pH 7.4, 2 mM EDTA, and 50 μL Triton X-100. The mitochondria were mechanically separated from the beads with a Pasteur pipette, the beads removed by a magnet, and the supernatant (P2; rich in mitochondria) was removed. Isolated mitochondria (P3) were broken (P4) by freeze fraction and sonication and a protein assay was performed on the samples. A normalized concentration of total protein was used to purify GLS from the mitochondria homogenate as described above. Gel electrophoresis was performed using 12% gel and separated employing the PhastSystem. Western blots were performed as described earlier.

Digitized images (600 dpi) of the Western blots (total and mitochondrial) were analyzed with Image Tool (UTHSCSA) to quantify the intensities of GLS bands. Digitized images were converted to grayscale, inverted, and a shadow north filter applied to enhance the contrast between the band and
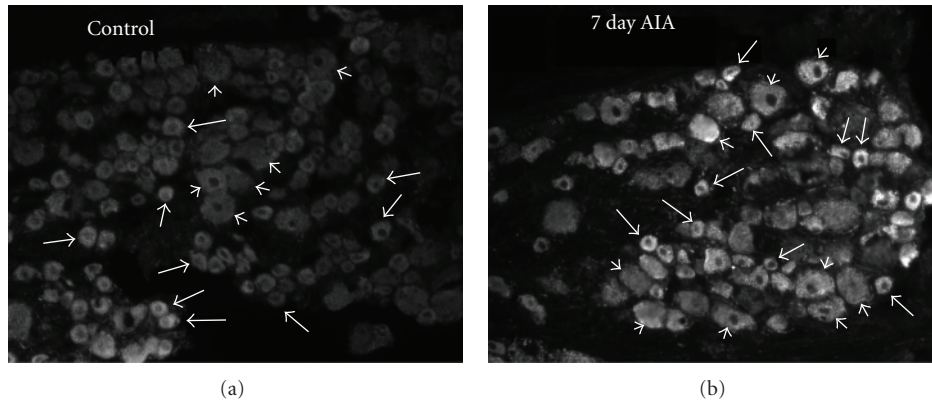
(a)

(b)

FIGURE 1: Glutaminase (GLS) immunoreactivity (ir) in rat L$_4$ dorsal root ganglia (DRG) following 7 days of adjuvant-induced arthritis (AIA) in the hindpaw. DRG sections were processed simultaneously with a rabbit polyclonal GLS antiserum and photographed under identical conditions. (a) In control sections, GLS-IR was light to moderate in all neuronal cell sizes, small (long arrows), and medium to large (short arrows). (b) Elevated GLS-ir in small (long arrows) and medium to large (short arrows) neurons occurred in the DRG following AIA.

background. Bands in each sample were traced separately with an interactive pen on a Cintiq 21UX (Wacom) tablet. Each band was traced three times to reduce bias and the mean calculated for each band.

2.7. Statistics. Data from the analyses are reported as mean value with standard error of the mean. A Student's *t*-test was used to determine differences between AIA and control groups (Prism version 5.01, GraphPad Software Inc., LaJolla, Calif). In all analyses, *P* values less than 0.05 were considered significant.

3. Results

Rats developed inflammation in the right hindpaw with redness and edema similar to previous descriptions [1]. Nociceptive responses to normally nonnociceptive pressures (allodynia) and decreased paw withdrawal latencies to thermal stimuli (hyperalgesia) were observed in the right hindpaw from rats with AIA (Table 1).

At 7 days, GLS-immunoreactivity in L$_4$ DRG neurons from AIA rats was increased over the control DRG neurons (Figure 1). The GLS-IR intensities of three different sizes of DRG cell bodies, therefore, were analyzed (Figure 2). The overall GLS-IR intensity of small (<600 μm^2) L$_4$ DRG cell bodies (Figure 2(a)) from the AIA rats (585.6 $\pm$ 7.7/μm^2) was greater (*P* < 0.01) than controls (484.6 $\pm$ 2.0/μm^2). This represented a 21% increase in small-sized DRG neurons at 7 days AIA compared to control. The GLS-IR intensity of medium-sized (600–1200 μm^2) L$_4$ DRG cell bodies (Figure 2(b)) from the AIA rats (556.9 $\pm$ 7.7/μm^2) was greater (*P* < 0.05) than controls (469.3 $\pm$ 4.9/μm^2). This represented a 19% increase in medium-sized L$_4$ DRG neurons at 7 days AIA compared to control. The GLS-IR intensity of large (>1200 μm^2) L$_4$ DRG cell bodies (Figure 2(c)) from the AIA rats (491.0 $\pm$ 5.8/μm^2) was greater (*P* < 0.001) than controls (431.6 $\pm$ 12.2/μm^2). This represented a 14% increase in large-sized DRG neurons at 7 days AIA compared to control.

Increased GLS enzyme activity in L$_4$ DRG's was observed in AIA rats compared to controls (Figure 3). There was a

TABLE 1: Mechanical and thermal sensitivities.

	Days	0	3	7
Pressure sensitivity (gm)	Control	66.6 $\pm$ 5.2	65.8 $\pm$ 4.7	64.1 $\pm$ 5.3
	AIA	61.6 $\pm$ 4.4	5.2 $\pm$ 0.5***	4.6 $\pm$ 0.1***
Thermal sensitivity (sec)	Control	9.5 $\pm$ 0.5	7.5 $\pm$ 0.6	8.5 $\pm$ 0.7
	AIA	10.0 $\pm$ 0.7	3.2 $\pm$ 0.2***	2.9 $\pm$ 0.8***

Pressure sensitivities determined with von Frey hairs are expressed as gm force. Pressure and thermal control values for each day were compared with AIA values with ANOVA. ***P < 0.0001.

29% increase in GLS enzyme activity from the AIA compared to control DRG. The GLS activity of the AIA rats (2.83 $\pm$ 0.30 moles/kg/hr) was greater (*P* < 0.05) than controls (2.20 $\pm$ 0.18 moles/kg/hr).

Western blots of brain, spinal cord, DRG, and kidney showed a characteristic, specific 65 kDa band for GLS (Figure 4) [37], as well as a large nonspecific IgG band in ~53 kDa range (data not shown) [37]. Analysis of the rat L$_4$ DRG (Figure 5) showed a significant increase (*P* < 0.05) in the AIA DRG (183.8 $\pm$ 11.05) compared to controls (154.4 $\pm$ 10.96; Figure 5). This represented a 19% difference between AIA and control DRG. Western blots of the isolated mitochondria from the L$_4$ DRG demonstrated a band of 65 kDa (Figure 6) corresponding to GLS immunoreactivity. There was a significant increase (*P* < 0.05) of GLS-immunoreactivity in the mitochondria from AIA rats (128 $\pm$ 4.163) compared to controls (100.3 $\pm$ 2.404). This represents a 21% increase in mitochondrial glutaminase concentration in AIA DRG (Figure 6).

4. Discussion

DRG neuronal cell bodies modify neuropeptide, receptor, and ion channel production during peripheral inflammation [2]. The current study further illustrates how primary sensory neurons are altered in regard to glutamate metabolism. In acute inflammation, glutamate release increases for 3 hrs in the spinal dorsal horn [13–16, 38, 39]. Increased
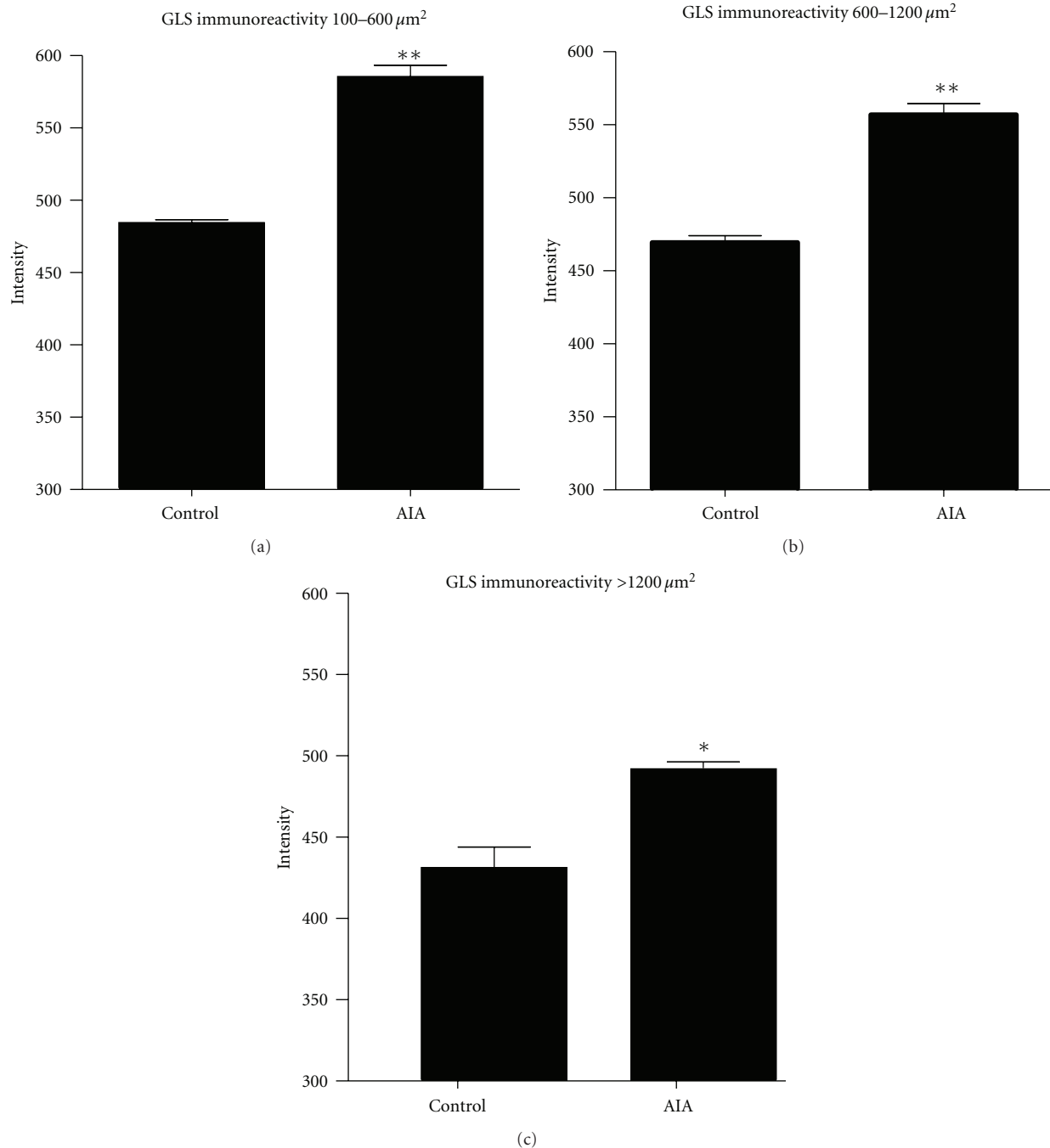
FIGURE 2: Image analysis of glutaminase (GLS) immunoreactivity (IR) in L_4 DRG neurons after 7 days of AIA in the hindpaw. Data are presented as intensity divided by the area of the cell. DRG neurons were categorized into three area size groups: (a) small: $100–600 \mu m^2$, (b) medium: $600–1200 \mu m^2$, (c) large: $>1200 \mu m^2$. (a) Small-sized neurons in DRG from AIA rats contained a significantly greater GLS immunoreactive signal ($**P < 0.01$) than controls. (b) Medium-sized neurons in DRG from AIA rats contained a significantly greater immunoreactive signal ($**P < 0.01$) than controls. (c) Large-sized neurons in DRG from AIA rats were more intensely stained than controls ($*P < 0.05$).

glutamate-immunoreactivity occurs in the dorsal horn 4–12 hr after AIA induction, but returns to normal levels by 24 hrs [17]. In peripheral nerve, glutamate-IR, unmyelinated and thinly myelinated axons increase in number by 2 hrs, peak between 4 and 6 hrs, but return to baseline by 8 hrs [40]. Acute alterations in terminals are likely to be caused

by local flux control mechanisms or allosteric modulation of glutamine cycle enzymes [18, 19, 41]. Phosphate-activated GLS [18, 41–43] has several regulatory sites and calcium (Ca^{2+}) and inorganic phosphate (P_i) are allosteric modulators of neurotransmitter glutamate levels [42, 43]. A stimulated nerve terminal during inflammation, therefore,
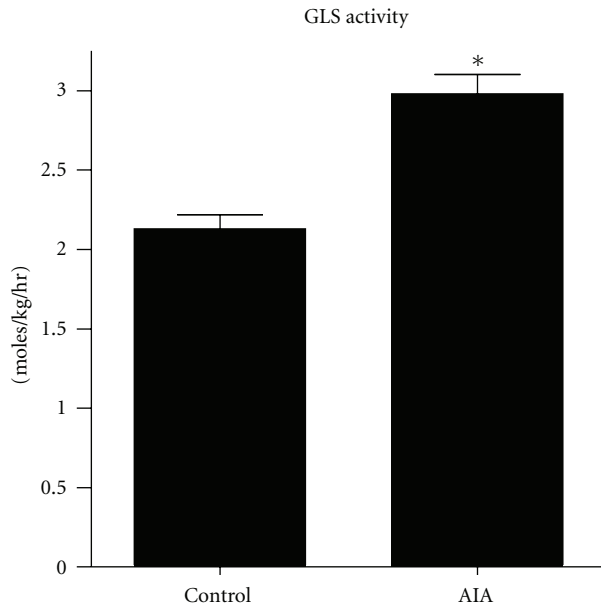
FIGURE 3: Glutaminase (GLS) enzyme activity in the L_4 DRG at 7 days AIA in the right hindpaw. GLS activity from the DRG of AIA rats (2.83 ± 0.30 moles/kg/hr) was elevated ($*P < 0.05$) over control values (2.20 ± 0.18 moles/kg/hr).
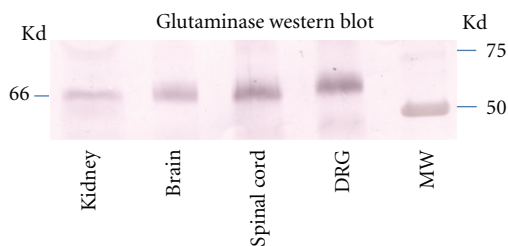

FIGURE 4: This figure represents the Western blots of glutaminase within the DRG, brain, spinal cord, and kidney. A characteristic 65 kDa band was visualized in all the samples confirming the specificity of the antibody and the presence of the kidney/brain glutaminase isoform within the spinal cord and DRG. Western blots have been cropped to exclude the nonspecific IgG 53 kDa band and enhance the presentation of the 65 kDa band.

FIGURE 5: This figure represents the Western blot studies of glutaminase (GLS) within the L_4 DRG from AIA and control rats. (a) Western blot represents GLS immunoreaction (65 kDa) from the L_4 DRG of AIA and control rats. Note the increase of GLS immunoreaction within the AIA animals. (b) This graph represents the statistical analysis of the GLS immunoreactivity between AIA and control rats. A significant increase ($*P < 0.05$) was found between the AIA rats when compared to controls.

would increase ATP use causing elevated P_i levels and the elevated P_i, in turn, would stimulate local GLS activity. Elevated Ca^{2+} concentration for synaptic vesicle fusion could augment the P_i stimulation of GLS [42, 43].

In addition to local mechanisms, the current study demonstrates an increase in GLS production in the neuronal cell body during inflammation. Increased GLS production could come from activity-dependent [44] or neurotrophic mechanisms [28, 45, 46]. The largest GLS increase occurred in small- and medium-sized DRG cell bodies. Neurons of these sizes are considered to include nociceptive neurons with unmyelinated C and lightly myelinated Aδ fibers [2]. Elevated amounts of GLS from the cell body are transported to axons [47] and are likely to increase production of glutamate in nociceptor terminals in the spinal cord and

periphery. SP and CGRP occur together with glutamate in spinal afferent terminals [48] and their corelease generates hypersensitivity of spinal neurons [2]. We postulate that an increase in the amount of GLS during chronic inflammation leads to increased production and release of glutamate along with SP and CGRP [49]. Increased production and release of these substances could sustain spinal hypersensitivity maintaining a state of chronic pain.

Increased GLS production could affect the peripheral terminals also. Glutamate release occurs from peripheral afferents and these terminals contain glutamate receptors [3]. Glutamate receptor agonists sensitize peripheral afferents and produce nociceptive reflexes/hyperalgesia [50, 51]. During inflammation, the number of peripheral axons increases with glutamate receptor immunoreactivity [52]. In chronic

Mitochondrial glutaminase concentration 7 days AIA

(a)

(b)

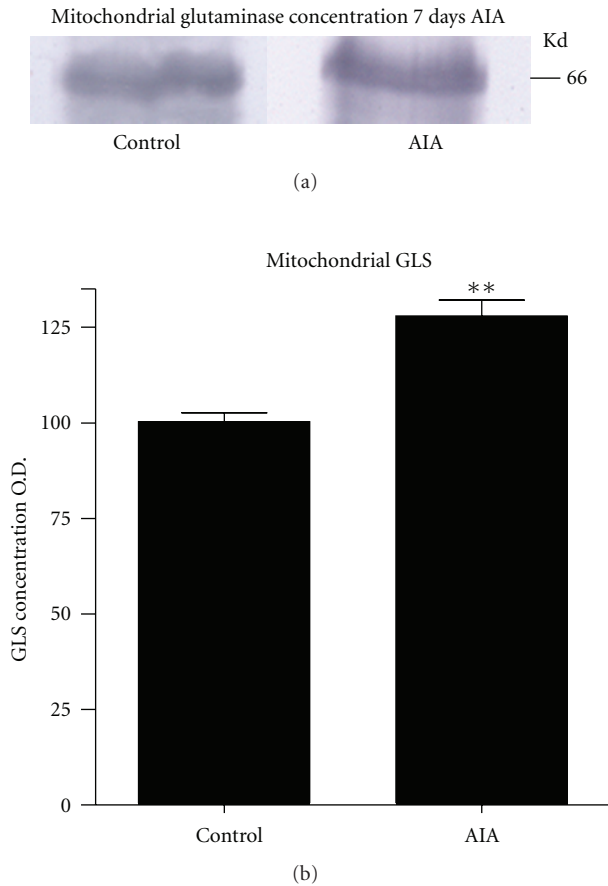FIGURE 6: These Western blots represent the mitochondrial glutaminase (GLS) immunoreaction (65 kDa) within the right L_4 DRG. (a) Note the increase of GLS immunoreaction from the L_4 DRG of AIA and control rats. (b) This graph represents the statistical analysis of mitochondrial GLS immunoreactivity between AIA and control rats. A significant increase (**$P < 0.05$) was found between the AIA rats when compared to controls.

inflammation, increased glutamate production and release could activate terminals with elevated number of glutamate receptors leading to ongoing sensitization of primary afferents [3]. A cycle of increased glutamate production and release, elevated numbers of axons with glutamate receptors, and maintenance of sensitization of peripheral nerve terminals would exacerbate the process of chronic pain in the periphery.

In the present study, long-term changes due to inflammation include an increase in glutaminase in the rat DRG cell body. This increase could lead to elevated production and release of glutamate at both peripheral and central terminals. An increase in glutamate metabolism in primary sensory neurons may be partly responsible for heightened nociceptive sensitivity in tonic pain models. Prevention of increased glutaminase production or inhibition of glutaminase enzyme activity, therefore, may reduce or block some nociceptive responses during chronic inflammation [53].

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Research Article

ASICs Do Not Play a Role in Maintaining Hyperalgesia Induced by Repeated Intramuscular Acid Injections

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Repeated intramuscular acid injections produce long-lasting mechanical hyperalgesia that depends on activation of ASICs. The present study investigated if pH-activated currents in sensory neurons innervating muscle were altered in response to repeated acid injections, and if blockade of ASICs reverses existing hyperalgesia. In muscle sensory neurons, the mean acid-evoked current amplitudes and the biophysical properties of the ASIC-like currents were unchanged following acidic saline injections when compared to neutral pH saline injections or uninjected controls. Moreover, increased mechanical sensitivity of the muscle and paw after the second acid injection was unaffected by local blockade of ASICs (A-317567) in the muscle. As a control, electron microscopic analysis showed that the tibial nerve was undamaged after acid injections. Our previous studies demonstrated that ASICs are important in the development of hyperalgesia to repeated acid injections. However, the current data suggest that ASICs are not involved in maintaining hyperalgesia to repeated intramuscular acid injections.

1. Introduction

Acid Sensing Ion Channels (ASICs) are found in peripheral neurons and play a significant role in modulation of nociceptive behavior following insult to muscle or joint. Decreases in pH activate ASICs, directly activate nociceptors, and produce pain in humans [1–4]. Four genes encoding six ASIC subunits (ASIC1a, ASIC1b, ASIC2a, ASIC2b, ASIC3, ASIC4) have been identified. Functional ASICs form as homotrimers or heterotrimers of three ASIC subunits, and in mouse DRG neurons the channels primarily exist as heteromers [5, 6]. Of the different isoforms, ASIC3 is found predominately in peripheral sensory neurons and has been shown to play a significant role in nociception.

After inflammation of the muscle or joint, there is a reduction in cutaneous secondary hyperalgesia of the paw in ASIC3−/− mice and a reduction in primary muscle hyperalgesia in ASIC1−/− mice [7–10]. Restoration of ASIC3 expression in primary afferent fibers innervating muscle of ASIC3−/− mice rescues mechanical hyperalgesia after muscle inflammation [7], suggesting a significant role for peripheral ASIC3 in inflammatory hyperalgesia from muscle. In contrast, cutaneous inflammatory hyperalgesia is unchanged or even enhanced in ASIC3−/− mice [11–13]. However, peripheral blockade of ASIC3 at the time of injection or siRNA knockdown of ASIC3 in DRG prevents the development of cutaneous inflammatory hyperalgesia in rats [14]. Inflammation also induces an increased mRNA (ASIC1 and ASIC3) and protein expression (ASIC3) in DRG and increased protein expression in peripheral terminals of nociceptors [8–10, 15–18]. This enhanced expression in DRG is manifested as an increased responsiveness to acidic pH [19]. Once developed, inflammatory hyperalgesia is reversed by blockade of ASICs nonselectively, or by selective blockade of ASIC3

intrathecally [10, 20, 21]. Together these data suggest an important role of ASIC1 and ASIC3 in the development and the maintenance of inflammatory musculoskeletal pain.

To model noninflammatory chronic muscle pain we developed a model induced by two injections of acidic saline, 5 days apart, into the gastrocnemius muscle [22]. Like wild-type mice, ASIC1−/− mice still develop secondary mechanical hyperalgesia of the paw; however, this response was completely abrogated in ASIC3−/− mice [23]. In addition, blockade of ASICs during the second acid injection with amiloride prevents the development of secondary hyperalgesia 24 hours later [23]. These data from our laboratory support that activation of ASICs, in particular ASIC3, is important for the development of secondary hyperalgesia in response to repeated acid injections. However, it is not known if there are changes in ASICs after the development of hyperalgesia in this model, or if ASICs in muscle afferents are important for maintaining the hyperalgesia. We therefore examined the properties of ASIC-like currents from retrogradely labeled muscle sensory neurons, and the effects of antagonism of ASICs on primary (muscle) and secondary (cutaneous) hyperalgesia 24 hours after the second injection of acidic saline.

2. Materials and Methods

2.1. Animals. C57BL/6 male mice (age 2–4 months; n58) (Jackson Laboratories, Bar Harbour, Maine) were used in these studies. The Animal Care and Use Committee at the University of Iowa approved all experiments (ACURF#0908193).

2.2. Labeling of Muscle Sensory Neurons. Sensory neurons innervating muscle were fluorescently labeled using the retrograde tracer DiI (1,1-dioctadecyl-3,3,3,3 tetramethylindocarbocyanine perchlorate; 17 mg/mL dissolved in 20% v/v ethanol and suspended in 80% v/v sterile saline). Animals were anesthetized with 2–5% isoflurane, a small incision was made in skin over the left gastrocnemious muscle, and 10 μL DiI was injected into the left gastrocnemius muscle as previously described ($n = 30$) [18]. After injection, saline-soaked sterile gauze was placed on the open incision for 10 minutes to prevent the dye from leaking to the overlying skin. The skin was then sutured closed and mice were allowed to recover for approximately 2 weeks.

2.3. Intramuscular Acid Injections. For recording experiments, mice were injected 2 weeks after DiI injection into the same gastrocnemius muscle with 20 μL of pH 4.0 ($n = 10$) or pH 7.2 ($n = 11$) saline while deeply anesthetized with isoflurane (5%). In approximately half the animals a second 20 μL of injection of pH 4.0 ($n = 6$) or pH 7.2 ($n = 6$) saline was reinjected into the gastrocnemius muscle 5 days later. 24 hours after a single injection of saline, or 24 hours after a second injection of saline, mice were euthanized and the L4-L6 DRG neurons were isolated and cultured. For behavioral experiments, mice received an initial injection of 20 μL of pH 4.0 ($n = 24$) while deeply anesthetized with isoflurane

(5%), immediately after baseline behavioral testing. A second injection was repeated 5 days later in all mice.

2.4. Isolation of DRG Neurons. The ipsilateral L4-L6 DRGs were collected and dissociated as previously described [6]. DRGs were treated with papain and collagenase/dispase and then gently triturated to isolate neurons. Neuron suspensions were then plated on 35 mm Petri dishes coated with poly L-lysine and laminin. Cells were cultured in F12 medium supplemented with 10% heat inactivated serum, penicillin-streptomycin, and 50 ng/mL NGF. 24 hours after plating we examined cells with whole-cell patch-clamp.

2.5. Electrophysiology of Cultured DRG Neurons. Whole-cell patch-clamp recordings of DiI labeled DRG neurons were performed at room temperature at a holding potential of −70 mV. Currents were filtered at 1 kHz and sampled at 2 kHz using the Axopatch 200B amplifier, Digidata 1200, and Clampex 8.2 (Axon instruments, Union city, CA). Micropipettes (3–5 MΩ) were filled with internal solution (mM): 100 KCl, 10 EGTA, 40 Hepes, 5 MgCl$_2$, pH 7.4 with KOH. External solutions contained (mM) 120 NaCl, 5 KCl, 1 MgCl$_2$, 2 CaCl$_2$, 10 HEPES, and 10 MES; pH was adjusted with tetramethylammonium hydroxide; osmolarity was adjusted with tetramethylammonium chloride. Extracellular pH solutions ((pH 7.4 (control)), 6.8, 6.5, 6.0, 5.0) were used to study ASIC currents. Whole cell capacitance was compensated and recorded. Solutions with different pH were applied directly to the cell by using a perfusion system BPS 8 (ALA scientific, Westbury NY), which was controlled by Digidata 1200 and Clampex 8 software (pClamp8).

To measure pH dose responses, pH currents activated by pH 5, 6, 6.5, and 6.8 solutions were normalized to pH 5 induced currents. Time constants for desensitization were measured from single exponential fit to the falling phase of the current evoked by pH application. The time course of recovery from desensitization was measured by completely desensitizing the ASIC current at pH 6 by a long desensitizing pulse followed by bathing in pH 7.4 for a defined time followed by a second stimulation at pH 6. Recovery is percentage of recovery of current evoked by second pulse by first pulse.

2.6. Behavioral Assessment. Mice were given one dose of 0.025 μmol A-317567 (C-(6-[2-(1-isopropyl-2-methyl-1,2, 3,4-tetrahydro-isoquinolin-7-yl)-cyclopropyl]-naphthalen-2-yl)-methanediamine) (10 μL) injected into the left gastrocnemius muscle 24 hours after induction of hyperalgesia. This dose was based on our prior study which showed a reduction in pain-behaviors after muscle inflammation [10]. Separate mice were used to test behavioral sensitivity. Muscle sensitivity was tested as follows: before the first injection of the muscle, before the second injection of the muscle, 24 hours after the second injection, and 15 minutes after A-317567 injection. A-317567 was injected intramuscularly immediately after the 24 hours behavioral test. C57BL/6 mice were acclimated for 2 days before testing for muscle sensitivity and cutaneous mechanical sensitivity, as described

previously [8] and separate groups of mice were used to test muscle sensitivity and cutaneous sensitivity. *Muscle mechanical sensitivity* was tested by squeezing the gastrocnemius muscle of the mice with a calibrated pair of tweezers until the mouse withdrew from the stimulus as previously described [10]. The force at which the mouse withdrew was measured in mN. A decrease in threshold was interpreted as muscle hyperalgesia. *Cutaneous mechanical sensitivity* was tested bilaterally by assessing the number of responses to repeated application of a 0.4 mN von Frey filament to the plantar surface of the paw as previously described [23]. The number of withdrawals out of 5 was assessed in 10 trials and an average of all 10 trials was determined for each time period. A significant increase in the number of responses was interpreted as cutaneous hyperalgesia.

2.7. Electron Microscopy.

Twenty-four hours after injection of acidic saline, mice were deeply anesthetized sodium pentobarbital (60 mg/kg, i.p.) and transcardially perfused with 2.5% gluteraldehyde in 0.1 M cacodylate buffer. The tibial nerve was dissected bilaterally and placed in the same fixative until processing. Post fixation was carried out for 1 hour at room temperature with a buffered 1% osmium tetroxide solution reduced with 1.5% potassium ferrocyanide. Samples were stained with 2.5% uranyl acetate. Blocks were then rinsed and dehydrated using gradually increasing concentrations of acetone to 100%. Infiltration of Spurr's epoxy resin and acetone were carried out over several days to 100% resin and cured 48 hours in a 60°C oven. Sections of 100 nm thickness were cut using a Leica UC-6 ultramicrotome and collected on 400 mesh copper grids. The grids were then counterstained with 5% uranyl acetate for 2 minutes and Reynold's lead citrate for 2 minutes. Samples were imaged using a JEOL 1230 transmission electron microscope at 120 KV.

2.8. Experimental Design.

Experiment 1 tested pH currents in DRG isolated from five groups of mice were used for the present study as follows: a control group that did not receive injections into the gastrocnemius muscle ($n = 9$ mice; 57 cells), a control group injected with pH 7.2 saline once ($n = 5$ mice, 28 cells), an experimental group injected with pH 4.0 saline once ($n = 4$ mice, 22 cells), a control group injected with pH 7.2 saline twice ($n = 6$ mice, n-48 cells), and an experimental group injected with pH 4.0 saline twice ($n = 6$ mice, 41 cells).

Experiment 2 tested the effects of blockade of ASIC channels, with A-317567, after the development of hyperalgesia. We tested the responses in two separate groups of animals. One group was tested for paw sensitivity ($n = 6$ A-317567, $n = 6$ vehicle) and one for muscle sensitivity ($n = 6$ A-317567, $n = 6$ vehicle). A-317567 was tested against a vehicle control with the tester blinded to drug or vehicle injection.

Experiment 3 tested if pH 4.0 saline produced nerve damage using electron microscopy in 2 mice per group 24 hours after injections as follows: (1) pH 4.0, single injection, (2) pH 7.2, single injection, (3) pH 4.0 two injections, (4) pH 7.2 two injections.

2.9. Statistical Analysis.

Patch clamp data were analyzed using Clampfit (Axon instruments), Microsoft Excel, and Origin 7 software (Northampton MA). A two-way ANOVA was used to study differences between groups and differences between pH using SPSS 17. Post hoc testing between groups was performed with a Tukey's test. $P < 0.05$ was considered significant. Behavioral data were analyzed with a repeated measures ANOVA for differences across time and between groups. Data are represented as mean ± SEM.

3. Results

3.1. Injection of pH 4.0 Does Not Alter the Number of DRG Neurons Expressing ASIC-Like Currents.

To test if ASICs current properties were altered after repeated acid injections, we performed whole-cell patch clamp of retrogradely labeled muscle afferents 24 hours after the second acid injection. Figure 1(a) shows representative traces of proton-activated currents recorded from a labeled muscle DRG neuron. Acidic pH evoked a rapidly activating transient current that then desensitized in the continued presence of acid. In some neurons pH 5 and pH 6 activated currents had a sustained current along with a transient component. The transient component properties are characteristic of ASICs [6, 22], whereas the sustained component can also represent activation of transient receptor potential subfamily vanilloid 1 (TRPV1) channels in DRG neurons [24]. We previously demonstrated that the transient component of pH-activated currents in muscle DRG neurons was blocked by the ASIC inhibitor, amiloride, and was unaffected by the TRPV1 inhibitor, capsazepine [18]. Therefore, we defined a neuron as expressing an ASIC-like current if pH 5 evoked a transient inward current of greater than 100 pA. We compared the 5 groups of mice (uninjected, one or two injections of pH 7.2 or 4.0) and found no statistical difference in the percentage of labeled muscle afferents that expressed ASIC-like currents (Figure 1(b)). Thus, intramuscular acid injections do not appear to alter the percentage of muscle afferents that express ASICs.

ASICs are expressed in small- and medium-sized neurons that can respond to noxious stimuli and are also expressed in larger neurons that correspond to low threshold mechanoreceptors [25]. Muscle sensory neurons that possessed ASIC-like currents were medium-size-neurons, and neurons that did not express ASIC-like currents were significantly smaller (Figure 1(c)) ($F_{1,195} = 11.2$, $P = 0.001$; sizes ranged in both groups from 20 to 37 μm). Furthermore, there was no difference in cell sizes between treatment groups, suggesting that intramuscular acid injection did not cause a shift in ASIC expression in neurons of a particular size.

3.2. Mean Current Amplitudes of pH-Evoked Currents Are Unchanged after Intramuscular Injection of Acid.

We next examined if there were increases in ASIC expression after inflammation by examining the amplitude of current in response to acidic pH. There was no significant difference between groups for the current amplitude. Twenty-four hours after the first acid injection the mean maximal current
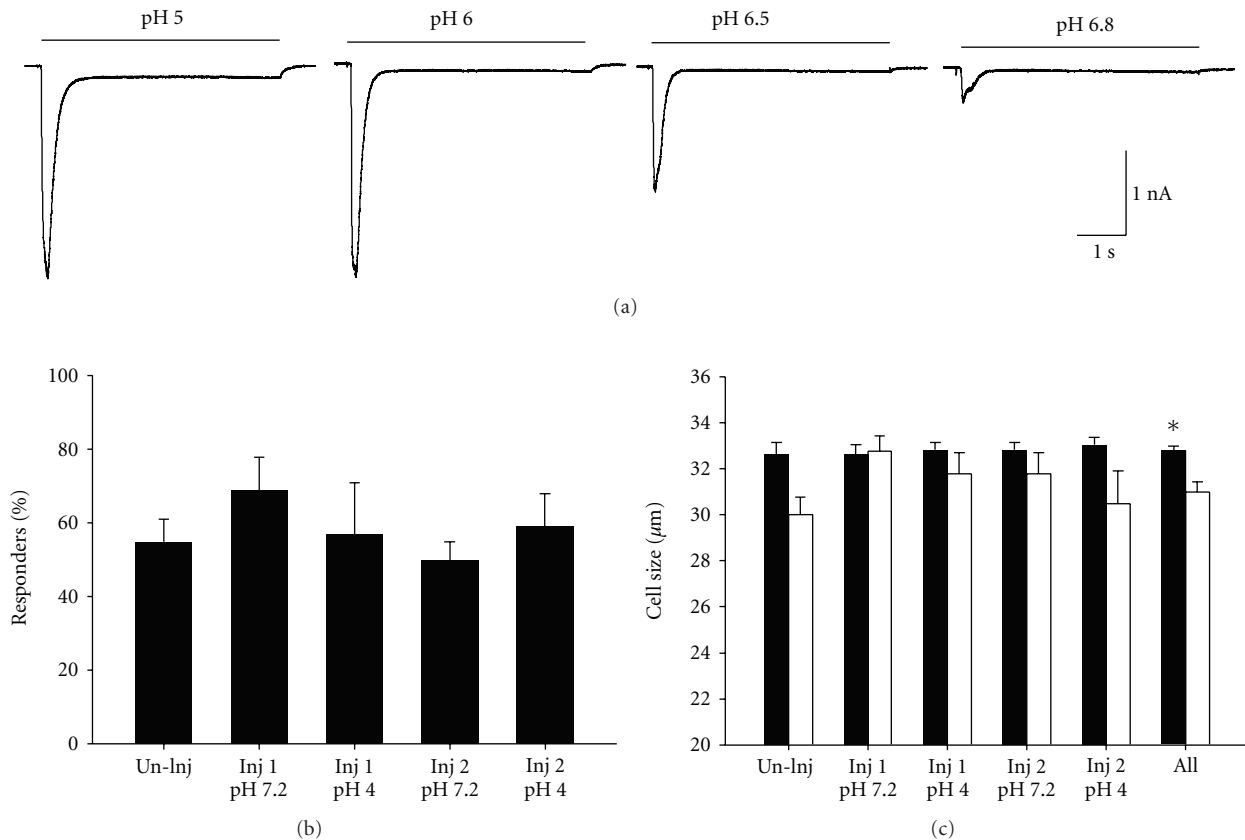
(a)

(b)

(c)

FIGURE 1: (a) Representative traces of pH currents recorded in DRG neurons innervating muscle. (b) The percentage of labeled muscle DRG neurons that responded to pH 5 application with a transient inward current (ASIC-like current) greater than 100 pA is shown for each experimental group. (c) The mean cell diameters of the muscle DRG neurons that responded to pH 5 application with a transient ASIC-like current (responders; black bars) are compared to those that did not respond (nonresponders; open bars) for each of the experimental groups. The number of responders in each group is as follows: uninjected = 29/57; Injection 1, pH 7.2 = 19/29; Injection 1, pH 4.0 = 22/35; Injection 2, pH 7.2 = 25/48; Injection 2, pH 4.0 = 24/36. $^*P < 0.05$.

amplitude was 1904 ± 446 pA ($n = 22$) (Figure 2(a)) and similar to that after the second acid injection (1521 ± 305, $n = 24$), or pH 7.2 injected controls (Injection 1: 2808 ± 474, $n = 19$; Injection 2: 2856 ± 376, $n = 25$), or uninjected (2228 ± 338, $n = 28$) controls.

3.3. ASIC Channel Properties Were Unaltered by Intramuscular Acid Injections. Sensory neurons have been found to express ASIC1, ASIC2, and ASIC3 isoforms and generally form as heteromers in mouse DRG neurons [6], and each of the different heteromeric combinations of channels displays different biophysical properties [6, 26]. To determine the relative distribution of isoforms we studied different properties of ASIC currents including pH sensitivity of activation, desensitization kinetics, and recovery from desensitization. We hypothesized that hyperalgesia associated with intramuscular acid injections might cause a change in the subunit composition of the ASIC channels, and we could detect this as a change in the biophysical properties. Figure 2(b) shows that the pH sensitivity of ASIC currents, measured by normalizing the current amplitude recorded at varying

pH (6.8–6.0) to the pH 5 current amplitude, did not change significantly after intramuscular acid injections when compared to uninjected and pH 7.2 injected controls.

ASIC currents desensitize in the continued presence of acidic pH. By fitting the desensitizing phase of the currents to single exponentials, the rates of desensitization (τ) were measured. Figure 3(a) demonstrates that intramuscular acid injections did not change the rate of desensitization of the ASIC-like currents compared to control groups injected with pH 7.2 or uninjected controls.

After ASIC channels desensitize, they need to be exposed again to a more alkaline pH for some period of time to allow the channels to "recover", before they can be activated again (see methods for protocol of how recovery was measured). Figure 3(b) shows that the intramuscular acid injections did not alter the rate of recovery from desensitization. In summary, we found no change in the distribution of muscle DRG neurons that expressed ASIC-like currents, nor were there changes in the current properties at either 24 hours after the first or 24 hours after the second injection of acidic saline.

(a)

- ● Uninjected
- ▽ Inj 1, pH 7.2
- ■ Inj 1, pH 4.0
- ◇ Inj 2, pH 7.2
- ▲ Inj 2, pH 4.0

(a)

(b)

- ● Uninjected
- ▽ Inj 1, pH 7.2
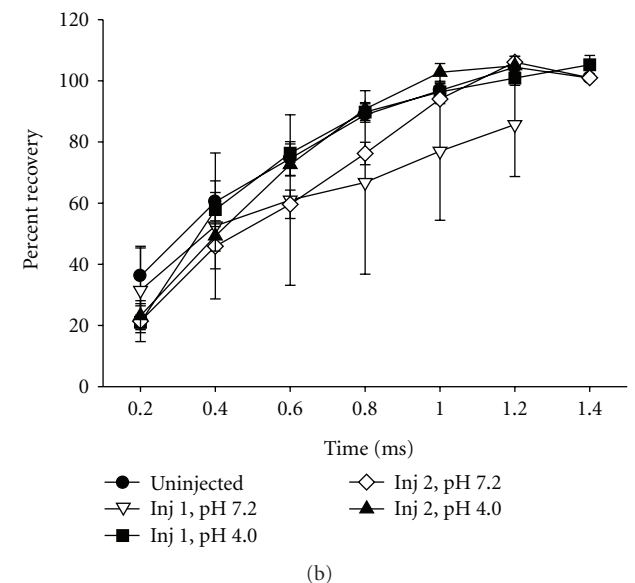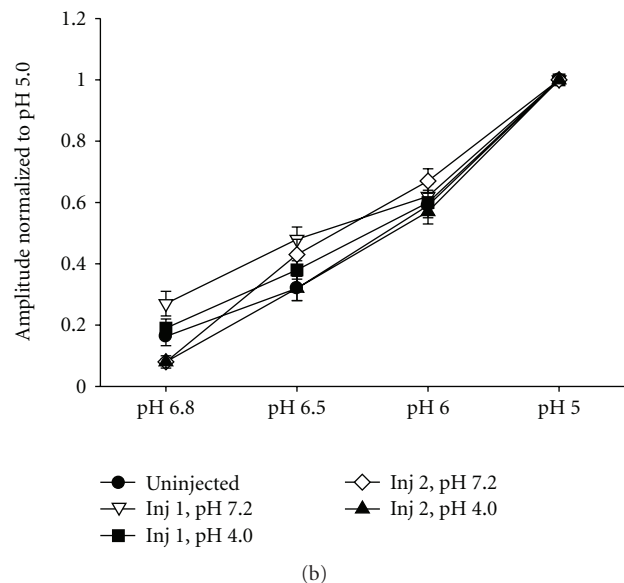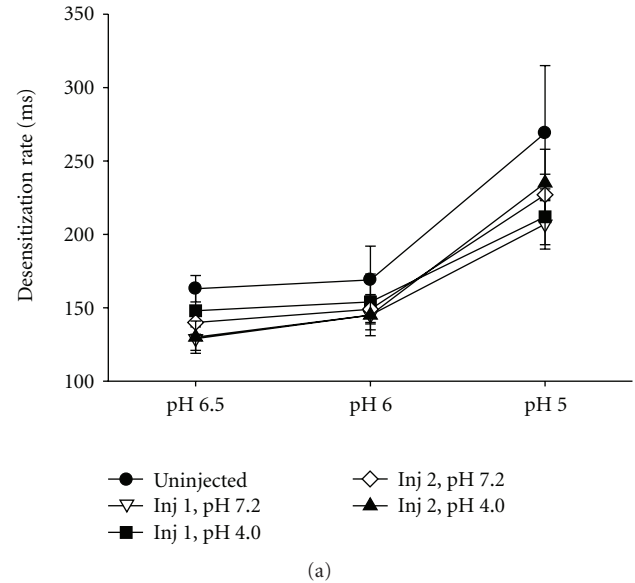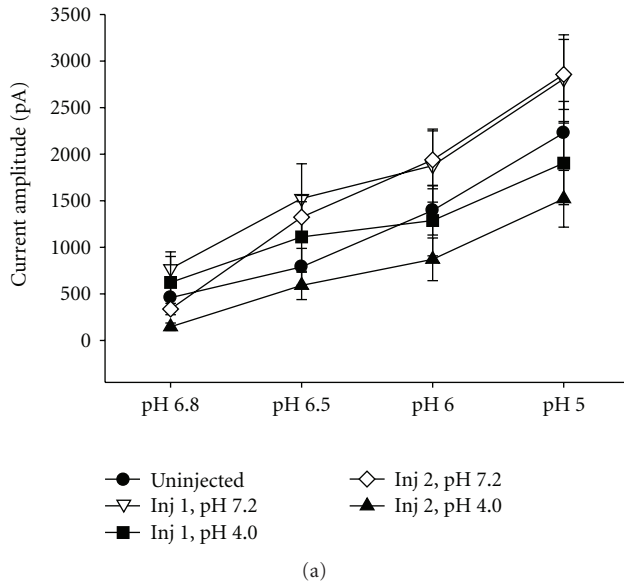- ■ Inj 1, pH 4.0
- ◇ Inj 2, pH 7.2
- ▲ Inj 2, pH 4.0

(b)

FIGURE 2: (a) The mean current amplitudes of pH currents at pH 5, 6, 6.5, and 6.8 are not significantly different between groups injected with pH 4.0 (Injection 1, $n = 22$; Injection 2, $n = 24$) and controls injected with pH 7.2 (Injection 1, $n = 19$; Injection 2, $n = 25$), or uninjected controls ($n = 28$). (b) Data from (2A) was normalized to the peak transient currents evoked by pH 5 to analyze pH dose responses. pH dose responses and were not different between groups injection with pH 4.0 (Injection 1, $n = 21$; Injection 2, $n = 24$) and those injected with pH 7.2 (Injection 1, $n = 19$; Injection 2, $n = 24$) or uninjected controls ($n = 27$).

FIGURE 3: (a) The mean time constants of desensitization of ASIC-like transient currents evoked by the indicated pH solutions were similar between uninjected ($n = 26$), controls injected with pH 7.2 (Injection 1, $n = 18$ Injection 2, $n = 22$) and those injected with pH 4.0 (Injection 1, $n = 19$; Injection 2, $n = 21$). (b) The rate of recovery from desensitization is similar between groups: uninjected ($n = 12$), controls injected with pH 7.2 (Injection 1, $n = 3$; Injection 2, $n = 15$) and those injected with pH 4.0 (Injection 1, $n = 7$; Injection 2, $n = 10$).

3.4. Muscle and Cutaneous Hyperalgesia Is Unaffected by Intramuscular Blockade of ASICs with A-317567. Our previous work demonstrated that ASICs are required for the development of secondary hyperalgesia of the paw after repeated intramuscular acid injections [23]. Here we tested if continued activation of ASICs was necessary to maintain the hyperalgesia after it had been developed. As previously shown, repeated intramuscular injection of pH 4.0 saline increases the number of responses of the paw to repeated stimulation (Figure 4(a)) and decreases the force threshold to withdrawal of the muscle (Figure 4(b)), 24 hours after the second injection. Interestingly, intramuscular injection of $0.025\,\mu$mol of A-317567, a dose previously shown to reverse muscle cutaneous sensitivity after muscle inflammation [10],
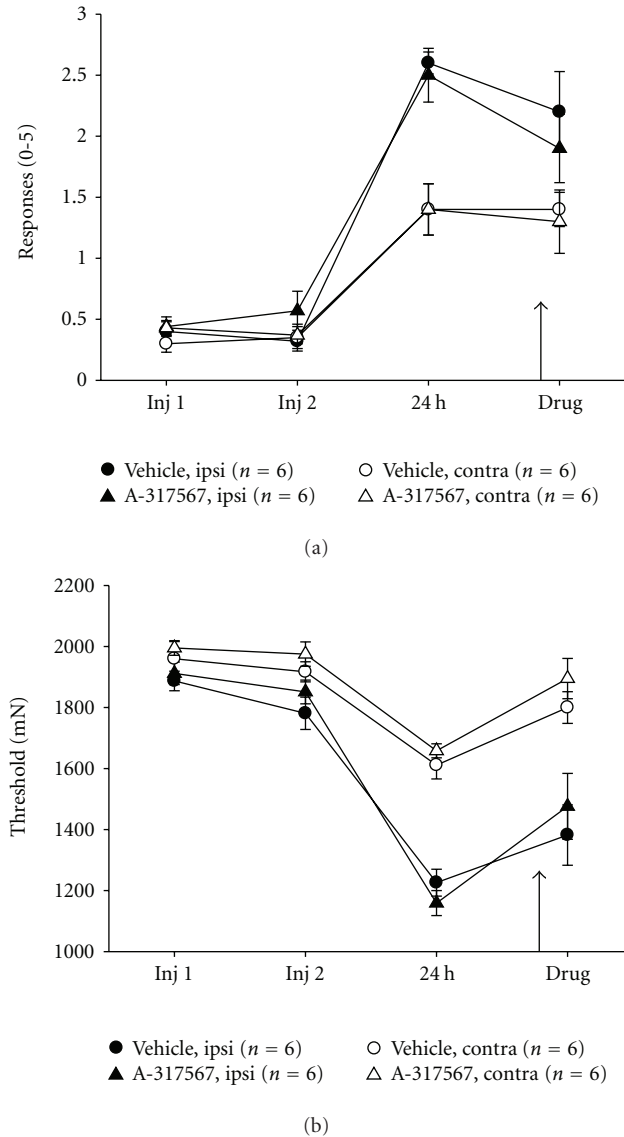
(a)

(b)

FIGURE 4: ASIC pharmacological antagonism has no effect upon primary (muscle) or secondary (cutaneous) hyperalgesia induced by repeated intramuscular acid injections. (a) Mean muscle withdrawal thresholds. (b) mean number of responses to repeated von Frey stimulation of the paw measured before first and second acid injections, 24 hours after second acid injection, and after intramuscular injection of A-317567 or control injection. While there was an increase in mechanical sensitivity of the paw and muscle after repeated acid injections, there was no difference in this sensitivity after intramuscular injection of the ASIC antagonist.

had no effect on the enhanced cutaneous and muscle sensitivity induced by repeated acid injections (Figures 4(a) and 4(b)).

3.5. Repeated Acid Injections Do Not Produce Damage to Tibial Nerve. To be certain that repeated acid injections do not produce damage to the tibial nerve, and hence cause neuropathic pain, we performed electromicrographic analysis of the tibial

nerve after injections of pH 4.0 compared to the tibial nerves from the contralateral hindlimb. As shown in Figure 5, there was no difference between the muscles injected with pH 4.0 and those injected with pH 7.2, or from the contralateral hindlimb. This was observed 24 hours after a single injection of pH 4.0 or 24 hours after the second injection of pH 4.0.

4. Discussion

We previously found that ASICs are necessary for the development of hyperalgesia after repeated intramuscular acid injection [23]. The current study shows that ASICs are not involved in maintaining the hyperalgesia once it has already developed. We found that pharmacological inhibition of ASICs 24 hours after a second intramuscular acid injection, at a time when hyperalgesia is well established, had no effect upon muscle or cutaneous hyperalgesia. Moreover, we found that repeated acid injections produced no change in the expression of ASIC-like currents or their properties in labeled muscle DRG neurons, suggesting that hyperalgesia in this model is not associated with changes in ASIC expression.

Prior studies from our laboratory show that ASIC3 is important for the induction of long-lasting mechanical hyperalgesia of the paw after repeated acid injections [23]. Specifically, ASIC3−/− mice do not develop mechanical hyperalgesia of the paw after repeated acid injections when compared to ASIC3+/+ mice [23]. Further, nonselective inhibition of ASICs, or selective blockade of ASIC3, given at the time of the second acid injection prevents the onset of hyperalgesia 24 hours later [23, 27]. This lack of hyperalgesia in ASIC3−/− mice is likely the result of a loss of central sensitization. Recordings from dorsal horn neurons show that ASIC3−/− mice do not show enhanced responsiveness to mechanical stimulation or expansion of receptive fields, measures of central sensitization, when compared to ASIC3+/+ mice [23]. These data, therefore suggest that ASIC3 is important in the induction of long-lasting hyperalgesia and central sensitization.

This noninflammatory pain model is unique and, once developed, likely depends on central mechanisms. Prior studies from our laboratory and others show that pharmacologically NSAIDs are ineffective while channel blockers and agents with central actions, opioids and pregabalin, reverse the hyperalgesia once developed [28–30]. After the second acid injection, we show that receptive fields of dorsal horn neurons expand to include the contralateral hindlimb, there is increased mechanical sensitivity of the paw bilaterally [23] and Miranda et al., show increased visceral sensitivity [31], all indicative of central changes. Mechanistically, we show an increase in glutamate release both spinally and supraspinally in response to the second, but not the first, intramuscular acid injection [32, 33]. Similarly, we show that local anesthetic blockade at the level of the rostral ventromedial medulla during the second, but not the first, acid injection prevents the development of hyperalgesia 24 hours later [34]. Together, these data suggest that induction of the hyperalgesia initially involves activation of ASICs in muscle afferents, that then send input to the central nervous system to result in enhanced excitability both spinally and supraspinally.
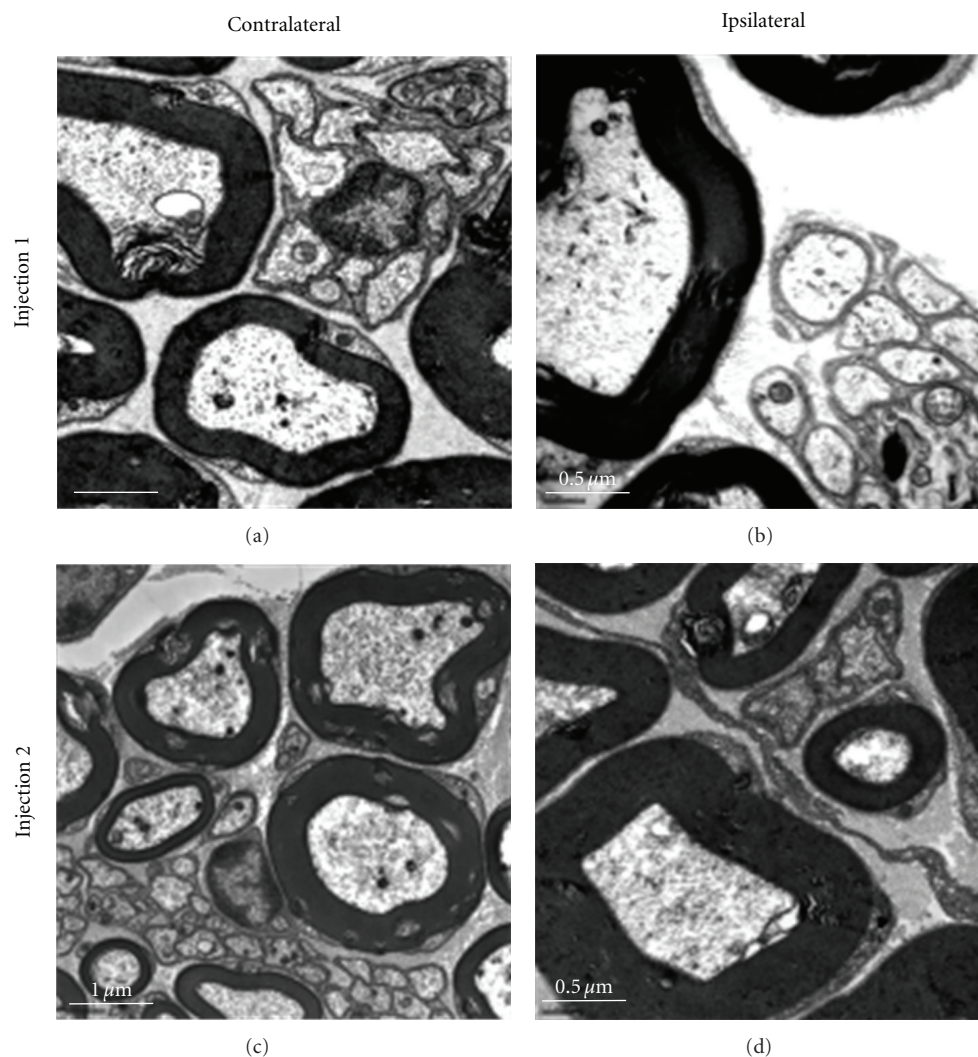
Contralateral

Ipsilateral

(a)

(b)

(c)

(d)

FIGURE 5: Electron micrograph showing the tibial nerve ipsilateral and contralateral to the site of injection of pH 4.0 saline. The tibial nerve was examined 24 hours after the first injection and 24 hours after the second injection for potential structural changes and inflammatory cell infiltration. There were no observable changes at either time point when compared to the contralateral hindlimb after repeated acid injections.

Once the hyperalgesia develops, however, local anesthetic or neurotrophin-3 delivered directly to the muscle has no effect [22, 35] suggesting that peripheral mechanisms do not maintain the hyperalgesia. Our data are consistent with the conclusion that peripheral mechanisms do not maintain the hyperalgesia since blockade of ASICs did not affect the ongoing hyperalgesia. Similarly, the selective ASIC3 antagonist APETx2 also has no effect on the hyperalgesia in this model once developed [27]. On the other hand, our laboratory showed that local anesthetic blockade supraspinally or blockade of NMDA receptors spinally and supraspinally reverses the hyperalgesia once developed [34, 36, 37]. This activation of NMDA receptors likely turns on second messenger systems to result in long-lasting sensitivity and hyperalgesia. In support we show an increase in phosphorylation of the transcription factor CREB and blockade of the cAMP pathway reverses the hyperalgesia once developed [38]. Thus, once the

hyperalgesia develops in the noninflammatory muscle pain model, there are no changes in the number or sensitivity of ASICs and blockade of ASICs has no effect on hyperalgesia.

In contrast inflammatory muscle pain clearly involves activation of ASICs for the maintenance of the hyperalgesia. Specifically, we show that pharmacological blockade of ASICs with the nonselective antagonist A-317567 reverses the hyperalgesia once developed [10]. In both muscle and paw inflammation models, we and others show an increase in the amplitude of pH responsiveness of DRG and an increase in mRNA for ASICs [10, 14]. Our laboratory also showed an increase in the number of joint afferents that express ASIC3 after joint inflammation [8]. This suggests that mechanisms underlying the maintenance of muscle inflammatory pain are uniquely different from those underlying the maintenance of noninflammatory muscle pain.

The hyperalgesia associated with the muscle is processed differently from that associated with the skin. For ASICs there is a greater expression of ASIC3 in small DRG neurons innervating muscle when compared to DRG neurons innervating skin [39]. In this noninflammatory model, fos expression in response to the muscle stimulation after the development of hyperalgesia increased in the superficial dorsal horn compared to controls and was increased after paw stimulation in acid-injected WT but not acid-injected NT-3 muscle-specific knockouts or NT-3 muscle-injected mice [35, 40]. The lack of injury to the tibial nerve shown in the current study after repeated acid injections is consistent with the fact that there is no peripheral tissue damage or inflammation in response to repeated acid injections [22]. Thus, the muscle hyperalgesia at the site of acid injection and in the contralateral muscle can be considered secondary hyperalgesia. Without peripheral damage to the nerve innervating the paw, we further conclude that the hyperalgesia of the paw is also secondary hyperalgesia.

It is unclear how repeated injections of acidic saline result in widespread hyperalgesia after the second but not the first injection. In addition to muscle and cutaneous hyperalgesia, this model is also associated with enhanced visceral hyperalgesia [31]. The widespread nature of the hyperalgesia suggests involvement in the central nervous system. Peles et al. show that a single injection of pH 4.0 saline enhances spontaneous firing of spinal neurons as well as the response to colorectal distension [41]. It is possible that this sensitization lasts for several days setting up the nervous system to respond in an exaggerated manner to the second acid injection. Indeed both spinally and supraspinally there is enhanced glutamate release during the second acid injection [32, 33] and sensitization of dorsal horn neurons in response to the second acid injection [23]. The increased glutamate at the spinal level is clearly important in the development of the hyperalgesia as spinal blockade of NMDA receptors during the second acid injection delays the onset of hyperalgesia [36]. Thus, the central sensitization induced by peripheral acid injections likely underlies the widespread nature of the hyperalgesia induced by this model. Activation of ASICs, in particular ASIC3, plays a critical role in the development of this central sensitization, which is absent in ASIC3−/− mice [23].

5. Conclusion

In summary, the current study shows that once developed the hyperalgesia associated with repeated acid injection does not depend on continued activation of ASICs. There are no changes in expression of ASIC channels in terms of the number or subunit composition and blockade of ASICs after development of hyperalgesia is ineffective. These data further support existing studies showing that the hyperalgesia induced by repeated acid injections is maintained by central mechanisms.

Acknowledgments

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Research Article

Peripheral Glutamate Receptors Are Required for Hyperalgesia Induced by Capsaicin

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Transient receptor potential vanilloid1 (TRPV1) and glutamate receptors (GluRs) are located in small diameter primary afferent neurons (nociceptors), and it was speculated that glutamate released in the peripheral tissue in response to activation of TRPV1 might activate nociceptors retrogradely. But, it was not clear which types of GluRs are functioning in the nociceptive sensory transmission. In the present study, we examined the c-Fos expression in spinal cord dorsal horn following injection of drugs associated with glutamate receptors with/without capsaicin into the hindpaw. The subcutaneous injection of capsaicin or glutamate remarkably evoked c-Fos expression in ipsilateral sides of spinal cord dorsal horn. This capsaicin evoked increase of c-Fos expression was significantly prevented by concomitant administration of MK801, CNQX, and CPCCOEt. On the other hand, there were not any significant changes in coinjection of capsaicin and MCCG or MSOP. These results reveal that the activation of iGluRs and group I mGluR in peripheral afferent nerves play an important role in mechanisms whereby capsaicin evokes/maintains nociceptive responses.

1. Introduction

Glutamate which is a major excitatory neurotransmitter in the central nervous system has been shown recently to play an important role in peripheral nociceptive transmission [1]. Concerning the existence of glutamate in the small-diameter afferent fibers, it has been reported that electrical stimulation of the sciatic nerve or noxious heat stimulation (50°C) caused an increase of the glutamate level in subcutaneous perfusate [2, 3].

Glutamate receptors (GluRs) are classified into ionotropic GluRs (iGluRs: NMDA, AMPA/kainate receptor) and metabotropic GluRs (mGluRs: group I, II, and III mGlu receptor). There is evidence that both iGluRs [4] and mGluRs [5] have been located in the peripheral processes of primary unmyelinated afferents. In this connection, electron microscope studies demonstrate that GluRs are transported from the DRG cell bodies into central and/or peripheral primary afferent terminals [6]. NMDA, AMPA, and kainate receptors (NMDA/AMPA-kainate receptors) are localized on unmyelinated axons at the dermal-epidermal junction in the glabrous and hairy skin of rats [7, 8] and human hairy skin [9]. The immunostain labeled unmyelinated fibers are assumed to be sensory fibers and not sympathetic efferents that are also unmyelinated.

Besides these anatomical data, behavioral evidences support a role for peripheral GluRs in normal nociceptive transmission. Intraplantar injection of L-glutamate or iGluR agonists into the hindpaw evokes thermal and mechanical hyperalgesia and allodynia, which can be blocked by appropriate antagonists [7, 10–12].

Transient receptor potential Vanilloid 1 (TRPV1) is also located in small- and medium-sized dorsal root ganglion (DRG) neurons. A broad range of stimuli such as noxious

heat, protons, lipid-derived endovanilloids, and inflammatory mediators either directly activate or modulate TRPV1 [13, 14], and stimulation of TRPV1 elicits the release of glutamate [12, 15, 16]. At the peripheral terminals of primary afferents, TRPV1-mediated Ca^{2+} influx triggers the release of neuropeptides and neurotransmitters, which is responsible for nociceptive processing [14]. TRPV1-expressing dorsal horn neurons in the spinal cord are revealed to be glutamatergic [17].

With regard to the interaction between TRPV1 and GluRs, there is some evidence supporting the modulation of TRPV1 function through GluRs. For example, group I mGluRs (especially mGluR5) are expressed together with TRPV1 in DRG neuron and increase thermal sensitivity by enhancing TRPV1 function at peripheral endings and central presynaptic terminals of nociceptors [18, 19]. Group II mGluRs are also coexpressed with TRPV1 and activation of mGluRs can inhibit nociceptive transmission induced by TRPV1 activation [20]. Furthermore, it was reported that the activation of peripheral iGluRs (NMDA receptors) may be important in the mechanisms whereby capsaicin evokes nociceptive trigeminal responses [21].

We also observed that GluRs, in particular, iGluRs and group I mGluR existing in peripheral endings of capsaicin-sensitive afferent fibers, play an important role in the development and maintenance of hyperalgesia following excitation of TRPV1 by determining the changes of glutamate levels in the extracellular space of rat hindpaw and pain behavior in the thermal withdrawal latency [12].

In this study, we are examining c-Fos levels concerning the neural activity in the spinal cord in response to peripheral activation of TRPV1, GluRs, or both in an attempt to see which GluRs drive highest levels of activity.

2. Materials and Methods

2.1. Experimental Procedures. All surgical and experimental procedures for animals were reviewed and approved by the Osaka University Faculty of Dentistry and the Ohu University Intramural Animal Care and Use Committees, and conformed to the guidelines of the International Association for the Study of Pain [14].

Adult male Sprague Dawley rats weighing 200 g–300 g (CLEA Japan, Inc. Tokyo) were used in all experiments. Rats were kept in a 12 h light/dark cycle and received food and water ad libitum.

2.2. Drug Administration. While animals were inside the small cage, drugs were administrated subcutaneously (s.c.) into the left hindpaw.

Drugs were administered in the volume of 50 μL, s.c., into the plantar surface of the left hindpaw using a 100 μL Hamilton syringe (Reno, Nev, USA) with a 30-gauge needle without anesthesia when animals were kept in a small cage to hold them. The needle was inserted into the plantar skin proximal to the midpoint of the hindpaw. The applied concentrations of L-glutamate and GluR antagonists were decided based on the result obtained from the previous study [12].

2.3. c-Fos Immunohistochemistry. Fos immunoreactivity (labeled nuclei of Fos-immunoreactive spinal neurons) ipsi- and contralateral to the stimulated side was detected according to a standard avidin–biotin–peroxidase technique [18, 22]. Briefly, two hours after the drug injection, animals were deeply anesthetized with sodium-pentobarbital and perfused transcardially with 100 mL of 0.9% saline followed by 500 mL of 4% paraformaldehyde in 0.1 M phosphate buffer (PB; pH 7.4) and the spinal cord was taken out, postfixed in the same fixative overnight at 4°C, and then immersed into 20% sucrose in 0.1 M PB at 4°C until it sank. Serial transverse 60–80 μm thick sections at L4-L6 were cut using a freezing microtome and collected in 0.02 M phosphate buffered saline (PBS). Sections were washed in PBS for 30 min and blocked with 1% normal goat serum for 30 min and then incubated in a rabbit antibody against c-Fos (1 : 7000 dilution; Santa Cruz Biotech, Santa Cruz, Calif, USA) for 60 min in room temperature and then for 12 h at 4°C. After washing in PBS for 30 min, sections were incubated in biotinylated goat antirabbit antiserum, and washed in PBS for 30 min and then immunohistochemically stained for 60 min using avidin-biotin-peroxidase complex (Vectastain, Vector Laboratories, Burlingame, Calif, USA). To visualize peroxidase activity, sections were immersed in 0.05% diaminobenzidine tetrahydrochloride, 0.1% ammonium nickel sulfate, and 0.01% hydrogen peroxide in 0.05 M Tris-HCl buffer (pH 7.2). Sections were washed in PBS for 30 min and then mounted on gelatin-coated slides, air-dried, and coverslipped.

2.4. Quantification of Number of c-Fos-Immunoreactive (ir) Cells. c-Fos-ir cells of laminae I-II and laminae III-IV of all sections (about 80–100) were counted in accordance with previously described methods [23]. In brief, among them, the best labeled 10 sections of the spinal cord segments from L4–L6 were chosen for the mean number per certain segment of labeled neurons.

Our quantification dealt only with the number and location of cell profiles with histologically detectable Fos immunoreactivity, where the intensity of positive cell profiles was not considered. The number of c-Fos-ir cell profiles in the spinal cord dorsal horn was counted only in laminae I-II and laminae III-IV, respectively, according to the method by which the dorsal horn was separated into two areas as follows: the lateral one-fourth of laminae I-II that is referred to as the posterior cutaneous (PC) territory in conjunction with the medial three-fourth of laminae I-II that corresponds to the terminal field of C fibers of primary neurons innervating the sciatic nerve, and laminae III-IV that is the terminal field of A_β fibers of primary neurons, [24, 25]. In all these tests, a double blind procedure was used to prevent the observers from knowing the experimental groups.

2.5. Drug Preparation. We used L-glutamate as GluR agonist. As GluR antagonists, the following drugs were used: selective noncompetitive NMDA-receptor antagonist, (5S,10R)-(+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclo-hepten-5,10-imine hydrogen maleate [(+)-MK-801 hydrogen

TABLE 1: Mean value of c-Fos-ir cells in the dorsal horn of L4-L5 2 h after s.c. injection of capsaicin, glutamate, and glutamate receptors antagonists with/without capsaicin. The value in each group was represented mean ± SEM obtained from at least 10 animals, and the difference of the means was analyzed with the Student's t-test.

Group	Ipsilateral		Contralateral	
	I/II-layer	III\IV-layer	I/II-layer	III\IV-layer
Vehicle	60 ± 5	22 ± 8	12 ± 4	7 ± 2
Capsaicin(cap)	489 ± 34*	63 ± 18	44 ±13	20 ± 9
Glutamate	283 ± 18*	36 ± 5	19 ± 8	12 ± 6
MK801	79 ± 3	11 ± 7	33 ± 12	9 ± 5
CNQX	70 ± 8	7 ± 3	14 ± 7	3 ± 2
CPCCOEt	59 ± 8	6 ± 3	10 ± 4	6 ± 2
MCCG	63 ± 10	5 ± 2	28 ± 11	5 ± 2
MSOP	66 ± 16	5 ± 3	9 ± 4	5 ± 3
Cap + MK801	227 ± 32#	14 ± 4	8 ± 6	3 ± 2
Cap + CNQX	205 ± 40#	11 ± 7	22 ± 12	3 ± 2
Cap + CPCCOEt	236 ± 58#	17 ± 11	12 ± 7	4 ± 3
Cap + MCCG	560 ± 85	27 ± 10	24 ± 9	3 ± 1
Cap + MSOP	383 ± 21	22 ± 3	18 ± 13	4 ± 1

* Significant difference at $P < 0.05$ between vehicle and capsaicin, or glutamate-treated group. # Significant difference at $P < 0.05$ between capsaicin and capsaicin + MK801, or capsaicin + CNQX, or capsaicin + CPCCOEt-treated group.

maleate]; competitive kainite/AMPA-receptor antagonist, 6-cyano-7-nitroquinoxaline-2,3-dione disodium (CNQX); group I mGluR selective noncompetitive mGlu1R antagonist, 7-(hydroxyimino) cyclopropa[b]chromen-1a-carboxylate ethyl ester (CPCCOEt); group II mGluR antagonist, (2S,3S,4S)-2-methyl-2-(carboxycyclopropyl)glycine (MCCG); selective group III mGluR antagonist, (R,S)-a-methylserine-O-phoephate (MSOP). These agonist and antagonists of the GluR were obtained from Tocris (Ballwin, MO, USA). 8-Methyl-N-vanillyl-6-noneamide (capsaicin) and N-[2-(4-chlorophenyl)ethyl]-1,3,4,5-tetrahydro-7,8-dihydroxy-2H-2benzazepine-2-carbothio-amide (capsazepine) were obtained from Sigma Chemical Co. (St. Louis, MO, USA), and Cayman Chemical (Ann Arbor, MI, USA), respectively. All the other chemicals were obtained from Wako Pure Chemical Industries, Ltd. (Osaka).

In accordance with the product material safety data sheets, L-glutamate was diluted in NaOH; MK801, MCCG, and MSOP were diluted in water. CNQX and CPCCOEt were diluted in dimethyl sulfoxide. The other drugs except for these were dissolved in saline. Capsaicin (Cap) was prepared as a 3 mM solution in saline containing 10% ethanol and 10% Tween 80. The pHs of all solutions were adjusted to 7.4.

2.6. Statistical Analysis. Statistical analysis of c-Fos-ir cells was performed by Student's t-test for unpaired values. All data was presented as a mean ± SEM. P values less than 0.05 or 0.01 were considered to indicate statistical significance.

3. Results

3.1. Time Course of Number of c-Fos-ir Cells in L4-L5 Spinal Dorsal Horn after Intraplantar Injection of Saline. Immunoreactivity for c-Fos appeared gray to black and homogeneously labeled the oval or roundish nucleus of cells in spinal

dorsal horn at L5 (Figures 1, 5, and 6). In the intact animals, the basal level of c-Fos expression on the both sides of the lumbar spinal cord (L4-L5) was very low (zero or one c-Fos-ir cell per 60–80 μm thick section), which was referred to as pretreatment value (pre). In animals administered with vehicle, c-Fos-ir cells were rarely distributed either in laminae I/II (60 ± 5) or in laminae III/IV (22 ± 8) on the ipsilateral side or on the contralateral side (I/II, 12 ± 4; III/IV, 7 ± 2) (Figure 2, Table 1).

3.2. Capsaicin- and Glutamate-Induced c-Fos Expression. In all the experimental tests with injection of capsaicin (3 mM) or glutamate (100 mM), the maximum number of labeled cells occurred consistently in laminae I and II (I/II) of the spinal dorsal horn on the ipsilateral side.

The maximum number of c-Fos-ir cells (400–500) evoked by capsaicin was observed in laminae I/II on the ipsilateral side 1 h–3 h after injection, and much smaller number of c-Fos-ir cells (<100) occurred in laminae III and IV. This remarkable increase in laminae I/II on the ipsilateral side was maintained for more than 6 h. However, there was no difference between the numbers of c-Fos-ir cells in III/IV on the ipsilateral side and, that in laminae I/II and III/IV on the contralateral side at any measuring time (Figure 3, Table 1).

In glutamate-treated animals, the increase of c-Fos-ir cells began 15 min after injection in laminae I/II on the ipsilateral side, and continued for 4 hours. The maximum number was observed (283 ± 18) 2 h after injection. The glutamate-induced c-Fos expression in laminae I/II and laminae III/IV on the ipsilateral side was lower than that with capsaicin. The numbers of c-Fos-immunopositive cells on the contralateral side was modest either with capsaicin (I/II, 44 ± 13; III/IV, 20 ± 9) or glutamate (I/II, 19 ± 8; III/IV, 12 ± 6) at each peak time on the ipsilateral side (Figure 4, Table 1).

(a)

(b)

(c)

(d)
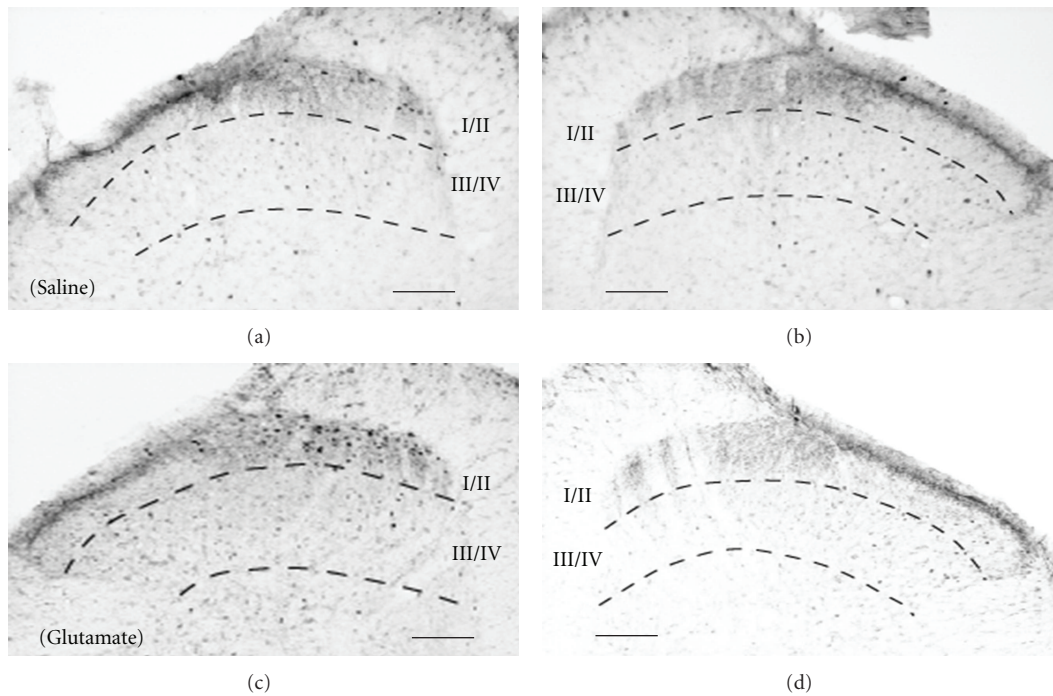
FIGURE 1: Photomicrographs showing saline-induced (a), (b) and glutamate-induced c-Fos-immunoreactive (ir) cells (c), (d) in the dorsal horn of L5 2 h after s.c. injection. (a) and (c) ipsilateral side. (b) and (d) contralateral side. Solid line indicates 100 μm.

(a)

(b)

FIGURE 2: Time course of number of c-Fos-ir cells in the dorsal horn of L4-L5 after s.c. injection of saline. All data presented are mean ± SEM obtained from six animals.
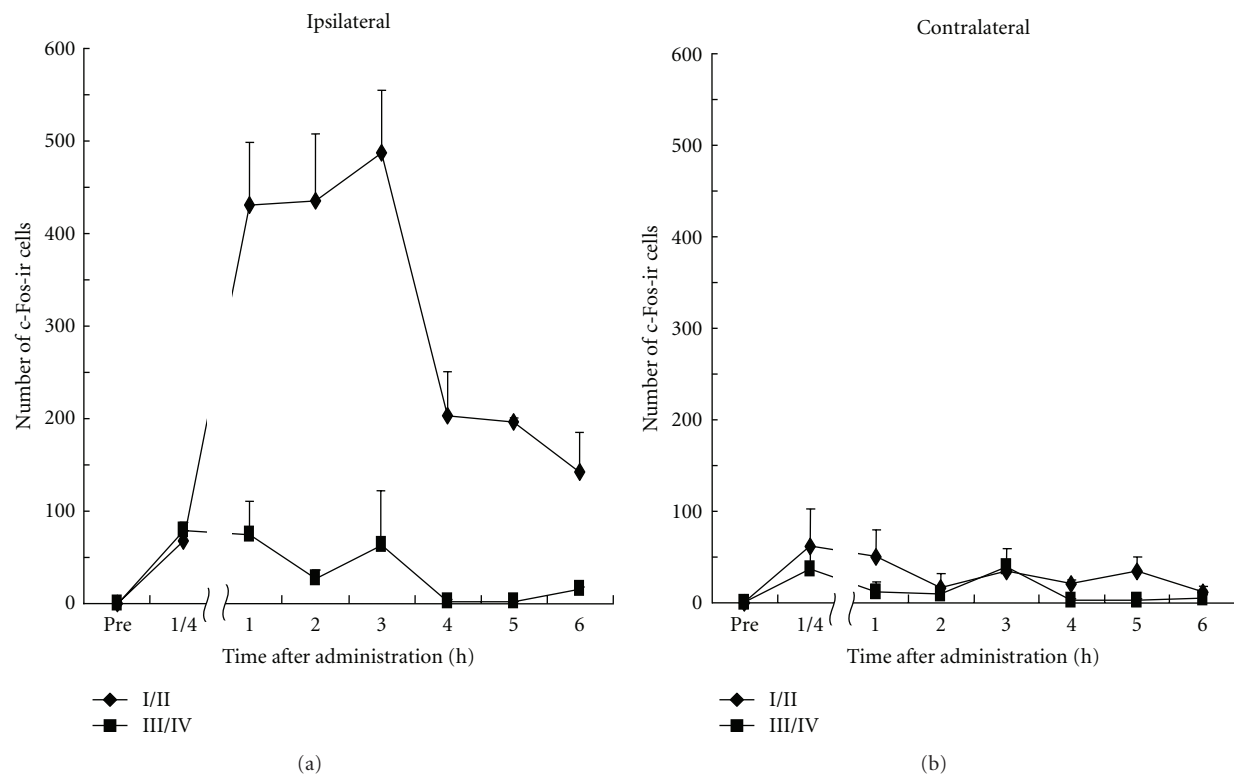
FIGURE 3: Time course of number of c-Fos-ir cells in the dorsal horn of L4-L5 after s.c. injection of capsaicin (3 mM). All data presented are mean ± SEM obtained from six animals.
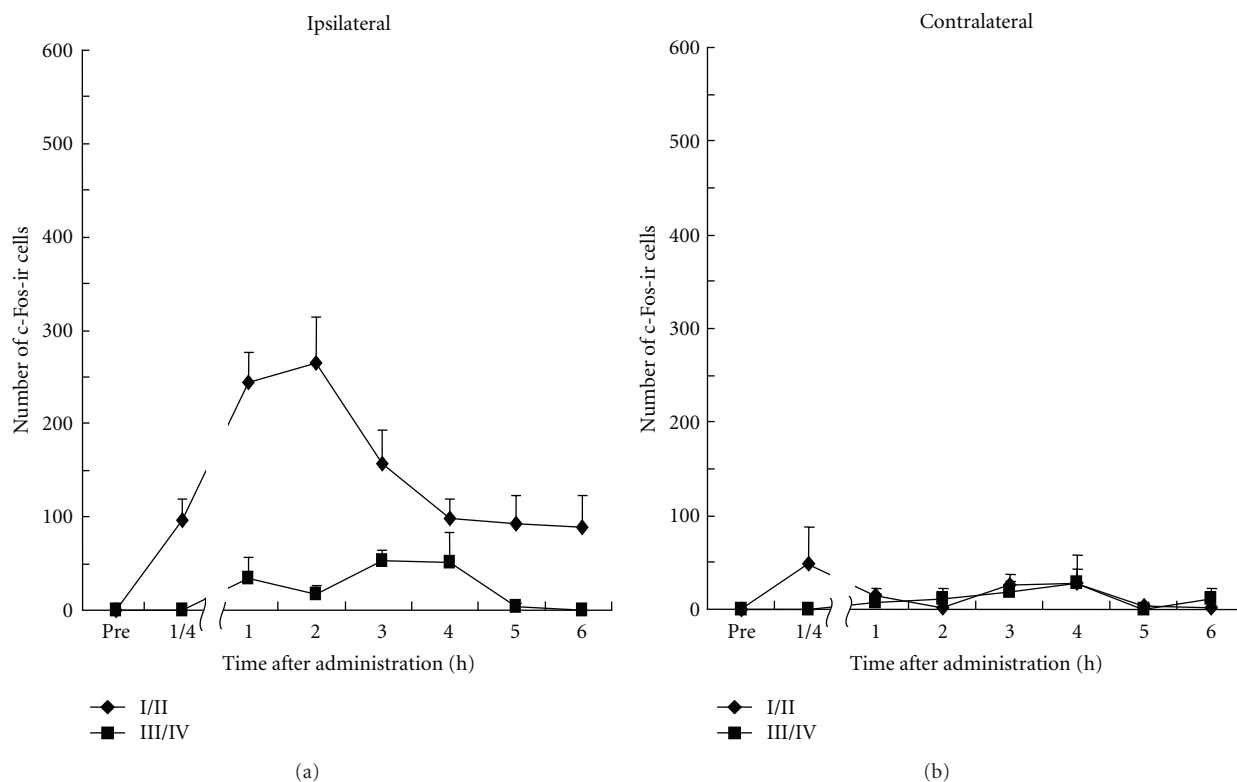
FIGURE 4: Time course of number of c-Fos-ir cells in the dorsal horn of L4-L5 after s.c. injection of glutamate. All data presented are mean ± SEM obtained from six animals.

(a)

(b)

(c)

(d)
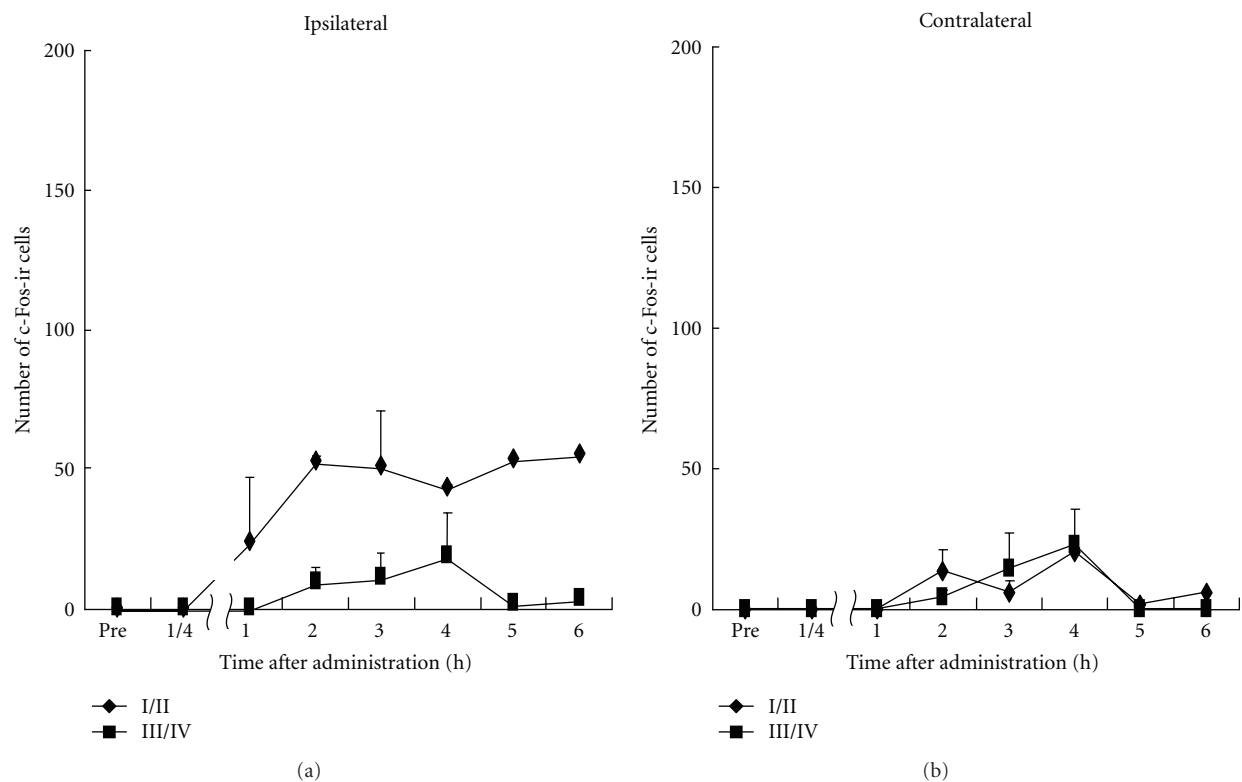
FIGURE 5: Photomicrographs showing capsaicin-induced c-Fos-ir cells in the dorsal horn of L5 2 h after s.c. injection of capsaicin alone (a), (b), or combined with MK801 ((c), 1 mM) or with CNQX ((d), 1 mM). All data presented are mean ± SEM obtained from six animals. (a), (c), and (d) ipsilateral side. (b) contralateral side. Solid line indicates $100\,\mu$m.

(a)

(b)

(c)

(d)

FIGURE 6: Photomicrographs showing capsaicin-induced c-Fos-ir cells in the dorsal horn of L5 h after s.c. injection of capsaicin alone (a), combined with CPCCOEt ((b), 5 mM), with MCCG ((c), 5 mM), an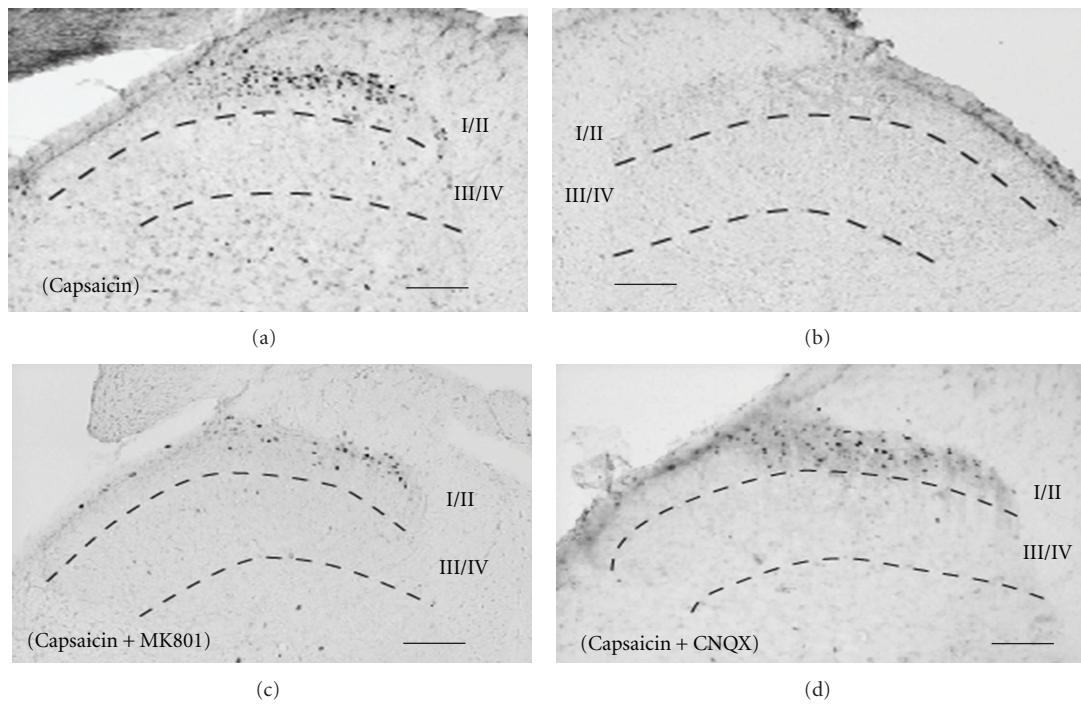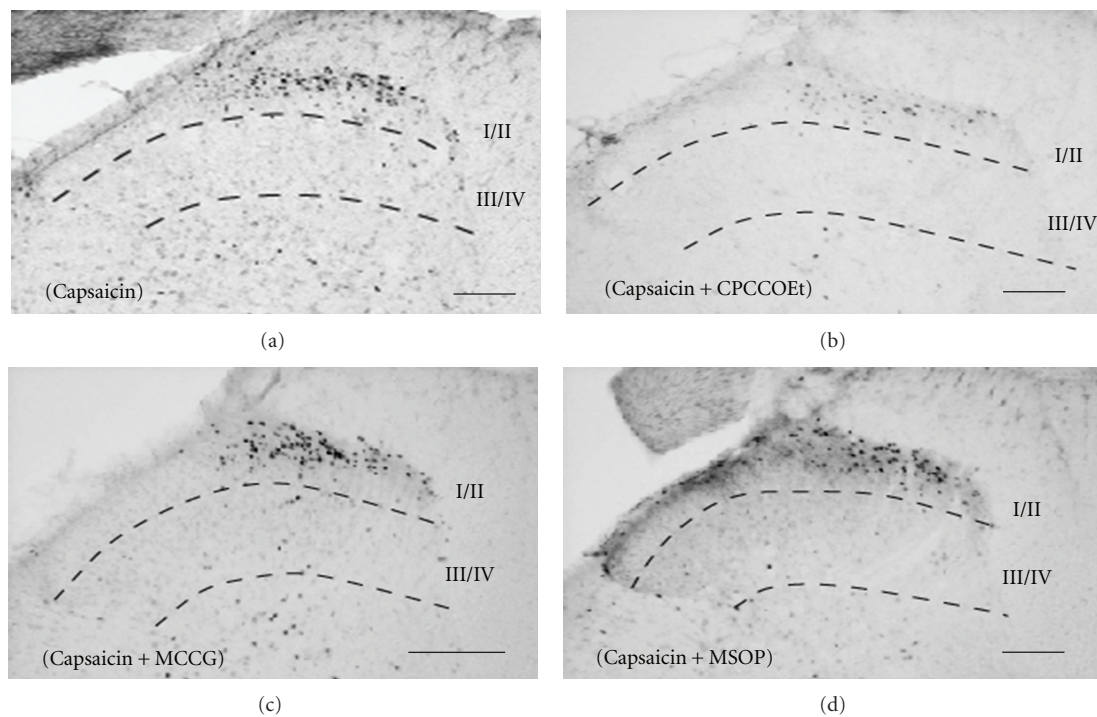d with MSOP ((d), 5 mM). All data presented are mean ± SEM obtained from six animals. (a), (b), (c), and (d) ipsilateral side. Solid line indicates $100\,\mu$m.

3.3. Effects of iGluRs Antagonists on the Capsaicin-Induced c-Fos Expression.

To investigate which types of GluRs were involved in the peripheral mechanisms whereby capsaicin evokes/maintains nociceptive responses, the effects of GluRs antagonists on the capsaicin-induced c-Fos expression were examined at 2 h after treatment, because the maximum number of labeled cells was observed 1 h–3 h after capsaicin injection.

Few c-Fos-ir cells were found in laminae I/II and laminae III/IV of the ipsilateral dorsal horn after each single injection of ionotropic glutamate receptors antagonists; MK-801 (1 mM) (I/II, 79 ± 3; III/IV, 11 ± 7) or CNQX (1 mM) (I/II, 70 ± 8; III/IV, 7 ± 3), similar to vehicle injection (I/II, 60 ± 5; III/IV, 22 ± 8). The numbers of capsaicin-induced c-Fos-ir cells in laminae I/II (489 ± 34), but not in laminae III/IV (63 ± 18), were significantly decreased, when MK801 and CNQX were injected with capsaicin (Cap + MK801, I/II, 227 ± 32, III/IV, 14 ± 4, Cap + CNQX, I/II, 205 ± 40, III/IV, and 11 ± 7) (Figure 5, Table 1). The numbers of capsaicin-induced c-Fos-ir cells on the contralateral sides did not significantly change by any of drugs with/without capsaicin.

3.4. Effects of mGluRs Antagonists on Capsaicin-Induced c-Fos Expression.

Few c-Fos-ir cells in the ipsilateral laminae I/II and III/IV, and fewer cells in the contralateral sides, were observed with single injection of CPCCOEt (5 mM) (I/II, 59 ± 8, III/IV, 1 ± 1), MCCG (5 mM) (I/II, 63 ± 10, III/IV, 3 ± 2), and MSOP (5 mM) (I/II, 66 ± 16, III/IV, 5 ± 3). Coinjection of CPCCOEt with capsaicin (Cap + CPCCOEt) significantly decreased the number of capsaicin-induced c-Fos-ir cells in the ipsilateral laminae I/II (236 ± 58), but not in laminae III/IV and contralateral laminae I/II and III/IV. There was no significant change in the number of c-Fos-ir cells in the ipsilateral laminae I/II, and III/IV by injection of MCCG combined with capsaicin (Cap + MCCG; I/II, 560 ± 85, III/IV, 27 ± 10) or by injection of MSOP combined with capsaicin (Cap + MSOP; I/II, 383 ± 21, III/IV, 22 ± 3) compared to single injection of capsaicin, respectively (Figure 6, Table 1).

4. Discussion

It has been previously reported that glutamate was released peripherally by electrical stimulation of the sciatic nerve, heat stimulation (50°C), or local application of capsaicin cream to the hind instep [2, 3]. High-dose repeated pretreatment with capsaicin leading to desensitization of small-diameter sensory neurons (nociceptors) [26–32], significantly attenuated the glutamate release.

Capsaicin significantly decreased thermal withdrawal latency to irradiation [12]. These effects of capsaicin were inhibited by not only pretreatment with capsazepine, but also coinjection of capsaicin with GluR antagonists (iGluRs and group I mGluR). These results suggest that glutamate is released from the peripheral endings of nociceptors by TRPV1 activation. Since DRG cells as well as their central and peripheral terminals express iGluRs (NMDA, AMPA/kainate receptor) and mGluRs (group I, II, and III mGluRs) [4–

9, 17], released glutamate could activate peripheral iGluRs and group I mGluR in development and/or maintenance of nociception.

In the present study, we examined the c-Fos expression as indicator of neuronal activity by pharmacological approach. To clarify the contribution of peripheral GluRs in development and/or maintenance of nociception, L4-L5 spinal dorsal horn neuronal activity, induced by intraplantar injection of drugs associated with glutamate receptors with/without capsaicin into the hindpaw, was investigated.

The subcutaneous injection of capsaicin or glutamate remarkably evoked c-Fos expression in ipsilateral sides of spinal cord dorsal horn and the maximum number of labeled cells occurred consistently in laminae I and II (I/II) of the spinal dorsal horn, where small-diameter DRG neurons terminate. The number of capsaicin-evoked c-Fos-immunoreactive (ir) cells was about twice as much as glutamate induced. A subset of capsaicin-sensitive nociceptors might express GluRs at the peripheral endings.

This capsaicin-evoked increase of c-Fos expression was significantly prevented by concomitant administration of MK801, CNQX, and CPCCOEt. These results were in agreement with the findings of the behavioral pharmacology, that is, capsaicin-induced thermal hypersensitivity was inhibited by the injection of the antagonists such as MK801, CNQX, or CPCCOEt in combination with capsaicin [12]. It should be noted that approximately 50% of the capsaicin-evoked c-Fos-ir cells were still positive, in spite of the complete inhibition of capsaicin-induced thermal hyperalgesia caused by injection of these antagonists [12]. On the other hand, there were not any significant changes in co-injection of capsaicin and MCCG or MSOP.

In conclusion, some of the capsaicin-sensitive neurons projected in laminae I and II (I/II) of the spinal cord play an essential role in mechanisms whereby capsaicin evokes/maintains nociceptive responses, though the function of the other capsaicin-sensitive neurons remained unclear. Ionotropic glutamate receptors and/or group I mGluR at the afferent terminals of these nociceptive neurons might be required for capsaicin-induced hyperalgesia, because blocking them resulted in remarkable analgesia [12].

Our present study not only supports the view that GluRs existing in peripheral3endings of capsaicin-sensitive afferent fibers are involved in the development and maintenance of hyperalgesia following excitation of TRPV1, but also suggests the probability of development of new medicines. Namely, the formulation of the peripheral iGluRs and group I mGluR antagonists that do not cross the blood brain barrier may be of potential benefit by reducing peripheral nociceptive excitability, and therefore they could provide a new therapeutic target to pain control in the periphery.

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Research Article

Aging Independently of the Hormonal Status Changes Pain Responses in Young Postmenopausal Women

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Both aging and hormonal status have an effect on pain perception. The goal of this study was to isolate as much as possible the effect of aging in postmenopausal women. Thirty-two women with regular menstrual cycles (RMW) and 18 postmenopausal women (PMW) underwent a 2-minute cold pressor test (CPT) to activate DNIC with a series of tonic heat pain stimulations with a contact thermode to assess ascending pain pathways. We found that this procedure induced much less pain during the first 15 seconds of stimulation the PMW group ($P = 0.03$), while the mean thermode pain ratings, pain tolerance, pain threshold, and DNIC analgesia were similar for both groups ($P > 0.05$). The absence of the peak pain in the PMW was probably due to reduced function of the myelinated Aδ fibers that naturally occurs with age.

1. Introduction

Aging brings a decline in the majority of sensory modalities including pain and touch, two sensory modalities involving A and C fibers [1]. These two types of primary afferent fibers carry touch-(A-β) and pain-related information (A-δ and C). Although the observed decline in function of the sensory system is observed above 65 years of age, there is evidence that pain-related functions start to decline around middle age ($\approx$50 years old) [2]. The results obtained by Larivière et al. showed a decline of function of the endogenous pain inhibitory system in middle age adults; this decline was similar to adults above 65 years old. Although these changes in pain perception can be due to aging, sex hormones could also be a factor influencing these changes since women reach menopause around 50 years of age and sex hormones levels significantly decrease. Although many biopsychosocial factors influence pain perception, it has been shown that sex hormones can influence many aspects of nociception [3]. This leads to the question: what happens to pain perception as women reach menopause?

Menopause, defined by the absence of menses for more than 12 months, usually occurs at about 50 years of age and results in significant changes in sex hormones levels: decreased progesterone (PRO) and estrogen (EST), increased luteinizing hormone (LH) and follicle-stimulating hormone (FSH). As we age, we generally tend to suffer from more pain-related pathologies [4], which are often more prevalent in women [5]. Comparable findings can be seen in midlife where postmenopausal women (PMW) are showing significant increases in clinical pain symptoms [6, 7]. Consequently, the changes in pain perception in this population of midlife women could be due to the decrease of sex hormones and/or aging.

We found no previous studies that examined specific aspects of nociception and pain perception in PMW compared to women with a regular menstrual cycle (RMW). Fillingim and Edwards [8] studied the effect of hormonal replacement therapy (HRT) on PMW and found that women on HRT had lower pain thresholds than PMW not on HRT. Although these results conflict with what is usually found in young healthy women, they do seem to indicate that

sex hormones could influence pain perception in an older population [8].

In our study, we used a cross-sectional design to examine pain perception in young PMW in comparison to RMW. Therefore, the study's main goal was to verify the influence of age on the ascending and descending pain mechanisms, while controlling for sex hormones levels, a potential confounding factor. To our knowledge, this has never been done and has significant clinical relevance for a large proportion of the population.

2. Methods

2.1. Participants. After approval from the hospital ethics review board, we collected data from 18 PMW (mean age 54.5 ± 5.4 years) and 32 RMW (mean age 34.3 ± 7.5 years). Subjects were recruited via local publicity and were all French-speaking women dwelling in the community. Brief initial phone interviews allowed for the screening of potential subjects and scheduling them for testing. On the day of testing, subjects were asked to refrain from smoking (only four RMW and three PMW self-reported as smokers) and/or drinking coffee one hour before testing. PMW were included if they met the Society of Obstetricians and Gynaecologists of Canada criteria for menopause (absence of menses during the past 12 months). Inclusion criteria for women in the RMW group were to have a regular menstrual cycle, which was defined as varying from 26 to 30 days in length. This criterion was confirmed by verifying menstrual-cycle length in the month before and after testing (self-reported). The average length was 28.4 ± 0.8 days. None of the RMW had any known disease or self-reported hypo/hypertension, or was taking pain medication. However, ten of the PMW reported having intermittent low back pain, but none were taking prescribed medication for this condition. Only three of them reported taking over-the-counter ibuprofen/acetaminophen as needed; when present, the mean intensity of their low back pain was 4/10 (numerical pain rating score).

All subjects signed an informed consent form and received $40 as compensation for taking part in the study. Each experimental procedure lasted about 90 minutes and took place at the *Centre de Recherche Clinique Étienne-LeBel du Centre Hospitalier Universitaire de Sherbrooke*, Sherbrooke, Québec, Canada.

2.2. Experimental Design. To control for a potential effect of sex hormones on pain responses, all RMW were tested during days 1 to 3 of their menstrual cycles, while PMW were tested at their convenience. We chose this time frame since sex hormones levels are at their lowest during menses. This enabled us to compare pain perception, while both groups have comparable sex hormones levels. The first day of menses was considered as day 1 of the menstrual cycle, which was obtained by self-reporting and confirmed by blood sampling, where we observed low levels of PRO, EST, and LH (all levels where within the normal reference values for this phase of the menstrual cycle). A qualified registered nurse took blood

samples for 17β-estradiol, PRO, FSH, LH, and testosterone dosage, prior to each experimental session.

2.3. Pain Procedures. All subjects underwent the experimental procedures in the same order (experimental heat pain, cold-pressor conditioning stimulus, and experimental heat pain).

2.3.1. Apparatus. The experimental heat pain was induced by a 9 cm^2 thermode (TSA II, NeuroSensory Analyzer, Medoc Instruments, North Carolina, USA). During this stimulus, pain perception was assessed with a computerized visual analogue scale (COVAS) linked to the thermode, which was graduated from 0 (absolutely no pain) to 100 (maximum tolerated pain). This allowed us to determine pain threshold (PTh), as measured by thermode temperature at which subjects reported initial pain sensation (visual analogue scale score: 1/100); pain tolerance (PTol), as measured by the maximum thermode temperature subjects could tolerate (visual analogue scale score: 100/100); and mean pain intensity of the noxious tonic stimulus. The conditioning stimulus was induced by a cold pressor test (CPT), which consisted of immersing the right arm (up to the elbow) in circulating cold water maintained at 12°C. During the CPT, subjects were asked to rate their pain intensity every 15 seconds with a numerical pain rating scale ranging from 0 to 100.

2.3.2. Pretest. Subjects were given a pretest for practicing pain rating with the visual analogue scale and to determine the temperature to be used for the heat pain test. The pretest was performed with the thermode applied to the right palm. For familiarization purposes, subjects were advised that the thermode temperature would gradually increase from 32°C to a maximum of 51°C (rising rate = 0.3°C/second). This procedure was repeated twice and the subjects verbally reported the point at which they actually began feeling pain (PTh) as well as PTol. On the third test, the thermode was placed on the volar aspect of the right forearm. Subjects were given the visual analogue scale and advised that they would have to start moving the cursor towards the right (towards the "100" mark) when they started to feel pain (PTh) and that the cursor had to be at the extreme right (at the "100" mark) when pain was intolerable (PTol) [9]. This procedure was repeated until the subject's pain reports were consistent between trials. The temperature used during the tonic experimental heat pain test was the temperature that the subject had rated pain intensity at 50/100 with the visual analogue scale during the pretest.

2.3.3. Tonic Experimental Heat Pain Stimulus. The tonic heat pain test was performed by applying the thermode at a constant temperature to the anterior (volar) aspect of the left forearm for two minutes [10]. Before the procedure, subjects were told that the thermode temperature could increase, remain stable, or decrease, and that they would have to evaluate their pain with the visual analogue scale throughout the test. In fact, after a constant rise (0.3°C/second) from

the baseline (32°C) to the predetermined temperature, the thermode temperature remained constant (mean = 46.1 ± 1.64°C—see Table 2 for mean thermode temperature used for each group) throughout the 120 seconds (ramp and hold). All subjects were blinded to the temperature used and to the study's hypothesis. Two observable events occurred during the tonic heat pain test [11, 12]. The first nociceptive event was characterized by a sharp but brief increase in pain intensity. This peak in pain intensity occurred when the thermode has reached its fixed temperature and lasted approximately 15 seconds. It was labeled "peak pain" because this is the interval in which heat sensitive A-delta fiber nociceptors display peak neuronal activity following constant stimulation at suprathreshold levels [13]. Peak pain has previously been described by Jensen and Petersen [14] using a similar design and repeated in our laboratories [9]. The second observable event was the rise in pain intensity that occurs during the last minute of stimulation. Since this increase in pain rating occurred at a set temperature, it clearly describes a temporal summation phenomenon (see Figure 1), which is known to depend on the summation of nociceptive inputs from primary afferent C-fibers. Granot et al. [15] also observed temporal summation effects using similar tonic heat stimulations. Previous research in our laboratory has shown that pain perception scores increase progressively during this tonic heat test, even if the temperature remains constant [9].

2.3.4. Activation of Diffuse Noxious Inhibitory Controls.

The conditioning stimulus (to induce DNIC) was applied to the opposite arm using the 12°C CPT. Pain intensity ratings were measured every 15 seconds during the test. If a subject removed her arm before the end of the 2 minutes, a pain intensity score of 100/100 was noted [16, 17]. The CPT enabled us to activate DNIC and was also used as a different type of tonic pain (cold pain versus heat pain; greater surface area than the 9 cm^2 thermode).

2.3.5. Assessment of DNIC Pain Modulation (Analgesia).

In order to measure the analgesic effect of the DNIC activated by the CPT, the experimental heat pain procedure was performed immediately after the immersion test using the same parameters. The amount of pain modulation produced by the CPT (DNIC efficacy) was calculated as the difference in pain score between the mean heat pain after and before the CPT. A negative score indicated a reduction in pain perception and therefore analgesia.

2.4. Statistical Analysis.

Descriptive statistics are presented as means and standard deviations (SD) in the text and as mean and standard error in the figures. Since our data were normally distributed, a Student's t-test was used for group comparisons of pain perception measurements and to assess the presence of DNIC analgesia, comparing the average pain score between the first and second experimental heat pain tests (thermode procedure). Afterwards, we compared the difference score (mean pain after minus mean pain before) for both groups, which also allowed us to quantify

TABLE 1: Sex hormones dosage by group.

	Sex Hormones: Mean ± SD (reference values)		
	RMW $n = 32$	PMW ($n = 18$)	P value*
Testosterone (total) (nmol/L)	1.15 ± 0.62 (0.7–2.8)	1.12 ± 0.57 (0.7–2.8)	0.86
Progesterone (nmol/L)	4.0 ± 4.1 (0.6–4.7)	2.51 ± 1.95 (0.3–2.5)	0.15
Estradiol (pmol/L)	151 ± 161 (46–607)	97.8 ± 66.0 (0–201)	0.18
FSH (IU/L)	6.15 ± 4.1 (3.5–12.5)	77.6 ± 36.4 (26–135)	<0.0001
LH (IU/L)	3.71 ± 1.19 (2.4–12.6)	40 ± 19.6 (8–59)	<0.0001

* RMW versus PMW.
Reference values for each hormone are reported in parenthesis.

DNIC analgesia. We used a t-test comparing the first and the last pain ratings during the last minute of the thermal stimulation with the thermode at constant temperature (T_{60} versus T_{120}) in order to confirm the presence of temporal summation. Temporal summation was then quantified by calculating the mean difference score between the first and the last pain ratings (pain rating at T_{120} minus T_{60}) [9]. This enabled us to obtain a delta score, which was compared to both groups. Peak pain was calculated by obtaining the mean pain score during this period (first 15 seconds of constant temperature stimulation). A P value of less than 0.05 was considered statistically significant.

3. Results

3.1. Sex Hormones Level.

The mean values for each sex hormone are illustrated in Table 1. All subjects had sex hormones within normal levels for each phase according to the reference values obtained from the biochemistry laboratory at the *Centre Hospitalier Universitaire de Sherbrooke* (http://www.lab.chus.qc.ca/). Sex hormones were comparable for both groups, except for FSH and LH, which were higher in the PMW group (a normal endocrine manifestation of menopause).

3.2. Pain Perception

3.2.1. Heat Pain Threshold and Heat Pain Tolerance.

Heat PTh and PTol, as measured by the thermode temperature (°C) at which a subject reports the onset of pain or tolerance, were similar for both groups (P = 0.46 and 0.29, resp.). When the elapsed time (sec) before reaching PTh was evaluated, the PMW took significantly longer to report the onset of pain (43.1 ± 7.69 seconds versus 36.3 ± 13.2 seconds; P = 0.05), indicating a trend towards higher PTh (sec) for PMW (see Table 2). Moreover, we found a significant correlation (r = 0.31; P = 0.02) between age and PTh (sec). No significant correlation was found between age and PTol, mean CPT pain intensity, or PTh (°C).
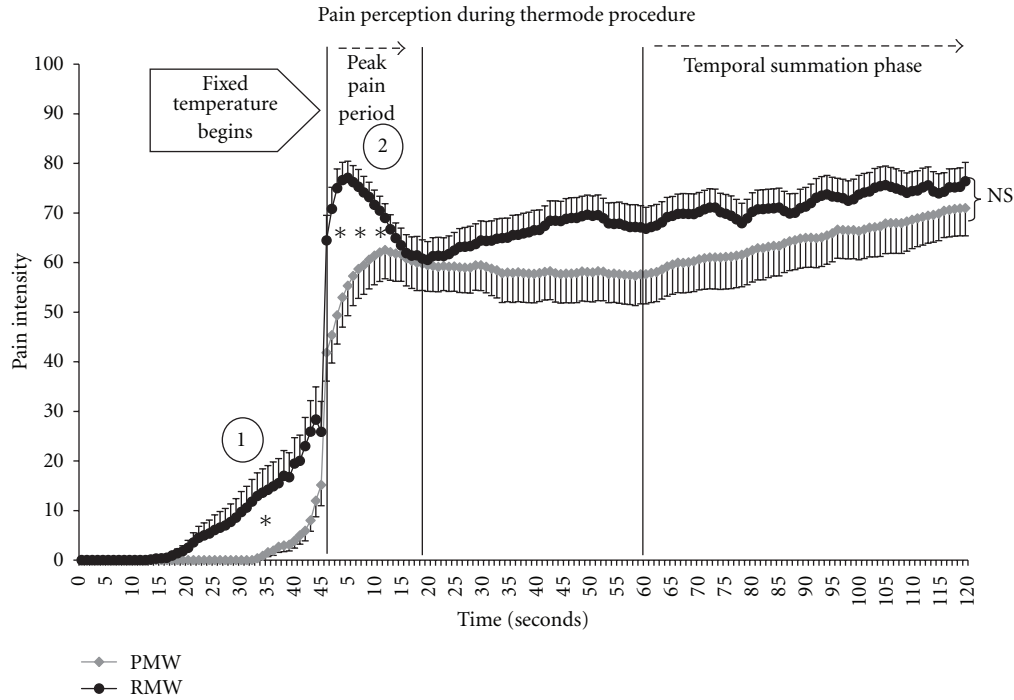
Pain perception during thermode procedure

FIGURE 1: Average heat pain (thermode) intensity for all subjects during the "before" session (mean ± SE). (1) The PMW group took longer to report initial pain and (2) the RMW had much higher peak pain than the PMW. Finally, the temporal summation phase (last minute of stimulation) was similar for both groups.

TABLE 2: Group comparison of each pain measurement (mean value ± standard deviation).

Variable	RMW ($n = 32$) (Mean ± SD)	PMW ($n = 18$) (Mean ± SD)	P value
Pain threshold (°C)	42.5 ± 3.01	43.2 ± 3.84	0.46
Pain threshold (seconds)	36.35 ± 13.2	43.1 ± 7.69	**0.05**
Pain tolerance	47.07 ± 1.77	46.7 ± 2.49	0.29
Fixed thermode temperature (°C)	46.3 ± 1.0	45.64 ± 2.37	0.18
Peak pain (mean T_0 to T_{15})	70.6 ± 15.3	57.44 ± 25.76	**0.03**
Last pain score (T_{120})	76.46 ± 19.76	70.66 ± 26.2	0.38
Thermode (before CPT) (mean T_0 to T_{120})	69.4 ± 17.08	60.75 ± 23.1	0.14
Thermode (after CPT) (mean T_0 to T_{120})	57.0 ± 21.6	48.89 ± 28.64	0.26
CPT mean pain intensity	53.8 ± 23.4	74.3 ± 24.8	**0.005**
TS delta score	9.57 ± 14.5	14.19 ± 17.34	0.39
DNIC delta score (T_{120} minus T_{60})	−12.4 ± 15.24	−11.85 ± 28.9	0.99

3.2.2. Tonic Pain Perception

(1) Cold Pressor Test. For an identical cold pain stimulus, the PMW reported significantly more pain during the CPT, where the mean pain intensity was 74.3 ± 24.8 compared to 57.44 ± 25.76 for the RMW ($P = 0.005$).

(2) Heat Pain Stimulus (Thermode). We found no significant group difference in the mean pain ratings during the heat pain test, where the mean intensity for the 120-second period was 60.75 ± 23.1 for the PMW and 69.4 ± 17.1 for the RMW ($P = 0.14$). This was expected since pain intensity was individually adjusted to a VAS of 50/100. Furthermore, the

thermode temperature used to evoke a pain score of 50/100 was similar for both groups ($P = 0.18$).

3.2.3. Peak Pain and Temporal Summation

(1) Peak Pain Period. The RMW reported significantly higher pain intensity during the peak pain period (first 15 seconds of constant stimulation) compared to the PMW, where the mean ratings for this period were 70.6 ± 15.3 for the RMW versus 57.44 ± 25.76 for the PMW ($P = 0.03$) (see Figure 1).

(2) Temporal Summation. Temporal summation did occur for both groups during the tonic heat pain test, where pain

Mean pain intensity ratings during
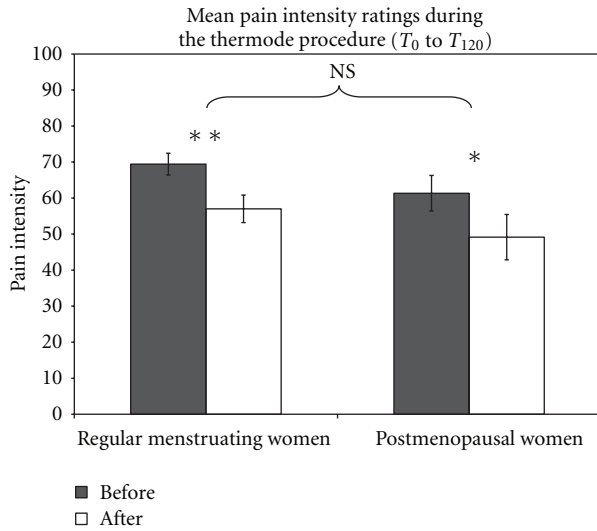the thermode procedure (T_0 to T_{120})

FIGURE 2: Mean heat pain ratings (thermode) were significantly lower after the CPT. Both groups had comparable changes (decrease) in pain intensity ratings following the CPT, indicating comparable DNIC analgesia.

intensity at T_{120} was significantly higher than at T_{60} (all $P < 0.05$). Furthermore, comparisons of the delta scores (T_{120} minus T_{60}) obtained for each group were also similar ($P = 0.39$), indicating that both groups had comparable temporal summation during the tonic heat pain procedure. Finally, pain ratings at the last point in time (T_{120}) were not significantly different ($P = 0.38$) (see Figure 1).

3.2.4. DNIC Analgesic Effect on Tonic Heat Pain. Mean pain ratings for both groups were significantly lower during the thermode procedure following the CPT compared to the first tonic heat pain. This indicates similar DNIC analgesia for both the RMW and PMW (see Figure 2). This was also confirmed by comparing the delta scores (mean difference in heat pain perception before and after the CPT), which were similar for both groups ($P = 0.99$). Detailed results of the above sections (Section 3.2) are presented in Table 2.

Finally, we reanalyzed the results for the variables where we found significant group differences (CPT pain, peak pain, and PTh) with covariance analysis controlling for FSH and LH (the only two sex hormones that significantly differed between both groups), only the "peak pain" remains significant ($P = 0.02$).

4. Discussion

In this study, we examined pain responses to different experimental type of nociceptive stimuli in a group of young PMW and compared the results to nonmenopaused women. We decided to proceed with the experiment while the RMW were during their menstrual phase, with the rational being that sex hormones levels are more comparable to PMW [18]; blood sampling enabled us to assure that both groups had comparable sex hormones levels for PRO and EST, the main female sex hormone.

The main finding of this study is that the PMW showed an absence of peak pain and a trend towards a delayed PTh (sec) during the heat pain procedure as clearly illustrated in Figure 1. Since female sex hormones were comparable between both groups at time of testing, it suggests that age, not PRO or EST, is the main factor for these changes in nociceptive activity. However, since FSH and LH were higher in the PMW group, we cannot exclude a potential effect of the sex hormones. However, when we analyzed the results for the variables where we found significant group differences (CPT pain, peak pain, and PTh) and statistically controlled for FSH and LH, only peak pain remained significant. This further supports that the observed group difference in peak pain is probably due to age rather than sex hormones.

On a physiological perspective, we think that these changes in pain perception derive from decreased Aδ-fiber function. Indeed, we and others have previously shown that the peak pain phase is always present during the tonic heat pain procedure; this has been demonstrated in young and middle-aged adults [9, 12, 14].

As Figure 1 clearly shows, the PMW took more time to reach their PTh (sec) but, most importantly, did not display the typical "rise and fall" pattern in pain perception observed during the first 15 seconds of constant stimulation. This difference in PTh (sec) is neither related to thermode temperature rate (both groups started at 32°C with a rising rate of 0.3°C/sec) nor to the thermode temperature used during the constant stimulation period (both groups had comparable temperatures). Therefore, an age-related factor would most likely account for these findings as suggested by the covariance analysis. Although a literature review concluded that there were no age-related changes in PTh or PTol [19], others previously demonstrated that aging produces an impairment in myelinated nociceptive fibers (i.e., Aδ fibers) [20], that experimental PTh does increase with age [4, 21], and that C-fiber activity remains intact with aging [22]. Most importantly, Chakour et al. demonstrated a differential age-related change in Aδ versus C-fiber pain perception. By blocking Aδ-fiber function in a group of young (20–40 years) and older subjects (>65 years), they found that both groups had comparable C-fiber function. The thermal PTh (sec), however, was affected only in the younger group, suggesting decreased Aδ-fiber function in the older subjects [20]. Although we found a significant correlation ($r = 0.31$; $P = 0.02$) between age and PTh (seconds), which indicated that only PTh increases with age, it only shows a modest association.

Moreover, Tucker et al. [23] also reported a decreased Aδ-fiber function as shown by an increased cutaneous pain threshold to the transcutaneous neuronal electrical stimulator. These findings closely relate to our study, since the peak pain period during the thermode test is most likely explained by Aδ-fiber activity [13]. To our knowledge, there is very few relevant literature than these results and the other studies mentioned supporting such changes in nociception and aging [24].

We also found that endogenous pain inhibitory mechanisms, more specifically diffuse noxious inhibitory controls (DNICs), were also equivalent between both groups. This

is somewhat different from what is found in the literature, since previous studies reported changes in DNIC with age [25, 26]. However, their samples were significantly older than our population of PMW. Furthermore, they compared their "older" groups to a group of young males and females, without controlling for menstrual-cycle phase, which is known to affect DNIC efficacy [27]. The effect of sex hormones on DNIC efficacy was significant only during the ovulatory phase, a phase which represents only 3 to 5 days of the complete menstrual cycle, while in the present study, the data were collected during the menstrual phase of the cycle [27].

A recent study conducted in our laboratories has also shown a decrease in DNIC efficacy after the age of 45 [2]. These results might be accounted for, in part, by the fact that a different experimental design was used and that sex hormones levels or menstrual-cycle phase was not controlled. Furthermore, although the PMW and RMW in our sample show comparable DNIC analgesia, the PMW reported more pain during the conditioning stimuli (cold pressor test) (see Section 3.2.2 (1)), yet they showed similar DNIC analgesia. This could suggest that the PMW would have had lower DNIC analgesia if they had lower pain score during the conditioning stimulus. However, we did not observe any significant interaction between DNIC efficacy (delta scores) and CPT pain intensity used as a covariable ($P = 0.60$). This observation strongly suggests that DNIC analgesia is therefore comparable in both groups. Consequently, we think that our PMW were probably too young to allow us to observe any age difference in DNIC.

The results regarding tonic painful procedures and PTol suggest that PMW have comparable C-fiber nociceptive activity. Although our sample of PMW reported greater pain ratings during the CPT, we observed no significant difference in temporal summation of heat pain, an event mostly related to C-fibers [15]. The fact that PMW had higher pain intensity ratings during the CPT, but not elsewhere, might be related to the greater affective component of this test. The cold pressor test has been shown to induce higher estimates of unpleasantness, and thus may better mimic clinical pain [28], which 10 women in the PMW group reported. Furthermore, the fact that PMW had higher pain intensity ratings during the CPT could also be explained by the specific effect of hormonal changes on mood by menopause, where PMW are at higher risk of depression [29]; depression is known to negatively influence pain perception [30]. Also, since the cold pressor test pain is mainly related to the activity of C-fibers [31], it may well be a separate effect between a-delta and c-fibers. Finally, aging has previously been demonstrated to be related to a reduction of cutaneous pain but an augmentation of deep pain [32]. Since the thermode produces a cutaneous pain and that the cold pressor test produces deep pain, it may explain the apparently contradictory results.

These results have important clinical implications. First, it shows that quantitative sensory testing, such as the heat pain procedure, brings useful information for the detection of impairments in the peripheral nervous system. Moreover, since our test seems to discriminate Aδ and C-fiber activity,

it could serve as an objective criterion for measuring the severity of pain-related disorders, such as neuropathic pain.

Our study has potential limitations. First, we know that LH and FSH levels were higher in the PMW group. Hence, we cannot ignore the possibility that the observed differences in LH and FSH are indeed responsible in the observed pain responses rather than age. There is, however, no way of controlling for these two specific sex hormones. As mentioned in the introduction, the higher LH and FSH levels are natural manifestations of menopause. Moreover, past studies on pain perception and sex hormones did not reveal that LH or FSH had any effect on pain perception [18, 33]. Finally, the fact that 10 PMW reported lower-back pain could be a potential confounding factor. In fact, studies show that low back pain can sensitize [34] the central nervous system which could then explain why PMW women had greater pain during the cold pressor test. However, this could not explain why PMW had lower pain ratings (peak pain) during the 2-minute heat pain test with the thermode. However, since low back pain symptoms are frequent in the general population, it adds to the external validity, since the prevalence of painful conditions (such as low back pain) usually increases with age [4].

The age effects we observed are probably not limited to women. Since there was no control group of men, it would be imprudent to imply that the external validity of our results applies to men. More research is needed to address this question.

In conclusion, age seems to be the main factor influencing changes in tonic pain perception in our group of midlife PMW. The absence of the peak pain in the PMW was probably due to a reduction of function in myelinated Aδ fibers that naturally occurs with age. Interestingly, these changes in pain perception occurred as early as 50 years old, which is congruent with recent literature [2]. These pain-related changes in postmenopausal women clearly demonstrate the importance of studying nociception and endogenous pain modulation in this population.

Acknowledgments

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Review Article

Estrogen and Visceral Nociception at the Level of Primary Sensory Neurons

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Clinical studies suggest the comorbidity of functional pain syndromes such as irritable bowel syndrome, painful bladder syndrome, chronic pelvic pain, and somatoform disorders approaches 40% to 60%. The incidence of episodic or persistent visceral pain associated with these "functional" disorders is two to three times higher in women than in men. One of the possible explanations for this phenomenon is estrogen modulation of viscerovisceral cross-sensitization. While a central site of this modulation has been shown previously, our studies suggest a peripheral site, the dorsal root ganglion (DRG). Estrogens have remarkably wide range of functions including modulation of voltage-gated calcium channels (VGCCs) and purinoreceptors (P2Xs). Significantly, inflammation dramatically alters purinoception by causing a several fold increase in ATP-activated current, alters the voltage dependence of P2X receptors, and enhances the expression of P2X receptors increasing neuronal hypersensitivity. Gonadal hormones are thought as indispensable cornerstones of the normal development and function, but it appears that no body region, no neuronal circuit, and virtually no cell is unaffected by them. Thus, increasing awareness toward estrogens appears to be obligatory.

1. DRG Neurons and Visceral Sensitization

Sex hormones and 17β-estradiol (E$_2$), in particular, directly influence the functions of primary afferent neurons. However, E$_2$ has a multiplicity of actions: membrane, cytoplasmic, and nuclear: E$_2$ modulates cellular activity by altering ion channel opening, G-protein signaling, and activation of trophic factor-like signal transduction pathways [1]. DRG neurons in culture express receptors of nociceptive signals [2] and retain most, if not all, their intracellular signaling cascades. DRG neurons *in vitro* are a valuable preparation because adult primary sensory neurons can be studied without the interference of modulation by central or peripheral messengers. Visceral afferents are sensitive to ATP [3], and several indirect pieces of evidence suggest that visceral afferents are E$_2$-sensitive: (i) visceral pain is affected by hormonal level in cycling females [4]; (ii) there are gender differences in the prevalence of functional disorders involving the viscera [5]; (iii) putative visceral afferents [6] fit into the population of DRG neurons that are E$_2$-sensitive. Although it is generally accepted that each primary afferent

neuron is a single sensory channel, several studies have challenged that view and demonstrate that a population of DRG neuron can innervate both the viscera and somatic tissues.

Both subtypes of estrogen receptors (ERα and ERβ) are present in small-diameter DRG neurons, presumably nociceptors. Relevantly, estrogen receptors are distributed in regions of the central and peripheral nervous system that mediate nociception. A large body of the literature supports the idea that E$_2$ modulates nociceptive responses in pelvic pain syndromes; however, whether E$_2$ is pro- or antinociceptive remains unresolved. Within the context of our hypothesis, E$_2$ modulation of nociceptive response depends on the type of pain, its durations, and the involvement of other antinociceptive mechanisms. Visceral nociception and nociceptor sensitization appear to be regulated by ATP and substance P [7], and DRG is an important site of visceral afferent convergence and cross-sensitization.

Mechanisms of viscerovisceral hyperalgesia between organs with documented partially common sensory projection probably involve sensitization of visceroviscero-somatic convergent neurons [8]. Within the context of our hypothesis,
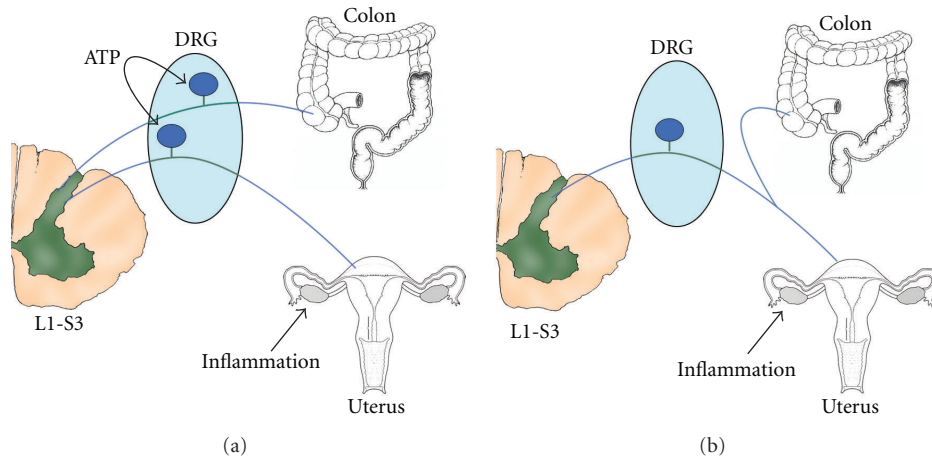
(a)　　　　　　　　　　　　　　　(b)

FIGURE 1: Models of alternative possibilities for viscero-visceral cross-sensitization in the DRG neuron. (a) ATP released by a neuron innervating the inflamed uterus acts on a neighboring neuron sensitizing its responses to colonic distention. (b) The same neuron innervates the uterus and colon. Uterus inflammation directly sensitizes the neuron to colonic distention.

sensory neurons can release an ATP intraganglionically during inflammation. The inflammation in the reproductive tract can cross-sensitize the response to ATP in colonic DRGs. Although it has been accepted that each primary afferent neuron is a single sensory channel (Figure 1(a)), several studies has challenged that view [9], and our own data using retrograde labeling demonstrate that population of DRG receive sensory input from different visceral organs: uterus and colon [10] and that inflammation in the uterus upregulated nociceptive signaling in the colon [11] (Figure 1(b)).

2. Transduction of Visceral Nociception

Pain is an unpleasant sensory and emotional experience associated with actual or potential damage or depicted in terms of such damage and normal transmitting pain system is absolutely essential to keep the integrity of our body [12]. The incidence of persistent, episodic, or chronic "functional" visceral pain associated with functional disorders such as irritable bowel syndrome, fibromyalgia, painful bladder syndrome, chronic pelvic pain, and others is much more prevalent in females [13, 14]. A meta-analysis of acute experimental pain studies (pressure pain and electrical stimulation) using a "box score" methodology in humans shows that females display greater sensitivity and that pain threshold and tolerance are modulated across the menstrual cycle [4]. 17β-estradiol (E_2) may account for these observed fluctuations in pain perception and symptoms [13]. Thus, a possible explanation is that E_2 may have different actions on nociceptive signaling depending on the origin of the noxious stimulus. The localization of both estrogen receptors $ER\alpha$ and $ER\beta$ in DRG neurons and the attenuation of ATP-induced $[Ca^{2+}]_i$ [15] strongly suggest that E_2 modulates visceral pain processing peripherally.

Nociceptors were defined as receptors by Sherrington more than a century ago (1906) that respond selectively to stimuli that cause tissue damage. The nociceptors can be divided into two main groups based on anatomical and functional properties: Activity in myelinated $A\delta$-fiber nociceptors gives rise to the initial sharp feeling of pain while on the contrary unmyelinated C-fiber nociceptors gives rise to the later, duller, burning pain sensation due to their different morphology. The cell bodies of these axons are located in the DRG.

The molecular and cellular mechanism of nociception has been defined during the past two decades. Molecular function of nociceptors depends on expression of different nociceptive receptors and ion channels that can be activated by noxious stimuli. These receptors and ion channels detect specific physical or chemical stimuli and induce membrane depolarization. Among these nociceptive receptors and ion channels, transient receptor potential ion channel (TRP)-related TRPV family and purinergic receptors ($P2X_2$ and $P2X_3$) have been very well studied and acknowledged as molecular detectors of noxious thermal, mechanical, or chemical stimuli [16]. Visceral nociceptive C-fibers are activated by ATP released by noxious stimuli from cells in target organs and have been implicated as mediators of noxious stimulus intensities [3]. Alteration in signal transduction of primary afferent neurons can result in enhanced perception of the visceral sensation, which is common in patients with different disorders resulting in elevated pain perception. Peripheral sensitization can develop in response to sustain stimulation, inflammation, and nerve injury.

3. Nongenomic Mechanism of Estrogen Action on Visceral Nociceptors

Both estrogen receptor α ($ER\alpha$) and estrogen receptor β ($ER\beta$) belong to nuclear receptors of the steroid/thyroid superfamily, in which the members have structural and functional similarities such as a ligand-dependent transcription factor that modulates gene expression. There is a high homology between $ER\alpha$ and $ER\beta$ in the DNA-binding domain

(97%), but a moderate homology in the ligand-binding domain (55%), resulting in somewhat lower affinities for endogenous 17α- and 17β-estradiol to ERβ than ERα.

A variety of cell types respond rapidly to E_2, making a nongenomic mechanism of action. Some nongenomic activities of estrogens may be explained by the presence of classic estrogen receptors [17–19], but a brain-specific ERX has also been suggested [20]. However, this putative new receptor has been expressed only in glial cells and can be activated by the inactive 17α-estradiol stereoisomer. Overall, the nongenomic estrogen effects are thought to be mediated by ERα that is sequestered to the cytoplasm or the plasma membrane within a signaling complex [1]. The initial evidence for this concept was provided by studies showing that signal transduction is initiated by estradiol conjugated to large, membrane impermeant molecules such as bovine serum albumin [15]. The local synthesis of estrogens in the brain provides high concentrations of estrogens locally. In fact, local synthesis of estrogens in the brain seems to be a requirement for the rapid signaling of estrogen receptor since the cyclic changes of plasma hormone levels are too slow to fit this rapid pattern of activation. Many effects of estradiol in the brain, such as part of the regulation of lordosis behavior [21] and signaling in the dorsal root ganglion [7, 18], are in the time range proposing nonclassic mechanisms.

ERs distributed through CNS and PNS including regions that mediate nociception. For example, ERs are expressed in dorsal horn neurons of the spinal cord [22, 23] and DRG neurons [24]. DRG neurons express both ERα and ERβ *in vivo* [25] and *in vitro* [10]. These findings suggest that E_2 may modulate sensory input at the primary afferent level. E_2 can alter gene transcription, resulting in pronociceptive (reducing β-endorphin expression) or antinociceptive (increasing enkephalin expression) changes of endogenous opioid peptides and opioid receptors [22, 26, 27]. E_2 can modulate cellular activity by altering ion channel opening and second messenger signaling by stimulating G-proteins [15, 28–30], the signal transduction pathways traditionally associated with membrane receptor activation. Many of these effects have been ascribed to membrane-associated receptors [31]. The results from other laboratories [28, 32] and our data [15] indicate that E_2 is acting to modulate L-type VGCC.

Adenosine 5′-triphosphate (ATP) is one of the most common chemical compounds in living cells. Neurons represent a remarkable source of ATP, as a neurotransmitter, which is widely used in both central and peripheral nervous systems [33]. ATP can be released from vesicle pools, or it can be coreleased and/or costored together with classical neurotransmitters in neurons. A decade ago, ATP was shown to be an extracellular signal involved in peripheral hyperalgesia [34]. One of ATP's best defined roles has been described in sensory transduction of noxious stimuli by activating purinergic, ATP-gated P2X receptors on primary afferent fibers [33]. ATP release from tissues during pathological conditions that cause tissue damage or inflammation activates P2X receptors on primary afferent fibers innervating the afferent organs [35].

Some molecular targets have been identified as key players in the activation and sensitization of visceral nociceptors, notably ASICs, TTX-resistant Na channels, and the TRPV1 receptor [36]. TRPV1 receptor is a sensory neuron-specific cation channel which belongs to the transient receptor potential subfamily 1 and plays an important role in transporting thermal and inflammatory pain signals. Evidence for TRPV1's role in their pathogenesis comes from studies showing that mice lacking TRP1R gene have deficits in thermal- or inflammatory-induced hyperalgesia [37]. Activation of both TRPV1 and P2X receptors induce mobilization of $[Ca^{2+}]_i$ in cultured DRG neurons [38]. Capsaicin-induced TPRV1 receptor-mediated changes in $[Ca^{2+}]_i$ may represent a level of DRG activation to noxious cutaneous stimulation, while ATP-induced changes in $[Ca^{2+}]_i$ may reflect the level of DRG neuron sensitization to noxious visceral stimuli since ATP is released by noxious stimuli and tissue damage near the primary afferent nerve terminals [39].

The expression of transient receptor potential vanilloid 1 (TRPV1) receptors is widespread in several areas of the nervous system, but it is particularly strong in DRG. TRPV1 receptors are at the cellular membrane as well as in the membrane of intracellular calcium stores (e.g., endoplasmatic reticulum). For pain transmission, the pain/temperature sensitive TRPV1 receptor is highly expressed in nociceptive neurons of the peripheral nerve system. TRPV1 receptors are activated by a wide variety of stimuli, both exogenous (capsaicin, noxious heat) and endogenous (protons, lipoxygenase products, anandamide, dopamine), and they mediate a nonselective cationic current with particularly high Ca^{2+} permeability [37]. The potentiating of TRPV1 activity can be quantified by measuring the differences of capsaicin-induced Ca^{2+} concentration changes before and after receptor activation [40]. Significantly, a subset of DRG neurons respond to both capsaicin and ATP [7] indicating that there may be cross-activation of these receptors that may underlie the sensitization of visceral nociceptors.

The action of estrogens at the level of primary sensory neurons is complex and determined by the characteristics of the target genes and coregulators, as well as, the regional availability of E_2. Several authors have suggested that the sex differences in pain sensitivity and the prevalence of chronic pain disorders may result from a malfunctioning endogenous pain inhibitory response rather than an increase nociceptive activity [41]. Many previous studies have established connections between estrogens and the modulation of different nociceptive pathways. One of the mechanisms may be estrogen inhibition of nociceptive signaling (mediated by both P2X3 and TRPV1) through interaction with mGluR$_{2/3}$. Painful stimuli initiate action potentials in the peripheral terminals of DRG neurons evoking release of excitatory neurotransmitters such as glutamate (Figure 2). Ligand-gated P2X receptors which can be activated by endogenous ATP during induction of action potentials are highly expressed in identified nociceptors [42]. Thus, E_2-modulated encoding of nociceptive stimuli at the level of primary sensory neurons may contribute to our understanding of complex mechanism of nongenomic effect of gonadal steroids.
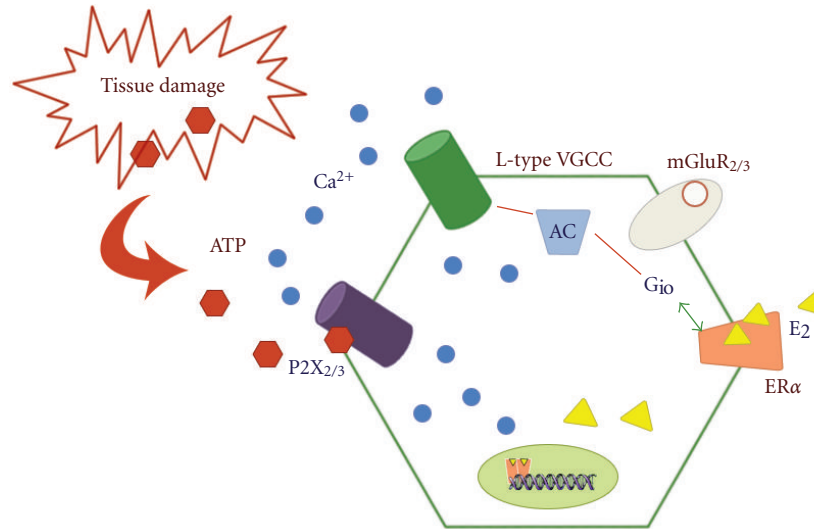
Figure 2: Proposed mechanism of estradiol effect on ATP-induced $[Ca^{2+}]_i$ signaling in primary sensory neurons. ATP released by tissue damage acts on $P2X_3$ receptor resulting in activation of the L-type voltage-gated calcium channel (VGCC). ERα activates mGluR2/3 which in turn activates Gi/o signaling resulting in inhibition of adenylate-cyclase (AC). Decreased cAMP concentration reduced PKA activation and decreased the conductance of the L-type VGCC.

4. Conclusions

The fact that homeostatic changes are similar in all "functional" disorders suggests a model in which alteration in the neuronal circuits in predisposed individuals is triggered by the similar pathophysiology. Both physiologic and psychological variables appear to play important roles in the development of functional syndromes, and psychologically oriented treatments have a role in their management. Patients have an altered perception of visceral sensation that is typically manifested as hyperalgesia, an enhanced perception of pain. Pain is the symptom that patients with functional disorders list as the most depressing and is a major factor for consulting a physician. The functional characteristics of primary sensory (small diameter, presumably nociceptors) neurons and their receptors are usually investigated by means of a wide diversity of experimental models, both *in vivo* and *in vitro*. Several *in vivo* animal models have been developed and characterized for specific sensory modalities, including nociception, particularly in rodents [2]. The use of these models suggests the advantage of a physiological/pathological condition, and it is hence a basic step in the understanding of the fundamental mechanisms underlying the function of sensory systems and for the development and proof of new possible strategies for their modulation. As far as the modulation of receptors involved in nociceptive input transmission is concerned, it is indeed possible to reproduce in rodents various painful states observed in humans. The deficiency of suitable animal models for certain pain syndromes requires the use of strictly controlled *in vitro* paradigms in order to dissect the fundamental properties of the basic processes. One possible approach to the study of the functional characteristics of neurotransmitter (i.e. ATP) release and activated ion channel receptor is the direct recording of the receptor response to its agonist and antag-

onist on isolated cells expressing the receptor, by means of calcium imaging or patch clamp recordings techniques. Histological and functional studies implied that the important receptors involved in the transmission and modulation of nociceptive inputs are expressed at the level of the peripheral and/or central terminals of DRG neurons (e.g., $P2X_3$ and TRPV1).

Although it is generally accepted that each primary afferent neuron is a single sensory channel, several studies have challenged that view and demonstrate that a population of DRG neuron can innervate both the viscera and somatic tissues. The design of the proposed studies using retrograde labeling from the uterus and colon will address the possibility that the same primary afferent can innervate both reproductive and gastrointestinal organs (DRG neuron will have both retrograde tract tracing dyes). This new subset of dichotomizing fibers provides a novel pathway for sensitization of one viscus by another. Adult DRG neurons in short-term culture retain the expression of receptors (P2X and TRPV1) [2] which mediate the response to putative nociceptive signals. They continue to respond to ER agonists mimicking *in vivo* activation. An important advantage is that these neurons can be studied apart from endogenous signals. A large body of literature supports the idea that E_2 modulates nociceptive responses in functional pain syndromes. Within the context of our hypothesis, E_2 modulation of nociceptive responses depends on the type of pain, its durations, and the involvement of other antinociceptive mechanisms. E_2 modulates DRG neurons response to ATP, suggesting that visceral afferent nociceptors are modulated by E_2, which may explain the observed clinical and animal gender differences in visceral hypersensitivity and suggests a potential target for mediating nociception. Future direction of research should include more experiments to study the role of peripheral ERs in visceral nociception using *in vivo* models. Thus, from

a public health perspective, the outcome of this study will have a substantial impact, because it will increase our knowledge of nociceptive functional diseases, such as IBS, interstitial cystitis, and chronic pelvic pain, and help achieve a deeper understanding of gender differences presented in clinical aspects of these symptoms associated with various psychiatric disorders. Only a thorough understanding of the mechanism implicated in these phenomena can truly contribute to the design of new and more efficient therapies. Many illnesses affect women and men differently. Some disorders are more common in women, and some express themselves with different symptoms. It is very well documented that differences between the sexes exist in the prevalence and severity of a broad range of diseases, disorders, and conditions. In calling for greater focus on gender-based biomedical research, the clinical and scientific communities will be better equipped to identify barriers to the achievement of knowledge about gender differences-including ethical, financial, sociological, and scientific factors. In developing countries, pain accounts for nearly 20% of all primary health care visits. Studies have shown that at least one-third of patients with pain also suffer from depression and it affects more women than men. The new approach in pain research and treatment should address a crucial question in women's health and in visceral nociception in particular. Reaching further, this research is a liaison between the basic science work and the clinical aspects that are addressed through other disciplines such as anesthesiology (pain management), gastroenterology, obstetrics, and gynecology.

Conflict of Interests

The author has delare that there is no conflict of interests.

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