Differential contributions of NMDA receptor subtypes to lamina II synaptic responses
across juvenile spinal cord development

by

Hadir Mahmoud

A thesis presented to the Faculty of Graduate and Postdoctoral Affairs in partial
fulfillment of the requirements for the degree of

Master of Science

in

Neuroscience

Carleton University
Ottawa, Ontario

© 2019

Hadir Mahmoud

Differential contributions of NMDA receptor subtypes to lamina II synaptic responses across juvenile spinal cord development

Abstract

NMDA receptors are heteromeric complexes crucial to the regulation of excitatory synaptic transmission, including in the spinal cord. The presence of specific subtypes of GluN2 subunits determines the kinetic properties of receptor activity. The Hildebrand lab has demonstrated that slow-decaying GluN2B and GluN2D dominate NMDAR responses at lamina I adult spinal synapses, which is unlike the fast GluN2A-dominated synapses found throughout most of the mature CNS. The functional contribution of specific GluN2 subunits is less characterized for synaptic NMDAR responses in lamina II neurons. We performed whole-cell patch clamp recordings of mEPSCs in the presence and absence of subtype-specific NMDAR pharmacological blockers. We observed a relatively equal and stable contribution of GluN2A and GluN2B throughout lamina II development, contrasting the shift in contribution from GluN2B to GluN2A commonly observed during postnatal development in the brain. We also identified a slower synaptic NMDAR component that is blocked by a GluN2D antagonist. These findings provide key insights into the differential roles of specific NMDAR subtypes in nociceptive signaling at juvenile spinal synapses.

Keywords: NMDA receptor, GluN2A, GluN2B, GluN2D, spinal cord development, lamina II, dorsal horn hyperexcitability, mEPSCs

Acknowledgements

Thank you to members of my lab: Rikesh Raichura, Shannon Tansley, and Newton Martin for their aid in preparation, imaging, and analysis, respectively.

Thank you to Dr. Hildebrand, one of the most supportive and compassionate mentors I've ever had. Your passion for research is truly inspiring and I am lucky to have witnessed it. Eternal gratitude for your continuous guidance, feedback, and encouragement.

Last but not least, I am grateful for the support of my family and friends during this trying time. This publication is as much yours as it is mine.

Table of Contents

Title Page	i
Abstract	ii
Keywords	ii
Acknowledgments	iii
Table of Contents	iv
Abbreviations	vi
List of Figures	vii
Introduction	1
The pain problem	1
Superficial dorsal horn of the spinal cord	3
NMDAR diversity and role in synaptic plasticity	4
NMDAR-GluN2 subunit expression throughout the mature rodent brain	6
NMDAR-GluN2 subunit expression across brain development	7
NMDAR-GluN2 subunit expression throughout the spinal cord	8
GluN2-subunit specific pharmacological inhibitors	9
Miniature excitatory postsynaptic currents	11
Study aims	12
Methods	13
Animals	13
Spinal cord isolation and tissue sectioning	13
Whole-cell patch-clamp electrophysiological recordings	14
Pharmacological inhibition of NMDAR-GluN2 subunits	15
Data analysis	16

Statistical analysis 17

Results 20

Discussion 30

References 37

Abbreviations:

AMPAR – α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor

CNS – Central Nervous System

DRG – Dorsal Root Ganglion

EPSC – Excitatory Postsynaptic Current

IASP – International Association for the Study of Pain

LTD – Long-term Depression

LTP – Long-term Potentiation

mEPSC - miniature Excitatory Postsynaptic Current

NMDAR – N-Methyl-D-aspartate receptor

SG – Substantia Gelatinosa

TTX – Tetrodotoxin

List of Figures	Page
Figure 1: Biological underpinnings of pain	2
Figure 2: Summary of protocol for data collection	19
Figure 3: Characterization of mEPSCs at juvenile lamina II synapses	22
Figure 4: GluN2B and GluN2A both contribute prominently to NMDAR responses at lamina II synapses throughout early development	24
Figure 5: GluN2D minimally contributes to NMDAR-components of juvenile lamina II mEPSCs	26
Figure 6: Characterization of slow decaying mEPSCs at juvenile lamina II synapses	28
Figure 7: GluN2D minimally contributes to slow decaying NMDAR-components of juvenile lamina II mEPSCs	29

Introduction

The pain problem

The concept of pain was officially defined in 1986 by the International Association for the Study of Pain (IASP) as ‘an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage’ (Merskey, 1986). This subjective feeling, coded neurally as ‘nociception’, is often accompanied by an emotional component (Merskey & Bogduk, 1994) rendering the experience memorable for future reference. For example, placing a finger on a hot plate is accompanied by a rapid reflex of withdrawal such that no further damage (burned skin) occurs, in addition to a rather memorable aversive sensation (‘pain’) serving as a reminder and deterrent of a future reoccurrence. Fittingly, pain and fear work together to signal the avoidance of potentially harmful or fatal situations, also known as self-preservation, ultimately increasing the chances of survival for the organism.

Unfortunately, in many instances this signaling has been known to become maladaptive, resulting in various forms of chronic pain pathologies. Chronic pain is clinically diagnosed following the appearance of persistent, symptomatic pain of moderate or severe intensity presenting for an extended period of time, which can be traced back to either known or unknown etiology such as that seen in fibromyalgia (Lannersten & Kosek, 2010). Due to varying etiology, differential clinical definitions, and minimal understanding of the underlying mechanisms at play, diagnoses tend to be non-specific, inaccurate, or simply discounted under the pretense that the pain will eventually self-resolve. Without proper treatment, pain experienced by these patients can become extremely distressing and often debilitating. Inadequate methods for diagnosing and managing chronic pain have

become a burden on societies in the form of increasing healthcare spending as well as severe or permanent loss of productivity (Lynch, 2011). This is a major cause of concern since chronic pain disorders impact one in every five adults (Schopflocher, Taenzer, & Jovey, 2011), contributing to a substantial loss of working-class contribution. Intriguingly, chronic pain becomes more common with increasing age and is more prevalent in females compared to males (Moulin, et al., 2002; Schopflocher, Taenzer, & Jovey, 2011). Depending on the pain-related condition diagnosed, patients are often prescribed an assortment of pharmaceutical interventions ranging from antidepressants and anticonvulsants to various analgesics (Binder & Baron, 2016). While some patients may find benefit from the proposed treatment interventions, others find little to no relief or are plagued by a host of hindering and critical side effects (Roques, Fournie-Zaluski, & Wurm, 2012). Identifying key targets in the pain pathway is a promising avenue for the development of personalized pharmaceutical treatments which can be more effective and specific to remedying problematic pain conditions.

Figure 1 – Biological underpinnings of pain. Pain perception begins with the activation of peripheral nociceptors in response to chemical, thermal, and mechanical stimulation. The resultant signal is transmitted to lamina I and lamina II in the superficial dorsal horn of the spinal cord for processing and immediate reflexive action. Projection neurons in lamina I transmit the signal to higher brain centers for conscious awareness while neurons in lamina II receive descending input from the brain regarding signal modulation.

The superficial dorsal horn of the spinal cord

The superficial dorsal horn of the spinal cord is an important site for the processing, integration, and transmission of noxious stimuli. It is comprised of cellular layers referred to as lamina I and II, which are the first level for the relay and modulation of information between primary sensory afferents and descending projections from higher pain processing centers (Cordero-Erausquin et al., 2016). Specifically, lamina I contains various populations of interneurons as well as a small population of projection neurons that are responsible for transmitting somatosensory information from the periphery to higher brain centers for conscious awareness (Todd, 2010). In contrast, lamina II contains only interneurons that receive synaptic inputs from peripheral afferents in addition to descending inputs from the brain, and are critical for the modulation of spinal somatosensory processing (Todd, 2010). The heterogeneous population of lamina II interneurons differ mainly in dendritic morphology and neurotransmitter used for signaling, and can be morphologically classified as islet, central, vertical, and radial cells. Inhibitory islet cells release GABA exclusively, while radial and the majority of vertical cells are excitatory, releasing glutamate and glycine (Maxwell et al., 2007). Central cells exhibit the ability to signal using either neurotransmitter (Maxwell et al., 2007). Hyperexcitability in superficial laminae is a prominent feature of many maladaptive pain disorders, commonly referred to as “central sensitization”. This feature characteristically increases in prevalence with age and can have clinical manifestations in the form of spontaneous pain, mechanical allodynia, as well as hyperalgesia (Binder & Baron, 2016). Spinal hyperexcitability is particularly problematic due to poor understanding of underlying neural circuitry. The superficial dorsal horn provides many functionally relevant potential

therapeutic targets involved in modulation of neuronal excitability and alteration of overall pain perception.

NMDAR diversity and roles in synaptic plasticity

The N-Methyl-D-aspartate receptor (NMDAR) is a crucial constituent in the regulation of excitatory synaptic transmission and plasticity throughout the central nervous system, including in the spinal cord. NMDARs have been associated extensively with the processing and transmission of noxious stimulation and are candidate contributors for the spinal hyperexcitability observed in many chronic pain disorders (Cull-Candy & Leszkiewicz, 2004). They are heteromeric complexes comprised of four homologous subunits, two of which are GluN1. GluN1 subunits are essential to the structure and function of NMDA and are encoded by one gene, *GRIN1* (Traynelis et al., 2010). The other two subunits can stem from either the GluN2 family, encoded by four possible genes (*GRIN2A*, *GRIN2B*, *GRIN2C*, *GRIN2D*), or GluN3 which are encoded by two genes, *GRIN3A* and *GRIN3B* (Traynelis et al., 2010). NMDARs can be classified into two types: diheteromeric or triheteromeric. Diheteromeric NMDARs are composed of two GluN1 subunits in addition to two subunits of the same type (e.g. GluN1/GluN2A). On the other hand, triheteromeric receptors contain two GluN1 subunits and two subunits of a different variety (e.g. GluN1/GluN2A/GluN2B). The presence of specific subtypes of GluN2 determines the kinetic properties of receptor activity (Paoletti, Bellone, & Zhou, 2013; Sheng et al., 1994). Accordingly, GluN2A- to GluN2D-containing NMDARs have varying deactivation (channel closing) rates and roles in synaptic integration (Monyer et al., 1994). GluN2A subunits show the quickest deactivation, with a progression of slower decays from GluN2B to 2C to 2D (Vicini et al., 1998). This unique property determines the magnitude

and duration of depolarization as well as calcium influx through the NMDA receptor resulting in differential contribution of each GluN2 subunit to synaptic plasticity.

NMDARs typically function as coincidence detectors for presynaptic glutamate release and postsynaptic depolarization through neighboring AMPA receptors (AMPA receptors). NMDAR activation occurs following co-agonist binding of glutamate and glycine in combination with a change in membrane voltage as a result of AMPAR-mediated depolarization, which electrostatically repels the Mg^{2+} ion obstructing the calcium-permeable NMDAR pore (Bliss & Collingridge, 1993). The specific subtype of GluN2 subunit expressed plays a significant role in determining NMDAR sensitivity to Mg^{2+} block (Monyer et al., 1994) as well as co-agonist binding potency (Traynelis et al., 2010). The resultant calcium influx through NMDARs is critical to neuronal processes underlying development, learning, and memory. GluN2A appears to contribute significantly to the induction of LTP in mature mice hippocampi while GluN2B plays a smaller role in LTP initiation (Berberich et al., 2007; Fox et al., 2006; Köhr et al., 2003; Li et al., 2007; Volianskis et al., 2015). This relationship seems to be reversed in younger, developing animals (Köhr et al., 2003), suggesting divergent roles of GluN2A and GluN2B expression in learning and development. The roles of GluN2 subunits in the induction of LTD remains controversial, although evidence suggests a minor involvement of GluN2A (Li et al., 2007; Liu et al., 2004; Shipton & Paulsen, 2013) in underlying processes. The role of GluN2B in LTD has been demonstrated using pharmacological inhibition in juvenile (P0-P28) hippocampal slices (Liu et al., 2004), as well as *in vivo* (P30-P40) (Fox et al., 2006); however, other studies suggest little to no involvement of GluN2B in the induction of LTD within the first three postnatal weeks (Bartlett et al., 2007; Li et al., 2007; Morishita et al., 2007). This

discrepancy could be accounted for by potential differences in the developmental time ranges investigated, as the molecular switch in subunit expression is suspected to occur approximately around the third postnatal week.

NMDAR-GluN2 subunit expression throughout the mature rodent brain

NMDARs are ubiquitously expressed throughout the mammalian brain and play an integral role in various neurological processes and disorders involving learning and memory. The functional presence of diheteromeric GluN1/GluN2A complexes has been demonstrated at forebrain synapses in postnatal mice, with the GluN2A-component of NMDAR-mediated mEPSCs dominating in adult forebrain synapses (Matta et al., 2011; Monyer et al., 1994). However, several studies suggest a functional role of GluN1/GluN2A/GluN2B triheteromeric complexes that is not explained by the presence of either diheteromeric NMDAR complex alone (Luo et al., 1997; Sheng et al., 1994; Tovar, Mcginley, & Westbrook, 2013), suggesting a functional role for both GluN2A- and GluN2B-mediated NMDAR activity in adult hippocampal circuitry (Rauner & Köhr, 2011). In comparison to the widespread expression of GluN2A and GluN2B, GluN2C is marginally expressed throughout the mature CNS (Cull-Candy & Leszkiewicz, 2004), with expression limited mainly to cerebellar granule cells (Monyer et al., 1994). A minimal role has been identified for GluN2D subunits in the adult rat brain (Cull-Candy & Leszkiewicz, 2004; Monyer et al., 1994), with regional expression confined primarily to the midbrain and hippocampus (Standaert et al., 1996). Moreover, GluN2D expression appears to be predominantly extrasynaptic and minimally contributes to synaptic responses (Misra et al., 2000; Paoletti, Bellone, & Zhou, 2013). The functional properties of NMDARs in the majority of adult brain synapses corresponds with subunit-specific expression, with faster

components GluN2A, and variably GluN2B, facilitating relatively fast NMDAR-mediated mEPSCs.

NMDAR-GluN2 subunit expression across rodent brain development

Robust differences in the expression of various GluN2 subunits have been noted throughout various regions of developing, juvenile brains. GluN2B is abundant embryonically and maintained postnatally, peaking around the first week, followed by a drop in expression thereafter (Matta et al., 2011; Quinlan, Olstein, & Bear, 1999; Rodenas-Ruano et al., 2012; Sheng et al., 1994). In contrast, GluN2A expression is first observed during the first postnatal week in low concentrations, where it steadily increases during development, eventually dominating at mature brain synapses. Moreover, Quinlan and colleagues demonstrated an activity-dependent upregulation of GluN2A subunits in juvenile rat visual cortex neurons, whereby GluN2A-containing NMDAR expression increased following periods of activity (exposure to light) and decreased following periods of inactivity (Quinlan, Olstein, & Bear, 1999). This suggests an activity-dependent switch from GluN2B- to GluN2A-mediated NMDAR responses during developmental critical periods (Carmignoto & Vicini, 1992; Quinlan et al., 1999). On the other hand, GluN2C is first detected during postnatal development around P10, maintaining a relatively low expression in mature CNS synapses (Akazawa et al., 1994; Momiyama, Feldmeyer, & Cull-Candy, 1996; Monyer et al., 1994). GluN2D mRNA expression has been observed primarily prenatally, peaking in the midbrain during the first postnatal week around P7 and exponentially decreasing to virtually negligent levels in adulthood (Momiyama et al., 1996; Monyer et al., 1994).

NMDAR-GluN2 subunit expression throughout the rodent spinal cord

Similar to the brain, glutamate is the primary facilitator of excitatory synaptic signaling in the spinal cord, acting on cation-permeable ion channels such as NMDARs and AMPARs. NMDAR slow deactivation and temporal integration allows for amplified and prolonged neurotransmission, such as that underlying pathological pain. Specifically, activation of synaptic NMDARs has been associated with the onset and maintenance of central sensitization due to the receptor's susceptibility to wind up (signal amplification) (Dickenson, Chapman, & Green, 1997).

Initial probing into GluN2-subunit-mediated NMDAR responses in the superficial dorsal horn through single-NMDAR channel current analysis in somatic outside-out patches described a ubiquitous presence of extrasynaptic GluN2B-type, and to a lesser extent GluN2D-type, conductance channels in adult substantia gelatinosa (SG) neurons (Momiya, 2000). They also cited faster kinetic responses at synaptic sites, attributing that to the primarily contribution of GluN2A-containing NMDARs to EPSCs in these neurons (Momiya, 2000). Tong & McDermott later proposed triheteromeric GluN1/GluN2A/GluN2B NMDAR complexes as primary contributors to NMDAR-mediated EPSCs at mature peripheral afferent synapses in lamina I (Tong & MacDermott, 2014). Immunohistochemical studies examining GluN2 distribution in superficial laminae (I and II) demonstrate robust GluN2B, moderate GluN2D, and minimal GluN2A or GluN2C expression (Hummel et al., 2008; Yung, 1998). The Hildebrand lab's previous research has combined subtype-specific pharmacology with recordings of miniature excitatory postsynaptic potentials (mEPSCs) to demonstrate that GluN2B and GluN2D dominate NMDAR responses at lamina I spinal synapses, which is unlike the GluN2A-

dominated synapses found throughout most of the mature CNS (Akazawa et al., 1994; Hildebrand et al., 2014; Quinlan, Olstein, & Bear, 1999). Taken together, it is speculated that the presence of slow decaying GluN2 subunits in NMDAR assemblies prompt the development of maladaptive spinal signaling, hence subunit-specific pharmacological interventions are a prospect of interest in clinical studies for the treatment of pathological pain disorders.

Given that differences in NMDAR expression and function is evident in the brain across maturation, there is a substantial interest in elucidating the role of NMDARs in spinal cord development and their contribution to increased vulnerability of maladaptive signaling in adulthood. Bardoni and colleagues were the first to explore NMDAR-mediated EPSCs at juvenile superficial dorsal horn synapses, noting a substantial contribution of NMDARs at both pure and mixed glutamatergic synapses during the first two postnatal weeks (Bardoni, Magherini, & MacDermott, 1998). They describe the presence of both fast and slow decaying components of NMDAR EPSCs that did not differ across development, and are comparable to previous reports of mature spinal synaptic responses (Bardoni et al., 1998). However, is it not clear how the functional contribution of particular GluN2 subunits to NMDAR responses at lamina II spinal synapses differs from that of lamina I and potentially across spinal cord development. This is essential to understanding the functional diversity of NMDARs in spinal circuitry in comparison to the rest of the CNS.

GluN2-subunit specific pharmacological inhibitors

The contributions of individual GluN2 subunits to synaptic NMDAR responses can be characterized using subtype-specific agonists and antagonists. Many of the antagonists

used act allosterically, not directly interfering with the binding of glutamate and activation of NMDARs as a whole (Hansen, Ogden, & Traynelis, 2012). Initial studies have utilized ifenprodil to antagonize GluN2B (Li et al., 2007; Luo et al., 1997; Momiyama, 2000; Williams, 1993); however it has been shown to be unselective in inhibiting GluN1/2B (Hatton & Paoletti, 2005). Ifenprodil-derived Ro-25-6981 is a specific, activity-dependent, GluN2B inhibitor with a 5000-fold selectivity for inhibiting GluN2B-components of NMDARs without interfering with the activity of other subunits (Fischer et al., 1997). It has a reported selectivity of GluN2B over GluN2A that is 25-fold greater than that of ifenprodil (Fischer et al., 1997). Administration of 1 μ M Ro25-6981 has been previously shown to block GluN2B to ~90% without interfering with GluN2A activity (Chazot, Lawrence, & Thompson, 2002; Fox et al., 2006; Hildebrand et al., 2014; Rauner & Kohr, 2010). Several GluN2A-specific inhibitors have also been utilized in previous studies, with Zn^{2+} being among the most popular (Hatton & Paoletti, 2005). One limitation of this antagonist is the inevitable contamination of Zn^{2+} and other heavy metals *in vivo* and *in vitro* (Paoletti, Ascher, & Neyton, 1997). More recently, TCN-201 is a non-competitive GluN2A subunit-specific blocker shown to inhibit GluN2A activity at 92-100% efficacy and 300-fold selectivity over GluN2B when used at a concentration of 3 to 10 μ M (Bettini et al., 2010; Hansen et al., 2012). TCN-201 does not block GluN1/GluN2D complexes at a concentration of 30 μ M, while its role at GluN2C-containing NMDARs has yet to be explored (Bettini et al., 2010). There is a lack of inhibitors that target the slower-decaying GluN2 subunits, however a highly-selective novel pharmacological inhibitor, DQP-1105, has recently been developed to preferentially block GluN2D and GluN2C NMDARs at neuronal synapses. It exhibits a 50-fold binding selectivity for inhibiting

GluN2D/GluN2C-mediated NMDAR responses over other GluN2 subunits, GluN2A and GluN2B (Acker et al., 2011). Therefore, DQP-1105 can be used in combination with Ro25-6981 and TCN-201 to assess the presence and contribution of GluN2D, GluN2B, and GluN2A-mediated NMDAR responses, respectively.

Miniature excitatory postsynaptic currents

Signaling throughout the dorsal horn of the spinal cord is comprised of inputs from primary afferents such as dorsal root ganglion (DRG), projections to and from the brain, as well as polysynaptic signaling between neighboring interneurons throughout laminae I-III (D'Mello & Dickenson, 2008; Todd, 2010). This interconnected nature of dorsal horn circuitry is not suited for evoked potential electrophysiology as the polysynaptic currents generated result in prolonged synaptic responses and NMDAR activation at synaptic sites other than on the neuron of interest. Hence, measuring spontaneous activity in superficial dorsal horn neurons is an appropriate alternative. Miniature excitatory postsynaptic currents (mEPSCs) allow for the measurement of synaptic responses produced by quantal release of glutamate onto specific lamina II neurons (Hildebrand et al., 2014). In order to isolate spontaneous excitatory synaptic responses, spinal sections are treated with various blockers that inhibit other possible sources of electrical activity. These include: inhibitory GABAergic receptors (10 μ M bicuculline), inhibitory glycine receptors (10 μ M strychnine), voltage-gated calcium channels (10 μ M Cd^{2+}), as well as voltage-gated sodium channels (0.5 μ M TTX). Taken together, the resultant synaptic activity recorded is presumed spontaneous and excitatory in nature, attributable to postsynaptic AMPARs and NMDARs on the recorded neuron.

Study aims

The global differences in spatial and temporal expression of specific GluN2 NMDAR subunits throughout the nervous system and across brain development indicate distinct roles for GluN2 subunits in physiological and pathological signaling. This study aims:

1. To investigate the contributions of GluN2A- and GluN2B- components to NMDAR responses at lamina II synapses across juvenile spinal cord developmental time ranges: P7-P11, P12-P16, and P17-P21. We plan to use whole-cell patch clamp recordings of miniature excitatory postsynaptic currents (mEPSCs) to assess spontaneous synaptic responses in the presence and absence of subtype-specific NMDAR antagonists, 1 μ M Ro25-6981 and 10 μ M TCN-201. We hypothesize that unlike the GluN2B to GluN2A synaptic switch commonly observed during juvenile brain development, GluN2B-mediated NMDAR responses will persist or even increase at lamina II synapses during juvenile development.

2. To elucidate the functional relevance of GluN2D subunits at juvenile lamina II synapses using the subunit-selective pharmacological inhibitor, 10 μ M DQP-1105. We hypothesize that GluN2D-mediated NMDAR responses play a minimal role at juvenile lamina II synapses, in contrast to their major role at adult spinal lamina I synapses (Hildebrand et al., 2014).

Ultimately, these findings will provide key insights into the role of specific NMDAR subtypes during spinal cord development, which could highlight potential targets for the increased prevalence of maladaptive pain signaling in aging organisms.

Methods

I. Animals

Juvenile (P7-P21) Sprague Dawley rats from Charles River were used for all experiments. Animals were anaesthetized using 20% urethane (1mL/10g). All performed experiments followed the guidelines set out by the Canadian Council on Animal Care regarding animal care and handling. Experiment-specific animal protocols were authorized by the Animal Care Committee at Carleton University and Animal Veterinary Services at The Heart Institute, where animals were housed.

II. Spinal cord isolation and tissue sectioning

Juvenile (P7-P21) Sprague Dawley rats were anaesthetized using intraperitoneal injections of 20% (wt/vol) urethane (Sigma-Aldrich) at 1mL/10g of weight. The spinal cord excision protocol that was followed here is the same as previously utilized by the Hildebrand lab to investigate NMDAR subunit composition contributing to synaptic responses in lamina I adult spinal cord neurons (Hildebrand et al., 2014) (also see Figure 1). Accordingly, the spinal cord was excised immediately following anesthesia and placed in an ice-cold, bubbled (5% CO₂, 95% O₂) sucrose cutting solution containing (in mM): 50 sucrose, 92 NaCl, 15 D-(+)-glucose, 26 NaCO₃, 5 KCl, 1.25 NaH₂PO₄, 0.5 CaCl₂, 7 MgSO₄. This solution also contained 1 mM kynurenic acid to inhibit NMDAR excitotoxicity, thereby increasing neuronal viability. The spinal cord was then cut to a ~ 1 cm portion containing lumbar spinal cord (L3-L6), from which meninges and ventral/dorsal roots were carefully removed under a Leica dissecting microscope. The trimmed spinal segment was mounted on its ventral surface to a piece of agar, which was

then glued vertically to a vibratome sectioning chuck. Transverse (400 μm) slices were generated using a Leica VT1200S vibratome. Tissue slices were placed in a sucrose cutting solution that did not contain kynurenic acid and was heated to $\sim 33.9^\circ\text{C}$ using a water bath (Fisher-Scientific). The slices were allowed to recover for 40 minutes before the solution was removed from the water bath and allowed to reach room temperature ($\sim 23^\circ\text{C}$) prior to being used for the electrophysiological recordings described below. These recordings were also performed on adult Sprague Dawley (250 g – 350 g) lamina II neurons using the same spinal cord isolation and electrophysiological protocols described in this experiment (Figure S1B).

III. Whole-cell patch-clamp electrophysiological recordings on lamina II juvenile spinal cord neurons

Spinal cord slices were visualized under a Zeiss microscope and acutely perfused with artificial cerebral spinal fluid (aCSF) containing (in mM): 125 NaCl, 3 KCl, 26 NaCO_3 , 1.25 NaH_2PO_4 , 2 CaCl_2 , 1 MgCl_2 , and 20 D-(+)-glucose. Additionally, 10 μM bicuculline (Tocris Bioscience), 10 μM strychnine, 10 μM Cd^{2+} , and 0.5 μM TTX (Alomone Labs) were added to block inhibitory GABAergic receptors, inhibitory glycine receptors, voltage-gated calcium channels, and voltage-gated sodium channels, respectively. This ensured that isolated events during recording are restricted to miniature excitatory post-synaptic currents (mEPSCs). Lamina II was visualized under the microscope at 10x magnification and clearly identified as a bright band located between the relatively darker laminae I and III. Lamina II neurons of interest were visualized under 40x magnification. Patch-clamp recording borosilicate glass pipettes of 5 to 11 $\text{M}\Omega$ resistance were pulled using a Sutter Flaming micropipette puller and fire-polished. Once ready for use, pipettes were filled with

an internal solution containing (in mM): 105 D-gluconic acid, 105 CsOH, 17.5 CsCl, 10 HEPES, 10 EGTA, 2 Mg-ATP, 0.5 Na₂-GTP (pH = 7.25; 295 mOsm). Voltage-clamp recordings were obtained from patched neurons at room temperature using a Multi-clamp 700 B amplifier (Molecular Devices, Sunnyvale, CA, USA), Digidata 1550 Data Acquisition System (Molecular Devices) and a personal computer running pClamp10.7 software. Initial recordings were obtained at -60 mV. Following 120 seconds of recording at -60 mV, the voltage was incrementally adjusted to +60 mV to remove blockade of NMDAR responses by magnesium ions. Pharmacological blockers were added following observation of a minimum of 20 mEPSC events in a 10-minute period.

IV. Pharmacological inhibition of specific NMDAR GluN2 subunits

Unless otherwise stated, all chemicals used in this experiment were obtained from Sigma-Aldrich. Three groups were used to assess the effects of subunit specific blockers on mEPSCs in juvenile lamina II neurons. 1 μ M Ro-25-6981 (Tocris Bioscience) (n=13) was added to inhibit GluN2B-containing NMDARs. Alternatively, 10 μ M TCN-201 (Tocris Bioscience) (n=14) was added to antagonize GluN2A-containing NMDARs. Additionally, 3 μ M TCN-201 (n=6) was added to a subset of slices prior to the escalation of concentration to 10 μ M in order to assess the optimal concentration for maximal TCN-201 effects. In an additional subset of experiments, both 1 μ M Ro-25-6981 and 10 μ M TCN-201 (n=7) were added to investigate potential additive effects and blocker-resistant NMDAR mEPSCs. The GluN2D-specific subunit inhibitor, 10 μ M DQP-1105 (Tocris Bioscience) (n=14), was used to assess the presence of GluN2D in juvenile lamina II

neurons. Each blocker was added to the aCSF during recording and allowed to perfuse for an average of 38.2 +/- 1.7 minutes (n=41).

V. Data analysis

Acquired recordings were studied using analysis software, Clampfit10. For -60 mV, the event detection criteria included a minimum amplitude of -7 pA but no more than -100 pA, and at least 200 ms between synaptic events. The criteria for events observed at +60 mV includes events with peak currents ranging from 9 pA to a maximum of 100 pA, and event decay to at least half of the peak amplitude within 500 ms. Additionally, selected events could not have occurred within 500 ms from a subsequent event. For averages of mEPSCs at -60 mV, a minimum of 10 events were required for a representative trace and measurement of peak current (pA) and time decay constant (τ_{decay} , ms). To establish a baseline for NMDAR mEPSCs at +60 mV, a minimum of 20 events were selected according to the inclusion criteria and then averaged. The same criteria were used for events observed following pharmacological inhibition, which are quantified in 10 minute increments to assess the effects of the blocker over time. Events observed during the first 20 minutes of blocker addition were not quantified to allow for a sufficient amount of time for the compound to reach the slice and act on its respective targets at synaptic sites within the tissue. The time period displaying maximal drug effects was identified and resultant average NMDAR mEPSC trace from this time period was compared against the relative NMDAR mEPSC trace at baseline. For each NMDAR mEPSC average trace, the peak amplitude (pA), charge transfer (pC), and τ_{decay} (ms) was calculated. Difference currents were calculated as a difference between the control average trace and the resultant trace

following addition of the pharmacological inhibitor to the aCSF solution. This yielded the portion of mEPSCs that was sensitive to the specific blocker used, which is often indicative of the specific subunit blocked based on the decay kinetics of the difference current. This protocol was repeated using 1000 ms analysis whereby spontaneous events were selected based on decay to $\sim 1/2$ peak current within 1000 ms. This was used to analyze the effect of 10 μM DQP-1105 on slower decaying mEPSCs, such that the slow decay of GluN2D commonly noted (1s – 4s) was not underestimated by 500 ms event selection.

Finally, to assess the potential differential expression of GluN2 subunits across the development period of interest (P7-P21), the postnatal time period was divided into 3 developmental time frames: P7-P11, P12-P16, and P17-P21. The correlation between each time frame and biophysical properties (charge transfer and τ_{decay}) was considered in the investigation of potential differences in NMDAR activity across development. Additionally, NMDAR charge transfer blockade (%) following 1 μM Ro25-6981 treatment, 10 μM TCN-201 treatment, and 1 μM Ro25-6981 + 10 μM TCN-201 treatment was segregated into the specific timeframes to elucidate possible patterns of expression for each subunit across juvenile development. The same parameters were calculated for neurons treated with 10 μM DQP-1105, using both 500 ms and 1000 ms analysis.

VI. Statistical analysis

All results are presented as average $\pm$ standard error of the mean (SEM). Statistical comparisons of data were performed using the following (where applicable): Student's paired t-test, student's unpaired t-test (unequal variance), and one- or two- way ANOVA followed by a Tukey's test for means comparisons. Paired t-test was used to analyze

statistical significance of treatment effect on charge transfer (Figure 3C, Figure 6B), as well as to compare τ_{decay} and current amplitude of the same population of cells at -60 mV versus at +60 mV (Figure 2A, Figure 5A). Unpaired t-tests assuming unequal variance were used to compare the difference in NMDAR block between different pharmacological inhibitors (Figure 3C_(inset)) and control populations (Figure 3C_(control), Figure S1A_(inset)). One-way ANOVAs were used to compare differences across specific developmental timeframes (Figure 2B, Figure 4D, Figure 5B, Figure 6C) as well as compare the difference in NMDAR block by each pharmacological inhibitor used (Figure 4C). A two-way ANOVA was used to analyze the difference in NMDAR block between 1 μM Ro25-6981 and 10 μM TCN-201 across the various developmental time frames (Figure 3D). Results were interpreted as statistically significant at $p < 0.05$.

Figure 2 – Summary of protocol for data collection. Transverse 400 μ M lumbar rat spinal cord slices are generated following spinal cord isolation from juvenile rats (P7-P21). The superficial dorsal horn in isolated spinal slices is visualized underneath 60x magnification to reveal lamina II spinal neurons. A patch-clamp protocol is utilized to achieve a whole cell patch between glass pipettes and lamina II juvenile neurons. Output current (pA) is measured using a voltage-clamp protocol at -60 mV and +60 mV. Observed miniature excitatory postsynaptic currents (mEPSCs) are chosen based on appropriate criteria and lined up using Clampfit. Averaged control and post-drug currents are computed and analyzed for τ_{decay} (ms), amplitude (pA), and charge transfer (pC). Drug effects are calculated based on residual charge transfer following drug treatment in comparison to control charge transfer; computed as average percent block (%).

Results

The glutamatergic synaptic responses (mEPSCs) of juvenile lamina II neurons are consistent across juvenile spinal cord development

To characterize postsynaptic glutamatergic responses at juvenile lamina II synapses, whole cell patch voltage-clamp electrophysiology was utilized to record mEPSCs at -60 mV and +60 mV from postnatal day 7 to 21 aged rats. Pharmacological antagonism of inhibitory GABAergic receptors (10 μ M bicuculline), inhibitory glycine receptors (10 μ M strychnine), voltage-gated calcium channels (10 μ M Cd^{2+}), and voltage-gated sodium channels (0.5 μ M TTX) produced isolated glutamatergic responses (mEPSCs). The average amplitude of mEPSCs at -60 mV was 15.9 $\pm$ 0.9 pA (n=62, Figure 2A). The average amplitude of mEPSCs at +60 mV was not significantly different from those observed at -60 mV (16.3 $\pm$ 0.7 pA, n=62, p=0.68, Figure 2A). The average τ_{decay} for mEPSCs observed at -60 mV was 11.1 $\pm$ 1.1 ms (n=62, Figure 2A), which is slightly slower than that previously reported for AMPARs in the spinal cord (6.7 ms) (Hildebrand et al., 2014). Contrastingly, the τ_{decay} for mEPSCs observed at +60 mV was 169.3 $\pm$ 7.1 ms (n=62, Figure 2A), comparable to previous reports of single-exponential τ_{decay} of NMDAR dorsal horn synaptic responses (Momiya, 2000). There was a significant difference between mEPSC τ_{decay} observed at -60 mV and +60 mV (n=62, p<0.001, Figure 2A), indicating a difference in molecular receptors mediating these two responses. We conclude that above threshold at +60 mV, both NMDAR and AMPAR are represented in observed mEPSCs, with a dominant role for NMDAR-mediated components in the prolonged phase of responses.

To quantify the NMDAR component of mEPSCs at juvenile lamina II synapses

across spinal cord development, we calculated charge transfer (pC) from 20 ms to 500 ms after onset of events at +60 mV. The Hildebrand lab previously showed that charge transfer measured during this range is exclusively mediated by NMDAR and not AMPAR activity (Hildebrand et al., 2014). The overall age range of animals (P7-P21) was evenly divided into 3 developmental bins: P7-P11 (n=22), P12-P16 (n=19), and P17-P21 (n=21). There was no significant difference ($p=0.42$) in average NMDAR charge transfer across each developmental timeframe, which was 2.58 ± 0.29 pC (n=22), 2.70 ± 0.22 pC (n=19), 2.22 ± 0.25 pC (n=21), respectively (Figure 2B). Similarly, there was no significant difference in average τ_{decay} of mEPSCs at juvenile lamina II synapses across the developmental timeframes (P7-P11: 158.7 ± 16.7 ms (n=22), P12-P16: 183.3 ± 16.3 ms (n=19), P17-P21: 167.9 ± 15.9 ms (n=21), $p=0.366$, Figure 2B). This data demonstrates consistency in overall NMDAR contribution and composition in lamina II neurons across development.

Figure 3 – Characterization of mEPSCs at juvenile lamina II synapses. (A) Average mEPSC responses at -60 mV (n=62) and +60 mV (n=62) in juvenile (P7-P21) lamina II neurons. (B) Peak current (pA) of mEPSCs at -60 mV and +60 mV did not significantly differ in juvenile lamina II neurons. (C) The decay constant (τ_{decay}) of juvenile lamina II mEPSCs is significantly different at +60 mV in comparison to -60 mV. (D) Average mEPSCs traces across specific developmental time frames: P7-P11 (n=22), P12-P16 (n=19), P17-P21 (n=21). (E) NMDAR charge transfer of mEPSC traces did not differ across specific developmental time frames. (F) Associated τ_{decay} also did not differ across specific developmental time frames. Axis (A, D): x = 100 ms, y = 5 pA. *p<0.05.

GluN2B and GluN2A significantly contribute to mEPSCs at juvenile lamina II synapses

To explore the relative contribution of GluN2 subtypes to NMDAR-mediated mEPSCs at juvenile lamina II neurons, we used subunit-specific pharmacological inhibitors and assessed their effects on NMDAR kinetic properties. The contribution of GluN2B was investigated using the GluN2B-specific inhibitor, 1 μ M Ro25-6981 (Fischer et al., 1997). NMDAR charge transfer was calculated for a 10-minute baseline control recording period and compared to a time period where antagonist effects reached a steady-

state level, typically after 40 minutes of drug administration. To quantify 1 μ M Ro25-6981-mediated blockade, the reduction in charge transfer following addition of 1 μ M Ro25-6981 was calculated by comparing the average control charge transfer to the remaining average charge transfer following drug treatment (Figure 3A). Accordingly, administration of 1 μ M Ro25-6981 resulted in a significant block of NMDAR-mediated mEPSCs at juvenile lamina II synapses (56.9 $\pm$ 7.1 %, n=13, p=0.0003, Figure 3C). To compare the contribution of GluN2A to NMDAR-mediated mEPSCs at lamina II spinal synapses, the GluN2A-specific antagonist, TCN-201, was applied. Administration of 10 μ M TCN-201 significantly blocked NMDAR-mediated charge transfer (41.6 $\pm$ 7.2 %, n=14, p=0.0006, Figure 3C). There was no significant difference between control group charge transfer for 1 μ M Ro25-6981- and 10 μ M TCN-201-treated neurons (p=0.903), suggesting that overall NMDAR properties did not diverge between lamina II neurons in the two treatment groups. To assess the selectivity of the specific pharmacological inhibitors, the drug-sensitive difference current was graphed and fitted for τ_{decay} . The 1 μ M Ro25-6981-sensitive difference current displayed a τ_{decay} of 354 ms (n=13, Figure 3A), which falls within the τ_{decay} range for GluN2B commonly reported (Hildebrand et al., 2014; Paoletti, Bellone, & Zhou, 2013). The 10 μ M TCN-201-sensitive difference current had a τ_{decay} of 158 ms (n=14, Figure 3B), consistent with a decay much faster than that of GluN2B (Hildebrand et al., 2014; Vicini et al., 1998).

We explored potential developmental switches in GluN2 subunit expression between days 7 to 21 of juvenile development by analyzing the effects of specific subunit pharmacological inhibition according to the aforementioned developmental time frames: P7-P11 (n_{Ro}=4, n_{TCN}=5), P12-P16 (n_{Ro}=5, n_{TCN}=5), P17-P21 (n_{Ro}=4, n_{TCN}=4). Two-way

ANOVA analysis revealed no significant difference in drug block for either 1 μ M Ro25-6981 ($p=0.24$) or 10 μ M TCN-201 ($p=0.72$) across development (Figure 3D). There was also no significant interaction between age group and drug used ($p=0.42$). We therefore conclude that the relative contributions of GluN2A versus GluN2B to synaptic NMDAR responses in lamina II neurons does not change across juvenile development (Figure 3D).

Figure 4 – GluN2B and GluN2A both contribute prominently to NMDAR responses at lamina II synapses throughout early development. (A) Averaged NMDAR mEPSCs before (black) and after (red) application of 1 μ M Ro25-6981 ($n=13$) in juvenile (P7-P21) lamina II neurons. 1 μ M Ro25-6981-sensitive NMDAR mEPSC difference current ($n=13$), with a decay constant of 354 ms (inset). (B) Averaged NMDAR mEPSCs before (black) and after (green) application of 10 μ M TCN-201 ($n=14$) in juvenile (P7-P21) lamina II neurons. 10 μ M TCN-201-sensitive NMDAR mEPSC difference current ($n=13$) illustrates the inhibition of a fast ($\tau_{\text{decay}} = 158$ ms) component of juvenile lamina II mEPSCs (inset). (C) Average NMDAR charge transfer (pC) before (black) and after application of 1 μ M Ro25-6981 (red) or 10 μ M TCN-201 (green). There was no significant difference in overall NMDAR block between 1 μ M Ro25-6981- and 10 μ M TCN-201-treated cells. (D) NMDAR block (%) at various developmental time frames: P7-P11 ($n=4,5$), P12-P16 ($n=5,5$), P17-21 ($n=4,4$). Axis (A, B): $x = 100$ ms, $y = 5$ pA. * $p < 0.05$.

GluN2D has a minor contribution to mEPSCs at juvenile lamina II synapses

Taken together, the average block observed following individual treatment with 1 μ M Ro25-6981 and 10 μ M TCN-201 sums up to ~98%, theoretically implying the sole presence of these two receptor subtypes in mediating NMDAR responses at juvenile lamina II spinal synapses. To directly test this, both 1 μ M Ro25-6981 and 10 μ M TCN-201 were added sequentially during recordings of NMDAR mEPSCs at +60 mV. Interestingly, when added together, 1 μ M Ro25-6981 + 10 μ M TCN-201 blocked 64.7 $\pm$ 11 % of overall NMDAR charge transfer (n=7, p=0.01, Figure 4A). Ultimately, this implies the presence of a Ro25-6981- and TCN-201- resistant component contributing to NMDAR-mediated mEPSCs at juvenile lamina II synapses.

To explore the molecular identity of NMDARs that mediate Ro25-6981- and TCN-201-resistant mEPSCs, the GluN2D-specific pharmacological inhibitor, DQP-1105, was applied. Treatment with 10 μ M DQP-1105 resulted in an average block of 23.7 $\pm$ 8.6 % of NMDAR-mediated mEPSCs at juvenile synapses (n=14, p=0.03, Figure 4B). The 10 μ M DQP-1105-sensitive difference current exhibited a τ_{decay} of 596 ms (n=14, Figure 4B) consistent with GluN2D-mediated decay, which is much slower than either GluN2A or GluN2B (Cull-Candy & Leszkiewicz, 2004; Hildebrand et al., 2014; Paoletti, Bellone, & Zhou, 2013).

Additionally, we investigated potential differences in the contribution of GluN2D to juvenile lamina II synaptic responses across development by calculating average DQP-mediated block for each developmental time frame: P7-11 (n=5), P12-P16 (n=5), and P17-P21 (n=4). One-way ANOVA analysis illustrated no significant difference in DQP-1105 inhibition of NMDAR-mediated mEPSCs across development (p=0.07). When comparing

all of the pharmacological inhibitors, there was no significant difference in overall NMDAR block between 1 μ M Ro25-6981 and 10 μ M TCN-201 ($p=0.35$, Figure 4C) or 10 μ M TCN-201 and 10 μ M DQP-1105 ($p=0.23$, Figure 4C). However, 1 μ M Ro25-6981 inhibited NMDAR charge transfer significantly more than 10 μ M DQP-1105 ($p=0.01$, Figure 4C).

Figure 5 – GluN2D minimally contributes to NMDAR-components of juvenile lamina II mEPSCs. (A) Averaged NMDAR mEPSCs before (black) and after (purple) application of 10 μ M Ro25-6981 + 10 μ M TCN-201 ($n=7$) in juvenile (P7-P21) lamina II neurons. (B) Averaged NMDAR mEPSCs before (black) and after (blue) application of 10 μ M DQP-1105 ($n=14$) in juvenile (P7-P21) lamina II neurons. 10 μ M DQP-1105-sensitive NMDAR mEPSC difference current ($n=14$) illustrates a decay constant of 596 ms (inset). (C) Average NMDAR charge transfer reduction (%) following application of 1 μ M Ro25-6981, 10 μ M TCN-201 or 10 μ M DQP-1105. (D) Average drug block (%) at various developmental time frames following 10 μ M DQP-1105 treatment: P7-P11 ($n=5$), P12-P16 ($n=5$), P17-21 ($n=4$). Axis (A, B): $x=100$ ms, $y=5$ pA. $*p<0.05$.

The relative contribution of slow decaying mEPSCs does not differ across development at juvenile lamina II synapses

Due to the slow decaying kinetics of GluN2D, we re-analyzed our data using criteria that allowed for the inclusion of slower decaying mEPSCs. Accordingly, events were chosen based on the criteria of current decay to at least half the peak current amplitude within 1000 ms instead of 500 ms, to ensure that slower NMDAR components of juvenile lamina II mEPSCs were not underestimated. Using this criteria, the average τ_{decay} of NMDAR-mediated components of juvenile synaptic responses measured 248.0 ± 9.6 ms ($n=53$, Figure 5A), much slower than that observed for 500 ms analysis (169.3 ± 7.1 ms, $n=62$, $p<0.0001$, Figure 2A). The average amplitude of mEPSCs at +60 mV was 16.4 ± 0.7 pA ($n=53$, Figure 5A). This was similar to that observed at +60 mV 500 ms analysis and not significantly different to the average amplitude of mEPSCs at -60 mV ($n=53$, $p=0.78$, Figure 5A). The τ_{decay} of juvenile lamina II mEPSCs was significantly different at +60 mV when compared to -60 mV ($p<0.0001$).

Similar to 500 ms analysis, we tested for potential differences in NMDAR kinetics across development for the 1000 ms analysis. Charge transfer was calculated from 20 ms to 1000 ms after event onset across specific timeframes: P7-P11 ($n=20$), P12-P16 ($n=15$), P17-P21 ($n=18$). There was no significant difference ($p=0.906$) in average charge transfer across each developmental timeframe: 3.91 ± 0.50 pC, 3.86 ± 0.30 pC (P12-P16), 4.15 ± 0.57 pC, respectively (Figure 5B). Furthermore, there was no significant difference ($p=0.955$) in average τ_{decay} of mEPSCs at juvenile lamina II synapses across development: 246.3 ± 14.7 ms, 252.7 ± 16.9 ms, and 245.9 ± 16.2 ms, respectively (Figure 5B). This data supports and augments our previous conclusion that there is no change in NMDAR

subunit contribution across lamina II development.

Figure 6 – Characterization of slow decaying mEPSCs at juvenile lamina II synapses. (A) Average mEPSC responses at +60 mV (n=53) in juvenile (P7-P21) lamina II neurons. (B) Peak current (pA) of mEPSCs at -60 mV and +60 mV did not significantly differ. (C) τ_{decay} of juvenile lamina II mEPSCs were significantly different at +60 mV when compared to -60 mV. (D) Average NMDAR mEPSC responses across specific developmental time frames: P7-P11 (n=20), P12-P16 (n=15), P17-P21 (n=18). Charge transfer of mEPSCs at +60 mV did not differ across development. The τ_{decay} did not differ across development. Axis (A, D): x = 100 ms, y = 5 pA. *p<0.05.

Inclusion of slow decaying mEPSCs does not increase the effects of the GluN2D antagonist, DQP-1105, at juvenile lamina II synapses

To examine whether the effect of DQP-1105 was under-represented by 500 ms analysis, 10 μ M DQP-1105-treated cells were analyzed using 1000 ms event analysis. Analysis revealed an average DQP-1105-mediated block of 21.2 $\pm$ 9.7 % of NMDAR-mediated mEPSCs at juvenile synapses (n=12, p=0.02, Figure 6A), which was not significantly different than that observed from 500 ms analysis (p=0.85). Furthermore, we investigated the differential contribution of GluN2D to juvenile lamina II synaptic responses

by calculating average block for each developmental time frame: P7-P11 (n=5), P12-P16 (n=4), P17-P21 (n=3). One-way ANOVA analysis revealed no significant difference in DQP-1105 inhibition of NMDAR-mediated mEPSCs across development ($p=0.737$, Figure 6B). Altogether, this suggests GluN2D is functionally present at juvenile lamina II synapses but does not contribute to NMDAR-mediated mEPSCs as much as GluN2B and does not vary in relative contribution across juvenile development.

Figure 7 – GluN2D minimally contributes to slow decaying NMDAR-components of juvenile lamina II mEPSCs. (A) Averaged NMDAR mEPSCs before (black) and after (blue) application of 10 μ M DQP-1105 (n=12) in juvenile (P7-P21) lamina II neurons. (B) Average remaining NMDAR charge transfers for control (n=12) and 10 μ M DQP-1105-treated cells (n=12) (left). (C) Percentage of NMDAR mEPSCs blocked at various developmental time frames following 10 μ M DQP-1105 treatment (right): P7-P11 (n=5), P12-P16 (n=4), P17-21 (n=3). Axis (A): x = 100 ms, y = 5 pA. * $p<0.05$.

Discussion

Whole-cell electrophysiological recordings of spontaneous synaptic responses enabled us to investigate the contributions of GluN2A- GluN2B-, and GluN2D- mediated NMDAR responses in juvenile lamina II neurons. Pharmacologically isolated mEPSCs at juvenile lamina II synapses exhibited a fast AMPAR-mediated current at -60 mV. A voltage-dependent NMDAR-mediated component was observed only at +60 mV, and identified according to decay kinetics that were significantly slower than that of currents at -60 mV, contributing to the majority of the observed charge transfer at +60 mV. Pharmacological inhibition of mEPSCs at +60 mV using GluN2-subunit specific antagonists, TCN-201 and Ro25-6981, revealed a significant and equal contribution of GluN2A and GluN2B to juvenile lamina II synaptic responses, in contrast to the dominance of GluN2B over GluN2A at mature lamina I synapses (Hildebrand et al., 2014). Like-wise, DQP-1105-treated neurons elucidated a functional presence of GluN2D at juvenile lamina II synapses that is less than that of GluN2B, in contrast to the dominant role of GluN2D contribution to mature lamina I synaptic responses (Hildebrand et al., 2014). Taken together, our data suggests divergent contribution of NMDARs to synaptic signaling in lamina II compared to lamina I, likely owing to their differing roles in spinal signal transmission and integration.

Our data illustrates an equal contribution of GluN2A- and GluN2B-mediated NMDAR responses at lamina II synapses, unlike the dominant role of GluN2B at early juvenile synapses in the brain. During brain development, the functional contribution of GluN2B peaks during the second postnatal week and decreases thereafter, eventually being replaced by GluN2A as the dominant contributor to NMDAR-mediated synaptic responses in the

adult brain (Paoletti, Bellone, & Zhou, 2013). In contrast, GluN2B has been shown to have a larger presence than GluN2A at adult lamina I spinal synapses (Hildebrand et al., 2014; Momiyama, 2000). This data suggests a developmental switch from GluN2B to GluN2A expression across brain development. Since the opposite expression is observed in adult spinal cord, it was hypothesized that GluN2B-mediated NMDAR responses may increase at lamina II synapses overtime. To explore this, we divided the animals into three age groups: P7-P11, P12-P16, and P17-P21. Interestingly, we observed no significant difference in the contribution of either GluN2A or GluN2B across each age group, unlike the GluN2B to GluN2A switch in synaptic expression and contribution commonly observed during juvenile brain development (Matta et al., 2011; Rodenas-Ruano et al., 2012; Sheng et al., 1994). Our data augments initial reports proposing the maturation of glutamatergic synapses in the superficial dorsal horn by birth (Bardoni et al., 1998). Ultimately, this suggests differing glutamatergic circuitry in juvenile spinal cord in comparison to the brain. The conservation of prominent slow-decaying GluN2B responses throughout spinal cord development into adulthood potentially mediates the increased likelihood of signal potentiation at spinal synapses, resulting in a susceptibility for the development of pathological pain.

When added together, the GluN2A and GluN2B antagonists (TCN-201 and Ro25-6981, respectively) did not block NMDAR-mediated synaptic responses entirely. This was not expected considering TCN-201 and Ro25-6981 each blocked about half of their respective mEPSC charge transfers, theoretically adding up to 100% in tandem. This result could be explained by differential maximal drug effects of these antagonists against diheteromers versus triheteromers. Triheteromeric receptors exhibit kinetic properties that

are distinct from diheteromeric receptors (Hatton & Paoletti, 2005; Rauner & Kohr, 2010). Ifenprodil-derived Ro25-6981 preferentially antagonizes diheteromeric GluN1/GluN2B NMDARs, with less potent inhibition of triheteromeric NMDARs containing GluN1/2A/2B (Hatton & Paoletti, 2005). In fact, the maximum inhibition of ifenprodil on these receptors is approximately 20% (Hatton & Paoletti, 2005; Rauner & Kohr, 2010). Moreover, if the composition of lamina II neurons is mainly triheteromeric receptors, then part of the substantial residual current that is resistant to GluN2A and GluN2B antagonists may be mediated by GluN1/GluN2A/GluN2B NMDARs (Hatton & Paoletti, 2005), resulting in an unclear representation of the relative contribution of each subunit to overall synaptic responses. The presence of triheteromeric receptors at synapses poses an issue where subunit discrimination is concerned, therefore the development of triheteromeric-specific pharmacological inhibitors is required to augment our current understanding of differential properties of NMDARs.

A component of the residual TCN-201+ Ro25-6981-resistant current can also be explained by the contribution of an additional subunit to NMDAR juvenile synaptic responses. Since GluN2D has been identified at adult lamina I synapses, a GluN2D-selective pharmacological inhibitor, DQP-1105, was used to elucidate the functional relevance of GluN2D subunits at juvenile lamina II synapses. Our data confirmed a small role of GluN2D-mediated NMDAR responses at juvenile lamina II synapses, in contrast to the major role of GluN2D at adult spinal lamina I synapses (Hildebrand et al., 2014). Although GluN2D contribution at juvenile lamina II synapses appears to be less than that of GluN2B, it is more than what is generally noted in the brain (Cull-Candy & Leszkiewicz, 2004; Monyer et al., 1994). To ensure the validity of our conclusions, event selection was

broadened from 500 ms to 1000 ms to include the detection of slower decaying currents such as those mediated by GluN2D (decay constant from 600 to 4000 ms (Monyer et al., 1994; Vicini et al., 1998). Analysis revealed a consistency in block of NMDAR synaptic responses by DQP-1105 between both criteria. These results confirm that the effects of DQP-1105 were not underestimated, and in fact GluN2D NMDARs mediate a small fraction of mEPSCs at juvenile lamina II spinal synapses.

Limitations

Although the results of statistical comparisons showed that NMDAR responses and decay constants were consistent across control treatment groups, heterogeneity in these values across lamina II neurons was apparent.

Recording mEPSCs entails analyzing averaged synaptic responses originating from one or more of the following: peripheral nociceptive afferents, local excitatory interneurons, and descending inputs from the brain. While pharmacological inhibition of receptors such as GABA, glycine, and voltage-gated sodium channels isolates postsynaptic responses, distinguishing between the different presynaptic inputs driving these averaged NMDAR synaptic responses is a challenge. Additionally, previously reported NMDAR EPSCs in juvenile lamina II neurons indicate increased heterogeneity in synaptic responses at mixed glutamatergic synapses (AMPA and NMDAR) in comparison to pure NMDA or AMPA synapses (Bardoni et al., 1998). Furthermore, various conformations of NMDARs could be present at any given synapses as a heterogeneous or homogenous population. Inevitably, this leads to differential drug effects since each slice (containing one patched neuron) is treated with only one drug at a time. The only exception was with the subsequent treatment of neurons with both 1 μ M Ro25-6981 and 10 μ M TCN-201.

Therefore, this electrophysiological data would be well suited to be combined with staining or western blot biochemical approaches to quantify overall GluN2 subunit expression and compare to what is observed functionally.

The heterogeneity in NMDAR mEPSCs between individual cells can also be attributable to the subpopulation of interneurons present in lamina II. There are four types of interneurons present in lamina II (islet, central, vertical, radial) that differ in morphology and excitability, and these distinct cell types could express different variants of NMDARs. An attempt was made at classifying patched lamina II neurons based on dendritic morphology. We aliquoted Lucifer yellow in the internal solution such that treated neurons could later be imaged under a confocal or two-photon microscope. Unfortunately, this technique was not successful, as the rate of successfully filling the patched neurons was approximately 25%. Of those that survived, the light sensitive properties of Lucifer yellow, as well the thickness (400 μm) and myelination of the tissue slice resulted in insufficient resolution for imaging.

It is important to note that heterogeneity among individual cells treated with different pharmacological inhibitors is potentially a product of small population numbers in each group ($n=12$ to 14). Performing pharmacology on mEPSCs is particularly challenging due to limited neuronal viability. The protocol employed in these experiments was refined by the Hildebrand lab to maximize neuronal viability and limit excitotoxicity. However, the length of time required for valid pharmacological analysis, 30-60 minutes for individual inhibitor treatment and up to two hours for double-pharmacology treatments, creates a demanding criteria of neuronal health for any neuronal patch-clamp recording, let alone for delicate dorsal horn neurons. A limited amount of neurons survive long enough

to be treated with a pharmacological inhibitor, and of those that do, not all survive the length of time required for the inhibitor to reach the cell and act on its respective receptors. Nevertheless, consistency in overall NMDAR control kinetics across the entire population of patched neurons (n=62) using different analysis methods justifies the generalization of our results to represent GluN2-subunit expression across the juvenile lamina II neuronal population.

Future direction

To test whether the composition of NMDAR mEPSCs at lamina II neurons was conserved across development into adult, we performed preliminary experiments on NMDAR-mediated synaptic responses in adult lamina II neurons. When compared to juvenile NMDAR-mediated mEPSCs, adult responses appear slower, with a τ_{decay} of 212 ms (n=7), in comparison to the 169 ms (n=60) measured for the decay of juvenile responses (Figure S1A). The relative contribution of GluN2D-contained NMDARs was assessed using analysis of residual current (%), computed as the ratio of current from 975 ms to 1000 ms with respect to peak current. This revealed a slightly higher but not significant residual current at adult lamina II synapses, 12.3 $\pm$ 0.4 % (n=7), in comparison to juvenile synapses (10.0 $\pm$ 0.7, n=60, p=0.237, Figure S1A). The adult lamina II neurons were also treated with 1 μ M Ro25-6981 to assess the contribution of GluN2B to adult NMDAR-mediated mEPSCs. 1 μ M Ro25-6981 had a prominent effect on lamina II synaptic responses: 40.1 $\pm$ 4.3% inhibition (n=4, p=0.02, Figure S1B). The remaining charge transfer following 1 μ M Ro25-6981 treatment revealed a prominent contribution of a slower decaying component to NMDAR-mediated lamina II spinal synapses, potentially mediated by GluN2D (Figure S1B). Additionally, a fast GluN2A-like component may also

contribute to this residual activity. There is a need for more research probing the composition of NMDARs in both juvenile lamina I and adult lamina II neurons such that a complete understanding of underlying regulation of NMDAR composition can be obtained across complete spinal cord development and spinal laminae. Understanding the differences between developing and mature spinal circuitry is potentially useful to identifying key molecular targets underlying the prevalence of maladaptive pain signaling in aging populations. Furthermore, the clinical prevalence of chronic pain is greater in females than males (Moulin et al., 2002; Schopflocher, Taenzer, & Jovey, 2011), suggesting a potential difference in underlying mechanisms. It is imperative that future studies include both female and male experimental groups such that specific molecular targets contributing to this clinical difference can be elucidated. Target identification is a crucial first step in the development of effective, personalized pharmacological inhibitors that alter spinal noxious signal transmission and intractable human pain.

References

- Acker, T. M., Yuan, H., Hansen, K. B., Vance, K. M., Ogden, K. K., Jensen, H. S., ... Traynelis, S. F. (2011). Mechanism for Noncompetitive Inhibition by Novel GluN2C / D N -Methyl- D -aspartate Receptor Subunit-Selective Modulators □, 80(5), 782–795. <https://doi.org/10.1124/mol.111.073239>.
- Akazawa, C., Shigemoto, R., Bessho, Y., Nakanishi, S., & Mizuno, N. (1994). Differential expression of five N-methyl-D-aspartate receptor subunit mRNAs in the cerebellum of developing and adult rats. *The Journal of Comparative Neurology*, 347(1), 150–160. <https://doi.org/10.1002/cne.903470112>
- Bardoni, R., Magherini, P. C., & MacDermott, a B. (1998). NMDA EPSCs at glutamatergic synapses in the spinal cord dorsal horn of the postnatal rat. *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience*, 18(16), 6558–67. Retrieved from <http://www.ncbi.nlm.nih.gov/pubmed/9698343>
- Bartlett, T. E., Bannister, N. J., Collett, V. J., Dargan, S. L., Massey, P. V., Bortolotto, Z. A., ... Lodge, D. (2007). Differential roles of NR2A and NR2B-containing NMDA receptors in LTP and LTD in the CA1 region of two-week old rat hippocampus. *Neuropharmacology*, 52(1), 60–70. <https://doi.org/10.1016/j.neuropharm.2006.07.013>
- Berberich, S., Jensen, V., Hvalby, Ø., Seeburg, P. H., & Köhr, G. (2007). The role of NMDAR subtypes and charge transfer during hippocampal LTP induction. *Neuropharmacology*, 52(1), 77–86. <https://doi.org/https://doi.org/10.1016/j.neuropharm.2006.07.016>
- Bettini, E., Sava, A., Griffante, C., Carignani, C., Buson, A., Capelli, A. M., ... Cardullo, F. (2010). Identification and characterization of novel NMDA receptor antagonists selective for NR2A- over NR2B-containing receptors. *The Journal of Pharmacology and Experimental Therapeutics*, 335(3), 636–644. <https://doi.org/10.1124/jpet.110.172544>
- Binder, A., & Baron, R. (2016). The Pharmacological Therapy of Chronic Neuropathic Pain, 616–627. <https://doi.org/10.3238/arztebl.2016.0616>
- Bliss, T. V. P., & Collingridge, G. L. (1993). A synaptic model of memory: long-term potentiation in the hippocampus. *Nature*, 361, 31. Retrieved from <http://dx.doi.org/10.1038/361031a0>
- Carmignoto, G., & Vicini, S. (1992). Activity-dependent decrease in NMDA receptor responses during development of the visual cortex. *Science (New York, N.Y.)*, 258(5084), 1007–1011.
- Chazot, P. L., Lawrence, S., & Thompson, C. L. (2002). Studies on the subtype selectivity of CP-101,606: evidence for two classes of NR2B-selective NMDA receptor antagonists. *Neuropharmacology*, 42(3), 319–324.
- Chazot, P. L., Sarah, K., Cik, M., & Stephenson, F. A. (1994). Molecular Characterization of N-Methyl-D-aspartate Receptors Expressed in Mammalian Cells Yields Evidence for the Coexistence of Three Subunit Types within a Discrete Receptor Molecule.
- Cordero-Erausquin, M., Inquimbert, P., Schlichter, R., & Hugel, S. (2016). Neuronal networks and nociceptive processing in the dorsal horn of the spinal cord.

- Neuroscience*, 338(Supplement C), 230–247.
<https://doi.org/https://doi.org/10.1016/j.neuroscience.2016.08.048>
- Cull-Candy, S. G., & Leszkiewicz, D. N. (2004). Role of distinct NMDA receptor subtypes at central synapses. *Science's STKE : Signal Transduction Knowledge Environment*, 2004(255), re16. <https://doi.org/10.1126/stke.2552004re16>
- D'Mello, R., & Dickenson, A. H. (2008). Spinal cord mechanisms of pain. *British Journal of Anaesthesia*, 101(1), 8–16. <https://doi.org/10.1093/bja/aen088>
- Dickenson, A. H., Chapman, V., & Green, G. M. (1997). The pharmacology of excitatory and inhibitory amino acid-mediated events in the transmission and modulation of pain in the spinal cord. *General Pharmacology*, 28(5), 633–638.
- Fischer, G., Mutel, V., Trube, G., Malherbe, P., Kew, J. N., Mohacsi, E., ... Kemp, J. A. (1997). Ro 25-6981, a highly potent and selective blocker of N-methyl-D-aspartate receptors containing the NR2B subunit. Characterization in vitro. *The Journal of Pharmacology and Experimental Therapeutics*, 283(3), 1285–1292.
- Fox, C. J., Russell, K. I., Wang, Y. T., & Christie, B. R. (2006). Contribution of NR2A and NR2B NMDA subunits to bidirectional synaptic plasticity in the hippocampus in vivo. *Hippocampus*, 16(11), 907–915. <https://doi.org/10.1002/hipo.20230>
- Hansen, K. B., Ogden, K. K., & Traynelis, S. F. (2012). Subunit-selective allosteric inhibition of glycine binding to NMDA receptors. *The Journal of Neuroscience*, 32(18), 6197–6208. <https://doi.org/10.1523/JNEUROSCI.5757-11.2012>
- Hatton, C. J., & Paoletti, P. (2005). Modulation of triheteromeric NMDA receptors by N-terminal domain ligands. *Neuron*, 46(2), 261–274.
<https://doi.org/10.1016/j.neuron.2005.03.005>
- Hildebrand, M. E., Pitcher, G. M., Harding, E. K., Li, H., Beggs, S., & Salter, M. W. (2014). GluN2B and GluN2D NMDARs dominate synaptic responses in the adult spinal cord. *Scientific Reports*, 4(1), 4094. <https://doi.org/10.1038/srep04094>
- Hummel, M., Strassle, B., Miller, S., Kaftan, E., Whiteside, G., & Neuroscience, D. (2008). NMDA-2D RECEPTOR SUBUNIT IN A RAT MODEL OF NEUROPATHIC PAIN, 155, 492–502.
<https://doi.org/10.1016/j.neuroscience.2008.05.041>
- Köhr, G., Jensen, V., Koester, H. J., Mihaljevic, A. L. a, Utvik, J. K., Kvellø, A., ... Hvalby, Ø. (2003). Intracellular domains of NMDA receptor subtypes are determinants for long-term potentiation induction. *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience*, 23(34), 10791–9.
<https://doi.org/23/34/10791> [pii]
- Lannersten, L., & Kosek, E. (2010). Dysfunction of endogenous pain inhibition during exercise with painful muscles in patients with shoulder myalgia and fibromyalgia. *Pain*, 151(1), 77–86. <https://doi.org/10.1016/j.pain.2010.06.021>
- Li, R., Huang, F.-S., Abbas, A.-K., & Wigstrom, H. (2007). Role of NMDA receptor subtypes in different forms of NMDA-dependent synaptic plasticity. *BMC Neuroscience*, 8, 55. <https://doi.org/10.1186/1471-2202-8-55>
- Liu, L., Wong, T. P., Pozza, M. F., Lingenhöhl, K., Wang, Y., Sheng, M., ... Wang, Y. T. (2004). Role of NMDA receptor subtypes in governing the direction of hippocampal synaptic plasticity. *Science (New York, N.Y.)*, 304(5673), 1021–1024.
<https://doi.org/10.1126/science.1096615>
- Luo, J., Wang, Y., Yasuda, R. P., Dunah, A. W., & Wolfe, B. B. (1997). The Majority of

- N -Methyl- D -Aspartate Receptor Complexes in Adult Rat Cerebral Cortex Contain at Least Three Different Subunits (NR1 / NR2A / NR2B), 86, 79–86.
- Lynch, M. E. (2011). The need for a Canadian pain strategy. *Pain Research & Management*, 16(2), 77–80. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/21499581>
- Matta, J. A., Ashby, M. C., Sanz-Clemente, A., Roche, K. W., & Isaac, J. T. R. (2011). mGluR5 and NMDA receptors drive the experience- and activity-dependent NMDA receptor NR2B to NR2A subunit switch. *Neuron*, 70(2), 339–351. <https://doi.org/10.1016/j.neuron.2011.02.045>
- Maxwell, D. J., Belle, M. D., Cheunsuang, O., Stewart, A., & Morris, R. (2007). Morphology of inhibitory and excitatory interneurons in superficial laminae of the rat dorsal horn. *The Journal of Physiology*, 584(Pt 2), 521–533. <https://doi.org/10.1113/jphysiol.2007.140996>
- Mayer, M. L., Westbrook, G. L., & Guthrie, P. B. (1984). Voltage-dependent block by Mg²⁺ of NMDA responses in spinal cord neurones. *Nature*, 309(5965), 261–263.
- Merskey, H. (1986). *Descriptions of chronic pain syndroms: first draft collection for the International Association for the Study of Pain. IASP PRESS.*
- Merskey, H., & Bogduk, N. (1994). *Classification of chronic pain: descriptions of chronic pain syndromes and definitions of pain. IASP PRESS.*
- Misra, C., Brickley, S. G., Wyllie, D. J. A., & Cull-candy, S. G. (2000). Rapid Report Slow deactivation kinetics of NMDA receptors containing NR1 and NR2D subunits in rat cerebellar Purkinje cells, 299–305.
- Momiyama, A. (2000). Distinct synaptic and extrasynaptic NMDA receptors identified in dorsal horn neurones of the adult rat spinal cord. *The Journal of Physiology*, 523 Pt 3, 621–8. <https://doi.org/10.1111/j.1469-7793.2000.t01-1-00621.x>
- Momiyama, A., Feldmeyer, D., & Cull-Candy, S. G. (1996). Identification of a native low-conductance NMDA channel with reduced sensitivity to Mg²⁺ in rat central neurones. *The Journal of Physiology*, 494(Pt 2), 479–492. Retrieved from <http://www.ncbi.nlm.nih.gov/pmc/articles/PMC1160649/>
- Monyer, H., Burnashev, N., Laurie, D. J., Sakmann, B., & Seeburg, P. H. (1994). Developmental and regional expression in the rat brain and functional properties of four NMDA receptors. *Neuron*, 12(3), 529–540.
- Morishita, W., Lu, W., Smith, G. B., Nicoll, R. A., Bear, M. F., & Malenka, R. C. (2007). Activation of NR2B-containing NMDA receptors is not required for NMDA receptor-dependent long-term depression. *Neuropharmacology*, 52(1), 71–76. <https://doi.org/10.1016/j.neuropharm.2006.07.005>
- Moulin, D. E., Clark, A. J., Speechley, M., & Morley-Forster, P. K. (2002). Chronic pain in Canada--prevalence, treatment, impact and the role of opioid analgesia. *Pain Research & Management*, 7(4), 179–184.
- Paoletti, P., Ascher, P., & Neyton, J. (1997). High-affinity zinc inhibition of NMDA NR1-NR2A receptors. *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience*, 17(15), 5711–5725.
- Paoletti, P., Bellone, C., & Zhou, Q. (2013). NMDA receptor subunit diversity: impact on receptor properties, synaptic plasticity and disease. *Nature Reviews. Neuroscience*, 14(6), 383–400. <https://doi.org/10.1038/nrn3504>
- Quinlan, E. M., Olstein, D. H., & Bear, M. F. (1999). Bidirectional, experience-

- dependent regulation of N-methyl-D-aspartate receptor subunit composition in the rat visual cortex during postnatal development. *Proceedings of the National Academy of Sciences of the United States of America*, 96(22), 12876–80. Retrieved from <http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=23143&tool=pmcentrez&rendertype=abstract>
- Rauner, C., & Kohr, G. (2010). Triheteromeric NR1 / NR2A / NR2B Receptors Constitute the Major N -Methyl- D -aspartate Receptor Population in Adult Hippocampal Synapses, 286(9), 7558–7566. <https://doi.org/10.1074/jbc.M110.182600>
- Rodenas-Ruano, A., Chavez, A. E., Cossio, M. J., Castillo, P. E., & Zukin, R. S. (2012). REST-dependent epigenetic remodeling promotes the developmental switch in synaptic NMDA receptors. *Nature Neuroscience*, 15(10), 1382–1390. <https://doi.org/10.1038/nn.3214>
- Roques, B. P., Fournie-Zaluski, M.-C., & Wurm, M. (2012). Inhibiting the breakdown of endogenous opioids and cannabinoids to alleviate pain. *Nature Reviews. Drug Discovery*, 11(4), 292–310. <https://doi.org/10.1038/nrd3673>
- Schopflocher, D., Taenzer, P., & Jovey, R. (2011). The prevalence of chronic pain in Canada, 16(6), 445–450.
- Sheng, M., Cmings, J., Roldan, L. A., Jan, Y. N., & Jan, L. Y. (1994). Changing subunit composition of heteromeric NMDA receptors during development of rat cortex. *Nature*, 368(6467), 144–147. <https://doi.org/10.1038/368144a0>
- Shipton, O. A., & Paulsen, O. (2013). GluN2A and GluN2B subunit-containing NMDA receptors in hippocampal plasticity. *Philosophical Transactions of the Royal Society B: Biological Sciences*, 369(1633), 20130163–20130163. <https://doi.org/10.1098/rstb.2013.0163>
- Standaert, D. G., Landwehrmeyer, G. B., Kerner, J. A., Penney, J. B. J., & Young, A. B. (1996). Expression of NMDAR2D glutamate receptor subunit mRNA in neurochemically identified interneurons in the rat neostriatum, neocortex and hippocampus. *Brain Research. Molecular Brain Research*, 42(1), 89–102.
- Todd, A. J. (2010). Neuronal circuitry for pain processing in the dorsal horn. *Nature Reviews Neuroscience*, 11(12), 823–836. <https://doi.org/10.1038/nrn2947>
- Tong, C.-K., & MacDermott, A. B. (2014). Synaptic GluN2A and GluN2B Containing NMDA Receptors within the Superficial Dorsal Horn Activated following Primary Afferent Stimulation. *Journal of Neuroscience*, 34(33), 10808–10820. <https://doi.org/10.1523/JNEUROSCI.0145-14.2014>
- Tovar, K. R., Mcginley, M. J., & Westbrook, G. L. (2013). NIH Public Access, 33(21), 9150–9160. <https://doi.org/10.1523/JNEUROSCI.0829-13.2013>
- Traynelis, S. F., Wollmuth, L. P., McBain, C. J., Menniti, F. S., Vance, K. M., Ogden, K. K., ... Dingledine, R. (2010). Glutamate receptor ion channels: structure, regulation, and function. *Pharmacological Reviews*, 62(3), 405–496. <https://doi.org/10.1124/pr.109.002451>
- Vicini, S., Wang, J. F., Li, J. H., Zhu, W. J., Wang, Y. H., Luo, J. H., ... Grayson, D. R. (1998). Functional and pharmacological differences between recombinant N-methyl-D-aspartate receptors. *Journal of Neurophysiology*, 79(2), 555–66. [https://doi.org/10.1016/0014-5793\(92\)80648-Z](https://doi.org/10.1016/0014-5793(92)80648-Z)

- Volianskis, A., France, G., Jensen, M. S., Bortolotto, Z. A., Jane, D. E., & Collingridge, G. L. (2015). Long-term potentiation and the role of N-methyl-D-aspartate receptors. *Brain Research*, 1621, 5–16.
<https://doi.org/10.1016/j.brainres.2015.01.016>
- Williams, K. (1993). Ifenprodil discriminates subtypes of the N-methyl-D-aspartate receptor: selectivity and mechanisms at recombinant heteromeric receptors. *Molecular Pharmacology*, 44(4), 851–859.
- Yung, K. K. (1998). Localization of glutamate receptors in dorsal horn of rat spinal cord. *Neuroreport*, 9(7), 1639–1644.