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Involvement of glia in central sensitization in trigeminal subnucleus caudalis (medullary dorsal horn)

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Abstract

Central sensitization is a crucial mechanism underlying the increased excitability of nociceptive pathways following peripheral tissue injury and inflammation. We have previously demonstrated that the small-fiber excitant and inflammatory irritant mustard oil (MO) applied to the tooth pulp produces glutamatergic- and purinergic-dependent central sensitization in brainstem nociceptive neurons of trigeminal subnucleus caudalis (Vc). Recent studies have implicated both astrocytes and microglia in spinal nociceptive mechanisms, showing, for example, that inhibition of spinal astroglial metabolism or spinal microglial p38MAPK activation can attenuate hyperalgesia in inflammatory pain models but have not tested effects of glial inhibitors on central sensitization in functionally identified spinal nociceptive neurons. The aim of the present study was to determine whether glial cells are involved in the MO-induced central sensitization in Vc nociceptive neurons, by examining the effects of intrathecally applied SB203580 (SB), an inhibitor of p38MAPK, and fluoroacetate (FA), an inhibitor of the astroglial metabolic enzyme aconitase. During continuous superfusion of phosphate-buffered saline over Vc, MO application to the pulp-induced central sensitization in Vc nociceptive neurons reflected in significant increases in cutaneous mechanoreceptive field (RF) size and responses to noxious mechanical stimuli and a decrease in mechanical activation threshold. The i.t. application of SB or FA markedly attenuated the MO-induced increases in pinch RF size and responses to noxious stimuli and the decrease in activation threshold. Neither SB nor FA application significantly affected the baseline (i.e., pre-MO application) RF and response properties. These results suggest that glial metabolic processes are important in the development of Vc central sensitization.
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1. Introduction

Noxious stimuli and tissue inflammation can produce pain hypersensitivity that may result both from peripheral sensitization (an increased excitability of the peripheral terminals of nociceptive primary sensory neurons) and central sensitization (an increased excitability of dorsal horn neurons) (for review, see Salter, 2004; Dubner, 2005; Woolf and Salter, 2005). There is accumulating evidence that glia (astrocytes and microglia) in the central nervous system are activated by inflammation or peripheral nerve injury and

are involved in spinal nociceptive transmission and central sensitization. For example, the use of markers for astrocytes (e.g., glial fibrillary acidic protein [GFAP]) and microglia (e.g., OX-42; Iba-1) reveal that astrocytes and microglia become activated in spinal models of both neuropathic and inflammatory pain, with the microglial activation usually preceding astrocyte activation (for review, see Ji and Strichartz, 2004; Ji, 2004; Tsuda et al., 2005; Watkins and Maier, 2003, 2005). However, there appears to have been no published report of the possible contribution of glial activation to central sensitization in functionally identified spinal nociceptive neurons.

Key processes in astroglial metabolism are the tricarboxylic acid (TCA) cycle, which converts glucose to glutamate through the action of the enzyme aconitase, and the

subsequent production and release of glutamine which is then trafficked through transporters into glutamatergic terminals where it is hydrolyzed to form glutamate to replenish the glutamate transmitter pool. Inhibitors of aconitase such as fluoroacetate (FA) and its more toxic metabolite fluorocitrate (FC) that selectively block the TCA cycle (Fonnum et al., 1997; Schousboe et al., 1997), and hence affect the production of glutamine from glutamate, have been reported to prevent formalin-induced hyperalgesia and nerve inflammation-induced pain (for review, see Ji, 2004; Watkins and Maier, 2003, 2005; Tsuda et al., 2005). These various findings suggest that aconitase and the astrocyte-dependent glutamine supply to neurons could be relevant in sustaining enhanced neuronal activity related to chronic pain mechanisms.

Microglia also utilize a number of intracellular processes that have been implicated in central sensitization and spinal hyperalgesia. For example, following peripheral inflammation or nerve injury, a marked increase occurs in the number of phosphorylated p38 mitogen-activated protein kinase (MAPK)-immunoreactive microglia in the spinal cord (Kim et al., 2002; Svensson et al., 2003). In addition, pre-treatment (i.t.) with p38MAPK inhibitors such as SB203580 (SB) or down-regulation of the isoforms of p38MAPKβ, which is distinctly expressed in spinal microglia, attenuates hyperalgesia in several nociceptive models associated with spinal sensitization (Ji, 2004; Svensson et al., 2005; Tsuda et al., 2005). These results suggest that spinal microglial p38MAPK is involved in inflammation-induced pain and plays an important role in spinal nociceptive processing and sensitization.

While glial cell/neuronal interactions represent a promising field of study to clarify central nociceptive mechanisms, very little attention has been given to the involvement of glia in central mechanisms related to orofacial pain other than reports that microglial activation occurs after facial inflammation (Yeo et al., 2001) and that glial inhibition can reduce inflammatory or nerve injury-induced facial allodynia (Yang et al., 2005; Piao et al., 2006). Our previous studies (Chiang et al., 1998, 2005) have demonstrated that the small-fiber excitant and inflammatory irritant mustard oil (MO) applied to the tooth pulp produces long-lasting central sensitization in brainstem nociceptive neurons of the trigeminal subnucleus caudalis (Vc). Because of its many anatomical and functional similarities with the spinal dorsal horn, Vc has been termed the "medullary dorsal horn", but a number of distinct differences between Vc and spinal dorsal horn have also been noted (Bereiter et al., 2000; Dubner and Bennett, 1983; Sessle, 2000, 2005). In view of the important roles played by aconitase and p38MAPK, respectively, in astroglial and microglial metabolic processes, the findings that modulation of these intracellular mechanisms can markedly influence spinal hyperalgesia, and the lack of studies testing for glial contributions to central sensitization in functionally identified spinal dorsal horn or Vc nociceptive neurons, we initiated a study to determine if glia might be involved in Vc central

sensitization. We tested if application to Vc of the p38MAPK inhibitor SB and the aconitase inhibitor FA can influence the MO-induced central sensitization in Vc nociceptive neurons. Preliminary results of this study have been reported in abstract form (Chiang et al., 2004; Xie et al., 2005).

2. Methods and materials

2.1. Animal preparation

The experiments were performed in 20 male Sprague–Dawley adult rats (300–380 g). The methods used for animal preparation, stimulation, and neuronal recording, classification and analyses were similar to those described previously in detail (Chiang et al., 1998, 2002, 2005; Hu et al., 2002; Park et al., 2001) and so will only be briefly outlined here. Each rat was anesthetized by a single i.p. injection of a mixture of α-chloralose (50 mg/kg) and urethane (1 g/kg). Then a tracheal cannula was inserted and the left external jugular vein was cannulated. To expose the pulp of the right maxillary first molar, an occlusal cavity was prepared with a dental drill (Rotex™ 780) and immediately filled with a small piece of cotton pellet soaked with normal saline. After the rat was placed in a stereotaxic apparatus, the caudal medulla was surgically exposed and the overlying dura and subarachnoid membrane were removed. Just before the recording session, a supplemental dose of urethane (200–300 mg/kg, i.v.) was administered and the rat was then immobilized with i.v. pancuronium bromide (initial dose, 0.4 ml of 1 mg/ml solution, followed by a continuous i.v. infusion of a mixture of 70% urethane solution [0.2 g/ml] and 30% pancuronium solution [1 mg/ml] at a rate of 0.4–0.5 ml/h) and artificially ventilated throughout the whole experimental period. An adequate level of anesthesia was confirmed periodically by the lack of spontaneous movements and responses to pinching the hindpaw when pancuronium-induced muscle paralysis was allowed to wear off. Heart rate, percentage expired CO_2, and rectal temperature were constantly monitored and maintained at physiological levels of 333–430 beats/min, 3.5–4.2%, and 37–37.5 °C, respectively. All surgeries and procedures were approved by the University of Toronto Animal Care Committee in accordance with the regulations of the Ontario Animal Research Act (Canada).

2.2. Recording and stimulation procedures

Single neuronal activity was recorded extracellularly by means of an epoxy resin-coated tungsten microelectrode (5–15 MΩ, FHC, ME,USA). As the microelectrode was advanced with a rostral inclination of 23° into the caudal medulla, 1.4–2.0 mm lateral to the midline and 1.5–2.0 mm behind the obex (Paxinos and Watson, 1986), natural stimuli (see below) were applied to the orofacial tissues to search for Vc neurons receiving an orofacial sensory input. Neuronal activity was amplified, displayed on oscilloscopes and also led to a window discriminator connected to an A/D converter (Cambridge Electronic Design, CED 1401 plus, UK) and a personal computer. Data were analyzed off-line with Spike 2 software (Cambridge Electronic Design, UK).

A wide range of mechanical (brush, pressure and pinch) and noxious thermal (radiant heat, 51–53 °C) stimuli were applied to the orofacial region to classify each neuron as low-threshold mechanoreceptive (LTM), wide dynamic range (WDR) or nociceptive-specific (NS) (Chiang et al., 1998). In accordance with our recent Vc study (Chiang et al., 2005), only NS neurons were studied in detail. The level of spontaneous activity of a NS neuron was determined over an initial 1 min recording period. As mentioned in our previous studies (Chiang et al., 1998, 2005; Hu et al., 2002; Park et al., 2001), the cutaneous perioral facial mechanoreceptive receptive field (RF) of each NS neuron was determined through the use of a blunt probe and a pair of non-serrated forceps. A burst response consisting of at least two spikes during each stimulus trial was accepted as the criterion for the RF boundary of the neuron tested. Noxious stimulation was used sparingly so as to avoid damage to the skin and peripheral sensitization. A

deep nociceptive input was considered to occur only if application of a blunt probe to the skin overlying muscle or bone evoked a neuronal response at a mechanical threshold above 5 g, but no response could be evoked by the wide range of mechanical pinch or thermal stimuli applied to the skin itself (Chiang et al., 1994; Yu et al., 1993). The activation threshold to a mechanical stimulus applied to the orofacial RF was assessed by a pair of force-monitoring forceps (with an attached strain gauge that monitored force levels up to 600 g/mm^2) or an electronic von Frey monofilament. The gradual increase in the mechanical force and the responses of the tested neuron were monitored and recorded simultaneously by the use of the Spike 2 program (CED 1401 plus, UK). The NS neuronal responses to graded pressure and pinch stimuli delivered to the neuronal orofacial RF by means of electronic von Frey monofilaments and the force-monitoring forceps were determined as previously described (Chiang et al., 2005). The forceps applied four graded mechanical pinch stimuli, the levels of which depended on the neuronal activation threshold (25, 50, 75, and 100 g; or 50, 75, 100, and 200 g; applied in ascending order, each stimulus for 5 s at an interval of >45 s). The pinch- or pressure-evoked responses of a given neuron were assessed by summing the number of spikes evoked by each of these four graded stimuli during application of the stimulus.

2.3. Chemicals

The chemicals used included MO (allyl isothiocyanate, 95%; Aldrich Chemical Co., USA), FA (Sigma/RBI), and SB (Sigma RBI;). FA and SB were freshly dissolved in phosphate-buffered saline (PBS) at pH 7.4 (Sigma–Aldrich, St. Louis, MO, USA) for application to Vc; PBS application to Vc was used as vehicle control.

2.4. Experimental paradigm

Only one neuron was studied in each experiment. After baseline values of NS neuronal RF size, threshold and responses to graded pressure or pinch were obtained, FA (1 mM, at 0.6 ml/h) was continuously superfused (i.t.) over the ipsilateral Vc throughout the observation period in one group of animals (FA/MO group). Another group of animals (PBS/MO group) instead received PBS (as vehicle control) continuously superfused over Vc. In the third group of animals (SB/MO group), a bolus of SB (2.65 nmol in 10 μl) was applied to Vc. After one or two assessments of neuronal properties, the saline-soaked cotton pellet in the exposed pulp cavity was carefully removed from the molar pulp cavity and replaced with a segment of dental paper point soaked with MO (0.2 μl). The cavity was promptly sealed with CAVIT (3 M ESPE, Germany) in order to prevent MO leaking out of the tooth and to ensure the chemical's sustained action on pulp afferents. Three min after MO application, the neuronal properties were re-assessed at 10-min intervals throughout a 50-min observation period. Recording sites were marked by electrolytic lesions (anodal current of 8 μA for 13 s), and verified histologically (e.g., see Fig. 1).
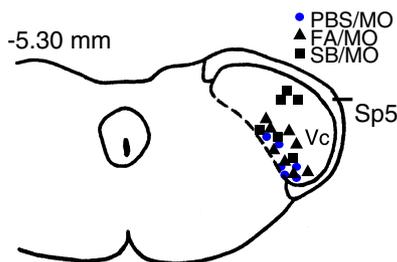

Fig. 1. Histologically confirmed neuronal recording sites. The recording sites were located at 1.5–2.0 mm caudal to obex, and for ease of depiction, they were all transferred into a single section plane corresponding to the −5.3 mm section (behind the interaural line) of the rat atlas (Paxinos and Watson, 1986). Abbreviations in this and subsequent figures: FA, fluoroacetate; PBS, phosphate-buffered saline; SB, SB203580; Sp 5, trigeminal spinal tract; Vc, trigeminal subnucleus caudalis.

2.5. Statistical analyses

All values are presented as means ± SE. Statistical analyses were based on the normalized data. Differences between the baseline value and values at different times after the chemical application (i.e., PBS, FA, SB or MO) were treated by repeated measures analysis of variance (RM ANOVA) on Ranks followed by Dunnett's test. Differences between the FA/MO or SB/MO group and the PBS/MO group were analyzed by two-way ANOVA followed by Dunnett's test. The level of significance was set at $P < .05$.

3. Results

A total of 20 functionally identified NS neurons were recorded and tested (six neurons in the PBS/MO and SB/MO groups, eight in the FA/MO group). The recording sites of all NS neurons were histologically verified and were mainly in the deep laminae of Vc (see Fig. 1).

3.1. Effects of PBS on the central sensitization evoked by MO

3.1.1. Orofacial RF size

All six NS neurons tested in the PBS/MO group had at baseline an ipsilateral RF with a mean size of $2.0 \pm 0.5 \, \text{cm}^2$. An example of a NS neuron in this group is illustrated in Fig. 2. Superfusion of PBS over Vc induced no significant change in RF size of these neurons ($1.9 \pm 0.5 \, \text{cm}^2$). MO application to the tooth pulp produced a significant, long-lasting increase in RF size in all neurons tested. Compared to the baseline value, the mean RF size increased significantly throughout the 50-min observation period after MO application ($P < .001$, RM ANOVA on Ranks), with its peak at 18 min as shown in Fig. 3. In addition, soon after MO application, a novel tactile RF appeared for 10–30 min in three of the six neurons.

3.1.2. Mechanical activation threshold

The six neurons tested in the PBS/MO group had at baseline a mean mechanical activation threshold of $100 \pm 38 \, \text{g}$. An example of a NS neuron in this group is illustrated in Fig. 2. PBS superfusion over Vc produced no significant change in threshold ($97 \pm 35 \, \text{g}$) in these neurons, but the threshold significantly decreased following MO application to the tooth pulp ($P = .002$, RM ANOVA on Ranks) and reached a peak decrease of $50 \pm 17 \, \text{g}$ around 18 min after MO application (see Fig. 4).

3.1.3. Responses to graded pinch/pressure stimuli

In the PBS/MO group, the six neurons had a mean baseline response to graded pinch/pressure stimuli of 52 ± 15 spikes. An example of a NS neuron in this group is illustrated in Fig. 2. PBS superfusion over Vc produced no significant change in the response (36 ± 10 spikes) of these neurons. The subsequent application of MO to the tooth pulp significantly increased the response ($P = .02$, RM ANOVA on Ranks; see Fig. 5), with a peak of 130 ± 41 spikes at 8 min after MO application; the response then slowly declined although it still had not reached baseline levels by 50 min.
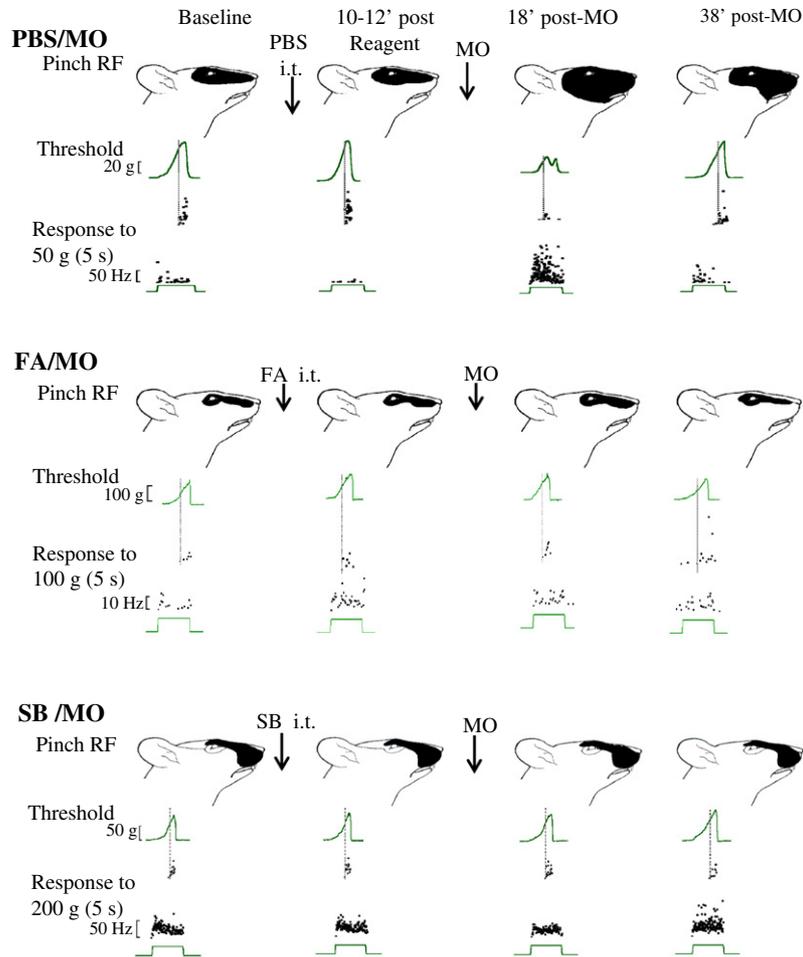
Fig. 2. Examples showing MO-induced neuroplastic changes reflecting central sensitization in NS neurons after i.t. application of PBS, SB or FA to Vc. For each example, the pinch RF (upper panel), mechanical activation threshold (middle panel) and responses to pinch/pressure stimuli (lower panel) are shown. Data of each neuron at baseline, 10–12 min after PBS, FA or SB application (i.e., prior to MO application) as well as at 18 and 38 min after MO application are arranged in columns from left to right. Note that the neuron illustrated from the PBS/MO group showed marked MO-induced neuroplastic changes, while no such marked changes occurred in the neurons of the FA and SB groups. Calibrations of stimulation intensities and neuronal responses to pinch/pressure stimuli are shown. The vertical cut-off line defines the activation threshold of a given neuron; the discharges related to the activation threshold and responses to pressure/pinch stimuli are displayed in a dot raster of inter-spike instantaneous frequency.

3.2. Effects of FA on the central sensitization evoked by MO

3.2.1. Orofacial RF size

The eight neurons in the FA/MO group had at baseline an ipsilateral RF with a mean size of $1.8 \pm 0.4 \, cm^2$. An example of a NS neuron in this group is illustrated in Fig. 2. FA continuous superfusion over Vc did not significantly affect the neuronal RF size, and the subsequent MO application also did not produce any increase in RF size of these neurons ($P > .05$, RM ANOVA on Ranks), as shown in Fig. 3. There were significant treatment ($F_{(1,84)} = 43.78$, $P < .001$, two-way ANOVA), time-point ($F_{(6,84)} = 5.18$, $P < .001$) and interaction ($F_{(6,84)} = 4.53$, $P < .001$) effects in this group compared to the PBS/MO group. A *post hoc* analysis indicated that there were significant differences at all the tested time-points after MO application ($P < .05$, Dunnett's *t*-test), as shown in Fig. 3. In addition, a novel tactile RF did not appear after MO application in the FA/MO group.

3.2.2. Mechanical activation threshold

The eight neurons in the FA/MO group had at baseline a mean mechanical activation threshold of $54 \pm 10 \, g$. An example of a NS neuron in the FA/MO group is illustrated in Fig. 2. FA superfusion over Vc did not significantly affect the threshold in these neurons. The subsequent application of MO also did not evoke any significant changes in threshold values ($P > .6$, RM ANOVA on Ranks; see Fig. 4). The difference in threshold between this group and the PBS/MO group was significant for treatment ($F_{(1,84)} = 8.78$, $P = .004$, two-way ANOVA). A *post hoc* analysis indicated that there was significant difference at 8, 28 min between these two groups ($P < .05$, Dunnett's *t*-test; see Fig. 4).

3.2.3. Responses to graded pinch/pressure stimuli

In the FA/MO group, the eight neurons had a mean baseline response of 131 ± 27 spikes to graded pinch/pressure stimuli. An example of a NS neuron in the FA/MO group is illustrated in Fig. 2. FA superfusion to Vc pro-
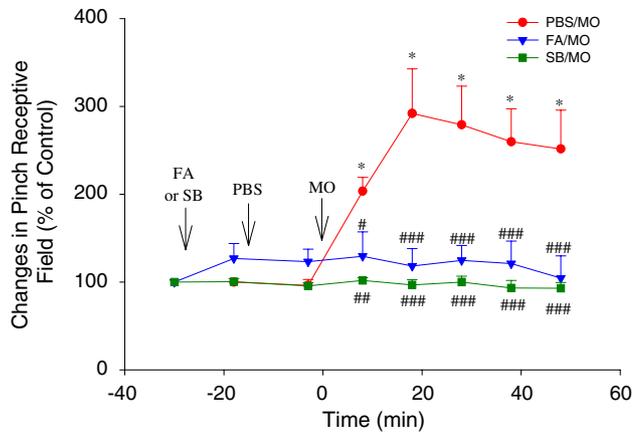
Fig. 3. Changes in neuronal pinch/pressure RF induced by MO application to the tooth pulp after i.t. application of PBS, FA or SB. Mean (±SE) values are shown for each group at the different time-points. *$P < .05$ compared to the baseline within the group (RM ANOVA on Ranks followed by Dunnett's test); #$P < .05$, ##$P < .01$, ###$P < .001$ compared to PBS/MO group at different time-points tested (two-way ANOVA followed by Dunnett's *t*-test). Arrows indicate the application time of drugs or PBS.
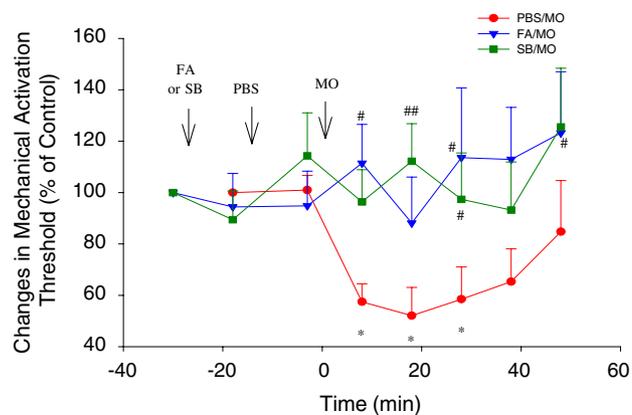

Fig. 4. Changes in neuronal mechanical activation threshold induced by MO application to the tooth pulp after i.t. application of PBS, FA or SB. Mean (±SE) values are shown for each group at the different time-points. *$P < .05$ compared to the baseline within the group (RM ANOVA on Ranks followed by Dunnett's test); #$P < .05$, ##$P < .01$ compared to PBS/MO group at different time-points tested (two-way ANOVA followed by Dunnett's *t*-test). Arrows indicate the application time of drugs or PBS.

duced no significant change in response in these neurons. The subsequent MO application did not produce any marked increase in responses ($P > .05$, RM ANOVA on Ranks; see Fig. 5). The difference in response between this group and the PBS/MO group was significant for treatment ($F_{(1,84)} = 5.27$, $P < .05$, two-way ANOVA). A *post hoc* analysis indicated that there was significant difference at 3 min between these two groups ($P < .05$, Dunnett's *t*-test; see Fig. 5).

3.3. Effects of SB on the central sensitization evoked by MO

In the SB group, three neurons were located in the deep Vc laminae and three more superficially (Fig. 1) but since there were no significant differences between both neuronal
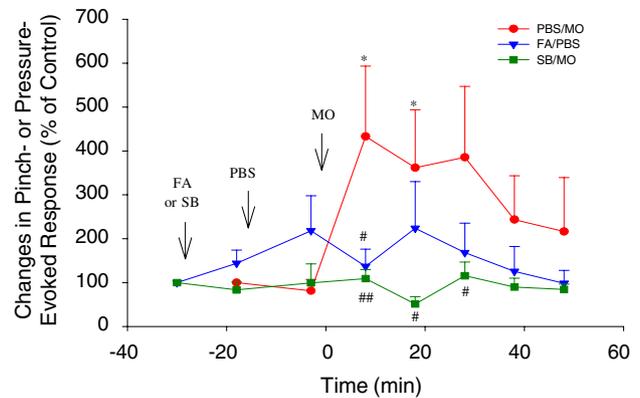

Fig. 5. Changes in neuronal responses to graded mechanical stimuli induced by MO application to the tooth pulp after i.t. application of PBS, FA or SB. Mean (±SE) values are shown for each group at the different time-points. *$P < .05$ compared to the baseline within the group (RM ANOVA on Ranks followed by Dunnett's test); #$P < .05$, ##$P < .01$ compared to PBS/MO group at different time-points tested (two-way ANOVA followed by Dunnett's *t*-test). Arrows indicate the application time of drugs or PBS.

groups in their baseline properties or effects of MO or SB, their data were pooled together.

3.3.1. Orofacial RF size

The six neurons in the SB/MO group had at baseline an ipsilateral RF with a mean size of $1.5 \pm 0.4 \, cm^2$. The neuronal RF was located in the maxillary and/or ophthalmic division, depending on whether the recording site was medial or lateral in Vc (e.g., Fig. 2). SB application to Vc did not affect the RF size, and the subsequent MO application failed to evoke any significant increase in RF size of these neurons ($P > .05$, RM ANOVA on Ranks), as shown in Fig. 3. There were significant treatment ($F_{(1,70)} = 76.65$, $P < .001$, two-way ANOVA), time-point ($F_{(6,70)} = 5.58$, $P < .001$) and interaction ($F_{(6,70)} = 5.79$, $P < .001$) effects in this group compared to the PBS/MO group. A *post hoc* analysis indicated that there were significant differences at all the tested time-points after MO application ($P < .01$, Dunnett's *t*-test; see Fig. 3). In addition, a novel tactile RF did not appear after MO application in the SB/MO group.

3.3.2. Mechanical activation threshold

The six neurons tested in the SB/MO group had at baseline a mean mechanical activation threshold of $176 \pm 72 \, g$. An example of a NS neuron in the SB/MO group is illustrated in Fig. 2. SB application to Vc itself did not affect the threshold, and the subsequent application of MO failed to evoke any significant changes in threshold values in these neurons ($P > .05$, RM ANOVA on Ranks; see Fig. 4). The difference in threshold between this group and the PBS/MO group was significant for treatment ($F_{(1,70)} = 17.43$, $P < .001$, two-way ANOVA). A *post hoc* analysis indicated that the differences between this group and the PBS/MO group were significant at the 18, 28, and 48 min time-points after MO application ($P < .01$, Dunnett's *t*-test; see Fig. 4).

3.3.3. Responses to graded pinch/pressure stimuli

In the SB/MO group, the six neurons had a mean baseline response of 116 ± 32 spikes to graded pinch/pressure stimuli. An example of a NS neuron in the SB/MO group is illustrated in Fig. 2. SB application to Vc itself produced no significant change in response, and the subsequent MO application also failed to produce any marked increases in responses in these neurons ($P > .4$, RM ANOVA on Ranks; see Fig. 5). The changes in response to graded pinch/pressure stimuli in the SB/MO group were significantly different from that of the PBS/MO group ($F_{(1,70)} = 13.87$, $P < .001$, two-way ANOVA). A *post hoc* analysis indicated that the differences were significant at the 8, 18, and 28 min time-points after MO application ($P < .05$, Dunnett's *t*-test; see Fig. 5).

4. Discussion

This study has provided the first documentation of the contribution of glia to central sensitization in functionally identified dorsal horn nociceptive neurons. The i.t. application of an astroglial aconitase blocker (FA) or a microglial p38MAPK inhibitor (SB) can completely abolish central sensitization induced by application of the small-fiber excitant and inflammatory irritant MO in functionally identified nociceptive neurons of Vc, the medullary dorsal horn. Although our data were limited to NS neurons located mainly in the deep laminae of Vc, the features of central sensitization are consistent with our previous studies (Chiang et al., 1998, 2005) revealing Vc central sensitization is reflected in a enlarged neuronal RF size, decreased mechanical activation threshold and increased responses to noxious mechanical stimuli. The possible mechanisms underlying these neuronal changes have previously been discussed (e.g., Hu et al., 1992; Chiang et al., 1998, 2005; Sessle, 2000). Also noteworthy are the present findings that FA and SB application did not affect the baseline RF and response properties of these Vc nociceptive neurons that contribute to ascending trigeminal nociceptive pathways (Dostrovsky and Sessle, 2006; Dubner and Bennett, 1983; Sessle, 2000, 2005). These findings collectively suggest that glial cells are important in the development of central sensitization in trigeminal nociceptive pathways but may not contribute to normal baseline nociceptive processing, consistent with findings in spinal nociceptive models (Meller et al., 1994; Watkins and Maier, 2003, 2005).

The present results implicating microglial p38MAPK in the central sensitization evoked in Vc nociceptive neurons by inflammatory irritants are consistent with recent reports (Yeo et al., 2001; Yang et al., 2005; Piao et al., 2006). p38MAPK is one family of serine/threonine protein kinases (Lee et al., 1999) and plays important roles in cellular responses to external stress signals via inflammatory mediators (Fonseca et al., 2005; Ji, 2004; Ji and Strichartz, 2004; Kumar et al., 2003; Watkins and Maier, 2003). Although there is evidence that neuronal p38MAPK may also be involved in nociceptive processing, this mainly involves primary sensory neurons (Mizushima et al., 2005; Schafers et al., 2003), and p38MAPK mediates hyperalgesia and spinal sensitization via the isoform p38β, which is distinctly expressed in spinal microglia, but not via p38α which is expressed in spinal neurons (Svensson et al., 2005). Furthermore, there are marked increases of p38MAPK in spinal microglia but not in neurons in many pain models, including formalin- or carrageenan-induced inflammation (Kim et al., 2002; Svensson et al., 2003) and inhibition of p38MAPK activation markedly attenuates expression of inflammatory mediators, hyperalgesia and spinal sensitization (Bhat and Fan, 2002; Fonseca et al., 2005; Hua et al., 2005; Kumar et al., 2003; Milligan et al., 2003). Consistent with these findings, the present study has documented that SB application to Vc completely abolishes the MO-induced central sensitization in Vc NS neurons. Although the present study could not exclude the possibility that modulation of neuronal p38MAPK may also have contributed, this finding suggests that microglial p38MAPK is an important element in the production of Vc central sensitization.

Over the last decade, it has also been recognized that astrocytes are involved in spinal nociceptive processing and sensitization (Salter, 2005; Watkins and Maier, 2003). Astrocytes convert glucose to glutamate through the action of aconitase in the TCA cycle, and then glutamate is converted to glutamine which is released into the extracellular space and taken up by presynaptic glutamatergic terminals (Fonseca et al., 2005; Hertz and Zielke, 2004; Keyser and Pellmar, 1994; Martinez-Hernandez et al., 1977). FA and its more toxic metabolite FC inhibit the action of aconitase in the astroglial TCA cycle, which leads to a reduction in the formation and release of glutamine by the astrocytes and eventually a reduction of glutamate utilized by neurons (Fonnum et al., 1997; Swanson and Graham, 1994; Schousboe et al., 1997). Previous studies have reported that FA or FC prevents formalin-induced hyperalgesia evoked by pro-inflammatory substances (Meller et al., 1994; Milligan et al., 2003; Watkins and Maier, 2003; Watkins et al., 1997), suppresses synaptic excitability and the frequency of spontaneous epileptiform activity in hippocampal neurons (Bacci et al., 2002) and rhythmic respiratory bursting activity in brainstem neurons (Hulsmann et al., 2000), effects that can be restored by glutamine. These various findings suggest that aconitase and the astroglial-dependent glutamine supply to neurons is important in the development of enhanced neuronal activity, and may therefore be an important factor in exaggerated pain states. The present study is consistent with this view since i.t. FA markedly attenuated the central sensitization induced by pulp application of MO in Vc nociceptive neurons, suggesting that astroglial TCA metabolism is involved in central sensitization in Vc which is a crucial element in trigeminal nociceptive pathways (Bereiter et al., 2000; Dostrovsky and Sessle, 2006; Dubner and Bennett, 1983; Sessle, 2000, 2005).

The role of glia in Vc central sensitization likely involves their interactions with glutamatergic and purinergic receptor mechanisms, both of which are critical processes in

central sensitization. Glia release glutamate and ATP and also have receptors for these chemical mediators (Allen and Barres, 2005; Ji and Strichartz, 2004; Watkins and Maier, 2003, 2005). Astrocytes may directly influence the state of presynaptic terminals through the activation of presynaptic NMDA receptors by increasing the probability of neurotransmitter release from nerve terminals and modulating synaptic transmission between cultured hippocampal neurons (Araque et al., 1998a,b). In addition, it has been shown that the NMDA antagonist MK-801 blocks both nerve injury-induced allodynia and hyperalgesia and glial activation (Garrison et al., 1994; Watkins and Maier, 2003, 2005). Furthermore, FA pre-treatment produces a substantial reduction in ATP in glial cells which can be prevented by simultaneous application with FA of the astroglial metabolic substrate isocitrate (Keyser and Pellmar, 1994). Also noteworthy are findings that pre-treatment with a p38MAPK inhibitor attenuates not only thermal hyperalgesia and mechanical allodynia but also the NMDA-evoked release of PGE2 (Jin et al., 2003; Svensson et al., 2003; Tsuda et al., 2004) and the ATP-evoked activation of p38MAPK and subsequent IL-6 production and release in microglial cell cultures (Shigemoto-Mogami et al., 2001). In addition, there are also reports that ATP potently stimulates inflammatory cytokine release in rat cultured brain microglia which can be inhibited by SB (Hide et al., 2000). These various findings, together with the present data and our previous studies documenting that the MO/pulp-induced central sensitization reflected in enhanced RF size and responses to noxious stimuli and decreased activation threshold in Vc nociceptive neurons can be modulated by NMDA and P2X receptor agonists or antagonists (Chiang et al., 1998, 2005), suggest that astrocytic and microglial metabolic processes may be involved in the central sensitization in dorsal horn nociceptive neurons that is dependent on NMDA and purinergic receptor mechanisms.

Acknowledgments

References

Allen, N.J., Barres, B.A., 2005. Signaling between glia and neurons: focus on synaptic plasticity. Curr. Opin. Neurobiol. 15, 542–548.

Araque, A., Parpura, V., Sanzgiri, R.P., Haydon, P.G., 1998a. Glutamate-dependent astrocyte modulation of synaptic transmission between cultured hippocampal neurons. Eur. J. Neurosci. 10, 2129–2142.

Araque, A., Sanzgiri, R.P., Parpura, V., Haydon, P.G., 1998b. Calcium elevation in astrocytes causes an NMDA receptor-dependent increase in the frequency of miniature synaptic currents in cultured hippocampal neurons. J. Neurosci. 18, 6822–6829.

Bacci, A., Sancini, G., Verderio, C., Armano, S., Pravettoni, E., Fesce, R., Franceschetti, S., Matteoli, M., 2002. Block of glutamate–glutamine cycle between astrocytes and neurons inhibits epileptiform activity in hippocampus. J. Neurophysiol. 88, 2302–2310.

Bereiter, D.A., Hirata, H., Hu, J.W., 2000. Trigeminal subnucleus caudalis: beyond homologies with the spinal dorsal horn. Pain 88, 221–224.

Bhat, N.R., Fan, F., 2002. Adenovirus infection induces microglial activation: involvement of mitogen-activated protein kinase pathways. Brain Res. 948, 93–101.

Chiang, C.Y., Hu, B., Hu, J.W., Dostrovsky, J.O., Sessle, B.J., 2002. Central sensitization of nociceptive neurons in trigeminal subnucleus oralis depends on integrity of subnucleus caudalis. J. Neurophysiol. 88, 256–264.

Chiang, C.Y., Hu, J.W., Sessle, B.J., 1994. Parabrachial area and nucleus raphe magnus-induced modulation of nociceptive and nonnociceptive trigeminal subnucleus caudalis neurons activated by cutaneous or deep inputs. J. Neurophysiol. 71, 2430–2445.

Chiang, C.Y., Park, S.J., Kwan, C.L., Hu, J.W., Sessle, B.J., 1998. NMDA receptor mechanisms contribute to neuroplasticity induced in caudalis nociceptive neurons by tooth pulp stimulation. J. Neurophysiol. 80, 2621–2631.

Chiang, C.Y., Zhang, S., Xie, Y.F., Hu, J.W., Dostrovsky, J.O., Salter, M.W., Sessle, B.J., 2005. Endogenous ATP involvement in mustard-oil-induced central sensitization in trigeminal subnucleus caudalis (medullary dorsal horn). J. Neurophysiol. 94, 1751–1760.

Chiang, C.Y., Zhang, S., Xie, Y.F., Hu, J.W., Dostrovsky, J.O., Sessle, B.J., 2004. Glia involvement in central sensitization in trigeminal subnucleus caudalis (medullary dorsal horn). Soc. Neurosci. Abstr. 30. 518.10.

Dostrovsky, J.O., Sessle, B.J., 2006. Nociceptive processing in the brainstem. In: Schmidt, R.F., Willis, W.D. (Eds.), Encyclopaedia of Pain, Section 10, Springer, Heidelberg, Germany, in press.

Dubner, R., 2005. Plasticity in central nociceptive pathways. In: Merskey, H., Loeser, J.D., Dubner, R. (Eds.), The Paths of Pain 1975–2005. IASP Press, Seattle, pp. 101–115.

Dubner, R., Bennett, G.J., 1983. Spinal and trigeminal mechanisms of nociception. Annu. Rev. Neurosci. 6, 381–418.

Fonnum, F., Johnsen, A., Hassel, B., 1997. Use of fluorocitrate and fluoroacetate in the study of brain metabolism. Glia 21, 106–113.

Fonseca, L.L., Monteiro, M.A., Alves, P.M., Carrondo, M.J., Santos, H., 2005. Cultures of rat astrocytes challenged with a steady supply of glutamate: new model to study flux distribution in the glutamate–glutamine cycle. Glia 51, 286–296.

Garrison, C.J., Dougherty, P.M., Carlton, S.M., 1994. GFAP expression in lumbar spinal cord of naive and neuropathic rats treated with MK-801. Exp. Neurol. 129, 237–243.

Hertz, L., Zielke, H.R., 2004. Astrocytic control of glutamatergic activity: astrocytes as stars of the show. Trends Neurosci. 27, 735–743.

Hide, I., Tanaka, M., Inoue, A., Nakajima, K., Kohsaka, S., Inoue, K., Nakata, Y., 2000. Extracellular ATP triggers tumor necrosis factor-alpha release from rat microglia. J. Neurochem. 75, 965–972.

Hu, B., Chiang, C.Y., Hu, J.W., Dostrovsky, J.O., Sessle, B.J., 2002. P2X receptors in trigeminal subnucleus caudalis modulate central sensitization in trigeminal subnucleus oralis. J. Neurophysiol. 88, 1614–1624.

Hu, J.W., Sessle, B.J., Raboisson, P., Dallel, R., Woda, A., 1992. Stimulation of craniofacial muscle afferents induces prolonged facilitatory effects in trigeminal nociceptive brain-stem neurones. Pain 48, 53–60.

Hua, X.Y., Svensson, C.I., Matsui, T., Fitzsimmons, B., Yaksh, T.L., Webb, M., 2005. Intrathecal minocycline attenuates peripheral inflammation-induced hyperalgesia by inhibiting p38MAPK in spinal microglia. Eur. J. Neurosci. 22, 2431–2440.

Hulsmann, S., Oku, Y., Zhang, W., Richter, D.W., 2000. Metabolic coupling between glia and neurons is necessary for maintaining respiratory activity in transverse medullary slices of neonatal mouse. Eur. J. Neurosci. 12, 856–862.

Ji, R.R., 2004. Mitogen-activated protein kinases as potential targets for pain killers. Curr. Opin. Investig. Drugs 5, 71–75.

Ji, R.R., Strichartz, G., 2004. Cell signaling and the genesis of neuropathic pain. Sci. STKE reE14.

Jin, S.X., Zhuang, Z.Y., Woolf, C.J., Ji, R.R., 2003. p38 mitogen-activated protein kinase is activated after a spinal nerve ligation in spinal cord microglia and dorsal root ganglion neurons and contributes to the generation of neuropathic pain. J. Neurosci. 23, 4017–4022.

Keyser, D.O., Pellmar, T.C., 1994. Synaptic transmission in the hippocampus: critical role for glial cells. Glia 10, 237–243.

Kim, S.Y., Bae, J.C., Kim, J.Y., Lee, H.L., Lee, K.M., Kim, D.S., Cho, H.J., 2002. Activation of p38 MAP kinase in the rat dorsal root ganglia and spinal cord following peripheral inflammation and nerve injury. Neuroreport 13, 2483–2486.

Kumar, S., Boehm, J., Lee, J.C., 2003. p38 MAP kinases: key signalling molecules as therapeutic targets for inflammatory diseases. Nat. Rev. Drug Discov. 2, 717–726.

Lee, J.C., Kassis, S., Kumar, S., Badger, A., Adams, J.L., 1999. p38 mitogen-activated protein kinase inhibitors–mechanisms and therapeutic potentials. Pharmacol. Ther. 82, 389–397.

Martinez-Hernandez, A., Bell, K.P., Norenberg, M.D., 1977. Glutamine synthetase: glial localization in brain. Science 195, 1356–1358.

Meller, S.T., Dykstra, C., Grzybycki, D., Murphy, S., Gebhart, G.F., 1994. The possible role of glia in nociceptive processing and hyperalgesia in the spinal cord of the rat. Neuropharmacology 33, 1471–1478.

Milligan, E.D., Twining, C., Chacur, M., Biedenkapp, J., O'Connor, K., Poole, S., Tracey, K., Martin, D., Maier, S.F., Watkins, L.R., 2003. Spinal glia and proinflammatory cytokines mediate mirror-image neuropathic pain in rats. J. Neurosci. 23, 1026–1040.

Mizushima, T., Obata, K., Yamanaka, H., Dai, Y., Fukuoka, T., Tokunaga, A., Mashimo, T., Noguchi, K., 2005. Activation of p38MAPK in primary afferent neurons by noxious stimulation and its involvement in the development of thermal hyperalgesia. Pain 113, 51–60.

Park, S.J., Chiang, C.Y., Hu, J.W., Sessle, B.J., 2001. Neuroplasticity induced by tooth pulp stimulation in trigeminal subnucleus oralis involves NMDA receptor mechanisms. J. Neurophysiol. 85, 1836–1846.

Paxinos, G., Watson, C., 1986. The Rat Brain in Stereotaxic Coordinates, second ed. Academic Press, New York.

Piao, Z.G., Cho, I.H., Park, C.K., Hong, J.P., Choi, S.Y., Lee, S.J., Lee, S., Park, K., Kim, J.S., Oh, S.B., 2006. Activation of glia and microglial p38MAPK in medullary dorsal horn contributes to tactile hypersensitivity following trigeminal sensory nerve injury. Pain 121, 219–231.

Salter, M.W., 2004. Cellular neuroplasticity mechanisms mediating pain persistence. J. Orofac. Pain 18, 318–324.

Salter, M.W., 2005. Cellular signalling pathways of spinal pain neuroplasticity as targets for analgesic development. Curr. Top. Med. Chem. 5, 557–567.

Schafers, M., Svensson, C.I., Sommer, C., Sorkin, L.S., 2003. Tumor necrosis factor-alpha induces mechanical allodynia after spinal nerve ligation by activation of p38MAPK in primary sensory neurons. J. Neurosci. 23, 2517–2521.

Schousboe, A., Sonnewald, U., Civenni, G., Gegelashvili, G., 1997. Role of astrocytes in glutamate homeostasis. Implications for excitotoxicity. Adv. Exp. Med. Biol. 429, 195–206.

Sessle, B.J., 2000. Acute and chronic craniofacial pain: brainstem mechanisms of nociceptive transmission and neuroplasticity, and their clinical correlates. Crit. Rev. Oral Biol. Med. 11, 57–91.

Sessle, B.J., 2005. Orofacial pain. In: Merskey, H., Loeser, J.D., Dubner, R. (Eds.), The Paths of Pain 1975–2005. IASP Press, Seattle, pp. 128–135.

Shigemoto-Mogami, Y., Koizumi, S., Tsuda, M., Ohsawa, K., Kohsaka, S., Inoue, K., 2001. Mechanisms underlying extracellular ATP-evoked interleukin-6 release in mouse microglial cell line, MG-5. J. Neurochem. 78, 1339–1349.

Svensson, C.I., Fitzsimmons, B., Azizi, S., Powell, H.C., Hua, X.Y., Yaksh, T.L., 2005. Spinal p38beta isoform mediates tissue injury-induced hyperalgesia and spinal sensitization. J. Neurochem. 92, 1508–1520.

Svensson, C.I., Marsala, M., Westerlund, A., Calcutt, N.A., Campana, W.M., Freshwater, J.D., Catalano, R., Feng, Y., Protter, A.A., Scott, B., Yaksh, T.L., 2003. Activation of p38 mitogen-activated protein kinase in spinal microglia is a critical link in inflammation-induced spinal pain processing. J. Neurochem. 86, 1534–1544.

Swanson, R.A., Graham, S.H., 1994. Fluorocitrate and fluoroacetate effects on astrocyte metabolism in vitro. Brain Res. 664, 94–100.

Tsuda, M., Inoue, K., Salter, M.W., 2005. Neuropathic pain and spinal microglia: a big problem from molecules in "small" glia. Trends Neurosci. 28, 101–107.

Tsuda, M., Mizokoshi, A., Shigemoto-Mogami, Y., Koizumi, S., Inoue, K., 2004. Activation of p38 mitogen-activated protein kinase in spinal hyperactive microglia contributes to pain hypersensitivity following peripheral nerve injury. Glia 45, 89–95.

Watkins, L.R., Maier, S.F., 2003. Glia: a novel drug discovery target for clinical pain. Nat. Rev. Drug Discov. 2, 973–985.

Watkins, L.R., Maier, S.F., 2005. Glia and pain: past, present, and future. In: Merskey, H., Loeser, J.D., Dubner, R. (Eds.), The Paths of Pain 1975–2005. IASP Press, Seattle, pp. 165–175.

Watkins, L.R., Martin, D., Ulrich, P., Tracey, K.J., Maier, S.F., 1997. Evidence for the involvement of spinal cord glia in subcutaneous formalin induced hyperalgesia in the rat. Pain 71, 225–235.

Woolf, C.J., Salter, M.W., 2005. Central nervous system mechanism of pain modulation. In: McMahon, S., Koltzenburg, M. (Eds.), Textbook of Pain, fifth ed. Churchill Livingstone, Edinburgh, pp. 91–105.

Xie, Y.F., Chiang, C.Y., Park, S.J., Hu, J.W., Dostrovsky, J.O., Sessle, B.J., 2005. Effect of p38 mitogen-activated protein kinase (mapk) inhibitor on central sensitization in trigeminal subnucleus caudalis (medullary dorsal horn). Soc. Neurosci. Abstr. 31.861.1.

Yang, C.S., Jung, C.Y., Ju, J.S., Lee, M.K., Ahn, D.K., 2005. Intracisternal administration of mitogen-activated protein kinase inhibitors reduced IL-1beta-induced mirror-image mechanical allodynia in the orofacial area of rats. Neurosci. Lett. 387, 32–37.

Yeo, J.F., Liu, H.P., Leong, S.K., 2001. Sustained microglial immunoreactivity in the caudal spinal trigeminal nucleus after formalin injection. J. Dent. Res. 80, 1524–1529.

Yu, X.M., Sessle, B.J., Hu, J.W., 1993. Differential effects of cutaneous and deep application of inflammatory irritant on mechanoreceptive field properties of trigeminal brain stem nociceptive neurons. J. Neurophysiol. 70, 1704–1707.