Chronic intrathecal infusion of mibefradil, ethosuximide and nickel attenuates nerve ligation-induced pain in rats

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Abstract

Background: T-type Ca2+ channels (TCC) are important for pain transmission, especially the CaV3.2 subtype. In this study, we examined the effects of intrathecal TCC blockers in the L5/6 spinal nerve ligation pain rat model.

Methods: Under isoflurane anaesthesia, rats received right L5/6 spinal nerve ligation and intrathecal catheters (attached to an infusion pump) were sited. After surgery, saline, mibefradil, ethosuximide or NiCl2 were given intrathecally for seven days. The right hindpaw withdrawal thresholds to von Frey hair stimuli and withdrawal latencies to radiant heat were measured before and once daily for seven days after surgery. Double immunofluorescence and western blotting were used to examine the expression of CaV3.2 in dorsal root ganglion (DRG) and spinal cord.

Results: On post-ligation day seven, rats receiving mibefradil, ethosuximide or NiCl2 had significant higher median withdrawal thresholds (15.0, 10.2, and 10.9 g) and latencies (8.0, 7.6 and 7.6 s) than saline-treated rats (1.6 g and 4.3 s, respectively). CaV3.2 was expressed in parvalbumin+, IB4+, CGRP+ and VR1+ neurones in DRG and most neurones in spinal dorsal horn. CaV3.2 was up-regulated in the right L5/6 DRG and spinal cord seven days after nerve ligation.

Conclusions: In this study, we demonstrated that intrathecal TCC blockers attenuate the development of nerve injury-induced mechanical allodynia and thermal hyperalgesia. Our data suggest that continuous intrathecal infusion of TCC or CaV3.2 blockers may be a promising alternative for the management of nerve injury-induced pain.

Key words: ca3,2; dorsal root ganglion; intrathecal; neuropathic pain; spinal cord

Neuropathic pain results from damage to the nervous system as a consequence of injury or disease. Of all the pain states, neuropathic pain is the most difficult to treat and is usually unresponsive to classical opioids and anti-inflammatory analgesics. In the last few decades, the involvement of neuronal and glial components in the formation of nerve injury-induced pain has been extensively studied. T-type Ca2+ channels (TCC) are important for pain transmission, especially the CaV3.2 subtype. In this study, we examined the effects of intrathecal TCC blockers in the L5/6 spinal nerve ligation pain rat model.
neuropathic pain has been described. Of the neuronal component, ion channels such as Na⁺, Ca²⁺ and K⁺ channels all have been reported to play a role in the pathogenesis of neuropathic pain. Unlike N- and P-type Ca²⁺ channels, T-type Ca²⁺ channels (TCC) are low-threshold activated Ca²⁺ channels and are involved in pain transmission. Up to now, three subtypes of TCC CaV3.1–3.3, have been identified, with CaV3.2 being most promising as a therapeutic target for neuropathic pain. Recent studies have also shown that pre-synaptic CaV3.2 channels are involved in the regulation of nociceptive transmission to the spinal dorsal horn. There are several different neuropathic animal pain models and the L5/6 spinal nerve ligation pain model is a classic nerve injury pain model. It induces characteristic chronic pain behaviours such as mechanical allodynia and thermal hyperalgesia. Using this pain model, we examined if chronic intrathecal infusion of mibefradil, ethosuximide or Ni²⁺, all of which have been reported to block TCC or CaV3.2 currents, could attenuate nerve injury-induced neuropathic pain. In addition, the expression pattern of CaV3.2 in the dorsal root ganglion (DRG) and the spinal cord were examined.

Methods

All the investigations adhered to the guidelines of the Committee for Research and Ethical Issues of the International Association for the Study of Pain and appropriate aspects of the ARRIVE guidelines, and were undertaken according to a protocol approved by the Institutional Animal Care and Use Committee of Mackay Memorial Hospital. Sample size calculations were used to estimate the smallest number of animals (n) needed to detect an arbitrarily chosen 20% difference in group mean, when significance was set at P=0.05 and statistical power at 0.9. Male Sprague-Dawley rats (200–250 g), purchased from BioLASCO Taiwan Co., Ltd., were housed in pairs with soft bedding at 20–24°C and maintained on a 12-h light/12-h dark cycle with free access to food and water.

L5/6 spinal nerve ligation

Surgery was carried out under isoflurane anaesthesia in 100% oxygen, induced at 5% and maintained at 2%. During the surgery, the percentage of isoflurane was increased if inadequate anaesthesia, indicated by the pedal withdrawal response to a nociceptive stimulus, was observed.

Right L5/6 spinal nerve ligation was performed as previously reported. Animals were anaesthetized and placed in a prone position. A midline incision was made on the lower back, and the right paraspinal muscles were detached from the spinal processes around the L4–S2 level. The L4–L5 spinal nerves were identified after the L5 transverse process was removed. The L5 nerve was firmly ligated with a 6-0 silk thread. The right L6 nerve was located medial to the sacroiliac joint and ligated with a silk thread. For sham surgery, the right L5/6 spinal nerves were exposed but not ligated. Postoperative analgesia was not provided because of the confounding effect of an analgesic on the assessment of neuropathic pain behaviours.

Intrathecal catheterization and implantation of infusion pump

Immediately after the surgery and for naïve rats, intrathecal catheterization and infusion pump implantation were performed as previously described. Under isoflurane anaesthesia, a PE-5 catheter (filled with normal saline or test agents) was introduced through an opening in the atlanto-occipital membrane, to a position eight cm caudal to the cisterna at the level of lumbar area. After the last behavioural test, laminectomy under anaesthesia was performed to confirm the positioning of intrathecal catheters. For chronic drug administration, an infusion pump (Model 2001, ALZET) with a flow rate of 1 µl h⁻¹ was filled with saline (1 µl h⁻¹), mibefradil (0.7 µg µl⁻¹), ethosuximide (60 µg µl⁻¹) or NiCl₂ (0.5 µg µl⁻¹) and attached to the intrathecal catheter (n=6–8 for each group). The pump was fixed subcutaneously and the wound closed with silk sutures. In this study, seven of the rats showed neurological deficits after surgery and were euthanized with isoflurane anaesthesia and intraperitoneal pentobarbital.

Drugs

Mibefradil and ethosuximide were purchased from Sigma (St Louis, Missouri) and hexahydrate NiCl₂ was from Riedel-de Haen GmbH (Seelze, Germany). All drugs were dissolved in saline. The infusion doses of tested drugs were determined according to our previous study (assuming the total cerebrospinal fluid volume was 400 µl) and preliminary experiments.

Behavioural assessments of mechanical and thermal sensitivity

The behavioural tests were performed between 9 a.m. and 3 p.m. by an investigator blinded to the treatment groups. Motor function was evaluated by testing the rat’s ability to ambulate in a normal posture and observing the righting and placing/stepping reflexes before the daily behaviour assessments.

The hindpaw withdrawal threshold (WT) in response to mechanical stimuli was measured by using the von Frey hair and up-down method. Rats were placed in a plastic cage with a metal-mesh floor, with acclimatization to the environment for at least 30 min. The von Frey filament was then pressed vertically to the plantar surface of right hindpaw, with adequate strength to induce slight buckling, for about six s. A positive response was observed if the hindpaw was sharply withdrawn. Flinching instantly on removal of the filament was also considered a positive response.

The hindpaw withdrawal latency (WL) to noxious thermal stimuli was determined using the Analgesia Meter apparatus (IITC-Life Science Instruments). Rats were placed on a glass floor under which a light box was located, with at least 30 min acclimatization. The movable radiant heat was focused on the plantar area of right hindpaw. WLs were calculated automatically using a photocell. To avoid tissue damage, a cut-off time of 20 s was set. Light intensity was predetermined to obtain a baseline latency around 10 s. The WLs were measured at a minimum of five min intervals and the middle six of the 10 latencies were averaged.
Double immunofluorescence study

Rats were terminally anaesthetized with isoflurane and intraperitoneal pentobarbital (100 mg kg\(^{-1}\)) and perfused via the ascending aorta with saline followed by 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 20 min. After the perfusion, the L5/6 DRG and spinal cord segment were removed and postfixed in the same fixative overnight. Sections were rinsed with 0.01 M PBS for 5 min three times at room temperature (RT), and cryoprotected in 50% sucrose in 0.01 M PBS overnight at 4°C. Sections were embedded in OCT solution and transverse slices were cut on a cryostat at 20 µm for spinal sections, or 15 µm for DRG sections and mounted directly onto gelatin-coated slides. All the slices were blocked with 2% goat serum, plus 0.3% Triton X-100 in a cryostat at 20 µm for spinal sections, or 15 µm for DRG sections.

Western blotting

Seven days after surgery, rats were killed with pentobarbital (100 mg kg\(^{-1}\), intraperitoneal (i.p.)). The right L5/6 DRG and spinal cord were extracted and stored in liquid nitrogen. Tissue samples [100 mg kg\(^{-1}\)] were homogenized by pestle (BioMasher, Japan) for 30 s in lysis buffer (Thermo, Illinois), plus 0.02% protease inhibitor cocktail tablets (Roche, Germany), on ice three times. After the addition of 0.1 g ceria stabilized zirconium oxide beads (Next Advance, NY), the homogenates were vortexed using a bullet blender for five min and centrifuged at 10 000 g for 30 min at 4°C. The supernatants were used for analysis and protein concentrations were determined using the BCA kit (Thermo, Illinois). Samples were run on (40 µg) NuPAGE Bis-Tris gradient gel (Life technologies, CA) and transferred onto a PVDF membrane followed by immunoblotting with antisera against CaV3.2 (1:1000, Santa Cruz) and actin (1:5000, Millipore). Images were captured and analysed using a cooled CCD system (FujiFilm).

Statistical analysis

All the behavioural data are presented as median and interquartile range (IQR). The behavioural data of naïve and other study groups were analysed using the Mann–Whitney U-test. The western blot data were analysed using Student’s t-test. P<0.05 was considered statistically significant.

Results

Figure 1 shows the sequential changes of the right hindpaw WT to von Frey hairs. There was no significant difference between groups pre-surgery. For the Ligation/Saline group, the pre-surgery WT decreased progressively to post-surgery day seven, such that compared with the WT of Sham/Saline group on day seven, there was a significant difference.

The WT of Ligation/Mibebradil, Ethosuximide or NiCl\(_2\) groups were significantly different from those of the Ligation/Saline group on post-surgery days 1–7 (P<0.05, Fig. 1A–C). These three agents did not affect the WT of naïve rats during the study period, compared with the pre-surgery value in each naïve group (P>0.05, Fig. 1A–C). No motor weakness was observed in any groups during the study period.

The sequential changes of hindpaw WL to radiant heat stimuli are shown in Figure 2. There was no significant difference between groups pre-surgery. For the Ligation/Saline group, the pre-surgery WL was 11.0 (9.6–11.0) s, decreased progressively to post-surgery day seven, such that the WL was significantly lower than the Sham/Saline group on post-surgery day seven.

![Fig 1](http://bja.oxfordjournals.org/)

**Fig 1** Effects of T-type Ca\(^{2+}\) channel blockers on the development of nerve ligation-induced mechanical allodynia. Intrathecal infusion of mibebradil, ethosuximide or NiCl\(_2\) attenuated the development of L5/6 spinal nerve ligation-induced mechanical allodynia (A–C), without affecting mechanical sensitivity in naïve rats. Bar above the X-axis represents intrathecal infusion with saline or tested agents. Data are presented as median and IQR. *P<0.05 vs Sham/Saline group; **P<0.05 vs Ligation/Saline group (Mann-Whitney U-test, n=6–8 per group).
The WLs of Ligation/Mibefradil, Ethosuximide or NiCl₂ groups were significantly different from those of Ligation/Saline group on post-surgery day 2–7 (P<0.05, Fig. 2A–C). Intrathecal infusion of all these three agents did not affect the WLs of naive rats during the study period, compared with the pre-surgery value in each naive group (Fig. 2A–C).

Figure 3 shows CaV3.2 expression in large, medium and small-sized L5/6 DRG neurones, which could be either parvalbumin⁺, IB4⁺, CGRP⁺ or VR1⁺ neurones. In the spinal dorsal horn, CaV3.2 was co-localized with most neurones, but not microglia or astrocytes (Fig. 4). The protein level of CaV3.2 in right L5/6 DRG and spinal cord of nerve-ligated rats seven days after surgery was significantly higher than that in the Sham group (P<0.05, Fig. 5).

Discussion

Nerve injury-induced pain is debilitating and refractory to traditional analgesics and the possible mechanisms involved and potential therapeutics have been actively explored. The L5/6 spinal nerve ligation model is an archetypal neuropathic pain model, consistently generating characteristic neuropathic pain behaviours and the data presented here also show this. In this study, we observed that intrathecal infusion of mibefradil, ethosuximide or NiCl₂ attenuated the development of the nerve ligation-induced mechanical allodynia and thermal hyperalgesia. Our findings suggest that intrathecal infusion of these three agents may be considered for the treatment of nerve injury-induced pain. To our knowledge, this is the first report demonstrating the effectiveness of continuous intrathecal infusion of TCC blockers in a nerve injury pain model.

Given systemically or intrathecally, mibefradil, ethosuximide and NiCl₂ have been reported to possess analgesic effects in various animal pain models. All three agents have been reported to block TCC or CaV3.2 currents and Ni²⁺ is known to inhibit CaV3.2 more efficiently than CaV3.1 and CaV3.3. Recently, mibefradil was found to normalize nerve injury-induced pain and neuronal hyper-excitability. In clinical practice, it has been used for the treatment of chronic angina pectoris and hypertension. However, as a result of some serious drug interactions, mibefradil was withdrawn from the market. The effectiveness of mibefradil infusion in the current study raised another possibility for intrathecal use in the management of nerve injury-induced pain.

Similarly, systemic ethosuximide given intraperitoneally has been reported to reverse chemotherapy- and chronic constriction
injury-induced neuropathic pain in animal models. Ethosuximide is used clinically for the treatment of absence epilepsy. However, oral administration of ethosuximide has been found to induce a lupus-like syndrome with cerebral and renal involvement and other side-effects, such as agranulocytosis, in patients. Our data suggest that intrathecal infusion could be considered as an alternative route of administration for ethosuximide, especially for the management of nerve injury-induced pain.

As a divalent cation, Ni²⁺ could block TCC at two different affinity binding sites. In our previous study, intrathecal NiCl₂ attenuated formalin-induced nociceptive behaviours. In both formalin and nerve injury-induced pain models, long-term potentiation in the spinal dorsal horn, which can be TCC-mediated, plays an important role in the pathogenesis of pain status. Therefore, it is possible that the TCC blockers used in this study achieve their antinociceptive effects via blocking the development of nerve injury-induced long-term potentiation in the spinal cord.

All three mRNA transcripts of CaV₃.1-3 are present in the spinal dorsal horn, whereas CaV₃.2 mRNAs are mostly limited to the superficial dorsal horn, which is involved in pain-related neurotransmission. Intrathecal injection of an antisense oligonucleotide targeted to the α₁-subunit of CaV₃.2, but not CaV₃.1 and CaV₃.3, produced an antinociceptive effect in both acute and neuropathic pain states. In the DRG compression pain model, CaV₃.2 mRNA was found to be increased, and CaV₃.2 antisense attenuated compression-induced pain. Therefore, it is likely that CaV₃.2, but not CaV₃.1 and CaV₃.3, channels are most involved in pain transmission. Recent studies also revealed CaV₃.2 gates transmitter release at the DRG sandwich synapse and is involved in diabetes-induced neuropathic pain.

The mRNA level of CaV₃.2 in DRG has been found to be increased after spinal nerve ligation. In the chronic constriction injury pain model, TCC current in small sensory neurones was up-regulated after sciatic nerve constriction. In the current study, we observed up-regulation of CaV₃.2 at the protein level in the ipsilateral DRG and spinal cord seven days after nerve ligation (Fig. 5). This up-regulation of CaV₃.2 may contribute to the development of nerve ligation-induced pain.

Different subsets of unmyelinated primary sensory fibres have been reported to mediate behavioural responses to noxious thermal and mechanical stimuli. In our study, we observed CaV₃.2 is co-localized in DRG with some parvalbumin⁺, IB4⁺ and...
CGRP+ neurones (Fig. 3); the last two have been proposed to primarily mediate mechanical and thermal nociception, respectively.\textsuperscript{24} Our finding that Ca\textsubscript{V}3.2 is co-localized with VR1\textsuperscript{+} DRG neurones (Fig. 3) also implies a potential role of Ca\textsubscript{V}3.2 in the transmission of thermal sensation. Using immunostaining within dissociated DRG cultures, Rose and colleagues recently found the expression of Ca\textsubscript{V}3.2 in unmyelinated and myelinated sensory DRG neurones.\textsuperscript{35} In our study, we did not find any effect of the three TCC blockers, at the doses tested, on acute mechanical and thermal nociception in naive rats (Figs 1 and 2). It is suggested that Ca\textsubscript{V}3.2 may play a more important role in the pathogenesis of nerve injury-induced pain than in acute physiological pain. This inference is supported by the observed up-regulation of Ca\textsubscript{V}3.2 in the ipsilateral DRG and spinal cord seven days after nerve ligation (Fig. 5).

Ion channels have been reported to be expressed in microglia and astrocytes.\textsuperscript{36,37} In our study, we found Ca\textsubscript{V}3.2 expression in spinal dorsal horn neurones, but not in microglia and astrocytes, implying that the neuronal Ca\textsubscript{V}3.2 could be the main action targets of the tested agents in this study. However, the three agents used in this study are non-selective Ca\textsubscript{V}3.2 blockers, and we cannot therefore rule out the possibility of off-target effects. For instance, mibefradil has been reported to block Na\textsuperscript{+} channels and other non-T-type Ca\textsuperscript{2+} channels.\textsuperscript{38} For Ni\textsuperscript{2+}, the Ca\textsuperscript{2+} channel subtype sensitivity is Ca\textsubscript{V}3.2>>Ca\textsubscript{V}1.2>Ca\textsubscript{V}3.3>Ca\textsubscript{V}3.1>Ca\textsubscript{V}2.3>Ca\textsubscript{V}2.1>>Ca\textsubscript{V}2.2.\textsuperscript{11} Recently, several novel selective Ca\textsubscript{V}3.2 blockers have been developed.\textsuperscript{39} Future studies may be performed to evaluate their antinociceptive effects in different animal pain models.

In summary, we have demonstrated that continuous intrathecal infusion of the TCC blockers mibefradil, ethosuximide and Ni\textsuperscript{2+} attenuated the development of nerve ligation-induced mechanical allodynia and thermal hyperalgesia in rats. Our findings suggest that intrathecal infusion of TCC or Ca\textsubscript{V}3.2 blockers may be considered as a promising alternative for the management of nerve injury-induced pain.

Authors’ contributions
Study design/planning: J.K.C.
Study conduct: Y.L.C., M.L.T.
Writing paper: Y.L.C., M.L.T., J.K.C.
Revising paper: all authors.

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Declaration of interest
None declared.

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References
4. Todorovic SM, Jevtovic-Todorovic V. Neuropathic pain: role for presynaptic T-type channels in nociceptive signaling. Pflugers Arch 2013; 465: 921–7
14. Perez-Reyes E, Van Deussen AL, Vitko I. Molecular pharmacology of human Ca\textsubscript{V}3.2 T-type Ca\textsuperscript{2+} channels: block by antihypertensives, antiarrhythmics, and their analogs. J Pharmacol Exp Ther 2009; 328: 621–7
15. Lacinova L. T-type calcium channel blockers - new and notable. Gen Physiol Biophys 2011; 30: 403–9
17. Tanaka H, Shigenobu K. Pathophysiological significance of T-type Ca\textsuperscript{2+} channels: T-type Ca\textsuperscript{2+} channels and drug development. J Pharmacol Sci 2005; 99: 214–20
monotherapy outcomes at 12 months. Epilepsia 2013; 54: 141–55

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