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Spinal NMDA Receptors and Nociception-evoked Release of Primary Afferent Substance P

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Abstract

Dorsal horn NMDA receptors contribute significantly to spinal nociceptive processing through an effect postsynaptic to non-primary glutamatergic axons, and perhaps presynaptic to the primary afferent terminals. The present study sought to examine the regulatory effects of NMDA receptors on primary afferent release of SP, as measured by neurokinin 1 receptor (NK1r) internalization in the spinal dorsal horn of rats. The effects of intrathecal NMDA alone or in combination with D-serine (a glycine site agonist) was initially examined on basal levels of NK1r internalization. NMDA alone or when co-administered with D-serine failed to induce NK1r internalization, whereas activation of spinal TRPV1 receptors by capsaicin resulted in a notable NK1r internalization. To determine whether NMDA receptor activation could potentiate NK1r internalization or pain behavior induced by a peripheral noxious stimulus, intrathecal NMDA was given prior to an intraplantar injection of formalin. NMDA did not alter the formalin-induced NK1r internalization nor did it enhance the formalin paw flinching behavior. To further characterize the effects of presynaptic NMDA receptors, the NMDA antagonists AP-5 and MK-801 were intrathecally administered to assess their regulatory effects on formalin-induced NK1r internalization and pain behavior. AP-5 had no effect on formalin-induced NK1r internalization, whereas MK-801 produced only a modest reduction. Both antagonists, however, reduced the formalin paw flinching behavior. In subsequent *in vitro* experiments, perfusion of NMDA in spinal cord slice preparations did not evoke basal release of SP or calcitonin gene-related peptide (CGRP). Likewise, perfusion of NMDA did not enhance capsaicin-evoked release of the two peptides. These results suggest that presynaptic NMDA receptors in the spinal cord play little if any role on the primary afferent release of SP.

Keywords

Neurokinin 1 receptor; internalization; dorsal horn; glutamate; C-fiber

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Small diameter primary afferents terminate superficially in lamina I and II and exert frequency-dependent depolarizing effects upon second order neurons. This effect is mediated in part through the release of excitatory transmitters such as glutamate and peptides such as substance P (SP). The magnitude of the postsynaptic effect is proportional to the magnitude of transmitter release (Marvizon et al., 2003). Thus, increasing stimulus intensity yields an increased frequency of afferent input and enhanced transmitter release as measured by extracellular concentrations of SP and enhanced internalization of neurokinin 1 receptors (NK1r; Allen et al., 1997; Marvizon et al., 1997; Cao et al., 1998; Honore et al., 1999).

Aside from afferent axonal traffic, transmitter release from primary afferents can be modulated by a variety of receptors present presynaptically on these afferent fibers. For instance, activation of mu- and delta-opioid receptors (Yaksh et al., 1980; Collin et al., 1991; Gu et al., 2005; Kondo et al., 2005), CB1 cannabinoid receptors (Lever and Malcangio, 2002) and GABA_B receptors (Malcangio and Bowery, 1993; Marvizon et al., 1999; Riley et al., 2001) have been shown to reduce evoked release of SP and/or calcitonin gene-related peptide (CGRP), likely by inactivating voltage sensitive calcium channels by beta-gamma subunits of G-proteins (Dolphin, 2003). Conversely, activation of serotonin 5-HT₃ receptors (Inoue et al., 1997), as well as agents involved in the inflammatory process, such as PGE₂ (Evans et al., 1996) and PGI₂ (Hingtgen and Vasko, 1994) enhance evoked release of SP and/or CGRP. On the other hand, activation of transient receptor potential vanilloid 1 (TRPV1) receptors is known to depolarize C-fiber terminals and elicit direct entry of calcium into primary afferent terminals, and thus, enhance the spontaneous release of SP (Aimone and Yaksh, 1989; Lever and Malcangio, 2002; Lao et al., 2003).

It has been reported that NMDA receptors are expressed on SP-containing primary afferent terminals (Shigemoto et al., 1992; Liu et al., 1994; Lu et al., 2003; Zeng et al., 2006). As a result, presynaptic NMDA receptors may intrinsically regulate primary afferent transmitter release by permitting direct entry of calcium into presynaptic terminals. Consistent with this proposal, spinal NMDA receptor stimulation has been shown to increase SP release following administration of NMDA to the spinal intrathecal space (Liu et al., 1997), or application of NMDA to spinal cord slices (Marvizon et al., 1997; Malcangio et al., 1998). Equally, NMDA antagonists have been reported to diminish the evoked release of SP (Liu et al., 1997; Marvizon et al., 1997; Afrah et al., 2001; Lao et al., 2003). However, recent work from our group unexpectedly found that an intrathecal administration of a competitive NMDA receptor antagonist (AP-5) did not attenuate NK1r internalization evoked by an administration of naloxone in chronic morphine treated rats (Gu et al., 2005). Consequently, we aimed to explore the role of NMDA receptors on SP release, induced *in vivo*, by: i) intrathecal administration of NMDA to assess the ability of NMDA to induce SP release; ii) intrathecal administration of NMDA to determine its potentiating effects on formalin-induced SP release; iii) determining whether NMDA antagonists can diminish formalin-induced SP release; and iv) comparing our *in vivo* findings with *in vitro* spinal cord slice preparations measuring SP and CGRP release upon perfusion of NMDA in the absence or presence of capsaicin. In the present *in vivo* study, SP release was measured by the internalization of NK1r. In comparison with the direct measurement of SP release by radioimmunoassay in spinal perfusates or dialysates (Yaksh et al., 1980; Aimone and Yaksh, 1989; Afrah et al., 2001), NK1r internalization provides segmental and laminar localization of SP release, which can be studied with *in vivo* dosing that permits the findings to be correlated with behavioral effects (Mantyh et al., 1995; Abbadie et al., 1997).

EXPERIMENTAL PROCEDURES

Subjects

Male Holtzman Sprague-Dawley rats (250–350g; Harlan, Indianapolis, IN, U.S.A.) were individually housed in standard cages and maintained on a 12-hr light/dark cycle (lights on at 7:00). Testing occurred during the light cycle. Animal care was in accordance with the Guide for the Care and Use of Laboratory Animals (National Institute of Health publication 85-23, Bethesda, MD, U.S.A.) and approved by the Institutional Animal Care and Use Committee of the University of California-San Diego and the Indiana University School of Medicine. For experiments on release of peptides from spinal cord slices, adult male Sprague-Dawley rats (200–250g) were utilized. The rats were housed in group cages in a light controlled room (light from 6:00 to 19:00) at a constant temperature of 22°C. Food and water were available *ad libitum* to all rats in the study.

Intrathecal Catheter Implantation

Rats were implanted with a single intrathecal (i.t.) catheter for drug delivery, as previously described (Yaksh and Rudy, 1976; Malkmus and Yaksh, 2004). 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 head-holder with the head flexed forward. A midline incision was made on the back of the occipital bone and the neck to expose the cisternal membrane. The membrane was carefully opened with a stab incision, and a single lumen polyethylene-5 (outer diameter 0.36 mm) catheter (8.5 cm) was inserted and passed into the intrathecal space to the level of the L1–L2 spinal segments. The other end of the catheter was jointed to a polyethylene-10 catheter, which was tunneled subcutaneously to exit through the top of the head. Catheters were flushed with 10 µl of saline, plugged and incisions were closed using 3-0 silk sutures. The rats were given 5 ml of lactated Ringer's solution subcutaneously and allowed to recover under a heat lamp; those showing motor weakness or signs of paresis on recovery from anesthesia were euthanized immediately. The rats were allowed to recover for 5–7 days prior to the experiment.

NMDA, D-serine, and Capsaicin on NK1r Internalization

Rats were anesthetized with sodium pentobarbital (50 mg/kg, i.p.). Five minutes after the anesthetic, rats received an intrathecal injection of NMDA (1 µg). Based on prior observations by our group, as well as others, intrathecal NMDA (1 µg) in awake rats and mice produce vocalizations and caudally directed biting and scratching, indicative of pain and discomfort (Aanonsen and Wilcox, 1987; Liu et al., 1997); therefore, intrathecal drugs were given to anesthetized rats in the present experiment. Another group of rats received an intrathecal injection of NMDA (1 µg) in combination with D-Serine (1 µg). D-serine is an agonist for the glycine site of the NMDA channel (Schell et al., 1997; Mothet et al., 2000). Occupation of the glycine site is necessary for glutamate to open the ion channel (Johnson and Ascher, 1992). Thus, D-serine was co-administered with NMDA to ensure excitation of NMDA receptors. As a positive control, another group of rats received an intrathecal administration of capsaicin (30 µg). Rats were transcardially exsanguinated and perfused 5 min after i.t. drug administration.

NMDA on Formalin-induced NK1r Internalization and Flinching Behavior

To assess the role of NMDA on formalin-induced NK1r internalization, sodium pentobarbital anesthetized rats were intrathecally administered NMDA (1 µg) 5 min before an intraplantar formalin injection (5%, 50 µl) to the left hind-paw. Rats were transcardially exsanguinated and perfused with fixative 8 min after the formalin injection. To measure the effect of NMDA on formalin flinching behavior, a different group of rats were tested on the formalin behavioral

paradigm using an automated formalin detection system (Yaksh et al., 2001). A soft metal band (10 mm wide and 27 mm long, shaped into a C, and weighting ~0.5g) was placed around the left hind paw of the animal being tested. Animals were allowed to acclimate in individual Plexiglas chambers for 30 min before experimental manipulations. Rats were then administered NMDA (1 μ g, i.t.) followed 5 min later by an injection of formalin (5%, 50 μ l, i.p.l.). Immediately after the formalin injection, rats were placed into the test chamber and nociceptive behavior was quantified by automatic counting of spontaneous flinching and shaking of the injected paw. Flinches were counted in 1-min intervals for 60 min. The data is expressed as total number of flinches observed during phase 1 (0–9 min) and phase 2 (10–60 min). Rats were sacrificed after the 1-hr testing session.

NMDA Antagonists on Formalin-induced NK1r Internalization and Flinching Behavior

To study the effects of NMDA antagonists and morphine on formalin-induced NK1r internalization, rats were intrathecally administered AP-5 (30 μ g), MK-801 (10 μ g), morphine (20 μ g), or saline. The doses of AP-5, MK-801 and morphine were chosen based on their analgesic efficacy in reducing formalin-induced flinching behavior in rats (Nishiyama, 2000; Yaksh et al., 2001; Hua et al., 2004). Five-minutes after i.t. drug administration, rats were anesthetized with sodium pentobarbital (50 mg/kg, i.p.). Intraplantar formalin injection (5%, 50 μ l) to the left hind-paw occurred 5 min after the anesthetic injection. Rats were transcardially perfused 8 min after the formalin injection. To confirm analgesic efficacy, AP-5, MK-801 and morphine were tested on the formalin behavioral paradigm using the automated formalin detection system (Yaksh et al., 2001). Rats were intrathecally administered AP-5 (10 or 30 μ g), MK-801 (10 μ g), or morphine (20 μ g) followed 10 min later by a formalin injection (5%, 50 μ l, i.p.l.). Immediately after the formalin injection, rats were placed into the test chamber and nociceptive behavior was quantified as described above.

Tissue Preparation and Immunocytochemistry

Anesthetized rats were transcardially exsanguinated and perfused with 0.9% NaCl followed by 4% paraformaldehyde in 0.1 M sodium phosphate buffer, pH 7.4. The Lumbar spinal cord was removed and post-fixed overnight. After cryoprotection in 20% sucrose, 30 μ m coronal sections were made using a microtome. Immunofluorescent staining was performed to examine NK1r expression in the spinal dorsal horn. In brief, sections were incubated in a rabbit anti-NK1r polyclonal antibody overnight at room temperature. The antibody was diluted to a concentration of 1:3000 in 0.01 M PBS containing 10% normal goat serum and 0.3% Triton X-100. After rinses in PBS, sections were then incubated for 90 min at room temperature in a goat anti-rabbit secondary antibody conjugated with Alexa 488 diluted at 1:1000 in 0.01 M PBS containing 10% normal goat serum and 0.3% Triton X-100. All sections were finally rinsed and mounted on glass slides and coverslipped with ProLong mounting medium (Invitrogen).

Quantification of NK1r Internalization

NK1r internalization was counted using an Olympus fluorescence microscope at 60X magnification and followed the standard of previous reports (Mantyh et al., 1995; Abbadie et al., 1997; Marvizon et al., 1997; Trafton et al., 1999). The total number of NK1r-immunoreactive neurons in lamina I, with or without NK1r internalization, was counted. The ratio of cells showing NK1r internalization vs. all NK1r-positive cells was determined and then converted into a percentage of NK1r-immunoreactive cells. Neuronal profiles that had 10 or more endosomes in their soma and the contiguous proximal dendrites were considered to have internalized NK1 receptors. NK1r positive neurons on both sides of the dorsal horn were counted. The person counting the neurons was blinded to the experimental treatments. Three

to five sections per segment of the lumbar spinal cord were counted and 3–4 animals were used for each group ($n = 3\text{--}4$).

Confocal Microscopy and Image Processing

Confocal images of representative NK1r cells were acquired by a Leica TCS SP2 confocal system equipped with AOBS with a 63X objective (1.4 numerical aperture) and an argon 488nm laser line with a pinhole of Airy 1 units. Images were acquired at a digital size of 1024×1024 pixels. Five to 10 adjacent optical sections (approximately $0.5 \mu\text{m}$ separation) along the z-axis were projected together for demonstration. Images were processed with Adobe Photoshop software (version 8.0) by using the “curves” option to adjust image contrast.

Spinal Cord Slice Preparation

Spinal cord slices were prepared as previously described (Pang and Vasko, 1986; Chen et al., 1996). In brief, rats were rendered unconscious with CO_2 and then decapitated and a 2 cm segment of the dorsal lumbar enlargement of the spinal cord, was dissected and sectioned first cross-sectionally and then parasagittally into $0.3 \times 0.3 \text{ mm}$ sections. The tissue was then weighed, placed into a cylindrical perfusion chamber with an internal volume of 0.5 ml, and perfused at a flow rate of 0.5 ml/min with a HEPES buffer, consisting of HEPES 25 mM, NaCl 135 mM, KCl 3.5 mM, MgSO_4 1 mM, CaCl_2 2.5 mM, dextrose 3.3 mM, bovine serum albumin 1%, ascorbic acid 200 μM , phe-ala 200 μM , p-chloromercuriphenyl sulfonic acid (PCMS) 50 μM and bacitracin 20 μM , aerated with 95% O_2 - 5% CO_2 , pH 7.4–7.5, and maintained at $36\text{--}37^\circ\text{C}$. After 20 min, the flow was switched to 0.25 ml/min for collection of perfusate samples and 1.5 ml samples were collected into test tubes containing 75 μl of 1M 2-(N-morpholino) ethanesulfonic acid (MES) buffer (pH 6.7–6.9) every six minutes. Basal release was established by first perfusing the tissue with HEPES buffer for 24 min. Tissue was then perfused for and additional 18 min with HEPES buffer in the absence or presence of 100 μM NMDA. To evoke CGRP and SP release from sensory neurons, the spinal cord slices were then exposed to HEPES buffer containing 500 nM capsaicin with or without 100 μM NMDA for 18 min. To demonstrate a return to basal release after stimulation, the tissue was perfused with HEPES buffer for another 36 minutes. The amount of peptide released was expressed as fmol/mg of tissue/min.

Radioimmunoassay of neuropeptides

Immunoreactive-substance P and immunoreactive-CGRP were assayed from perfusates and tissue aliquots by radioimmunoassay as previously described (Chen et al., 1996). In brief, 450 μl of each of the perfusate samples were assayed directly for SP. To each sample, 25 μl of a 1:160,000 dilution of rabbit anti-SP antiserum and 25 μl of ^{125}I -[^8Tyr]-SP containing 6000–8000 cpm was added. For radioimmunoassay of iCGRP, 300 μl aliquots of the perfusate was assayed. To measure the amount of iCGRP released, 25 μl of a 1: 1,000,000 dilution of CGRP antibody and 25 μl of ^{125}I -[Tyr]-CGRP (28–37) containing 4000–6000 cpm were added to each sample and to standards. The samples were incubated 16–20 h at 4°C . The unbound tracer was separated from that bound to antibodies by adding 0.5 ml of a 0.1 M phosphate buffer (pH 7.4) containing 1% Norite charcoal, 50 mM NaCl, and 1 % bovine serum albumin. This mixture was centrifuged at $2500\times g$ for 20 min at 4°C , the supernatant was decanted to new tubes and the radioactivity in these tubes was measured by gamma scintillation spectrometry. The amount of iSP and iCGRP in unknown samples was estimated by comparing the percent bound radioactivity in unknowns to a standard curve using a 4 point non-linear least squares regression analysis. Using this method, the minimal detectable amount of iSP and iCGRP was approximately 5 fmol (95% confidence interval). NMDA at the concentration used in these experiments did not alter the radioimmunoassays.

Drugs and Antibodies

Capsaicin, MK-801, AP-5, NMDA, D-serine and peptidase inhibitors were purchased from Sigma Chemicals (St. Louise, MO). Morphine sulfate was provided by Merck Pharmaceuticals (Rahway, NJ). All drugs except capsaicin were dissolved in sterile 0.9% saline on the day of experiment. For the intrathecal *in vivo* study, capsaicin was dissolved in 10% 2-hydroxypropyl-beta-cyclodextrin, purchased from Wacker-Chemie AG (Munich, Germany), followed by sonication. All intrathecal injections were administered in a volume of 10 μ L followed by a 10 μ L saline flush. For the *in vitro* spinal cord slice preparations, capsaicin was initially dissolved in 1-methyl, 2-pyrrolidinone (MPL) purchased from Aldrich Chemical Company (Milwaukee, WI) to a concentration of 10 mM, and then diluted to 500 nM with HEPES buffer. CGRP and SP were obtained from Peninsula Laboratory (Belmont, CA). Substance P antiserum was raised in the laboratory of Dr. M. R. Vasko at Indiana University (Pang and Vasko, 1986), whereas CGRP antiserum was a generous gift from Michael J. Iadarola (NIH). The anti-NK1r polyclonal antibody was purchased from Advanced Targeting Systems (San Diego, CA). Secondary Alexa 488 conjugated antibody was purchased from Invitrogen (Eugene, OR).

Statistical Analysis

Statistically significant differences for the NK1r internalization data were detected using a one-way or a two-way ANOVA to detect differences between groups. Tukey's *post-hoc* pairwise comparisons were made when necessary. The formalin behavioral data was analyzed using individual one-way ANOVAs for phase 1 and phase 2 data. Upon reaching a significant effect, Dunnett's *post-hoc* analysis was performed to compare the difference between control and experimental groups.

For the analyses of the spinal cord slice release data, each chamber contained the spinal cord tissue obtained from one animal and is reported as one experimental sample. All data are presented as mean $\pm$ standard error of the mean (S.E.M.) of the fractional release of peptide per 6-min intervals. Results are expressed as fmol of peptide release per mg of tissue per minute. To compare the tissues treated with NMDA and controls, evoked release was calculated by taking the mean of the three collections in the presence of capsaicin (stimulated release) and the mean of the three collections prior to capsaicin exposure (basal release) and subtracting the values of the stimulated release from the basal release. Student's t-test was used for comparison of the effects of acute exposure to 100 μ M NMDA on evoked release with significance set at $p < 0.05$.

RESULTS

Effects of Intrathecal NMDA on Basal NK1r internalization

The effects of NMDA, NMDA and D-serine, or capsaicin on NK1r internalization is illustrated in Figure 1. In rats given intrathecal administration of vehicle, only negligible levels of NK1r internalization were detected ($\leq 5\%$ NK1r internalization; Fig 1A, B). Intrathecal administration of NMDA (1 μ g) alone or in combination with D-serine (1 μ g) did not alter basal levels of NK1r internalization in lamina I of the lumbar spinal cord (Fig 1A, C). On the other hand, intrathecal capsaicin (30 μ g) administered under the same conditions produced robust internalization of NK1r as compared to vehicle, $F(3,11) = 68.63$, $p < 0.001$ (Fig 1A, D).

Effects of Intrathecal NMDA on Intraplantar formalin-induced NK1r Internalization and Flinching Behavior

To determine whether intraplantar formalin-induced NK1r internalization would be enhanced by a pretreatment of NMDA, intrathecal NMDA (1 μ g) was administered 5 min prior to the formalin injection into the hindpaw (Figure 2). Intraplantar formalin (5%), produced a large

increase in the incidence of NK1r internalization. Ipsilateral, but not contralateral lamina I cells labeled for NK1r showed the presence of immunoreactive endosomes, indicating marked internalization, $F(2,22) = 16.86$, $p < 0.001$ (Fig 2A). However, pretreatment with intrathecal NMDA did not alter formalin-induced NK1r internalization (Fig 2B). Consistent with the lack of NMDA effect upon formalin-induced NK1r internalization, in a separate experiment, the magnitude of the bi-phasic formalin-induced flinching behavior was not altered by pretreatment with intrathecal NMDA (1ug) compared to saline control (Fig 2C).

Effects of NMDA Antagonists on Formalin-induced NK1r Internalization and Flinching Behavior

The effect of NMDA antagonists and morphine on formalin-induced NK1r internalization is illustrated in Figure 3. Intraplantar formalin induced NK1r internalization in lumbar lamina I cells (Fig 3A, B). The induction of NK1r internalization by formalin was modestly attenuated by intrathecal MK-801 (10ug), while intrathecal AP-5 (30ug) had no effect on the formalin-induced NK1r internalization (Fig 3A, C). As a positive control, intrathecal morphine almost completely suppressed the formalin-induced NK1r internalization, $F(6,45) = 4.973$, $p < 0.001$ (Fig 3A, D).

In a separate experiment, the behavioral analgesic relevance of morphine (20ug), MK-801 (10ug), and AP-5 (10 & 30ug) were confirmed (Fig 4). Unilateral intraplantar injection of formalin (5%) produced a characteristic bi-phasic (phase 1 and phase 2) paw flinching behavior. During phase 1, both doses of AP-5 significantly reduced the formalin-induced paw flinches compared to saline controls, $F(4,26) = 11.06$, $p < 0.001$, while in phase 2, morphine, MK-801 and both doses of AP-5 reduced the formalin-induced paw flinches, $F(4,26) = 6.314$, $p < 0.002$. These behavioral findings indicate that the doses of AP-5, MK-801 and morphine used for the internalization study were adequate for antinociception.

Effects of NMDA on SP and CGRP release in spinal cord slices

The effects of NMDA perfusion on the release of SP and CGRP from spinal cord slice preparations are shown on Figures 5 and 6, respectively. Capsaicin (500nM) exposure enhanced SP release compared to basal release (Fig 5A). Perfusion of NMDA (100 μ M) to the spinal cord slices did not alter the basal release of SP, nor did it enhance the capsaicin stimulated release of SP (Fig 5B). Therefore, when measuring evoked release of SP (stimulated release minus basal release), NMDA did not enhance the capsaicin-evoked release of SP, $t(4) = 1.365$, $p > 0.05$, with a mean value for SP release of 2.98 ± 0.68 fmol/mg/18min for control and 1.70 ± 0.24 fmol/mg/18min for NMDA (Fig 5C). When assessing CGRP release, capsaicin (500nM) exposure enhanced the CGRP release compared to basal release (Fig 6A). Once again, exposure of NMDA (100 μ M) to the spinal cord slices did not alter the basal release of CGRP, nor did it enhance the capsaicin-stimulated release of SP (Fig 6B). NMDA did not enhance the capsaicin-evoked release of CGRP, $t(4) = 0.822$, $p > 0.05$, with a mean value for CGRP release of 16.80 ± 3.70 fmol/mg/18min for control and 11.10 ± 0.90 fmol/mg/18min for NMDA treatments (Fig 6C).

DISCUSSION

In the present series of experiments, intrathecal NMDA, at a dose that produces potent signs of excitation and pain-like behavior (Liu et al., 1997), did not induce NK1r internalization. It is known that occupation of the glycine site is necessary for the NMDA receptors to be activated (Johnson and Ascher, 1992), however, co-administration of NMDA and D-serine (a glycine site agonist) also failed to induce NK1r internalization. On the other hand, intrathecal capsaicin produced significant NK1r internalization, indicating that the *in vivo* internalization paradigm was able to demonstrate spinal SP release induced by the local activation of TRPV1 receptors

located presynaptically on peptidergic primary afferent terminals. Due to the lack of NMDA effect, we sought to determine if the effects of intrathecal NMDA might be revealed as an augmentation of release induced by a peripheral noxious stimulus, particularly an injection of formalin into the hindpaw. Intraplantar injection of formalin induced significant NK1r internalization, and produced a characteristic paw flinching pain behavior. However, intrathecal NMDA administration prior to formalin injection had no effect on the formalin-induced NK1r internalization nor did it alter the formalin-induced paw flinching behavior.

In a subsequent study, the effects of NMDA receptors on SP and CGRP release were tested on *in vitro* spinal cord slice preparations. Spinal cord slices were pretreated in the absence or presence of capsaicin to measure the effects of NMDA receptor activation of basal and capsaicin-evoked release of SP and CGRP. Similar to the *in vivo* findings, NMDA did not increase SP or CGRP release under basal conditions, nor did it enhance capsaicin-evoked release of SP or CGRP.

To further characterize the contribution of spinal NMDA receptors on nociceptive transmission, the effects of intrathecal NMDA antagonists on formalin-induced flinching behavior and NK1r internalization was assessed. In the behavioral study, AP-5, MK-801, and morphine significantly reduced the formalin-induced paw flinches. Interestingly, pretreatment with intrathecal AP-5 had no effect on reducing the formalin-induced NK1r internalization. Meanwhile, MK-801 produced a modest, but statistically significant reduction on the formalin-induced NK1r internalization. As a positive control, intrathecal morphine almost completely suppressed the formalin-induced NK1r internalization, indicating that the formalin-induced NK1r internalization paradigm was indeed sensitive to reduction or suppression. In the current set of data, AP-5 did not decrease formalin-induced NK1r internalization. It is possible that formalin produced a significant release of glutamate from primary afferents, which might saturate glutamate binding on presynaptic NMDA receptors and therefore render AP-5 ineffective, due to its competitive antagonistic actions. This limitation would not apply to MK-801 due to its non-competitive channel blocking properties. It is noteworthy that if such competition did occur for AP-5, then it would have also been expected to manifest in a lack of behavioral inhibition. Since both AP-5 and MK-801 were analgesic in the formalin behavioral model, it can be suggested that the analgesic actions of AP-5 and MK-801 in the formalin model may be mediated through a postsynaptic mechanism, independent of modulating the release of SP from primary afferent terminals. The presynaptic inhibitory effects of MK-801 might be due to the channel blocking effects of the drug on channels other than NMDA, such as those of the nicotinic acetylcholine type (Ramoa et al., 1990; Amador and Dani, 1991; Arias et al., 2001). Ultimately, the present findings demonstrate that activation of NMDA receptors did not induce NK1r internalization. The observation that both NMDA antagonists were analgesic, yet only the non-competitive antagonist (MK-801) diminished the formalin-induced NK1r internalization could further indicate the physiological limitations of the role of NMDA receptors in altering primary afferent SP release.

The issue of presynaptic effects of NMDA on primary afferent SP release has proven to be unexpectedly complicated, with several conflicting reports. For example, in *in vitro* spinal cord slice preparations, NMDA perfusion produced a dose-dependent biphasic response in SP release, as 1 μ M NMDA enhanced basal outflow of SP, while 10 μ M NMDA reduced basal outflow and electrically-evoked release of SP (Malcangio et al., 1998). Using a similar slice preparation, perfusion of 100 μ M NMDA enhanced NK1r internalization, and by extension, SP release (Marvizon et al., 1997). On the other hand, perfusion of the NMDA agonist L-CCG-IV did not enhance SP release from trigeminal slices preparations (Cuesta et al., 1999).

In vivo studies assessing the role of NMDA on primary afferent SP release are limited and also controversial. That is, Liu et al. (1997) reported that intrathecal administration of NMDA (6.8

nmol = 1 µg, the same dose used in the present study) evoked NK1r internalization in the superficial laminae of the dorsal spinal cord. Whereas, in agreement with the present findings, Afrah et al. (2001) used an *in vivo* spinal dialysis model to show that perfusion of a wide dose range of NMDA (10–1000 µM) through dialysis membrane did not induce the release of SP in the lumbar spinal dorsal horn. The discrepancy in the *in vivo* studies maybe due to the administration of NMDA in awake, rather than anesthetized rats. It appears that in the study by Liu et al. (1997), rats were given intrathecal NMDA in the awake state. To our knowledge, and also reported by others (Aanonsen and Wilcox, 1987; Liu et al., 1997), intrathecal NMDA (1 µg) produces immediate and significant vocalizations, biting and scratching, indicative of pain, stress and discomfort. In the current study and that by Afrah et al. (2001), spinal NMDA was applied to anesthetized rats, which resulted in a lack of NMDA-induced SP release and NK1r internalization. Although plausible, it is unlikely that the anesthetic was directly responsible for the differences in NK1r internalization, because i) the same anesthetic protocol did not prevent release from peripheral noxious stimuli, such as paw compression or intraplantar formalin (Honore et al., 1999; Trafton et al., 2001; Gu et al., 2005; Kondo et al., 2005); and ii) in the present study, intrathecal morphine did reduce the formalin-induced NK1r internalization, whereas, intrathecal capsaicin evoked NK1r internalization, indicating that presynaptic modulation of SP release has not been abolished by the anesthetic. Thus, NMDA-induced NK1r internalization demonstrated by Liu et al. (1997) could be due to a secondary effect of NMDA-induced hyperexcitability at spinal and super-spinal levels in awake rats.

Although there is little doubt that presynaptic NMDA receptors exist on peptidergic afferents, their contribution to the excitation of the afferent terminal is not clear. In addition to the lack of peptide release, the absence of excitatory effects of presynaptic NMDA receptors on primary afferent glutamate release has also been suggested. Electrophysiological studies have demonstrated that activation of primary afferent presynaptic NMDA receptors reduce, rather than enhance, spontaneous as well as stimulation-induced glutamate release (Bardoni et al., 2004; Zeng et al., 2006). In conclusion, our present findings do not support a stimulatory role of presynaptic NMDA receptors on primary afferent SP release. When considered together with the behavioral results, the present data suggests that glutamate-mediated nociceptive processing in the spinal cord may primarily be dependent on postsynaptic NMDA receptors expressed on second order dorsal horn neurons.

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Abbreviations

NMDA	N-methyl-D-aspartate
SP	Substance P
CGRP	Calcitonin gene-related peptide
NK1r	Neurokinin 1 receptor
TRPV1	

	Transient receptor potential vanilloid 1
AP-5	DL-2-Amino-5-phosphonopentanoic acid
MK-801	(5R,10S)-(+)-5-Methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine hydrogen maleate
PGE₂	Prostaglandin E ₂
PGI₂	Prostaglandin I ₂
AOBS	Acousto-optical beam splitter
ANOVA	Analysis of variance
i.t	intrathecal
i.p	intraperitoneal
i.p.l	intraplantar
PBS	Sodium phosphate buffer
HEPES	(4-(2-hydroxyethyl)-1-piperazine

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Figure 1.

The effect of intrathecal NMDA (1ug), NMDA co-administered with D-serine (both 1ug), or capsaicin (30ug) on NK1r internalization. **A**- NMDA or NMDA with D-serine did not enhance NK1r internalization compared to vehicle treated rats; whereas capsaicin produced a notable enhancement in NK1r internalization. The vehicle for capsaicin was 10% 2-hydroxypropyl-beta-cyclodextrin, while saline was the vehicle for NMDA and D-serine. Due to the lack of differences in NK1r internalization between the two vehicles, the data were combined. **B**, **C**, & **D**- are representative confocal images of NK1r internalization of rats intrathecally administered with vehicle, NMDA or capsaicin and sacrificed 5 min later by transcardial fixation. **B**- an image of a vehicle treated rat. Notice the absence of NK1r internalization shown by the existence of a homogeneous cell membrane and the lack of NK1r containing endosomes internalizing into the cytoplasm. **C**- an image of a NMDA treated rat. Again, there is no evidence of NK1r internalization. **D**- evidence of NK1r internalization from a rat treated with capsaicin. Notice the lack of a homogeneous cell membrane and the presence of NK1r containing endosomes internalizing into the cytoplasm. Confocal images were taken using a 63X objective with 5–10 optical sections along the z-axis separated by approximately 0.5 μ m. Scale bar is 20 μ m. NK1r internalization data is presented as mean ($\pm$ S.E.M.) percentages. NK1r internalization was quantified using a one-way ANOVA for drug treatment, while data for spinal segments were collapsed. * Signifies a difference between capsaicin and vehicle treatments, $p < 0.001$ ($n = 3$ –5 per group).

Figure 2.

The effects of intrathecal NMDA on formalin-induced NK1r internalization and pain behavior. **A-** Unilateral intraplantar injection of formalin (5%) produced robust NK1r internalization on ipsilateral but not contralateral lamina I (n=3–4). **B-** Intrathecal administration of NMDA (1ug) did not modulate the formalin-induced NK1r internalization on the ipsilateral side (n=3–4). **C-** In a different group of rats, formalin produced a characteristic biphasic (phase 1 & 2) paw flinching behavior that was measured for 60 min. The behavioral response to formalin was not altered by an intrathecal pretreatment with NMDA (1ug) 5 min prior to the formalin injection compared to their intrathecal saline treated controls (n=9–11). NK1r internalization data is presented as mean (±S.E.M.) percentages, and formalin flinching behavior is presented as

means ($\pm$ S.E.M.) of total flinches in Phase 1 & 2. * Signifies a difference in NK1r internalization between ipsilateral and contralateral lamina I cells.

Figure 3.

Modulatory role of NMDA antagonists and morphine on formalin-induced NK1r internalization. **A-** intrathecal AP-5 (30ug), MK-801 (10ug) or morphine (20ug) was administered 10 min prior to an intraplantar injection of formalin (5%). Rats were transcardially perfused 8 min after formalin injection. Formalin produced a significant NK1r internalization, which was reduced by MK-801 and completely suppressed by morphine at all segments of the lumbar spinal cord. AP-5 did not attenuate the formalin-induced NK1r internalization. **B, C, & D** are representative confocal images of rats pretreated with saline, AP-5 or morphine and then injected with formalin to produce NK1r internalization. **B-** an image of a saline pretreated rat given formalin into the hindpaw. Formalin produced NK1r internalization as evident from NK1r containing endosomes internalizing into the cytoplasm. **C-** an image of an AP-5 pretreated rat given formalin into the hindpaw. AP-5 was unable to block the formalin-induced NK1r internalization. **D-** an image of a morphine pretreated rat given formalin into the hindpaw. Morphine very effectively blocked the formalin-induced NK1r internalization, as there were no NK1r containing endosomes internalizing into the cytoplasm. Data are presented as means ($\pm$ S.E.M.) of percent NK1r internalization. Scale bar is 20 μ m. *Signifies a difference between drug treated and saline treated rats at a given spinal segment. ($p < 0.05$), ($n=3-4$).

Figure 4.

The doses of AP-5, MK-801, and morphine used in this study are analgesic in the formalin behavioral model. AP-5 (10 or 30 ug), MK-801 (10ug) or morphine (20ug) were intrathecally administered 10 min prior to an intraplantar injection of formalin (5%). Behavioral testing occurred immediately after formalin injection and lasted for 60 min. Formalin produced a characteristic biphasic (phase 1 & 2) flinching response. During phase 1, both doses of AP-5 significantly reduced the flinching behavior compared to saline control. During phase 2, all drugs reduced the formalin paw flinching behavior compared to saline. Data are presented as means ($\pm$ S.E.M.) of paw flinches for phase 1 & 2. *Signifies a significant difference between drug treatment and saline control within phase 1 or phase 2. Analysis utilized a separate one-way ANOVA for each phase, followed by a Dunnett's *posthoc* test ($p < 0.05$), ($n=4-8$).

Figure 5.

The effects of NMDA on SP release from spinal cord slice preparations. **A-**capsaicin (500nM) stimulated SP release from spinal cord slices during the time of its exposure. **B-** perfusion of 100 μ M NMDA (horizontal bar) did not increase SP release prior to capsaicin (basal state) or during capsaicin exposure (stimulated state). **C-** capsaicin-evoked release of SP (stimulated release minus basal release) did not significantly differ between NMDA treated and untreated samples. Data presented as means ($\pm$ S.E.M.) of the amount of peptide release as fmol/mg of tissue/min ($n=5$).

Figure 6.

The effects of NMDA on CGRP release from spinal cord slice preparations. **A-** capsaicin (500nM) stimulated CGRP release from spinal cord slices during the time of its exposure. **B-** perfusion of 100μM NMDA (horizontal bar) did not increase CGRP release prior to capsaicin (basal state) or during capsaicin exposure (stimulated state). **C-** capsaicin-evoked release of CGRP (stimulated release minus basal release) did not significantly differ between NMDA treated and untreated samples. Data presented as means ($\pm$ S.E.M.) of the amount of peptide release as fmol/mg of tissue/min ($n=5$).