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Intrathecal P/Q- and R-type calcium channel blockades on spinal substance P release and c-Fos expression

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Abstract

Intrathecal (IT) studies have shown that several voltage sensitive calcium channels (VSCCs), such as the L-, N- and T-type may play roles in nociception and that of these only the N-type regulates primary afferent substance P (SP) release. However, the actions of other VSCCs at the spinal level are not well known. We investigated the roles of spinal P/Q- and R-type VSCCs, by IT administration of R-type (SNX-482) and P/Q-type (ω-agatoxin IVA) VSCC blockers on intraplantar formalin-evoked flinching, SP release from primary afferents and c-Fos expression in spinal dorsal horn. Intraplantar injection of formalin (2.5%, 50 μL) produced an intense, characteristic biphasic paw flinching response. In rats with IT catheters, IT SNX-482 (0.5 μg) reduced formalin-evoked paw flinching in both phase 1 and 2 compared with vehicle. Intraplantar formalin caused robust neurokinin 1 receptor (NK1r) internalization (indicating SP release) and c-Fos expression in the ipsilateral dorsal horn, which were blocked by IT SNX-482. IT ω-agatoxin IVA (0.03, 0.125 and 0.5 μg) did not reduce formalin-evoked paw flinching or c-Fos expression at any doses, with higher doses resulting in motor dysfunction. Thus, we demonstrated that blockade

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of spinal R-type, but not P/Q type VSCCs attenuated formalin-induced pain behavior, NK1r internalization and c-Fos expression in the superficial dorsal horn. This study supports a role for Cav2.3 in presynaptic neurotransmitter release from peptidergic nociceptive afferents and pain behaviors.

Keywords

Voltage sensitive calcium channels; SNX-482; ω-agatoxin IVA; neurokinin 1 receptor; c-Fos; spinal cord

1. Introduction

Voltage-sensitive calcium channels (VSCCs) facilitate calcium influx and play an important role in the regulation of neurotransmitter release, synaptic transmission and neuronal excitability (Catterall, 2000; Catterall and Few, 2008). The a1 is the pore-forming subunit and dictates the VSCC's major characteristics of pharmacology, electrophysiology and kinetic activity, representing the basis for the calcium channel subtype. Ten different a1 have been identified, namely Cav1.1–1.4, Cav2.1–2.3 and Cav3.1–3.3. These are distributed into five subgroups, L-(Cav1.1–1.4), P/Q-(Cav2.1), N-(Cav2.2), R-(Cav2.3) and T-type (Cav3.1–3.3). Based on their voltage activation properties, VSCCs are divided into two classes, high voltage-activated channels and low voltage-activated channels (Ertel et al., 2000). High voltage-activated channels include L-, P/Q-, N- and R-types. These are heteromeric complexes consisting of an a1 subunit along with auxiliary subunits such as α2δ, β and γ subunit. Low voltage-activated channels or T-type are activated by much more negative membrane potentials (Carbone and Lux, 1984; Nowycky et al., 1985) and are not known to interact with auxiliary subunits.

VSCCs are expressed in the dorsal root ganglion (DRG) and spinal cord dorsal horn (Murakami et al., 2001; Westenbroek et al., 1998; Yusaf et al., 2001), suggestive of their critical role in nociception. Intrathecal (IT) ziconotide (Prialt®), a selective N-type blocker is approved for treatment of severe chronic pain. Consistent with location of N-type VSCCs on peptidergic primary afferents, N-type VSCC blockade attenuates neurotransmitter release from primary afferents, as defined in in vitro and in vivo models (Evans et al., 1996; Maggi et al., 1990; Santicioli et al., 1992; Takasusuki and Yaksh, 2011). However, the role of other VSCCs subtypes in afferent neurotransmitter release remains unclear. We found previously that intrathecal L- and T-type blockers minimally affected intraplantar formalin-evoked release of substance P (SP) in vivo as measured by neurokinin 1 receptor (NK1r) internalization (Takasusuki and Yaksh, 2011). Those results suggested L- and T-type VSCCs were not involved in stimulus-evoked SP release. In the present study we focus on the role of P/Q- and R-types.

Cav2.1 (P/Q-type) channels contain the a1A subunit and are distributed in the nervous system including DRG and spinal dorsal horn (Catterall and Few, 2008; Kulik et al., 2004; Urban et al., 2005). P/Q-type VSCCs are inhibited by ω-agatoxin IVA, a 48- amino acid peptide isolated from the venom of funnel web spider, Agelenopsis aperta (Mintz et al., 1992). ω-agatoxin IVA blocks P-type with high affinity and Q-type with lower affinity (Adams, 2004). IT ω-agatoxin IVA reduces the number of flinches in formalin phase 2, but did not alter thermal escape latencies (Malmberg and Yaksh, 1994). Cav2.3 (R-type) channels contain the α1E subunit, and are detected in spinal cord and DRG (Murakami et al., 2001; Saegusa et al., 2000; Westenbroek et al., 1998; Yusaf et al., 2001). Cav2.3 knockout mice exhibited reduced pain behaviors in the formalin test (Saegusa et al., 2000). In another study, Cav2.3 was shown to participate in nerve injury-induced hypersensitivity

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(Matthews et al., 2007). Dense labeling of Cav2.3 was observed in the superficial layers of the dorsal horn (Saegusa et al., 2000). However, whether Cav2.3 is involved in primary afferent neurotransmitter release has not been established.

Here, we investigated intrathecal R- and P/Q-type channel blockers and spinal release of SP evoked by intraplantar formalin. Increase in local extracellular SP due to its release (induced by intraplantar formalin) from small peptidergic, transient receptor potential protein vanilloid 1 (TRPV1) (+) C-fibers evokes NK1r internalization (Kondo et al., 2005; Mantyh, 2002). Thus NK1r internalization provides a powerful tool to evaluate in vivo the effects of VSCCs blockade on afferent terminal release of SP. Furthermore, intraplantar formalin induces c-Fos protein expression in dorsal horn neurons, which receive input from small diameter Aδ and C primary afferents (Bullitt, 1990; Hunt et al., 1987; Long et al., 2012), causing c-Fos expression to be a useful index of spinal activation. This in vivo methodology allows us to assess the effects of R- and P/Q- type VSCCs antagonists upon SP release, dorsal horn neuron activation and assess the covariance of these effects with pain behaviors (flinching) at the corresponding drug doses.

2. Material and Methods

2.1 Animals

Male Holtzman Sprague-Dawley rats (250–300g; Harlan Indianapolis, IN) were individually housed in standard cages and maintained on a 12-h light/dark cycle (lights on at 07:00 h). Testing occurred during the light cycle. Food and water were available ad libitum to all rats in the study. Animal care was in accordance with the Guide for the Care and Use of Laboratory Animals (National Institutes of Health publication 85–23, Bethesda, MD) and as approved by the institutional Animal Care and Use Committee of the University of California, San Diego. All efforts were made to minimize animal suffering, to reduce the number of animals used.

2.2 Intrathecal catheter implantation

Rats were implanted with a single intrathecal catheter for drug delivery (Malkmus and Yaksh, 2004; Yaksh and Rudy, 1976). In brief, rats were anesthetized by induction with 4% isoflurane in a mixture of air and 100% oxygen (1:1). Anesthesia was maintained with 2% isoflurane delivery by mask. The animal was placed on a stereotaxic head holder, a midline incision was made on the back of the occipital bone and the cisternal membrane was exposed by blunt dissection. A small opening was made on the cisternal membrane by a 22 gauge needle, and an 8.5 cm single-lumen polyethylene (OD 0.36 mm) catheter was inserted through the opening and passed into the intrathecal space to the level of the L2 to L3 spinal segments. The other end of the catheter was tunneled subcutaneously to exit through the top of the head, flushed with 10 μL of saline and plugged. Rats were given subcutaneously 5 mL of lactated Ringer's solution, to which was added 1.25 mg/mL of carprofen and recovered in a warmed chamber. Rats with any motor weakness or signs of paresis on recovery from anesthesia were euthanized immediately. Animals were allowed to recover for 5 to 7 days prior to other studies.

2.3 Voltage-sensitive calcium channel (VSCC) blockers on formalin-induced paw flinching behavior

Formalin-induced flinching behavior was analyzed by a paw movement detection system (Yaksh et al., 2001). Briefly, a soft metal band was placed around the left hind paw and secured with a drop of adhesive. Animals were allowed to acclimate in individual acrylic glass chambers for 30 min before experimental manipulation. Rats were intrathecally treated with saline, SNX-482 (0.5 μg) or ω-agatoxin IVA (0.03, 0.125 and 0.5 μg) in a volume of 10

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μL, followed by 10 μL of saline. 10 min after the IT injection, rats received a subcutaneous injection of 50 μL of formalin (2.5%) into the dorsal side of the banded paw. Rats were immediately, placed into test chambers and paw flinching assessed over the ensuing 60 min interval with an automated device (Department of Anesthesiology, University of California, San Diego, CA) for 60 min.

2.4 VSCC blockers on formalin-induced NK-1r internalization and c-Fos expression

Rats with catheter received IT saline or antagonist ten minutes before intraplantar formalin (50μl, 2.5%) injection. For NK1r internalization, rats were transcardially perfused 10 min after formalin. For c-Fos expression, rats were perfused 120 min after formalin.

2.5 Tissue preparation and Immunohistochemistry

Anesthetized rats were transcardially perfused with NaCl (0.9%) followed by paraformaldehyde (4%) in 0.1 M phosphate buffer (PB), pH 7.4. The lumbar spinal cord was removed and post-fixed in paraformaldehyde (4%) overnight. After cryoprotection in 30% sucrose, coronal sections (30 μm) were cut on a sliding microtome (HM 450; Thermo Scientific, Kalamazoo, Ml).

For NK1r, sections were incubated in a rabbit anti–NK-1r polyclonal antibody ab6 (Abeam, Eugene, OR, 1:3000) and mouse anti-NeuN MAB377 (Millipore, Temecula, CA, 1:500) overnight at room temperature. After the sections were rinsed in PBS, they were incubated for 2 hours at room temperature in a goat-anti-rabbit secondary antibody conjugated with Alexa 488 (Invitrogen, Carlsbad, CA, 1:500) to identify NK-1r and a goat-anti-mouse secondary antibody conjugated with Alexa 555 (Invitrogen, Carlsbad, CA, 1:500) to identify NeuN. All sections were finally rinsed and mounted on glass slides and coversliped with ProLong mounting medium (Invitrogen, Carlsbad, CA).

Immunohistochemistry of c-Fos was performed using the avidin-biotin complex (ABC) method. In short, free-floating spinal cord sections were treated with 3% hydrogen peroxide (Sigma, St. Louis, MO) for 10 min and then incubated in primary antibody solution containing 0.5% Triton X-100, 10% goat serum in phosphate buffered saline, and anti-c-Fos (sc-52, Santa Cruz Biotechnology, Santa Cruz, CA, 1:10,000) overnight at room temperature. After sections were rinsed in PBS, incubated with a biotinylated goat-anti-rabbit secondary antibody (BA-1000, Vector laboratories, Burlingame, CA,1:500) for 120 min. Sections were incubated in ABC solution for 1 hr (PK-6100, Vector laboratories, Burlingame, CA) and subsequently DAB substrate solution (SK-4100, Vector laboratories, Burlingame, CA) for an appropriate amount of time. Following mounting and dehydration, slides were coversliped with DPX (Electron Microscopy Science, Hatfield, PA).

2.6 Quantification of NK1r internalization and c-Fos expression

The amount of NK1r internalization was quantified using a standard method (Abbadie et al., 1997a; Kondo et al., 2005; Mantyh et al., 1995). NK1r-positive neurons in lamina l/ll on both sides of the dorsal horn were counted using an Olympus BX-51 fluorescence microscope (Olympus Optical, Tokyo, Japan) at × 60 magnification. Neurons with 10 or more endosomes in their soma and the conterminous proximal dendrites were deemed as having internalized NK1r. The person counting the neurons was blinded to the experimental treatment. In each segment, three to four randomly selected sections were counted. The sum of the number of NK1r-positive neurons with and without NK1r internalization taken from a segment were used to calculate the percentage of NK1r internalization to represent the particular spinal segment for a given animal. Four animals per each treatment group were used for statistical analysis. The ratio of cells showing NK-1r internalization versus all NK1r-positive cells was reported.

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We quantified the c-Fos-immunoreactive (IR) neurons under the Olympus BX-51 microscope at ×10 magnification in the ipsilateral and contralateral spinal dorsal horn. The number of c-Fos-IR neurons in lamina I/II and lamina III/V was determined by a blinded observer. Means counted from six to nine randomly selected sections at L3-L6 level spinal cord were employed for each animal. At least one spinal section was selected for each spinal segment. Five animals per treatment group (for the ω-agatoxin IVA study, two to five animals) were used for statistical analysis. Light microscopic images were taken using MagnaFire SP (Optronics, Goleta, CA) and processed by Photoshop CS5 (Adobe, San Jose, CA).

2.7 Behavioral and motor effects of intrathecal VSCC blockers

Behavioral and motor functions were carefully observed (Malmberg and Yaksh, 1994) after the intrathecal administration of VSCCs blockers. Behavior tests include irritability after stimuli by gently touching the flank and back with a plastic tubing (touch-evoked agitation and touch-evoked vocalization), reflective muscle jerk in any of the extremities evoked by click sound from behind (startle response) and toe pinching (withdrawal response). Motor function was assessed by: i) the placing /stepping reflex (upward lifting of the paw caused by drawing the dorsum of the hindpaw across the edge of the table) and ii) the righting reflex (placing the rat on its back, which induces a quick coordinated twisting. Catalepsy was assessed by placing the forelimb on a horizontal bar kept at 4 cm from a table surface. Failure to move from the bar within 30 sec was judged as a positive. Rats without any behavioral or motor dysfunction were used in the formalin test.

2.8 Antagonists

SNX-482 and ω-agatoxin IVA were purchased from Peptide institute (Osaka, Japan). Both drugs were dissolved in saline and administered in a volume of 10 μL followed by a 10-μL saline flush. Nomenclature for drugs and receptors conforms with the guide to receptors and channels of the British Journal of Pharmacology (Alexander et al., 2008).

2.9 Statistical Analysis

Statistical analysis was performed by Prism GraphPad 5 (GraphPad, La Jolla, CA). Changes in formalin-induced paw-flinching behavior, NK-1r internalization and c-Fos expression were analyzed by t test or one-way ANOVA. Data in table 2 was analyzed with two-way ANOVA with repeated measures. In t test, P value was expressed using the two-tailed test. In all analyses, probability to detect the difference was set at the 5% level ($P < 0.05$).

3. Results

3.1 Behavior and motor effects of intrathecal SNX-482

IT administration of R-type VSCCs blocker SNX-482 (0.5 and 4.6 μg) in rats induced various adverse effects such as agitation, spontaneous and touch-evoked vocalization and motor deficits (Table 1). At the low dose (0.5 μg) these effects were minor and the incidence was low. The following behaviors were noticed within 2 hours after the IT drug injection: vocalization evoked by gently touching the flank and back with a plastic tubing (2/14), facilitated startle response (2/14), absence of righting reflex (1/14), pinna reflex (1/14) and cornel reflex (1/14). The adverse effects became more severe and occurred at a higher incidence when a high dose (4.6 μg) of IT SNX-482 was injected. Behaviors observed included agitation (2/3), loss of pinna/cornel reflex (1/3), deficits in the placing /stepping test (2/3), trunk rigidity (1/3), intense whole-body shaking (1/3) and tail biting (1/3). Thus we concluded the highest tolerable dose of IT SNX-482 was 0.5 μg for the current study.

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3.2 Effects of intrathecal SNX-482 on formalin-induced paw flinching behavior

Intraplantar injection of formalin (2.5%, 50 μl) in rats produced a characteristic biphasic paw flinching response over a 60 min period (Figure 1). Pretreatment with IT SNX-482 (0.5 μg) before formalin significantly inhibited the number of flinches in both phase 1 (saline: 191 ± 20 vs. SNX-482: 131 ± 16, $P < 0.05$) and phase 2 (saline: 1362 ± 159 vs. SNX-482: 902 ± 61, $P < 0.05$).

3.3 Effects of intrathecal SNX-482 on formalin-induced NK1r internalization

NK1r is readily detected in the superficial dorsal horn neurons (Figure 2A and B). In naïve spinal cord, NK1r immunoreactivity is concentrated on the cell membrane and the incidence of intracellular NK1r is low. The number of NK1r-positive neurons per spinal cord section (showing internalization or not) was shown in table 2. As indicated, on average there were 8.2 ±1.1 and 6.7±1.1 NK1r-positive cells per section in the lumber spinal cord. Two-way ANOVA with repeated measures revealed no difference between the ipsilateral and contralateral sides. Hindpaw intraplantar injection of formalin resulted in a robust NK1r internalization in the ipsilateral laminae I/II at the L3-L6 level (L3/4: 56 ± 4%, L4/5: 63 ± 6% and L5/6: 69 ± 2%, figure 2B and C), but not on the contralateral side (L3/4: 10 ± 4%, L4/5: 10 ± 2% and L5/6: 11 ± 2, figure 2A and C). This formalin-induced NK1r internalization on the ipsilateral side was greatly inhibited by an IT pretreatment of SNX-482 (0.5 μg) (SNX-482, L3/4: 26 ± 2%; $P < 0.001$, L4/5: 27 ± 2%; $P < 0.01$ and L5/6: 30 ± 2%; $P < 0.001$, compared with IT saline ipsilateral, respectively). On the contralateral side, there was no significant difference in percentage of NK1r internalization between SNX-482 (0.5 μg) and saline groups (SNX-482, L3/4: 11 ± 2%; L4/5: 7 ± 3% and L5/6: 12 ± 3% vs. saline: L3/4: 10 ± 4%, L4/5: 10 ± 2% and L5/6: 11 ± 2, figure 2C). In addition, no change was detected in the number of NK1r-positive neurons per section after SNX-482 treatment compared to saline controls.

3.4 Effects of intrathecal SNX-482 on formalin-induced c-Fos expression

c-Fos is quickly induced in the dorsal horn neurons after peripheral noxious stimuli and often used as a marker for neuronal activation. Two hours following intraplantar formalin, we observed in the ipsilateral lumbar dorsal horn a significant increase in the number of neurons expressing c-Fos, compared to the contralateral side (Figure 3A and B) or naïve tissue (not shown). Pretreatment with IT SNX-482 (0.5 μg) significantly inhibited formalin-induced c-Fos expression in laminae l/II (vehicle: 63 ± 8 vs. SNX-482: 30 ± 2, $P < 0.01$). There was a trend of c-Fos reduction in lamina III/V although the change did not reach statistical significance (vehicle: 45 ± 7 vs. SNX-482: 29 ± 6, $P > 0.05$, figure 3D). In the contralateral dorsal horn, there was no significant difference in c-Fos expression between saline and SNX-482 (0.5 μg) groups in laminae l/ll (saline: 11 ± 3 vs. SNX-482: 12 ± 1, $P > 0.05$) and laminae III/V (saline: 16 ± 4 vs. SNX-482: 14 ± 1, $P > 0.05$).

3.5 Effects of intrathecal ω-agatoxin IVA on behavior, formalin-induced paw flinching and c-Fos expression

Next we studied the effects of P/Q type VSCC antagonist ω-agatoxin IVA after intrathecal administration. At low doses (0.03 and 0.125 μg), ω-agatoxin IVA did not induce any abnormal behavioral or motor problem during the 2 hours observation period (Table 1). In contrast, 0.5 μg of ω-agatoxin IVA caused behavioral changes such as agitation (2/5), startle response (1/5), trunk rigidity (1/5) and motor dysfunction in the placing/stepping response (2/5) and righting reflex (2/5). Only one rat received the highest dose (1.0 μg). We observed significant side effects, including intense whole-body shaking, motor coordination problems, self-biting, circling behavior and serpentine-like movements of its tail. We concluded the highest tolerable dose of IT ω-agatoxin IVA for this study was 0.5 μg.

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The effects of IT ω-agatoxin IVA on formalin-induced paw flinching were shown in Figure 4. Essentially ω-agatoxin IVA had no effects on the number of flinches at any of the doses (0.03, 0.125 and 0.5 μg) tested.

Consistent with the flinching behavior results, ω-agatoxin IVA did not change the number of c-Fos expressing neurons in the lumbar spinal cord dorsal horn after formalin (Figure 5). We note that these studies shown in Figure 3D were not run concurrently with the studies in Figure 5 and for that reason separate vechile controls were performed for the respective comparsions. The reason for differences in baseline counts is not known.

4. Discussion

Voltage sensitive calcium channels have received much attention in pain research. A well-defined role for VSCCs, specifically N-type VSCCs, is to mediate neurotransmitter release from primary sensory neurons in response to noxious stimuli (Evans et al., 1996; Maggi et al., 1990; Santicioli et al., 1992). Spinal blockade of N-type VSCCs results in potent analgesia in preclinical models (Chaplan et al., 1994; Lewis et al., 2000), which led to the development of ziconotide (Prialt®), an FDA approved drug to treat severe chronic pain. However, N-type VSCCs is widely distributed in the nervous system and its antagonism often causes adverse side effects, which directly limits the therapeutic window for ziconotide. Although other subtypes of VSCCs are detected in the primary sensory neurons, their involvement in presynaptic neurotransmitter release is less characterized. In this study, we used SNX-482 and ω-agatoxin IVA, selective antagonists for R- and P/Q-type VSCCs respectively, to investigate their contribution to afferent transmitter release and tissue injury-induced pain. We report here for the first time that spinal SNX-482 attenuated formalin-induced NK1r internalization, an indicator for substance P release. This effect was observed in parallel with an inhibition of formalin-evoked flinching behavior and c-Fos expression in the lumbar spinal dorsal horn. ω-agatoxin IVA, on the other hand, showed no effects in the above experiments.

4.1 Dorsal horn NK1 receptor internalization

Small unmyelinated fibers are a principal route for the input initiated by high intensity stimuli and local chemical irritants. A subgroup of the small fibers or peptidergic fibers, release glutamate and small peptides such as calcitonin gene-related peptide (CGRP) and SP in response to high intensity or irritant stimuli. In the spinal cord dorsal horn, SP mainly signals through NK1 receptors, which are heavily expressed on projection neurons (Todd et al., 2002). NK1r belongs to the G protein-coupled receptor (GPCR) superfamily. Agonist (i.e. SP) binding to NK1r may lead to activation of protein kinase A, protein kinase C, extracellular signal-regulated kinase and/or non-selective cation channels (Barber and Vasko, 1996; Fehrenbacher et al., 2003; Ito et al., 2002) and render the neurons more excitable. Events blocking spinal SP release usually correlate with reduced afferent/dorsal horn excitability and attenuated pain behaviors (Yaksh et al., 1980). Selective ablation of spinal NK1r-positive neurons by intrathecal SP-saporin blocked hyperpathia following tissue or nerve injury (Mantyh et al., 1997; Nichols et al., 1999; Suzuki et al., 2002). These data emphasize a critical role for the SP-NK1r pathway nociceptive processing. SP release therefore represents a well-defined model system for the study of factors governing the excitability of a defined population of high threshold afferents.

Typical for a GPCR, ligand (i.e. SP) binding to NK1r induces rapid endosomal internalization of the receptor, which can be reliably visualized and quantified by immunohistochemistry. Several lines of evidence support the generally accepted assertion that the extent of NK1r internalization reflects extracellular SP deriving from primary afferents in dorsal horn (Kondo et al., 2005; Marvizon et al., 2003). 1) Capsaicin

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pretreatment, which depletes SP in TRPV1-positive primary afferents, prevented NK1r internalization evoked by noxious stimuli (Kondo et al., 2005). 2) Intrathecal opiates, known to reduce SP release from primary afferents via presynaptic mechanisms, attenuated NK1r internalization following noxious stimuli (Marvizon et al., 2003; Yaksh et al., 1980). 3) There was a positive relationship ex vivo between the topical concentration of SP and NK1r internalization in spinal dorsal horn (Marvizon et al., 2003). From these examinations, we regard spinal NK1r internalization as a suitable marker for the measurement of SP release from primary afferent terminals. Further, this in situ method allows us to map the site of release (e.g. lamina I/II vs. lamina V; L4 vs. L6) and establish the anatomy of pain transmission pathway at the spinal level.

4.2 R-type calcium channels and release

Cav2.3, the major subunit of the R-type VSCCs, is readily detectable in small-, medium- and large-diameter primary sensory neurons in adult rodent DRG (Murakami et al., 2001; Westenbroek et al., 1998; Yusaf et al., 2001). Although six splice variants of Cav2.3 (Cav2.3a to Cav2.3f) have been identified in mammalian tissues, only Cav2.3a and Cav2.3e are detected in trigeminal and DRG neurons, with Cav2.3e being the predominant isoform (Fang et al., 2007; Fang et al., 2010). Single-cell RT-PCR revealed that the majority of Cav2.3e mRNA was confined to tyrosine kinase receptor A (tkrA) positive/isolectin B4 (IB4)-negative and TRPV1-positive neurons (Fang et al., 2007; Fang et al., 2010). These neurons are known to be peptidergic nociceptors. The same laboratory further demonstrated that Cav2.3e mRNA expression was associated with SNX-482-sensitive R-type calcium currents in trigeminal neurons (Fang et al., 2007). Although a direct link between Cav2.3e and neurotransmitter release from peptidergic nociceptors has not been established, the previous evidence strongly supported such a possibility. In this study, we use intraplantar formalin to elicit spinal release of SP in the spinal cord, which leads to significant NK1r internalization in dorsal horn neurons. Our results clearly showed that SNX482, the selective R-type VSCC blocker, strongly attenuated NK1r internalization. The data suggested there is a high probability that Cav2.3e, as does Cav 2.2 in previous work, plays a major role in noxious stimuli-induced presynaptic neurotransmitter release from peptidergic primary afferents.

4.3 Formalin evoked flinching

Formalin injected subcutaneously into the paw results in an immediate and intense increase in the primary afferents activity and paw flinching behavior. The flinching displays a biphasic time course. Phase 1 (0 – 10min) is considered to reflect the direct effect of algogen on nociceptors and phase 2 (11 – 60 min) is a reflection of a spinal sensitization (Dubuisson and Dennis, 1978; Puig and Sorkin, 1996; Tjolsen et al., 1992). In the current study, IT SNX-482 produced a significant inhibitory effect upon both phase 1 and phase 2 flinching, at a dose that produced minimal motor and behavior side effects. The result is in agreement with previous mouse studies that Cav2.3 knockout (Saegusa et al., 2000) or intrathecal SNX-482 (Murakami et al., 2004) attenuated formalin-induced pain behavior in phase 2. Further, spinal SNX-482 attenuated thermal and mechanical pain hypersensitivity in a neuropathic pain model (Matthews et al., 2007). However, the role of Cav2.3 in pain could be complicated. In the same paper, Murakami et al. also demonstrated that SNX-482 increased phase 1 flinching (Murakami et al., 2004). The hypersensitivity was only observed at high doses and thought to reflect the attenuation of the inhibitory effects of antinociceptive pathways. In certain neurons, Cav2.3 could act to attenuate neuronal excitability by inhibiting activation of N-methyl-D-aspartate (NMDA) receptors (Bloodgood and Sabatini, 2007). Cav2.3 might also reduce the induction of long-term potentiation by producing trains of back propagating action potentials (Yasuda et al., 2003). More studies are required to elucidate the role of Cav2.3 in pain.

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4.4 Formalin-evoked dorsal horn c-Fos

The proto-oncogene c-Fos is rapidly induced in spinal dorsal horn neurons following noxious stimuli, such as formalin injection to the paw, and commonly used as a maker for neural activation (Hunt et al., 1987). There is a positive correlation between c-Fos expression and formalin-evoked pain behavior (Gogas et al., 1996). Two hours after formalin injection, c-Fos was observed throughout the ipsilateral lumbar dorsal horn. This pattern of neuron activation correlated with peripheral input from both C-fiber and A-fiber, the majority of which terminate in the superficial and deep dorsal horn respectively. It is well established that paw formalin activates both groups of afferents (Puig and Sorkin, 1996). In the current study, we noted for the first time that intrathecal SNX-482 reduced formalin-evoked c-Fos in ipsilateral dorsal horn in laminae I/II, but not laminae III/V. Our observation supports a role of Cav2.3 in nociceptors but not in the deeply projecting large afferents A-fibers. This result is also in agreement with an electrophysiology study in which SNX-482 inhibited spinal neuronal responses evoked by monosynaptic C- and Aδ-fiber, but not Aβ-fiber, in neuropathic rat (Matthews et al., 2007).

In the current study, intrathecal SNX-482 at the analgesic dose (0.5 μg) produced some minor motor dysfunction and adverse effects, which occurred at a very low rate and disappeared within 2 hours. However, a higher dose (4.6 μg) produced severe motor dysfunction and other adverse behaviors. These effects could be attributed to Cav2.3 in the spinal motor neurons in the ventral horn (Murakami et al., 2004; Saegusa et al., 2000; Westenbroek et al., 1998) and/or neurons in supraspinal sites. Indeed, Cav2.3 knockout mice exhibited reduced spontaneous locomotor activities and signs of “timidity” (Saegusa et al., 2000a).

4.5 Spinal P/Q-type calcium channels

Although there have been some reports regarding P/Q-type calcium channels, the analgesic effects of the blockers are controversial. Previous studies have shown that intrathecal delivery of the antagonist for P/Q-type VSCCs, ω-agatoxin IVA, produced anti-nociceptive effects on formalin-evoked pain behavior (Malmberg and Yaksh, 1994; Murakami et al., 2004) and burn-induced hyperalgesia. Conversely, several spinal ω-agatoxin IVA studies have failed to note anti-nociceptive effects on high-threshold thermal stimuli (Malmberg and Yaksh, 1994), tactile allodynia (Chaplan et al., 1994) or in a hyperalgesia model (Yamamoto and Sakashita, 1998). Despite an early report by our group (Malmberg and Yaksh, 1994), we could not in the present detect any effects of ω-agatoxin IVA on formalin-evoked flinching behavior or c-Fos expression. Whether this was due to differences in animal strain, method of data collection, difference in the ω-agatoxin IVA used and/or a narrow therapeutic window of ω-agatoxin IVA is not known. Correspondence with the supplier indicated data showing purity and appropriate amino acid content (Dr. Watanabe from Peptide Institute, Inc.). We noticed that doses exceeding 0.5 μg caused severe side effects such as involuntary spasm, sudden burst of scratching, jumping followed by motor weakness and depression of breathing, symptoms also reported in previous studies (Malmberg and Yaksh, 1994; Sorkin et al., 2008). The motor side effects clearly show that the test article had biological activity and are consistent with the distribution of P/Q-type (α1A) calcium channel subunits in the ventral horn motor neurons (Kim et al., 2001; Murakami et al., 2004).

In conclusion, we demonstrate that spinal administration of R-type calcium channel blocker attenuated formalin-induced pain behavior, NK1r internalization and c-Fos expression in the superficial dorsal horn. This study strongly supports a role for Cav2.3 in presynaptic neurotransmitter release from peptidergic nociceptive afferents. Our study does not exclude a role for Cav2.3 in regulating post-synaptic dorsal horn neurons. Some conflicts with previous reports warrant further study for the involvement of VSCCs in pain.

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Abbreviations

CGRP	calcitonin gene-related peptide
GPCR	G protein-coupled receptor
DRG	dorsal root ganglia
IB4	isolectin B4
IT	Intrathecal
NK1r	neurokinin 1 receptor
NMDA	N-methyl-D-aspartate
SP	substance P
trkA	tropomyosin receptor kinase A
TRPV1	transient receptor potential protein vanilloid 1
IR	immunoreactive
VSCCs	voltage-sensitive calcium channels

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Highlights

1. IT SNX-482 (R-type antagonist) blocked formalin-evoked flinches in phase 1 and 2.
2. IT SNX-482 blocked formalin-induced spinal dorsal horn NK1 receptor internalization.
3. IT SNX-482 blocked formalin-induced spinal dorsal horn c-Fos expression.

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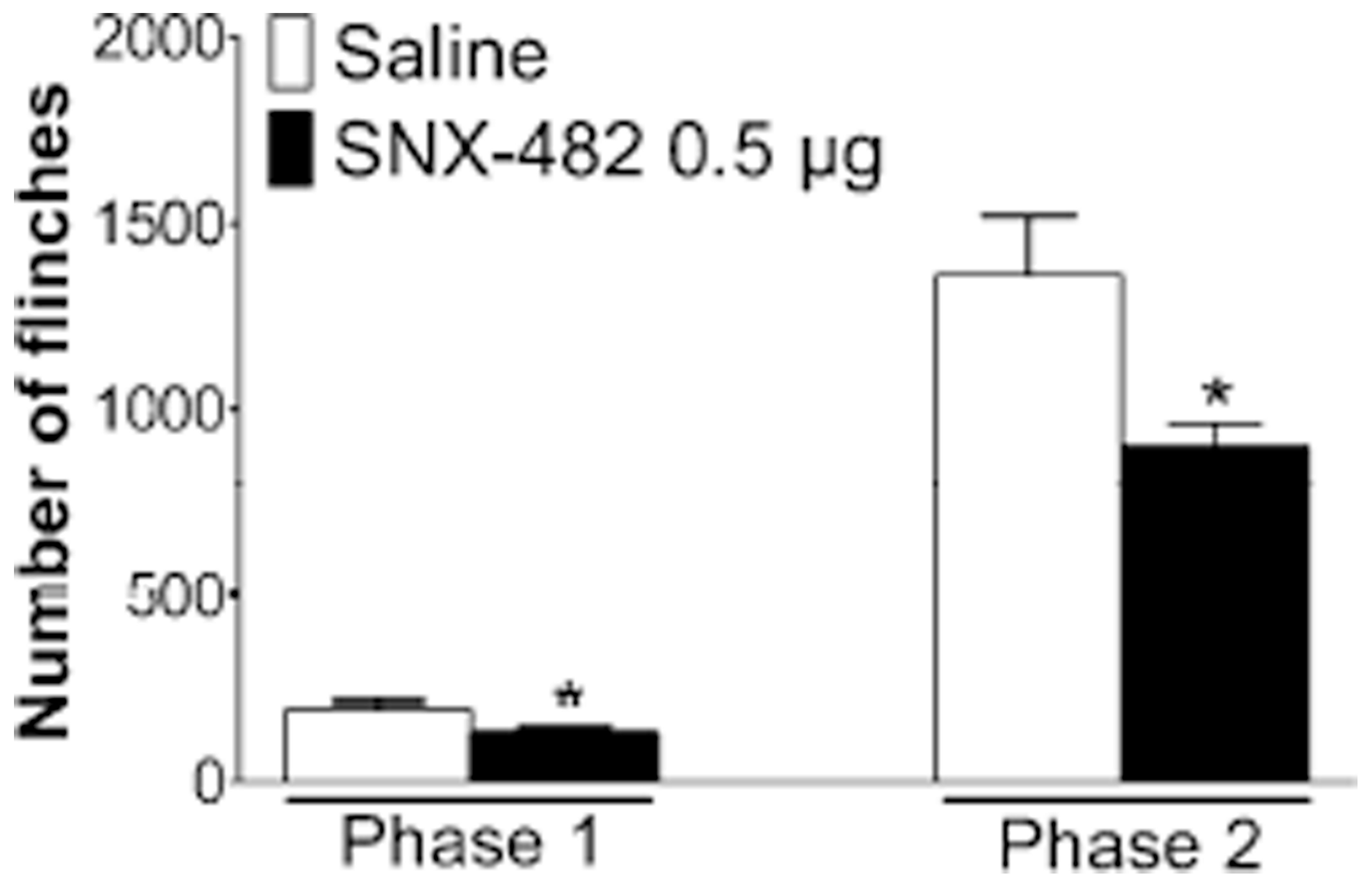

Figure 1.

Effects of intrathecal (IT) SNX482 on formalin-induced paw flinching behavior. Rats received IT injection of SNX-482 (0.5 μg) or saline 15 min before intraplantar formalin (2.5%, 50 μL) injection. The evoked flinching behavior was recorded for 60 min by an automated device. The total number of flinches in phase 1 (0–10 min) and phase 2 (11–60 min) were shown in the histogram. Data are expressed as Mean ± SEM. *$P < 0.05$ compared with saline by t-test. N = 5 rats per group.

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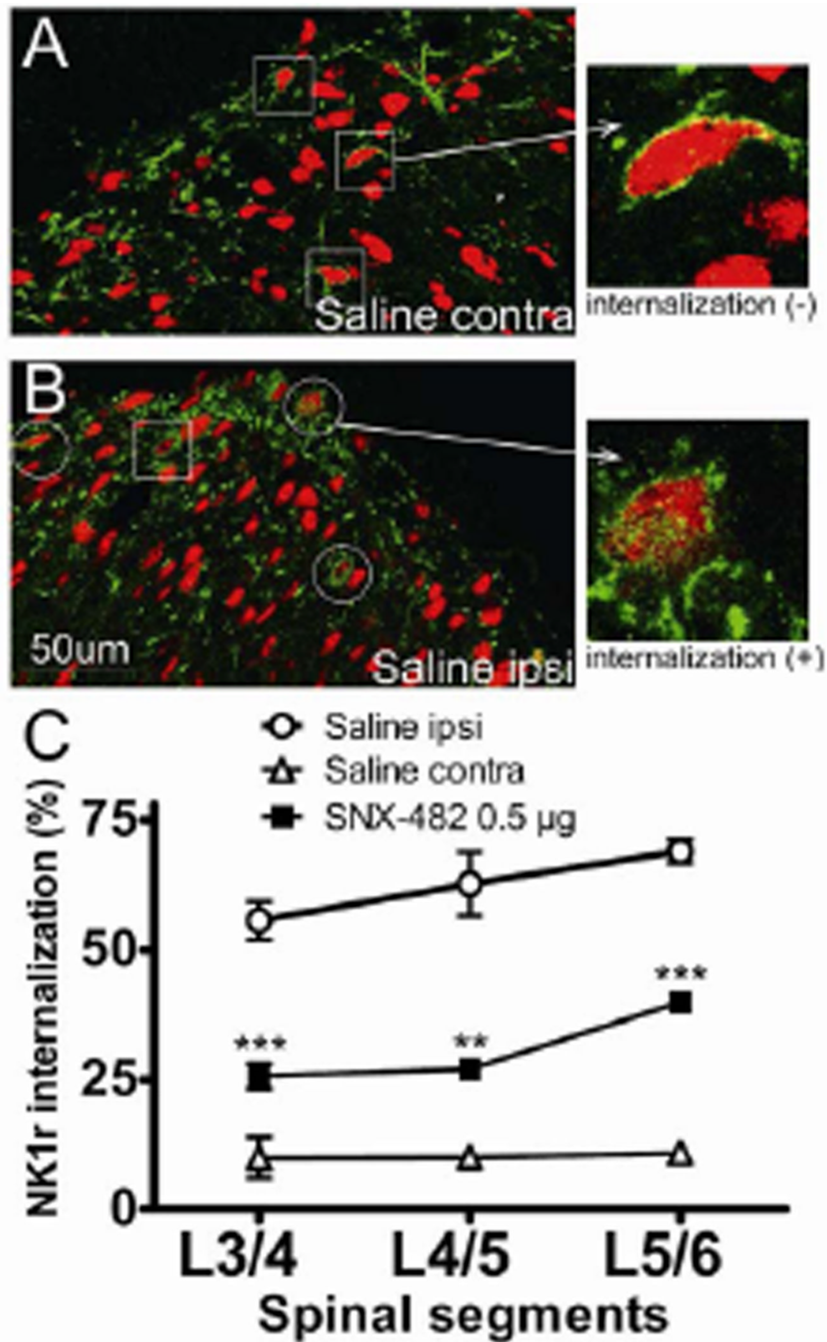

Figure 2.

Effects of intrathecal (IT) SNX-482 on formalin-induced neurokinin 1 receptor (NK1r) internalization. Rats received IT SNX-482 or saline 15 min before intraplantar formalin (2.5%, 50 μL) injection. (A–B) Representative confocal images of formalin-induced NK1r internalization in L5 superficial dorsal horn (green: NK1r; red: NeuN) in an animal received intrathecal saline pretreatment. Squares indicate NK1r immunoreactive neurons without NK1r internalization. Circles indicate neurons showing NK1r internalization. (A, right) NK1r immunoreactivity in the contralateral spinal lamina l/lI. Note the presence of a homogeneous cell membrane and the lack of NK1r-containing endosomes internalizing into the cytoplasm. (B, right) NK1r immunoreactivity in the ipsilateral spinal lamina l/ll. Note

the lack of a homogeneous cell membrane and the presence of NK1r-containing endosomes internalizing into the cytoplasm. (C) Line graph depicts percentage of NK1r internalization at each spinal segment. Formalin induced a robust NK1r internalization in the ipsilateral L3-L6 spinal cord dorsal horn, which was significantly blocked by IT SNX-482 pretreatment. Data are expressed as Mean ± SEM. **$P < 0.01$ and ***$P < 0.001$ compared with saline by one-way ANOVA. N = 4 rats per group. Scale bar is 50 μm. Ipsi, ipsilateral; contra, contralateral.

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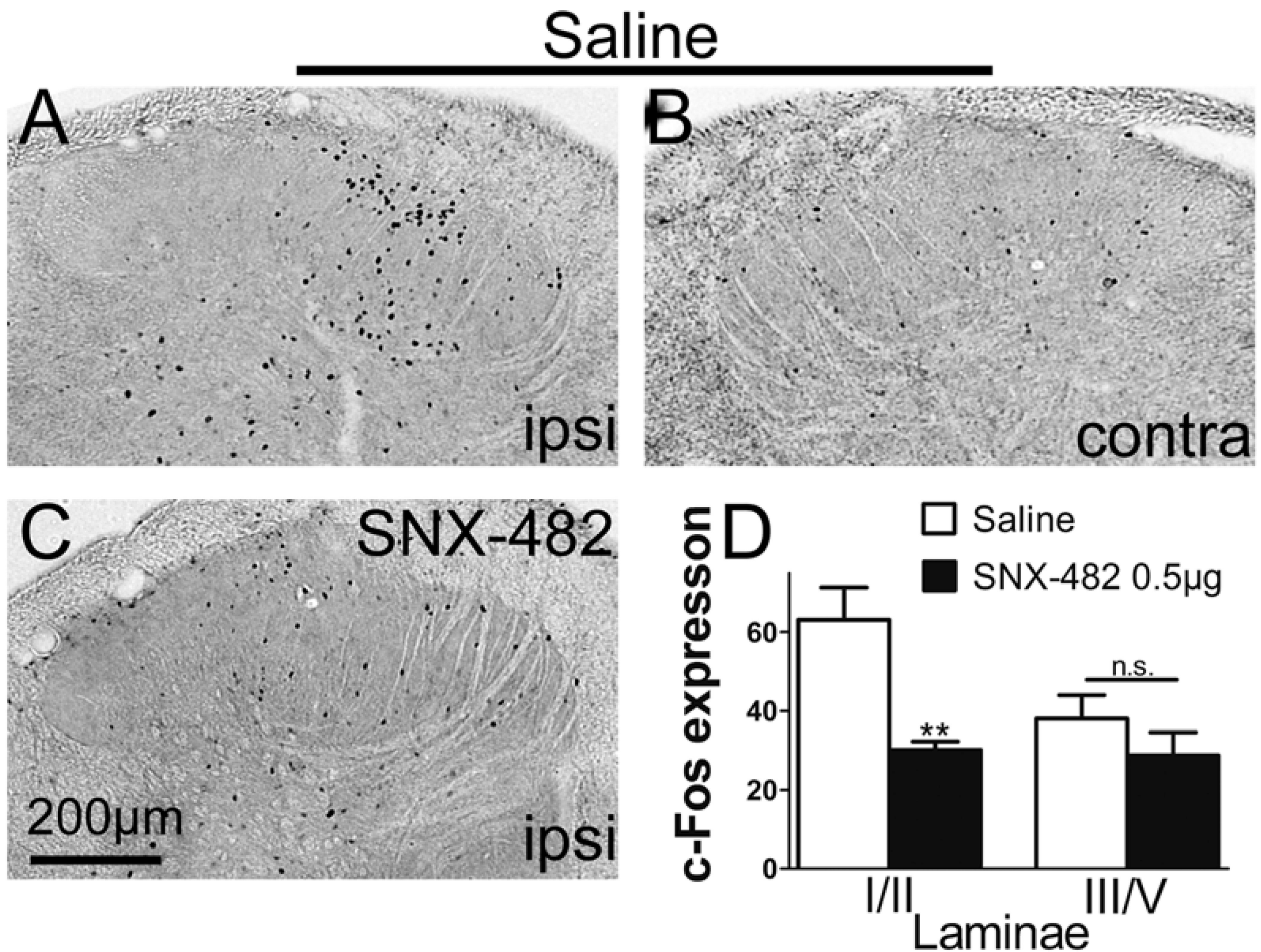

Figure 3.

Effects of intrathecal (IT) SNX-482 on formalin-induced c-Fos expression. (A–C) Representative light microscopic images of c-Fos expression in the lumbar spinal cord dorsal horn 2 h following intraplantar formalin (2.5%, 50 μL) injection. Rats were pretreated with IT (A and B) saline or (C) SNX-482 15 min before intraplantar formalin. (D) (D) Histogram represents mean counts of c-Fos positive neurons in lamina l/ll and lamina III/V per spinal section in the ipsilateral dorsal horn. Data from randomly selected slices at L3-L6 spinal segment were employed (Eight to nine slices per rat). Scale bar is 200 μm. Data are expressed as Mean ± SEM. $**P < 0.01$ compared with saline by t-test. N = 5 rats per group. Scale bar is 50 μm. Ipsi, ipsilateral; contra, contralateral.

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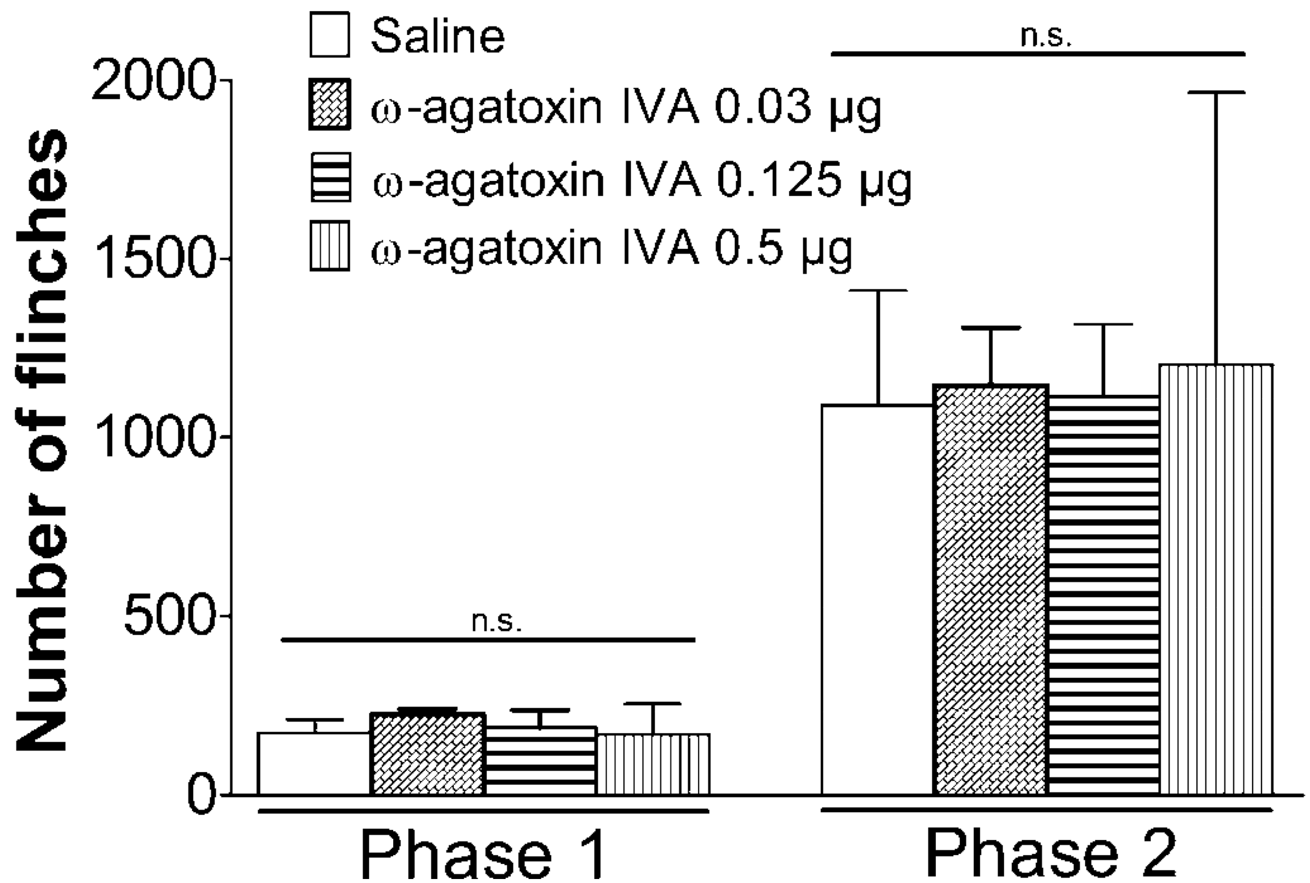

Figure 4.

Effects of intrathecal (IT) ω-agatoxin IVA on formalin-induced paw flinching behavior. Rats received IT injection of ω-agatoxin IVA (0.03, 0.125 or 0.5 μg) or saline 15 min before intraplantar formalin (2.5%, 50 μL) injection. The evoked flinching behavior was recorded for 60 min by an automated device. The total number of flinches in phase 1 (0–10 min) and phase 2 (11–60 min) were shown in the histogram. Data are expressed as Mean ± SEM. N = 2–5 rats per group.

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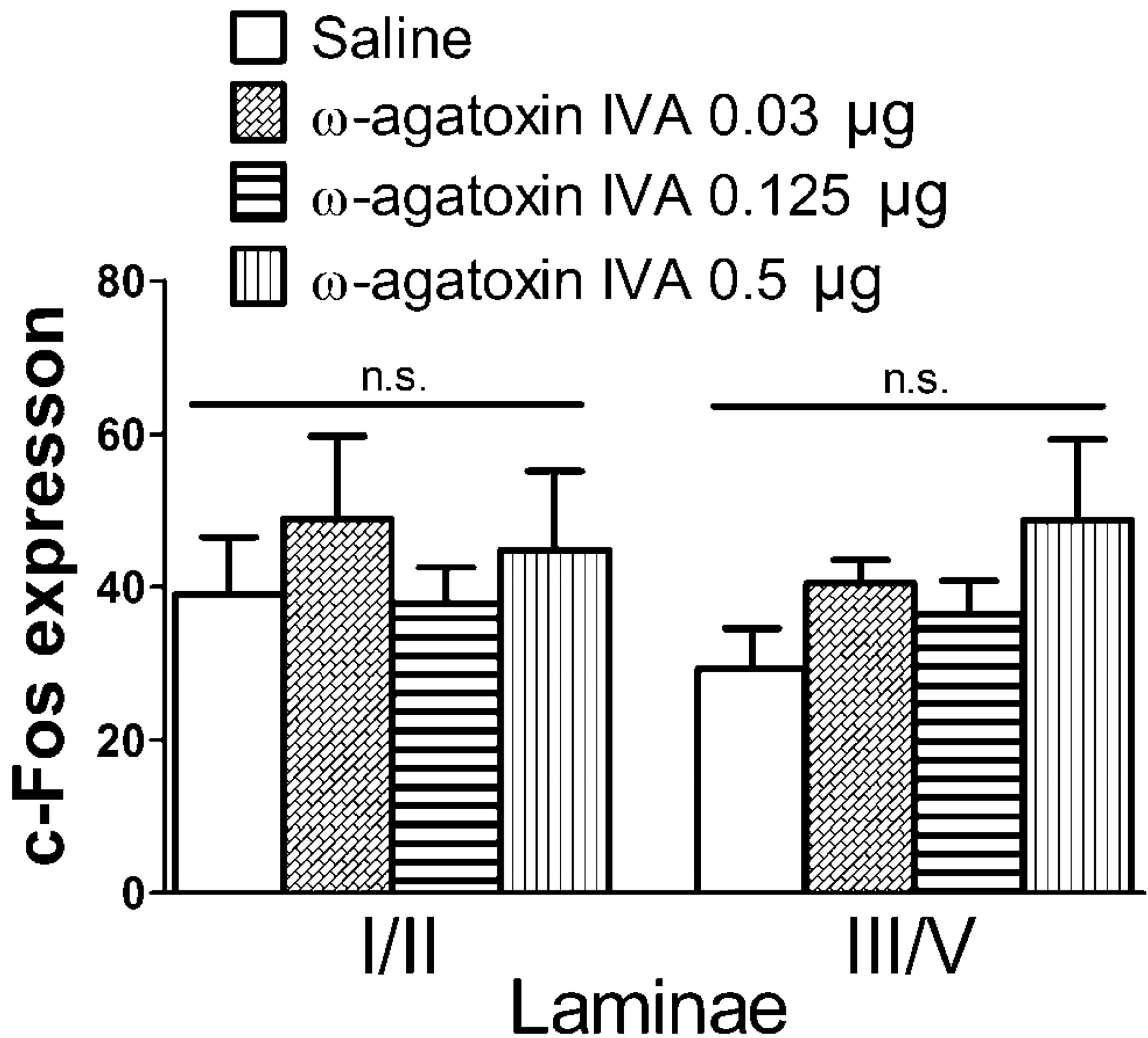

Figure 5.

Effects of intrathecal (IT) ω-agatoxin IVA on formalin-induced c-Fos expression. Histogram represents the number of c-Fos positive neurons in lamina I/II and lamina III/V of ipsilateral dorsal horn. Data from L3-L6 spinal segments were pooled. There was no significant difference between treatment groups. Data are expressed as Mean ± SEM. N = 2–5 rats per group.

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Table 1

Behavioral and Motor Effects of Intrathecal Voltage-Sensitive Calcium Channel Blockers

SNX-482 (0.5 and 4.6μg) and ω-agatoxin IVA (0.03, 0.125, 0.5 and 1.0μg) were administered intrathecally. Behavior and motor functions (see Material and Methods) were monitored for 2 hours. Percentage of rats with the indicated behavior was shown in the table.

Drug	Dose (μg)	n	Agitation/ Vocalization	Minor motor dysfunction	Severe motor dysfunction
SNX-482	0.5	14	14%	7%	0%
	4.6	3	67%	0%	67%
ω-agatoxin IVA	0.03	2	0%	0%	0%
	0.125	6	0%	0%	0%
	0.5	5	40%	20%	20%
	1	1	100%	0%	100%

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Table 2

Number of NK1r-positive neurons per section in the superficial dorsal horn of lumbar segments (L3-L6) of rats after unilateral intraplantar formalin injection

For each rat, 3–4 sections from lumbar segment were counted and the mean was taken as representative of that segment. The mean ± SEM of these individual counts for the 4 rats is presented in the table. Two-way ANOVA with repeated measures revealed no significant differences across ipsilateral and contralateral side ($p = 0.13$) and between drug treatments ($p = 0.43$).

Treatment	Number of rats	L3/4		L/4/5		L5/6		Overall mean ± SEM	
		Ipsi	Contra	Ipsi	Contra	Ipsi	Contra	Ipsi	Contra
IT saline	4	8.8 ± 1.6	5.7 ± 1.2	9.2 ± 1.1	7.6 ± 1.5	7.0 ± 0.7	6.8 ± 0.9	8.2 ± 1.1	6.7 ± 1.1
IT SNX-482	4	9.7 ± 1.3	8.0 ± 1.1	9.6 ± 2.0	6.5 ± 1.0	9.3 ± 0.4	7.5 ± 1.7	9.5 ± 1.2	7.3 ± 1.2