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IB4-Saporin Attenuates Acute and Eliminates Chronic Muscle Pain in the Rat

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Abstract

The function of populations of nociceptors in muscle pain syndromes remain poorly understood. We compared the contribution of two major classes, isolectin B4-positive (IB4(+)) and IB4-negative (IB4(−)) nociceptors, in acute and chronic inflammatory and ergonomic muscle pain. Baseline mechanical nociceptive threshold was assessed in the gastrocnemius muscle of rats treated with IB4-saporin, which selectively destroys IB4(+) nociceptors. Rats were then submitted to models of acute inflammatory (intramuscular carrageenan)- or ergonomic intervention (eccentric exercise or vibration)-induced muscle pain, and each of the three models also evaluated for the transition from acute to chronic pain, manifest as prolongation of prostaglandin E2 (PGE₂)-induced hyperalgesia, after recovery from the hyperalgesia induced by acute inflammation or ergonomic interventions. IB4-saporin treatment did not affect baseline mechanical nociceptive threshold. However, compared to controls, IB4-saporin treated rats exhibited shorter duration mechanical hyperalgesia in all three models and attenuated peak hyperalgesia in the ergonomic pain models. And, IB4-saporin treatment completely prevented prolongation of PGE₂-induced mechanical hyperalgesia. Thus, IB4(+) and IB4(−) neurons contribute to acute muscle hyperalgesia induced by diverse insults. However, only IB4+ nociceptors participate in the long term consequence of acute hyperalgesia. Finally, using retrograde labelling we found that approximately 70% of sensory neurons innervating the gastrocnemius muscle are IB4(+).

Keywords

Myalgia; nociceptor; isolectin B4; chronic muscle pain

Introduction

Discrete subsets of nociceptors are not only connected to different anatomical pathways but also exhibit distinct functional properties (Braz et al., 2005; Zylka et al., 2005; Joseph and Levine, 2010). One main subset of nociceptors are those that bind the plant lectin, isolectin B4 (IB4) (Molliver et al., 1997). IB4-positive (IB4(+)) nociceptors comprise small- to

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medium-diameter dorsal root ganglion (DRG) neurons which project mainly to the middle/inner part of lamina II of the dorsal horn (Snider and McMahon, 1998; Zylka et al., 2005). In contrast, IB4(−) nociceptors project to lamina I and outer lamina II (Snider and McMahon, 1998). At their peripheral terminals in the skin, IB4(+) nociceptors terminate more superficially in the *stratum granulosum*, while IB4(−) nociceptors terminate more deeply in the *stratum spinosum* (Zylka et al., 2005). Additionally, most IB4(+) neurons also express the enzyme fluoride resistant acid phosphatase (FRAP, also called thiamine monophosphatase, TMP), which has been recently shown to correspond to prostatic acid phosphatase (Molander et al., 1987; Bradbury et al., 1998; Taylor-Blake and Zylka, 2010), and the P2X₃ purinoceptor (Snider and McMahon, 1998; Bradbury et al., 1998).

IB4(+) DRG neurons have been described as representing between 5 and 40% of muscle afferents (Ambalavanar et al., 2003; Pierce et al., 2006), and other markers of this subset of sensory neurons such as TMP (Molander et al., 1987; O'Brien et al., 1989) and P2X₃ (Shin et al., 2008) are expressed in roughly 15% of DRG neurons. Certainly, most studies indicate a much larger percentage of IB4(+) neurons in the skin (Molander et al., 1987; O'Brien et al., 1989; Plenderleith and Snow, 1993; Ambalavanar et al., 2003; Pierce et al., 2006). While this raises the possibility that they are less important in muscle pain syndromes, there is a lack of consensus regarding the role of IB4(+) neurons (Ambalavanar et al., 2003; Pierce et al., 2006). However, relay of muscle sensory information to the main target of IB4(+) nociceptors in the dorsal horn, the inner lamina II (Della Torre et al., 1996) does suggest their role in muscle nociception. Indeed, a selective responsiveness of gastrocnemius nerve to C-fiber antidromic stimuli delivered to inner lamina II has been reported (McMahon and Wall, 1985). Furthermore, studies have shown that unmyelinated C-fibers that innervate the gastrocnemius muscle project densely to inner lamina II of the spinal dorsal horn (Ling et al., 2003; Panneton et al., 2005). And, intense muscular activity induces a selective Fos expression in the inner lamina II dorsal horn (Jasmin et al., 1994). At least some of this disparity may be due to the fact that nerve injury used in some studies for retrograde labelling of muscle afferents (Molander et al., 1987; Plenderleith and Snow, 1993) produces a down regulation of IB4 binding and markers related to this neuronal subset (Reid et al., 2011; Shehab et al., 2004; Sant'Anna-da-Costa et al., 1999; Knyihár and Csillik, 1976).

Recent studies have begun to elucidate the contribution of IB4(+) and IB4(−) neurons in cutaneous nociception and related animal models of clinical pain syndromes (Bogen et al., 2009; Ferrari et al., 2010; Joseph and Levine et al., 2010). For example, while IB4(+) and IB4(−) neurons participate in acute inflammatory pain in the skin (Ferrari et al., 2010; Joseph and Levine et al., 2010) only IB4(+) nociceptors mediate the latent enhancement of prostaglandin E₂ (PGE₂)-induced hyperalgesia observed after recovery from inflammation, a model of the transition from acute to chronic pain (Joseph and Levine et al., 2010). And, IB4(+) nociceptors are required for full expression of cutaneous symptoms observed in models of spinal nerve injury- and cancer chemotherapy-induced neuropathic pain (Tarpley et al., 2004; Joseph et al., 2008).

Thus, while morphological studies have identified a population, albeit believed to be small, of IB4(+) sensory neurons innervating skeletal muscle (Molander et al., 1987; O'Brien et al., 1989; Plenderleith and Snow, 1993; Ambalavanar et al., 2003; Pierce et al., 2006), their contribution in muscle pain syndromes has not been explored. In the present study we evaluated the role of IB4+ neurons in models of acute- and chronic-muscle pain. To accomplish this, we administered isolectin B4 conjugated to the neurotoxin saporin (IB4-saporin), to selectively destroy IB4(+) nociceptors (Vulchanova et al., 2001; Nishiguchi et al., 2004; Tarpley et al., 2004; Bogen et al., 2008; Joseph et al., 2008) in rats that were then submitted to inflammation or ergonomic interventions that produce acute and chronic muscle pain.

Materials and methods

Animals

Adult male Sprague Dawley rats (250–330 g; Charles River, Hollister, CA, USA) were used in these experiments. They were housed in the Animal Care Facility at the University of California San Francisco, under environmentally controlled conditions (lights on 07:00–19:00 h; room temperature 21–23°C) with food and water available *ad libitum*. Upon completion of experiments, rats were killed by pentobarbital overdose (>250 mg/kg) followed by cervical dislocation. Animal care and use conformed to NIH guidelines. The University of California San Francisco Committee on Animal Research approved all experimental protocols, which adhered to the ethical guidelines published by the International Association for the Study of Pain (Zimmerman, 1983). Every effort was made to minimize number and suffering of animals used in the experiments.

Drugs

Unless otherwise stated, all chemicals were obtained from Sigma-Aldrich (St. Louis, MO, USA).

Eccentric exercise

The method used to eccentrically exercise the rat hind limb (Alvarez et al., 2010) was similar to that described by Kano et al. (2004), and Mizumura and colleagues (Taguchi et al., 2005). Briefly, isoflurane-anesthetized rats were placed in the supine position, on a heating pad (to maintain body temperature at 37°C), and the right hind paw affixed to the foot bracket of the exercise apparatus (Model RU-72, NEC Medical Systems, Tokyo, Japan) with 3M Micropore® surgical paper tape, such that the angle of the knee and ankle joints was ~90° (the paw 30° from vertical). The gastrocnemius muscle was stimulated, via subcutaneous needle-type electrodes attached to a Model DPS-07 stimulator (Dia Medical System Inc., Tokyo, Japan) that delivered trains of rectangular pulses (100 Hz, 700 ms, 3 V) every 3 s, to give a total of 300 contractions. During these stimulus-induced contractions of the gastrocnemius muscle, the electromotor system rotated the foot to produce extension of the gastrocnemius muscle.

Mechanical vibration

The hind limbs of rats were vibrated with a laboratory vortex mixer (Digital Vortex Genie II, Fisher Scientific, Waltham, MA, USA), which has a variable-speed motor with a real-time digital readout of the vibration speed. Rats were anesthetized with 3% isoflurane in oxygen and a hind leg affixed to the platform with 3M Micropore® surgical tape so that the knee and ankle joint angles were both 90°, without longitudinal rotational torque on the leg. As previously described (Dina et al., 2010), the leg was vibrated for 15 min at a frequency of 60 to 80 Hz, with a 5-mm peak-to-peak displacement amplitude.

Carrageenan-induced myositis

Carrageenan (λ -carrageenan, 1% in 0.9% NaCl) was injected into the belly of the gastrocnemius muscle. The 100 μ g/10 μ l dose of carrageenan was determined in pilot studies as sufficient to produce robust muscle mechanical hyperalgesia, in the range of our previous observations (Dina et al., 2008).

Intramuscular injections

Rats were briefly anesthetized with 2.5 % isoflurane to facilitate the administration of carrageenan (1% in 0.9% NaCl, 10 μ l), prostaglandin E₂ (PGE₂, 1 μ g/20 μ l) or vehicle (0.9% NaCl, 20 μ l), into the belly of the gastrocnemius muscle. The injection site was

previously shaved and scrubbed with alcohol. Immediately after injections the skin puncture site was marked with a fine-tip indelible ink pen, so that the mechanical nociceptive threshold of the underlying injection site in the muscle could be repeatedly tested.

Intrathecal injection of IB4-saporin

The destruction of the IB4(+) population of dorsal root ganglion neurons by intrathecal injection of a cytotoxin, saporin, conjugated to IB4 (IB4-saporin) has been established previously (Vulchanova et al., 2001; Nishiguchi et al., 2004; Bogen et al., 2008; Joseph et al., 2008). We have previously provided evidence about the selective neurotoxic effects of the IB4-saporin conjugate: in contrast to unconjugated saporin, intrathecal injection of IB4-saporin produces a dramatic decrease in the IB4 labeling in lamina II of the dorsal horn (Joseph et al., 2008). These results fully agree with those obtained by Nishiguchi et al. (2004), who also verified a selective decrease of IB4 staining in dorsal root ganglion neurons after intrathecal injection of IB4-saporin. Using anti-saporin antibodies, Nishiguchi et al. (2004) also noted that saporin labeling was not detected in IB4(−) neurons, indicating that IB4-saporin is selectively internalized in IB4(+) neurons after intrathecal injection. Furthermore, from a functional point of view IB4-saporin abolishes GDNF, but not NGF-induced, hyperalgesia (Bogen et al., 2008; Bogen et al., 2009; Joseph and Levine, 2010).

IB4-saporin (Advanced Targeting Systems, San Diego, CA, USA) was diluted with saline and a dose of 3.2 µg in 20 µl administered intrathecally 10 days prior to experiments (Joseph et al., 2008; Joseph and Levine, 2010). Rats were briefly anaesthetized with 2.5% isoflurane (Phoenix Pharmaceuticals, St. Joseph, MO, USA) in 97.5% O₂. Then, a 30-gauge hypodermic needle was inserted into the subarachnoid space on the midline, between the L4 and L5 vertebrae. Control treatment consisted of intrathecal injection of saline (20 µl). Animals regained consciousness approximately 1 min after the injection.

Measurement of muscle hyperalgesia

Mechanical nociceptive threshold in the gastrocnemius muscle was quantified using a digital force transducer (Chatillon DFI2; Amtek Inc., Largo, FL) with a custom-made 7 mm-diameter probe (Alvarez et al., 2010). It has been shown that the use of a probe with a tip diameter ≥ 2.6 mm allows reliable measurements of mechanical nociceptive threshold in muscle, even when overlying cutaneous hyperalgesia is present (Takahashi et al., 2005; Nasu et al., 2010; Murase et al., 2010). Rats were lightly restrained in a cylindrical acrylic holder with lateral slats that allows for easy access to the hind limb and application of the transducer probe to the belly of the gastrocnemius muscle. The nociceptive threshold was defined as the force, in Newtons, required to produce a flexion reflex in the hind leg. Baseline limb-withdrawal threshold was defined as the mean of 3 readings taken at 5-minute intervals. Each hind limb was treated as an independent measure and each experiment performed on a separate group of rats.

Statistical analysis

A *t*-test was employed to determine if there were significant differences in mechanical thresholds between IB4-saporin- and saline-treated rats. A three-way repeated measures ANOVA with one within subjects-factor (time) and two between-subjects factors: intrathecal treatment with two levels (IB4-saporin-treated or saline-treated), and side with two levels (ipsilateral or contralateral). If the three-way ANOVA showed a significant three-way interaction, two way repeated measures ANOVAs were performed to determine the basis of the differences. For within-subjects effects the Mauchly criterion was used to determine if the assumption of sphericity was met; if not, Greenhouse-Geisser *p*-values are presented. Statistical significance (i.e. the α -level) was set at $p < 0.05$. To determine the times when nociceptive thresholds returned to baseline after vibration treatment, one-way

repeated measures ANOVAs with simple contrasts were performed. Because this analysis involved 10 comparisons, a Bonferroni-type correction was applied to the alpha level. That is, $p = 0.05 \div 10 = 0.005$ for these tests. Data were plotted as mean $\pm$ S.E.M.

Results

To determine if IB4(+) nociceptors play a role in pain syndromes, groups of rats experiencing one of three models of muscle hyperalgesia (i.e., hyperalgesia induced by carrageenan administration into the gastrocnemius muscle, eccentric exercise, or hind limb vibration) were pre-treated intrathecally (i.t.) with either IB4-saporin, to reduce the population of IB4(+) neurons or saline vehicle as a control. Ten days later, all rats underwent unilateral model induction, with the contralateral side serving as a control.

Prior to model induction rats were tested to determine if i.t. IB4-saporin treatment alone affects mechanical nociceptive thresholds. No significant difference was observed between IB4-saporin-treated and saline-treated rats ($t_{78} = -1.291$, $p = 0.208$).

Carrageenan-induced muscle hyperalgesia

Unilaterally administered carrageenan significantly reduced mechanical nociceptive threshold in both IB4-saporin- and saline-treated rats (Fig. 1A); however, the saline-treated rats showed significantly greater hyperalgesia than did the IB4-saporin-treated rats, especially with respect to duration of hyperalgesia, indicating that IB4(+) neurons play a statistically significant, but not a dominant, role in carrageenan-induced muscle hyperalgesia. The saline-treated contralateral side showed slight but statistically significant hyperalgesia; however, there were no significant differences between IB4-saporin-treated rats and saline-treated rats.

Ten days after carrageenan administration, when nociceptive thresholds returned to baseline, rats were assessed for hyperalgesic priming (Dina et al., 2008). The inflammatory cytokine PGE₂ (1 μ g/20 μ l) was injected bilaterally into the gastrocnemius muscles and nociceptive threshold measured at 1, 4 and 24 hours. In rats previously treated with intrathecally administered saline, PGE₂ injected into the saline-treated gastrocnemius muscle induced hyperalgesia that lasted less than 4 hours; in the carrageenan-treated side, however, PGE₂ induced prolonged hyperalgesia that lasted at least 24 hours, indicating the induction of hyperalgesic priming by carrageenan (Aley et al., 2000; Dina et al., 2008). In IB4-saporin-treated rats PGE₂ induced significant hyperalgesia that resolved within four hours on both the saline- and the carrageenan-treated sides. The prolonged hyperalgesic effect on the carrageenan treated side was markedly attenuated, indicating that carrageenan-induced hyperalgesic priming is mediated by IB4(+) nociceptors.

Eccentric exercise-induced muscle hyperalgesia

Unilateral eccentric exercise significantly reduced nociceptive thresholds in both IB4-saporin- and saline-treated rats (Fig. 2A); however, the saline-treated rats showed significantly greater hyperalgesia than did the IB4-saporin-treated rats, indicating that IB4(+) neurons contribute to eccentric exercise-induced muscle hyperalgesia. The untreated contralateral side showed slight but statistically significant hyperalgesia, and there were statistically significant differences between the IB4-saporin-treated and the saline-treated rats, with the IB4-saporin-treated rats returning to baseline a couple days earlier than the saline-treated rats.

After return to pre-exercise baseline threshold, rats were assessed for hyperalgesic priming as above. In rats previously treated with intrathecally administered saline, PGE₂ injected into the saline-treated gastrocnemius muscle of the non-exercised side induced hyperalgesia

that lasted about 4 hours; in the exercised side, however, PGE₂ induced prolonged hyperalgesia that lasted at least 24 hours, indicating the induction of hyperalgesic priming by eccentric exercise. In IB4-saporin-treated rats PGE₂ induced significant hyperalgesia that resolved within four hours on both the exercised and non-exercised sides. There was no prolongation of the hyperalgesic effect of PGE₂ on the exercised side, indicating that eccentric exercise-induced hyperalgesic priming is completely mediated by IB4(+) nociceptors.

Vibration-induced muscle hyperalgesia

Unilateral hind limb vibration significantly reduced nociceptive thresholds in both IB4-saporin- and saline-treated rats (Fig. 3A); however, the saline-treated rats showed significantly greater hyperalgesia than did the IB4-saporin-treated rats, indicating that IB4(+) neurons provide a major contribution to vibration-induced muscle hyperalgesia. Nociceptive thresholds for the IB4-saporin-treated rats returned to baseline by day seven, but the saline-treated rats remained hyperalgesic until day 21. The unvibrated contralateral side also showed significant hyperalgesia, and there were also differences between the IB4-saporin-treated rats and the saline-treated rats. Although this contralateral effect is likely to reflect the fact that vibration is transmitted throughout the body, an eventual contribution of central sensitization cannot be ruled out. Nociceptive thresholds for the IB4-saporin-treated rats returned to baseline by day 3 but remained significantly above baseline until day 18 for the saline-treated rats. Thus, IB4(+) nociceptors seem to play a bigger role in vibration-induced hyperalgesia than in either carrageenan-induced hyperalgesia or eccentric exercise-induced hyperalgesia.

After return to pre-vibration baseline nociceptive threshold, rats were assessed for hyperalgesic priming as above. In rats previously treated with intrathecally administered saline, PGE₂ induced hyperalgesia that lasted less than 4 hours in the non-vibrated side; in the vibrated side, however, PGE₂ induced prolonged hyperalgesia that lasted at least 24 hours, indicating the induction of hyperalgesic priming by limb vibration. In IB4-saporin-treated rats PGE₂ induced significant hyperalgesia that resolved within four hours on both the saline- and the vibrated sides. The prolonged hyperalgesic effect on the vibrated side was markedly attenuated, indicating that vibration-induced hyperalgesic priming is completely mediated by IB4(+) nociceptors.

Discussion

To assess the role of muscle IB4(+) nociceptors in muscle pain and to elucidate whether these nociceptors play a general role in persistent muscle hyperalgesia, the experimental approach used here involved the exposure to diverse muscle insults.

The carrageenan-induced muscle hyperalgesia model used here involves a self-limited inflammatory process which is likely to reproduce clinical myositis related to diverse insults, including muscle trauma (Dina et al., 2008). Our previous studies show that this model results in a state of chronic-latent hyperalgesia, suggesting that it may help reveal cellular processes by which acute muscle inflammation turns into chronic muscle pain (Dina et al., 2008).

The model of eccentric exercise-induced muscle hyperalgesia used here is likely to reproduce muscle soreness observed after unaccustomed exercise. Since most episodes of muscle soreness induced by eccentric exercise resolve in 4–5 days without any intervention, they usually do not represent a serious clinical problem. However, our previous studies indicate that it can also induce a state of chronic latent hyperalgesia, also suggesting cellular

mechanisms underlying exercise or work-related chronic musculoskeletal pain syndromes (Alvarez et al., 2010).

The model of muscle hyperalgesia induced by vibration exposure is likely to reproduce the muscle pain observed after occupational exposure to vibration. It is well established that occupational exposure to vibration is an important cause of acute and chronic musculoskeletal pain. Using this model, we have identified a role for pro-inflammatory cytokine receptors and second messengers in nociceptors implicated in the induction and maintenance of chronic muscle pain in the rat (Dina et al., 2010).

The selective destruction of IB4(+) nociceptors did not significantly affect mechanical nociceptive threshold in the gastrocnemius muscle, in the rat. Since IB4(+) and IB4(-) nociceptors respond to mechanical stimuli (Ferrari et al., 2010; Joseph and Levine, 2010), the lack of effect on mechanical nociceptive threshold appears to reflect an adequate redundancy such that either population of nociceptors can adequately detect a noxious stimulus.

The present results are in close agreement with previous studies that demonstrated that destruction of IB4(+) nociceptors produces little or no change in baseline thermal and mechanical nociceptive thresholds in the skin (Tarpley et al., 2004; Joseph et al., 2008; Joseph and Levine, 2008).

IB4-saporin did, however, attenuate both the duration of the acute mechanical hyperalgesia induced by inflammatory and ergonomic interventions, the magnitude of the mechanical hyperalgesia induced by the ergonomic interventions especially that induced by vibration. IB4-saporin also eliminated the long-lasting prolongation of PGE₂ hyperalgesia after recovery from acute hyperalgesia induced by inflammation and ergonomic interventions. The acute mechanical hyperalgesia in the gastrocnemius muscle induced by inflammatory and ergonomic interventions are models of pain conditions that induce different degrees of muscle injury (Armstrong et al., 1991; Necking et al., 2004; Dina et al., 2008). These results provide direct evidence of the involvement of IB4(+) nociceptors in both acute and chronic muscle pain syndromes in the rat, especially those associated with ergonomic insults.

The shortened time course of muscle hyperalgesia and the inhibition of nociceptive priming observed in animals treated with IB4-saporin in all models of muscle pain used here suggests that, irrespective of the type of muscle injury, IB4(+) nociceptors play a role in long lasting nociceptive responses, a hallmark of muscle pain observed in clinical practice.

The selective sensitizer of IB4(+) nociceptors, glial-derived neurotrophic factor (GDNF) (Albers et al., 2006; Bogen et al., 2008; Joseph and Levine, 2010; Ferrari et al., 2010), is highly expressed in the vicinity of the plasma membrane of skeletal muscle cells (Suzuki et al., 1998a; Suzuki et al., 1998b; Wehrwein et al., 2002). In addition, patients affected by painful neuromuscular diseases such as polymyositis and Duchenne type neuromuscular dystrophy exhibit increased levels of GDNF in their muscles (Suzuki et al., 1998a) and complain of pain in affected muscles (Zebracki and Drotar, 2008). GDNF, released by muscle damage, might therefore induce sensitization of IB4(+) nociceptors, contributing to the mechanical hyperalgesia in muscle. Previous studies have also shown that the destruction of IB4(+) nociceptors by administration of IB4-saporin markedly attenuates the acute cutaneous hyperalgesia induced by GDNF, monocyte chemoattractant protein 1, and bladder overactivity induced by irritants, and partially attenuates that induced by NGF (Tarpley et al., 2004; Nishiguchi et al., 2004; Bogen et al., 2009; Joseph and Levine, 2010).

The primary hyperalgesia mediated by IB4(+) nociceptors appears to be, at least in part, related to an activation of the epsilon isoform of the protein kinase C (PKC ϵ). Indeed, G-

protein coupled receptor activation by hyperalgesic mediators only induces PKC ϵ membrane translocation in IB4(+) nociceptors (Vellani et al., 2004; Hucho et al., 2005; Kuhn et al., 2008). This is consistent with the enhanced responsiveness to algogens such as bradykinin and capsaicin observed in IB4(+), compared to IB4(-) nociceptors, induced by inflammation (Breese et al., 2005). These observations are also in agreement with results of our previous studies showing an important contribution of PKC ϵ signalling in nociceptors to the primary muscle hyperalgesia induced by eccentric-exercise (Alvarez et al., 2010) and vibration (Dina et al., 2010), and to latent long-lasting muscle hyperalgesia (i.e., hyperalgesic priming), which was dependent on IB4(+) neurons (see below).

We have previously provided evidence that cutaneous inflammation produces a long-lasting change in the signalling pathways mediating subsequent cytokine-induced nociceptor sensitization and mechanical hyperalgesia, at the previously inflamed site (Aley et al., 2000; Reichling and Levine, 2009). Such neuroplastic changes, referred to as hyperalgesic priming, is evidenced as a PKC ϵ -dependent prolonged mechanical hyperalgesia to an otherwise short-lasting proalgesic effect of PGE₂, serotonin or adenosine (Aley et al., 2000; Parada et al., 2003) that normally signal via cAMP/PKA to produce mechanical hyperalgesia (Reichling and Levine, 2009). Hyperalgesic priming is also induced in skeletal muscle after inflammatory (e.g., intramuscular carrageenan) or ergonomic interventions (e.g., eccentric exercise or vibration) (Dina et al., 2008; Alvarez et al., 2010; Dina et al., 2010). In spite of muscle and cutaneous hyperalgesic priming sharing the same mechanisms (i.e., shift in dependence to PKC ϵ for the development, expression, and maintenance of priming) and that G-protein coupled receptor activation of PKC ϵ causes its translocation to plasma membrane only in IB4(+) neurons (Vellani et al., 2004; Hucho et al., 2005; Kuhn et al., 2008), evidence about the identity of nociceptors underlying the transition from acute to chronic muscle pain has been lacking. The data presented here shows that the selective destruction of IB4(+) neurons completely eliminated the ability of either inflammatory or ergonomic interventions to induce hyperalgesic priming in skeletal muscle (i.e., PGE₂-induced muscle hyperalgesia was no longer present at 4 h). These results are consistent with our previous studies regarding the role of IB4(+) nociceptors in hyperalgesic priming in the skin; their selective destruction prevented the long-lasting latent mechanical hyperalgesia produced by the intradermal injection of NGF (Ferrari et al., 2010; Joseph and Levine, 2010), GDNF (Ferrari et al., 2010; Joseph and Levine, 2010) or the highly selective activator of PKC ϵ , Ψ εRACK (Joseph and Levine, 2010).

Conclusions

While a role for IB4(+) neurons in nociception in skeletal muscle has been questioned (Ambalavanar et al., 2003), our results implicate them not only in acute muscle hyperalgesia, but also as the major player in hyperalgesic priming, a phenomenon suggested to contribute to the transition from acute to chronic pain (Reichling and Levine, 2009). The findings of the present study provide support for the role of IB4(+) nociceptors to diverse forms of acute pain and to the mechanisms underlying the transition of acute to chronic muscle pain. These data may lead to novel approaches for the prevention and treatment of chronic musculoskeletal pain.

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Highlights

Inflammatory and ergonomic injuries induce acute and chronic muscle pain in naïve rats.

Destruction of IB4(+) nociceptors attenuated acute skeletal muscle hyperalgesia.

It also disrupted the transition from acute to chronic pain in skeletal muscle.

Thus, IB4(+) muscle nociceptors may play a role in the onset of chronic musculoskeletal pain.

Figure 1. Role of IB4(+) nociceptors in carrageenan-induced muscle hyperalgesia. The neurotoxin saporin conjugated to IB4 (IB4-saporin) or saline were administered intrathecally 10 days prior to unilateral carrageenan administration into the gastrocnemius muscle. The contralateral gastrocnemius muscle was injected with saline as a control

(A) Nociceptive thresholds were tested until they returned to baseline, 6 days after injection of carrageenan. A three-way ANOVA showed a significant three-way interaction ($F_{7,140}=2.826$; $p=0.034$), significant main effect of intrathecal treatment ($F_{1,20}=16.951$; $p=0.001$), significant main effect of side ($F_{1,20}=395.999$; $p<0.001$), and a significant side $\times$ intrathecal treatment interaction ($F_{1,20}=12.435$; $p=0.002$). Based on the significant three-way interaction a two-way repeated measures ANOVA (time and intrathecal treatment) was performed for each side. For the carrageenan-treated side there was a significant two-way interaction ($F_{7,70}=3.345$; $p=0.036$), main effect of group ($F_{1,10}=21.645$; $p=0.001$), and main effect of time ($F_{7,70}=212.665$; $p<0.001$). For the saline-treated side there was only a significant main effect of time ($F_{7,70}=30.125$; $p<0.001$).

(B) When nociceptive thresholds returned to pre-carrageenan baseline, groups were tested for the presence of hyperalgesic priming. After bilateral PGE₂ administration, nociceptive thresholds were tested at 1, 4, and 24 hours. Only the group that received intrathecally administered saline demonstrated priming hyperalgesia. The IB4-saporin-treated group did not show hyperalgesic priming, indicating that IB4(+) nociceptors mediate hyperalgesic priming. A three-way ANOVA showed a significant three-way interaction ($F_{3,60}=52.385$; $p<0.001$), significant main effect of intrathecal treatment ($F_{1,20}=162.360$; $p<0.001$), significant main effect of side ($F_{1,20}=119.159$; $p<0.001$), and a significant side $\times$ intrathecal treatment interaction ($F_{1,20}=83.312$; $p<0.001$). Based on the significant three-way interaction a two-way repeated measures ANOVA (time and intrathecal treatment) was performed for each side. For the carrageenan-treated side there was a significant two-way interaction ($F_{3,30}=82.831$; $p<0.001$), main effect of group ($F_{1,10}=204.869$; $p<0.001$), and a significant main effect of time ($F_{3,30}=339.672$; $p<0.001$). For the saline-treated side there was a significant two-way interaction ($F_{3,30}=3.324$; $p=0.047$), main effect of group ($F_{1,10}=7.845$; $p=0.019$), and a significant main effect of time ($F_{3,30}=812.267$; $p<0.001$).

Figure 2. Role of IB4(+) nociceptors in eccentric exercise-induced muscle hyperalgesia. IB4-saporin or saline was administered intrathecally 10 days prior to unilateral hind limb eccentric exercise. The contralateral hind limb was left unexercised as a control

(A) Nociceptive thresholds were tested until they returned to baseline, 5 days. A three-way ANOVA showed a significant three-way interaction ($F_{6,168}=8.158$; $p<0.001$), significant main effect of intrathecal treatment ($F_{1,28}=92.704$; $p<0.001$), significant main effect of side ($F_{1,28}=445.679$; $p<0.001$), and a significant side $\times$ intrathecal treatment interaction ($F_{1,28}=12.435$; $p<0.001$). Based on the significant three-way interaction a two-way repeated measures ANOVA (time and intrathecal treatment) was performed for each side. For the exercise-treated side there was a significant two-way interaction ($F_{6,84}=14.231$; $p<0.001$), main effect of group ($F_{1,14}=93.120$; $p=0.001$), and a significant main effect of time ($F_{6,84}=164.182$; $p<0.001$). For the non-exercised side there was a significant main effect of group ($F_{1,14}=11.274$; $p=0.005$) as well as a significant main effect of time ($F_{6,84}=32.231$; $p<0.001$).

(B) When nociceptive thresholds returned to pre-exercise baseline, groups were tested for the presence of hyperalgesic priming as above. Only the group that received intrathecally administered saline demonstrated priming hyperalgesia. The IB4-saporin-treated group did not show hyperalgesic priming, indicating that IB4(+) nociceptors mediate hyperalgesic priming. A three-way ANOVA showed a significant three-way interaction ($F_{3,84}=801.600$; $p<0.001$), significant main effect of intrathecal treatment ($F_{1,28}=174.014$; $p<0.001$), significant main effect of side ($F_{1,28}=113.154$; $p<0.001$), and a significant side $\times$ intrathecal treatment interaction ($F_{1,28}=104.300$; $p<0.001$). Based on the significant three-way interaction a two-way repeated measures ANOVA (time and intrathecal treatment) was performed for each side. For the exercise-treated side there was a significant two-way interaction ($F_{3,42}=215.836$; $p<0.001$), main effect of group ($F_{1,14}=540.756$; $p<0.001$), and a significant main effect of time ($F_{3,42}=571.062$; $p<0.001$). For the non-exercised side there was a significant two-way interaction ($F_{3,42}=5.192$; $p=0.005$) and a significant main effect of time ($F_{3,30}=383.706$; $p<0.001$); the main effect of group was not significant ($F_{1,14}=2.970$; $p=0.107$).

Figure 3. Role of IB4(+) nociceptors in vibration-induced muscle hyperalgesia. IB4-saporin or saline was administered intrathecally 10 days prior to unilateral hind limb vibration. The contralateral hind limb was not vibrated as a control

(A) Nociceptive thresholds were tested until they returned to baseline, 21 days. A three-way ANOVA showed a significant three-way interaction ($F_{10,200}=7.424$; $p<0.001$), significant main effect of intrathecal treatment ($F_{1,20}=253.421$; $p<0.001$), significant main effect of side ($F_{1,20}=215.084$; $p<0.001$), and a significant side $\times$ intrathecal treatment interaction ($F_{1,20}=42.586$; $p<0.001$). Based on the significant three-way interaction a two-way repeated measures ANOVA (time and intrathecal treatment) was performed for each side. For the vibrated side there was a significant two-way interaction ($F_{10,100}=28.960$; $p<0.001$), main effect of group ($F_{1,10}=167.778$; $p<0.001$), and a significant main effect of time ($F_{10,100}=86.820$; $p<0.001$). For the non-vibrated side there was a significant main effect of time $\times$ intrathecal treatment interaction ($F_{10,100}=15.018$; $p<0.001$), a significant main effect of group ($F_{1,10}=88.470$; $p<0.001$), and a significant main effect of time ($F_{10,100}=25.881$; $p<0.001$). To determine when nociceptive thresholds for each group returned to baseline levels, one-way ANOVAs with simple contrasts were performed. For the vibrated side, the

IB4-saporin-treated group returned to baseline by day 5, and the saline-treated group returned to baseline on day 18; for the non-vibrated control side, the IB4-saporin-treated group returned to baseline on day 3, and the saline-treated group returned to baseline on day 18.

(B) When nociceptive thresholds returned to pre-vibration baseline levels, groups were tested for hyperalgesic priming as above. Only the group that received intrathecally administered saline demonstrated priming hyperalgesia. The IB4-saporin-treated group did not show hyperalgesic priming, indicating that IB4(+) nociceptors mediate priming. A three-way ANOVA showed a significant three-way interaction ($F_{3,60}=52.385$; $p<0.001$), significant main effect of intrathecal treatment ($F_{1,20}=162.360$; $p<0.001$), significant main effect of side ($F_{1,20}=119.159$; $p<0.001$), and a significant side $\times$ intrathecal treatment interaction ($F_{1,20}=83.312$; $p<0.001$). Based on the significant three-way interaction a two-way repeated measures ANOVA (time and intrathecal treatment) was performed for each side. For the carrageenan-treated side there was a significant two-way interaction ($F_{3,30}=82.831$; $p<0.001$), main effect of group ($F_{1,10}=204.869$; $p<0.001$), and a significant main effect of time ($F_{3,30}=339.672$; $p<0.001$).