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Activation of spinal microglia in a murine model of peripheral inflammation-induced, long-lasting contralateral allodynia

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Abstract

Increased sensitivity contralateral to an injury has been described in humans and in various models of neuropathic pain in rats. The mechanism underlying contralateral hypersensitivity is as yet unclear, although previous studies have implicated involvement of both spinal neurons and glia. We describe the development of a temporally delayed, robust and long-lasting contralateral allodynia in mice after hindpaw injection with 4% carrageenan. Both ipsilateral and contralateral allodynia could be inhibited temporarily by intrathecally administered morphine, clonidine, or neostigmine. The delayed development of contralateral allodynia correlated with an increase in OX-42, but not GFAP immunoreactivity in the contralateral dorsal horn. Furthermore, intrathecal treatment with minocycline inhibited the development of contralateral allodynia, suggesting that microglial activation plays a key role in contralateralization, and may be a potential target for clinical intervention *after* injury or inflammation has occurred, to eliminate the subsequent development of extraterritorial pain.

Keywords

contralateral allodynia; inflammatory pain; microglia; mouse; spinal cord; carrageenan

Introduction

Altered sensitivity in contralateral structures has been observed in many animal models of neuropathic and inflammatory pain (review [17]; nerve ligation [28]; radiculopathy [13]), leading to the proposal of a neurological basis for these phenomena, including changes in the contralateral peripheral nerves and spinal cord dorsal horn neurons [26]. Electrophysiological investigations of dorsal horn neurons show that chronic nerve injury/inflammation enhances responses to contralateral as well as ipsilateral stimuli [8,30].

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Activation of immunocompetent cells such as microglia occurs in the CNS in response to peripheral nerve injury or inflammation [6,35]. Spinal microglia are activated under diverse conditions that result in pain-like states including subcutaneous inflammation, spinal or peripheral nerve trauma, or nerve inflammation [4,5,9,10,31,11,20,15,32,7]. Drugs that disrupt glial activation prevent or reverse pain-like states [9,19,21,22,32,24,18,12], and blockade of cytokine production, cytokine receptors, or spinal gap junctions inhibits hyperalgesia that develops acutely after ipsilateral nerve inflammation in the rat [22,32]. In addition to activation of microglia in the ipsilateral spinal cord, contralateral microglial proliferation occurs in association with nerve injury [36,37,18] and injection of 5% formalin in the hindpaw [7].

Previous studies of microglial involvement and contralateralization have employed rats as the model organism, raising the question of whether this is a species-specific effect. In the present study, we describe a slowly developing change in contralateral mechanical sensitivity accompanied by increased spinal OX-42 immunoreactivity that follows unilateral implantation of carrageenan in mice; we also report the temporary inhibition of this allodynia by three spinally administered analgesics, and the prevention of contralateral allodynia by spinal administration of the microglial inhibitor, minocycline.

Materials and Methods

Animals

Outbred male ICR mice between 6–10 weeks old (25–35g) were used for all procedures, according to IACUC-approved protocols.

Carrageenan injection

Mice were anesthetized with isoflurane, and 50 μ L of 4% carrageenan or sterile saline was injected in between the 2nd and 3rd toes on the plantar side of the left hindpaw.

Assessment of Mechanical Hypersensitivity

Mechanical sensitivity was measured using a von Frey filament (#2.44, 0.4 mN force) applied 6 times to the plantar surface of each hind paw, beginning with the injected (ipsilateral) hindpaw first, followed immediately by the contralateral hindpaw. Mechanical sensitivity was determined at baseline (x2), 3 hours, 1, 3, 7, 10, 14, and 21 days after injection.

Spinal analgesia

In order to establish mechanical hypersensitivity as allodynia, mice were administered spinal analgesics at 3 hours and 8 days after carrageenan injection. After obtaining a baseline level of hypersensitivity, mice that received intrathecal injection via lumbar puncture [14] of morphine (10 nmol), clonidine (10 nmol), neostigmine (0.01 nmol) or normal saline (5 μ L) were re-tested 30 minutes later. The % inhibition of hypersensitivity was calculated as follows: $((\text{pre-injection \% response} - \text{post-injection \% response}) / \text{pre-injection \% response}) * 100$.

Microglial inhibition

In order to determine the role of microglia in allodynia development, we intrathecally administered minocycline (10 nmol) or saline (5 μ L) on days 3, 4, and 5 after carrageenan injection. Mice were tested both before and 1 hour after injection on each of these days, and on days 7, 14, and 21.

Immunohistochemistry

Mice were perfused with Lana's fixative followed by 10% sucrose for cryoprotection. In preliminary studies, sections from cervical, thoracic, and lumbar were sampled, and afterwards

only spinal cord segments L3-L6 were identified, excised and frozen for immunocytochemistry. Thaw-mounted cryostat sections (20 μ m) sections from the L4/5 segments were used for quantitative analysis. Primary antisera used were against integrin alphaM [CD11b], clone OX-42 (Chemicon, 1:100) and GFAP (Dako, 1:2000), followed by Cy3-conjugated secondary antisera (Jackson ImmunoResearch, West Grove, PA, 1:400). Omission of primary antibodies served as a negative control. Images were captured using a Bio-Rad MRC-1000 Confocal Imaging System (Bio-Rad Microscience Division, Cambridge, MA). Quantification of immunoreactivity within the dorsal horn area was performed using NIH image 1.63 under blinded conditions. Five representative background areas within the dorsal horn were chosen from each section to determine average background value; threshold was then calculated by adding a fixed amount of 35 units. The dorsal horn area was traced, the total area measured and the number of positive pixels (pixels above threshold) within the traced area determined and expressed as % pixels positive for immunoreactivity. Three random sections determined the average for each mouse, and 3–4 mice included in each treatment group. Significant differences between treatment groups were determined by one-way ANOVA followed by Newman-Keuls Multiple Comparison Test.

Results

Unilateral injection of 4% carrageenan into mouse plantar hindpaw resulted in hindpaw edema, which resolved between 3–7 days post-injection (unquantified observation). We observed a robust increase in ipsilateral hindpaw mechanical sensitivity at 3 hours after injection. Moreover, mechanical sensitivity of the carrageenan-injected paw continued to be significantly different from that in saline-injected controls for at least 21 days (Figure 1). Mechanical testing of the contralateral paw revealed a delayed increase in sensitivity, which was first significant at 3 days after injection, continued increasing to 7 days after injection, and lasted until the end of the study (Figure 1).

At 3 hours post-carrageenan injection, significant inhibition of hypersensitivity was achieved by intrathecal morphine ($86.2 \pm 6.9\%$ inhibition, $n=10$), neostigmine ($55.2 \pm 9.5\%$ inhibition, $n=10$) and clonidine ($79.8 \pm 7.8\%$ inhibition, $n=10$) compared to saline ($14.9 \pm 1.6\%$ inhibition, $n=6$) (* $p < 0.05$, ANOVA with Bonferroni post-hoc).

We next tested the reversibility of persistent mechanical sensitivity at 8 days. Both the ipsilateral (Fig 2A), and the by now fully developed contralateral hypersensitivity (Fig 2B) were attenuated by morphine and clonidine, suggesting that this persistent hypersensitivity represents allodynia.

Interestingly, on day 1 after carrageenan injection, OX42 immunoreactivity was not increased in either ipsilateral or contralateral dorsal horn compared to saline controls (Figure 4A), despite the robust ipsilateral mechanical allodynia at this time. However, on day 5 after injection, when contralateral mechanical allodynia has become apparent, OX42 immunoreactivity was increased in both the ipsilateral and contralateral dorsal horn of carrageenan-injected compared to controls (Figure 3 & 4B). Quantification of immunoreactivity within the ipsilateral and contralateral dorsal horn revealed a significant increase in OX42, but not in GFAP, at day 5 after injection (Figure 4 B&C).

In order to determine the extent to which microglial activation contributed to the development of contralateral allodynia, we treated carrageenan-injected mice intrathecally with minocycline (50 nmol) or saline on days 3, 4, and 5. Minocycline treatment had no acute anti-allodynic effect (data not shown), but did prevent the development of subsequent long-lasting allodynia in the contralateral hindpaw (Figure 5). Although there was a trend towards decreased allodynia in the ipsilateral hindpaw, this did not reach statistical significance.

Discussion

This study investigated the timing and territoriality of allodynia and spinal glial activation induced by unilateral injection of 4% carrageenan in the mouse hindpaw. We observed contralateral allodynia 3 days after ipsilateral carrageenan injection, which reached a plateau after 7 days, could also be temporarily reversed with spinal analgesics, and persisted at least 21 days. Spinal microglial activation was evident in both the ipsilateral and contralateral dorsal spinal cord at 5 days, but not at 1 day after carrageenan injection. Intrathecal minocycline treatment on days 3, 4, and 5 prevented the development of persistent contralateral, but not ipsilateral, allodynia, suggesting a role for microglial activation in the contralateral spread of allodynia.

Contralateral hyperalgesia

The manifestation of contralateral hypersensitivity appears to be in proportion to the intensity and duration of the ipsilateral stimulus, as higher concentrations of intramuscular acidic saline [29], zymosan [2], or carrageenan [23], or high intensity electrical stimulation [26] lead to contralateralization. Both the longevity and contralateralization of allodynia observed after 4% carrageenan could be due in part to a relatively intense and long-lasting tissue inflammation, as edema persists in the injected hindpaw for 3–7 days post-injection. However, allodynia persisted long past the resolution of this edema.

Microglial activation

OX42 immunoreactivity increased at lumbar levels 4 and 5, most prominently in the dorsal horn, in concurrence with many studies showing a correlation between microglial activation and hypersensitivity. In contrast to studies that implicate a role for astrocytic activation in the development of persistent hypersensitivity, we observed no increase in GFAP immunoreactivity at either day 1 or day 5 after carrageenan injection. This result is consistent with the view that microglia are more involved in initiation of hypersensitivity, while astrocytic involvement, if present, occurs later [6,7].

In some previous studies in rat, microglial inhibitors reversed hypersensitivity as early as three hours after induction, indicating an important role for early microglial activation [21]. Moreover, this reversal was observed soon (1 hour) after administration. We did not observe microglial activation on day 1 post-injection. This apparent discrepancy in the timing of microglial activation may result from the requirement of de novo protein synthesis for expression of the “activation” marker OX-42, whereas other, more acute processes, such as p38MAPK phosphorylation, mediate earlier microglial effects [25,12]. It is likely that the timing with which persistent hypersensitivity depends on microglial activation may vary in different types of injury or inflammation [3], or may differ between mice and rats.

Given our observation that OX42 immunoreactivity was increased bilaterally on day 5, the goal in intervening during the presumably critical period before full contralateralization (i.e. on days 3, 4, and 5) was to test the ability of microglial inhibition to preempt the spread of allodynia to the contralateral side. Indeed, intrathecal minocycline inhibited the development of contralateral allodynia, indicating that microglial activation is present at these time points and plays a key role in the development of contralateral allodynia.

We did not observe an acute inhibitory effect of minocycline at one hour after injection on days 3, 4 and 5. This is likely due to the fact that ipsilateral allodynia was already well-established and maintained by other mechanisms, and contralateral hyperalgesia was not yet robust enough to allow statistical detection of inhibition at these time points. It is also possible that the dose of minocycline was insufficient to completely inhibit microglial activation.

However, the dose used (50 nmol) exceeded that used by Narita et al (22) (1 nmol) in mice, and was more comparable to that used in rats to produce acute inhibition (100–50 nmol) (10,19).

Mechanisms of contralateral hypersensitivity: Peripheral vs. central

Carrageenan injection does not lead to histological evidence of inflammation in contralateral structures, despite the development of contralateral hyperalgesia [21]. Furthermore, extraterritorial pain is restricted to contralateral structures in the same dermatome, rather than generalizing rostrally or caudally [16,27]. Indeed, bilateral changes in TNF- α , IL-1 β , and p38 MAPK expression are activated after hindpaw, but not forepaw carrageenan injection, suggesting a segmental mechanism [1]. Similarly, changes in OX42 immunoreactivity tend to be segmentally isolated; we observed no microglial activation at cervical or thoracic levels (data not shown). Locally applied lidocaine relieves ipsilateral hyperalgesia without affecting contralateral hyperalgesia [28]. The ability of central (intrathecal) administration of a microglial inhibitor to prevent contralateral allodynia in the present study is further evidence that central, spinal mechanisms, presumably involving microglial activation, are important to the manifestation of contralateral hypersensitivity.

The microglial inhibitor minocycline did not significantly inhibit ipsilateral allodynia when given as a post-treatment (days 3, 4, and 5 after injection) in this model. However, minocycline treatment did inhibit contralateral allodynia at days 7, 14, and 21. The timing of administration could be viewed as preventive for contralateral allodynia, which emerges between days 3 and 7 (Figs. 1 & 5). This result is consistent with previous reports in rat (17,23) and mouse (22), in which minocycline inhibited ipsilateral hypersensitivity when administered preventively or early after injury, but also indicates that the window of possible intervention to prevent contralateral hypersensitivity may be later.

Clinically, persistent and mirror-image pain are often delayed in their onset. Using this model of delayed-onset contralateral pain, we showed that inhibition of microglia at a key time after injury can inhibit the persistent spread of allodynia to the contralateral side. This result suggests that clinical interventions targeting microglia might be applied *after* injury or inflammation has occurred, to prevent chronic extraterritorial pain.

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Figure 1. Unilateral carrageenan produces robust and long-lasting ipsilateral and contralateral mechanical hypersensitivity in mice

Mice were injected unilaterally with 4% carrageenan (N=65) or saline alone (N=12) and tested for mechanical sensitivity (% Response) of ipsilateral and contralateral hindpaws. * $p < 0.05$, ANOVA with Bonferonni post-hoc

Figure 2. Inhibition of persistent A) ipsilateral and B) contralateral mechanical hypersensitivity by intrathecally administered analgesics

At 8 days post-carrageenan injection, morphine- (n=7) and clonidine- (n=6) treated mice showed significant inhibition of hyperalgesia compared to saline-treated mice (n=6). *p<0.05, ANOVA with Bonferonni post-hoc

Figure 3. Increase in lumbar dorsal horn OX42 immunofluorescence at day 5 after carrageenan or saline injection

Representative 20X fluorescent images of OX42 immunoreactivity in ipsilateral (A,B) and contralateral (C,D) lumbar dorsal horn of saline- (A,C) and carrageenan- (B,D) injected mice at 5 days after injection.

Figure 4. Quantification of OX42 and GFAP immunoreactivity in dorsal horn at day 5 after carrageenan or saline injection

OX42 and GFAP immunoreactivities at 5 days after carrageenan or saline injection, expressed as the % of pixels within the dorsal horn positive for immunoreactivity. Significant differences were observed in OX42, but not GFAP, on day 5 after injection. # $p < 0.05$ different from naïve, * $p < 0.05$ different from saline control.

Figure 5. Microglial activation mediates contralateralization

Mice were injected unilaterally with 4% carrageenan (n=26) or saline (n=11) and tested for mechanical sensitivity (% Response) of ipsilateral and contralateral hindpaws. Intrathecal injection of mice with minocycline (50nmol/5μL) (n=13, “carra-mino”) at days 3, 4, and 5 resulted in a decreased contralateral mechanical allodynia compared to mice receiving intrathecal saline (n=13, “carra-sal”). *p<0.05, ANOVA with Bonferonni post-hoc