Regulation of NMDA Receptor Subtypes in Pain Processing Neurons Within Lamina I of the Spinal Cord

by

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Abstract

Chronic pain is a condition affecting 18.9% of Canadians over the age of 18 (Schopflocher et al., 2011). Despite the prevalence of chronic pain, there are currently no effective and safe treatment options. In lamina I of the spinal cord dorsal horn, excitatory N-methyl-D-aspartate receptors (NMDARs) that contain the GluN2B subunit are phosphorylated and potentiated by Src family kinase (SFK) through BDNF signalling in chronic pain models. However, the contribution of GluN2B to synaptic responses in the brain makes it a difficult target to specifically treat pain. The GluN2D subunit of NMDAR is highly expressed embryonically but uncommon at mature synapses in the brain, while playing a functional role in mature lamina I neurons. This study aimed to test whether GluN2D-containing NMDARs are necessary for BDNF-mediated potentiation of NMDARs, whether NMDARs containing the GluN2D subunit are functionally present at juvenile lamina I synapses and whether these receptors are directly potentiated by SFKs. Pharmacological inhibition of either GluN2D or GluN2B completely prevented potentiation by BDNF in adult spinal cord slices. Inhibition of GluN2A- and GluN2B-containing NMDARs resulted in significant remaining charge transfer through NMDARs, attributed to GluN2D-containing NMDARs. When GluN2B- or GluN2D-containing NMDARs were pharmacologically isolated, SFK activation failed to potentiate either isolated receptor subtype. We propose that lamina I of the spinal cord dorsal horn may contain triheteromeric GluN2B/D NMDARs which do not undergo potentiation in the presence of antagonists of GluN2B or GluN2D. Targeting the GluN2D subunit may provide a useful therapeutic strategy to ameliorate pain hypersensitivity as a result of BDNF-mediated potentiation.

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Table of Contents

Introduction

The Burden of Chronic Pain

Chronic pain is the persistent experience of pain, lasting 12 or more weeks. While acute pain can be adaptive and often provides information about processes causing bodily harm, chronic pain is often maladaptive. Chronic pain is a prevalent condition, occurring in 18.9% of Canadians over the age of 18 (Schopflocher et al., 2011). Despite the relatively short time before persistent pain is considered chronic, about 50% of individuals experiencing chronic pain report to have been suffering for over 10 years. Chronic pain can be debilitating and many patients are unable to work, causing a massive financial burden on individual families and the economy (Phillips, 2009). Current treatment options for chronic pain can often be ineffective, have unwanted side effects, and many pose a risk for addiction. This has created a demand for safe and effective treatment options for chronic pain.

Spinal Cord Physiology

The spinal cord is responsible for the bi-directional communication between the periphery and brain. This includes the transmission of pain signals, which can undergo modification within the spinal cord. The spinal cord can be partitioned into multiple laminae, which are involved in sensory input or motor control. The dorsal horn region of the spinal cord receives afferent inputs from peripheral sensory neurons. Pain signalling originates in the periphery, when pain sensing (nociceptive) sensory neurons are excited by a tissue-damaging (noxious) stimulus. These afferent neurons synapse onto central nervous system neurons within the superficial dorsal horn of the spinal cord, specifically onto neurons within laminae I and II (Braz et al., 2014). These synapses may be onto projection neurons, which have axons that extend into the brain, but it is more common for afferent neurons to synapse onto interneurons,

which have axons that remain within the spinal cord. Within the dorsal horn, interneurons may be excitatory or inhibitory. Inhibitory interneurons are mostly GABAergic; it has been found that many GABAergic interneurons in the dorsal horn are also glycinergic. It is hypothesized that all excitatory interneurons in the dorsal horn are glutamatergic (Todd, 2010). Physiological pain signalling requires a balance between excitatory and inhibitory input within the dorsal horn network. Since projection neurons which relay pain signals to the brain are exclusively excitatory, it would seem that modifications to the dorsal horn network which increase its excitability would increase pain perception, and vice versa. Indeed, many mechanisms which result in hyperexcitability or disinhibition have been implicated in chronic pain (Woolf and Salter, 2000; Todd, 2010; Bourinet et al., 2014).

Dorsal Horn Neurons Become Sensitized by Painful Stimuli

The experience of a painful stimulus can in itself alter the nociceptive network, increasing its sensitivity to further stimuli. Multiple models of pain have shown increased activity of dorsal horn neurons after repeated painful stimuli in a phenomenon referred to as central sensitization. Intradermal injection of capsaicin results in a decreased threshold for pain perception (hyperalgesia) and is associated with increased activity of dorsal horn neurons (Simone et al., 1991; Dougherty and Willis, 1992; Lin et al., 1997; Palec et al., 1999). Similar to capsaicin, cutaneous administration of mustard oil also induces hyperalgesia and prolonged increases in the responses of dorsal horn neurons to mechanical stimulation (Woolf and King, 1990). Both inflammation and nerve injury (neuropathy) are associated with increased pain perception and appear to depend on an increase in the sensitivity of dorsal horn neurons to external stimuli (Dickenson and Sullivan, 1987; Diaz and Dickenson, 1997; Laird and Bennett, 1993).

NMDARs and their Properties

The N-methyl-D-aspartate receptor (NMDAR) is critical in modulating neuronal excitability and has been implicated as a key mediator of central sensitization. NMDARs are tetrameric ion channels composed of two GluN1 subunits and two GluN2 subunits, or two GluN3 subunits (Paoletti et al., 2013). For the scope of this work, NMDAR will refer specifically to a GluN1/2 containing NMDAR. NMDARs are ligand gated ion channels, requiring the binding of glycine to the GluN1 subunit and glutamate to the GluN2 subunit. They are permeable to calcium and monovalent cations. While NMDARs are not voltage-gated, a magnesium ion blocks the channel at resting potential and membrane depolarization releases the magnesium ion, creating a voltage-dependent conductance. There are four types of GluN2 subunit: GluN2A, GluN2B, GluN2C and GluN2D. The synaptic release of glutamate induces currents through NMDARs which are well approximated by an exponential decay. Each GluN2 subtype has distinct decay properties. The decay constant of each subtype can vary substantially, but the general speed of decay constants is GluN2A(~40ms) > GluN2B(~300ms) = GluN2C > GluN2D(~1000ms) (Vicini et al., 1998; Paoletti et al., 2013). GluN2 subtypes can also be pharmacologically inhibited using specific antagonists. TCN-201 is a potent antagonist of GluN2A, with an IC_{50} of 0.446 μM and has minimal effects on GluN2B (Edman et al., 2012; Hansen et al., 2012). It should be noted that TCN-201's antagonism has a dependence on glycine concentration and that no studies to date have characterized its effect on GluN2D. Ro25-6981 is an antagonist used to block GluN2B, with an IC_{50} of 0.009 μM for GluN2B, and an IC_{50} of 52 μM for GluN2A (Fischer et al., 1997). Again, no studies have taken into account whether Ro25-6981 has effects on GluN2D. GluN2D containing NMDARs can be inhibited using DQP-1105, which has an IC_{50} of 1.5 μM for GluN2D, 121 μM for GluN2B and 206 μM for GluN2A (Acker

et al., 2011). A summary of different GluN2 subunit time constants and antagonists is seen in Table 1.

Table 1: **Summary of GluN2 subunit properties and selective antagonists**

Subunit	**τ (ms)**	**Antagonist**
GluN2A	~40	TCN-201
GluN2B	~ 300	Ro25-6981
GluN2C	~ 300	
GluN2D	~ 1000	DQP-1105

NMDARs are Present in the Dorsal Horn and are Necessary for the Induction of Central Sensitization

In situ hybridization studies have shown expression of NMDAR subunit mRNA within lamina I and II of the spinal dorsal horn (Tölle et al., 1993; Watanabe et al., 1994; Shibata et al., 1999; Stegenga and Kalb, 2001). In addition to this, electrophysiological recordings have shown many dorsal horn neurons which are sensitive to noxious stimuli are also excited by NMDAR agonists (Sher and Mitchell, 1990; Radhakrishnan and Henry, 1993; Yoshimura and Jessell, 1990; Baba et al., 2000; Bardoni et al., 1998; Suzuki et al., 2001; Tong et al., 2008; Shiokawa et al., 2010). During central sensitization, dorsal horn neurons experience an increased response to glutamate and NMDA, suggesting at least a partial role of NMDARs in central sensitization (Dougherty and Willis, 1992). In addition, blockade of NMDARs prevents the increased activity seen in superficial dorsal horn neurons in central sensitization (Torsney and MacDermott, 2006; Ikeda et al., 2006). The knockout of the GluN1 subunit of the NMDAR in the spinal cord prevents the induction of pain hypersensitivity through injury, but does not affect pain thresholds in uninjured animals (South et al., 2003; Weyerbacher et al., 2011; Garraway et al., 2007).

Clinically, the NMDAR antagonist ketamine has shown efficacy as an analgesic agent, however its interactions with NMDARs in non-pain related areas of the nervous system produce unwanted side effects (Ilkjaer et al., 1996). These studies collectively show that NMDARs are crucial for the process of central sensitization. The dorsal horn possesses a different NMDAR subtype composition than the brain, which may provide unique targets within the dorsal horn for treatment of pain without an impact on NMDAR signalling in the brain.

Levels of GluN2D Containing NMDARs are Insignificant in the Mature Brain

The composition of NMDARs in the brain varies during development. Rats are born with high levels of GluN2B mRNA in their cerebellums, and this diminishes significantly by postnatal day 21 (p21) (Akazawa et al., 1994). The levels of GluN2A mRNA in newborn rat cerebellums is insignificant and increases by p21. Similar to GluN2B, cerebellar levels of GluN2D mRNA are high at p3, but decrease rapidly. GluN2A and GluN2B dominate at synapses in the mature brain, with a minimal role for GluN2D (Cull-Candy and Leszkiewicz, 2004; Paoletti et al., 2013). GluN2D has been shown to be significant in the substantia nigra pars compacta and hippocampal synapses of juvenile mice, but very low levels are found in the adult hippocampus (Perszyk et al., 2016; von Engelhardt et al., 2015; Monyer et al., 1994; Morris et al., 2018). There are also high levels of GluN2D in the globus pallidus, thalamus, subthalamic nucleus and superior colliculus of embryonic and neonatal mice (Watanabe et al., 1992). The decrease in levels of GluN2D found in the brain through aging suggests GluN2D may be important developmentally, but it does not appear to play a significant role in mature brains.

GluN2D Containing NMDARs Have a Prominent Contribution to Overall NMDAR Activity in the Spinal Cord

While GluN2A is the predominant GluN2 subunit in the mature brain, GluN2B is also prevalent (Cull-Candy and Leszkiewicz, 2004). The spinal cord possesses a unique proportion of NMDARs which differs from that seen in the brain. Single channel recordings in the dorsal horn have demonstrated somatic GluN2D, suggesting it can be found extrasynaptically (Momiyama et al., 1996; Momiyama, 2000). Identification of low magnesium-sensitive NMDARs in the dorsal horn also provides evidence for GluN2C or GluN2D, since this is a property of those channels (Shiokawa et al., 2010; Tong et al., 2008). Immunoreactivity and mRNA expression of GluN2C in the spinal cord appear minimal, meaning the low magnesium-sensitive NMDARs likely contain GluN2D (Yung, 1998; Karlsson et al., 2002). The GluN2D subunit has also been found post-synaptically in dorsal horn neurons (Hummel et al., 2008).

A study in lamina I of the adult rat spinal cord showed that GluN2A, GluN2B and GluN2D containing NMDARs are responsible for approximately 5%, 50% and 45% of the charge transfer through NMDARs, respectively (Hildebrand et al., 2014). Peak currents passing through the subunits were 16%, 46% and 16% for GluN2A, GluN2B and GluN2D, respectively. The large charge transfer through GluN2D despite its relatively low peak current amplitude can be explained by its much slower decay constant.

The role of the GluN2D subunit in pain has been investigated using GluN2D knockout mice. These mice have not demonstrated any differences in pain thresholds using plantar formalin injection, spinal nerve transection and plantar incision models of chronic pain (Abe et al., 2005; Hizue et al., 2005; Nishimura et al., 2004). GluN2D knockout did appear to reverse pain hypersensitivity in mice with partial sciatic nerve ligation (Hizue et al., 2005). A recent study has shown that GluN2B compensates for the lack of GluN2D in GluN2D knockout mice,

suggesting these transgenic animal studies may underestimate the role of GluN2D in pain signalling (Morris et al., 2018).

While GluN2B has been implicated in pathological pain, its high prevalence in the brain makes it a difficult target for pharmacological treatment without many side effects. Despite GluN2D being expressed in some regions of the brain, its relative role in synaptic signalling compared to GluN2A/B appears to be minor. In contrast, GluN2D accounts for a large proportion of the charge passing through synaptic NMDARs in lamina I of the adult spinal cord. This may make GluN2D an excellent pharmacological target for treating pathological pain, since it is a condition which is most prevalent in adults.

Modification of NMDARs by Phosphorylation

Increased NMDAR activity is strongly associated with pathological pain states (Bourinet et al., 2014). Mechanisms which are associated with increased NMDAR activity should therefore be targeted for treatment of pathological pain. One such mechanism is phosphorylation of the NMDAR by tyrosine kinases. Inhibiting the phosphorylation of tyrosine residues results in a reduction in the current passing through NMDARs at brain synapses (Wang and Salter, 1994). The Src family kinases (SFKs) are a group of nine tyrosine kinases which have been implicated in NMDAR modulation. The peptide EPQ(pY)EEIPIA has been found to activate SFKs, and increases the channel opening probability of NMDARs during single channel recordings (Yu et al., 1997). Src(40-58) is a peptide which interferes with the interaction of Src with the NMDAR complex, one of the nine members of the SFKs (Macdonald et al., 2005). Addition of the SFK activating peptide EPQ(pY)EEIPIA as well as the Src interfering peptide has demonstrated that Src preferentially potentiates currents through GluN2A-containing NMDARs (Yang et al., 2012). A similar experiment was also carried out which replaced Src(40-58) with Fyn(39-57).

The Fyn(39-57) peptide interferes with the coupling of Fyn (another member of the SFKs) with the NMDAR complex. These results demonstrated that Fyn preferentially potentiates GluN2B containing NMDARs (Yang et al., 2012). These experiments were carried out in hippocampal neurons which lack GluN2D and therefore the role of SFKs in potentiating GluN2D remains unclear.

A study using a neuropathic pain model showed that GluN2B is phosphorylated at Tyr1472 after neuropathic pain (Abe et al., 2005). The pain and phosphorylation is diminished in mice without Fyn kinase, a member of the SFKs. Further work on modulation of NMDARs by SFKs has used the Src(40-49) peptide (which was found to uncouple Src from the NMDAR complex) to modulate pain, delivered intracellularly by attaching it to a TAT peptide (used to deliver the peptide through the plasma membrane) derived from HIV (Liu et al., 2008). The Src(40-49)TAT peptide reduced currents through NMDARs, and reduced pain hypersensitivity in models of neuropathic and inflammatory pain. These findings strongly suggest that phosphorylation of NMDARs by SFKs contributes to pathological pain, but its effect on GluN2D remains unclear.

It has also been found that BDNF acting on the TrkB receptor causes potentiation of synaptic NMDAR currents within lamina I of the spinal dorsal horn in a model of neuropathic pain (Hildebrand et al., 2016). BDNF-TrkB signalling resulted in activation of Fyn kinase, a member of the SFKs, which is necessary to potentiate NMDARs. Western blot analysis compared phosphorylation of the GluN2A and GluN2B subunits in response to Fyn. GluN2B was found to be phosphorylated while GluN2A was not, suggesting the phosphorylation of GluN2B is responsible for the NMDAR potentiation in response to Fyn. Despite the prevalence of GluN2D in lamina I, phosphorylation of GluN2D was not measured, and it remains unclear

whether GluN2D is phosphorylated and whether that phosphorylation contributes to NMDAR potentiation at lamina I synapses in pathological pain states.

The aim of this study is to determine if GluN2D containing NMDARs are required for the potentiation of NMDARs by BDNF and if GluN2D containing NMDARs are potentiated by SFKs in the context of pain. This will be accomplished by pharmacologically isolating GluN2D containing NMDARs during electrophysiological recordings of miniature excitatory postsynaptic currents (mEPSCs) and intracellularly administering the EPQ(pY)EEIPIA peptide, which activates SFKs. Many connections between neurons in the dorsal horn are polysynaptic, making it difficult to determine whether an evoked response occurred from one quantal vesicular release of glutamate or multiple. To circumvent this issue, mEPSCs are recorded while preventing action potentials and only measure currents resulting from the spontaneous release of glutamate. As NMDARs may be found in presynaptic terminals and postsynaptically, measuring evoked currents in the presence of NMDAR antagonists would include effects on both presynaptic and postsynaptic NMDARs. Recording mEPSCs prevents the concomitant contribution of presynaptic NMDARs to synaptic glutamate release. The intracellular administration of the EPQ(pY)EEIPIA peptide requires an hour long recording to allow the peptide to diffuse into the cell, and hence requires a stable medium. Since electrophysiological recordings are more stable in rat pups, they are a preferred medium to conduct this study. As there is no research to date on the developmental profile of GluN2D in lamina I in rats, the study must begin by confirming the functional contribution of GluN2D to NMDAR currents. If GluN2D is functionally present, the study can proceed to evaluate whether GluN2D is potentiated by SFKs. Provided that GluN2D is potentiated by SFKs, inhibitors of Src or Fyn will be administered to determine which specific member of the SFKs is responsible for potentiation.

Purpose

The aim of this study is to determine whether mechanisms which potentiate GluN2B containing NMDARs also potentiate GluN2D containing NMDARs in the dorsal horn of the spinal cord. To determine this, electrophysiological recordings were carried out on juvenile rat spinal cord tissue in the presence of various pharmacological treatments. The project was broken down into multiple aims: (1) to determine whether the GluN2D subunit is necessary for BDNF-mediated potentiation of NMDARs; (2) to determine whether slow-decaying GluN2D mediates a component of synaptic NMDAR responses in lamina I neurons of juvenile rats; (3A) to determine whether GluN2D-mediated NMDAR responses are potentiated by phosphorylation by SFKs; (3B) to test whether pharmacologically isolated GluN2B containing NMDARs are potentiated by SFKs.

Aim 1

To determine whether the GluN2D subunit is necessary for potentiation of NMDARs by BDNF in lamina I neurons, spinal cord slices were treated with the GluN2D antagonist DQP-1105, or with DQP-1105 and BDNF. Charge transfer through NMDARs was compared between the DQP-1105 treatment and DQP-1105 treatment with BDNF. The charge transfer was also compared with DQP-1105 treatment with BDNF and after BDNF washout. As negative and positive controls, respectively, similar approaches were used with antagonists of GluN2A (TCN-201) and GluN2B (Ro25-6981).

Aim 2

In order to determine whether GluN2D is present in the spinal cord of juvenile rats, electrophysiological recordings were performed on lamina I neurons of rats between the ages of

p8 and p21. These recordings included pre-administration of TCN-201 and Ro25-6981, selective antagonists of GluN2A and GluN2B, respectively. The residual mEPSC current at +60 mV would be composed of AMPA and GluN2D NMDARs, if this NMDAR subtype is present in the juvenile dorsal horn.

Aim 3A

To determine if GluN2D was potentiated by SFKs, the same electrophysiological recordings that were carried out in *Aim 1* were used. In addition, the EPQ(pY)EEIPIA peptide activator of SFKs was included in the intracellular solution. Throughout recording, the peptide diffused through the recording micropipette into the cell and if it potentiated GluN2D, an increase in NMDAR charge transfer over the course of the recording would be seen. To confirm the EPQ(pY)EEIPIA peptide potentiates NMDARs under these experimental conditions, recordings will first be carried out in the absence of NMDAR antagonists.

Aim 3B

To test the hypothesis that GluN2B containing NMDARs are potentiated by SFKs, the same protocol used in Aim 2A will be used, with the intracellular dialysis of the EPQ(pY)EEIPIA peptide during recording. This procedure will use different antagonists to isolate GluN2B containing NMDARs, administering TCN-201 and DQP-1105 to block currents through GluN2A- and GluN2D-containng NMDARs, respectively.

Methods

Animals

This study used male juvenile Sprague Dawley rats, aged postnatal day 8 (p8) to p21 supplied by Charles River, as well as adult male Sprague Dawley rats weighing about 350 g (approximately 3 months old). Policies and user protocols approved by the Animal Care Committees of Carleton University, the University of Toronto and the University of Ottawa Heart Institute were strictly followed for the care of the animals.

Spinal Slice Preparation

Rats were anaesthetized using an intraperitoneal injection of 3g/kg urethane and sacrificed by cervical transection. A dorsal laminectomy was performed to isolate the spinal cord, which was transferred to an ice cold protective artificial cerebrospinal fluid (pACSF) which contained (in mM) 50 sucrose, 92 NaCl, 15 D-glucose, 26 $NaHCO_3$, 5 KCl, 1.25 NaH_2PO_4, 0.5 $CaCl_2$, 7 $MgSO_4$, 1 kynurenate, bubbled with carbogen. The dorsal and ventral roots were removed and only the L3-L6 lumbar region was used. The spinal cord was glued onto an agarose base and transverse slices of the cord were cut at 400 μm using a Leica VT1200S microtome and used for electrophysiological recording. The slices were then allowed to rest for 40 minutes at 34°C in a similar pACSF to the one used previously, but lacking any kynurenate.

BDNF Pre-treatment

All data involving BDNF pre-treatment was collected at SickKids Hospital. BDNF pre-treatment was performed on spinal cord tissue isolated from adult Sprague Dawley rats weighing about 350 g. The same slice preparation was used as mentioned above, with the difference that 300 μm parasagittal slices were acquired. These were then treated with either saline, 10 μM

TCN-201 (Tocris), 1 μM Ro25-6981 (Tocris), or 10 μM DQP-1105 (Tocris) for five minutes, at which point 50 ng/mL BDNF (Alomone Labs) was administered for 60 minutes before undergoing electrophysiological recording. The same antagonist treatment that was administered before BDNF treatment was included in the pACSF during electrophysiological recording, but BDNF was not.

Electrophysiological Recording

Prior to recording, slices were allowed to warm to room temperature in pACSF and were then transferred to ACSF containing (in mM) 125 NaCl, 20 D-glucose, 26 $NaHCO_3$, 3 KCl, 1.25 NaH_2PO_4, 2 $CaCl_2$, and 1 $MgCl_2$ in addition to 500 nM TTX (Alomone Labs), 10 μM Cd^{2+}, 10 μM strychnine (Fisher) and 10 μM bicuculline (Tocris). The purpose of the antagonists TTX, cadmium, strychnine and bicuculline was to block voltage gated sodium channels, voltage gated calcium channels, glycine receptors and $GABA_A$ receptors, respectively. 10μM TCN-201 (Tocris) and 1μM Ro25-6981 (Tocris) or 10 μM DQP-1105 (Tocris) were added to the pACSF solution 20 minutes prior to recording, as well as the ACSF solution during recording. Spinal cord slices were placed under a brightfield microscope using oblique illumination to generate contrast. The slices were kept in ACSF at room temperature bubbled with carbogen throughout the recording procedure. Lamina I neurons were identified as neurons within 50 μm ventral of the white matter.

Whole cell voltage clamp recordings were carried out using borosilicate micropipettes filled with a solution which controls the intracellular environment and contains (in mM) 105 Cs-gluconate, 17.5 CsCl, 10 BAPTA, 10 HEPES, 2 MgATP, 0.5 Na_2GTP with a pH of 7.25 and an osmolarity of 295 mOsm. The intracellular solution included the phosphopeptide EPQ(pY)EEIPIA (Genscript). Neurons were patched onto at a holding potential of -60 mV while

stimulating with 5 mV pulses to measure membrane capacitance, membrane resistance and access resistance. Once a suitable seal was formed, on the order of 1 GΩ, the membrane was ruptured by applying light suction through the pipette holder. At this point the membrane voltage was gradually increased to +60 mV to relieve magnesium blockade of NMDARs and mEPSCs containing a prominent NMDAR component were recorded for 60 minutes.

Data was analyzed using Clampfit 10, using a threshold search. mEPSC events were characterized as those having an amplitude greater than 10 pA from the baseline and which possessed an approximately exponential decay. Any events which overlapped were rejected from further analysis. The duration of the sampling sweeps was 1000 ms from the initiation of the mEPSC for recordings for rats aged p8-p21 and 500 ms for adult rats. To evaluate the time courses of treatments, mEPSC events were binned into 5- or 10-minute intervals and averaged for the duration of the 60-minute recordings. As a metric of potentiation, charge transfer through NMDARs was used. This is determined by integrating the current through NMDARs over time

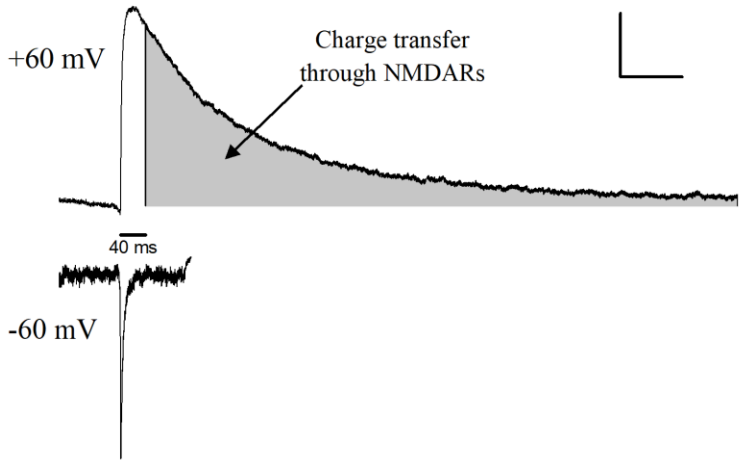

Figure 1: **Charge transfer through NMDARs in lamina I of rat transverse spinal cord slices.** *Top*, average mEPSC at +60 mV. The shaded region represents the charge transfer from 40 ms to 1 s after onset of the mEPSC. *Bottom*, representative average mEPSC at -60 mV. This demonstrates that current through AMPA has ceased within 40 ms of mEPSC onset, suggesting only current through NMDARs remains between 40 ms and 1 s. Scale bar x axis = 100 ms, y axis = 5 pA.

to determine the total amount of current passed through NMDARs in that time. The current was integrated from 40 ms after the onset of the mEPSC to 1000 ms after the onset for rats aged p8-p21 and 40 ms after mEPSC onset to 500 ms after onset for adults. The initial 40 ms allows currents through AMPARs to decay, leaving only currents through NMDARs after that time, demonstrated in Figure 1. The mEPSCs for each cell were averaged to create representative traces for each cell, which were then subsequently averaged to create average traces for each treatment group.

Biocytin Staining

To image neurons that had been recorded from, 3% biocytin (Sigma) was included in the intracellular solution for a small subset of experiments. After recording for 60 minutes, the patch pipette was withdrawn and the slices were placed in 4% paraformaldehyde for 24 hours. Slices were then transferred to phosphate buffer (PB) containing 10% sucrose for 24 hours, and this solution was replaced after 6 hours. The tissue was then transferred to PB containing 30% sucrose. In order to image the tissue, the 400 µm slices used for electrophysiology were re-sectioned to 40 µm. In order to re-section the slices, they were placed on filter paper, which created an adherent surface to flatten the slices. The tissue was then embedded in cryomatrix (Fisher) at -18 °C and sectioned at 40 µm using a Thermo Scientific cryostat, mounting slices directly onto coverslips. To stain biocytin, slices, were washed 3 times for 5 minutes in phosphate buffered saline (PBS), followed by 60 minutes of incubation with blocker solution (5% normal goat serum, 0.3% bovine serum albumin and 0.3% Triton-X in PBS). Slices were incubated overnight in streptavidin-conjugated alexafluor 594 (1:1000, Invitrogen #S32356) and mouse anti-CGRP (1:5000, Sigma Aldrich #C7113) diluted in blocker solution. Samples were then washed 3 times for 5 minutes in PBS, followed by a 120-minute incubation in goat anti-

mouse IGG alexafluor 647 (Invitrogen #A21235) in blocker solution. Finally, samples were mounted with Fluoromount aqueous mounting medium (Sigma) on Fisherbrand Superfrost Microscope Slides with 0.15 mm Fisherbrand coverslips. Imaging was performed using a Zeiss LSM 800 laser scanning confocal microscope.

Where not stated explicitly, the manufacturer of any products will by default be assumed to be Sigma-Aldrich.

Statistics

Findings are presented as means ± standard error of the mean (SEM) unless stated otherwise. Statistical tests for significance used one factor ANOVAs with Tukey's post-hoc analysis, as well as Student's paired and unpaired t-tests. A p-value less than 0.05 will be considered significant. All statistics were performed using Origin 2019 software.

Results

Lamina I of the spinal cord dorsal horn contains polysynaptic circuitry, making the study of synaptic responses using evoked currents problematic. In order to accurately measure postsynaptic currents, we recorded mEPSCs arising from the release of a single vesicle of glutamate using whole-cell voltage clamp. Holding the voltage at +60 mV allowed currents through AMPARs and NMDARs, while using the longer decay time of the NMDAR component of mEPSCs to isolate currents through NMDARs (Figure 1).

In order to determine which GluN2 subunit is necessary for BDNF mediated potentiation of NMDARs, recombinant BDNF (50 ng/mL) was co-administered with GluN2 selective antagonists before electrophysiological recordings of mEPSCs at +60 mV. To determine the extent to which BDNF potentiated NMDAR responses, charge transfer was compared between

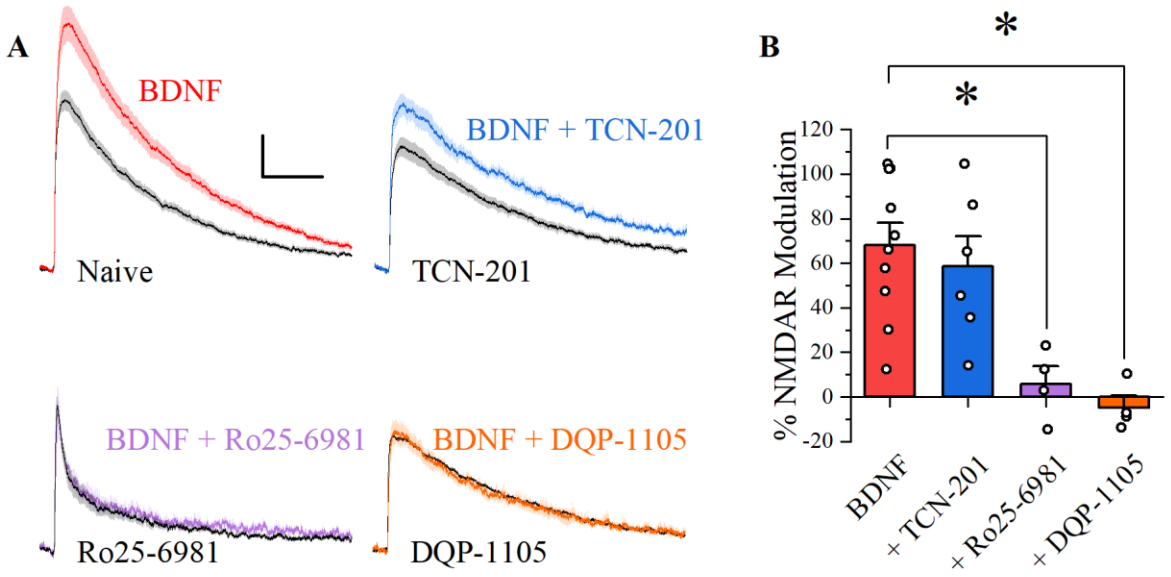

Figure 2: **BDNF potentiation of NMDAR mEPSCs in lamina I of rat spinal cord slices.** A) *Red*, pre-treatment of spinal cord tissue in 50 ng/mL BDNF (n = 14) potentiates NMDARs compared with naïve tissue (n = 20). *Blue*, pre-treatment of spinal cord tissue in BDNF and 3 µM of the GluN2A antagonist TCN-201 (n = 8) potentiates NMDARs compared with TCN-201 alone (n = 12). *Purple*, co-pre-treatment of spinal cord tissue in BDNF and 1 µM of the GluN2B antagonist Ro25-6981 (n = 8) potentiates NMDARs compared with Ro25-6981 alone (n = 12). *Orange*, co-pre-treatment of spinal cord tissue in BDNF and 10 µM of the GluN2D antagonist DQP-1105 (n = 6) potentiates NMDARs compared with DQP-1105 alone (n = 8). Shaded regions represent SEM. Scale bar x axis = 100 ms, y axis = 5 pA.
B) Modulation of NMDARs, comparing charge transfer with BDNF pre-treatment to charge transfer after BDNF washout. *$p < 0.05$.

mEPSCs recorded within 15 minutes of BDNF pre-treatment, and after BDNF washout. BDNF treatment without antagonists potentiated the overall NMDAR charge transfer by 68 ± 10 % when comparing mEPSCs immediately after BDNF pre-treatment to mEPSCs after BDNF washout (Figure 2B *red*). BDNF treatment also significantly ($p = 0.000088$) increased the charge transfer through NMDARs (Figure 2A *red*) from 3199 ± 219 fC (n = 20) in cells treated with saline to 5029 ± 375 fC (n = 14) in BDNF-treated cells. Inhibiting NMDARs containing the GluN2A subunit with TCN-201 still resulted in BDNF increasing NMDAR charge transfer by 59 ± 14 %, not significantly different from BDNF treatment alone ($p = 0.91$; Figure 2B *blue*). Cells

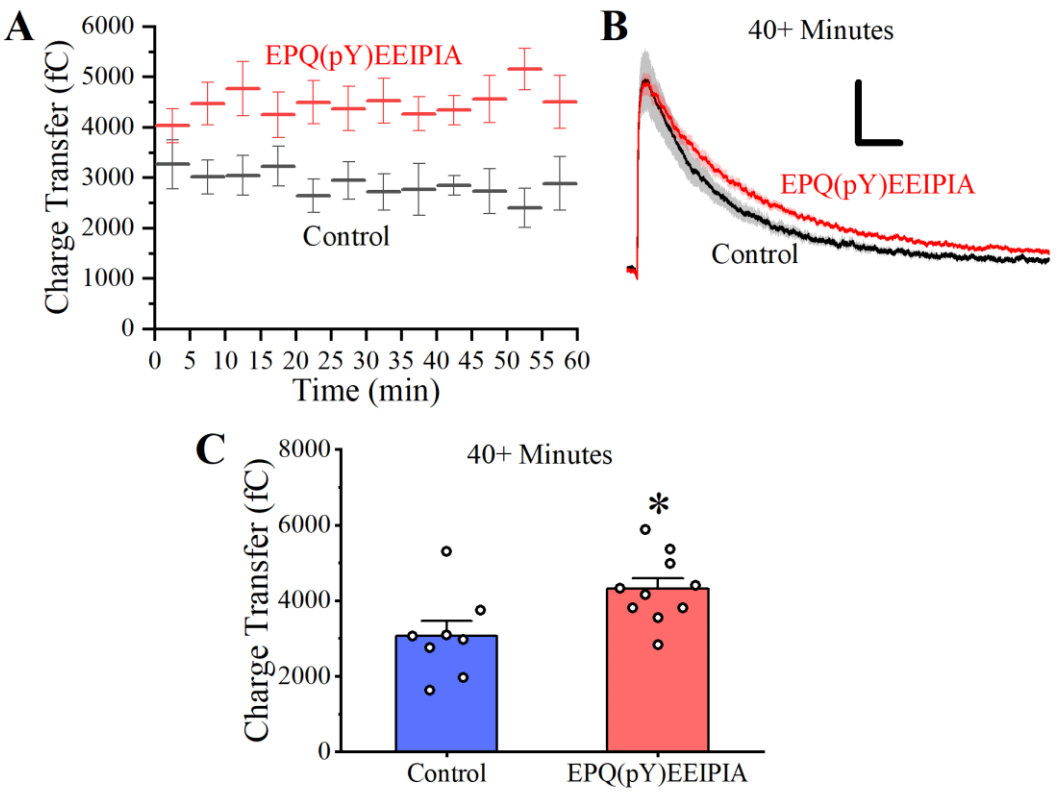

Figure 3: **The SFK activating peptide EPQ(pY)EEIPIA potentiates total NMDAR mEPSCs in lamina I of transverse rats spinal cord slices.** A) mEPSC charge transfer through total NMDARs with the EPQ(pY)EEIPIA SFK activating peptide (1 mM) included in the intracellular solution (n = 10) compared with control intracellular solution (n = 9). B) Average mEPSC trace between 40 and 60 minutes of recording with either EPQ(pY)EEIPIA (n = 10) in the patch pipette or control intracellular solution (n = 8). Shaded regions represent SEM. C) Charge transfer of mEPSCs through NMDARs between 40 and 60 min after patching with either control intracellular solution (n = 8) or intracellular solution containing 1mM EPQ(pY)EEIPIA (n = 10). *p < 0.05.Whole-cell voltage clamp recordings were performed at +60 mV. Scale bar x axis = 100 ms, y axis = 5 pA.

treated with TCN-201 possessed a charge transfer of 2857 ± 252 fC (n = 12), while cells treated with TCN-201 and BDNF (Figure 2A *blue*) had significantly larger charge transfers of 4237 ± 220 fC (n = 8, p = 0.0012). Inhibition of GluN2B containing NMDARs with Ro25-6981 prevented the potentiation seen with BDNF treatment alone, resulting in a relative change in charge transfer of 5.9 ± 8.0 % (n = 4, p = 0.0065; Figure 2B *purple*). Administration of BDNF with Ro25-6981 (Figure 2A *purple*) did not produce significantly different charge transfers

(1544 ± 216 fC, n = 6) through NMDARs, when compared with Ro25-6981 treatment alone (1230 ± 175 fC, n = 12, p = 0.30). Inhibition of GluN2D containing NMDARs with DQP-1105 completely abolished the potentiation seen with BDNF pre-treatment alone, with a relative change in charge transfer of -4.8 ± 5.3 % (n = 4, p = 0.0015; Figure 2B *orange*) Similarly, administration of DQP-1105 with BDNF (Figure 2A *orange*) did not alter the charge transfer through NMDARs (2522 ± 279 fC, n = 6) when compared to DQP-1105 alone (2595 ± 165 fC, n = 8, p = 0.81). These findings suggest that inhibition of NMDARs containing GluN2B or GluN2D is sufficient to completely prevent BDNF-mediated potentiation of NMDARs.

Knowing that the GluN2D subunit is necessary for BDNF-mediated potentiation of NMDARs, we aimed to test if NMDARs containing the GluN2D subunit were potentiated by kinase activity downstream of BDNF. In order to test for modulation of NMDARs through dialysis with SFK activators, long electrophysiological recordings were required, which are more feasible in juvenile rats, aged postnatal day 8 (p8) to 21 (p21). Since no information exists on the role of GluN2D at this developmental time point, we first tested whether GluN2D-containing NMDARs are present at this age. To determine the functional contributions of GluN2D-containing NMDARs in synapses of rats aged p8 and p21, voltage-clamp recordings were carried out on lamina I neurons in the presence of the GluN2A antagonist TCN-201 (10 μM) and GluN2B antagonist Ro25-6981 (1 μM). Residual current with these antagonists is proposed to be due to NMDARs containing the GluN2D subunit (Hildebrand et al., 2014). In the presence of these antagonists, currents through NMDARs still existed (Figure 4A,B), with a total charge transfer of 772 ± 141 fC (n = 8), compared to a total charge transfer of 3232 ± 487 fC (n = 8) for recordings with no antagonists. This suggests that GluN2D-containing NMDARs are responsible for a significant portion of the charge passed through NMDARs in rats aged p8-p21. As an

additional metric, the decay kinetics were examined of mEPSCs without antagonists present. The measured decay constant for control recordings was 196 ± 18 ms (n = 8). Previous findings report an overall NMDAR decay constant of 320 ± 40 ms for mEPSCs in adult lamina I synapses, and a contribution of GluN2D containing NMDARs to charge transfer of 890 ± 110 fC, compared to an overall charge transfer of 3470 ± 220 fC (Hildebrand et al., 2014). These results suggest that GluN2D-containing NMDARs in rats aged p8-p21 contribute significantly to synaptic currents in lamina I, which is similar to findings in adults.

Since inhibition of NMDARs containing the GluN2B subunit prevented BDNF mediated potentiation and inhibition of NMDARs containing the GluN2B subunit is necessary to pharmacologically isolate GluN2D-containing NMDARs, we activated a downstream target of BDNF in order to determine which GluN2 subunit of NMDAR it potentiates. SFKs act downstream of BDNF and are endogenously in an inactive state (Hildebrand et al., 2016). The peptide EPQ(pY)EEIPIA can be administered to activate endogenous SFKs by including the peptide in the intracellular solution during whole-cell recordings, resulting in subsequent cellular dialysis and potentiation of NMDAR currents during recording (Liu et al., 1993; Yu et al., 1997; Hildebrand et al., 2016). To evaluate the degree of potentiation and time course as the peptide diffuses into the cell under our experimental conditions, whole-cell voltage clamp recordings at +60 mV were initially carried out on untreated spinal cord tissue. mEPSCs were binned at 5-minute intervals and averaged over 60 minutes to produce 12 bins describing the time course of the peptide's activity. Administration of the EPQ(pY)EEIPIA peptide did not significantly increase charge transfer through NMDARs when comparing the first 5 minutes of recording to 40-60 minutes after patching (n = 10, p = 0.54). To test whether the peptide acted quickly enough to produce an effect within the first 5 minutes, control charge transfer (n = 8) was

compared to charge transfer with the EPQ(pY)EEIPIA peptide (n = 10) and showed no difference in the charge transfer (p = 0.19). Figure 3A suggests that the EPQ(pY)EEIPIA peptide may produce a modest, but insignificant increase in charge transfer between 0 and 5 minutes and a modest, but insignificant increase in charge transfer over the time course. The control charge transfer also appears to possess a small, but insignificant decrease over the course of the recording (p = 0.86). To test whether the apparent modest changes in charge transfer combine to produce a significant difference, we compared the charge transfer between 40 and 60 minutes of cells treated with EPQ(pY)EEIPIA and control intracellular solution (Figure 3B). Indeed, this showed a significant difference between cells treated with EPQ(pY)EEIPIA and control intracellular solution (p = 0.019; Figure 3C). While the time course of the increase in charge transfer resulting from the peptide may not be visible with this temporal resolution, comparing charge transfer after 40 minutes demonstrates the peptide does in fact potentiate the overall NMDAR response.

In order to determine which NMDAR subunit is the target of SFK activity, we pharmacologically isolated the GluN2D subunit by treating spinal cord slices with TCN-201 and Ro25-6981 and included the SFK activating peptide EPQ(pY)EEIPIA in the patch pipette (Figure 4). The time course did not demonstrate any change in the charge transfer through NMDARs with the inclusion of the EPQ(pY)EEIPIA peptide in the pipette, when comparing 0-10 minutes (n = 7) to 40-60 minutes (n = 6, p = 0.20). Finally, there was no difference in the charge transfer through isolated GluN2D-containing NMDARs between control intracellular solution and intracellular solution containing the EPQ(pY)EEIPIA peptide (Figure 4B,C, p = 0.59). Together, these results suggest SFKs do not potentiate GluN2D-containing NMDARs in the presence of antagonists of GluN2A and GluN2B.

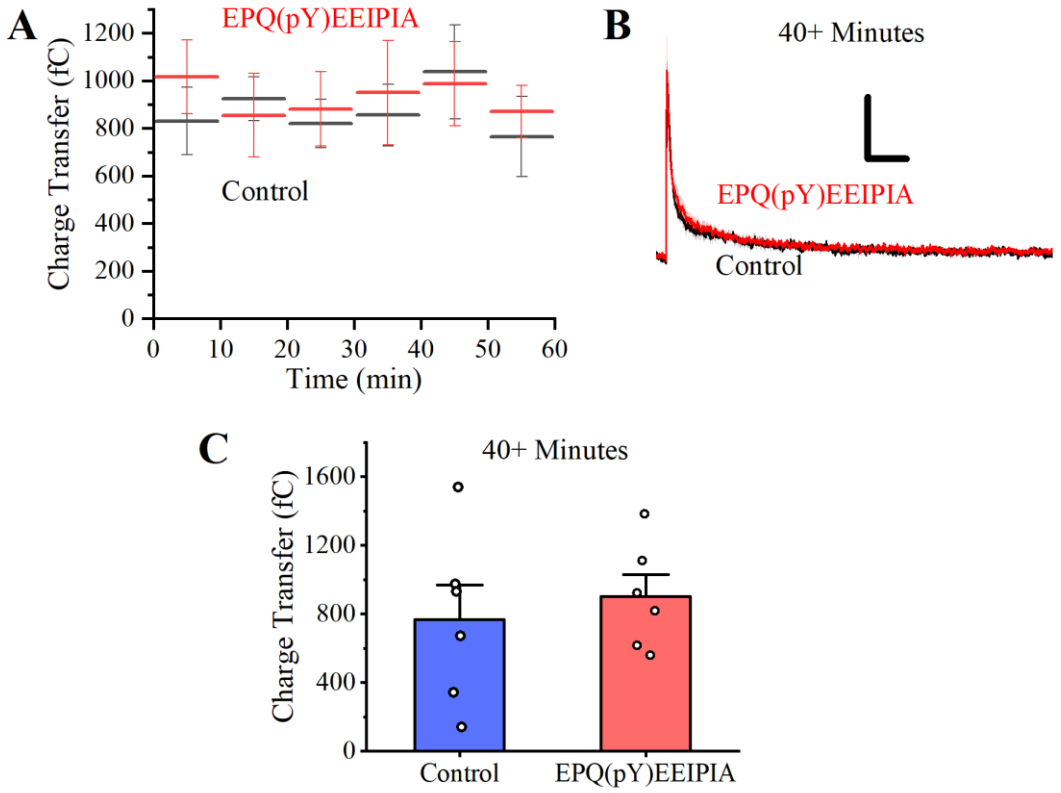

Figure 4: **The SFK activating peptide EPQ(pY)EEIPIA does not potentiate GluN2D-containing NMDAR mEPSCs in the presence of GluN2A and GluN2B antagonists in lamina I of transverse rats spinal cord slices.** Whole-cell voltage clamp recordings at +60 mV in lumbar spinal cords slices pretreated with the GluN2A antagonist TCN-201 (10 µM) and the GluN2B antagonist Ro25-6981 (1 µM).
A) mEPSC charge transfer through NMDARs. The EPQ(pY)EEIPIA SFK activating peptide (1 mM) was included in the intracellular solution (n = 7) and compared with control intracellular solution (n = 8). B) Average mEPSC trace for neurons with intracellular dialysis of EPQ(pY)EEIPIA after 40 minutes of recording (n = 7) or control intracellular solution after 40 minutes of recording (n = 8). Shaded regions represent SEM. C) Charge transfer of mEPSCs through NMDARs between 40 and 60 min after patching with either control intracellular solution or intracellular solution containing 1mM EPQ(pY)EEIPIA. Scale bar x axis = 100 ms, y axis = 5 pA.

With previous studies strongly suggesting the GluN2B subunit is implicated in NMDAR potentiation by SFKs, we aimed to test this directly by pharmacologically isolating NMDARs containing the GluN2B subunit in the presence of the SFK activating EPQ(pY)EEIPIA peptide (Hildebrand et al., 2016). Recording mEPSCs through NMDARs in the presence of TCN-201 and DQP-1105 demonstrated no difference in the charge transfer through NMDARs over the

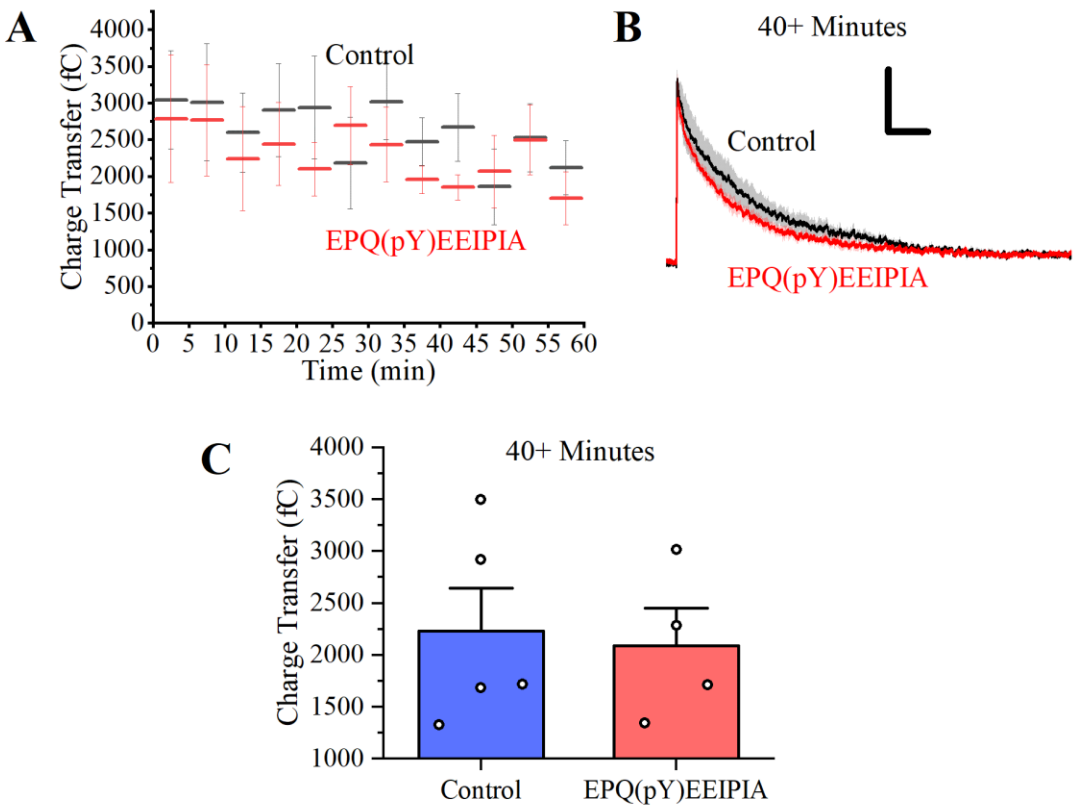

Figure 5: **The SFK activating peptide EPQ(pY)EEIPIA does not potentiate GluN2B-containing NMDAR mEPSCs in the presence of GluN2A and GluN2D antagonists in lamina I of transverse rats spinal cord slices.** Whole-cell voltage clamp recordings at +60 mV in lumbar spinal cords slices pretreated with the GluN2A antagonist TCN-201 (10 µM) and the GluN2D antagonist DQP-1105 (10 µM). A) mEPSC charge transfer over 60 minutes. The EPQ(pY)EEIPIA SFK activating peptide (1 mM) was included in the intracellular solution (n = 4) and compared with control intracellular solution (n = 5). B) Average mEPSC trace for neurons with intracellular dialysis of EPQ(pY)EEIPIA after 40 minutes of recording (n = 4) or control intracellular solution after 40 minutes of recording (n = 5). Shaded regions represent SEM. C) Charge transfer of mEPSCs through NMDARs between 40 and 60 min after patching with either control intracellular solution or intracellular solution containing 1mM EPQ(pY)EEIPIA. Scale bar x axis = 100 ms, y axis = 5 pA.

course of 60 minutes in the presence of the EPQ(pY)EEIPIA peptide ($p = 0.49$) compared to cells with control intracellular solution. mEPSCs after 40 minutes of treatment with EPQ(pY)EEIPIA did not appear larger than control, as would be expected if GluN2B was potentiated (Figure 5B). This was confirmed by examining charge transfer through isolated NMDARs containing the GluN2B subunit, showing that treatment with the SFK activating

peptide did not change the total charge passing through GluN2B-containing NMDARs (Figure 5C $p = 0.68$).

To confirm that compounds could readily diffuse from the patch pipette throughout the neuron of interest, biocytin was included in the patch pipette and imaged (Figure 6). Imaging showed biocytin in many processes of the neurons of interest, demonstrating that compounds in the pipette could diffuse throughout the neuron. Upon examination of the morphology of the stained neurons, it became clear that different subtypes of neuron exist within the population of neurons observed. With few ventral projections extending from the cell body, the neuron

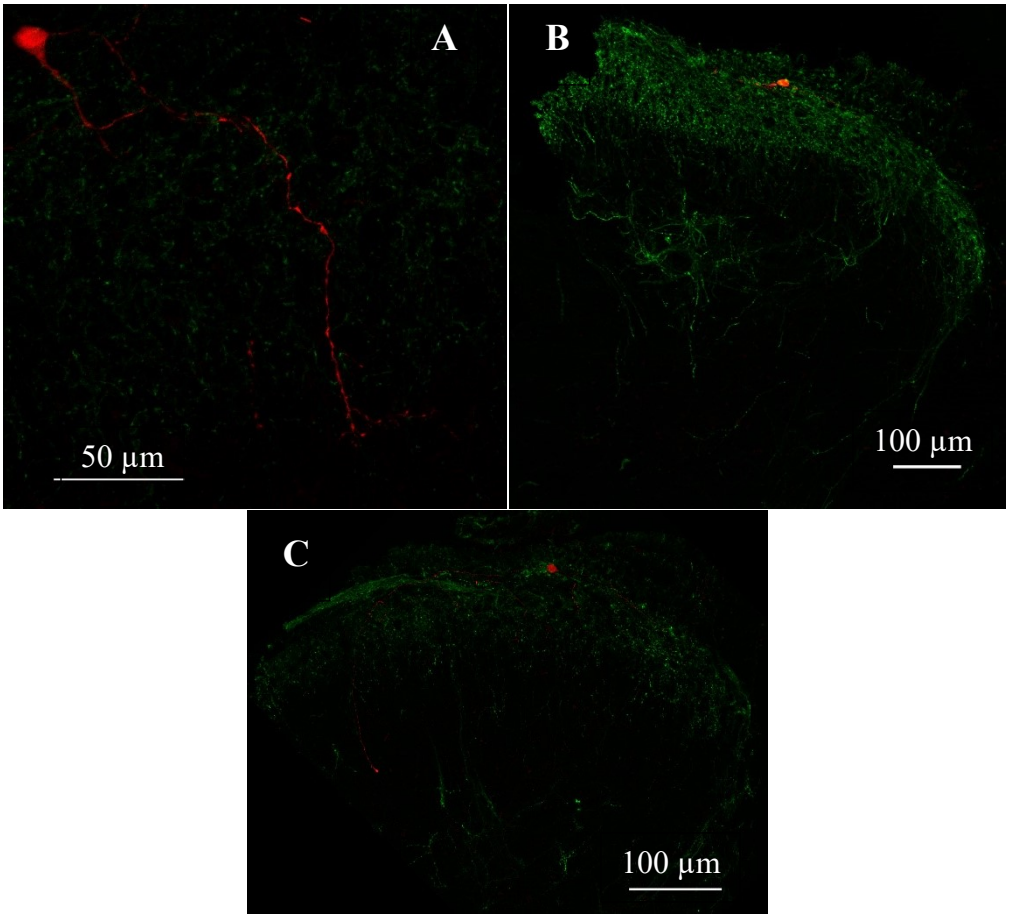

Figure 6: **The population of recorded lamina I neurons includes diverse morphological subtypes.** Neurons were filled with 3% biocytin, included in the patch pipette during electrophysiological recording (red). Analysis of cell morphology revealed different morphological subtypes, including a type IV neuron (A), type IA neuron (B) and type Ib neuron (C). Staining of CGRP (green) confirmed neurons were located in the superficial dorsal horn.

depicted in Figure 6A appears to be a pyramidal (type IV) neuron. Not shown in Figure 6B are sections up to 160 μm away showing longitudinal projections, paired with minimal ventral arborisation this is characteristic of fusiform neuron with longitudinal arbors (type IA). Figure 6C shows a neuron with projections extending very deep in the ventral direction and with few branches at the level of the soma, characteristic of a fusiform neuron with longitudinal and ventral arbors (type IB) (Lima and Coimbra, 1986). Despite only having three neurons imaged with biocytin, there appears to be a heterogenous population of neurons sampled from lamina I in this dataset.

Discussion

The results of this study have demonstrated a co-dependence between the GluN2B and GluN2D subunits of the NMDAR in rat lamina I neurons for modulation by BDNF. Inhibition of either GluN2B or GluN2D completely abolished BDNF-mediated potentiation of NMDARs, a surprising result, since one would expect that even if both subtypes were involved in potentiation, pharmacological blockade of one subtype of NMDAR would still allow potentiation of the other. This result was reconciled by proposing that NMDAR function may provide feedback necessary for BDNF-mediated potentiation. Indeed, Ca^{2+} is essential for potentiation of NMDARs by BDNF and Ca^{2+} concentrations are largely regulated by NMDARs. BDNF does not elicit potentiation of NMDARs during electrophysiological recordings due to strong Ca^{2+} buffering in the intracellular solution, but could be elicited by using a weaker Ca^{2+} buffer and higher Ca^{2+} conditions (Hildebrand et al., 2016). From this, we postulated that inhibition of GluN2B or GluN2D, which are the NMDAR subunits contributing to the greatest amount of charge transfer in lamina I neurons, may completely prevent BDNF-mediated potentiation.

In order to circumvent any Ca^{2+} dependencies, we used downstream targets of BDNF to probe for the direct modulation of the GluN2B and GluN2D subunits. The SFK, Fyn, acts downstream of BDNF and was activated using the general SFK activating peptide EPQ(pY)EEIPIA (Hildebrand et al., 2016). This resulted in potentiation of total NMDARs when no antagonists were present, even in the presence of the Ca^{2+} buffer BAPTA. This suggests that SFKs are capable of potentiating NMDARs independently of the intracellular concentration of Ca^{2+} and therefore that inhibition of GluN2B- or GluN2D-containing NMDARs would not prevent NMDAR potentiation by means of decreased intracellular concentrations of Ca^{2+}. It is then surprising that pharmacologically isolated NMDARs containing the GluN2B subunit or GluN2D subunit were not potentiated by SFKs.

The pharmacological isolation strategy for NMDARs containing GluN2B or GluN2D subunits that was used in this study relies on the notion that these subunits exist in diheteromeric NMDARs comprised of two GluN1 subunits and two identical GluN2 subunits. As illustrated in Figure 7A,B, combined antagonists of GluN2A and GluN2D would result in isolation of GluN2B-containing NMDARs and combined antagonists of GluN2A and GluN2B would result in isolation of GluN2D. If this were the case, whichever type of diheteromeric NMDAR is acted on by the SFK Fyn should be potentiated. However, we saw potentiation of neither type of pharmacologically isolated NMDAR. This may be explained by the existence of triheteromeric NMDARs containing two GluN1 subunits, one GluN2B subunit and one GluN2D subunit.

Triheteromeric NMDARs are known to be present in the brain, with GluN2A/B triheteromeric NMDARs playing a significant role in the brain (Paoletti et al., 2013; Stroebel et al., 2018). Interestingly, there is evidence of GluN2B/D triheteromeric NMDARs in the brain within the substantia nigra pars compacta, cerebellum and hippocampus (Brickley et al., 2003;

Brothwell et al., 2008; von Engelhardt et al., 2015; Perszyk et al., 2016). However, the roles and properties of GluN2B/D triheteromers remain elusive and it is unknown how subunit selective antagonists act on these receptors. GluN2A/B triheteromers have been more extensively studied and have been isolated in *Xenopus* oocytes and human embryonic kidney (HEK) 293 cells by engineering the GluN2A and GluN2B subunits to possess complimentary retention signals derived from GABA receptors, which only allow GluN2A/B triheteromers to be expressed in the plasma membrane (Hansen et al., 2014). The GluN2A/B triheteromers take on decay kinetics more characteristic of GluN2A. The triheteromers also having a decreased, but significant inhibition by the GluN2A selective antagonist TCN-201 compared to diheteromeric GluN2A NMDARs. Triheteromeric GluN2A/B NMDARs also have significantly reduced sensitivity to the GluN2B selective antagonist ifenprodil compared to diheteromeric GluN2B NMDARs.

While properties of triheteromeric GluN2A/B NMDARs may not directly carry over to GluN2B/D NMDARs, it does suggest the possibility that a fraction of triheteromeric GluN2B/D NMDARs will be blocked in the presence of GluN2B- or GluN2D-selective antagonists. Moreover, for the remaining GluN2B/D NMDARs, binding of DQP-1105 or Ro25-6981 to the extracellular domain of GluN2D or GluN2B may interfere with intracellular phosphorylation of triheteromeric GluN2B/D NMDARs by Fyn, preventing the potentiation of residual currents in the presence of either antagonist. Proof of interaction between intracellular and extracellular modulators of NMDARs has recently been shown by cleaving the intracellular domain of GluN2A-D subunits, resulting in decreased potentiation by positive allosteric modulators of GluN2 subunits (Sapkota et al., 2019). It was also shown that cleaving the intracellular domain of the GluN1 subunit affected potentiation by positive allosteric modulators of GluN2 subunits, suggesting that the intracellular domain of one subunit in a tetrameric NMDAR can interact with

the extracellular domain of another subunit in the tetrameric assembly. These findings support the idea proposed here, where extracellular inhibitors of GluN2B or GluN2D may prevent potentiation of the intracellular domain of GluN2B/D triheteromeric receptors. Interestingly, a study by Yang et al. (2012) proposing that Fyn acts on GluN2B-containing NMDARs was performed on isolated CA1 hippocampal neurons from GluN2A$^{-/-}$ mice. Later studies demonstrated that the GluN2D subunit is expressed in CA1 hippocampal neurons up to 58 days postnatally, which includes the ages used to test Fyn's modulation of GluN2B. This makes it ambiguous as to whether Fyn may be acting on diheteromeric GluN2B or triheteromeric GluN2B/D NMDARs (von Engelhardt et al., 2015; Perszyk et al., 2016).

To study whether triheteromeric GluN2B/D NMDARs exist within lamina I of the spinal cord, it may be useful to study the properties of isolated GluN2B/D NMDARs using retention sequences similar to those used for triheteromeric GluN2A/B NMDARs by Hansen et al. (2014). Comparing decay kinetics and sensitivities to the inhibitors DQP-1105 and Ro25-6981 between isolated GluN2B/D triheteromers and the NMDAR currents observed in lamina I may provide some evidence as to the nature of the NMDARs present in lamina I. Triheteromeric receptors could also be tested using co-immunoprecipitation, a technique previously used to probe triheteromeric GluN2A/B NMDARs (Sheng et al., 1994). Immunoprecipitating GluN2D and subsequent blotting for GluN2B would provide evidence of their coexistence within an NMDAR. This technique may run the risk of precipitating the entire NMDAR complex, which could be tested for by also probing other targets within the NMDAR complex, such as GluN2A.

Although the SFK activating peptide EPQ(pY)EEIPIA did cause potentiation of total NMDARs in this study, the time course appeared different from previous findings in lamina I.

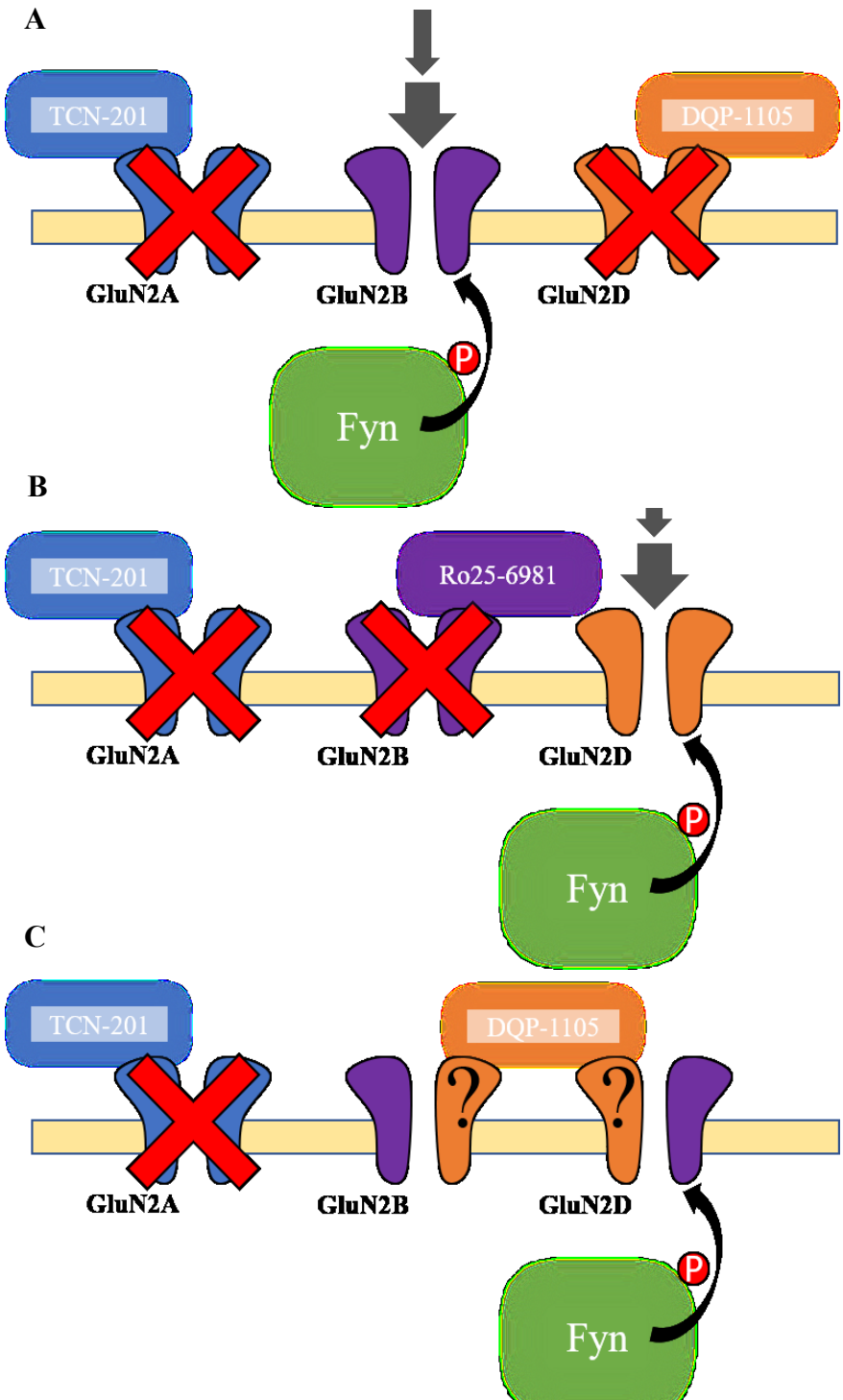

Figure 7: **Models of NMDAR subunit composition in lamina I neurons.** A) If GluN2B diheteromeric receptors exist, inhibiting GluN2A and GluN2D should result in potentiation of GluN2B currents (*grey*) by the SFK Fyn. B) If GluN2D diheteromeric receptors exist, inhibiting GluN2A and GluN2B may result in potentiation of GluN2D if Fyn acts on this subunit. C) If triheteromeric GluN2B/D receptors dominate at these synapses, inhibition of either subunit could interfere with potentiation by Fyn.

Previously, EPQ(pY)EEIPIA was found to elicit its peak effect on NMDAR mEPSCs after 40 minutes of recording with the peptide in the intracellular solution (Hildebrand et al., 2016). However, we saw a modest effect within 5 minutes and an apparent plateau in charge transfer after 15 minutes. One difference between these studies is that previous studies were performed on parasagittal slices, while we used transverse slices. It has been shown that neurons from parasagittal slices respond to glutamatergic input from more distal regions than neurons in transverse slices (Kato et al., 2009). The difference in the time course of the SFK activating peptide between this study and previous findings may be the result of the peptide diffusing to synapses faster in transverse slices, since the synapses in transverse slices appear closer to the soma, which is the source of diffusion of the peptide. The effect of dendritic arbour size on the SFK activating peptide's time course can be considered using the biocytin staining protocol developed in this study.

Previous findings with the EPQ(pY)EEIPIA peptide in the dorsal horn also demonstrated a greater peak effect of the peptide on NMDAR charge transfer in parasagittal slices (~1.8-fold compared to initial) (Hildebrand et al., 2016). Comparing charge transfer between cells with the EPQ(pY)EEIPIA peptide and control after 40 minutes demonstrated a ~1.4-fold increase in charge transfer. It should be noted that this study measured charge transfer between 40 ms and 1000 ms after the onset of mEPSCs, while the previous study measured between 40 ms and 500 ms and the difference in potentiation between the studies may be the result of different periods used for charge transfer. Despite this, differences between parasagittal and transverse results brings into question whether differences exist in NMDAR modulation between different regions of the same cell. Further work could increase the sample size of neurons recorded from with the EPQ(pY)EEIPIA peptide, including biocytin to examine cell morphology in both transverse and

parasagittal slices while comparing the potentiation time course and degree of potentiation to the neuronal morphology.

The findings presented in this study demonstrate that BDNF-mediated potentiation of lamina I pain processing neurons depends on both the GluN2B and GluN2D subunits of NMDAR, though the exact nature of this dependence remains unclear. While further work needs to be done to untangle the roles of the individual subunits, this finding alone could prove useful clinically. It is possible that the administration of a GluN2D antagonist to individuals with hyperalgesia would normalize pain signalling. Unfortunately, no antagonists exist that can be administered *in vivo* which are highly selective for the GluN2D subunit. Even the DQP-1105 used in this study has effects on the GluN2C NMDAR subunit (Acker et al., 2011). Thus, new pharmacological tools would need to be developed to test this. Together, our results call into question whether diheteromeric GluN2B NMDARs alone are responsible for BDNF-mediated potentiation of synaptic NMDAR responses, since the GluN2D subunit is also necessary to induce potentiation. This finding opens an avenue for potential therapeutics which could prevent BDNF-mediated potentiation by targeting the GluN2D subunit.

References

Abe, T., S. Matsumura, T. Katano, T. Mabuchi, K. Takagi, L. Xu, A. Yamamoto, K. Hattori, T. Yagi, M. Watanabe, T. Nakazawa, T. Yamamoto, M. Mishina, Y. Nakai, and S. Ito. 2005. Fyn kinase-mediated phosphorylation of NMDA receptor NR2B subunit at Tyr1472 is essential for maintenance of neuropathic pain. *Eur. J. Neurosci.* 22:1445–1454. doi:10.1111/j.1460-9568.2005.04340.x.

Acker, T.M., H. Yuan, K.B. Hansen, K.M. Vance, K.K. Ogden, H.S. Jensen, P.B. Burger, P. Mullasseril, J.P. Snyder, D.C. Liotta, and S.F. Traynelis. 2011. Mechanism for non-competitive inhibition by novel GluN2C/D NMDA receptor subunit-selective modulators. *Mol. Pharmacol. Fast Forward. Publ.* doi:10.1124/mol.111.073239.

Akazawa, C., R. Shigemoto, Y. Bessho, S. Nakanishi, and N. Mizuno. 1994. Differential expression of five N-methyl-D-aspartate receptor subunit mRNAs in the cerebellum of developing and adult rats. *J. Comp. Neurol.* 347:150–160. doi:10.1002/cne.903470112.

Baba, H., T.P. Doubell, K.A. Moore, and C.J. Woolf. 2000. Silent NMDA receptor-mediated synapses are developmentally regulated in the dorsal horn of the rat spinal cord. *J. Neurophysiol.* 83:955–62. doi:10.1152/jn.2000.83.2.955.

Bardoni, R., P.C. Magherini, and A.B. MacDermott. 1998. NMDA EPSCs at glutamatergic synapses in the spinal cord dorsal horn of the postnatal rat. *J. Neurosci.* 18:6558–6567.

Bourinet, E., C. Altier, M.E. Hildebrand, T. Trang, M.W. Salter, and G.W. Zamponi. 2014. Calcium-Permeable Ion Channels in Pain Signaling. *Physiol. Rev.* 94:81–140. doi:10.1152/physrev.00023.2013.

Braz, J., C. Solorzano, X. Wang, and A.I. Basbaum. 2014. Transmitting Pain and Itch Messages: A Contemporary View of the Spinal Cord Circuits that Generate Gate Control. *Neuron*.

82:522–536. doi:10.1016/J.NEURON.2014.01.018.

Brickley, S.G., C. Misra, M.H.S. Mok, M. Mishina, and S.G. Cull-Candy. 2003. NR2B and NR2D subunits coassemble in cerebellar Golgi cells to form a distinct NMDA receptor subtype restricted to extrasynaptic sites. *J. Neurosci.* 23:4958–66. doi:10.1523/JNEUROSCI.23-12-04958.2003.

Brothwell, S.L.C., J.L. Barber, D.T. Monaghan, D.E. Jane, A.J. Gibb, and S. Jones. 2008. NR2B- and NR2D-containing synaptic NMDA receptors in developing rat substantia nigra pars compacta dopaminergic neurones. *J. Physiol.* 586:739–50. doi:10.1113/jphysiol.2007.144618.

Cull-Candy, S.G., and D.N. Leszkiewicz. 2004. Role of Distinct NMDA Receptor Subtypes at Central Synapses. *Sci. Signal.* doi:10.1126/stke.2552004re16.

Diaz, A., and A.H. Dickenson. 1997. Blockade of spinal N- and P- type but not L-type calcium channels inhibits the excitability of rat dorsal horn neurones produced by subcutaneous formalin inflammation. *Pain*. 69:93–100.

Dickenson, A.H., and A.F. Sullivan. 1987. Peripheral origins and central modulation of subcutaneous formalin-induced activity of rat dorsal horn neurones. *Neurosci. Lett.* 83:207–211. doi:10.1016/0304-3940(87)90242-4.

Dougherty, P., and W.D. Willis. 1992. Enhanced responses of spinothalamic tract neurons to excitatory amino acids accompany capsaicin-induced sensitization in the monkey. *J. Neurosci.* 12:883–94.

Edman, S., S. McKay, L.J. Macdonald, M. Samadi, M.R. Livesey, G.E. Hardingham, and D.J.A. Wyllie. 2012. TCN 201 selectively blocks GluN2A-containing NMDARs in a GluN1 co-agonist dependent but non-competitive manner. *Neuropharmacology*. 63:441–9.

doi:10.1016/j.neuropharm.2012.04.027.

von Engelhardt, J., C. Bocklisch, L. Tönges, A. Herb, M. Mishina, and H. Monyer. 2015. GluN2D-containing NMDA receptors-mediate synaptic currents in hippocampal interneurons and pyramidal cells in juvenile mice. *Front. Cell. Neurosci.* 9:95. doi:10.3389/fncel.2015.00095.

Fischer, G., V. Mutel, G. Trube, P. Malherbe, J.N.C. Kew, E. Mohacsi, M.P. Heitz, and J.A. Kemp. 1997. Ro 25-6981, a Highly Potent and Selective Blocker of N-Methyl-D-aspartate Receptors Containing the NR2B Subunit. Characterization in Vitro. *Pharmacol. Exp. Ther.* 283:1285–1292.

Garraway, S.M., Q. Xu, and C.E. Inturrisi. 2007. Design and Evaluation of Small Interfering RNAs That Target Expression of the N-Methyl-D-aspartate Receptor NR1 Subunit Gene in the Spinal Cord Dorsal Horn. *Pharmacology*. 322:982–988. doi:10.1124/jpet.107.123125.

Hansen, K.B., K.K. Ogden, and S.F. Traynelis. 2012. Subunit-Selective Allosteric Inhibition of Glycine Binding to NMDA Receptors. *J. Neurosci.* 32:6197–6208. doi:10.1523/JNEUROSCI.5757-11.2012.

Hansen, K.B., K.K. Ogden, H. Yuan, and S.F. Traynelis. 2014. Distinct functional and pharmacological properties of Triheteromeric GluN1/GluN2A/GluN2B NMDA receptors. *Neuron*. 81:1084–1096. doi:10.1016/j.neuron.2014.01.035.

Hildebrand, M.E., G.M. Pitcher, E.K. Harding, H. Li, S. Beggs, and M.W. Salter. 2014. GluN2B and GluN2D NMDARs dominate synaptic responses in the adult spinal cord. *Sci. Rep.* 4:1–12. doi:10.1038/srep04094.

Hildebrand, M.E., J. Xu, A. Dedek, S. Beggs, P.J. Lombroso, M.W. Salter Correspondence, Y. Li, A.S. Sengar, and M.W. Salter. 2016. Potentiation of Synaptic GluN2B NMDAR

Currents by Fyn Kinase Is Gated through BDNF-Mediated Disinhibition in Spinal Pain Processing Article Potentiation of Synaptic GluN2B NMDAR Currents by Fyn Kinase Is Gated through BDNF-Mediated Disinhibition in Spinal. *Cell Rep.* 17:2753–2765. doi:10.1016/j.celrep.2016.11.024.

Hizue, M., C.H. Pang, and M. Yokoyama. 2005. Involvement of N-methyl-D-aspartate-type glutamate receptor epsilonl and epsilon4 subunits in tonic inflammatory pain and neuropathic pain. *Neuroreport*. 16:1667–1670. doi:10.1097/01.wnr.0000183328.05994.9e.

Hummel, M., B. Strassle, S. Miller, E. Kaftan, and G. Whiteside. 2008. Anatomical localization and expression pattern for the NMDA-2D receptor subunit in a rat model of neuropathic pain. *Neuroscience*. 155:492–502. doi:10.1016/J.NEUROSCIENCE.2008.05.041.

Ikeda, H., J. Stark, H. Fischer, M. Wagner, R. Drdla, T. Jäger, and J. Sandkühler. 2006. Synaptic amplifier of inflammatory pain in the spinal dorsal horn. *Science (80-.).* 312:1659–1662. doi:10.1126/science.1127233.

Ilkjaer, S., K.L. Petersen, J. Brennum, M. Wernberg, and J.B. Dahl. 1996. Effect of systemic N-methyl-D-aspartate receptor antagonist (ketamine) on primary and secondary hyperalgesia in humans. *Br. J. Anaesth.* 76:829–34.

Karlsson, U., J. Sjödin, L. Wikström, J. Näsström, and J. Näsström. 2002. Glutamate-induced currents reveal three functionally distinct NMDA receptor populations in rat dorsal horn - Effects of peripheral nerve lesion and inflammation. *Neuroscience*. 112:861–868. doi:10.1016/S0306-4522(02)00140-9.

Kato, G., Y. Kawasaki, K. Koga, D. Uta, M. Kosugi, T. Yasaka, M. Yoshimura, R.-R. Ji, and A.M. Strassman. 2009. Organization of Intralaminar and Translaminar Neuronal Connectivity in the Superficial Spinal Dorsal Horn. doi:10.1523/JNEUROSCI.6175-

08.2009.

Laird, J.M., and G.J. Bennett. 1993. An electrophysiological study of dorsal horn neurons in the spinal cord of rats with an experimental peripheral neuropathy. *J. Neurophysiol.* 69:2072–2085. doi:10.1152/jn.1993.69.6.2072.

Lima, D., and A. Coimbra. 1986. A Golgi study of the neuronal population of the marginal zone (lamina I) of the rat spinal cord. *J. Comp. Neurol.* 244:53–71. doi:10.1002/cne.902440105.

Lin, Q., Y.B. Peng, J. Wu, and W.D. Willis. 1997. Involvement of cGMP in nociceptive processing by and sensitization of spinothalamic neurons in primates. *J. Neurosci.* 17:3293–3302.

Liu, X., S.R. Brodeur, G. Gish, Z. Songyang, L.C. Cantley, A.P. Laudano, and T. Pawson. 1993. Regulation of c-Src tyrosine kinase activity by the Src SH2 domain. *Oncogene*. 8:1119–1126.

Liu, X.J., J.R. Gingrich, M. Vargas-Caballero, Y.N. Dong, A. Sengar, S. Beggs, S.-H. Wang, H.K. Ding, P.W. Frankland, and M.W. Salter. 2008. Treatment of inflammatory and neuropathic pain by uncoupling Src from the NMDA receptor complex. *Nat. Med.* 14:1325–1332. doi:10.1038/nm.1883.

Macdonald, D.S., M. Weerapura, M.A. Beazely, L. Martin, W. Czerwinski, J.C. Roder, B.A. Orser, and J.F. Macdonald. 2005. Modulation of NMDA Receptors by Pituitary Adenylate Cyclase Activating Peptide in CA1 Neurons Requires G q , Protein Kinase C, and Activation of Src. doi:10.1523/JNEUROSCI.3871-05.2005.

Momiyama, A. 2000. Distinct synaptic and extrasynaptic NMDA receptors identified in dorsal horn neurones of the adult rat spinal cord. *J. Physiol.* 523 Pt 3:621–8. doi:10.1111/J.1469-7793.2000.T01-1-00621.X.

Momiyama, A., D. Feldmeyer, and S.G. Cull-Candy. 1996. Identification of a native low-conductance NMDA channel with reduced sensitivity to Mg2" in rat central neurones. 479–492 pp.

Monyer, H., N. Burnashev, D.J. Laurie, B. Sakmann, and P.H. Seeburg. 1994. Developmental and Regional Expression in the Rat Brain and Functional Properties of Four NMDA Receptors. 12. 529–540 pp.

Morris, P.G., M. Mishina, and S. Jones. 2018. Altered Synaptic and Extrasynaptic NMDA Receptor Properties in Substantia Nigra Dopaminergic Neurons From Mice Lacking the GluN2D Subunit. *Front. Cell. Neurosci.* 12:354. doi:10.3389/fncel.2018.00354.

Nishimura, W., T. Muratani, S. Tatsumi, K. Sakimura, M. Mishina, T. Minami, and S. Ito. 2004. Characterization of N-methyl-D-aspartate receptor subunits responsible for postoperative pain. *Eur. J. Pharmacol.* 503:71–75. doi:10.1016/j.ejphar.2004.09.033.

Palec, V., Y.B.O. Peng, J. Wu, M. Cui, Q. Lin, J. Palec, W.D. Willis, and Y.B. Peng. 1999. Nitric Oxide Mediates the Central Sensitization of Primate Spinothalamic Tract Neurons. *Nitric Oxide*. 83:1075–1085. doi:10.1152/jn.1999.81.3.1075.

Paoletti, P., C. Bellone, and Q. Zhou. 2013. NMDA receptor subunit diversity: Impact on receptor properties, synaptic plasticity and disease. *Nat. Rev. Neurosci.* 14:383–400. doi:10.1038/nrn3504.

Perszyk, R.E., J.O. DiRaddo, K.L. Strong, C.-M. Low, K.K. Ogden, A. Khatri, G.A. Vargish, K.A. Pelkey, L. Tricoire, D.C. Liotta, Y. Smith, C.J. McBain, and S.F. Traynelis. 2016. GluN2D-Containing N-methyl-d-Aspartate Receptors Mediate Synaptic Transmission in Hippocampal Interneurons and Regulate Interneuron Activity. *Mol. Pharmacol.* 90:689–702. doi:10.1124/mol.116.105130.

Phillips, C.J. 2009. The Cost and Burden of Chronic Pain. *Rev. pain*. 3:2–5. doi:10.1177/204946370900300102.

Radhakrishnan, V., and J.L. Henry. 1993. Excitatory amino acid receptor mediation of sensory inputs to functionally identified dorsal horn neurons in cat spinal cord. *Neuroscience*. 55:531–544. doi:10.1016/0306-4522(93)90522-H.

Sapkota, K., K. Dore, K. Tang, M. Irvine, G. Fang, E.S. Burnell, R. Malinow, D.E. Jane, and D.T. Monaghan. 2019. The NMDA receptor intracellular C-terminal domains reciprocally interact with allosteric modulators. *Biochem. Pharmacol.* 159:140–153. doi:10.1016/J.BCP.2018.11.018.

Schopflocher, D., P. Taenzer, and R. Jovey. 2011. The prevalence of chronic pain in Canada. *Pain Res. Manag.* 16:445–50.

Sheng, M., J. Cummings, L.A. Roldan, Y.N. Jan, and L.Y. Jan. 1994. Changing subunit composition of heteromeric NMDA receptors during development of rat cortex. *Nature*. 368:144–147. doi:10.1038/368144a0.

Sher, G.D., and D. Mitchell. 1990. Intrathecal N-methyl-d-aspartate induces hyperexcitability in rat dorsal horn convergent neurones. *Neurosci. Lett.* 119:199–202. doi:10.1016/0304-3940(90)90833-U.

Shibata, T., M. Watanabe, R. Ichikawa, Y. Inoue, and T. Koyanagi. 1999. Different expressions of alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid andN-methyl-D-aspartate receptor subunit mRNAs between visceromotor and somatomotor neurons of the rat lumbosacral spinal cord. *J. Comp. Neurol.* 404:172–182. doi:10.1002/(SICI)1096-9861(19990208)404:2<172::AID-CNE3>3.0.CO;2-U.

Shiokawa, H., E.J. Kaftan, A.B. MacDermott, and C.K. Tong. 2010. NR2 subunits and NMDA

receptors on lamina II inhibitory and excitatory interneurons of the mouse dorsal horn. *Mol. Pain*. 6:1–15. doi:10.1186/1744-8069-6-26.

Simone, D.A., L.S. Sorkin, U. Oh, J.M. Chung, C. Owens, R.H. LaMotte, and W.D. Willis. 1991. Neurogenic hyperalgesia: central neural correlates in responses of spinothalamic tract neurons. *J. Neurophysiol.* 66:228–246. doi:10.1152/jn.1991.66.1.228.

South, S.M., T. Kohno, B.K. Kaspar, D. Hegarty, B. Vissel, C.T. Drake, M. Ohata, S. Jenab, A.W. Sailer, S. Malkmus, T. Masuyama, P. Horner, J. Bogulavsky, F.H. Gage, T.L. Yaksh, C.J. Woolf, S.F. Heinemann, and C.E. Inturrisi. 2003. A Conditional Deletion of the NR1 Subunit of the NMDA Receptor in Adult Spinal Cord Dorsal Horn Reduces NMDA Currents and Injury-Induced Pain. *Neuroscience*. 23:5031–5040.

Stegenga, S.., and R.. Kalb. 2001. Developmental regulation of N-methyl-D-aspartate- and kainate-type glutamate receptor expression in the rat spinal cord. *Neuroscience*. 105:499–507. doi:10.1016/S0306-4522(01)00143-9.

Stroebel, D., M. Casado, and P. Paoletti. 2018. Triheteromeric NMDA receptors: from structure to synaptic physiology. *Curr. Opin. Physiol.* 2:1–12. doi:10.1016/j.cophys.2017.12.004.

Suzuki, R., E.A. Matthews, and A.H. Dickenson. 2001. Comparison of the effects of MK-801, ketamine and memantine on responses of spinal dorsal horn neurones in a rat model of mononeuropathy. *Pain*. 91:101–109. doi:10.1016/S0304-3959(00)00423-1.

Todd, A.J. 2010. Neuronal circuitry for pain processing in the dorsal horn. *Nat. Rev. Neurosci.* 11:823–36. doi:10.1038/nrn2947.

Tölle, T.R., A. Berthele, W. Zieglgänsberger, P.H. Seeburg, and W. Wisden. 1993. The differential expression of 16 NMDA and non-NMDA receptor subunits in the rat spinal cord and in periaqueductal gray. *J. Neurosci.* 13:5009–28. doi:10.1523/JNEUROSCI.13-12-

05009.1993.

Tong, C.K., E.J. Kaftan, and A.B. MacDermott. 2008. Functional identification of NR2 subunits contributing to NMDA receptors on substance P receptor-expressing dorsal horn neurons. *Mol. Pain*. 4:1–11. doi:10.1186/1744-8069-4-44.

Torsney, C., and A.B. MacDermott. 2006. Disinhibition Opens the Gate to Pathological Pain Signaling in Superficial Neurokinin 1 Receptor-Expressing Neurons in Rat Spinal Cord. *J. Neurosci.* 26:1833–1843. doi:10.1523/JNEUROSCI.4584-05.2006.

Vicini, S., J.F. Wang, J.H. Li, W.J. Zhu, Y.H. Wang, J.H. Luo, B.B. Wolfe, and D.R. Grayson. 1998. Functional and Pharmacological Differences Between Recombinant N-Methyl- d-Aspartate Receptors. *J. Neurophysiol.* 79:555–566. doi:10.1152/jn.1998.79.2.555.

Wang, Y.T., and M.W. Salter. 1994. Regulation of NMDA receptors by tyrosine kinases and phosphatases. *Nature*. 369:233–235. doi:10.1038/369233a0.

Watanabe, M., Y. Inoue, K. Sakimura, and M. Mishina. 1992. Developmental changes in distribution of NMDA receptor channel subunit mRNAs. *Neuroreport*. 3:1138–40.

Watanabe, M., M. Mishina, and Y. Inoue. 1994. Distinct spatiotemporal distributions of the N-Methyl-D-aspartate receptor channel subunit mRNAs in the mouse cervical cord. *J. Comp. Neurol.* 345:314–319. doi:10.1002/cne.903450212.

Weyerbacher, A.R., Q. Xu, C. Tamasdan, S.J. Shin, and E. Inturrisi. 2011. N-Methyl-D-Aspartate Receptor (NMDAR) independent maintenance of inflammatory pain. *Pain*. 148:237–246. doi:10.1016/j.pain.2009.11.003.N-Methyl-D-Aspartate.

Woolf, C.J., and A.E. King. 1990. Dynamic alterations in the cutaneous mechanoreceptive fields of dorsal horn neurons in the rat spinal cord. *J. Neurosci.* 10:2717–2726. doi:10.1152/jn.1989.62.4.907.

Woolf, C.J., and M.W. Salter. 2000. Neuronal Plasticity: Increasing the Gain in Pain. *Science (80-.).* 288:1765–1768.

Yang, K., C. Trepanier, B. Sidhu, Y.F. Xie, H. Li, G. Lei, M.W. Salter, B.A. Orser, T. Nakazawa, T. Yamamoto, M.F. Jackson, and J.F. MacDonald. 2012. Metaplasticity gated through differential regulation of GluN2A versus GluN2B receptors by Src family kinases. *EMBO J.* 31:805–816. doi:10.1038/emboj.2011.453.

Yoshimura, B.Y.M., and T. Jessell. 1990. Amino Acid-Mediated EPSPs at Primary Afferent Synapses with Sunstantia Gelatinosa Neurones in the Rat Spinal Cord. *J. Physiol.* 430:315–335.

Yu, X.M., R. Askalan, G.J. Keil, and M.W. Salter. 1997. NMDA channel regulation by channel-associated protein tyrosine kinase Src. *Science (80-.).* 275:674–678. doi:10.1126/science.275.5300.674.

Yung, K.K.L. 1998. Localization of glutamate receptors in dorsal horn of rat spinal cord. *Neuroreport*. 9:1639–1644. doi:10.1097/00001756-199805110-00069.