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Systemic Pregabalin Attenuates Sensorimotor Responses and Medullary Glutamate Release in Inflammatory Tooth Pain Model

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Abstract

Our previous studies have demonstrated that application to the tooth pulp of the inflammatory irritant mustard oil (MO) induces medullary glutamate release and central sensitization in the rat medullary dorsal horn (MDH), as well as nociceptive sensorimotor responses in craniofacial muscles in rats. There is recent evidence that anticonvulsant drugs such as pregabalin that influence glutamatergic neurotransmission are effective in several pain states. The aim of this study was to examine whether systemic administration of pregabalin attenuated glutamate release in the medulla as well as these nociceptive effects reflected in increased electromyographic (EMG) activity induced by MO application to the tooth pulp. Male adult rats were anesthetized with isoflurane (1.0–1.2 %), and jaw and tongue muscle EMG activities were recorded by needle electrodes inserted bilaterally into masseter and anterior digastric muscles and into the genioglossus muscle, and also the medullary release of glutamate was assessed by *in vivo* microdialysis. Pregabalin or vehicle control (isotonic saline) was administered 30 min before the pulpal application of MO or vehicle control (mineral oil). Application of mineral oil to the maxillary first molar tooth pulp produced no change in baseline EMG activity and glutamate release. However, application of MO to the pulp significantly increased both the medullary release of glutamate and EMG activity in the jaw and tongue muscles for several minutes. In contrast, pre-medication with pregabalin, but not vehicle control, significantly and dose-dependently attenuated the medullary glutamate release and EMG activity in these muscles after MO application to the tooth pulp (ANOVA, $p < 0.05$). These results suggest that pregabalin may attenuate the medullary release of glutamate and associated nociceptive sensorimotor responses in this acute inflammatory pulpal pain model, and that it may prove useful for the treatment of orofacial inflammatory pain states.

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Introduction

The application of inflammatory irritants such as mustard oil (MO) or capsaicin to orofacial tissues including the tooth pulp can evoke nociceptive sensorimotor responses reflected in sustained electromyographic (EMG) activity in craniofacial muscles which is associated with the medullary release from nociceptive primary afferents of neurochemicals such as glutamate (Yu et al., 1994, 1996; Bereiter and Benetti, 1996; Cairns et al., 1998; Sunakawa et al., 1999; Tsai et al., 1999; Lam et al., 2005; Cherkas et al., 2010). Furthermore, neuroplastic changes reflecting central sensitization also occur in nociceptive neurons of trigeminal subnucleus caudalis (also termed the medullary dorsal horn, MDH) following the application of MO or capsaicin to these tissues and involve NMDA as well as non-NMDA glutamatergic receptors and several second messenger systems (Hu et al., 1992; Yu et al., 1993; Bereiter and Benetti, 1996; Yu et al., 1996; Cairns et al., 1998; Chiang et al., 1998, 2008; Sunakawa et al., 1999; Sessle, 2000; Capra and Ro, 2004; Dubner and Ren, 2004; Bereiter, 2007; Honda et al., 2008; Noma et al., 2008; Ro, 2008; Lam et al., 2009). The trigeminal central sensitization is considered a crucial process underlying the development and maintenance of orofacial pain states (for review, see Sessle, 2000, 2005; Dubner and Ren, 2004).

Pregabalin is an anticonvulsant drug that is known to decrease glutamate release by binding to the $\alpha 2\delta$ subunit of voltage-dependent calcium channels (Kumar et al., 2010; Quintero et al., 2011). Pregabalin is increasingly used clinically for the treatment of especially neuropathic pain conditions (Moulin et al., 2007; Freeman et al., 2008; Cappuzzo 2009; Finnerup et al., 2010; Teasell et al., 2010; Tzellos et al., 2010; Lindsay et al., 2010), and its related compound gabapentin has also been shown effective clinically in some orofacial pain states (e.g.; Hill et al, 2001 Serpell, 2002; Heckmann et al, 2006; Obermann et al, 2008), as well as reducing nociceptive behaviors in animal models of orofacial neuropathic or inflammatory pain (Christensen et al. 2001; Grabow and Dougherty 2002). However, pregabalin has not been tested in animal models of acute orofacial inflammatory pain. Therefore, the aim of this study was to examine whether the nociceptive sensorimotor responses and the medullary release of glutamate evoked by MO application to the tooth pulp can be attenuated by systemic administration of pregabalin. Brief outlines of the findings have been published in abstract form (Cherkas et al, 2010; Narita et al., 2010)

Methods

Animal Preparation

Experimentation on all animals conformed to the regulations of the Canadian Council on Animal Care and the Ontario Animals for Research Act, and was approved by the University of Toronto Animal Care Committee. The study was carried out on male Sprague–Dawley rats weighing between 250 and 350 g and housed in groups of 3–4, on a 12:12 h light/dark cycle; food and water were available ad libitum. General anesthesia was induced by isoflurane (1.0–1.2 %) mixed with O₂, so that a tracheal cannula could be inserted. Surgical anesthesia was maintained with a mixture of O₂ and isoflurane (1.0–1.2 %) with the aid of an artificial ventilator (Yu et al., 1994, Sunakawa et al., 1999). 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. Animals were placed on a heating pad and in the ventral position, with the mouth full-opened so that chemicals could be applied to the tooth pulp. The other methods used for animal preparation, stimulation, and EMG recordings and their processing were similar to those described previously (Yu et al., 1994, 1996; Sunakawa et al., 1999), and so only a brief description follows.

To allow for the pulpal application of MO, the left or right maxillary first molar pulp was exposed by the use of a low-speed dental drill with a round tungsten carbide bur (#1) and water cooling. The pulp surface was covered with a small piece of cotton soaked with physiological saline until MO or vehicle (see below) could be applied. After the surgery, the isofluorane concentration was reduced (0.9–1.0%) until noxious pressure applied to the hind-paw could induce a weak flexion reflex of the hind limb to ensure that an adequate level of general anesthesia was maintained for the duration of the experiment.

EMG Recordings

As previously described, nociceptive sensorimotor responses were recorded as evoked EMG activity (Yu et al, 1994,1996; Cairns et al, 1998; Sunakawa et al, 1999). In a total of 30 rats, a pair of bipolar EMG electrodes (36–40 gauge, single-stranded, Teflon®-coated stainless steel wire; inter-polar distance: 5 mm; exposed tips: 0.8mm) was inserted in the bilateral masseter (Mm) and anterior digastric muscles (AD) and genioglossus muscle (GG). The EMG electrode locations in each muscle were confirmed by post-mortem dissection.

A rest period of 0.5–1 h was allowed after the surgery in order to obtain a stable level of EMG activities. Then the EMG activities were monitored continuously, first at baseline for 15 min, and then during pregabalin (1, 10 and 100 mg/kg, i.p; Pfizer Canada) or vehicle control (isotonic saline) systemic administration, and then 30 min later, during and after MO (0.2 µl, allyl isothiocyanate, 95%) or vehicle (mineral oil) application to the exposed pulp. MO or mineral oil was applied to the pulp by a small piece of dental paper point (diameter, 0.3 mm; length 0.5 mm); the cavity was then quickly sealed with temporary dental filling material (Cavit, ESPE, Germany) to prevent any possible leakage of the chemical to other oral tissues. There were 5 groups (n=6 rats/group), each group receiving one dose of pregabalin or isotonic saline.

The EMG activity of each muscle was amplified (gain: 1000–5000; bandwidth: 30–3000 Hz) and displayed on an oscilloscope, and was directly processed, i.e. rectified and integrated, by the computer interface 1401/program Spike 2 (CED, Cambridge, UK). Increases in EMG activity evoked by the application of MO were regarded as significant if one or more EMG area bins (mV/min) increased at least two standard deviations (SD) above the mean baseline level. All EMG activities during the experimental session were measured and transformed into percentage values by the division of the averaged baseline activity (mV/min) for the first 5 minutes in the experimental sessions. Two-way repeated measures (RM) ANOVA and pair-wise multiple comparisons with Tukey's Test were used to compare the effects of MO versus its vehicle (mineral oil), and also the effects of pre-medication with pregabalin (1, 10 and 100 mg/kg) on the relative MO-evoked EMG activities in Mm, AD, and GG muscles, as compared with those following pre-medication with isotonic saline. In addition, t-tests or Mann-Whitney Rank Sum tests were used to evaluate the effects of pre-medication with pregabalin (1, 10 and 100 mg/kg) to EMG burst duration, area and peak amplitude of MO-evoked sustained EMG activities as compared to isotonic saline. P values less than 0.05 (two-tailed) were regarded as significant.

Microdialysis Experiments

The medullary release of glutamate was examined in another 36 rats. Each animal was prepared as previously described (Chiang et al, 1998, 2007). Under general anesthesia, the head of each rat was fixed in a stereotaxic apparatus, and the neck muscles dissected. Then the dura mater was opened to expose the obex, which served as a reference point for the lateral and anterior-posterior coordinates of the microdialysis probe. The probe (1 mm active length, 0.3 mm diameter, crescent type; Bio-analytic Systems, West Lafayette, IN), was inserted and held by a stereotaxic needle holder in a perpendicular direction on the right side

of the medulla overlying the MDH (L:1.4 mm; P:1.4 mm relative to obex). Artificial cerebrospinal fluid (CSF) was infused through the probe fiber at a flow rate of 2 μ l/min. The artificial CSF contained 154.7 mM Na⁺, 2.9 mM K⁺, 1.1 mM Ca²⁺, 0.82 mM Mg²⁺ and 132.49 mM Cl⁻, and was bubbled with 95% carbon dioxide and 5 % oxygen at the start of the experiment in order to adjust the pH to 7.40. The exposed medulla with the microdialysis probe was kept wet by a continuous perfusion of phosphate-buffered saline (PBS) solution at a flow rate 0.6 ml/h. After a 1 h stabilization period, microdialysis samples were collected in the anesthetized rats (n=6, each group) every 5 min at a flow rate of 2 μ l/min; these included 3 basal samples, 6 samples after administration of pregabalin (1, 10, 30 or 100 mg/kg, i.p.) or vehicle control (isotonic saline), and 12 samples following application (0.2 μ l) of MO or mineral oil to the exposed pulp. The doses of pregabalin were based on previous behavioral findings (Yokoyama et al 2007; Kumar et al 2010) and our EMG data. Dialysis was performed using an infusion pump (BioAnalytical 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). At the end of the sample collection, the anesthetized rat was then quickly perfused following a standard fixation protocol for subsequent examination to confirm the medullary location of the probe fiber (Chiang et al 1998).

The glutamate content in each sample was determined using gradient, reverse-phase HPLC with fluorescent detection as detailed by Böttcher et al (2003). In brief, pre-column derivatization of glutamate in samples was carried out in the autoinjector of the HPLC system (Waters Alliance 2690, Waters Corp., Milford, MA). Samples of 10 μ L microdialysis perfusate were incubated with 10 μ L of derivatizing reagent consisting of o-phthalaldehyde (OPA-incomplete reagent) and 2- mercaptoethanol 0.4% (v/v) (both Sigma, St. Louis, MO, USA) for 1.5 min, and then injected into the HPLC system. The system was fitted with a C18 ODS Supelco column (15 cm $\times$ 4.6 mm, 5 μ m particle size (ODS Supelco, Bellefonte, PA). The derivatized samples were eluted with a mobile phase at a flow rate of 1 ml/min. The mobile phase consisted of 70 mM sodium acetate (pH 6.95), 1.5% of tetrahydrofuran, and 11% methanol. An isocratic elution was maintained for 8.5 min to obtain a clear separation of glutamate. The temperatures of the column heater and sample chamber were kept at 37°C and 5°C, respectively. The eluted derivatives were detected with a fluorescent detector (Waters 474, Waters Corp., Milford, MA) at excitation and emission wavelengths of 330 and 450 nm, respectively. Peak area was used to calculate the concentration of each analyte, and quantification of glutamate was determined from a standard curve derived by using external standards. The sensitivity of the assay for glutamate was 1 ng/ml in the sample. The accuracy and precision of the estimation method was acceptable as the inter-day and intra-day assay coefficients were 5.42 % and 4.31 %, respectively. The recovery of glutamate from the probe was 36%.

For the analyses of the glutamate release data, baseline glutamate concentrations were normalized and all values, post-drug and post-MO application to the tooth pulp, were expressed as percentage change from baseline. Data from the groups receiving pregabalin were compared to those from the saline control group by two-way Repeated measures ANOVA followed by Fisher's least-squared difference post-hoc comparisons. One-way repeated measures ANOVA was used for post-MO values compared with baseline values in each group.

Results

1. The features of EMG activities evoked by MO application to tooth pulp

Pulpal application of mineral oil produced no change in EMG activity in any of the muscles following pre-medication (i.p.) with isotonic saline (Fig. 1). In contrast, MO application to

the tooth pulp following pre-medication with isotonic saline evoked marked EMG activities in the jaw (Mm and AD) and tongue (GG) muscles (Fig. 1). The MO-evoked relative EMG activities in GG and bilaterally in Mm and AD were increased significantly (two-way ANOVA, $p < 0.05$) compared with the relative EMG activity levels following pulp application of mineral oil. (Fig. 1).

2. Effects of pregabalin on EMG activities evoked by MO application to tooth pulp

Table 1 shows the incidence of EMG activities evoked by MO application to the tooth pulp following pre-medication with pregabalin (1, 10 or 100 mg/kg) and Figure 2 shows the effects of 1 mg/kg pregabalin on the MO-evoked EMG activities in Mm, AD and GG. Note there were no MO-evoked EMG activities in the Mm, AD, and GG when the MO pulp application was preceded by pregabalin doses of 10 and 100 mg/kg, whereas pre-medication with 1 mg/kg pregabalin was less effective in reducing the incidence of MO-evoked EMG activities (Table 1). Nonetheless, the burst durations of the MO-evoked Mm and AD EMG activities were significantly ($p < 0.05$) decreased by pre-medication with 1 mg/kg pregabalin as compared to isotonic saline (Fig. 2), and the areas of evoked AD and Mm EMG activities and the peak amplitude of evoked AD EMG activity showed trends of decreased activities following pre-medication with 1 mg/kg pregabalin as compared to isotonic saline (Fig. 2). Pre-medication with all 3 doses of pregabalin significantly (two-way ANOVA, $p < 0.05$) and dose-dependently decreased the relative values of right AD and bilateral Mm and GG EMG activities evoked by the MO application to the pulp and pre-medication with 10 and 100 mg/kg of pregabalin significantly (two-way ANOVA, $p < 0.05$) and dose dependently decreased the relative value of MO-evoked left AD EMG activity, as compared to isotonic saline (Figs 3).

3. Effects of pregabalin on glutamate release evoked by MO application to tooth pulp

Compared with baseline and the post-saline period, glutamate release was increased significantly (RM ANOVA, $p < 0.05$) during the first 10 min following MO application to the tooth pulp, and then returned to baseline levels (Fig. 4). Administration of PBS (starting 30 min prior to MO application) did not significantly affect baseline values, and application of mineral oil (instead of MO) to the pulp also did not produce any significant change in glutamate levels. However, a single dose of pregabalin (1 mg/kg i.p.) significantly ($p < 0.05$) attenuated the MO-induced glutamate release as compared to vehicle (isotonic saline) treatment (Fig. 4). Administration of higher doses of pregabalin (10, 30, or 100 mg/kg, i.p.) significantly ($p < 0.05$) reduced the MO-induced glutamate release and also reduced baseline glutamate levels during the 15-min post-drug period (Fig. 4).

Discussion

This is the first report to document that pregabalin is effective in attenuating the nociceptive sensorimotor responses and medullary release of glutamate that are evoked by the application of inflammatory algescic agents such as MO to orofacial tissues. Previously, it has been reported that application of MO to the rat maxillary molar tooth pulp induces significant and prolonged increases in nociceptive sensorimotor responses reflected by increases in jaw muscle EMG activity that may be related to trigeminal central sensitization (Sunakawa et al., 1999; Sessle, 2000, 2005). The present findings have confirmed and extended this earlier observation in showing that MO application to the rat molar pulp produces marked increases in tongue, as well as jaw, EMG activities that are accompanied by increases in the medullary release of glutamate. In addition, we have also shown that pregabalin dose-dependently attenuates this sensorimotor activity and release of glutamate evoked by MO application to the rat tooth pulp. These findings are consistent with our other recent data that the systemic administration of pregabalin markedly reduces the MO-induced

central sensitization in functionally identified nociceptive neurons in the MDH (Cherkas et al., 2010). We have also recently found that systemic administration of pregabalin, is effective in markedly reducing both the nociceptive behavior (facial mechanical hypersensitivity) and the MDH central sensitization that are features of our trigeminal neuropathic pain model (Cao et al., 2011).

A limitation of the study was that we could not provide details of the exact site of glutamate release in the medulla. However, many trigeminal primary afferents supplying the tooth pulp terminate in the MDH and contain glutamate, which is the major excitatory neurotransmitter in the MDH. Also, it has previously been shown that noxious orofacial stimulation evokes glutamate release in the MDH, and local MDH application of glutamate activates nociceptive MDH neurons receiving tooth pulp afferent inputs (e.g., Henry et al., 1980; Azerad et al., 1992; Bereiter and Benetti, 1996; see Sessle, 2000; Bereiter, 2007). This, the release of glutamate was expected to be mainly from afferents ending in the MDH. In addition, all doses of pregabalin, used in the present study (including the lowest dose of 1 mg/kg) were effective in attenuating MO-evoked glutamate release in the medulla. The 1 mg/kg dose of pregabalin produced a somewhat more limited on the incidence of MO-evoked EMG activities, although it did significantly reduce EMG burst duration, area or peak amplitude in all muscles. These findings suggest that pregabalin, at least at the lower doses, was not acting non-specifically in producing a generalized reduction in motor activity, and this is supported by a finding in our recent study (Cao et al, 2011), where various pregabalin doses were assessed for their effects on general motor behavior and we found that only the 100 mg/kg dose produced any evidence of a slight lethargy and reduced general motor behavior, consistent with earlier pregabalin studies in rats (eg, Yokoyama et al, 2007). It is also noteworthy that the 1 mg/kg dose of pregabalin only had partial effects on the medullary release of glutamate, in that this low dose did not decrease baseline levels of glutamate, which only occurred with the higher pregabalin doses. The effectiveness of the higher doses of pregabalin in attenuating the baseline levels of glutamate release suggests that a tonic release of glutamate may occur in our model, possibly from tooth pulp afferents or other afferent inputs to the MDH or adjacent medullary regions associated with the surgeries required to implant the dialysis fiber or to expose the tooth pulp for MO application.

Further investigations are needed to determine the specific mechanisms and site of action of pregabalin in the trigeminal system; this was not the aim of the present study which was designed to replicate the mode of administration (systemic) by which pregabalin is used clinically. The mechanism of action of pregabalin is not completely clear, but is thought to be similar to that of gabapentin. Pregabalin is a structural analog of GABA, but it is inactive at GABA-A or -B receptors, and is not converted into GABA or a GABA antagonist, and it does not affect GABA uptake (Sabatowski, et al., 2004; Frampton, et al., 2005; Cada, et., 2006). Pregabalin may alter the release of several neurotransmitters by selectively binding to the $\alpha 2\delta$ protein subunit of voltage-gated calcium channels. By tightly binding to the $\alpha 2\delta$ protein, pregabalin reduces the influx of calcium, thereby reducing the release of neurotransmitters, including glutamate, norepinephrine, and substance P (Fink, 2002; Dooley et al., 2000; Taylor et al., 2003). These mechanisms are thought to result in the anticonvulsant, anxiolytic, and analgesic properties exhibited by pregabalin (Field et al., 2006; Bauer et al., 2009; Bauer et al., 2010; Kumar et al 2010; Offord et al., 2010; Lotarski et al., 2011). In the spinal nociceptive system, the spinal dorsal horn (the spinal cord analog of the MDH) has been shown to be an important site of action for pregabalin and its related compound gabapentin, which includes attenuation of both spinal release of glutamate and spinal dorsal horn neuronal activity in rat pain models (Wallin et al, 2002;Coderre et al., 2007; Kumar et al., 2010; Bannister et al., 2011). Thus, it is likely that the marked effects of pregabalin on EMG activities found here can be explained, at least in part, by a decrease of

medullary glutamate release. Released glutamate would then produce an activation of trigeminal interneuronal reflex circuits, which depend upon the functional integrity of MDH and on NMDA and non-NMDA receptors in the MDH, and activate the medullary and pontine cranial nerve motoneuron pools supplying the craniofacial muscles (e.g. Cairns et al., 1998, 2001; Tsai et al., 1999). An action by pregabalin directly on these motoneuron pools cannot also be discounted, especially since glutamate is a major transmitter in these sites (Lund et al, 2009) but is unlikely since our recent study in a trigeminal neuropathic pain model (Cao et al, 2011) found no effect of low doses of pregabalin (eg, 10mg/kg) on baseline orofacial behavior in rats with no nerve injury. It should also be kept in mind that MO-induced EMG responses depend on a multisynaptic medullary/pontine pathway that may utilize several transmitters in addition to glutamate (eg, Cairns et al, 1998, 2001; Tsai et al, 1999; Sessle, 2000), and so the small differences noted above between EMG responses vs medullary release of glutamate for low doses of pregabalin are not unexpected. It is also important to note that systemic administration of pregabalin can suppress also ectopic discharges from injured primary afferents (Chen et al., 2001), so we cannot rule out the possibility that the pregabalin effects on medullary glutamate release and EMG responses may depend, at least in part, on a reduction of MO-evoked tooth pulp afferent activity.

Pregabalin has been reported clinically to have significant analgesic effects on acute pain following third molar extraction (Hill et al., 2001), and on paresthesia following inferior alveolar nerve damage (Lopez-Lopez et al., 2011). These observations, together with the present pre-clinical findings in our acute tooth pulp inflammatory pain model, and our recent data of pregabalin effectiveness in our trigeminal neuropathic pain model (see above), suggest that pregabalin may prove to be effective in a range of orofacial inflammatory pain states as well as neuropathic pain conditions in humans, and warrants further studies.

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HIGHLIGHTS

1. The inflammatory irritant mustard oil was applied to the rat molar tooth pulp.
2. Pregabalin dose-dependently attenuated orofacial EMG activity evoked by the mustard oil application.
3. Pregabalin also attenuated the medullary release of glutamate evoked by the mustard oil application.
4. Based on these findings, pregabalin may prove useful clinically in orofacial inflammatory pain states.

Fig. 1.

Diagram shows at top some examples of EMG traces following MO or mineral oil (vehicle control) application to molar pulp (A and B). The five traces show the EMG activities (mV) of genioglossus (GG), anterior digastric (AD), and masseter (Mm) muscles (A and B). Below are shown relative EMG activities induced by MO application to tooth pulp following systemic injection of isotonic saline (C). Significant increase in relative EMG activities occurred in bilateral Mm and AD and in GG muscles immediately after MO application (D). Relative EMG activity was normalized to the EMG activity (mV) in the first baseline 5 min. R: right, L: left.

Fig. 2.

Parameters of MO-induced sustained EMG activities, such as burst duration, area, and peak amplitude were markedly decreased by pre-medication with 1mg/kg pregabalin (PG) as compared with control condition of pre-medication with isotonic saline. The data related to GG and Mm (L) EMG activities was not statistically analyzed since their low incidence resulted in sample sizes too small for analysis (see Table 1). Values shown as mean $\pm$ SD; t-test, (*) and Mann-Whitney Rank Sum Test, (†); R: right; L: left.

Fig. 3.

Pre-medication with pregabalin (1mg/kg, 10mg/kg, and 100 mg/kg, i.p.) as compared to isotonic saline significantly ($p < 0.05$) decreased relative MO-evoked EMG activities (mean $\pm$ SD) in GG, Mm(R/L) and AD(R) muscles. A significant difference ($p < 0.05$) also occurred in relative MO-evoked EMG activities of AD(L) muscle, as compared to isotonic saline following pre-medication with pregabalin doses of 10mg/kg and 100mg/kg. R: right; L: left.

Fig. 4.

Effect of pregabalin pretreatment on medullary glutamate release induced by MO application to the molar tooth pulp. Graphs show basal, post-drug and post-MO glutamate levels (mean $\pm$ SE) for groups of rats (each $n=6$) injected (i.p.) with a single dose of gabalin or vehicle. The first arrow indicates the start of injection of the 4 different doses of pregabalin, (1, 10, 30, or 100 mg/kg, i.p.) or vehicle (isotonic saline i.p.) at -30 min, and the second arrow indicates MO application at 0 min. RM ANOVA revealed a significant main effect of time, $F(4, 25) = 6.93$, $p < 0.0007$, drug $F(20, 60) = 7.39$, $p < 0.0001$, and a significant drug $\times$ time interaction, $F(80, 500) = 2.28$, $p < 0.0001$. Post-hoc comparisons (Fisher's test) indicated that MO application significantly increased glutamate concentration at 5 and 10 min as compared to baseline ($\dagger p < 0.05$ and $\dagger\dagger p < 0.01$). Pretreatment with the low dose of pregabalin attenuated the MO-induced glutamate release compared to control ($* p < 0.05$, $** p < 0.01$, A). Higher doses of pregabalin (10, 30 or 100 mg/kg i.p.) also significantly reduced glutamate levels, post-drug and post-MO, 85 min, -15 min to 60 min.

Table 1

The effects of pre-medication of isotonic saline and pregabalin on the incidence of MO-induced EMG activities.

Muscle	Isotonic saline	PG 1mg/kg	PG 10mg/kg	PG 100mg/kg
Mm (L)	100%(6)	33%(2)	0%(0)	0%(0)
Mm (R)	67%(4)	100%(4)	0%(0)	0%(0)
AD (L)	67%(4)	100%(4)	0%(0)	0%(0)
AD (R)	100%(6)	67%(4)	0%(0)	0%(0)
GG	100%(6)	33%(2)	0%(0)	0%(0)

PG: pregabalin; R: right, L: left