Modulation of astroglial glutamine synthetase activity affects nociceptive behaviour and central sensitization of medullary dorsal horn nociceptive neurons in a rat model of chronic pulpitis

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Keywords: antinociceptive, glutamate, hyperalgesia, trigeminal

Abstract
Previous studies indicate that the astroglial glutamate–glutamine shuttle may be involved in acute pulpal inflammatory pain by influencing central sensitization induced in nociceptive neurons in the trigeminal subnucleus caudalis [the medullary dorsal horn (MDH)] by application of an inflammatory irritant to the rat tooth pulp. The aim of this study was to test if intrathecal application to the rat medulla of the astroglial glutamine synthetase inhibitor methionine sulfoximine (MSO) can influence the central sensitization of MDH nociceptive neurons and the animal’s associated behaviour that are manifested in a model of chronic pulpitis pain induced by exposure of a mandibular molar pulp. This model was found to be associated with nocifensive behaviour and enhanced reflex activity evoked by mechanical stimulation of the rat’s facial skin and with immunocytochemical evidence of astroglial activation in the MDH. These features were apparent for up to 28 days post-operatively. During this post-operative period, the nocifensive behaviour and enhanced reflex activity were significantly attenuated by intrathecal application of MSO (5 μL, 10 mM) but not by vehicle application. In electrophysiological recordings of nociceptive neuronal activity in the MDH, central sensitization was also evident in pulp-exposed rats but not in intact rats and could be significantly attenuated by MSO application but not by vehicle application. These behavioural and neuronal findings suggest that the astroglial glutamate–glutamine shuttle is responsible for the maintenance of inflammation-induced nocifensive behavioural changes and the accompanying central sensitization in MDH nociceptive neurons in this chronic pulpitis pain model.

Introduction
Toothache is a very common pain condition, and recent studies have provided some insights into central neural processes related to dental pain (for review, see Byers & Näärhi, 1999; Sessle, 2000; Hu, 2004). These include our findings that application to the rat molar pulp of the small-fibre excitant and inflammatory irritant mustard oil (MO) produces a glutamatergic-dependent central sensitization in nociceptive neurons in the trigeminal spinal subnucleus caudalis [also termed the medullary dorsal horn (MDH)] (Chiang et al., 1998, 2005, 2007, 2008, 2010a,b; Hu et al., 2002; Xie et al., 2007). Central sensitization is considered to be a crucial mechanism underlying the increased excitability of nociceptive pathways following peripheral tissue injury and inflammation, and has been implicated in the development and maintenance of persistent pain states (see Dubner & Basbaum, 1994; Sessle, 2000; Woolf & Salter, 2006). Recent studies have provided evidence that non-neural (glial) mechanisms may contribute to MDH central sensitization. Glia–neuron interactions are known to be involved in mechanisms underlying the development of chronic inflammatory and neuropathic pain (see Raivich, 2005; Watkins & Maier, 2005; Scholz & Woolf, 2007; Milligan & Watkins, 2009; Gao & Ji, 2010; Jarvis, 2010; Chiang et al., 2011). The glutamate–glutamine shuttle in astroglia may play a key role as it involves astroglial uptake of extrasynaptic glutamate followed by the production of glutamine from glutamate through the action of the astroglial enzyme glutamine synthetase (GS), and then glutamine is released from astroglia after which it is taken up by neuronal elements to replenish the supply of glutamate (Suarez et al., 1997; Zwingmann & Leibfritz, 2003; Hertz & Zielke, 2004; Fonseca et al., 2005; Woolf & Salter, 2006). A potent inhibitor of GS is methionine sulfoximine (MSO), and its inhibitory effects can be counteracted by exogenous application of glutamine (Bacci et al., 2002; Blin et al., 2002; Shin et al., 2003; Gibbs & Hertz, 2005; Tanigami et al., 2005; Liang et al., 2006; Okada-Ogawa et al., 2009). In our acute MO/pulp pain model, superfusion over the MDH of MSO or the astroglial metabolic...
inhibitor fluoroacetate or a glutamine transporter blocker can attenuate the MO-induced central sensitization in MDH nociceptive neurons, whereas basal neuronal properties remain unchanged (Chiang et al., 2007, 2008; Xie et al., 2007), consistent with findings that glia may not affect basal nociceptive processing but rather participate in exaggerated pain states (Watkins & Maier, 2005).

Although our previous MO/pulp-related studies have provided important insights into the role of medullary non-neuronal cells (glia) as well as neural processes in nociceptive mechanisms associated with acute pulpitis, the mechanisms underlying chronic dental pain are unclear. In the present study, we have used a chronic pulp inflammatory model (Torneck et al., 1996; Chudler & Byers, 2005) to test if intrathecal (i.t.) application to the rat medulla of the astroglial GS inhibitor MSO can influence central sensitization of MDH nociceptive neurons and the animal's associated behaviour in this model of chronic pulpitis pain. Some of the data have been published in abstract form (Hu et al., 2006; Miyamoto et al., 2010).

Materials and methods

Experimental animals

Adult male Sprague-Dawley rats (250–350 g) were kept in their home cages and provided with food and water ad libitum for at least 5 days before any behavioural testing or pulpal exposure operation. 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).

Tooth pulp exposure procedure

In accordance with our previously published procedures, which produce a chronic inflammation of the pulp (Torneck et al., 1996), the rat was briefly anaesthetized with halothane and the mandibular left first molar pulp was exposed by a slowly rotating dental burr (with #1 round tip), left exposed without any further treatment and then the rat was returned to its home cage. Some rats did not receive the pulp exposure and served as intact controls (i.e. with an intact pulp). Rats were monitored post-operatively to ensure that they were not experiencing discomfort leading to a disruption of feeding and to a loss in weight (e.g. Fig. S1).

Behavioural and reflex testing

In the behavioural experiments, two groups of rats with (n = 9) or without (n = 8) pulp exposure were tested for their withdrawal threshold (WT) to mechanical stimulation of the facial skin (see below). At least three behavioural sessions on successive days were given to provide baseline values before the pulp-exposure operation. Each rat was then tested behaviourally at day 0 (1 h before the pulp exposure surgery) and at 1, 3, 5, 7, 14, 21 and 28 days after surgery. Escape responses to mechanical stimulation of the mandibular skin (2 mm below the lower lip) and maxillary skin (whisker pad) ipsilateral and contralateral to the pulp exposure side were also then determined in these rats, as previously described (Iwata et al., 2001; Saito et al., 2008). For this testing, we employed a black plastic tube (5 cm diameter) with a black cover at one end that also contained a 1.6 cm diameter opening reinforced with dental cement to discourage gnawing. The rats had a natural tendency to enter the tube and remain quiet and still in the tube for a long period of time (> 30 min). The rats adapted well to this position and remained quiet and still in the tube for the testing period. They placed their nose, mouth and

whisker pad through the 1.6 cm diameter opening, thus allowing the experimenter to readily probe the exposed facial region with different von Frey filaments (1, 1.4, 2, 4, 6, 8, 10, 16, 26 and 60 g; North Coast Medical, Inc., Morgan Hill, CA, USA). Although 60 g was the maximal force applied to the maxillary skin, we limited the maximal force tested on the mandibular skin to 16 g because a von Frey filament force of 26 g or more applied to the mandibular skin produced a potentially confounding passive movement of the mandible in intact rats. In cases where there was no withdrawal at forces below 16 g, the WT was rated as 16. Escape responses to the mechanical stimulation were manifested as a sudden backward withdrawal movement of the head from the opening. The WT at each mandibular and maxillary site was defined as the lowest filament intensity that evoked two or more escapes out of five stimulation trials with intertrial intervals of more than 10 s.

Another series of behavioural experiments was carried out in 20 rats at 10–14 days after the pulp exposure. It involved two i.t. injections (separated by > 3 days) of L-MSO (5 μL; at 1 mM (n = 6) or 10 mM (n = 8), freshly dissolved in phosphate-buffered saline (PBS); Sigma-Aldrich Canada, Oakville, ON, Canada) or vehicle (PBS, as control; n = 6). Behavioural testing was carried out beforehand and for 20–120 min and 24 h after each injection. The i.t. injection was performed under brief halothane anaesthesia (4% for rapid induction and 1.5% for maintenance, a total time of < 5 min); a 27-gauge dental needle (45° bevel) covered with a plastic sleeve except for the distal 10 mm (which typically was the distance between the skin at the posterior edge of the skull and surface of the medulla) was inserted into the subarachnoid space overlying the caudal medulla. This needle was connected with PE-10 tubing to a Hamilton microsyringe. This procedure was similar to that described by Fischer et al. (2005) except that the needle was encased in a fixed length of tubing to prevent its penetration into the medulla. The injection procedure took less than 5 min and aspiration of cerebrospinal fluid without any evidence of blood contamination was used to assist the correct placement of the needle. To further confirm the integrity of the medulla during the i.t. injection procedure, Evans Blue dye was injected i.t. (instead of MSO or PBS) in two intact rats that were killed 20 min later with i.p. 2.5 mg/g urethane. At autopsy, the dye distribution was visualized only on the surface of the medulla and first three cervical spinal segments, with the highest concentration of dye near the obex region. In order to rule out the possibility of motor disturbances associated with the MSO injection (Giniefi-Gayet & Gayet, 1988), three additional groups of rats (n = 5 each) received i.t. injection of MSO (1 or 10 mM) or PBS and were subjected to the rota-rod test (UGO BASILE, VA, Italy) immediately before the injection and at 20, 40 and 60 min after the injection. The rat was trained to stay on the rotating rod (at a speed of 6 rpm) for 2 min, and the animal’s test score was based on the time that it remained on the rod when the rotating speed was changed from 6 to 60 rpm. In a series of reflex behavioural experiments, rats were anaesthetized with halothane mixed with O2 and N2O (O2, 0.3–0.4 L/min; N2O, 0.6 L/min; halothane, 1.5–2.5%) via a face mask, and then artificially ventilated for the duration of the experiment. Bipolar electrodes were fabricated from 40-gauge Teflon-coated, single-stranded stainless-steel wires and inserted into the left neck to record the electromyographic activity of the splenius capitis muscle, as previously described (Yu et al., 1995; Saito et al., 2008). The halothane concentration was then adjusted (0.8–1.0%) until the flexion reflex response to mechanical pressure applied to the hindpaw disappeared, to ensure that an adequate level of anaesthesia was maintained for the duration of the experiment. The threshold for evoking a reflex response in the neck muscle was next determined by applying a noxious mechanical stimulus to the ipsilateral or contralateral mandibular skin with a rodent pincher (RP-1; BIOSEB,
Ghavile Cedex, France). In each rat, the stimulus was progressively increased, and the threshold was assessed as the lowest stimulus intensity (in g) evoking a reflex response more than 2 SD above the baseline (pre-stimulation) electromyographic activity (see Fig. 3B). The reflex activation threshold was measured on the day before, as well as at 7, 14 and 28 days after the pulp exposure (n = 6). Rats with intact tooth pulps served as controls (n = 6). In order to test the effect of MSO on the reflex response, the activation threshold in rats with pulp exposure was measured at 20, 40 and 60 min after i.t. application of MSO (5 μL bolus, 1 mM, n = 7 or 10 mM, n = 9) or PBS (n = 7) on day 7 after the exposure.

Neuronal recording

Another series of experiments used 15 intact rats as well as 39 rats that had received pulp exposure at 7 (n = 13), 14 (n = 12) and 28 (n = 14) days previously. All of these rats had undergone WT behavioural testing (but not i.t. MSO injection) to ensure the presence of hyperalgesia in the pulp-exposed rats. Each of these rats was anaesthetized by a single intraperitoneal injection of a mixture of α-chloralose (50 mg/kg) and urethane (1 g/kg). A tracheal cannula was inserted and each rat was artificially ventilated throughout the whole experimental period. During each recording session, the rats were immobilized with pancuronium bromide (1 mg/kg, 0.4–0.5 mL/h) and artificially ventilated. The heart rate, percentage expired CO₂ 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.

Single neuronal activity was recorded extracellularly by methods similar to those described in detail in our previous publications (Hu, 1990; Chiang et al., 1998, 2005, 2007, 2008, 2010a,b). As the immunohistochemical analyses (see below) indicated that astroglia were mainly activated in the dorsomedial portion of the MDH, i.e. the MDH region where neurons with mandibular or mandibular plus maxillary mechanoreceptive fields (RFs) are found (e.g. Hu & Sessle, 1984; Chiang et al., 1998) and where mandibular pulp afferents principally terminate in the MDH (Takemura et al., 1991), our microelectrode tracks were targeted to this particular region of the MDH. Briefly, as the microelectrode was advanced into the MDH, stimuli (see below) were applied to the orofacial tissues to search for MDH nociceptive neurons receiving an orofacial sensory input. Neuronal activity was amplified, displayed on oscilloscopes and also fed to a window discriminator connected to an A/D converter (CED 1401 plus, Cambridge Electronic Design, Cambridge, UK) and a personal computer. Data were analysed off-line with Spike 2 software (Cambridge Electronic Design). 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, wide dynamic range (WDR) or nociceptive-specific (NS) as previously described (Chiang et al., 1998, 2005, 2007, 2008, 2010a,b; Hu, 1990; Hu et al., 1992). Only WDR and NS neurons were studied in detail as we have previously documented that low-threshold mechanoreceptive neurons show no evidence of central sensitization that may be reflected in increases in spontaneous activity, RF size and responses to noxious stimuli, and in a decreased activation threshold. The average spontaneous activity (in Hz) of a nociceptive neuron was determined over a 2 min period without any stimulation at the early stage of neuronal recording as well as between the assessments of the RF after MSO application. The cutaneous tactile and nociceptive RF of each WDR neuron and the cutaneous nociceptive RF of each NS neuron were determined and outlined on a life-sized drawing of a rat’s head. The stimulus-evoked responses of each nociceptive neuron were determined by applying an ascending staircase series of 10, 20, 40, 80, 100 and 200 g forces, each for 3 s, with a pair of force-monitoring forceps, and the average response frequency (Hz) was counted at each force level. For WDR neurons, a series of 10 brush (< 1 g force) stimulations was also swept through the tactile RF and the total number of evoked spikes noted. Any afterdischarges to noxious radiant heat (duration 3 s) and mechanical stimulation were recorded for up to 10 s after the offset of the stimulation.

Because WDR but not NS neurons responded over a wide range of mechanical stimulus intensities ranging from tactile to noxious, the stimulus–response (S-R) relationships and the effect of MSO on these relationships were only studied in WDR neurons in rats receiving the pulp exposure 10–14 days previously. After the baseline neuronal RF and response data were recorded, MSO (5 μL, 1 mM, n = 5 and 10 mM, n = 5) or PBS (n = 5) was applied i.t. over the exposed caudal medulla. The neuronal properties were assessed prior to and at 20, 40 and 60 min after the MSO or PBS application. The i.t. application of MSO or PBS was carried out only once in each experiment. At the conclusion of neuronal recording, the neuronal recording sites were marked by electrolytic lesions (anodal current of 8 μA for 10 s) and verified histologically as previously described (Hu, 1990; Chiang et al., 1998).

Immunohistochemical analyses

Our previously described procedures (Okada-Ogawa et al., 2009) were used for glial fibrillary acidic protein (GFAP) and GS immunohistochemistry. Four groups of rats (intact, 7, 14 and 28 days after pulp exposure; n = 3 in each group) were anaesthetized with sodium pentobarbital (80 mg/kg, i.p.) and perfused through the aorta with isotonic saline (100 mL) followed by 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4, 500 mL). The medulla and upper cervical spinal cord were removed and post-fixed in 4% paraformaldehyde for 3 days at 4 °C. The tissues were then transferred to 20% sucrose (w/v) in PBS overnight for cryoprotection, and then sections (30 μm thick) were cut with a freezing microtome and processed for GFAP (a specific marker of astroglia) and GS immunohistochemistry, alongside negative controls. Free-floating tissue sections were rinsed in PBS and 10% normal goat serum in PBS for 1 h and then incubated in mouse monoclonal anti-GFAP antibody (1 : 1000; Chemicon, Temecula, CA, USA) and rabbit anti-GS antibody (1 : 5000; Abcam, Cambridge, UK) for 2 days at 4 °C. Sections were then incubated in anti-mouse Alexa Fluor 568 IgG (1 : 200; Invitrogen, Eugene, OR, USA) and anti-rabbit Alexa Fluor 488 IgG (1 : 1000; Invitrogen) for 2 h at room temperature (23 °C) in a dark room. After washing they were mounted and cover slipped. Immunopositive cells were detected using a fluorescence microscope (BX 9000; KEYENCE, Tokyo, Japan). From three consecutive slices of the rostral end of the MDH in each rat, two photomicrographic images were taken from the dorsomedial and lateral aspects of the superficial MDH that receive, respectively, mandibular cutaneous and mandibular and maxillary pulp afferents, and maxillary cutaneous afferents (Takemura et al., 1991; Noma et al., 2008). The areas occupied by GFAP-positive cells (expressed as a percent of the 20 × 20 μm² image selected from each photomicrograph of the dorsomedial and lateral aspects of the superficial MDH) in intact and 7-, 14- and 28-day pulp-exposure rats (all n = 3) were analysed with an imaging analysis system (Image J, version 1.44; National Institute of Health, Bethesda, MD, USA). Similarly, in these groups of rats, the areas occupied by GS-labelled

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GFAP-positive immunoproducts were also processed for imaging analysis.

**Statistical analyses**

Data are reported as mean ± SEM or median (25 : 75% interquartile range). The neuronal properties, nociceptive behaviour, reflex electromyographic responses and areas occupied by GS-labelled GFAP-positive cells were analysed by ANOVA or ANOVA on ranks followed by Dunnett’s test. Differences between baseline values and values at different post-MSO or PBS time points in each group were treated by repeated-measures (RM) ANOVA or RM ANOVA on ranks, followed by Dunnett’s test or Dunn’s test. The areas occupied by GFAP-labelled cells and the S-R function of neurons in rats treated with PBS or two doses of MSO were compared by two-way ANOVA followed by a Tukey test. The level of significance was set at $P < 0.05$.

**Results**

**Astroglia in medullary dorsal horn after pulp exposure**

Glia fibrillary acidic protein expression was detected in both intact (Fig. 1B) and pulp-exposed (Fig. 1A,C–F) rats but, compared with intact rats, astroglial activation was evident at 7 days (Fig. 1C) and 14 days (Fig. 1E) after pulp exposure ($P < 0.01$, two-way ANOVA, Tukey tests). The labelled cells were hypertrophied in pulp-exposed rats (Fig. 1C), and the area occupied by GFAP-labelled cells in the superficial laminae of the dorsomedial aspect of the MDH of these rats was significantly enlarged compared with that in intact rats and with the lateral aspect of the superficial MDH in the 7- and 14-day pulp-exposed rats ($P < 0.01$, two-way ANOVA, Tukey tests, Fig. 1J). GS expression was detected in both intact (Fig. 1G,H) and pulp-exposed (Fig. 1I) rats. The area occupied by GS-labelled GFAP-positive cells in the dorsomedial aspect of the superficial MDH was also significantly enlarged in the 7- and 14-day pulp-exposed rats ($P < 0.01$, one-way ANOVA, Dunnett’s test, Fig. 1K).

**Behavioural and reflex tests**

Gains in body weight were similar in both intact (control) and pulp-exposed (see Fig. S1) rats. Figure 2A shows that the baseline and subsequent daily WTs to mechanical stimulation of the ipsilateral mandibular skin in the intact rats were approximately 16 g, i.e. the cut-off level [median (25 : 75%), 16 (10 : 6) g; $n = 9$]. The WTs were reduced to around 4 g within 3 days after pulp exposure compared with the baseline WTs; this reduction was significant at 5 days and lasted at least 28 days ($P < 0.05$, one-way RM ANOVA on ranks, Dunnett’s test; Fig. 2A). Furthermore, the WTs to mechanical stimulation of the contralateral mandibular skin also showed a significant reduction compared with the baseline ($P < 0.05$, one-way RM ANOVA on ranks, Dunnott’s test) at 5–21 days after pulp exposure. The WTs to mechanical stimulation of the maxillary skin ipsilateral to the pulp exposure also showed a significant reduction at 14 days after surgery compared with the WTs contralateral to the pulp exposure in the same rats ($P < 0.05$, one-way RM ANOVA on ranks; Fig. 2B). After tooth pulp exposure, the activation threshold for the nociceptive neck reflex was significantly decreased (Fig. 2C; $P < 0.05$, RM ANOVA, Dunnett’s test, $n = 6$). The mean (±SEM) threshold decreased from $477 ± 60.7$ g at pre-pulp exposure to $184 ± 13.9$, $169 ± 25.3$ and $145 ± 24.8$ g, at 7, 14 and 28 days after pulp exposure, respectively. However, the activation threshold for the reflex evoked by contralateral facial noxious mechanical stimulation did not change ($P > 0.05$, RM ANOVA; Fig. 2C).

**Methionine sulfoximine effects on behavioural and reflex thresholds**

The effects of MSO were tested at 10–14 days after the pulp-exposure procedure. The vehicle (PBS, i.t., $n = 6$) injection did not affect the WT ($P > 0.05$, one-way RM ANOVA on ranks, Fig. 3A). In contrast, a significant increase in WT from 3.0 to 16 g occurred at 20 min after i.t. injection of 10 mM MSO ($n = 8$, one-way RM ANOVA on ranks, $P < 0.01$) and this effect lasted more than 40 min; 1 mM MSO i.t. injection produced a smaller and shorter but also significant increase in WT (up to 20 min after i.t. injection, $n = 6$) (Fig. 3A). There was no apparent change in the animal’s general behaviour or health condition on the day of the injection or the following day. Moreover, two repeated injections of 10 mM MSO carried out (24 h apart) in two rats produced an increase in WT without any evidence of deleterious behavioural effects (data not shown). Furthermore, in the rota-rod test to rule out any possible motor disturbance as the cause of the antinocifensive behavioural effects of MSO over the 60-min period, rats receiving 1 or 10 mM MSO i.t. injection showed no difference from those receiving PBS (vehicle) i.t. injection up to 60 min post-injection ($P > 0.05$, one-way RM ANOVA on rank for each of PBS, 1 and 10 mM MSO; rectal temperature was also maintained at around 37.5°C over this period following MSO application). In five additional rats tested at later time periods (2 and 3 h after MSO injection), a drop in rectal temperature of approximately 1.5°C and a reduction in rota-rod scores to < 30% of control were observed.

Similarly, the activation threshold for the nociceptive neck reflex was also modulated by i.t. MSO (Fig. 3B). The threshold was significantly increased at 20 min after the application of 10 mM MSO ($n = 9$) compared with that pre-application baseline ($n = 7$; Fig. 3B, $P < 0.05$, one-way RM ANOVA, Dunnett’s test) but not significantly changed at both 40 and 60 min after MSO application. Application of either 1 mM MSO ($n = 7$) or PBS ($n = 7$) was ineffective in significantly influencing the threshold (Fig. 3B).

**Neuronal activity after pulp exposure**

A total of 130 MDH nociceptive neurons (in 54 rats) was recorded in rats at 1, 2 or 4 weeks after pulp exposure and in intact rats. The number of neurons in each of these four groups is presented in Table 1. Neurons were recorded in superficial and deep MDH laminae, and the majority of neurons were WDR neurons that were recorded in the deeper laminae of the medial portion of the MDH and first cervical segment of the dorsal horn (see Fig. 4). All neurons had an RF located in the peri-oral region (within the mandibular and/or maxillary trigeminal divisions) and the centres of their RFs were located within the mandibular division. In the pulp-exposed rats, there was an additional small number ($n = 8, 8.8\%$) of neurons with the unusual property of a rapidly adapting response to tactile stimulation of their RF (median for von Frey filament activation threshold < 1 g, or brush stimulation) in addition to a slowly adapting response to noxious mechanical stimulation (median threshold of 20 g).

In the 2- and 4-week, but not 1-week, pulp-exposed groups compared with the intact group, the high-threshold RFs of WDR neurons were significantly enlarged ($P < 0.05$, ANOVA on ranks and Dunnett’s test; Fig. 5A,D) although the size of the tactile RF of the WDR neurons (Fig. 5B) and the high-threshold RF of NS neurons (Fig. 5C) were not significantly changed after pulp exposure (one-way ANOVA).
ANOVA on ranks, $P > 0.05$). There were also no significant changes in the NS neurons’ evoked responses and afterdischarges to noxious mechanical or heat stimulation, or in their spontaneous activity. There was, however, an overall significant increase in the mechanical S-R function of the WDR neurons in all three pulp-exposed groups compared with the intact group (two-way RM ANOVA; $P < 0.01$ for intensity; $P < 0.001$ for post-operative periods; $P < 0.05$ for interaction of intensity); further comparisons between different post-operative
time periods (Tukey test, $P < 0.05$) revealed a significant increase in response magnitude to increased noxious mechanical stimulation in the 1-week (for 100 g), 2-week (for 40, 80, 100 and 200 g) and 4-week (for 80, 100 and 200 g) pulp-exposed groups compared with the intact group (Fig. 6A). In addition, there were significantly increased afterdischarges of the WDR neurons to noxious mechanical stimulation ($P < 0.05$, one-way ANOVA on ranks, Dunn’s test) compared with the intact group. The responses of the WDR neurons to tactile stimulation were also significantly enhanced in the 1-, 2- and 4-week pulp-exposed groups ($P < 0.05$, ANOVA on ranks, Dunn’s test, Fig. 6C). Their responses to radiant heat

![Fig. 2. Median value of WTs of the mandibular skin (A) and maxillary skin (B) (both within the whisker pad) in intact and pulp-exposed groups. Each box indicates the median (short horizontal line inside box when less than the 16 or 60 g cut-off values) and interquartile ranges (25 and 75%); the error bar indicates the 5 or 95% confidence interval. a, ipsilateral to pulp exposure vs. ipsilateral to intact tooth; b, ipsilateral to pulp exposure vs. contralateral to exposed pulp; c, contralateral to exposed pulp vs. contralateral to intact pulp ($*P < 0.05$, $#P < 0.01$). (C) Activation threshold of neck reflex response evoked by noxious mechanical stimulation of ipsilateral mandibular skin at 7, 14 and 28 days after pulp exposure compared with the pre-pulp exposure (Pre) threshold. $*P < 0.05$, Dunn’s test. EMG, electromyographic.](https://example.com/fig2.png)

![Fig. 3. (A) In rats at 10–14 days after pulp exposure, the effects of MSO (compared with PBS) were tested on the WT. Note that the higher dose (10 mM) of MSO produced a larger and longer elevation of WT ($P < 0.01$ at 20 and 40 min and $P < 0.05$ at 60 min) than the lower dose (1 mM) of MSO ($P < 0.05$ at 20 min only). (B) In the same pulp-exposed rats, the effects of MSO were tested on the neck reflex evoked by stimulation of ipsilateral mandibular skin. Only the higher dose of MSO significantly ($P < 0.05$) increased the reflex activation threshold, at 20 min. The insets in B show the threshold reflex responses at –10 min (Baseline) and at 20 and 60 min after MSO application. EMG, electromyographic. $*P < 0.05$.](https://example.com/fig3.png)

![Table 1. Number of nociceptive neurons in intact rats and rats at 1, 2 and 4 weeks after pulp exposure](https://example.com/table1.png)

<table>
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<th>Intact ($n = 15$)</th>
<th>1 week ($n = 13$)</th>
<th>2 week ($n = 12$)</th>
<th>4 week ($n = 14$)</th>
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<tbody>
<tr>
<td>WDR</td>
<td>24 (19)</td>
<td>28 (20)</td>
<td>27 (15)</td>
<td>25 (14)</td>
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<tr>
<td>NS</td>
<td>7 (5)</td>
<td>5 (3)</td>
<td>3 (3)</td>
<td>3 (3)</td>
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As well as 122 WDR and NS neurons, there were an additional eight neurons with unusual properties that are included in the table (three in the 1-week group, two in the 2-week group and three in the 4-week group). Numbers in parentheses indicate the number of WDR and NS neurons responding to radiant heat. $n$, number of rats in each group.
were significantly enhanced in both the 2- and 4-week pulp-exposed groups ($P < 0.05$, one-way ANOVA on ranks, Dunn's test, Fig. 6D), although their afterdischarges for the 10 s after the offset of the radiant heat stimulus were not significantly altered ($P > 0.05$, one-way ANOVA on ranks, Dunn's test; data not shown). The spontaneous activity of WDR neurons was significantly enhanced only in the 1-week pulp-exposed group (one-way ANOVA, $P < 0.05$; Fig. 6E).

**Methionine sulfoximine effects on neuronal activity**

Intrathecal application of MSO (10 mM) produced no changes in neuronal properties in rats with an intact pulp (WDR neurons, $P > 0.05$, one-way ANOVA; $n = 7$; see Fig. S2). In 15 rats that received the pulp-exposure procedure at 10–14 days prior to neuronal recording, the activity of 15 WDR neurons in the MDH was monitored before and after a bolus application of vehicle (PBS, $n = 5$), 1 mM MSO ($n = 5$) or 10 mM MSO ($n = 5$) over the caudal medulla. Before the application of MSO or vehicle, 10 of the 15 neurons had a very low rate of spontaneous activity (< 0.2 Hz), and there were no significant differences between the 15 neurons in terms of pinch RF size, responses to tactile and noxious mechanical stimulation and radiant heat, and afterdischarges following noxious stimulation. In addition, the mechanical S-R functions of the WDR neurons in the animals receiving vehicle, 1 mM MSO or 10 mM MSO were also similar ($P > 0.05$; two-way RM ANOVA, Fig. 7A, baseline S-R function). Note that all four S-R functions (i.e. at baseline, and at 20, 40 and 60 min) were similar after vehicle (PBS) application ($P > 0.05$; two-way RM ANOVA, interaction (time × doses) was not significant, Fig. 7A–D), but they were altered after the application of MSO. Compared with S-R responses following vehicle application, the responses to mechanical stimulus intensities of 100 and 200 g ($P < 0.05$) for the 1 mM dose of MSO and of 80, 100 and 200 g intensities for the 10 mM dose of MSO were significantly depressed at 20 min after MSO application ($P < 0.01$; two-way RM ANOVA) (Fig. 7B). Moreover, these reduced responses after MSO (e.g. 17.3 ± 5.1 spikes/s for 200 g) were smaller than but not significantly different ($P > 0.05$ for the treatment effect and interaction (treatment × force), but $P < 0.01$ for the force, two-way RM ANOVA performed between intact and 10 mM at 20 min post-MSO injection) from the responses evoked in intact (control) rats.
suggesting that MSO was not affecting the basal nociceptive neuronal activity but rather only the enhanced responses observed in the pulp-exposed animals. Some of these effects were also apparent at 40 and 60 min after MSO application (Fig. 7C,D).

Discussion
This study has provided the first documentation suggesting a role for astroglia and the glutamate–glutamine shuttle in mediating nociceptive responses associated with chronic inflammation of the tooth pulp. This chronic pulpitis pain model manifested nocifensive behaviour and enhanced reflex activity evoked by mechanical stimulation of the rat’s facial skin as well as MDH central sensitization and immunohistochemical evidence of astroglial activation in the dorsomedial aspect of the MDH. The behavioural and reflex changes lasted at least 28 days and could be reversed in a dose-related manner by i.t. application of the astroglial GS inhibitor MSO. In addition, the accompanying neuroplastic changes reflecting MDH central sensitization in functionally identified nociceptive neurons were also obvious at 1, 2 and 4 weeks after pulp exposure, and i.t. application of MSO produced a significant attenuation of the neuronal S-R function to mechanical stimulation especially in the noxious range, consistent with the MSO-induced reduction in the WT and nociceptive reflex. The lack of MSO effects on baseline nociceptive neuronal properties in intact rats is consistent with our findings of a lack of MSO effects on baseline neuronal properties in our acute pulpitis pain model (Chiang et al., 2007), and suggests that the action of MSO was limited to reversal of the neuronal hyperexcitability indicative of central sensitization.

In a previous study of the effects of pulp injury, the authors reported behavioural findings suggesting spontaneous pain that lasted up to 3 days (Chudler & Byers, 2005). The observed hyperalgesic changes reflected for the central sensitization, manifested in evoked nocicep-

Fig. 6. The WDR neuronal properties before and after pulp exposure. (A) The mechanical S-R functions in WDR neurons. The intact rats showed consistently smaller responses, whereas pulp exposure led to significantly larger responses. (B) Afterdischarges within 10 s of offset of 200 g stimulation. (C) WDR neuronal responses to 10 consecutive brush stimuli. (D) Response to radiant heat. (E) Spontaneous activity of MDH nociceptive neurons. *Significant difference between 4 weeks and intact; +significant difference between 2 weeks and contralateral; #significant difference between 1 week and intact. *, + and #P < 0.05, Tukey test.
tive behaviour, reflex and MDH nociceptive neuronal hyperexcitabil-
ity, lasted much longer than this in the present study, consistent with
the several weeks of nociceptive behaviour previously reported in
trigeminal nerve injury models, such as that following inferior alveolar
nerve transection (IANX) or infraorbital nerve constriction (Iwata
et al., 1998, 2005, 2007, 2008, 2010a,b; Xie et al., 2007); this
possibility requires further investigation.

Chronic pulp exposure induces an inflammatory response but may
also provoke expression of GFAP within trigeminal ganglion satellite
cells and damage the nerve endings within the pulp (Ajima et al.,
2001; Byers & Närhi, 1999; Byers et al., 2004; Stephenson & Byers,
1995). The present data, as shown by the activated astroglia in the
MDH (in terms of hypertrophied cells that also expressed GS as well as
GFAP) and the MSO-induced modulation of the behavioural hyper-
algesia, nociceptive reflex and central sensitization of MDH nocicep-
tive neurons, along with our previous findings (Chiang et al., 2007,
2008; Guo et al., 2007; Xie et al., 2007; Okada-Ogawa et al., 2009;
Piao et al., 2006; Yeo et al., 2001; for review see Chiang et al., 2011),
demonstrate that a pro-inflammatory response can occur within the
central processing of sensory information from the tooth pulp and
other oral tissues, which are frequent sites of inflammatory pain.

A key process of astroglial function is the glutamate–glutamine
shuttle, which includes the uptake of excess extracellular glutamate,
and the production (via the astroglial enzyme GS) and release from
astroglia of glutamine that is then taken up by neuronal elements to
replenish the supply of glutamate (Zwingmann & Leibfritz, 2003;
Hertz & Zielke, 2004; Fonseca et al., 2005), which plays a major role
in central sensitization (Woolf, 1992; Dubner & Basbaum, 1994;
Chiang et al., 1998; Salter, 2004; Woolf & Salter, 2006). We found
that i.t. application of MSO reduced mechanical hyperalgesia and
the S-R function of WDR neurons; these effects lasted only up to 60 min,
possibly due to the cerebrospinal fluid flushing away the MSO applied
to the medulla. These findings are consistent with earlier spinal and
trigeminal behavioural studies (Watkins & Maier, 2005; Piao et al.,
2006; Guo et al., 2007; Lan et al., 2007). These earlier studies
included those using i.t. administration of an astroglial or microglial
inhibitor and demonstrated that astroglial as well as microglial
activation is involved in the hyperalgesia induced in inflammatory or
neuropathic pain models. In the present study, the astroglial glutamate–glutamine shuttle in the MDH represented the likely target
for MSO as MSO has been documented to be a potent selective
inhibitor of the shuttle enzyme GS in astroglia (Bacci et al., 2002;
Blin et al., 2002; Shin et al., 2003; Gibbs & Hertz, 2005; Tanigami
et al., 2005; Liang et al., 2008). This view is further supported by
earlier findings that the suppressive effect of MSO on central MDH
sensitization can be reversed by superfusion of glutamine in orofacial
pain models (Chiang et al., 2008; Okada-Ogawa et al., 2009), and that
MSO-induced antiepileptiform discharge effects in hippocampus
slices (Bacci et al., 2002) and the MSO-induced reduction in

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European Journal of Neuroscience, 34, 292–302
GABA-evoked inhibitory post-synaptic currents (Liang et al., 2006) can be reversed by application of glutamate. Earlier reports have indicated that intracerebroventricular or intraperitoneal application of MSO may prevent GABA synthesis, thus producing convulsions, hypothermia and ataxia (Stransky, 1969; Ginefri-Gayet & Gayet, 1988) but, if this was the case in the present study, MSO would have been expected to facilitate the pulp-exposure-induced hyperalgesia and MDH neuronal hypersensitivity rather than produce suppression as documented in the present study. Furthermore, no obvious motor disturbances were apparent up to 1 h after i.t. MSO in the present study, consistent with Ginefri-Gayet & Gayet (1988). These various findings collectively suggest that MSO produces inhibition of the astroglial glutamate–glutamine shuttle that leads to a reduction of nociceptive and reflex behaviour as well as attenuation of the associated MDH central sensitization, which has been shown to be crucial in other orofacial chronic inflammatory or neuropathic pain models (Sessle, 2000; Iwata et al., 2001; Salter, 2004; Guo et al., 2007) and in acute orofacial and tooth pulp pain processing in trigeminal nociceptive pathways (Chiang et al., 1998, 2005, 2007, 2008, 2010a,b; Hu et al., 2002; Hu, 1990; Hu et al., 1992; Xie et al., 2007; see Salter, 2004; Sessle, 2000).

In accordance with our previous findings that i.t. superfusion of the MDH by the astroglial inhibitor MSO, or by SB203580 or minocycline (which target microglia), can block the initiation of MDH central sensitization induced by the application of MO to the pulp in our acute pulp inflammatory pain model (Chiang et al., 2007, 2008, 2010a,b; Xie et al., 2007), the present MSO findings from the chronic pulp inflammatory pain model extend these earlier findings by providing novel data that glial cells, specifically astroglia, may also modulate the maintenance phase of central sensitization. Also noteworthy is that the present study employed a single bolus injection of MSO at a much higher dose (1 or 10 mM), instead of a constant superfusion (0.1 mM) for over 0.5 h as used in our acute pulpitis studies (Chiang et al., 2007, 2008; Xie et al., 2007), and the higher concentration of MSO (10 mM) produced a longer and stronger antinociceptive action than the lower concentration (1 mM); neither dose affected the baseline neuronal properties. Thus, a critical concentration of MSO may be needed for its antinociceptive action through the astroglial modulation of glutamine release. Furthermore, all of our present and previous studies (Chiang et al., 2005, 2007, 2010a,b; Xie et al., 2007) have demonstrated that the suppressive effects of glial modulators, such as fluoroacetate, MSO, minocycline, capsaicin and SB203580, were limited to the increased excitability manifested in the MDH nociceptive neurons, and in the hyperalgesic component of nociceptive responses, but did not affect basal nociceptive activity. In addition, MSO at the dose used in the present study did not produce any notable effect on the properties of nociceptive neurons in intact rats. This is consistent with findings that glia may not affect basal nociceptive processing but rather participate in exaggerated pain states (Watkins & Maier, 2005).

Supporting Information

Additional supporting information can be found in the online version of this article:

Fig. S1. The time course change in body weight following tooth pulp exposure. No differences were found between intact (control) and pulp-exposed rats (P > 0.05; two-way RM ANOVA). Pre, before pulp-exposure surgery.

Fig. S2. Effect of MSO on evoked responses of MDH nociceptive neurons in the rats with intact tooth pulp. Intrathecal MSO (10 mM) application produced no changes in neuronal activities for different intensities of mechanical stimulation of WDR neurons. One-way ANOVA revealed no changes over time (P > 0.05).

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Acknowledgements

Supported by CIHR grants (MOP-43095 and MOP-82831) to J.W.H. and J.O.D., respectively, NIH grant DE04786 to B.J.S., and Japan–Canada Joint Health Research Program 167458 to B.J.S. and K.I. Grant-in-Aid for Scientific Research (C) (#22592051 to Y.T.). The authors gratefully acknowledge the technical assistance of Mr K. MacLeod, Ms S. Carter and Ms I. Suzuki.

Abbreviations

GFAP, glial fibrillary acidic protein; GS, glutamine synthetase; i.t., intrathecal; IANX, inferior alveolar nerve transection; MDH, medullary dorsal horn; MO, mustard oil; MSO, methionine sulfoximine; NS, nociceptive neuron; PBS, phosphate-buffered saline; RF, mechanoreceptive field; RM, repeated-measures; S-R, stimulus–response; WDR, wide dynamic range; WT, withdrawal threshold.

References


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