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Systemic pregabalin attenuates facial hypersensitivity and noxious stimulus-evoked release of glutamate in medullary dorsal horn in a rodent model of trigeminal neuropathic pain

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Abstract

Pregabalin is effective in treating many neuropathic pain conditions. However, the mechanisms of its analgesic effects remain poorly understood. The aim of the present study was to determine whether pregabalin suppresses facial mechanical hypersensitivity and evoked glutamate release in the medullary dorsal horn (MDH) in a rodent model of trigeminal neuropathic pain. Nociceptive mechanical sensitivity was assessed pre-operatively, and then post-operatively 1 h following pregabalin or vehicle (saline) treatment on post-operative days 2 and 5 following infraorbital nerve transection (IONX). In addition, an *in vivo* microdialysis probe was inserted into the exposed medulla post-operatively and dialysate samples were collected. Glutamate release was then evoked by mustard oil (MO) application to the tooth pulp, and the effects of pregabalin or vehicle were examined on the MDH glutamate release. Glutamate concentrations in the dialysated samples were determined by HPLC, and data analysed by ANOVA. IONX animals (but not control animals) showed facial mechanical hypersensitivity for several days post-operatively. In addition, tooth pulp stimulation with MO evoked a transient release of glutamate in the MDH in IONX animals. Compared to vehicle, administration of pregabalin significantly attenuated the facial mechanical hypersensitivity as well as the MO-evoked glutamate release in MDH. This

¹Abbreviations: cerebrospinal fluid (CSF); high performance liquid chromatography (HPLC); intraperitoneal (i.p.); intravenous (i.v.); medullary dorsal horn (MDH); mustard oil (MO); partial transection of the infraorbital nerve (IONX); standard error of the mean (SEM).

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study provides evidence in support of recent findings pointing to the usefulness of pregabalin in the treatment of orofacial neuropathic pain.¹

Keywords

nociception; glutamate; medullary dorsal horn; pregabalin; tooth pulp; neuropathic pain

1. Introduction

The prevalence of acute and chronic pain conditions in the population is high (12–30%), and although the mechanisms underlying chronic pain are not fully understood, many pain conditions can be managed through the use of antiepileptic drugs (Iwata et al, 2011; Sessle, 2011). Our previous studies have shown that application of the algescic chemical mustard oil (MO) to the tooth pulp causes nociceptive sensorimotor behavior and central sensitization in the trigeminal subnucleus caudalis, also known as the medullary dorsal horn (MDH) (eg, Chiang et al., 1998, 2007, 2011; Narita et al., 2012). Central sensitization is considered to be a crucial process underlying the development and maintenance of chronic pain states, and indeed has been documented in animal models of chronic inflammatory or neuropathic orofacial pain (Iwata et al., 2011; Sessle, 2011). Also, the excitatory amino acid glutamate is a key neurotransmitter involved in producing central sensitization of trigeminal as well as spinal cord dorsal horn neurons (Coderre, 2006; Iwata et al., 2011; Sessle, 2011).

The antiepileptic drug pregabalin, and its related compound gabapentin, have been found to be clinically effective in alleviating various neuropathic and other pain conditions including orofacial pain, as well as reducing central sensitization and nociceptive behaviors in animal models of orofacial neuropathic or inflammatory pain (Cao et al., 2013; Cheng and Chiou, 2006; Krzyzanowska and Avendano, 2012; Narita et al., 2012; Obermann et al., 2008). *In vitro* investigations have shown that pregabalin reduces transmission between dorsal root ganglion and the spinal cord dorsal horn neurons in cultured slices (Hendrich et al., 2012). *In vivo* studies have also shown that pregabalin attenuates glutamate release in the spinal cord dorsal horn of animals with neuropathic pain (Kumar et al., 2010). Furthermore although pregabalin has been shown to attenuate glutamate release in the MDH in association with a reduction in nociceptive sensorimotor behavior in an acute dental inflammatory pain model (Narita et al., 2012), the effect of pregabalin on glutamate release in orofacial neuropathic pain models has not been studied. The aim of this study was to determine whether pregabalin suppresses evoked glutamate release in the MDH and nociceptive behavior in a rodent model of trigeminal neuropathic pain.

2. Materials and Methods

2.1. Animals

The experiments were performed in male C57BL/6J (BL6) mice (18–24 g, Charles River) and male Sprague-Dawley rats (300–370 g, Charles River), housed in groups of 3–4, on a 12:12 h light-dark cycle; food and water were available *ad libitum*. Mice were used for the behavioral study and rats for the microdialysis study since it was not possible technically to carry out the latter study in mice. 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. IONX surgery

The left infraorbital nerve (ION) was exposed, at its entry into the infraorbital foramen, by an intra-oral incision (2 mm) made in the oral mucosa of the left fronto-lateral maxillary

vestibulum in anesthetized (isofluorane 4–5% induction, 2–2.5 % maintenance) mice and rats. The ION was lifted from the maxillary bone and cut (IONX) without damaging other nerves and vessels in the vicinity. After the surgery, the wounds were closed by sutures. The animals were returned to their home cages and fed with mash and chow. Naïve or sham-operated animals served as controls. The animals were monitored daily post-operatively.

2.3. Assessing Mechanical Sensitivity

Animals were acclimatized, trained and tested for facial mechanical sensitivity (see below) at least for 1–3 days prior to the surgery to obtain baseline values, and then again tested on post-surgery days 2, 5 or 7. Testing was also carried out on these post-operative days (on which the animals showed facial mechanical hypersensitivity; see Results) at 1 h after treatment with a single dose of pregabalin (25 mg/kg, i.p., n=7) or isotonic saline (i.p., n=7) in mice. Facial mechanical sensitivity was evaluated as the animals' head-withdrawal thresholds which were obtained by slowly applying graded von Frey filament hairs to the ipsilateral facial skin area 2 mm below the lower lip, as described previously (Saito et al. 2008). Escape responses to the mechanical stimulation were demonstrated as a sudden backward withdrawal movement of the head. The head-withdrawal threshold at the lower lip skin was defined as the lowest filament intensity that evoked three or more escapes out of five stimulation trials with intertribal intervals of more than 10 s.

2.4. Microdialysis

On post-operative day 7, IONX rats showed a significant ($P < 0.05$, ANOVA) reduction in withdrawal thresholds (0.41 ± 0.03 mg, mean $\pm$ SEM) from pre-operative levels (0.50 ± 0.01 mg, mean $\pm$ SEM), consistent with recent findings (Cao et al, 2013). At this time point, the IONX or sham rats were anesthetized with urethane and α -chloralose (1 g/kg and 50 mg/kg i.p., respectively), then immobilized by intravenous (i.v.) pancuronium 1 mg/ml (initial dose, 0.3 ml), followed by a continuous i.v. infusion of a mixture of 70% urethane (0.2 g/ml) and 30% pancuronium (1 mg/ml) at a rate of 0.4–0.5 ml/h, and artificially ventilated throughout the whole experimental period. 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.

To acutely stimulate the tooth pulp in IONX and sham rats, dental paper points soaked in mustard oil (MO, 0.2 μ l, allyl isothiocyanate 95%, Aldrich Chemical, USA) was applied to an occlusal cavity drilled in the right maxillary first molar, as previously described (Chiang et al., 1998). We did not use the vehicle (mineral oil) for MO in the present study, since we have previously shown that it is ineffective in inducing nociceptive effects or MDH glutamate release (Chiang et al., 1998; Narita et al., 2012). After exposing the pulp, a microdialysis fiber was inserted into the right MDH (L:1.4 mm; P:1.4 mm relative to obex), and artificial cerebrospinal fluid (CSF, see below) was infused through the probe fiber at a flow rate of 2 μ l/min, and phosphate-buffered saline (PBS) was superfused (i.t.) over the exposed MDH at a rate of 0.6 ml/h, as previously described (Narita et al., 2012). Microdialysis samples were then collected every 5 min at a flow rate of 2 μ l/min; these included 3 basal samples, 6 samples after treatment with pregabalin (1 or 25 mg/kg i.p. (both n=6), from Pfizer USA) or vehicle (isotonic saline i.p., (n=6)), and 6 samples following application of MO to the exposed molar pulp. The doses of pregabalin were chosen based on our previous studies (Cao et al., 2013; Narita et al., 2012), which used various doses of pregabalin and documented those doses effective in blocking MO-induced MDH central sensitization or nociceptive behavior without producing motor or sedative effects. Dialysis was performed using an infusion pump (Bio-Analytical Systems, West Lafayette, IN), and samples were sorted in a fraction collector kept at 4°C. Samples were stored at –80°C until assayed by high performance liquid chromatography (HPLC) technique. At the end of the

sample collection, the anesthetized rats were transcardially perfused following a standard fixation protocol for subsequent histological examination to confirm the site of the microdialysis probe in the MDH (Chiang et al., 1998, 2007).

2.5. Estimation of glutamate concentration by HPLC

Glutamate was quantitated in each sample by using a gradient, reverse-phase HPLC technique with fluorescent detection (Waters Alliance 2690 system & 474 detector, Waters Corp., Milford, MA) as previously described (Kumar et al., 2010). Briefly, samples were derivatized using o-phthalaldehyde and 2-mercaptoethanol (both Sigma, St. Louis, MO, USA), and eluted using a C18 ODS Supelco column (15 cm × 4.6 mm, 5 µm-particle size, ODS Supelco, Bellefonte, PA, USA). Peak area was used to calculate the concentration of each sample, and quantification of glutamate was determined from a standard curve derived using external standards. The sensitivity limit for detecting glutamate in the sample was 1 ng/ml, and the inter-day and intra-day assay coefficients were 5.42% and 4.31%, respectively.

2.6 Statistical analysis

In behavioral studies, statistical differences between the naïve, IONX vehicle (saline)-treated and IONX pregabalin-treated groups at different days were tested by 2-way ANOVA followed by Bonferroni post-hoc test. In the glutamate release studies, glutamate concentrations (mean + SEM) were normalized with all post-drug and post-MO values expressed as a percentage of baseline. Statistical differences between IONX groups that received pregabalin (1 or 25mg/kg) or vehicle (saline), and an analogous group of sham rats that received vehicle were compared by two-way repeated measures (RM) ANOVA followed by Fisher's least squared difference post-hoc comparisons. One-way repeated measures ANOVA was used to compare post-MO values with baseline values in each group.

3. Results

3.1. Effect of pregabalin on mechanical hypersensitivity in IONX mice

A significant decrease in mechanical head-withdrawal thresholds (g) for the ipsilateral lower lip occurred in the IONX mice on day 2 and 5 post-IONX as compared to the naïve mice (Fig. 1). In the IONX group, administration (i.p.) of pregabalin (but not isotonic saline) significantly decreased the mechanical hypersensitivity on post-operative days 2 and 5 (Fig. 1).

3.2. Increase in glutamate release in the MDH induced by MO application to tooth pulp

Compared to baseline and to administration (i.p.) of isotonic saline, extracellular glutamate release in the MDH was increased significantly ($p < 0.01$) during the first 5 min following MO application in both IONX and sham rats (Fig. 2A), and then returned to baseline levels by the next time point. There was no difference in MO-induced glutamate levels between sham-operated and IONX rats (Fig. 2A). Administration of isotonic saline alone (i.e., without MO application to the pulp) did not significantly alter glutamate levels relative to baseline levels (data not shown).

3.3. Effects of pregabalin on MO-induced glutamate release in the MDH

Administration of the low dose of pregabalin (1 mg/kg i.p.) reduced MO-induced glutamate release in IONX rats, such that MO no longer significantly increased glutamate levels compared to baseline (Fig. 2B). The higher dose of pregabalin (25 mg/kg i.p.) also significantly attenuated MO-induced glutamate levels in IONX rats in the first 5 min after

MO, but also significantly reduced glutamate release below basal levels at the 30 min time-point post-MO, as compared with isotonic saline administration ($P < 0.01$, Fig. 2C).

4. Discussion

The present *in vivo* behavioral and microdialysis studies in a rodent model of facial neuropathic pain have shown that pregabalin reduces both the facial mechanical hypersensitivity and the increased glutamate release in the MDH induced by the application of MO to the tooth pulp in IONX animals. These findings support recent documentation that pregabalin attenuates MO-evoked glutamate release in the MDH of naïve rats (Narita et al., 2012), and also reverses mechanical hyperalgesia and central sensitisation in IONX rats (Cao et al., 2013). In this orofacial neuropathic pain model, we found that the low systemic dose of pregabalin attenuated the MO-evoked glutamate release, but not tonically-released glutamate, whereas the higher systemic dose of pregabalin attenuated both the MO-evoked glutamate release, as well as the tonic glutamate levels, consistent with previous findings in naïve rats (Narita et al., 2012). This tonic level of glutamate release in the MDH might have been evoked by the trauma associated with the surgical procedure required for implanting the microdialysis probe and/or reflected synaptic activity induced by the IONX itself.

Previous studies have reported that various orofacial noxious stimuli trigger glutamate release and central sensitisation in the MDH. For example, application of MO to the molar pulp produces MDH central sensitization and increases glutamate in the MDH (Chiang et al., 1998, 2007; Narita et al., 2012). In addition to neural processes, activated glial cells in the MDH may also play a role in the increased extracellular glutamate levels in the MDH and the associated MDH central sensitization after such types of orofacial injury (Chiang et al., 2011).

Pregabalin has been shown to be an effective analgesic in patients with neuropathic orofacial pain, and like gabapentin, is also effective in various animal pain models, including orofacial pain models (Cao et al., 2013; Cheng and Chiou, 2006; Narita et al., 2012; Obermann et al., 2008). Like gabapentin, pregabalin is an adjuvant antiepileptic drug that is a structural analog of GABA, but has no action at GABA receptors, and does not affect GABA synthesis (Dolphin, 2012; Hendrich et al., 2012; Li et al., 2011). Pregabalin has been shown to modulate the release of several neurotransmitters by selectively binding to the $\alpha_2\delta$ auxiliary subunit of calcium channels to reduce the influx of Ca^{2+} ions, thereby reducing the release of glutamate, norepinephrine and substance P (Li et al., 2011; Dolphin, 2012). There is an increase in $\alpha_2\delta-1$ at presynaptic terminals in the superficial dorsal horn in neuropathic rats that have allodynia and hyperalgesia, which is inhibited by pregabalin (Li et al., 2011). Pregabalin also inhibits the trafficking of calcium channels in dorsal root ganglion cells (Hoppa et al., 2012), and pregabalin binding at $\alpha_2\delta$ channels reduces synaptic transmission between rat dorsal root ganglion and dorsal horn neurons in culture (Hendrich et al., 2012).

In support of previous studies on the effects of pregabalin, the present study in the IONX model of facial neuropathic pain suggests that pregabalin's effectiveness in attenuating facial mechanical hypersensitivity in this model and in treating chronic orofacial neuropathic pain may involve its attenuation of enhanced release of glutamate. This is consistent with recent reports showing that systemic pregabalin administration in rodents reduces MO-induced sensorimotor behavior (Narita et al., 2012) and mechanical hypersensitivity in trigeminal neuropathic pain models (Cao et al., 2013) as well as chronic orofacial pain in humans (eg, Obermann et al., 2008). Similarly, peripheral noxious stimulus-induced increase of glutamate in the spinal dorsal horn and the accompanying allodynia in rats with chronic constriction injury of the sciatic nerve are attenuated by pregabalin (Kumar et al., 2010). Pregabalin also inhibits pre-synaptic glutamate release *in vitro* and *in vivo* both in the

spinal cord dorsal horn (Hendrich et al., 2012; Kumar et al., 2010) and the MDH (Narita et al., 2012). Further investigation is needed to clarify whether pregabalin's attenuation of MDH glutamate release seen here depends on its activity on pre-synaptic glutamatergic primary afferent terminals, or on other processes, such as glutamate release from other neural elements (Dolphin, 2012; Hoppa et al., 2012), or through an action on the glutamate-glutamine cycle, which is a key metabolic feature of astroglia (Chiang et al., 2011).

4.1. Conclusions

This study has demonstrated that systemic administration of pregabalin reduces the facial mechanical hypersensitivity and the MDH release of glutamate in a trigeminal neuropathic pain model, and provides support to recent findings pointing to the usefulness of pregabalin in the treatment of orofacial neuropathic pain.

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Fig. 1.

Effect of systemic (i.p.) pregabalin treatment on facial mechanical sensitivity following IONX. Two-way ANOVA indicated significant main effects of treatment ($F(2,4) = 24.41$, $P < 0.0001$), time ($F(2,54) = 23.74$, $p < 0.0001$), and a significant treatment X time interaction ($F(4,54) = 5.61$, $p = 0.0013$). Post hoc Bonferroni test revealed that IONX mice showed a significantly lower head-withdrawal thresholds (g) as compared to naive mice ($\dagger p < 0.001$), and compared to vehicle showed a significant reduction in the mechanical hypersensitivity with pregabalin treatment on days 2 and 5 after IONX ($*p < 0.05$).

Fig. 2.

Comparison of the effects of mustard oil (MO) application to the tooth pulp on glutamate release in the medullary dorsal horn (MDH) of IONX and sham rats pretreated with i.p. saline (A), as well as the effect of either 1 mg/kg i.p. (B) or 25 mg/kg, i.p. (C) of pregabalin pretreatment on MO-evoked MDH glutamate release in IONX rats ($n=6$ per group). These graphs (A, B and C) show basal, post-drug and post-MO glutamate levels for groups of IONX rats treated with pregabalin or saline (as vehicle control). The first arrow indicates when i.p. pregabalin or saline vehicle was administered at -30 min, and the second arrow indicates when MO was applied to the tooth pulp at 0 min. In A, MO evoked a significant increase in glutamate release in the first 5 min after its application ($p < 0.01$), but there was no significant difference between the IONX and sham rats (Fig. 2A). In B–C, RM-ANOVA revealed significant main effects of drug ($F(2, 15) = 10.64$, $p < 0.001$) and time ($F(14, 210) = 3.87$, $p < 0.0001$), as well as a significant drug $\times$ time interaction ($F(28, 210) = 2.90$, $P < 0.0001$). Post-hoc comparisons (Fisher's test) indicated that MO application to the tooth pulp significantly increased glutamate concentration at 5 min as compared to baseline ($\dagger p < 0.01$). While the 1 mg/kg dose of pregabalin reduced the MO-induced glutamate level in IONX rats, so that glutamate levels at 5 min did not differ from baseline (Fig. 2B), pretreatment with 25 mg/kg pregabalin significantly attenuated the MO-induced glutamate release, as well as significantly depressing tonic glutamate levels at 30 min post-MO, as compared with vehicle treatment ($*p < 0.01$, Fig. 2C).