Regulation of Spinal Substance P Release by Intrathecal Calcium Channel Blockade

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ABSTRACT

Background: The authors investigated the role of different voltage-sensitive calcium channels expressed at presynaptic afferent terminals in substance P release and on nociceptive behavior evoked by intraplantar formalin by examining the effects of intrathecally delivered N- (ziconotide), T- (mibefradil), and L-type voltage-sensitive calcium channel blockers (diltiazem and verapamil).

Methods: Rats received intrathecal pretreatment with saline or doses of morphine, ziconotide, mibefradil, diltiazem, or verapamil. The effect of these injections upon flinching evoked by intraplantar formalin (5%, 50 μl) was quantified. To assess substance P release, the incidence of neurokinin-1 receptor internalization in the ipsilateral and contralateral lamina I was determined in immunofluorescent-stained tissues.

Results: Intrathecal morphine (20 μg), ziconotide (0.3, 0.6, and 1 μg), mibefradil (100 μg, but not 50 μg), diltiazem (500 μg, but not 300 μg), and verapamil (200 μg, but not 50 and 100 μg) reduced paw flinching in phase 2 compared with vehicle control ($P < 0.05$), with no effect on phase 1. Ziconotide (0.3, 0.6, and 1 μg) and morphine (20 μg) significantly inhibited neurokinin-1 receptor internalization ($P < 0.05$), but mibefradil, diltiazem, and verapamil at the highest doses had no effect.

Conclusion: These results emphasize the role *in vivo* of N-type but not T- and L-type voltage-sensitive calcium channel blockers in mediating the stimulus-evoked substance P release from small primary afferents and suggest that T- and L-type voltage-sensitive calcium channel blockers exert antihyperalgesic effects by an action on other populations of afferents or mechanisms involving postsynaptic excitability.

What We Already Know about This Topic

- Ziconotide, an approved intrathecal drug for treating neuropathic pain, inhibits N-type voltage-gated calcium channels as its presumed mechanism of action

What This Article Tells Us That Is New

- In rats, intrathecal ziconotide blocked neurokinin-1 receptor (NK-1r) internalization, a measure of substance P release from small primary afferents
- Surprisingly, other spinal voltage-gated calcium channel blockers produced antinociception but did not reduce NK-1r internalization

SMALL primary afferents are activated by a variety of high-intensity thermal, mechanical, and chemical stimuli. A subpopulation of these high threshold afferents contain and release excitatory amino acids and a variety of peptides. One population of such high threshold afferents, notably those that contain and release the peptide transmitter substance P[1], project largely into the superficial dorsal horn, where they make synaptic contact with projection neurons that densely express neurokinin-1 receptors (NK-1r).[2] Importantly, specific destruction of these NK-1r(+) cells with substance P-saporin attenuated hyperpathic states initiated with tissue and nerve injury, emphasizing the functional relevance of these NK-1r(+) cells to nociceptive processing.[3–5]

The release of substance P from these spinal terminals onto the NK-1r(+) neurons is initiated by an increase in intracellular calcium secondary to the opening of voltage-sensitive calcium channels (VSCCs) located on the central terminals, from which this substance P release originates. VSCCs are classified into high-voltage–activated and low-voltage–activated channels. High-voltage–activated channels are further classified into L- (Cav1.1–1.4), P/Q- (Cav2.1), N- (Cav2.2), and R- (Cav2.3) types based on their activation kinetics, pharmacologic sensitivities, and α_1-subunit sequences. A low-voltage–activated channel includes T-type VSCCs Cav (Ca3.1–3.3), which are activated in response to a small membrane depolarization.[6–10]

An important question is which, if any, of these channels plays a role in mediating the release of transmitters from the small peptidergic afferents. With regard to their locations on

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primary afferents, L-type channels have been reported in myelinated and unmyelinated sensory axons.[11,12] In the dorsal horn, the L-type channel protein predominantly locates with neuronal soma and dendrites.[13] N-type VSCCs predominate in lamina I, largely located presynaptically on terminals and dendrites.[13] Many substance P(+) nerve terminals also show colocalization with N-type VSCCs.[13] Binding studies with ω-conotoxins indicate that the associated N-type channel is concentrated in laminae I and II on the superficial dorsal horn, where small high-threshold afferents terminate.[14,15] T-type channel (Cav3.2 and Cav3.3) messenger RNA is present in dorsal root ganglion neurons. Although some report transcripts to be only in small- and medium-size neurons,[16] others find Cav3.3 to be equally present in large dorsal root ganglion neurons.[17] All members of the T-type VSCC family are prominently expressed in lamina I.[18,19] The role of the respective channels in afferent transmitter release may be assessed by the use of calcium channel antagonists. N-type channels are blocked by agents such as ω-conotoxin GVIA and their homologs, notably the commercially available ziconotide.[20] L-type VSCCs are selectively blocked by 1,4-dihydropyridines (such as nimodipine and nifedipine), phenylalkylamines (such as verapamil), and benzothiazepines (such as diltiazem).[21] T-type channels are blocked by mibefradil.[22]

Despite the apparent presence of many of the VSCC species in afferents, electrophysiologic studies in spinal slice preparations find that the monosynaptic postsynaptic depolarization of the superficial dorsal horn neurons in slices after root activation is diminished by N-type channel block and minimally by T- and L-type channel blocks.[19,23] These observations on localization and electrophysiology raise the possibility that the N-, T-, and L-type channels may contribute to varying degrees to release from peptidergic sensory neurons. Direct studies on peptide release (as a marker of small afferent terminal activity) have reported that N-type VSCC blockers will prevent substance P release from primary afferents in *ex vivo* models.[24–26] In contrast, L-type VSCC blockers were reported to be without effect.[25]

In the current work, we examined the effects of intrathecally delivered N-, T-, and L-type channel blockers to determine the effects on dorsal horn substance P release evoked by intraplantar formalin. To determine changes in extracellular substance P, we examined the internalization of the NK-1r. Previous work has shown that NK-1r internalization is a robust index of extracellular substance P released from primary afferents.[27–29] This methodology, in contrast to other *in vivo* (superfusion, dialysis) or *in vitro* (slice, culture) release approaches, allows us to assess directly the effects of treatment on the release of substance P onto neurons known to be important in the spinal nociceptive pathway. Because it is carried out *in vivo*, we can assess the relationship between drug effects upon release and the corresponding changes in behavior. Thus, the current studies will define the effects of the respective antagonists for N-, T-, and L-type VSCCs given intrathecally on substance P release from small peptidergic primary afferents and the effects of these drugs on pain behavior at corresponding doses.

Materials and Methods

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 7:00 AM). Testing occurred during the light cycle. Food and water were available *ad libitum*. 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.

Intrathecal Catheter Implantation

Rats were implanted with a single intrathecal catheter for drug delivery, as described previously.[30,31] In brief, rats were anesthetized by induction with 4% isoflurane in a room air/oxygen mixture (1:1), and the anesthesia was maintained with 2% isoflurane delivery by mask. The animal was placed in a stereotaxic headholder, and a midline incision was made on the back of the occipital bone to expose the cisternal membrane. The membrane was incised with a stab blade, and a single-lumen polyethylene (OD 0.36 mm) catheter was inserted and passed into the intrathecal space to the level of the L2–L3 spinal segments (8.5 cm). The other end of the catheter was joined to a polyethylene-10 catheter, which was tunneled subcutaneously to exit through the top of the head. The catheters were flushed with 10 μl saline and plugged. Rats were given 5 ml lactated Ringer's solution subcutaneously and allowed to recover under a heat lamp. If any showed motor weakness or signs of paresis on recovery from anesthesia, they were euthanized immediately. Animals were allowed to recover for 5–7 days before the experiment.

VSCC Blockade on Formalin-induced Paw Flinching

To assess formalin-induced flinching, a soft metal band (10 mm wide) weighing ~ 0.5 g was placed around the left hind paw of the animal being tested. Animals were allowed to acclimate in individual acrylic glass chambers for 30 min before experimental manipulation. For the VSCC blockade studies, rats were administered saline, ziconotide (0.3, 0.6, or 1 μg), mibefradil (50 or 100 μg), diltiazem (300 or 500 μg), or verapamil (50, 100, or 200 μg) 10 min before a subcutaneous injection of 50 μl formalin (5%) into the dorsal side of the banded paw. Intrathecal morphine (20 μg) was used as an active control. All drugs were injected intrathecally in a volume of 10 μl, followed by a 10-μl saline flush. Immediately after the formalin injection, rats were placed individually into separate test chambers, and nociceptive

behavior (flinching and shaking of the injected paw) was quantified by an automatic flinch-counting device (UARDG; Department of Anesthesiology, University of California, San Diego, CA).[32] Flinches were counted in 1-min intervals for 60 min. The data were expressed as total number of flinches observed during phase 1 (0–10 min) and phase 2 (11–60 min). Animals were then killed.

VSCC Blockade on Formalin-induced NK-1r Internalization

After recovery from intrathecal catheter implantation, rats received intrathecally saline, ziconotide (0.3, 0.6, or 1 μg), mibefradil (50, 100, or 300 μg), diltiazem (300 or 500 μg), or verapamil (300 μg). Intrathecal morphine (20 μg) was used as an active control. Ten minutes after intrathecal drug administration, rats were anesthetized with 4% isoflurane in a room air/oxygen mixture (1:1) and injected with 50 μl formalin (5%) to the left hind paw. Rats were then transcardially perfused with fixative 10 min after the formalin injection.

Tissue Preparation and Immunocytochemistry

Anesthetized rats were transcardially perfused with NaCl (0.9%) followed by paraformaldehyde (4%) in 0.1 M sodium phosphate buffered saline, pH 7.4. The lumbar spinal cord was removed and postfixed overnight. After cryoprotection in 30% sucrose was performed, coronal sections were made using a sliding microtome (30 μm). Immunofluorescent staining was performed to examine NK-1r expression in the spinal cord dorsal horn. In brief, sections were incubated in a rabbit anti–NK-1r polyclonal antibody overnight at room temperature. The antibody was diluted to a concentration of 1:3,000 in 0.01 M phosphate buffered saline containing 10% normal goat serum and 10% Triton X-100. After the sections were rinsed in phosphate buffered saline, they were incubated for 120 min at room temperature in a goat antirabbit secondary antibody conjugated with Alexa 488 to identify NK-1rs and a goat antimouse secondary antibody conjugated with Alexa 594 to identify NeuN diluted at 1:1,000 in 0.0 1 M phosphate buffered saline containing 10% goat serum and 10% Triton X-100. All sections were finally rinsed and mounted on glass slides and covered with a coverslip with ProLong mounting medium (Invitrogen, Carlsbad, CA).

Quantification of NK-1r Internalization

Neurokinin-1 receptor internalization was counted using an Olympus BX-51 fluorescence microscope (Olympus Optical, Tokyo, Japan) at × 60 magnification and followed the standards outlined in previous reports.[33,34] The total number of NK-1r immunoreactive neurons an lamina I, with or without NK-1r internalization, was counted and taken as a ratio of cells showing NK-1r internalization *versus* all NK-1r(+) cells and then converted into a percentage of NK-1r–immunoreactive cells. Neuronal profiles that had 10 or more endosomes in their soma and the contiguous proximal dendrites were considered to have internalized NK-1rs. NK-1r(+) neurons in both sides of the dorsal horn were counted. The person counting the neurons was blinded to the experimental treatments. Mean counts from two to five sections per segment of the lumbar spinal cord were used as representative counts for a given animal. Three to five animals per drug treatment group were used for statistical analysis (n = 3–5). Light microscopic images were taken using MagnaFire SP (Optronics, Goleta, CA) and processed by Photoshop CS4 (Adobe, San Jose, CA).

Effects of Intrathecal Ziconotide on NK-1r Internalization Induced by Exogenous Substance P

To rule out the possibility that ziconotide directly blocks the NK-1r internalization mechanism, the effect of ziconotide on internalization induced by exogenous substance P (intrathecal injection) was examined. Rats were administered intrathecal saline or ziconotide (0.6 μg) 10 min before intrathecal substance P (30 nmol). Thirty minutes after intrathecal substance P, rats were killed and fixed for examination. The total number of NK-1r immunoreactive neurons in lamina I, with or without NK-1r internalization, was counted.

Behavioral and Motor Effects of Intrathecal VSCC Blockade

Behavioral and motor effects of intrathecal VSCC blockade were examined after the pretreatment according to methods described previously.[21] Behavioral effects were assessed in a quiet environment and after stimuli, such as handling, a hand clap from 25 cm (startle response), and toe pinching (withdrawal response). Motor function was examined by assessing the placing/stepping reflex, where normal behavior is a stepping reflex when the hind paws are drawn across the edge of a table. Righting reflex was assessed by placing the rat horizontally with its back on the table, which normally gives rise to an immediate coordinated twisting of the body to an upright position. Before the examinations, behavioral and motor effects were assessed again. Rats with behavioral and motor dysfunction were removed.

Drug, Antibody, and Materials

Ziconotide, mibefradil, diltiazem, and verapamil were purchased from Sigma Chemical (St. Louis, MO). Morphine sulfate was provided by Merck Pharmaceuticals (Rahway, NJ). Substance P was obtained from Peninsula Laboratory (Belmont, CA). All drugs were dissolved in saline and administered in a volume of 10 μl followed by a 10-μl saline flush. The rabbit anti–NK-1r polyclonal antibody was purchased from Advanced Targeting Systems (San Diego, CA). Secondary Alexa 488 conjugated antibody and Alexa 594 conjugated antibody were purchased from Invitrogen (Eugene, OR). ProLong mounting medium was obtained from Fisher Scientific (Pittsburgh, PA). Nomenclature for drugs and re-

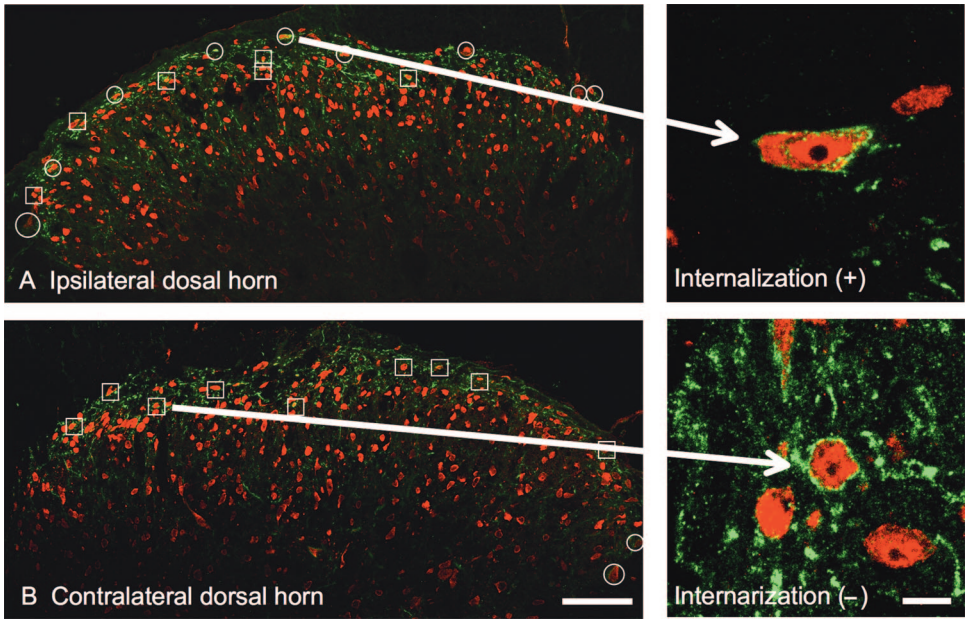

Fig. 1. Representative confocal images of immune stained (*red*: NeuN; *green*: neurokinin-1 receptor (NK-1r) L6 dorsal horns ipsilateral (*A*) and contralateral (*B*) to the paw receiving intraplantar formalin in a rat administered intrathecal saline. *Squares* indicate the NK-1r immunoreactive neurons without NK-1r internalization. *Circles* indicate neurons showing NK-1r internalization. In the ipsilateral dorsal horn, there are 15 NK-1(+) neurons, with nine showing NK-1r internalizations. In the contralateral dorsal horn, there are 11 NK-1r(+) neurons, with 2 showing NK-1r internalizations. *Left,* Confocal images taken at ×20 magnification. Scale bar is 100 μm. *Right,* Confocal images of NK-1r immunoreactive neurons with (*A*) or without (*B*) NK-1r internalization; taken at ×63 magnification. Scale bar is 10 μm.

ceptors conforms with the guide to receptors and channels of the *British Journal of Pharmacology*.[35]

Statistical Analysis

Statistical analysis was performed by Prism 4 (GraphPad, La Jolla, CA). Changes in formalin-induced, paw-flinching behavior were analyzed using *t* test or one-way ANOVA for phases 1 and 2. Upon detection of a significant ANOVA, Tukey *post hoc* tests were performed for pairwise comparisons of drug-treated groups with their phase 1 or 2. The analysis for NK-1r internalization data consisted of *t* test or one-way ANOVA. To detect the differences in the presence of a significant one-way ANOVA, Tukey *post hoc* analysis was conducted. 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 ($P < 0.05$).

Results

Intraplantar Formalin-injection–evoked Dorsal Horn NK-1r Internalization

Neurokinin-1 receptor immunoreactivity was constitutively expressed in superficial dorsal horn neurons (fig. 1, A and B, left). Examination of these sections at ×63 magnification revealed that, in the absence of stimulation, most of these NK-1r(+) cells showed immunoreactivity distributed on the membrane surface (fig. 1B). Unilateral intraplantar injection of 50 μl formalin (5%) produced robust ipsilateral NK-1r internalization, as evidenced by the appearance of NK-1r(+) endosomes (fig. 1A, right). This internalization typically was most evident in NK-1r(+) endosomes in lamina I at the L4–L6 levels of the lumbar spinal cord (fig. 1A). NK-1r internalization was not observed on the contralateral side to the formalin-injected paw (fig. 1B, right).

Effects of Intrathecal Morphine on Formalin-induced, Paw-flinching Behavior and NK-1r Internalization

The effects of intrathecal morphine on formalin-induced, paw-flinching behavior and NK-1r internalization in spinal lamina I are shown in figure 2, A–F. Administration of 20 μg intrathecal morphine significantly reduced the formalin-induced, paw-flinching behavior in phase 2 (saline: 976 ± 56, morphine 20 μg: 47 ± 19, $P < 0.0001$) but not phase 1 (saline: 83 ± 27, morphine 20 μg: 29 ± 13, $P = 0.13$) (fig. 2, A and B). Intraplantar formalin (5%, 50 μl) injection produced robust ipsilateral NK-1r internalization at L5 and L6 compared with the contralateral side (L4: ipsilateral 26 ± 4%, contralateral 10 ± 4%, $P = 0.026$; L5: ipsilateral 58 ± 6%, contralateral 4 ± 2%, $P < 0.0001$; L6: ipsilateral 58 ± 7%, contralateral 11 ± 6%, $P = 0.0011$) (fig. 2, C–E). Administration of 20 μg morphine also significantly reduced the formalin-induced NK-1r internalization at the L5 and L6 levels of ipsilateral spinal cord dorsal horn compared with vehicle control (L4: saline 26 ± 4%, morphine 12 ± 8%, $P = 0.14$; L5: saline 58 ± 6%, morphine 24 ± 2%, $P = 0.0035$; L6: saline 58 ± 7%, morphine 28 ± 3%, $P = 0.025$) (fig. 2, C and F).

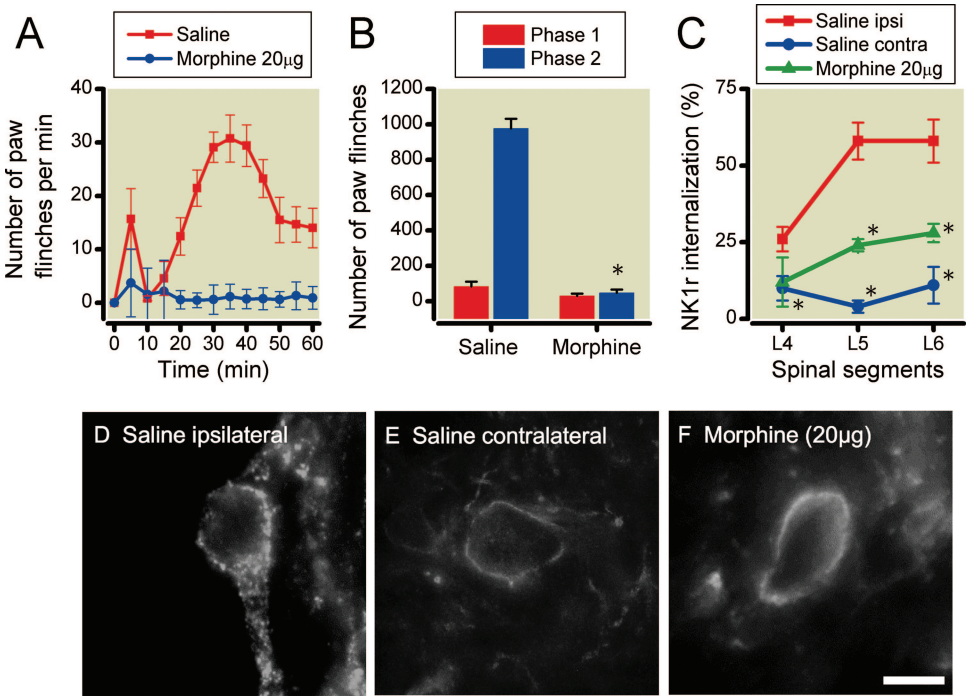

Fig. 2. Effects of intrathecal 20 μg morphine on formalin-induced, paw-flinching behavior and neurokinin-1 receptor (NK-1r) internalization. Time-effect curves of intrathecal morphine and saline on formalin-induced paw flinching (*A*). Histogram showing cumulative flinching during phase 1 and phase 2 (*B*). Intrathecal morphine significantly reduced phase 2 of formalin-induced paw flinching. The percentage of NK-1r(+) neurons showing internalization *versus* spinal segment as a function of intrathecal treatment (saline vehicle ipsilateral, saline vehicle contralateral, and morphine treatment ipsilateral) (*C*). Unilateral intraplantar injection of formalin (5%) produced a robust ipsilateral NK-1r internalization at L4–L6 levels of lumbar lamina I compared with the contralateral side. Intrathecal morphine significantly reduced formalin-induced NK-1r internalization in the spinal segments L5 and L6 compared with saline. Representative light microscopic images of NK-1r internalization induced by unilateral injection of formalin (5%) into the hind paw (*D–F*). Image of formalin-induced NK-1r internalization in the ipsilateral spinal lamina I from a rat administered intrathecal saline (*D*). Note the lack of a homogeneous cell membrane and the presence of NK-1–containing endosomes internalizing into the cytoplasm. Image of formalin-induced NK-1r internalization in the contralateral spinal lamina I (*E*). Note the presence of a homogeneous cell membrane and the lack of NK-1–containing endosomes internalizing into the cytoplasm. Intrathecal morphine (20 μg) blocked formalin-induced NK-1r internalization (*F*). Data are presented as mean number of paw flinching and percentage of NK-1r internalization with vertical bars showing SEM. * Significant difference between saline-treated and drug-treated animals, $P < 0.05$. Magnification, ×60. Scale bar is 10 μm. Saline, n = 6; morphine, n = 5 (*A* and *B*). Saline ipsilateral, n = 5; contralateral, n = 5; morphine; n = 3 (*C*).

Effects of VSCC Blockade on Formalin-induced, Paw-flinching Behavior and NK-1r Internalization

The effects of intrathecal ziconotide, mibefradil, diltiazem, and verapamil on formalin-induced paw flinching and NK-1r internalization are shown in figures 3, 4, 5, and 6, respectively. Ziconotide (0.3, 0.6, and 1 μg) did not reduce the number of formalin-induced, paw-flinching episodes in phase 1 (0.3 μg: 80 ± 30, $P > 0.05$; 0.6 μg: 112 ± 39, $P > 0.05$; 1 μg: 81 ± 22, $P > 0.05$) but reduced phase 2 formalin-induced paw flinching in a dose-dependent manner compared with vehicle control (0.3 μg: 556 ± 140, $P < 0.05$; 0.6 μg: 163 ± 69, $P < 0.0001$; 1 μg: 126 ± 73, $P < 0.0001$) (fig. 3, A and B). Ziconotide reduced formalin-induced NK-1r internalization at the L5 and L6 levels of spinal lamina I compared with vehicle control (L4: 0.3 μg 19 ± 2%, $P > 0.05$, 0.6 μg 17 ± 8%, $P > 0.05$, 1 μg 18 ± 10%, $P > 0.05$; L5: 0.3 μg 30 ± 7%, $P < 0.05$, 0.6 μg 26 ± 8%, $P < 0.05$, 1 μg 15 ± 4%, $P < 0.01$; L6: 0.3 μg 35 ± 1%, $P < 0.05$, 0.6 μg 31 ± 4%, $P < 0.05$, 1 μg 23 ± 4%, $P < 0.01$) (fig. 3C). Mibefradil (100 but not 50 μg) reduced formalin-induced paw flinching in phase 2 (50 μg: 813 ± 180, $P > 0.05$; 100 μg: 464 ± 115, $P < 0.05$) but not phase 1 (50 μg: 122 ± 34, $P > 0.05$; 100 μg: 79 ± 23, $P > 0.05$) (fig. 4, A and B). Mibefradil at the highest dose did not reduce formalin-induced NK-1r internalization (L4: 50 μg 37 ± 9%, $P > 0.05$, 100 μg 33 ± 11%, $P > 0.05$, 300 μg 32 ± 10%, $P > 0.05$; L5: 50 μg 54 ± 7%, $P > 0.05$, 100 μg 49 ± 2%, $P > 0.05$, 300 μg 52 ± 11%, $P > 0.05$; L6: 50 μg 58 ± 7%, $P > 0.05$, 100 μg 59 ± 8%, $P > 0.05$, 300 μg 61 ± 10%, $P > 0.05$) (fig. 4C). Diltiazem (500 but not 300 μg) significantly reduced formalin-induced paw flinching in phase 2 (300 μg: 806 ± 194, $P > 0.05$; 500 μg: 486 ± 126, $P < 0.05$) but not phase 1 (300 μg: 128 ± 46, $P > 0.05$; 500 μg: 80 ± 21, $P > 0.05$). Diltiazem at the highest dose had no effect on formalin-induced paw flinching in phases 1 and 2 (fig. 5, A and B) or upon formalin-induced NK-1r internalization (L4: 300 μg 27 ± 2%, $P > 0.05$, 500 μg 32 ± 12%, $P > 0.05$; L5: 300 μg 55 ±

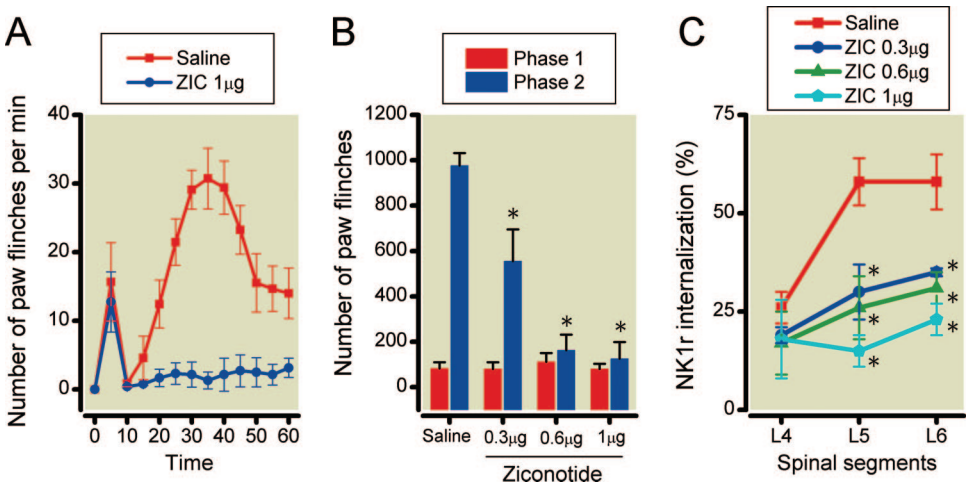

Fig. 3. Effects of intrathecal ziconotide on formalin-induced, paw-flinching behavior and neurokinin-1 receptor (NK-1r) internalization. Time-effect curves of intrathecal 1 μg ziconotide and saline on formalin-induced paw flinching (*A*). Intrathecal 0.3, 0.6, and 1 μg ziconotide reduced phase 2 of formalin-induced paw flinching (*B*). Intrathecal 0.3, 0.6, and 1 μg ziconotide reduced the formalin-induced NK-1r internalization in the spinal segments L5 and L6 compared with saline (*C*). Data are presented as mean number of paw flinching and percentage of NK-1r internalization with vertical bars showing SEM. * Significant difference between saline-treated and drug-treated animals, $P < 0.05$. Saline, n = 6; 0.3 μg, n = 5; 0.6 μg, n = 4; 1 μg, n = 4 (*A* and *B*). Saline, n = 5; 0.3 μg, n = 3; 0.6 μg, n = 3; 1 μg, n = 3 (*C*).

9%, $P > 0.05$, 500 μg 59 ± 6%, $P > 0.05$; L6: 300 μg 62 ± 8%, $P > 0.05$, 500 μg 61 ± 9%, $P > 0.05$) (fig. 5C). Verapamil (200 but not 50 or 100 μg) reduced formalin-induced paw flinching in phase 2 (50 μg: 690 ± 217, $P > 0.05$; 100 μg: 612 ± 118, $P > 0.05$; 200 μg: 386 ± 149, $P < 0.05$) but not phase 1 (50 μg: 126 ± 28, $P > 0.05$; 100 μg: 97 ± 22, $P > 0.05$; 200 μg: 106 ± 36, $P > 0.05$) (fig. 6, A and B). Verapamil, even at 300 μg, did not reduce formalin-induced NK-1r internalization (L4: 31 ± 5%, $P = 0.41$; L5: 52 ± 4%, $P = 0.43$; L6: 63 ± 3%, $P = 0.65$) (fig. 6C).

Effects of Intrathecal Ziconotide on NK-1r Internalization Induced by Exogenous Substance P

To determine if agents preventing internalization were acting by a presynaptic action, we examined whether intrathecal ziconotide would alter NK-1r internalization independent of a presynaptic mechanism. Accordingly, we showed that intrathecal substance P (30 nmol) produced widespread formalin-induced NK-1r internalization at the L4–L6 levels of spinal cord lamina I compared with intrathecal saline (L4: saline 10 ± 4%, substance P 66 ± 5%, $P < 0.001$; L5: saline 4 ± 2%, substance P 64 ± 13%, $P < 0.01$; L6: saline 11 ±

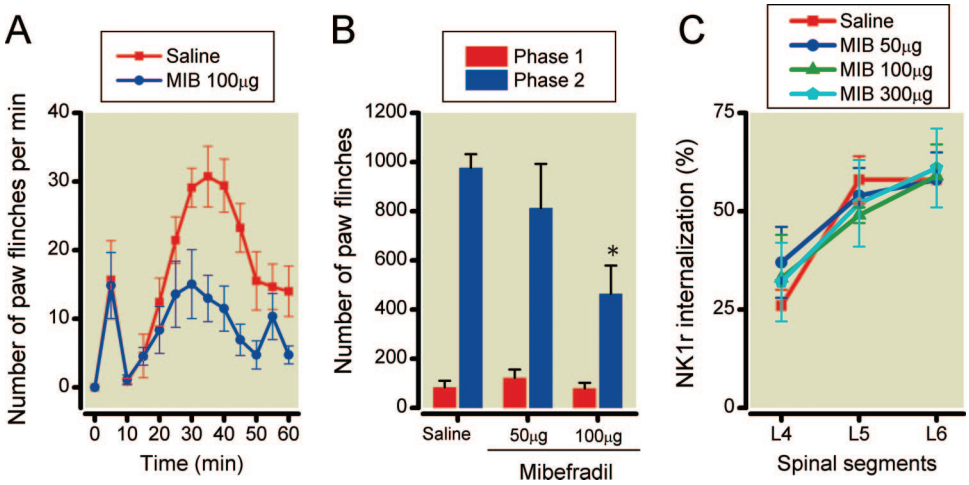

Fig. 4. Effects of intrathecal mibefradil on formalin-induced, paw-flinching behavior and neurokinin-1 receptor (NK-1r) internalization. Time-effect curves of intrathecal 100 μg mibefradil and saline on formalin-induced paw flinching (*A*). Intrathecal 100 μg, but not 50 μg, mibefradil significantly reduced phase 1 of formalin-induced paw flinching (*B*). Intrathecal 50 and 100 μg mibefradil did not reduce the formalin-induced NK-1r internalization (*C*). Data are presented as mean number of paw flinching and percentage of NK-1r internalization with vertical bars showing SEM. * Significant difference between saline-treated and drug-treated animals, $P < 0.05$. Saline, n = 6; 50 μg, n = 5; 100 μg, n = 5 (*A* and *B*). Saline, n = 5; 50 μg, n = 3; 100 μg, n = 3; 300 μg, n = 3 (*C*).

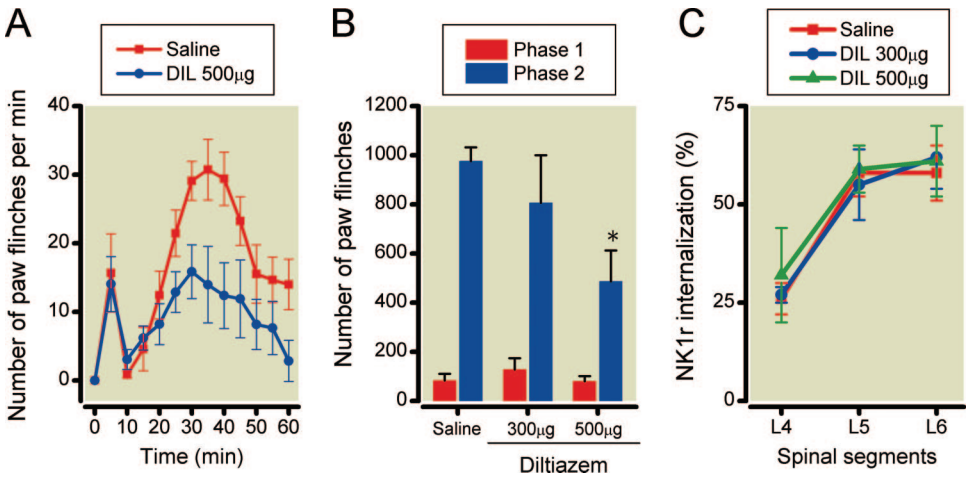

Fig. 5. Effects of intrathecal diltiazem on formalin-induced, paw-flinching behavior and neurokinin-1 receptor (NK-1r) internalization. Time-effect curves of intrathecal 500 μg diltiazem and saline on formalin-induced paw flinching (*A*). Intrathecal 500, but not 300 μg, diltiazem significantly reduced phase 2 of formalin-induced paw flinching (*B*). Intrathecal 300 and 500 μg diltiazem did not reduce the formalin-induced NK-1r internalization (*C*). Data are presented as mean number of paw flinching and percentage of NK-1r internalization with vertical bars showing SEM. * Significant difference between saline-treated and drug-treated animals, $P < 0.05$. Saline, n = 6; 300 μg, n = 5; 500 μg, n = 5 (*A* and *B*). Saline, n = 5; 300 μg, n = 3; 500 μg, n = 3 (*C*).

6%, substance P 84 ± 13%, $P < 0.001$) (fig. 7A). Administration of 0.6 μg intrathecal ziconotide, a dose that completely blocked formalin-induced NK-1r internalization, did not alter the exogenous substance-P–induced NK-1r internalization (L4: 65 ± 11%, $P > 0.05$; L5: 66 ± 12%, $P > 0.05$; L6: 61 ± 2%, $P > 0.05$) (fig. 7, B–D).

Behavioral and Motor Effects of Intrathecal VSCC Blockade

During the experiment, ziconotide, mibefradil, diltiazem, and verapamil caused dose-dependent adverse effects on motor function (table 1). In general, the adverse motor effects were dose dependent, showed an immediate onset, and typically declined over the course of the experiment. As previously reported,[36] ziconotide produced, in a dose-dependent manner, some adverse effects, such as whole-body shaking, serpentine-like movement of the tail, and ataxia. Mibefradil, diltiazem, and verapamil typically produced a loss of hind paw function at the highest doses used. It should be noted that although motor function was disturbed, this change in function did not impair the ability to flinch, as evidenced by the lack of any effect on phase 1 behavior. Moreover, flinching behavior was comparably observed in animals in which there was little, if any, observable effect on motor function. Morphine produced no adverse effects on behavior or motor function.

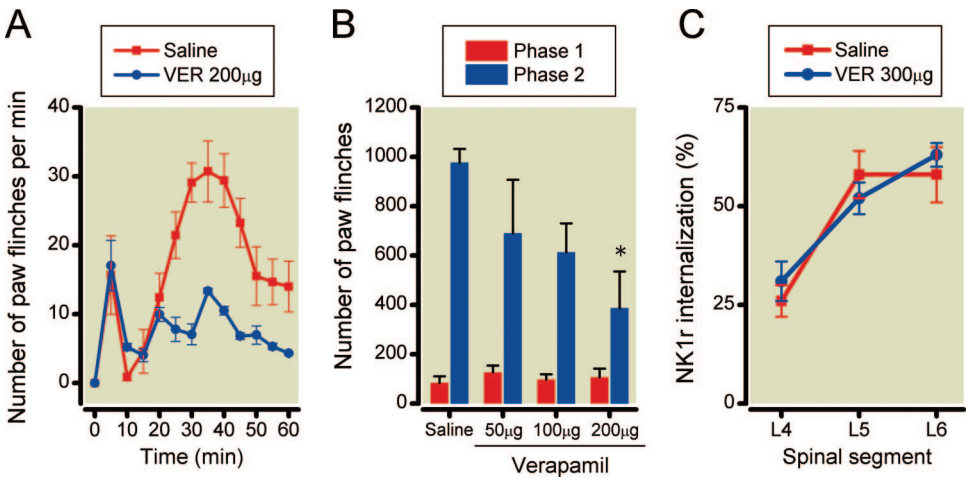

Fig. 6. Effects of intrathecal verapamil on formalin-induced, paw-flinching behavior and neurokinin-1 receptor (NK-1r) internalization. Time-effect curves of intrathecal 200 μg verapamil and saline on formalin-induced paw flinching (*A*). Intrathecal 200, but not 50 or 100 μg, verapamil significantly reduced phase 2 of formalin-induced paw flinching (*B*). Intrathecal 300 μg verapamil did not reduce formalin-induced NK-1r internalization (*C*). Data are presented as mean number of paw flinching and percentage of NK-1r internalization with vertical bars showing SEM. * Significant difference between saline-treated and drug-treated animals, $P < 0.05$. Saline, n = 6; 50 μg, n = 3; 100 μg, n = 4; 200 μg, n = 4 (*A* and *B*). Saline, n = 5; 300 μg, n = 3 (*C*).

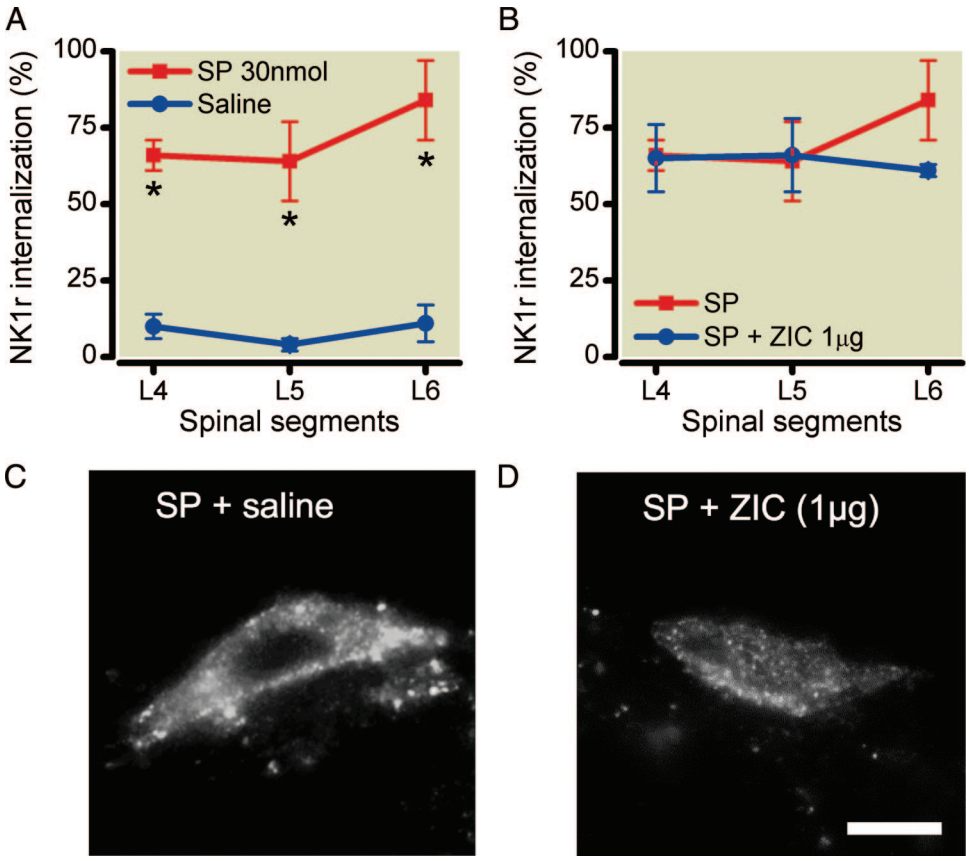

Fig. 7. The effects of ziconotide on the neurokinin-1 receptor (NK-1r) internalization induced by exogenous substance P. Intrathecal substance P (30 nmol) produced a robust bilateral NK-1r internalization at L4–L6 levels of lumbar lamina I compared with saline (*A*). Pretreatment of intrathecal ziconotide (0.6 μg) did not block NK-1r internalization induced by intrathecal substance P (*B*). Representative light microscopic images of NK-1r internalization in lamina I after substance P alone (*C*), substance P after ziconotide (*D*). Data are presented as mean percentage of NK-1r internalization with vertical bars showing SEM. * Significant difference in NK-1r internalization between saline-treated and drug-treated animals, $P < 0.05$. Magnification, ×60. Scale bar is 10 μm. Saline, n = 5; substance P, n = 3; substance P + ziconotide, n = 3.

Discussion

Tissue injury leads to the activation of small, high-threshold primary afferents, which induces transmitter release from the dorsal horn terminals of those afferents. This terminal release is mediated by the opening of VSCC, which leads to increased intracellular calcium and mobilization of transmitter vesicles, leading to exocytosis.[37] An important component of this process is the identity of the VSCCs that must be involved in this process. As noted, the three channel classes examined here are all present on small afferents. However,

Table 1. Behavioral and Motor Effects of Intrathecal Voltage-sensitive Calcium Channel Blockers

Drug	Route	Dose (μg)	n	% Showing Side Effect	Side Effects
Ziconotide	Intrathecal	0.3	8	38	Body shaking, serpentine-like movement of the tail, ataxia
—	—	0.6	7	43	
—	—	1	9	56	
Mibefradil	Intrathecal	50	3	0	Irreversible hind paw paralysis (> 2 h)
—	—	100	3	0	
—	—	300	4	75	
Diltiazem	Intrathecal	300	6	67	Reversible hind paw paralysis (< 10 min)
—	—	500	5	80	
Verapamil	Intrathecal	50	3	0	Reversible hind paw paralysis (< 10 min)
—	—	100	5	40	
—	—	200	6	67	
Morphine	Intrathecal	20	5	0	—

electrophysiologic studies in slices have indicated that monosynaptic excitation evoked by root stimulation in slices is most strongly attenuated by N- and less so by L- and T-type channels.[38] Such studies likely reflect the depolarization evoked by glutamate and not necessarily just by substance P. However, in the current studies we found that release of substance P evoked by intraplantar formalin was blocked by doses of N-type channel blocker that blocked formalin-induced flinching, whereas L- and T-type channel blockades had significant effects upon flinching but no effect, even at higher doses, on substance P release. In the following sections we consider several issues relevant to the interpretation of these studies.

Use of Internalization to Define Substance P Release

The NK-1r is a G-protein–coupled receptor that internalizes when occupied by an agonist. The assertion that the degree of internalization reflects extracellular substance P derived from primary afferents is supported by several observations: (1) evoked internalization is lost in animals pretreated with doses of capsaicin, which depletes the substance P in transient receptor potential vanilloid 1(+) afferent[29]; (2) in spinal cord slices, there is a marked covariance between extracellular substance P and the fraction of cells showing internalization[27]; (3) spinal opiates, which reduce extracellular substance P release through presynaptic action, reduce the fraction of spinal neurons that show NK-1r internalization after stimulation with a noxious stimulus[29,39]; (4) conversely, intrathecal capsaicin, which is known to evoke substance P release through activation of transient receptor potential vanilloid 1, increases spinal NK-1r internalization[40,41]; and (5) we demonstrated that intrathecal ziconotide at a dose that blocked formalin-evoked internalization had no effect on the internalization evoked by direct NK-1r activation using intrathecal substance P. Based on these observations, we consider NK-1r internalization to be a robust index of substance P release from spinal primary afferents and reduction of that internalization to be a marker for reduced release of substance P from those afferent terminals.

Role of N-, T-, and L-type Channels on Formalin-evoked Pain Behavior

In the current study, we characterized the effect of VSCC blockers on formalin-induced, paw-flinching behavior and *in vivo* substance P release from small primary afferents using NK-1r internalization. Intrathecal ziconotide, mibefradil, diltiazem, and verapamil reduced paw flinching behavior in phase 2 of the current study. Previous work has shown that intrathecal N-type calcium channel blockers, such as ziconotide, are effective in a variety of models, including those initiated by peripheral inflammation and nerve injury.[21,24,36,42,43] T-type VSCC blockers, such as mibefradil, have been reported to display analgesic effects in both phases of formalin-induced, paw-flinching behavior.[5,44] Previous work suggested that the intrathecal L-type VSCC blockers nimodipine and nifedipine had no effect on formalin-induced, paw-flinching behavior, whereas verapamil and diltiazem produced modest, but significant, inhibition.[21] These behavioral effects were observed at doses that did not produce motor dysfunction.

Effects on Spinal Substance P Release

Previous studies have shown that inhibition of VSCCs *via* the activation of presynaptic μ-opioid receptors serve to reduce the release from small primary afferents of nociceptive transmitters.[29,39,45–47] In the current work, despite the reported presynaptic disposition of all three families of calcium channels, only the N-type channel blocker was found to be clearly effective in blocking release, suggesting its location on the terminals of small peptidergic primary afferents (fig. 8). These results are consistent with electrophysiologic studies examining the effects of calcium channel blocker on monosynaptic-evoked dorsal horn depolarization in spinal slices, where the N-type channel blocker was highly effective compared with the T- and L-types.[38]

Previous studies have shown that intrathecal N-type VSCC blockers reduced phase 2 of the formalin-induced paw flinches and hyperalgesia initiated by knee joint inflammation[48] and intraplantar injection of capsaicin.[49] Similarly, N-type VSCC blockers suppressed the allodynia initiated by nerve ligation.[6,36,42] Spinal N-type VSCCs are closely allied with processes that serve to augment the responses evoked by afferent input under the allodynic states.[36] Results of the current study demonstrate that ziconotide suppresses, in a dose-dependent manner, phase 2, not but phase 1, of formalin-induced, paw-flinching behavior. Within the same dose range, intrathecal ziconotide significantly reduced spinal substance P release.

The absence of effect of T- and L-type VSCC blockers on release in the face of a significant effect on formalin-induced flinching may reflect effects on nonsubstance-P–releasing afferents or a postsynaptic effect. As noted, the distribution of these calcium channels is not limited to the primary afferent but is also noted on dorsal horn neuronal soma. Thus, T-type VSCCs exist in both presynaptic and postsynaptic sites of spinal sensory neurons and modulate synaptic transmission in the spinal cord dorsal horn.[19,50,51] T-type VSCCs play an important role in the initiation of long-term potentiation at synapses between afferent C fibers and lamina I projection neurons.[37,50] Drdla and Sandkühler reported that spinal administration of mibefradil completely prevented long-term potentiation induction that was induced by low-frequency stimulation of C fibers in the sciatic nerve.[52] Todorovic *et al.*[53] reported work indicating that T-type VSCCs facilitated pain signals in peripheral terminals of nociceptors. In the current work looking at the central terminals of substance P(+) afferents, mibefradil had no effect on release, suggesting a possible difference between the central and peripheral roles for this channel (fig. 8). With regard to L-type channels,

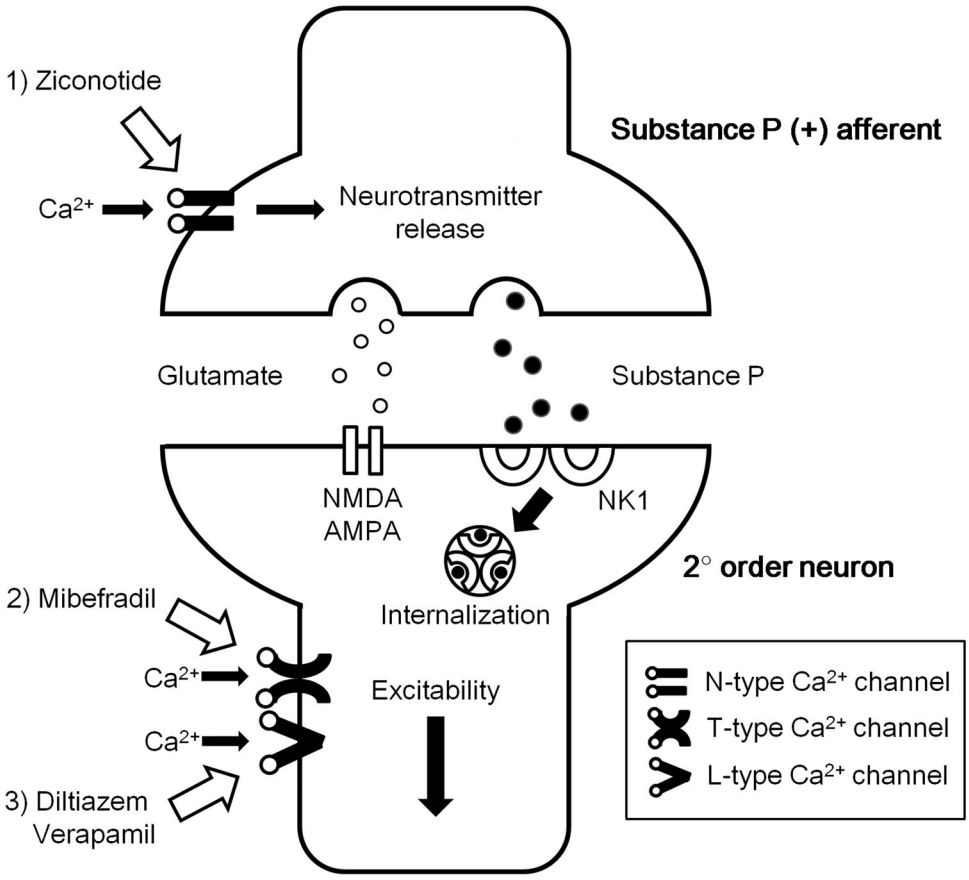

Fig. 8. The role of voltage-sensitive calcium channels (VSCCs) in nociceptive pathway in the spinal cord dorsal horn. Ziconotide (N-type VSCC blocker) inhibits Ca^{2+} influx then reduces, releasing neurotransmitters such as substance P and glutamate at presynaptic terminals (*1*). Mibefradil (T-type VSCC blocker) inhibits Ca^{2+} influx at the dorsal root ganglion. Inhibition of Ca^{2+} influx inactivates a small depolarization and attenuates neuronal excitability and/or postsynaptic neurotransmitter release (*2*). L-type VSCCs mainly locate on neuronal soma and dendrites. Diltiazem and verapamil (L-type VSCC blockers) postsynaptically block Ca^{2+} influx and reduce excitability and/or postsynaptic neurotransmitter release (*3*). AMPA = α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid; NK1 = neurokinin-1; NMDA = *N*-methyl-D-aspartate.

previous work in slices reported that bradykinin-stimulated release of substance P and calcitonin gene-related peptide were unaffected by the blockade of L-type VSCCs (nifedipine). In contrast, potassium-stimulated release of peptides was inhibited by nifedipine.[54] The current work suggests that the postsynaptic effects of L-type VSCCs are important for the observed facilitatory actions (fig. 8).[19,55,56]

Important elements of these effects are that none of the agents, including ziconotide, had a measurable effect on phase 1, even at the highest usable dose. This was unexpected and distinguished these effects from those of agents that block substance P release, such as morphine, which reduced phase 1 flinching in a dose-dependent manner.[32] This distinction for ziconotide suggests that the effect of this agent, despite the wide distribution of N-type channel, is surprisingly selective. Conversely, the effects on phase 1 may reflect the pre- and postsynaptic actions of agents such as morphine. The predominate effects on phase 1 *versus* phase 2 behavior reflect the profile of antihyperalgesic actions associated with the intrathecal effect of NK-1 antagonists and the destruction of the superficial NK-1(+) lamina I neurons.[3,4,5,57]

Motor Effects

In the current study, at the highest doses, these agents produce reversible hind paw paralysis that was observed immediately after injection. It has been reported that large doses of intrathecal diltiazem produced a reversible hind paw paralysis that may be attributed to the local anesthetic action caused by the blocking of Na^+ channels.[58] L-type VSCCs such as verapamil and diltiazem produce a local anesthetic effect as a result of inhibiting the fast Na^+ inward current by Na^+ channel blockade.[59] Intrathecal ziconotide was approved by the Food and Drug Administration in 2004 for management of severe chronic pain. In patients with cancer or acquired immune deficiency syndrome, significant pain relief was observed after titrated intrathecal infusion of ziconotide .[37] Because of the serious adverse effects, it has been recommended that ziconotide be used only for severe chronic pain refractory to other therapies.[60,61] In humans, serious adverse effects, such as nausea, dizziness, blurred vision, nystagmus, somnolence, and asthenia, have been reported with the use of intrathecal ziconotide.[60–62] These adverse side effects vanished after ziconotide was discontinued.[60] In animals, intra-

thecal administration of ziconotide produced dose-dependent reversible adverse effects.[21,63,64] In this study, intrathecal ziconotide produced adverse effects such as body shaking, serpentine-like movement of the tail, and ataxia (0.3 μg: 38%, 0.6 μg: 43%, 1 μg: 56%). Consistent with these observations, the therapeutic index of intrathecal ziconotide is indeed narrow in the clinical setting.[24]

In conclusion, the current results show *in vivo* that the spinal delivery of N-type calcium channel blocker will reduce substance P release at doses that approximate those required to block the facilitated state in the formalin model. Blockers for the T- and L-type channels also had inhibitory effects on formalin-induced paw flinching, but at the highest doses examined, there were no effects on substance P release. This suggests that T- and L-type channels may contribute to dorsal-horn–facilitated processing by mechanisms not involving the primary afferents.

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