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N-METHYL-D-ASPARTATE RECEPTORS AND LARGE CONDUCTANCE CALCIUM-SENSITIVE POTASSIUM CHANNELS INHIBIT THE RELEASE OF OPIOID PEPTIDES THAT INDUCE μ -OPIOID RECEPTOR INTERNALIZATION IN THE RAT SPINAL CORD

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Abstract

Endogenous opioids in the spinal cord play an important role in nociception, but the mechanisms that control their release are poorly understood. To simultaneously detect all opioids able to activate the μ -opioid receptor, we measured μ -opioid receptor internalization in rat spinal cord slices stimulated electrically or chemically to evoke opioid release. Electrical stimulation of the dorsal horn in the presence of peptidase inhibitors produced μ -opioid receptor internalization in half of the μ -opioid receptor neurons. This internalization was rapidly abolished by *N*-methyl-D-aspartate ($IC_{50}=2 \mu M$), and *N*-methyl-D-aspartate antagonists prevented this effect. μ -Opioid receptor internalization evoked by high K^+ or veratridine was also inhibited by *N*-methyl-D-aspartate receptor activation. *N*-methyl-D-aspartate did not affect μ -opioid receptor internalization induced by exogenous endomorphins, confirming that the effect of *N*-methyl-D-aspartate was on opioid release. We hypothesized that this inhibition was mediated by large conductance Ca^{2+} -sensitive K^+ channels BK (Ca^{2+}). Indeed, inhibition by *N*-methyl-D-aspartate was prevented by tetraethylammonium and by the selective BK(Ca^{2+}) blockers paxilline, penitrem A and verruculogen. Paxilline did not increase μ -opioid receptor internalization in the absence of *N*-methyl-D-aspartate, indicating that it does not produce an increase in opioid release unrelated to the inhibition by *N*-methyl-D-aspartate. The BK (Ca^{2+}) involved appears to be a subtype with slow association kinetics for iberiotoxin, which was effective only with long incubations. The BK(Ca^{2+}) opener NS-1619 also inhibited the evoked μ -opioid receptor internalization, and iberiotoxin prevented this effect. We concluded that Ca^{2+} influx through *N*-methyl-D-aspartate receptors causes the opening of BK(Ca^{2+}) and hyperpolarization in opioid-containing dorsal horn neurons, resulting in the inhibition of opioid release. Since μ -opioid receptors in the dorsal horn mediate analgesia, inhibition of spinal opioid release could contribute to the hyperalgesic actions of spinal *N*-methyl-D-aspartate receptors.

Keywords

dorsal horn; dynorphin; enkephalin; internalization; μ -opioid receptor; opioid

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Abbreviations

aCSF, artificial cerebrospinal fluid; ANOVA, analysis of variance; AP-5, DL-2-amino-5-phosphonopentanoic acid; BK(Ca²⁺), large conductance Ca²⁺-sensitive K⁺ channels; CCK, cholecystokinin; CCK-8, cholecystokinin-8; C.I., confidence interval; CPP, (RS)-3-(2-carboxypiperazin-4-yl)-propyl-1-phosphonic acid; DAMGO, [D-Ala², NMe-Phe⁴, Gly-ol⁵]enkephalin; DCG-IV, (2S,2'R,3'R)-2-(2',3'-dicarboxycyclo-propyl)-glycine; DHPG, (RS)-3,5-dihydroxyphenylglycine; DPDPE, [2-D-penicillamine, 5-D-penicillamine]-enkephalin; IC₅₀, effective concentration of drug for 50% of the inhibition; K⁺-aCSF, aCSF with 5 mM KCl; L-AP4, L-(+)-2-amino-4-phosphonobutyric acid; LY-341495, (2S)-2-amino-2-[(1S,2S)-2-carboxycycloprop-1-yl]-3-(xanth-9-yl) propanoic acid; mGluR, metabotropic glutamate receptor; MK-801, dizocilpine, (5R,10S)-(+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine hydrogen maleate; MOR, μ -opioid receptor; NBQX, 2,3-dioxo-6-nitro-1,2,3,4,-tetrahydrobenzo[f]quinoxaline-7-sulfonamide; n_H, Hill coefficient; NMDA, N-methyl-D-aspartate; NS-1619, 1,3-dihydro-1-[2-hydroxy-5-(trifluoromethyl)-phenyl]-5-(trifluoromethyl)-2H-benzimidazol-2-one; SDZ-220-040, (S)- α -amino-2',4'-dichloro-4-hydroxy-5-(phosphonomethyl)-[1,1'-biphenyl]-3-propanoic acid; sucrose-aCSF, artificial cerebrospinal fluid with 5 mM KCl and 215 mM sucrose instead of NaCl; TEA, tetraethylammonium

Alkaloid opiates acting on μ -opioid receptors (MORs) are the most powerful analgesics available, but they produce tolerance and addiction. Physiologically, MORs are activated by opioid peptides, and strategies that increase the availability of these opioids by inhibiting their degradation have been shown to produce analgesia (Chou et al., 1984; Fournie-Zaluski et al., 1992; Noble et al., 1992b). Moreover, there is some evidence that this approach produces little tolerance (Noble et al., 1992c) and dependence (Noble et al., 1992a). One way to increase opioid availability would be by targeting neurotransmitter receptors that control opioid release; however, these are largely unknown. One group has reported that Met-enkephalin release in the spinal cord is increased by neuropeptide FF (Ballet et al., 1999; Mauborgne et al., 2001) and inhibited by μ and δ autoreceptors (Bourgoin et al., 1991; Collin et al., 1994; Mauborgne et al., 2001). Other investigators (Przewlocka et al., 1990) found that spinal release of α -neoendorphin was increased by noradrenaline and inhibited by GABA_A receptors. However, the physiological relevance of these effects remains unclear.

Our previous studies (Song and Marvizon, 2003a,b) indicated that internalization of MORs in dorsal horn neurons evoked by high K⁺, veratridine or electrical stimulation reflects the release of enkephalins and dynorphins from other dorsal horn interneurons (Todd and Spike, 1993). Studying opioid release is particularly challenging because, whereas post-translational processing of opioid gene products produces many active peptides (Yaksh et al., 1983), the immunoassays commonly used to measure opioid release detect just one of them, and therefore are poor predictors of opioid receptor activation. In contrast, MOR internalization can be used to simultaneously detect the release of all opioid peptides able to activate this receptor (Eckersell et al., 1998; Marvizon et al., 1999; Trafton et al., 2000; Song and Marvizon, 2003a,b; Mills et al., 2004). Although morphine and other alkaloid opiates can activate the MOR without inducing its internalization (Whistler et al., 1999), all physiologically-occurring opioids tested produce MOR internalization (Trafton et al., 2000; Song and Marvizon, 2003a). Further evidence that MOR internalization follows its activation by peptides is that the potency of [D-Ala²,NMe-Phe⁴,Gly-ol⁵]-enkephalin (DAMGO) to produce MOR internalization is the same as its potency to increase [γ -³⁵S]GTP binding and to inhibit adenylyl cyclase (Marvizon et al., 1999), and that DAMGO injected intrathecally produced spinal MOR internalization and behavioral analgesia at the same doses (Trafton et al., 2000).

In the present study we used stimulus-evoked MOR internalization in dorsal horn neurons to identify neurotransmitter receptors that modulate spinal opioid release. We found that activation of GABA_A, GABA_B, δ -opioid, cholecystokinin (CCK) and metabotropic glutamate receptors (mGluRs) does not affect spinal opioid release. However, activation of *N*-methyl-D-aspartate (NMDA) receptors produces a robust inhibition of spinal opioid release by opening large conductance Ca²⁺-dependent K⁺ channels (maxi-K or BK(Ca²⁺)). Because MORs in the dorsal horn mediate analgesia (Yaksh, 1997), this is consistent with the sensitization to pain produced by spinal NMDA receptors (Dingledine et al., 1999; Brauner-Osborne et al., 2000; South et al., 2003).

EXPERIMENTAL PROCEDURES

All animal procedures were approved by the Chancellor's Animal Research Committee at UCLA and conform to NIH guidelines. Efforts were made to minimize the number of animals and their suffering.

Chemicals

Baclofen, 1,3-dihydro-1-[2-hydroxy-5-(trifluoromethyl)-phenyl]-5-(trifluoromethyl)-2*H*-benzimidazol-2-one (NS-1619), endomorphin-2, L-glutamic acid, ibenitoxin, ifenprodil, isoguvacine, ketamine, MK-801, NMDA, paxilline, [2-D-penicillamine, 5-D-penicillamine]-enkephalin (DPDPE), penitrem A, D-serine, tetraethylammonium (TEA), veratridine, and verruculogen were purchased from Sigma-RBI (St. Louis, MO, USA). (2*S*)-2-amino-2-[(1*S*, 2*S*)-2-carboxycycloprop-1-yl]-3-(xanth-9-yl) propanoic acid (LY-341495), (*S*)- α -amino-2', 4'-dichloro-4-hydroxy-5-(phosphonomethyl)-[1,1'-biphenyl]-3-propanoic acid (SDZ-220-040), L-(+)-2-amino-4-phosphonobutyric acid (L-AP4), DL-2-amino-5-phosphonopentanoic acid (AP-5), (*R,S*)-3-(2-carboxypiperazin-4-yl)-propyl-1-phosphonic acid (CPP), (2*S*,2'*R*,3'*R*)-2-(2',3'-dicarboxycyclopropyl)-glycine (DCG-IV), 5,7-dichloro-kynurenic acid, (*R,S*)-3,5-dihydroxyphenylglycine (DHPG), and 2,3-dioxo-6-nitro-1,2,3,4-tetrahydrobenzo[*f*]quinoxaline-7-sulfonamide (NBQX) were purchased from Tocris (Ellisville, MO, USA). Cholecystokinin-8 (CCK-8) was a gift from Dr. Joseph Reeves, Division of Digestive Diseases, UCLA (Los Angeles, CA, USA). Isoflurane was from Halocarbon Laboratories, River Edge, NJ, USA.

Spinal cord slices

Media used for the slices were: artificial cerebrospinal fluid (aCSF), containing (in mM) 124 NaCl, 1.9 KCl, 26 NaHCO₃, 1.2 KH₂PO₄, 1.3 MgSO₄, 2.4 CaCl₂ and 10 glucose; K⁺-aCSF, containing 5 mM of KCl, and sucrose-aCSF, the same as K⁺-aCSF except that NaCl was iso-osmotically replaced with sucrose (215 mM, as assessed with an osmometer). Coronal spinal cord slices were prepared as previously described (Lao et al., 2003; Song and Marvizon, 2003a,b). Briefly, the spinal cord was extracted from 3 to 4 weeks old male Sprague-Dawley rats (Harlan, Indianapolis, IN, USA) under isoflurane anesthesia. Coronal slices (400 μ m) without roots were cut with a Vibratome (Technical Products International, St. Louis, MO, USA) in ice-cold sucrose-aCSF. Up to six slices from each animal were cut sequentially in the L1-L4 region.

Slice stimulation

Slices were stimulated electrically at the dorsal horn as described (Song and Marvizon, 2003b). Briefly, a coronal slice was held vertically in a superfusion chamber with stainless steel insect pins and a stimulating electrode was placed with its poles on either side of one dorsal horn (i.e. current flow is oriented rostro-caudally). The shape and size of the stimulating electrode was such that it completely covered one of the dorsal horns ("hook" shape with 1 mm diameter, made from two 0.25 mm platinum/iridium parallel wires separated 1 mm;

purchased from Frederick Haer & Co., Bowdoinham, ME, USA). The non-stimulated side of the slice was marked with a round hole in the ventral horn. Electrical stimulation typically consisted of 1000 square pulses (30 V, 0.4 ms) delivered at 500 Hz; frequencies of 10 Hz, 30 Hz or 100 Hz were used in some experiments. Peptidase inhibitors (10 μ M actinonin, captopril and phosphoramidon, with 6 μ M dithiothreitol) and other drugs were superfused starting 5 min before and ending 5 min after the stimulation. NMDA and D-serine were superfused for 2.5 min, ending with the stimulation. Other slices were stimulated chemically by incubating them with 50 mM KCl for 2 min or with 20 μ M veratridine for 1 or 2 min (Song and Marvizon, 2003a). Slices were fixed 5 min after the end of the stimulation. Treatments were randomized between slices, and no more than two slices from the same animal received the same treatment.

Immunohistochemistry

Histological sections of 25 μ m were cut from the slices and labeled as previously described (Marvizon et al., 1999; Song and Marvizon, 2003a,b). To label MORs we used a rabbit antiserum (1:7000 dilution) raised against amino acids 384–398 of the cloned rat MOR-1 (DiaSorin, Stillwater, MN, USA, catalog no. 24216). This antiserum has been characterized (Arvidsson et al., 1995) and shown to label dorsal horn neurons (Spike et al., 2002). Pre-absorption of the MOR antibody with its immunizing peptide (10 μ g/ml) abolished the staining. The secondary antibody was Alexa-488 goat anti-rabbit IgG (Molecular Probes, Eugene, OR, USA), used at 1:2000 dilution for 2 h at room temperature. Sections were mounted in Prolong (Molecular Probes).

Confocal microscopy

Confocal images were acquired at UCLA's Carol Moss Spivak Cell Imaging Facility with a Leica TCS-SP confocal microscope. Low magnification images were obtained with a 20 $\times$ objective and consist of one optical section 2.53 μ m thick (full width half maximum), or two optical sections 2.53 μ m thick separated 2.48 μ m. High magnification images were obtained with a 100 $\times$ objective and consist of two to three optical sections 0.62 μ m thick, separated 0.57 μ m. The pinhole was 1.0 Airy units. Optical sections were averaged four times to reduce noise. Images were processed using Adobe Photoshop 5.5. The "curves" feature of the program was used to adjust the contrast. Images were acquired at a digital size of 1024 $\times$ 1024 pixels and were later cropped to the relevant part of the field.

Quantification of MOR internalization

MOR internalization was quantified as previously described (Marvizon et al., 1999; Song and Marvizon, 2003a,b). A Zeiss Axiovert 135 (Carl Zeiss, Inc., Thornwood, NY, USA) conventional fluorescence microscope with a 100 $\times$ objective was used to visually count MOR neurons, classifying them as with or without MOR internalization. Counting was done blind to the treatment. Neuronal somata with five or more endosomes were considered as having internalization. All MOR neurons of the stimulated and the contralateral dorsal horns were counted for each histological section, and three to five sections per slice (chosen randomly) were used. There were 20–40 MOR neurons/dorsal horn in the 25 μ m sections; therefore we counted 60–200 MOR neurons per hemi-slice (stimulated or contralateral side of a slice). Data from one hemi-slice were considered as one replicate measure for statistical purposes. MOR internalization in the contralateral hemi-slice was always low and unaffected by drugs, and is not shown in the figures.

Data analysis

Data were analyzed using Prism 4 (GraphPad Software, San Diego, CA, USA) (Marvizon et al., 2003; Song and Marvizon, 2003a,b). Data are the mean $\pm$ standard error (S.E.) of three to 13 slices. Statistical analyses consisted of one-way or two-way analysis of variance (ANOVA)

and Bonferroni's post-test, with significance set at 0.05. Two-way ANOVA was used with electrical stimulation of the dorsal horn; the two variables were "drugs" (drug combinations or concentrations) and "side of the slice" (ipsilateral or contralateral to electrical stimulation) and the Bonferroni's post-test was applied to the variable "drugs" to compare effects on the ipsilateral side to the control, unless indicated otherwise. The absence of asterisks in the figure indicates that the difference with control is not significant. MOR internalization in the contralateral side was always negligible and unaffected by drugs. The time course of the inhibition by NMDA was fitted to an exponential decay function: $Y = Span \times \exp(-K \times X) + \text{plateau}$; with half life($t_{1/2}$) = $0.69/K$. Concentration-response data were fitted by a sigmoidal dose-response function: $Y = \text{bottom} + (\text{top} - \text{bottom}) / (1 + 10^{(\text{Log}(\text{IC}_{50}) - \text{Log} X) \times n_H})$, where "top" and "bottom" are the maximum and minimum values of the response (Y), respectively, IC_{50} is the concentration (X) that produces half of the maximum inhibition, and n_H is the Hill coefficient. The statistical error of the IC_{50} was expressed as 95% confidence interval (C.I.). Akaike's Information Criterion (Motulsky and Christopoulos, 2003) was used to determine whether the value of one of the parameters differed from a hypothetical value. Parameter constraints were: $0\% < \text{top} < 100\%$, $0\% < \text{bottom}$, $n_H = 1$ (except for DCG-IV in Fig. 8B). We assumed that concentration-response curves were monotonic and sigmoidal. Baseline measures (zero concentration of drug) were included in the non-linear regression by assigning them a concentration value about 3 log units lower than the IC_{50} . This was necessary because the log of zero is undefined, and is based on the assumption that very low concentrations of drug produce a response indistinguishable from baseline.

RESULTS

The release of opioid peptides that bind to MORs in the spinal dorsal horn was measured through the internalization of MORs in laminae I–II neurons (Fig. 1A, Fig. 2A) (Marvizon et al., 1999; Trafton et al., 2000; Song and Marvizon, 2003a,b). MOR internalization was likely caused by the release of pro-enkephalin and pro-dynorphin gene products from dorsal horn interneurons (Yaksh et al., 1983; Todd and Spike, 1993; Song and Marvizon, 2003a,b). Opioid release was evoked by stimulating rat spinal cord slices electrically at the dorsal horn, or chemically with high K^+ or veratridine. To block opioid degradation, the peptidase inhibitors actinonin, captopril and phosphoramidon ($10 \mu\text{M}$, together with $6 \mu\text{M}$ dithiothreitol) were included in all experiments (Song and Marvizon, 2003a).

NMDA inhibited MOR internalization evoked by electrical stimulation

In most experiments, the dorsal horn was stimulated electrically at 500 Hz because this high frequency maximizes opioid release (Song and Marvizon, 2003b) while minimizing substance P and glutamate release from C-fibers, which are unable to follow it (Raymond et al., 1990). Stimulation at 500 Hz produced MOR internalization in $53 \pm 2\%$ ($n=15$ slices) of MOR neurons in the stimulated dorsal horn (Fig. 1A), but only in $6 \pm 2\%$ ($n=15$) of MOR neurons in the contralateral dorsal horn. Neurons with internalization were found in medial and central laminae I–II (Fig. 2A), but were scarce in the lateral dorsal horn.

NMDA applied for 2.5 min before stimulation inhibited MOR internalization in the stimulated dorsal horn in a concentration-dependent fashion (Fig. 1A, Fig. 2B). D-Serine ($10 \mu\text{M}$), a selective agonist of the NMDA receptor glycine site (McBain et al., 1989), decreased the evoked MOR internalization by itself (Fig. 1A, Fig. 2C). This indicates that the electrical stimulation was able to release some glutamate, because NMDA receptor activation requires the binding of both glutamate and glycine (Marvizon and Baudry, 1993; Dingledine et al., 1999; Brauner-Osborne et al., 2000). Likewise, the fact that NMDA inhibited the evoked MOR internalization by itself suggests the presence of some glycine in the slices. D-Serine did not increase the inhibition produced by most concentrations of NMDA. The IC_{50} values for NMDA

in the presence and absence of 10 μM D-serine did not differ significantly, and were 10 μM and 2 μM , respectively (Fig. 1A). These IC_{50} values for NMDA are consistent with its reported affinity for NMDA receptors, particularly those containing the NR2D subunit, that have an affinity for NMDA (9 μM) somewhat higher than NMDA receptors with other NR2 subunits (22–36 μM) (Brauner-Osborne et al., 2000).

The inhibition produced by NMDA plus D-serine was quite rapid: it was statistically significant in 30 s and essentially complete in 60 s (Fig. 1B). This indicates that the inhibition was not caused by excitotoxic damage, which requires longer exposures to NMDA. In any case, in most experiments slices were exposed to NMDA for only 2.5 min to avoid excitotoxic damage.

NMDA did not inhibit MOR internalization evoked by exogenous opioids

Importantly, the inhibition produced by NMDA receptors was not of MOR internalization itself, and therefore has to be attributed to inhibition of opioid release. Thus, MOR internalization produced by incubating the slices with endomorphin-2 (100 nM for 10 min) (Song and Marvizon, 2003a) was the same in the absence ($99.7 \pm 0.3\%$ MOR neurons with internalization, $n=3$ slices) and in the presence of 10 μM NMDA and D-serine for 10 min ($97.6 \pm 0.3\%$, $n=3$). MOR internalization produced by a lower concentration (30 nM for 10 min) of endomorphin-1 was also the same in the absence ($34.6 \pm 6.2\%$, $n=4$) and in the presence of 10 μM NMDA and D-serine ($34.6 \pm 6.8\%$, $n=4$), showing that NMDA receptors do not affect MOR internalization even when it is driven by a less powerful stimulus. These results also indicate that NMDA did not have a toxic effect on the MOR neurons, because this would have affected the internalization mechanism.

NMDA inhibited MOR internalization evoked by chemical stimulation

MOR internalization evoked by chemical stimulation (Song and Marvizon, 2003a) was also inhibited by NMDA receptors. Internalization evoked by incubating the slices in 50 mM KCl for 2 min was inhibited by 30 μM NMDA, and the NMDA receptor channel blocker MK-801 (1 μM) prevented this inhibition (Fig. 1C). Similarly, MOR internalization evoked by the Na^+ channel opener veratridine (20 μM for 1 min) was decreased by 30 μM NMDA, and the competitive NMDA receptor antagonist CPP (5 μM) attenuated this inhibition (Fig. 1D).

NMDA receptors antagonists prevented the inhibition by NMDA

The inhibition by NMDA and D-serine (both 10 μM) of MOR internalization evoked by electrical stimulation was prevented by several NMDA receptor antagonists (Fig. 3). These included the glutamate site competitive antagonists CPP and SDZ-220-040, the glycine site antagonist 5,7-dichloro-kynurenic acid, and the channel blockers MK-801 and ketamine. For reviews on NMDA receptor pharmacology, please see (Dingledine et al., 1999; Brauner-Osborne et al., 2000). MOR neurons representative of these treatments are shown in Fig. 4. These results demonstrate that the inhibition of opioid release produced by NMDA is indeed caused by activation of NMDA receptors. However, the AMPA/kainate receptor antagonist NBQX (Brauner-Osborne et al., 2000) was also able to prevent the inhibition of MOR internalization by NMDA and D-serine, suggesting that AMPA/kainate receptors contribute to the inhibition produced by NMDA. This is likely due to the need for the depolarization produced by AMPA/kainate receptors (activated by glutamate released by the stimulus) to relieve the Mg^{2+} blockade of the NMDA receptor channel. Ifenprodil at 10 μM , a concentration at which it selectively blocks NMDA receptors possessing the NR2B subunit (Kew et al., 1998), did not affect the inhibition produced by NMDA plus D-serine (Fig. 3, Fig. 4F). Hence, the NMDA receptors that inhibit spinal opioid release do not appear to have the NR2B subunit.

NMDA receptors antagonists did not increase evoked MOR internalization

Since the inhibition produced by D-serine or NMDA alone (Fig. 1A) indicated that the electrical stimulus released some glutamate and glycine, we considered the possibility that the evoked opioid release was already partly inhibited by these NMDA receptors. If this were the case, then NMDA antagonists would increase the evoked MOR internalization. Nevertheless, the NMDA antagonists CPP and MK-801 did not increase MOR internalization evoked by electrical stimulation (Fig. 5). This was observed over a wide frequency range, including frequencies that C-fibers are able to follow (10 Hz and 30 Hz), which is important because C-fiber terminals are a significant source of glutamate in the dorsal horn (Teoh et al., 1996). Therefore, the amounts of glutamate and glycine released by the stimulus were not high enough to produce a noticeable decrease in opioid release, unless the co-agonist was present. Incidentally, data in Fig. 5 also confirm that opioid release increased with the frequency of electrical stimulation, as we previously reported (Song and Marvizon, 2003b).

BK(Ca²⁺) channels mediate the inhibitory effect of NMDA receptors

It is somewhat surprising that NMDA receptors inhibit spinal opioid release, since these receptors normally have an excitatory function. For example, NMDA receptors facilitate substance P release from the central terminals of primary afferents (Liu et al., 1997; Marvizon et al., 1997; Malcangio et al., 1998) and opioid release in the gut (Patierno et al., 2005). However, it was recently reported (Isaacson and Murphy, 2001) that in olfactory bulb neurons NMDA receptors produce hyperpolarization and inhibitory postsynaptic currents mediated by the opening of BK(Ca²⁺) (or maxi-K) closely associated with them. Accordingly, we investigated whether BK(Ca²⁺) channels mediated the inhibition of spinal opioid release produced by NMDA by assessing whether BK(Ca²⁺) channel blockers prevented this inhibition. Indeed, the non-selective K⁺ channel blocker TEA (2 mM) completely prevented the inhibition of electrically-evoked MOR internalization by NMDA and D-serine (Fig. 6A, Fig. 7A). Moreover, the selective BK(Ca²⁺) channel blockers paxilline (Fig. 7B) (Li and Cheung, 1999), penitrem A (Fig. 7C) (Knaus et al., 1994; Hollywood et al., 2000) and verruculogen (Norris et al., 1980; Aoki and Baraban, 2000) also prevented this inhibition (Fig. 6A). The effect of the BK(Ca²⁺) channel blockers was a true reversal of the inhibition produced by NMDA and not an increase in MOR internalization unrelated to the effect of NMDA, because paxilline did not increase MOR internalization evoked by stimulation at 30 Hz or 500 Hz in the absence of NMDA (Fig. 5). A concentration-response for paxilline (Fig. 6B) yielded an approximate EC₅₀ of 100 nM, comparable to that obtained for its inhibition of BK(Ca²⁺) channels in vascular smooth muscle cells (36 nM (Li and Cheung, 1999)).

However, BK(Ca²⁺) blockers only partially attenuated the inhibition by NMDA receptors. Thus, although paxilline, penitrem A and verruculogen significantly reversed the inhibition by NMDA plus D-serine, they did so to values still significantly lower ($P < 0.01$, Bonferroni's posttest) than the control in the absence of NMDA agonists (dotted line in Fig. 6A). The concentration at which these blockers were used (1 μ M) should be sufficient to saturate the BK(Ca²⁺) channels. This was confirmed by the concentration-response curve for paxilline in Fig. 6B, showing that in the presence of NMDA plus D-serine saturating concentrations of paxilline ("top" parameter = $40 \pm 4\%$) produced lower MOR internalization than the control in the absence of NMDA agonists ($53 \pm 2\%$, see legend of Fig. 6B for statistics).

Another selective BK(Ca²⁺) channel blocker, the scorpion toxin iberiotoxin, did not prevent the inhibition produced by NMDA receptors at 0.3 μ M (Fig. 6A, Fig. 7D), a concentration at which it is generally used to block BK(Ca²⁺) channels (Isaacson and Murphy, 2001). Importantly, some neuronal BK(Ca²⁺) channels appear to be insensitive to iberiotoxin because they contain the $\beta 4$ subunit (Meera et al., 2000), which markedly slows down the association and dissociation of iberiotoxin. However, a prolonged incubation with a higher concentration

of iberiotoxin can produce a virtually irreversible blockade these $BK(Ca^{2+})$ channels (Meera et al., 2000). This provided a way to test the involvement of $\beta 4$ -containing $BK(Ca^{2+})$ channels in the effect of NMDA receptors: we preincubated the slices with 3 μM iberiotoxin for 30 min, and then stimulated them in medium that contained NMDA and D-serine but no iberiotoxin. We found that this treatment prevented the inhibition produced by NMDA and D-serine to the same extent as the other $BK(Ca^{2+})$ channel blockers (Fig. 6A, Fig. 7E). This indicates that the $BK(Ca^{2+})$ channels that mediate the effect of NMDA have the $\beta 4$ subunit or other subunit composition that slows down the association and dissociation of iberiotoxin.

A $BK(Ca^{2+})$ channel opener mimics the inhibitory effect of NMDA

If inhibition of spinal opioid release by NMDA receptors is mediated by $BK(Ca^{2+})$ channels, the direct opening of these channels should also inhibit opioid release and subsequent MOR internalization. Accordingly, we determined whether the $BK(Ca^{2+})$ channel opener NS-1619 inhibited MOR internalization evoked by electrical stimulation of the dorsal horn in the absence of NMDA. NS-1619 did inhibit the evoked MOR internalization (Fig. 7F) with an IC_{50} of 9 μM (Fig. 6C), which is consistent with its potency in other functional $BK(Ca^{2+})$ channel assays (Sheldon et al., 1997; Malysz et al., 2004). However, in addition to opening $BK(Ca^{2+})$ channels, NS-1619 also inhibits L-type voltage-gated Ca^{2+} channels in smooth muscle (Sheldon et al., 1997). This could also explain its inhibition of opioid release, which likely involves Ca^{2+} entry through voltage-gated Ca^{2+} channels. To rule out this possibility, we pre-incubated slices with 3 μM iberiotoxin for 30 min to block the $BK(Ca^{2+})$ channels. In these slices inhibition of the evoked MOR internalization by 100 μM NS-1619 was abolished, showing that this inhibition was indeed caused by the opening of $BK(Ca^{2+})$ channels.

Neurotransmitter receptors without effect on spinal opioid release

The relevance of the inhibitory effect of NMDA receptors on spinal opioid release is underscored by the lack of effect of several other neurotransmitter systems. Thus, the ubiquitous inhibitory neurotransmitter GABA did not inhibit spinal opioid release (Table 1), as evidenced by the lack of effect on evoked MOR internalization of the selective $GABA_A$ receptors agonist isoguvacine (100 μM) and the selective $GABA_B$ receptor agonist baclofen (20 μM). Isoguvacine and baclofen (as well as all the other compounds tested) did not increase MOR internalization in the contralateral side of the slices (data not shown), indicating that basal opioid release is not affected by these drugs.

The neuropeptide CCK has been attributed anti-opioid actions (Noble and Roques, 1999), which could be caused by inhibition of opioid release. However, CCK-8, an agonist with sub-nanomolar affinities at CCK-A and CCK-B receptors (Noble and Roques, 1999), did not affect MOR internalization evoked by 500 Hz stimulation at one and 10 nM (Table 1). Therefore, the anti-opioid effects of CCK are not due to inhibition of spinal opioid release, and may be mediated postsynaptically.

A previous study (Collin et al., 1994) suggested that δ -opioid autoreceptors inhibit spinal opioid release. To investigate this possibility, we determined whether the selective δ -opioid receptor agonist DPDPE (1 μM) inhibited MOR internalization evoked by 10 μM veratridine or electrical stimulation of the dorsal horn. No inhibition was found with either stimulus.

MOR internalization evoked by veratridine or electrical stimulation was not affected by the AMPA receptor antagonist NBQX (5 or 10 μM), either. This suggests that these stimuli recruit opioid-containing neurons directly, and not through excitatory synapses that they receive from other neurons.

We also investigated whether mGluRs inhibited spinal opioid release evoked by electrical stimulation of the dorsal horn. L- Glutamate at 10 μM partially inhibited the evoked MOR internalization (Fig. 8A), an effect probably mediated by NMDA receptors. No effect was found (Fig. 8A) with the selective agonist of group I mGluRs (mGlu₁ and mGlu₅ receptors) DHPG (30 μM), or the agonist of group III mGluRs (mGlu₄, mGlu₆, mGlu₇ and mGlu₈ receptors) L- AP4 (10 μM). In contrast, the agonist of group II mGluR (mGlu₂ and mGlu₃ receptors) DCG-IV (10 μM) completely abolished the evoked MOR internalization with an IC_{50} of 6.2 μM (Fig. 8B). However, the inhibition by 10 μM DCG-IV was not prevented by LY-341495 (1 μM , Fig. 8A), a group II mGluR antagonist with nanomolar affinity (Kingston et al., 1998; Johnson et al., 1999), but it was prevented by the NMDA antagonist AP-5 (100 μM). Therefore, the effect of DCG-IV was mediated by NMDA receptors and not by group II mGluRs. Previous studies (Breakwell et al., 1997; Uyama et al., 1997) have shown that this compound is an agonist at both receptors. We concluded that none of the mGluR subtypes modulate spinal opioid release.

DISCUSSION

This study demonstrates that NMDA receptors inhibit opioid release in the dorsal horn. The likely mechanism for this inhibition is that Ca^{2+} influx through NMDA receptors causes the opening of BK(Ca^{2+}) channels in their vicinity and subsequent hyperpolarization of the opioid-containing dorsal horn neurons or presynaptic terminals.

Possible artifacts

The inhibition by NMDA receptors of spinal opioid release likely reflects a physiologically relevant mechanism and is not an artifact. First, MOR internalization evoked by electrical stimulation of the dorsal horn reflects physiological opioid release. Thus, it was abolished by lidocaine, by the absence of Ca^{2+} , by the absence of peptidase inhibitors and by a MOR antagonist; indicating that it required neuronal firing, Ca^{2+} -dependent peptide release, and agonist binding to MORs (Song and Marvizon, 2003b). Second, the decrease in evoked MOR internalization by NMDA reflects a suppression of opioid release and not a direct inhibition of the internalization process, because NMDA did not affect MOR internalization produced by exogenous endomorphins. Third, inhibition of opioid release by NMDA was not due to excitotoxic damage (Rothman and Olney, 1987) of the dorsal horn neurons that release opioids. Excitotoxicity typically requires high agonist concentrations or long incubation times; for example, 500 μM glutamate for 5 min (Choi et al., 1987, 1988) or 30 μM NMDA for 60 min (Sattler et al., 2000). In contrast, NMDA inhibited opioid release with an IC_{50} < 10 μM , and this inhibition took place in less than one minute. Moreover, NMDA application to the slices did not produce any visible damage in neurons with MORs (Fig. 2B) or neurokinin 1 receptors (Marvizon et al., 1997). Indeed, in the experiments with endomorphins NMDA for 10 min did not affect the ability of neurons to internalize MORs, which shows that their metabolism was not impaired. Finally, it is highly unlikely that blocking BK(Ca^{2+}) channels would prevent NMDA excitotoxicity, which is mediated by depolarization and Ca^{2+} entry (Rothman and Olney, 1987); if anything, BK(Ca^{2+}) blockade would contribute to this depolarization.

Effect of other receptors on opioid release

We also found that a variety of receptors known to modulate opioid signaling does not affect spinal opioid release. These include GABA_A, GABA_B, δ -opioid, CCK and mGluRs. More recently, we found that CGRP receptors also inhibit spinal opioid release (Marvizon et al., 2005). To our knowledge, NMDA receptors have not been reported to increase CGRP release, as they do with substance P release. Even if that was the case, our results could not be mediated by CGRP receptors, because our experiments were done in the presence of dithiothreitol, which inactivates CGRP by breaking its disulfide bridge.

Release of endogenous glutamate

Electrical stimulation of the dorsal horn should have produced glutamate release, which would activate the NMDA receptors that inhibit opioid release. Indeed, the presence of glutamate in the slices can be inferred from the inhibition produced by D-serine (Fig. 1A). It is a well-known fact that NMDA receptor activation requires agonist binding to both the glutamate and glycine sites (Marvizon and Baudry, 1993; Dingledine et al., 1999; Brauner-Osborne et al., 2000); hence, an effect of D-serine implies the presence of a certain amount of glutamate. Conversely, the fact that NMDA inhibited opioid release in the absence of D-serine implies the presence of a certain amount of glycine in the slices. Glycine and glutamate mutually increase their affinities at NMDA receptors by allosteric mechanisms (Marvizon and Baudry, 1993); hence, as the concentration of one of the co-agonists is increased, the effect of small amounts of the other co-agonist becomes more noticeable. Further evidence for the release of endogenous glutamate is provided by the fact that the AMPA/kainate antagonist NBQX prevented the inhibition produced by NMDA, which suggests that the inhibition produced by NMDA receptors requires the activation of AMPA/kainate receptors by endogenous glutamate. However, the fact that NMDA receptor antagonists did not increase the evoked MOR internalization indicates that the amount of glutamate released was not enough to inhibit opioid release. This may be due to limitations of the slice preparation. For example, slices may allow released glutamate to readily diffuse out of the tissue, which does not happen physiologically. Moreover, glutamate uptake systems may limit the effect of endogenous glutamate, as suggested by the fact that the inhibition of evoked MOR internalization produced by 10 μ M exogenous glutamate (Fig. 8A) was not as pronounced as that produced by the same concentrations of NMDA (Fig. 1A) or DCG-IV (Fig. 8A).

BK(Ca²⁺) channels mediate the inhibition by NMDA receptors

We hypothesized that the inhibition of opioid release by NMDA receptors was caused by the opening of BK(Ca²⁺) channels triggered by Ca²⁺ influx (Fig. 9). This hypothesis was based in a recent study in the olfactory bulb (Isaacson and Murphy, 2001) reporting that NMDA receptors produce hyperpolarization and inhibitory postsynaptic currents by opening BK (Ca²⁺) channels. These BK(Ca²⁺) channels are closely associated with the NMDA receptors, because they could be detected in the same excised patches. Our data provide substantial evidence for the hypothesis. First, the non-selective K⁺ channel blocker TEA and several selective blockers of BK(Ca²⁺) channels (paxilline, penitrem A and verruculogen) prevented the inhibition produced by NMDA and D-serine. Second, this was a true reversal of the inhibition and not an unrelated increase in opioid release (caused, for example, by an increase in neuronal firing when K⁺ are blocked) because, in the absence of NMDA, paxilline did not increase MOR internalization evoked by electrical stimulation. Third, although iberiotoxin did not prevent the inhibition produced by NMDA when applied to the slices for 5 min, it did when applied to the slices for 30 min at higher concentrations, indicating the involvement of particular subtypes of BK(Ca²⁺) channels with slow association rates for iberiotoxin, for example, those containing the β 4 subunit (Meera et al., 2000). Fourth, the BK(Ca²⁺) channel opener NS-1619 inhibited the evoked MOR internalization with a potency similar to that found in other functional BK(Ca²⁺) channel assays (Sheldon et al., 1997; Malysz et al., 2004). NS-1619 has effects other than opening BK(Ca²⁺) channels: it inhibits L-type voltage-gated Ca²⁺ channels in smooth muscle (Sheldon et al., 1997) and releases Ca²⁺ from intra-cellular stores (Yamamura et al., 2001). However, the inhibition of evoked MOR internalization by NS-1619 was fully prevented in slices preincubated with iberiotoxin, showing that it was indeed mediated by the opening of BK(Ca²⁺) channels.

In agreement with our findings, BK(Ca²⁺) channels have been shown to regulate secretion in exocrine tissues (Petersen and Maruyama, 1984; Dopico et al., 1999), as well as regulate transmitter release at the frog neuromuscular junction (Robitaille and Charlton, 1992) and at

rat hippocampal synapses (Raffaelli et al., 2004). There is evidence that BK(Ca²⁺) channels are located in presynaptic nerve endings, where they are closely associated with Ca²⁺ channels (Robitaille et al., 1993; Raffaelli et al., 2004).

The fact that NMDA inhibits MOR internalization evoked with 50 mM KCl may seem inconsistent with a mechanism involving K⁺ channel opening. However, it should be noted that the large conductance of the BK(Ca²⁺) channels is very effective to clamp the membrane potential to the equilibrium potential for K⁺. Thus, when the BK channels are open the permeability for K⁺ becomes so large compared with that of other ions that the membrane potential would be practically the same as the equilibrium potential for K⁺, i.e. -104 mV in normal aCSF and -29 mV in aCSF with 50 mM KCl (calculated from the Nernst equation assuming [K⁺]_i=155 mM (Hille, 1991)). When BK channels are closed, the equilibrium potential of Na⁺ also contributes to the membrane potential, making it more positive than the K⁺ equilibrium potential. Therefore, whereas with BK(Ca²⁺) channels closed 50 mM KCl depolarizes the membrane to a potential much more positive than -29 mV, with BK(Ca²⁺) channels open 50 mM KCl depolarizes the membrane only to -29 mV, which probably is not enough to evoke opioid release.

It should be noted, however, that the reversal of NMDA receptor inhibition by BK(Ca²⁺) channels blockers does not appear to be complete, even in the presence of relatively high concentrations of paxilline or after preincubation with high concentrations of iberiotoxin. This suggests that additional mechanisms may contribute to NMDA receptor inhibition of opioid release. Since TEA fully prevented this inhibition, it is possible that other K⁺ channels participate in this effect. Other possible mechanisms that we are currently investigating include activation by NMDA receptors of the nitric oxide synthase (Malmberg and Yaksh, 1993; Prast and Philippu, 2001) or the phospholipase A₂/cyclooxygenase signaling pathways (Svensson and Yaksh, 2002).

Neuronal pathways involved in NMDA receptor inhibition of opioid release

The neuronal pathways responsible for the release of opioids in the dorsal horn were investigated in a previous study (Song and Marvizon, 2003b). Electrical stimulation of the dorsal root or the dorsolateral funiculus failed to evoke MOR internalization in spinal cord slices, indicating that in our conditions opioids are not released by primary afferents, second order neurons or medullary descending axons. Hence, in the present study MOR internalization evoked by dorsal horn stimulation likely reflects the release of opioids from dorsal horn interneurons, which have been shown to contain enkephalins and dynorphins (Todd and Spike, 1993).

It remains to be clarified what is the location of the NMDA receptors and BK(Ca²⁺) channels involved in the inhibition of opioid release. One possibility is that they are located extrasynaptically in the soma and dendrites of the opioid-releasing neurons (Fig. 9), as happens in the olfactory bulb (Isaacson and Murphy, 2001). Opioid release from these neurons requires high frequency firing of action potentials (Song and Marvizon, 2003b), which would be prevented by the hyperpolarization produced by BK(Ca²⁺) channel opening. These extrasynaptic NMDA receptor would be activated by volume transmission, perhaps by "spillover" glutamate released by nociceptive primary afferents signaling intense noxious stimuli. Indeed, many dorsal horn neurons have extrasynaptic NMDA receptors (Momiya, 2000; Cull-Candy et al., 2001). Another possibility is that the NMDA receptors and the BK (Ca²⁺) channels are located presynaptically (Fig. 9), where hyperpolarization could prevent the opening of voltage-gated Ca²⁺ channels that mediate opioid release. However, this possibility is less likely because Ca²⁺ entry through the NMDA receptors would contribute to opioid release.

A related question is what neural pathways activate these NMDA receptors. One possibility is that they are activated by primary afferents (Fig. 9), either because opioid-containing dorsal horn neurons receive synapses from C-fibers, or because intense noxious stimulation produces enough glutamate release from nociceptors to activate extrasynaptic NMDA receptors by volume transmission. Alternatively, these NMDA receptors may be activated by medullary descending pathways (although it is not known whether they are glutamatergic) or intrinsic spinal circuits (Fig. 9). The first possibility is interesting because it could explain why some noxious stimuli produce opioid release only in spinal cord segments other than the one receiving the noxious signal (i.e. “heterosegmental” release). Thus, Met-enkephalin release occurred in the lumbar but not in the cervical spinal cord during noxious mechanical stimulation of the muzzle or forepaw, whereas the opposite was found during stimulation of the hindpaw (Le Bars et al., 1987). These results were attributed to the existence of “diffuse noxious inhibitory controls” from the brainstem that drive opioid release throughout the spinal cord (Rivot et al., 1979; Morgan et al., 1991; Budai and Fields, 1998). However, it was left unexplained why opioid release did not occur in the spinal segment receiving the noxious stimulus. A possible explanation is that glutamate released from nociceptive afferents during intense noxious stimulation activates NMDA receptors that inhibit opioid release. This possibility could be explored by assessing whether NMDA antagonists increase MOR internalization in spinal segments receiving noxious stimulation.

Possible role in pain modulation

Spinal NMDA receptors produce sensitization to pain (Dingledine et al., 1999; Brauner-Osborne et al., 2000; South et al., 2003), participate in the “wind-up” of dorsal horn neurons (Dickenson and Sullivan, 1987; Kristensen et al., 1992; Yaksh, 1993) and mediate long-term potentiation of synapses between primary afferents and second order neurons (Randic et al., 1993). Because MORs in the dorsal horn produce analgesia (Yaksh, 1997), inhibition of spinal opioid release could contribute to the hyperalgesic actions of spinal NMDA receptors. If this is true, then NMDA antagonists or BK(Ca²⁺) channel blockers may produce analgesia by disinhibiting spinal opioid release. Although the therapeutic use of NMDA receptor antagonists presents numerous problems (Dingledine et al., 1999; Brauner-Osborne et al., 2000), it may be possible to target the particular subtype of NMDA receptors responsible for inhibiting opioid release. Thus, whereas extrasynaptic NMDA receptors in dorsal horn neurons contain the NR2B and NR2D subunits (Momiya, 2000; Cull-Candy et al., 2001), we have ruled out the involvement of NR2B-containing NMDA receptors because of the lack of effect of the NR2B-selective antagonist ifenprodil, leaving NR2D-containing NMDA receptors as the candidates to mediate inhibition of opioid release. This idea is supported by the high potency of NMDA in our experiments (2–10 μM), more consistent with its potency at NR2D-containing NMDA receptors (9 μM) than at other NMDA receptors (22–36 μM) (Brauner-Osborne et al., 2000). Likewise, BK(Ca²⁺) channel blockers have important cardiovascular effects (Calderone, 2002), but our results suggest the involvement of a particular “iberiotoxin-insensitive” subtype that could be selectively targeted by new drugs to produce analgesia.

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References

- Aoki T, Baraban SC. Properties of a calcium-activated K(+) current on interneurons in the developing rat hippocampus. *J Neurophysiol* 2000;83:3453–3461. [PubMed: 10848561]

- Arvidsson U, Riedl M, Chakrabarti S, Lee JH, Nakano AH, Dado RJ, Loh HH, Law PY, Wessendorf MW, Elde R. Distribution and targeting of a mu-opioid receptor MOR1 in brain and spinal cord. *J Neurosci* 1995;15:3328–3341. [PubMed: 7751913]
- Ballet S, Mauborgne A, Gouarderes C, Bourgoin AS, Zajac JM, Hamon M, Cesselin F. The neuropeptide FF analogue, 1DME, enhances in vivo met-enkephalin release from the rat spinal cord. *Neuropharmacology* 1999;38:1317–1324. [PubMed: 10471085]
- Bourgoin S, Collin E, Benoliel JJ, Chantrel D, Mauborgne A, Pohl M, Hamon M, Cesselin F. Opioid control of the release of Met-enkephalin-like material from the rat spinal cord. *Brain Res* 1991;551:178–184. [PubMed: 1655159]
- Brauner-Osborne H, Egebjerg J, Nielsen EO, Madsen U, Krosgaard-Larsen P. Ligands for glutamate receptors: design and therapeutic prospects. *J Med Chem* 2000;43:2609–2645. [PubMed: 10893301]
- Breakwell NA, Huang L, Rowan MJ, Anwyl R. DCG-IV inhibits synaptic transmission by activation of NMDA receptors in area CA1 of rat hippocampus. *Eur J Pharmacol* 1997;322:173–178. [PubMed: 9098684]
- Budai D, Fields HL. Endogenous opioid peptides acting at mu-opioid receptors in the dorsal horn contribute to midbrain modulation of spinal nociceptive neurons. *J Neurophysiol* 1998;79:677–687. [PubMed: 9463431]
- Calderone V. Large-conductance, Ca(2+)-activated K(+) channels: function, pharmacology and drugs. *Curr Med Chem* 2002;9:1385–1395. [PubMed: 12132994]
- Choi DW, Koh JY, Peters S. Pharmacology of glutamate neurotoxicity in cortical cell culture: attenuation by NMDA antagonists. *J Neurosci* 1988;8:185–196. [PubMed: 2892896]
- Choi DW, Maulucci-Gedde M, Kriegstein AR. Glutamate neurotoxicity in cortical cell culture. *J Neurosci* 1987;7:357–368. [PubMed: 2880937]
- Chou J, Tang J, Del Rio J, Yang HY, Costa E. Action of peptidase inhibitors on methionine5-enkephalin-arginine6-phenyl-alanine7 (YGGFMRF) and methionine5-enkephalin (YGGFM) metabolism and on electroacupuncture antinociception. *J Pharmacol Exp Ther* 1984;230:349–352. [PubMed: 6205138]
- Collin E, Mauborgne A, Bourgoin S, Benoliel JJ, Hamon M, Cesselin F. Morphine reduces the release of met-enkephalin-like material from the rat spinal cord in vivo by acting at delta opioid receptors. *Neuropeptides* 1994;27:75–83. [PubMed: 7969822]
- Cull-Candy S, Brickley S, Farrant M. NMDA receptor subunits: diversity, development and disease. *Curr Opin Neurobiol* 2001;11:327–335. [PubMed: 11399431]
- Dickenson AH, Sullivan AF. Evidence for a role of the NMDA receptor in the frequency dependent potentiation of deep rat dorsal horn nociceptive neurones following C fibre stimulation. *Neuropharmacology* 1987;26:1235–1238. [PubMed: 2821443]
- Dingledine R, Borges K, Bowie D, Traynelis SF. The glutamate receptor ion channels. *Pharmacol Rev* 1999;51:7–61. [PubMed: 10049997]
- Dopico AM, Widmer H, Wang G, Lemos JR, Treistman SN. Rat supraoptic magnocellular neurones show distinct large conductance, Ca2+-activated K+channel subtypes in cell bodies versus nerve endings. *J Physiol* 1999;519 (Pt 1):101–114. [PubMed: 10432342]
- Eckersell CB, Popper P, Micevych PE. Estrogen-induced alteration of μ -opioid receptor immunoreactivity in the medial preoptic nucleus and medial amygdala. *J Neurosci* 1998;18:3967–3976. [PubMed: 9570823]
- Fournie-Zaluski MC, Coric P, Turcaud S, Lucas E, Noble F, Maldonado R, Roques BP. “Mixed inhibitor-prodrug” as a new approach toward systemically active inhibitors of enkephalin-degrading enzymes”. *J Med Chem* 1992;35:2473–2481. [PubMed: 1352352]
- Hille B (1991) Ionic channels of excitable membranes. Sunderland, MA: Sinauer Associates Inc.
- Hollywood MA, McCloskey KD, McHale NG, Thornbury KD. Characterization of outward K(+) currents in isolated smooth muscle cells from sheep urethra. *Am J Physiol Cell Physiol* 2000;279:C420–C428. [PubMed: 10913009]
- Isaacson JS, Murphy GJ. Glutamate-mediated extrasynaptic inhibition: direct coupling of NMDA receptors to Ca(2+)-activated K+ channels. *Neuron* 2001;31:1027–1034. [PubMed: 11580901]
- Johnson BG, Wright RA, Arnold MB, Wheeler WJ, Ornstein PL, Schoepp DD. [3H]-LY341495 as a novel antagonist radioligand for group II metabotropic glutamate (mGlu) receptors: characterization

- of binding to membranes of mGlu receptor subtype expressing cells. *Neuropharmacology* 1999;38:1519–1529. [PubMed: 10530814]
- Kew JNC, Richards JG, Mutel V, Kemp JA. Developmental changes in NMDA receptor glycine affinity and ifenprodil sensitivity reveal three distinct populations of NMDA receptors in individual rat cortical neurons. *J Neurosci* 1998;18:1935–1943. [PubMed: 9482779]
- Kingston AE, Ornstein PL, Wright RA, Johnson BG, Mayne NG, Burnett JP, Belagaje R, Wu S, Schoepp DD. LY341495 is a nanomolar potent and selective antagonist of group II metabotropic glutamate receptors. *Neuropharmacology* 1998;37:1–12. [PubMed: 9680254]
- Knaus HG, McManus OB, Lee SH, Schmalhofer WA, Garcia-Calvo M, Helms LM, Sanchez M, Giangiacomo K, Reuben JP, Smith AB III. Tremorgenic indole alkaloids potently inhibit smooth muscle high-conductance calcium-activated potassium channels. *Biochemistry* 1994;33:5819–5828. [PubMed: 7514038]
- Kristensen JD, Svensson B, Gordh TJ. The NMDA-receptor antagonist CPP abolishes neurogenic “wind-up pain” after intrathecal administration in humans. *Pain* 1992;51:249–253. [PubMed: 1484720]
- Lao L, Song B, Marvizon JCG. Neurokinin release produced by capsaicin acting on the central terminals and axons of primary afferents: relationship with NMDA and GABA_B receptors. *Neuroscience* 2003;121:667–680. [PubMed: 14568027]
- Le Bars D, Bourgoin S, Clot AM, Hamon M, Cesselin F. Noxious mechanical stimuli increase the release of Met-enkephalin-like material heterosegmentally in the rat spinal cord. *Brain Res* 1987;402:188–192. [PubMed: 3828785]
- Li G, Cheung DW. Effects of paxilline on K⁺ channels in rat mesenteric arterial cells. *Eur J Pharmacol* 1999;372:103–107. [PubMed: 10374720]
- Liu H, Mantyh PW, Basbaum AI. NMDA-receptor regulation of substance P release from primary afferent nociceptors. *Nature* 1997;386:721–724. [PubMed: 9109489]
- Malcangio M, Fernandes K, Tomlinson DR. NMDA receptor activation modulates evoked release of substance P from rat spinal cord. *Br J Pharmacol* 1998;125:1625–1626. [PubMed: 9886751]
- Malmberg AB, Yaksh TL. Spinal nitric oxide synthesis inhibition blocks NMDA-induced thermal hyperalgesia and produces antinociception in the formalin test in rats. *Pain* 1993;54:291–300. [PubMed: 8233543]
- Malysz J, Buckner SA, Daza AV, Milicic I, Perez-Medrano A, Gopalakrishnan M. Functional characterization of large conductance calcium-activated K(+) channel openers in bladder and vascular smooth muscle. *Naunyn Schmiedebergs Arch Pharmacol* 2004;369:481–489. [PubMed: 15095032]
- Marvizon JC, Baudry M. Receptor activation by two agonists: analysis by nonlinear regression and application to N-methyl-D-aspartate receptors. *Anal Biochem* 1993;213:3–11. [PubMed: 7902027]
- Marvizon JC, Grady EF, Waszak-McGee J, Mayer EA. Internalization of μ -opioid receptors in rat spinal cord slices. *Neuroreport* 1999;10:2329–2334. [PubMed: 10439458]
- Marvizon JC, Martinez V, Grady EF, Bunnett NW, Mayer EA. Neurokinin 1 receptor internalization in spinal cord slices induced by dorsal root stimulation is mediated by NMDA receptors. *J Neurosci* 1997;17:8129–8136. [PubMed: 9334388]
- Marvizon JC, Song B, Grady E, Bunnett N. CGRP receptors inhibit opioid release in the rat spinal cord. *Soc Neurosci Abstr* 2005;31:982.19.
- Marvizon JCG, Wang X, Lao L, Song B. Effect of peptidases on the ability of exogenous and endogenous neurokinins to produce neurokinin 1 receptor internalization in the rat spinal cord. *Br J Pharmacol* 2003;140:1389–1398. [PubMed: 14623771]
- Mauborgne A, Bourgoin S, Polienor H, Roumy M, Simonnet G, Zajac JM, Cesselin F. The neuropeptide FF analogue, 1DMe, acts as a functional opioid autoreceptor antagonist in the rat spinal cord. *Eur J Pharmacol* 2001;430:273–276. [PubMed: 11711042]
- McBain CJ, Kleckner NW, Wyrick S, Dingledine R. Structural requirements for activation of the glycine coagonist site of N-methyl-D-aspartate receptors expressed in *Xenopus* oocytes. *Mol Pharmacol* 1989;36:556–565. [PubMed: 2554111]
- Meera P, Wallner M, Toro L. A neuronal beta subunit (KCNMB4) makes the large conductance, voltage- and Ca²⁺-activated K⁺ channel resistant to charybdotoxin and iberiotoxin. *Proc Natl Acad Sci U S A* 2000;97:5562–5567. [PubMed: 10792058]

- Mills RH, Sohn RK, Micevych PE. Estrogen-induced μ -opioid receptor internalization in the medial preoptic nucleus is mediated via neuropeptide Y-Y1 receptor activation in the arcuate nucleus of female rats. *J Neurosci* 2004;24:947–955. [PubMed: 14749439]
- Momiyama A. Distinct synaptic and extrasynaptic NMDA receptors identified in dorsal horn neurones of the adult rat spinal cord. *J Physiol* 2000;523 (Pt 3):621–628. [PubMed: 10718742]
- Morgan MM, Gold MS, Liebeskind JC, Stein C. Periaqueductal gray stimulation produces a spinally mediated, opioid antinociception for the inflamed hindpaw of the rat. *Brain Res* 1991;545:17–23. [PubMed: 1860042]
- Motulsky H, Christopoulos A (2003) Fitting models to biological data using linear and nonlinear regression. San Diego, CA: GraphPad Software, Inc.
- Noble F, Coric P, Fournie-Zaluski MC, Roques BP. Lack of physical dependence in mice after repeated systemic administration of the mixed inhibitor prodrug of enkephalin-degrading enzymes, RB101. *Eur J Pharmacol* 1992a;223:91–96. [PubMed: 1478261]
- Noble F, Soleilhac JM, Soroca-Lucas E, Turcaud S, Fournie-Zaluski MC, Roques BP. Inhibition of the enkephalin-metabolizing enzymes by the first systemically active mixed inhibitor prodrug RB 101 induces potent analgesic responses in mice and rats. *J Pharmacol Exp Ther* 1992b;261:181–190. [PubMed: 1560364]
- Noble F, Turcaud S, Fournie-Zaluski MC, Roques BP. Repeated systemic administration of the mixed inhibitor of enkephalin-degrading enzymes, RB101, does not induce either antinociceptive tolerance or cross-tolerance with morphine. *Eur J Pharmacol* 1992c;223:83–89. [PubMed: 1478260]
- Noble F, Roques BP. CCK-B receptor: chemistry, molecular biology, biochemistry and pharmacology. *Prog Neurobiol* 1999;58:349–379. [PubMed: 10368033]
- Norris PJ, Smith CC, De Belleruche J, Bradford HF, Mantle PG, Thomas AJ, Penny RH. Actions of tremorgenic fungal toxins on neurotransmitter release. *J Neurochem* 1980;34:33–42. [PubMed: 6108984]
- Patierno S, Zellalem W, Ho A, Parsons CG, Lloyd KC, Tonini M, Sternini C. N-methyl-D-aspartate receptors mediate endogenous opioid release in enteric neurons after abdominal surgery. *Gastroenterology* 2005;128:2009–2019. [PubMed: 15940633]
- Petersen OH, Maruyama Y. Calcium-activated potassium channels and their role in secretion. *Nature* 1984;307:693–696. [PubMed: 6321995]
- Prast H, Philippu A. Nitric oxide as modulator of neuronal function. *Prog Neurobiol* 2001;64:51–68. [PubMed: 11250062]
- Przewlocka B, Lason W, Dziedzicka M. Modulation of prodynorphin peptides release from the rat spinal cord in vitro. *Neuropeptides* 1990;16:201–206. [PubMed: 1980349]
- Raffaelli G, Saviane C, Mohajerani MH, Pedarzani P, Cherubini E. BK potassium channels control transmitter release at CA3-CA3 synapses in the rat hippocampus. *J Physiol* 2004;557:147–157. [PubMed: 15034127]
- Randic M, Jiang MC, Cerne R. Long-term potentiation and long-term depression of primary afferent neurotransmission in the rat spinal cord. *J Neurosci* 1993;13:5228–5241. [PubMed: 8254370]
- Raymond SA, Thalhammer JG, Popitz-Bergez F, Strichartz GR. Changes in axonal impulse conduction correlate with sensory modality in primary afferent fibers in the rat. *Brain Res* 1990;526:318–321. [PubMed: 2257488]
- Rivot JP, Chaouch A, Besson JM. The influence of naloxone on the C fiber response of dorsal horn neurons and their inhibitory control by raphe magnus stimulation. *Brain Res* 1979;176:355–364. [PubMed: 227529]
- Robitaille R, Charlton MP. Presynaptic calcium signals and transmitter release are modulated by calcium-activated potassium channels. *J Neurosci* 1992;12:297–305. [PubMed: 1370323]
- Robitaille R, Garcia ML, Kaczorowski GJ, Charlton MP. Functional colocalization of calcium and calcium-gated potassium channels in control of transmitter release. *Neuron* 1993;11:645–655. [PubMed: 7691106]
- Rothman SM, Olney JW. Excitotoxicity and the NMDA receptor. *Trends Neurosci* 1987;10[7]:299–302.
- Sattler R, Xiong Z, Lu WY, MacDonald JF, Tymianski M. Distinct roles of synaptic and extrasynaptic NMDA receptors in excitotoxicity. *J Neurosci* 2000;20:22–33. [PubMed: 10627577]

- Sheldon JH, Norton NW, Argentieri TM. Inhibition of guinea pig detrusor contraction by NS-1619 is associated with activation of BKCa and inhibition of calcium currents. *J Pharmacol Exp Ther* 1997;283:1193–1200. [PubMed: 9399993]
- Song B, Marvizon JC. Peptidases prevent mu-opioid receptor internalization in dorsal horn neurons by endogenously released opioids. *J Neurosci* 2003a;23:1847–1858. [PubMed: 12629189]
- Song B, Marvizon JCG. Dorsal horn neurons firing at high frequency, but not primary afferents, release opioid peptides that produce μ -opioid receptor internalization in the rat spinal cord. *J Neurosci* 2003b; 23:9171–9184. [PubMed: 14534251]
- South SM, Kohno T, Kaspar BK, Hegarty D, Vissel B, Drake CT, Ohata M, Jenab S, Sailer AW, Malkmus S, Masuyama T, Horner P, Bogulavsky J, Gage FH, Yaksh TL, Woolf CJ, Heinemann SF, Inturrisi CE. A conditional deletion of the NR1 subunit of the NMDA receptor in adult spinal cord dorsal horn reduces NMDA currents and injury-induced pain. *J Neurosci* 2003;23:5031. [PubMed: 12832526]
- Spike RC, Puskas Z, Sakamoto H, Stewart W, Watt C, Todd AJ. MOR-1-immunoreactive neurons in the dorsal horn of the rat spinal cord: evidence for nonsynaptic innervation by substance P-containing primary afferents and for selective activation by noxious thermal stimuli. *Eur J Neurosci* 2002;15:1306–1316. [PubMed: 11994125]
- Svensson CI, Yaksh TL. The spinal phospholipase-cyclooxygenase-prostanoid cascade in nociceptive processing. *Annu Rev Pharmacol Toxicol* 2002;42:553–583. [PubMed: 11807183]
- Teoh H, Malcangio M, Fowler LJ, Bowery NG. Evidence for release of glutamic acid, aspartic acid and substance P but not gamma-aminobutyric acid from primary afferent fibres in rat spinal cord. *Eur J Pharmacol* 1996;302:27–36. [PubMed: 8790988]
- Todd AJ, Spike RC. The localization of classical transmitters and neuropeptides within neurons in laminae I–II of the mammalian spinal dorsal horn. *Prog Neurobiol* 1993;41:609–645. [PubMed: 7904359]
- Trafton JA, Abbadie C, Marek K, Basbaum AI. Postsynaptic signaling via the mu-opioid receptor: Responses of dorsal horn neurons to exogenous opioids and noxious stimulation. *J Neurosci* 2000;20:8578–8584. [PubMed: 11102461]
- Uyama Y, Ishida M, Shinozaki H. DCG-IV, a potent metabotropic glutamate receptor agonist, as an NMDA receptor agonist in the rat cortical slice. *Brain Res* 1997;752:327–330. [PubMed: 9106476]
- Whistler JL, Chuang HH, Chu P, Jan LY, von Zastrow M. Functional dissociation of mu opioid receptor signaling and endocytosis: implications for the biology of opiate tolerance and addiction. *Neuron* 1999;23:737–746. [PubMed: 10482240]
- Yaksh TL. The spinal pharmacology of facilitation of afferent processing evoked by high-threshold afferent input of the postinjury pain state. *Curr Opin Neurol Neurosurg* 1993;6:250–256. [PubMed: 8097639]
- Yaksh TL. Pharmacology and mechanisms of opioid analgesic activity. *Acta Anaesthesiol Scand* 1997;41:94–111. [PubMed: 9061092]
- Yaksh TL, Terenius L, Nyberg F, Jhamandas K, Wang JY. Studies on the release by somatic stimulation from rat and cat spinal cord of active materials which displace dihydromorphine in an opiate-binding assay. *Brain Res* 1983;268:119–128. [PubMed: 6860955]
- Yamamura H, Ohi Y, Muraki K, Watanabe M, Imaizumi Y. BK channel activation by NS-1619 is partially mediated by intracellular Ca^{2+} release in smooth muscle cells of porcine coronary artery. *Br J Pharmacol* 2001;132:828–834. [PubMed: 11181423]

Fig. 1.

NMDA receptor agonists inhibit opioid release. (A) MOR internalization evoked by dorsal horn stimulation at 500 Hz was inhibited by NMDA alone ($IC_{50}=2 \mu\text{M}$, 95% C.I. 1–5 μM) or in the presence of 10 μM D-serine ($IC_{50}=10 \mu\text{M}$, 95% C.I. 4–26 μM). The effect of D-serine was significant ($P<0.001$, Bonferroni's post-test of a two-way ANOVA) in the absence of NMDA, but not significant at any other concentration of NMDA. NMDA and D-serine were superfused for 2.5 min before stimulation. (B) Time course of the inhibition by NMDA plus D-serine (both 10 μM), which were superfused to the slices for the times indicated, ending with the stimulation (no NMDA and D-serine were applied for the "0 s" time point). The curve represents fitting of the data to an exponential decay function ($K=0.025\pm0.011 \text{ s}^{-1}$, $t_{1/2}=28 \text{ s}$).

(C) MOR internalization evoked by incubating the slices with 50 mM KCl was inhibited by 30 μ M NMDA, and this inhibition was prevented by 1 μ M MK-801. NMDA, MK-801 and 50 mM KCl were applied together for 2 min. (D) MOR internalization evoked by incubating the slices with 20 μ M veratridine was inhibited by 30 μ M NMDA, and this inhibition was attenuated by 5 μ M CPP. NMDA and CPP were applied for 2 min, first 1 min alone and then 1 min with veratridine. Numbers indicate the number of slices used for each data point. * $P < 0.05$, *** $P < 0.001$ compared with control (0 s in B); $\dagger\dagger$ $P < 0.01$ compared with NMDA.

Fig. 2.

Confocal images of MOR neurons in the dorsal horn after electrical stimulation. Slices were stimulated at the dorsal horn at 500 Hz while being superfused with aCSF alone (A), with 10 μ M NMDA (B), or 10 μ M D-serine (C). MOR neurons with internalization are indicated by “*,” and those without internalization by “#.” Roman numerals indicate the approximate position of laminae I–III, and “central” and “medial” the location of these areas of the dorsal horn. Images in the main panels were taken with a 20 $\times$ objective (scale bars=50 μ m), and those in the insets with a 100 $\times$ objective (scale bars=10 μ m). Images correspond to the medial and central dorsal horn because few MOR neurons with internalization were usually found in the lateral dorsal horn.

Fig. 3.

Reversal by NMDA receptor antagonists of the inhibition of MOR internalization by NMDA. Dorsal horn stimulation at 500 Hz evoked MOR internalization (dotted line) that was inhibited by 10 μ M NMDA and D-serine (control) superfused for 2.5 min before stimulation. This inhibition was prevented by the NMDA receptor competitive antagonists CPP (1 μ M) and SDZ-220-040 (0.1 μ M), the glycine site antagonist 5,7-dichloro-kynurenic acid (5,7-Cl-Kyn, 10 μ M), and the channel blockers MK-801 (1 μ M) and ketamine (10 μ M), but not by the NR2B selective antagonist ifenprodil (10 μ M). It was also prevented by the AMPA/kainate receptor antagonist NBQX (10 μ M). Antagonists were superfused starting 5 min before and ending 5 min after the stimulation. ** $P < 0.01$, *** $P < 0.001$, compared with control. Numbers indicate the number of slices used for each bar.

Fig. 4.

MOR neurons representative of the effect of NMDA receptor antagonists. Slices were stimulated at 500 Hz at the dorsal horn in the presence of 10 μ M NMDA and D-serine and no other addition (A, control), or 1 μ M CPP (B), 10 μ M 5,7-dichloro-kynurenic acid (5,7-Cl-Kyn, C), 1 μ M MK-801 (D), 10 μ M ketamine (E), or 10 μ M ifenprodil (F). MOR neurons with internalization are indicated by “*,” and those without internalization by “#.” The inhibition of MOR internalization produced by NMDA plus D-serine was prevented by all the antagonists except ifenprodil. Scale bar=10 μ m.

Fig. 5.

NMDA receptor antagonists and paxilline did not increase MOR internalization evoked by different stimulation frequencies. Slices were stimulated at the dorsal horn with 1000 pulses delivered at 10, 30 or 500 Hz, while being superfused with aCSF alone (control), NMDA receptor antagonists (1 μ M MK-801 for 10 Hz and 500 Hz, 1 μ M CPP for 30 Hz) or the BK (Ca^{2+}) channel blocker paxilline (1 μ M, 30 Hz and 500 Hz only). MOR internalization increased with the frequency of stimulation ($P < 0.0001$), but was not affected by the NMDA receptor antagonists or paxilline. Numbers indicate the number of slices used for each data point. NMDA antagonists and paxilline were superfused starting 5 min before and ending 5 min after the stimulation.

Fig. 6.

Effects of BK(Ca²⁺) channels blockers and opener. (A) Dorsal horn stimulation at 500 Hz evoked MOR internalization (dotted line), which was inhibited by 10 μ M NMDA and D-serine (“control”) superfused for 2.5 min ending with the stimulation. This inhibition was prevented by the non-selective K⁺ channel blocker TEA (2 mM), and by the selective BK(Ca²⁺) channel blockers paxilline, penitrem A and verruculogen (all 1 μ M). Vehicle used for paxilline, penitrem A and verruculogen (0.1% DMSO) did not affect the inhibition produced by NMDA and D-serine. These blockers were superfused starting 5 min before and ending 5 min after stimulation. Iberitoxin (IbTx) superfused to the slices at 0.3 μ M using the same time line (IbTx 5 min) did not prevent the inhibition. However, IbTx prevented the inhibition when preincubated with the slices at 3 μ M for 30 min and then omitted from the superfusate (IbTx 30 min). (B) Concentration-response for paxilline. Conditions are the same as in panel A. EC₅₀=101 nM (95% C.I. 13–782 nM); “top” = 40 $\pm$ 4%. The value of “top” was significantly lower than in the absence of NMDA and D-serine (53 $\pm$ 2%, dotted line), as determined by using Akaike’s Information Criterion (Motulsky and Christopoulos, 2003) to compare the fitting with “top” constrained to 53% (12% probability of being correct) to the fitting with “top” unconstrained (88% probability of being correct). (C) The BK(Ca²⁺) channel opener NS-1619, superfused for 5 min before stimulation, inhibited MOR internalization with an IC₅₀ of 9 μ M (95% C.I. 2–48 μ M). In slices preincubated for 30 min with 3 μ M IbTx, 100 μ M NS-1619 no longer inhibited MOR internalization ($^{\dagger\dagger}P<0.01$ compared with 100 μ M NS-1619). Two-way ANOVA and Bonferroni’s post-test for each panel: ** $P<0.01$, *** $P<0.001$ compared with “control” or “0 M.” Numbers indicate the number of slices used for each data point.

Fig. 7.

MOR neurons representative of the effect of BK(Ca²⁺) channel blockers and opener. (A–E) Slices were stimulated at 500 Hz at the dorsal horn in the presence of 10 μ M NMDA and D-serine. TEA (2 mM, A), paxilline (1 μ M, B) and penitrem A (1 μ M, C) prevented the inhibition of MOR internalization by NMDA and D-serine. Iberitoxin (IbTx) did not prevent the inhibition when superfused to the slices at 0.3 μ M for 5 min (D), but it did when preincubated with the slices at 3 μ M for 30 min (E). (F) Slice stimulated at 500 Hz at the dorsal horn in the presence of the BK(Ca²⁺) channel opener NS-1619 (30 μ M), which inhibited the evoked MOR internalization. MOR neurons with internalization are indicated by “*,” and those without internalization by “#.” Scale bar=10 μ m.

Fig. 8.

Lack of effect of mGluRs on evoked MOR internalization. The dorsal horn was electrically stimulated at 500 Hz, and the compounds indicated were superfused starting 5 min before and ending 5 min after stimulation. (A) L-Glutamate (L-Glu, 10 μM) and the group II mGluR agonist DCG-IV (10 μM) inhibited the evoked MOR internalization, whereas the group I mGluR agonist DHPG (30 μM) and the group III mGluR agonist L-AP4 (10 μM) produced no effect. Inhibition by 10 μM DCG-IV was prevented by the NMDA receptor antagonist AP5 (100 μM), but not by the selective group II mGluR antagonist LY-341495 (1 μM). *** $P < 0.001$ compared with control; ††† $P < 0.001$ compared with DCG-IV. (B) Concentration-response for DCG-IV, yielding an IC₅₀ of 6.2 μM (95% C.I. 4.7–8.1 μM) and a Hill coefficient of 2.4 ± 0.6 . Numbers indicate the number of slices used for each data point.

Fig. 9.

Diagram of the proposed mechanism for NMDA receptor inhibition of opioid release. Somata are represented by stars, presynaptic terminals by rounded triangles. Opioids (opi) are released from dorsal horn interneurons, activating MORs in other interneurons. Glutamate (Glu) released from C-fiber terminals activate NMDA receptors (NMDA-R) in the somata or in the presynaptic terminals of the opioid-containing neurons. Ca²⁺ entry through the NMDA receptors triggers the opening of BK(Ca²⁺) channels (BK), which produce hyperpolarization, inhibiting opioid release. Glu might also be released by the spinal terminals of ON-cells of the nucleus raphe magnus (NRM).

Table 1

Drugs without effect on MOR internalization

Stimulus	Drug	[]	Drug action	% MOR internalization	n
Veratridine	Control	—	—	77±4	8
	Isoguvacine	100 μ M	GABA _A agonist	57±15	5
	Baclofen	20 μ M	GABA _B agonist	82±5	3
	DPDPE	1 μ M	δ -Opioid agonist	66±4	5
100 Hz	NBQX	10 μ M	AMPA antagonist	86±1	3
	Control	—	—	56±8	6
	Isoguvacine	100 μ M	GABA _A agonist	57±17	3
	DPDPE	1 μ M	δ -Opioid agonist	64±2	4
500 Hz	NBQX	5 μ M	AMPA antagonist	69±11	3
	Control	—	—	53±2	13
	CCK-8	1 nM	CCK-A and CCK-B agonist	43±7	3
	CCK-8	10 nM	—	54±5	3

Opioid release and subsequent MOR internalization were evoked in spinal cord slices using veratridine (10 μ M for 2 min), or electrical stimulation of the dorsal horn (3000 pulses at 100 Hz, or 1000 pulses at 500 Hz). “% MOR internalization” refers to the percentage of MOR neurons in lamina II with internalization. For each stimulation mode, one-way ANOVA and Bonferroni’s post-tests revealed no significant effects of any of the drugs ($P>0.05$). When using electrical stimulation, MOR internalization was routinely measured in the contralateral dorsal horn, and it was not affected by any of these drugs.