Immunohistochemical characterization of GluN2 NMDA receptor subunit expression in the dorsal horn of rats and humans

by

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Expression of NMDAR subunits in the dorsal horn of rats and humans

Abstract

NMDA receptors (NMDARs) are excitatory ionotropic glutamate receptors expressed throughout the CNS, including in the superficial dorsal horn (SDH) of the spinal cord. The GluN2 subtypes of NMDAR subunit, GluN2A, GluN2B and GluN2D, confer NMDARs with structural and functional variability, enabling heterogeneity in synaptic transmission and plasticity. Despite essential roles for NMDARs in physiological and pathological pain processing within the SDH, the distribution and function of specific GluN2 isoforms across SDH laminae remains poorly understood. Surprisingly, there is a complete lack of knowledge on GluN2 expression in female rodent or human spinal cord. In this study we therefore aimed to investigate the relative expression of specific GluN2 variants in the L4/L5 lumbar SDH of both male and female rats and humans. To detect synaptic GluN2 isoforms that are expressed in the SDH (GluN2A, 2B and 2D), we used a spinal cord immunohistochemistry approach combined with pepsin antigen-retrieval to unmask these highly cross-linked protein complexes. We found a dominant expression of both GluN2B and GluN2D subunits in the SDH of male rats, while only GluN2B was preferentially localized to the SDH of females. Surprisingly, we also identified that the GluN2B NMDAR subtype was more abundantly expressed in the medial compared to the lateral, but in male rats only. Finally, we successfully adapted the staining approaches from rodent to human spinal tissue in order to investigate whether the specific expression patterns for NMDAR GluN2 subtypes are conserved in the human SDH in future studies. These specific localization patterns of GluN2-NMDARs subtypes to pain-processing regions of the SDH has important implications for both the understanding and treatment of pain.
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1. Introduction

1.1. Pain, dorsal horn and NMDARs

1.1.1. Pain: definition and brief classification

Pain states, from acute to chronic, are among the major health conditions that affect humans. Despite this, the underlying molecular mechanisms are still poorly understood and pain conditions remain inefficiently treated, especially for chronic pain. Nociceptive pain is an adaptive acute physiological condition that occurs in response to a noxious stimulus that temporarily activates peripheral nociceptors. This physiological form of pain alerts the organism of potential or ongoing danger and prompts it to respond immediately to prevent serious damage. However, there are forms of pain that are maladaptive as they persist beyond initial activation of nociceptive process. These pain conditions fall under the definition of pathological pain. Pathological pain may occur as a consequence of another pre-existent chronic disease or be due to an intrinsic lesion of the somatosensory system (Jensen et al., 2011; Treede et al., 2008). The latter form of pain in particular is known as neuropathic pain.

1.1.2. Pain processing and circuitry in the spinal cord: the dorsal horn

1.1.2.1. Nociceptive pathway

The nociceptive pathway and neuronal circuitry have been studied extensively; however, given its complexity, the underlying molecular mechanisms within neuronal pain circuits have not been fully understood and further investigation is still needed. The pain pathway is initiated when a noxious stimulus activates peripheral nociceptors. The noxious
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stimulus is then relayed to the dorsal horn of the spinal cord where an intricate network of interneurons receives and processes the incoming stimulus. The dorsal horn projecting neurons, through ascending pathways, relay the nociceptive information to supraspinal regions to be ultimately processed by cortical somatosensory areas resulting in the unpleasant emotional and sensory experience of pain (Li et al., 2019; Todd, 2010, 2017; Woolf & Salter, 2000). From cortical regions, descending pathways project again into the dorsal horn to modulate pain sensation. Therefore, among the central nervous system (CNS) areas involved in pain processing, the dorsal horn of the spinal cord is a principle region where this modulation occurs.

1.1.2.2. Dorsal horn: anatomical organization

The dorsal horn has a laminar organization which was described for the first time in the cat, in 1952, by Rexed (Rexed, 1952) and then studied also in other species, especially rats and mice (Molander et al., 1984; Molander et al., 1989). Based on the neuronal cytological organization (neuronal size and neuronal density) of the dorsal horn, Rexed identified a total of six laminae: laminae I-VI. Of particular interest for pain processing is the superficial dorsal horn (SDH), the most marginal dorsal part of the dorsal horn which includes laminae I and II. While lamina I is a thin veil of grey matter (Rexed, 1952), lamina II is approximately double the thickness of lamina I (Rexed, 1952). Lamina I neurons are of medium and large sizes and are loosely distributed, while lamina II neurons are relatively smaller in size and more densely packed (Molander et al., 1984; Rexed, 1952). The observation of a heterogeneous appearance of neurons within lamina II dates back to the studies of the first neuroanatomists Golgi and Cajal reported in Rexed’s studies. Therefore, lamina II was further subdivided into lamina II outer (LIIo) and lamina II inner (LIIi) for the presence of smaller and more dense number of neurons in the outer part respect to the inner part of lamina II. Laminae III-VI instead are part of the deep
dorsal horn (DDH) whose function is to process preferentially non-noxious somatosensory (mechanical) information and to a less extent noxious information (Schoffnegger et al., 2008).

1.1.2.3. SDH: neuronal diversity and inputs

The SDH has been the main focus for studying pain as it is the integrative spinal cord area that receives inputs from nociceptive primary afferent fibers (Aδ and C fibers) (Li et al., 2019). Lamina I and IIo receive the unmyelinated C-fibers releasing substance P (SP) or calcitonin-gene related peptide (CGRP) neuropeptides along with glutamate; these neurons express the neurokinin 1 receptor (NK-1) that is activated by presynaptic release of the peptide agonist, substance P. The peptidergic afferent fibers expressing CGRP are concentrated in SDH. Thus, the CGRP peptide is a marker of nociceptive afferent fibers concentrated in the SDH, specifically in laminae I and IIo. However, it should be noted that a small proportion of collaterals in the DDH laminae also release CGRP (Traub et al., 1990). Lamina IIIi, instead, receives C-fibers that are labelled by the non-peptidergic marker, lectin IB4 (Bleakman et al., 2006; Pan & Pan, 2004; Todd, 2017). Besides unmyelinated fibers, myelinated Aδ nociceptive axons also synapse onto lamina I neurons (Pan & Pan, 2004). The specific modulation of nociceptive signals is determined by the presence of a heterogeneous number of neurons and neuropeptides/neurotransmitters released in this area which contribute to the fine and complex neuronal circuitry.

The SDH is composed of two broad classes of neurons, projection neurons and interneurons, which can be either excitatory (glutamatergic) or inhibitory (GABAergic and glycinergic) and can be further subdivided into different subpopulations. These SDH neurons have been described according to different criteria: their morphology (Lima & Coimbra, 1983, 1986; A. J. Todd & Lewis, 1986), the type of neuropeptide/neurotransmitter they contain and/or
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receive from primary afferent fibers (Boyle et al., 2017), the receptor types they express on their surface (Almarestani et al., 2007; Littlewood et al., 1995; Todd et al., 2000; Yu et al., 1999), the area of the brain for projection neurons, and their functional features (Prescott & De Koninck, 2002; Ruscheweyh & Sandkühler, 2002). Specifically, within the SDH, lamina I is the lamina with the major representation of projection neurons, yet they are in a very limited number (around 5%) (Spike et al., 2003; Todd, 2017).

According to morphological classification, there are three types of projection neurons: multipolar, fusiform and pyramidal (Lima & Coimbra, 1983). At a receptor level, the majority of lamina I projection neurons (~80%) express the NK-1 receptor, which is activated by substance P (Al-Khater et al., 2008; Spike et al., 2003). In physiological conditions, NK-1 receptors have been identified on multipolar and fusiform neuron subtypes, but not on pyramidal neurons (Almarestani et al., 2007). However, chronic conditions (e.g. peripheral inflammation) may induce the expression de novo of NK-1 receptors also in pyramidal cells (Almarestani et al., 2007; Almarestani et al., 2009). This indicates therefore that receptor expression patterns of SDH neurons can change upon physiological versus pathological conditions. NK-1 projection neurons can be further divided into subpopulations based on neurochemical composition identified through immunohistochemical, biochemical and, more recently, RNA-sequencing approaches.

Beyond projection neurons, the majority of neurons found in both lamina I (~95%) and II (virtually all) are interneurons. Based on rat studies, around 70% are excitatory, of the glutamate subtype (Polgár et al., 2013). The remaining ~30% are inhibitory GABAergic interneurons (Todd & Sullivan, 1990). Among the interneurons of laminae I and II, there are four different morphological types: islet, central, radial and vertical (aka stalked) cells (Grudt & Perl, 2002). Islet cells are exclusively inhibitory (Gobel et al., 1980; Todd & McKenzie, 1989) as they
are GABAergic (a subset also contain glycine), vertical and radial cells are mostly excitatory (glutamatergic), and central cells are both inhibitory and excitatory (Maxwell et al., 2007; Yasaka et al., 2007; Yasaka et al., 2010). Subpopulation of non-overlapping GABAergic interneurons (~65% in lamina I and ~50% in lamina II) (Sardella et al., 2011) have been identified based on the expression of specific neurochemical markers: galanin (Simmons et al., 1995; Tiong et al., 2011), neuropeptide Y (Rowan et al., 1993), nNOS (Sardella et al., 2011), and parvalbumin (Laing et al., 1994).

In order to understand the circuitry in the SDH and therefore pain processing, scientists have tried to determine if morphological and/or neurochemical features of SDH neurons correlate with functional properties. In particular, these studies predominantly took into consideration features like post-synaptic responses and firing patterns of SDH neurons. Overall, it has been observed that the correlation between morphology and functionality is not absolute, but it exists to some extent. Prescott and De Koninck proposed the existence of tonic, phasic, delayed onset and single spike neuronal types based on the firing membrane properties of lamina I neurons of adult rats (Prescott & De Koninck, 2002). The firing pattern of these neurons matched to a specific neuronal morphology so that tonic-firing neurons were mostly of the fusiform morphological type, phasic-firing neurons were of the pyramidal morphology, and delayed onset and single spike - firing neurons were multipolar cells (Prescott & De Koninck, 2002). However, a subset of neurons could not be included in any group. Maxwell et al. also analyzed the functional properties of neurons in both laminae (Maxwell et al., 2007). The researchers found that there was correlation between morphology and post-synaptic action only for lamina II islet cells, while the other morphological subtypes showed mixed electrophysiological signatures. The inhibitory islet cells in fact displayed tonic firing in
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response to depolarization (to depolarizing currents) (Maxwell et al., 2007). In hamster laminae I and II neurons, Grudt and Perl reached similar conclusions as they found variability in action potential firing within individual morphological types as well as functional similarities among different morphological subtypes (Grudt & Perl, 2002).

As mentioned above, among the neurotransmitters involved in spinal nociceptive transmission and processing, glutamate is a major one. It is the ligand of several metabotropic and ligand-gated ionotropic receptors such as α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPARs) and N-Methyl-D-Aspartate receptors (NMDARs). Electrophysiological studies have shown that the membrane of post-synaptic second-order neurons in the SDH (Bardoni et al., 1998), both projection neurons and interneurons, contain these ionotropic receptors.

1.1.3. Molecular mechanisms of pain: the NMDA receptors

At a molecular level, chronic pain is due to an increased excitability (hyperexcitability) of the membrane of post-synaptic neurons of the dorsal horn, mediated by the activation of several receptors, among which NMDARs play a central role. In physiological acute pain states, a nociceptive stimulus triggers glutamate to be released from central afferents of nociceptors onto second-order dorsal horn neurons, where it generates excitatory post-synaptic currents (EPSCs) (Basbaum et al., 2009; Dickenson et al., 1997). In the presence of acute noxious stimuli, AMPA receptors are activated (Dickenson et al., 1997), while NMDARs remain silent due to blockade of the NMDAR pore by extracellular magnesium cations (Mg$^{2+}$). If the release of glutamate from the nociceptive fibers increases due to the presence of a sustained noxious stimulus, the depolarization of the post-synaptic neuron increases and is sufficient to remove the Mg$^{2+}$ block from activated NMDARs, leading to a rise in output from nociceptive SDH neurons (Basbaum et
al., 2009). This phenomenon is known as windup, a physiological protective and reversible mechanism of pain amplification (Baranauskas & Nistri, 1998). However, in some situations the increased activation of NMDARs may lead to central sensitization, which involves molecular mechanisms that lead to sustained hyperexcitability in SDH neurons and a pathological form of pain response (McMahon et al., 1993; Woolf, 1983). In this way, NMDARs are central in determining pain hypersensitivity and modulating synaptic plasticity. A potentiation in NMDARs activity is therefore at the basis of determining the switch from physiological to pathological forms of chronic pain (Basbaum et al., 2009). This critical role for NMDARs is also driven by the fact that the activation of NMDARs leads to an increase in the levels of intracellular Ca\(^{2+}\) which alters the synaptic strength (Li et al., 2019). Transient elevated levels of cytoplasmic Ca\(^{2+}\) in turn activate intracellular pathways which are critical for the induction of post-synaptic long-term potentiation (LTP) in the SDH (Li et al., 2019). LTP consists of an increase in the strength of synaptic transmission which tend not to be static but it is finely modulated by variations in pre- and post-synaptic activity (Youn at al., 2013). One mechanism that may promote LTP is the phosphorylation of NMDARs by protein kinases A and C (PKA, PKC) and calcium-dependent calmodulin kinase II (CaMKII) (Youn et al., 2013).

The contribution of NMDARs to hyperexcitability and central sensitization may also originate from the activation of pre-synaptic receptors. Pre-synaptic NMDARs located on presynaptic afferents have been shown to enhance glutamate release; they act as auto-receptors enhancing the release of glutamate (Bouvier et al., 2015) and thus contributing to the hyperexcitability of SDH neurons (Bouvier et al., 2015; Liu et al., 1997). These findings however are in contrast with those of other researchers (Bardoni, 2013; Bardoni et al., 2004) that have shown that the activation of pre-synaptic NMDARs at specific subtypes of central terminals
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slows or depresses, instead of potentiates, the evoked release of glutamate onto lamina II dorsal horn neurons (Bardoni, 2013; Bardoni et al., 2004).

Besides the activation of synaptic NMDARs, an excess of glutamate may activate NMDARs located outside the synaptic cleft: the extra-synaptic NMDARs. The main extra-synaptic source of glutamate is the spillover from synapses due to altered functionality of reuptake systems mediated by glutamate transporters located in the membrane of neurons (Nie & Weng, 2009) and/or glial cells (Nie & Weng, 2010; Pál, 2018). In healthy rats, the experimental pharmacological blockade of both neuronal and glial glutamate transporters in lamina II of the dorsal horn leads to increased duration and amplitude of NMDA EPSCs proposed to be mediated by activation of extra-synaptic NMDAR by spillover of glutamate (Nie & Weng, 2009). Similarly, in neuropathic rats, impeding the diffusion of glutamate to extra-synaptic sites mitigates the increased activation of NMDARs, while the inhibition of glial transporters enhances their activation (Nie & Weng, 2010). In this context, as NMDARs are composed of several subunits of different subtypes, the discrimination between synaptic and extra-synaptic NMDARs is based on the type of subunit they preferentially express.

In the next paragraphs I will describe the structure of NMDAR complexes and their component subunits, specifically of the GluN2 subtype. The identity of the GluN2 subtype will affect receptor properties and the role that NMDARs play in physiological and chronic pain conditions.
1.2. NMDARs: structure, subunits and properties

1.2.1. Structural organization of NMDARs and subunits

NMDARs are glutamate - and glycine - gated ion channel receptors found throughout the CNS, and they are involved in numerous vital physiological processes in a variety of tissues. NMDARs are conserved transmembrane protein complexes, consisting of an extracellular N-terminal domain, three transmembrane domains and an intracellular C-terminal domain. The N-terminal domain contains the ligand (glutamate) binding site (in GluN2), the site for the binding of the co-agonist glycine (in GluN1) and other allosteric sites that allow the modulation of some receptor properties, such as channel opening. The three membrane-spanning domains are M1, M3 and M4 and between M1 and M3 there is a membrane re-entrant pore-loop, M2, involved in the formation of the channel pore (Furukawa & Gouaux, 2003). The M2 loop acts as specificity filter for the channel’s ionic permeability. The C-terminal domain has phosphorylation domains (which affect receptor localization, activity and mobility) and interacts with cytoplasmic proteins involved in the activation of various signaling pathways (Chen & Roche, 2007).

NMDARs are excitatory receptors as they contribute to excitatory neurotransmission at glutamatergic synapses. The conductance of cations through NMDARs is dependent on the simultaneous binding of L-glutamate and glycine to their respective binding sites along with the requirement of a post-synaptic membrane depolarization, strong enough to allow the removal of the cation Mg$^{2+}$ from its specific location within the channel (Lee et al., 2014), which makes them coincidence detectors (Lee et al., 2014). To be functional, NMDARs assemble in heterotetrameric complexes (Laube et al., 1998) formed by different subunits. The GluN1 subunit is the obligatory subunit, required for the functionality of the receptor. This subunit can combine with either GluN2 or GluN3 subunits. The most common combination is two GluN1 and two GluN2.
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or, less common, two GluN1 and two GluN3. The former combination is activated by glutamate and glycine; the latter is activated exclusively by glycine as GluN3 contains the binding site for glycine and not glutamate.

GluN2 subunits exist in four isoforms: GluN2A, GluN2B, GluN2C and GluN2D encoded by four different genes. In addition, the variability of each isoform is further increased by alternative splicing (Sugihara et al., 1992). The presence of such variability within GluN2-NMDAR subunits makes them an interesting object of study as it influences the overall properties of the NMDAR complex. Among the GluN2-NMDAR subunits, the least conserved region is the C-terminal domain (Ryan et al., 2008). The C-terminus varies in amino acid sequence and length (Franchini et al., 2020; Paoletti at al., 2013; Traynelis et al., 2010; Wyllie et al., 2013) and is subject to modulation by intracellular signals which alter receptor trafficking, protein interactions and properties (Traynelis et al., 2010).

1.2.2. Properties of NMDARs

The role of the C-domain has been studied in GluN2A- and GluN2B- containing NMDARs. For instance, the C-domain of GluN2B-NMDARs compared to the C-domain of GluN2A-NMDARs renders GluN2B-containing NMDARs more toxic to Ca\(^{2+}\) influx than GluN2A-NMDARs, promoting neuronal death more efficiently (Martel et al., 2012). The C-domain of GluN2 subtypes seem to also influence NMDARs gating kinetics (Maki et al., 2012); indeed, receptors lacking the C-terminal of GluN2A or GluN2B have longer receptor openings and larger desensitization intervals (Maki et al., 2012). NMDARs display some unique functional properties compared to other ligand-gated ion channels: voltage-dependent block by Mg\(^{2+}\) cations, highly permeability to Ca\(^{2+}\), and relatively slow activation and deactivation kinetics. These properties of the NMDARs are strictly dependent on NMDAR subunit
expression. It is in particular the GluN2 family that confers unique and differential properties to the NMDARs. GluN2A- and GluN2B- containing NMDARs show higher sensitivity to voltage-dependent Mg\textsuperscript{2+} block. In addition, GluN2A and GluN2B have slow Mg\textsuperscript{2+} unblock rates, whereas GluN2C and GluN2D exhibit fast unblock rates (Traynelis et al., 2010). In terms of Ca\textsuperscript{2+} permeability, GluN2A and GluN2B have higher Ca\textsuperscript{2+} permeability and higher channel conductance than GluN2C- and GluN2D - containing NMDARs (Traynelis et al., 2010; Wyllie et al., 2013). In contrast, the agonist and co-agonist potency is lower for GluN2A and GluN2B and higher for GluN2C and GluN2D (Wyllie et al., 2013). The deactivation kinetics of the receptor goes from fast to slow progressively among the four GluN2 subunits: GluN2A have the fastest deactivation rate, while GluN2D have the slowest.

### 1.3. Plasticity of NMDARs in the brain and spinal cord: GluN2A, GluN2B and GluN2D subunits

#### 1.3.1. Spatiotemporal expression of GluN2 subunits

The subunit composition of NMDARs in the brain tends to change significantly throughout life. Of particular interest are the subunits belonging to the GluN2 family, which undergo major changes from development to adulthood. The subunits of the GluN2 type change based on developmental stages, but also based on sensory experiences (Kopp et al., 2007; Paoletti et al., 2013; Philpot at al., 2001) and neuronal activity (Paoletti et al., 2013). Variation in GluN2 expression is also dependent on the region of the CNS involved. Furthermore, variations in subunit composition are accompanied by changes in receptor kinetics, from slower NMDAR responses mediated by GluN2B and GluN2D subunits to faster responses mediated by GluN2A subunits (Paoletti et al., 2013). From *in situ* hybridization, immunohistochemistry and electrophysiological studies, it has emerged that GluN2-containing NMDARs display a
variability in the expression pattern within the CNS, with major differences between the brain and the spinal cord. In the rodent brain (including the hippocampus and visual cortex), at synaptic sites, GluN2B is the dominant subunit during embryonic stages and early postnatal days. During postnatal development, synaptic GluN2B presence reduces but it remains expressed during adulthood to a less extent. While GluN2B diminishes, GluN2A expression increases and dominates the adult rodent brain (Paoletti et al., 2013). In addition, GluN2C subunit expression starts during the late stages of development (p7) and is restricted to specific areas of the brain, such as granule cells of the cerebellum and olfactory bulb. The GluN2D subtype is highly dominant in embryos and drops after birth and in the adult is weakly expressed in diencephalon and brain stem (Paoletti, 2011).

In the spinal cord, GluN2A and GluN2B subunits are expressed at glutamatergic synapses throughout the entire dorsal horn (Nagy et al., 2004) and each receptor subunit has a different temporal expression compared to the brain. In lamina I of the SDH, GluN2A contribution to NMDARs in the adult is minor when compared to GluN2B subunits (Hildebrand et al., 2014). It seems indeed that GluN2B and GluN2D are the major subtypes in mediating the lamina I neuron’s synaptic responses in adult rats (Hildebrand et al., 2014). However, in juvenile rats (p14-20), both GluN2A and GluN2B mediate synaptic responses evoked by afferent C-fiber stimulation in NK-1 positive lamina I neurons (Tong & MacDermott, 2014). The mRNA (Shibata et al., 1999) and protein (Yung, 1998) for GluN2C have not been detected in the spinal cord for many research groups and will not be taken into account in this study. However, other researchers have identified that the GluN2C transcript is also expressed (Tolle at al., 1993) in the spinal cord. Differences in NMDAR subunit distribution and functionality observed among various research groups may be due to the approach used, the splice isoform identified, the
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neuronal subpopulation within the dorsal horn and/or the level of the spinal cord taken into consideration for the study. Similarly, the developmental stage, the sex and the species taken into account in the studies are other key potential factors that may influence the results obtained. For example, a pharmacological and functional study showed that a subpopulation of lamina I neurons expressing substance P in p14-19 rats preferentially express either GluN2C or GluN2D receptor subtypes (Tong et al., 2008) over either GluN2A or GluN2B, based on their Mg$^{2+}$ sensitivity and GluN2-subtype antagonist selectivity. Furthermore, despite the finding that all subunits are expressed in NK1R- and NK1R+ lamina I neurons, there is a difference in their proportion: GluN2C/GluN2D subunits are strongly expressed by NK1R+ lamina I neurons, whereas the GluN2A/GluN2B receptor subtypes dominate more strongly NK1R- neurons (Tong et al., 2008). In an early study conducted on adult rat lamina II neurons, functional GluN2B-NMDARs were identified ubiquitously in the soma as well as at extra-synaptic sites (Momiyama, 2000). At extra-synaptic sites GluN2D-NMDARs were rarely present (Momiyama, 2000). Synapses of lamina II neurons instead seemed to be mainly contain the GluN2A receptor subtype, while GluN2D contribution is low at these synapses (Momiyama, 2000). Another study, based on Mg$^{2+}$ sensitivity and pharmacological properties of NMDARs, found that lamina II GABAergic interneurons in mice (p14-21) express both groups of high and low Mg$^{2+}$-sensitivity (Shiokawa et al., 2010), whereas lamina II excitatory interneurons showed a dominance of high Mg$^{2+}$-sensitivity (Shiokawa et al., 2010).

In humans, in vitro experiments on neurons obtained from reprogrammed fibroblasts have shown that GluN2B subunits dominate NMDAR complexes (Zhang et al., 2016). In early ex vivo experiments, GluN2A, GluN2C and GluN2D proteins were detected in the dorsal horn (Sundström at al., 1997) of adult donors and all four GluN2A, GluN2B, GluN2C and GluN2D
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subunits were identified in first trimester aborted embryos (Åkesson et al., 2000) in immunoblotting experiments. These findings are based on biochemical assays from whole tissues rather than immunohistochemical approaches, and therefore the specific localization of GluN2 subtypes in the human SDH remains unknown. Furthermore, the difficulties in obtaining human spinal cord samples has limited further investigation and understanding of the specific laminar distribution, contribution and functionality of GluN2A, 2B and 2D in humans. In addition, it is not clear how potential subunit differences may impact physiological and pathological pain modulation.

**1.3.2. LTP and LTD: forms of synaptic plasticity**

Beyond the differences in the spatiotemporal expression of the GluN2-NMDAR subunits, other molecular mechanisms that mediate synaptic plasticity are LTP and LTD. LTP and LTD can be induced experimentally according to different protocols. Some protocols include the use of chemical activators, while others are evoked by electrical stimulation. Electrical stimulation can include either high (e.g. tetanic) or low frequency stimulation.

NMDAR-mediated synaptic plasticity in the SDH shares similarities to NMDAR-mediated synaptic plasticity described in cortical areas of the CNS as well as in the hippocampus. However, the SDH has some unique properties. In the SDH, induction of LTP requires the presence of NMDARs and/or the activation of NK-1 receptors by substance P (Li et al., 2019); when induced, LTP is long-lasting (4 hours max) but reversible, whereas LTP in the hippocampus or more generally, in the cortex, are generally more permanent (Ji et al., 2003; Latremoliere & Woolf, 2009). In addition, in SDH neurons, LTP can be either monosynaptic or, more frequently, heterosynaptic, where stimulation of one synapse enhances the activity of multiple synapses and sensitizes the entire neuron (Ji et al., 2003; Latremoliere & Woolf, 2009).
In contrast, LTP is exclusively homosynaptic in the cortex (Latremoliere & Woolf, 2009). In SDH neurons, the maintenance of LTP requires the recruitment of new potentiated NMDARs, whereas in the hippocampus, the insertion of AMPA receptors into the post-synaptic membrane is required for the maintenance of synaptic strengthening (Bourinet et al., 2014).

The contribution of specific GluN2-NMDAR subunits to LTP and LTD in the dorsal spinal cord are not known. All information concerning GluN2-NMDAR subtypes and LTP or LTD derive from studies in brain areas. In this context, the most studied subunits for LTP are GluN2A and GluN2B. GluN2B does not seem to be required for the induction of LTP in many brain regions (ACC, amygdala, CA1 of hippocampus) (Zhuo, 2009). Indeed, the application of GluN2B antagonists reduces LTP but does not fully block it (Pedersen & Gjerstad, 2008; Zhuo, 2009). In forebrain areas involved in pain processing (anterior cingulate cortex and insular cortex), GluN2B was found to be upregulated in chronic pain conditions, but not in physiological pain (Wei et al., 2001; Wu et al., 2005; Yang et al., 2015) in mice. At the spinal level, in the SDH, new emerging studies have shown that the potentiation of GluN2B-containing NMDAR subunits contributes to pathological pain processes (Dedek et al., 2019; Hildebrand et al., 2016) in rats and humans (Hildebrand et al., 2014; Qu et al., 2009). The GluN2A subunit seems to be necessary for the induction of LTP in hippocampal areas (Franchini et al., 2020). On the contrary, GluN2A-containing NMDARs are less involved in the potentiation of SDH neurons (Li et al., 2017). In the SDH, a critical aspect for determining whether synaptic transmission will be potentiated or not depends on the intensity of the post-synaptic depolarization (Li et al., 2019). If the intensity of the depolarization remains low, the strength of synaptic transmission may be reduced according to a phenomenon that is known as long term depression (LTD). Despite this, both mechanisms, LTP and LTD, may be implicated in the manifestation of chronic pain. Indeed,
it was observed that the same intense peripheral stimulus that induces LTP on spinothalamic tract neurons, a population of spinal pain projection neurons, induces LTD on GABAergic interneurons of the dorsal horn (Kim et al., 2015). In either case, for LTP and LTD to happen, both NMDAR activation and intracellular Ca\(^{2+}\) increase are required to occur in the two neuronal populations (Kim et al., 2015).

1.4. Pain and NMDARs in males and females

1.4.1. Role of sex hormones in pain modulation

Like many models of pathology, the majority of scientific literature has been focusing on studying pain and NMDARs mainly in male animal models. Despite the fact that many forms of acute and chronic pain are prevalent in women (Bartley & Fillingim, 2013; Berkley, 1997; Fillingim, 2000; Mogil, 2012), studies on the role that NMDARs may play in nociceptive circuitry in females have been mostly overlooked. Increasing evidence suggests that differences in nociception are mediated by sex hormones. In particular, estrogens and testosterone seem to play major roles in modulating pain sensitivity (Aloisi & Ceccarelli, 2008).

The effects of estradiol, one of the most common estrogen, on pain processing have been studied both in the peripheral nervous system (DRG neurons) and in the CNS (spinal cord neurons). Administration of 17-β-estradiol to primary cultures of DRG neurons (McRoberts et al., 2007) and to spinal SDH neurons (Zhang et al., 2012) produces a greater increase in NMDAR currents in females compared to males in a dose-dependent manner; estradiol also acts as a modulator of excitatory synaptic transmission and enhances LTP (Zhang et al., 2012). The potentiation of synaptic transmission by estrogens is associated with an increased expression of GluN2B-containing NMDARs in females (McRoberts et al., 2007). More recently, it has
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emerged that in male rats with increased levels of estrogen, enhanced pain sensitivity is mediated by the upregulation of GluN1 NMDARs in DRG neurons; administration of the antagonist AP-5 decreases both GluN1 NMDAR expression and the pain threshold (Deng et al., 2017). In agreement with these findings, in ovariectomized (OVX) female adult rats, the injection of estradiol augments spinal processing of visceral nociception again by increasing the expression of GluN1 NMDAR receptor and its phosphorylation (Tang et al., 2008). Thus, estrogen-mediated regulation of NMDARs may be one of the mechanisms that give higher propensity for pain in females compared to males.

Beyond estrogens, some studies have also emphasized the importance of testosterone in pain modulation. In particular, in experimental models of non-opioid stress-induced analgesia, male and female adult mice show a dimorphic mechanism in regulating chronic pain which involves NMDAR activity. In males, the blockade of NMDARs with the antagonist MK-801 elicits the reversal of cold-induced analgesia (Marek et al., 1992; Marek et al., 1991; Vaccarino et al., 1992) and ethanol-induced analgesia (Mogil et al., 1993), whereas in females the blockade of NMDARs does not produce any effect (Marek et al., 1992, 1991). Therefore, in males, analgesia seems to be mediated by NMDARs, while in females it is NMDAR-independent for the above specific pain phenomena.

To investigate whether steroid sex hormones could have had a role in this divergent mechanism between sexes, a similar study was conducted in female mice that were injected with testosterone during the neonatal phase (Sternberg et al., 1995). As previously observed in males, NMDARs mediated the reversal of the analgesic effects through the antagonism of MK-801 (Sternberg et al., 1995). Therefore, in female mice, testosterone showed an organizational effect as the exposure to it during early development is able to persistently switch the circuitry towards
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the NMDAR-mediation of pain, even after the production of estrogens in adult females (Craft et al., 2004; Sternberg et al., 1995). The presence or absence of testosterone seems to be responsible to the NMDAR-dependence or independence neuronal circuitry in females. In the absence of testosterone, induced experimentally, females recruit the melanocortin-1 system to reverse analgesia and mediate pain (Mogil et al., 2003). The melanocortin-1 mechanism is not exclusive, in fact, the higher level of testosterone or the administration of exogenous testosterone as well as low levels of estrogens are capable to switch the circuitry to the male NMDAR mechanism of pain processing (Mogil, 2018). For instance, the drug MK-801 in ovariectomized mice induces NMDAR-antagonism and pain through the reversal of stress-induced analgesia similar to males. On the contrary, the supplementation of estrogens restores the insensitivity to the NMDAR system in females (Mogil et al., 1993). In a parallel experiment, gonadectomized male rats displayed increased pain sensitivity after repetitive nociceptive stimulation through formalin injection compared to intact rats (Aloisi et al., 2003; Ceccarelli et al., 2003). The plasma levels of testosterone in the gonadectomized rats were decreased, while estrogens were high (Aloisi et al., 2003), thus showing a potential protective role of testosterone (Fischer et al., 2007).

Despite the availability of scientific literature on the role of sex hormones in pain modulation, the basic understanding of whether there are differences in GluN2-NMDAR subunits in baseline SDH functioning between sexes is still unknown and will be elucidated in this study.
1.4.2. The immune-system

1.4.2.1. Microglia and nociception

Microglia are highly dynamic cells of the innate immune system of the CNS equivalent to the macrophages of the periphery. In the last years, the study of microglia has become prominent for its role in mediating pain hypersensitivity. The activation of microglia in response to an exogenous insult in fact initiates not only an immune response but also a nociceptive signaling pathway involving the interaction between spinal microglial cells and spinal SDH neurons. The most striking and unexpected findings about the microglia-neuron signaling pathway is the existence of a sexual dimorphic mechanism in nociceptive processing observed primarily in rodents. Despite the microglial immunoreactivity is activated in both sexes after a damage to peripheral nerves or inflammation, glial-mediated pain hypersensitivity seems to occur exclusively in males. In mice models of neuropathic pain, the activation of microglia induces pain (mechanical allodynia) solely in male mice (Mapplebeck et al., 2018; Sorge et al., 2011). Furthermore, the use of drugs which disrupt the function of glia, thus acting as inhibitors of microglia, alleviates or reverses pain hypersensitivity in males but not in females. Given the fact that microglial nociceptive signaling occurs in males, it is likely that testosterone could have a role in the differences observed between sexes. Experiments in which circulating testosterone was removed in adult male rats resulted in absence of allodynia (Sorge et al., 2011). Furthermore, the supplementation of testosterone in gonadectomized male mice and in female mice induced allodynia in both sexes (Sorge et al., 2011).

In summary, spinal microglia may not be crucial for the development of pain in females. Some authors postulate that females would preferentially use a different pathway that involves adaptive immunity instead of innate immunity, specifically the T-lymphocytes (Mapplebeck et
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al., 2016; Sorge et al., 2015). Indeed, prevention of infiltration of T-cells in the spinal cord is effective in reducing pain exclusively in female mice (Sorge et al., 2015). It seems, however, that female can switch to the microglial pathway depending on the level of estrogens and testosterone and based on the functioning of the adaptive immune cells (Mapplebeck et al., 2016).

1.4.2.2. Microglia and dorsal horn NMDARs

Despite the identification of microglia and neuron signaling in mediating pain selectively in males, it is still not clear how NMDARs are involved in this mechanism. Recent findings from our lab and others have shown that BDNF, released by microglia, potentiates NMDARs responses in lamina I neurons through the KCC2/STEP61/Fyn pathway in male rats and humans (Coull et al., 2005; Dedek et al., 2019; Hildebrand et al., 2016). Our labs’ preliminary data, however, seems to suggest that the pathological potentiation of NMDARs by BDNF does not occur in the spinal cord of female rats or humans (Dedek A PhD thesis project, unpublished data). Another study aimed at investigating the link between microglia and NMDARs demonstrated that NMDARs participate in the mediation of spinal microglia activation (Li et al., 2017). The blockade of NMDARs in the dorsal horn of the spinal cord with the selective antagonist MK-801 attenuated pain hypersensitivity (Li et al., 2017) in male rats pre-treated with bee venom to induce inflammation. Indeed, while venom injection induces hyperalgesia and changes in the morphology of microglial cells as well as the overexpression of the microglial marker CD11b/c, the inactivation of NMDARs reverses all these effects (Li et al., 2017). Another study using only male rats revealed that one of the initiating factors in the glia-neuron nociceptive signaling, interferon γ (INFγ), enhances NMDAR activity (shown as an increase in inward currents) in lamina II neurons through a process involving the release of the CCL2 agonist acting on microglial CCR2 (Sonekatsu et al., 2016). Due to the lack of research on
female models, it has not been disentangled what neurobiological mechanism occurs in females. However, since the general antagonism of NMDAR activity alleviates pain hypersensitivity in both sexes (Mapplebeck et al., 2017; Sorge et al., 2015), it is proposed that differences between the sexes are restricted to upstream microglia-neuron signaling cascades (Mapplebeck et al., 2017). However, according to our lab findings, the regulation of NMDARs appears to diverge in males and females as the potentiation of NMDARs by BDNF occurs only in males (Dedek et al., 2019; Hildebrand et al., 2016) and not in females (Dedek A PhD thesis project, unpublished data).

Further research is required to elucidate the roles of NMDAR subunit composition in the functioning of the entire NMDARs complex in the SDH of males and females. It is also worth investigating the upstream pathway that is responsible for the dimorphism in nociception observed between the sexes. Moreover, a fundamental problem for potential translation to humans is whether the neurobiology of nociception observed in rodents is evolutionary conserved or not in other species such as humans.

1.5. **Humans vs rodents: NMDARs and pain processing**

Despite the fact that the anatomy of the nociceptive pathway and the specific role of spinal cord in relaying pain signals is evolutionary conserved between humans and rodents, it is possible that molecular mechanisms in pain processing involving NMDARs significantly diverge between the two species. For instance, in a comparative study between humans and mice (Rostock et al., 2018), the relative fraction of two subtypes of voltage-gated sodium channels, Nav1.8 and Nav1.9, involved in mediating peripheral chronic pain, was higher in humans than in mice. Similarly, TrkA peptidergic nociceptors of peripheral/DRG sensory neurons present some differences in expression between the two species (Rostock et al., 2018). Other species-related
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differences have been observed for the cannabinoid receptor CB2. For example, rats, mice and humans show significant differences in CB2 gene structures, transcripts and isoforms (Liu et al., 2009); divergence in pharmacological profiles such as in ligand-specificity between rats and humans has been observed as well (Mukherjee et al., 2004). It is therefore plausible to think that similar variations may occur in central areas implicated in pain processing and modulation, such as in the SDH. As it has not been investigated whether GluN2-containing NMDAR expression is conserved across the two species, this poses the need to characterize the expression and role of NMDARs in humans.

1.6. Aims and hypotheses

The role that each specific GluN2-NMDAR subunit has in determining the NMDAR properties in pain processing and in the excitability of spinal cord neurons is unknown. Therefore, in this study, we investigated the relative expression of specific NMDAR-GluN2 subunits, GluN2A, 2B and 2D, in physiological conditions in rats and humans of both sexes in the SDH of the spinal cord. This study therefore sets the foundation for further investigations and comparisons involving pathological pain conditions, including both rodent in vivo and human ex vivo chronic pain models.

Aim 1:

Investigation of GluN2-NMDARs across laminae in male rats

We aimed to study the expression of GluN2A, 2B and 2D NMDARs across the whole dorsal horn of the lumbar portion of the spinal cord in rats. We hypothesized that GluN2 subunits are heterogeneously distributed between the SDH and the DDH. Given the current evidence from electrophysiological studies (Hildebrand et al., 2014; Mahmoud et al., 2020) we predicted a
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prevalent expression of GluN2B and GluN2D in the SDH (laminae I and IIo), the area devoted to pain processing, compared to the DDH in rats.

Aim 2:

Comparison of GluN2 subtypes across SDH in females and males

We aimed to investigate GluN2A, GluN2B and GluN2D expression in male versus female rats. We hypothesized that all three subunits are expressed in the lumbar SDH of males and female rodents. However, on the premise that pain states and pain sensitivity seem to be more pronounced in females rather than in males (Fillingim et al., 2009), and that GluN2B-containing NMDARs have been implicated in the molecular mechanisms of nociception (Hildebrand et al., 2014, 2016; Tong & MacDermott, 2014), we hypothesized that GluN2B is more preferentially expressed in the dorsal horn of females versus males.

Aim 3:

Establishment of staining procedures to test if GluN2 molecular identity is conserved between rodents and humans

We aimed to characterize whether GluN2A, GluN2B and GluN2D subunit expression across the dorsal horn is species-specific by first establishing immunohistochemical protocols for labelling NMDAR subtypes in fixed human spinal cord tissue. Given that recent research has shown some differences in the expression pattern of peptidergic nociceptors in DRG as well as in subtypes of voltage-gated sodium channels between rodents and humans (Rostock et al., 2018), we hypothesized that similar differences may exist in the pattern of NMDAR receptor subunit expression between rats and humans.
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2. Materials and Methods

2.1. Animals

The experimental procedures involving the use of animals have been approved by the Carleton University Animal Care Committee and conducted in accordance to the guidelines of the Canadian Council for Animal Care. For this study, postnatal day 21 (p21) Sprague-Dawley rats of both sexes were purchased from Charles River Laboratories.

2.2. Rat spinal cord isolation

Twelve Sprague-Dawley p21 juvenile rats, 6 females and 6 males were anesthetized with an intraperitoneal (ip) injection of 3g/kg urethane (Sigma Aldrich). As previously described (Hildebrand et al., 2014), the lumbar spinal cord was dissected through ventral laminectomy and placed in an ice-cold, oxygenated protective sucrose solution containing 50 mM sucrose, 92 mM NaCl, 17 mM D-glucose, 26 mM NaHCO3, 5 mM KCl, 1.25 mM Na2HPO4, 0.5 mM CaCl2, 7 mM MgSO4. Spinal cord segments were removed of the meninges and blood vessels under a dissecting microscope within 10 minutes to preserve tissue integrity. Only the lumbar segments of the spinal cord were selected for the current study. After dissection, the tissue was fixed in 4% paraformaldehyde (PFA) for 24 h at 4°C, then placed in subsequent steps of 10% (one wash for 24 h and a second wash for at least 6 h) and 30% sucrose solutions (in PB) for 72 h at 4°C. The tissue was then embedded in OCT for cryopreservation, frozen in isopentane (chilled in liquid nitrogen) and stored at -80°C prior to sectioning. The lumbar portion of the spinal cord was cut in 25 μm thick transverse sections using a microtome cryostat (ThermoScientific). Each section was sequentially mounted on pre-treated slides (FisherBrand™ SuperFrost™ Plus) and ready for the immunohistochemistry experiments or for storage at -80 °C. In order to have approximately a
representation of the same spinal cord section in each slide, we placed each new slice in a serial fashion on new, previously numbered, slides. The immunostaining was run in triplicate or quadruplicate for each animal and analyzed as described below. All the spinal cord tissue from male and female rats were collected and processed in parallel. After the immunohistochemistry staining, the slides were cover-slipped with the Fluoromount™ Aqueous Mounting Medium (Sigma, F4680-25ML) before the imaging.

2.3. Human donors and spinal cord isolation

The spinal cord tissue was collected from adult (18-69 years old) male and female human neurological determination of death organ donors, identified by the Trillium Gift of Life Network. Approval to collect and conduct experiments with human tissue was obtained from the Ottawa Health Science Network Research Ethics Board.

The donors deceased for subarachnoid hemorrhage or ischemia. Donors were pre-screened to exclude any blood-borne disease such as HIV, hepatitis and syphilis. From the study, we also excluded all the donors with chronic diseases and lesions of the spinal cord as well as donors who were under treatment for chronic pain to prevent any interference or confounding source with our experimental aims.

A cooling bed was used to induce hypothermia and the body was perfused with high magnesium protective solution. The spinal cord was isolated via ventral laminectomy after cross-clamping the heart. The lumbar portion was then excised and immediately placed in an ice-cold, oxygenated saline solution (the same used for rats and described above). The lumbar spinal cords were removed of the meninges and blood vessels under a dissecting microscope within 10 minutes to preserve tissue viability. The tissue was then fixed in 4% PFA for 24 h at 4°C, washed with 10% sucrose for 24 h, re-washed in 10% sucrose for at least 6 h and then in 30% sucrose (in
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PB) for 72 h at 4°C. The tissue was then embedded in OCT, frozen in isopentane (chilled in liquid nitrogen) and stored at -80°C prior to sectioning. The lumbar portion of the spinal cord was cut in 25 μm thick transverse sections using a microtome cryostat (ThermoScientific). Each section was mounted according to the same procedure adopted for the rats (see above) for subsequent immunohistochemical experiments.

2.4. Immunohistochemistry

To investigate the distribution of the three NMDAR subunits: GluN2A, 2B and 2D, we employed a modified immunohistochemistry method based on the pepsin antigen-retrieval technique. Pepsin treatment allows the labelling of all the receptor subunits of interest, including those localized to protein complexes at synapses. It unmaskes and exposes the epitope/antigens (aka receptor subunits) of highly cross-linked proteins in fixed tissues, such as those found in the postsynaptic density, and, thus, facilitates the binding of the antibody to the target (Nagy et al., 2004). After incubation in a peroxidase solution (50% methanol, 1.8% hydrogen peroxide in PBS) for 30 minutes at room temperature (RT, around 22°C) to block the endogenous peroxidases, we pre-treated the slices of the lumbar spinal cord with the protease pepsin, followed by digestion in the incubator at 37°C for 5 minutes. After incubation, the sections were washed three times with PBS solution pre-warmed at 37°C.

In separate sets of double-immunofluorescence experiments, and after blocking in a PBS solution containing 5% NGS, 0.3% Triton-X100 and 0.3% BSA, the sections were incubated in antibodies against the NMDAR subunits: rabbit anti-GluN2A (1:2000; Alomone Labs, AGC-002), rabbit anti-GluN2B (1:1000; Alomone Labs, AGC-003) or rabbit anti-GluN2D (1:1000; Alomone Labs, AGC-020), at RT overnight. In parallel, we immunostained for the calcitonin gene1-related peptide (CGRP) as it marks exclusively the nociceptive peptidergic afferent fibers
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localized in the SDH and, thus, it represents an optimal reference to identify the area that define the laminae I and IIi (Carlton et al., 1988; Chung et al., 1988; Gibson et al., 1984). We incubated the sections with mouse anti-CGRP antibody (1:5000; Sigma, C7113). Following washes in PBS 1X, the slices were incubated for 2 hours at RT with the following secondary antibodies diluted in the blocking solution: goat anti-mouse AlexaFluor647 (1:1000; Invitrogen, A21235) and goat anti-rabbit IgG conjugated with horseradish peroxidase (HRP) H+L (1:500; Jackson ImmunoResearch Laboratories). We then added the tyramide substrate for the enzyme HRP using Tyramide Signal Amplification (TSA) fluorescent kit conjugated with the fluorophore cyanine 3 (cy3) (TSA plus cyanine 3 system, Perkin Elmer, Inc., NEL744001KT) diluted (1:50) in the TSA amplification buffer for 2.5 min (GluN2A) or 7.5 min (GluN2B and GluN2D). The enzymatic reaction allows the cy3 to be deposited and the detection of the NMDAR subunits for an optimal visualization of the staining (Brenner et al., 2004; Willcockson & Valtscchanoff, 2008). A Hoechst 33258 Staining Dye Solution (1:1000; Abcam, ab228550) was used to stain for nuclei.

2.5. Image acquisition

The immunofluorescent sections were acquired using the confocal microscope Zeiss LSM 800 Airyscan at 20x objective with Zeiss ZEN 3.1 imaging software. Stacks of horizontal plane images and orthogonal projections of the entire dorsal horn were acquired for each L4/L5 lumbar section. L4/L5 lumbar sections have been identified as described in the next paragraph. We tiled the region corresponding to the dorsal horn (SDH and DDH laminae). The acquisition of the images was performed applying the same laser settings to all the sections.
2.6. Analysis

To analyze NMDAR staining specifically in the L4 or L5 lumbar segments of the spinal cord, we identified spinal sections from these specific lumbar segments under a light microscope, before fluorescent images were acquired. Two experimenters independently classified and identified the same spinal lumbar sections as L4/L5.

Specifically, we distinguished the L4 and L5 lumbar sections based on grey matter and dorsal column white matter anatomical characteristics as illustrated in a rodent spinal cord atlas (Sengul et al., 2012). One major feature was that the overall size tended to vary among the six lumbar segments. L4 and L5 transverse section segments were slightly wider than L1, L2, L3 segments and largely wider than the L6 segment. In terms of cellular features, the morphology of the grey and white matters differed along the lumbar segments as well. While the dorsal horns appeared similarly rounded and curved (medially and laterally) in every lumbar segment, the ventral horns of L4, L5 and L6 presented two main caudolateral enlarged areas with two distinguishable clusters of neurons. Moreover, the dorsal column white matter of L1/L2 and L3/L6 morphologically resembled a heart and an inverted cone with rounded bases, respectively. On the contrary, the dorsal column white matter of L4 and L5 appeared very narrow and extended longitudinally towards the central canal.

In this study we took into account two main regions, the SDH and DDH. Our primary region of interest was the SDH: lamina I, LIIO and LIi as it is critical for pain processing (Schoffnegger et al., 2008). The SDH has been compared to the DDH, which is the area right below (ventral to) the SDH, and it is involved in processing preferentially non-nociceptive somatosensory information (Schoffnegger et al., 2008). As mentioned in the introduction, the region corresponding to the SDH is crossed by a multitude of peptidergic fibers expressing...
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CGRP. Therefore, in this study we used CGRP as a marker for the SDH. Despite the majority of the afferent fibers terminate in the SDH, a few penetrate and synapse into the deeper laminae (laminae III-VI) giving a minor positive staining. For simplicity, considering that a strong localized CGRP-positive (CGRP+) staining is visualized in the SDH, we referred to the SDH as CGRP+ region, whereas we referred to the DDH as CGRP-negative (CGRP-) region. For the selection of the CGRP+ area, we have drawn a contour along the dorsal edge of the dorsal horn down (excluding the dorsal root and the deeper laminae). We then made a second selection corresponding to the CGRP- area, right below the SDH, which terminate in proximity of the beginning of the central canal.

In order to understand the expression of the GluN2-NMDARs subunits, we measured the optical density (O.D.) of the cy-3 stained areas in the CGRP+ and CGRP- regions using the software Fiji ImageJ. We normalized the CGRP+ and CGRP- O.D. to their respective areas. Further, we normalized to the background (BG) values by positioning a square selection (55x55 µm) region in the deep dorsal column of the white matter and measuring the O.D. of that region, with normalization to the BG area. We measured the BG values in the white matter instead of the grey matter as the GluN2 subunit staining was extensive throughout the grey matter regions and so prevented selection of a GluN2-negatively stained BG region in the grey matter.

To investigate the potential differential expression of NMDAR subunits across the dorsal horn, we selected one side of the spinal cord for quantification. The selection of either the right or left dorsal horn was based primarily on the integrity and quality of the spinal cord tissue section. When the integrity of the entire section was optimal for both the left and right dorsal horn, we randomly selected one of the two dorsal horns.
We also made selections in the medial, central and lateral regions of either the left or right dorsal horn. Specifically, we placed oval shaped selections of fixed parameters (40x100 µm) along the most marginal edge of the CGRP+ region of the dorsal horn. The medial and lateral ovals were positioned approximately at the “corners” where the dorsal horn curves medially and laterally, whereas the central oval was positioned in the middle of the SDH mediolateral axis.

All the measurements were conducted in triplicate or quadruplicate for each animal. The raw O.D. values were averaged for each animal.

2.7. Statistical analysis

We used the IBM SPSS Statistics software for the statistical analysis. The results are presented as average ± standard error of the mean (SEM). To assess the normality of the data we performed the Shapiro-Wilk test. T-test for paired or independent samples and one-way ANOVA for paired or independent samples have been performed to assess the significance of the differences object of study. P<0.05 was considered statistically significant.
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3. Results

3.1. GluN2A, GluN2B and GluN2D distribution across SDH laminae in males

3.1.1. GluN2B and GluN2D is preferentially expressed in the SDH compared to the DDH in male rats

To investigate the basal expression of GluN2A, GluN2B and GluN2D NMDAR subunits across the SDH and DDH of the lumbar (L4/L5) spinal cord, we compared the relative expression of GluN2A, GluN2B and GluN2D in the SDH and DDH of juvenile male rats. In order to accomplish this, we selected the CGRP+ and the CGRP- regions, corresponding to the SDH and DDH respectively, and then measured the O.D. for each receptor subunit in both areas. The obtained O.D. values for GluN2A, GluN2B and GluN2D were normalized to the background fluorescence quantified from the dorsal column white matter region above the central canal (Fig. 1A). When we compared the GluN2-subunit distribution in the SDH and DDH, we observed a different qualitative pattern in immunoreactivity for each receptor subunit. Specifically, GluN2A showed an intense but widely diffused expression pattern across both the SDH and DDH laminae (Fig.1A and Suppl. Fig.1). We found that the GluN2B subunit was also expressed both in the SDH and DDH, however, its expression was more localized to the SDH than the DDH (Fig.2A and Suppl. Fig.3). Overall, GluN2B distribution appeared to be punctate and strongly present in the soma of the cells rather than homogenously diffused as found for GluN2A (Suppl. Fig.3). We also identified some cell body staining in the dorsal column region for GluN2B. To a lesser extent, a similar pattern of punctate distribution was also observed for GluN2D as found for GluN2B, especially in the DDH, while GluN2D immunoreactivity in the SDH was more diffuse (Fig. 3A and Suppl. Fig.5). Although we found that the mean O.D. was significantly greater in the SDH versus DDH for GluN2A (p = 0.012 , n = 6, Fig. 1B), GluN2B
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(p = 0.014, n = 5, Fig. 2B) and GluN2D (p = 0.039, n = 5, Fig. 3B) subunits, the relative difference in expression between the two regions was greatest for GluN2B and GluN2D (Fig. 5A). However, this SDH versus DDH staining intensity ratio was not found to be significantly different between the GluN2A, GluN2B and GluN2D subunits in male juvenile rats. Taken together, our results suggest that both GluN2B and GluN2D are preferentially expressed in the SDH, with staining that is more punctate and localized to cell bodies, compared to the more ubiquitous and diffuse labelling for the GluN2A subunit across the dorsal horn of juvenile male rats.

Fig. 1: GluN2A in SDH and DDH of male rats. (A) Representative confocal image showing the immunostaining for GluN2A. Fluorescence was quantified in visually selected regions, including the superficial dorsal horn (SDH), deep dorsal horn (DDH), and background (BG) region in the central canal, as shown. GluN2A immune-positive cells are detected diffusely throughout the SDH and DDH laminae. Scale bar: 200 µm. (B) Quantitative statistical analysis of mean O.D./BG for GluN2A NMDAR subunit in the SDH versus DDH. *p<0.05
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**Fig. 2: GluN2B in SDH and DDH of male rats.** (A) Representative confocal image showing the immunostaining for GluN2B. GluN2B punctate immunoreactivity is observable in both the SDH and DDH, with a more localized distribution detected in the SDH. Scale bar: 200 µm. (B) Quantitative statistical analysis of mean O.D./BG for GluN2B NMDAR subunit, indicating a significant higher distribution of GluN2B in SDH. *p<0.05

**Fig. 3: GluN2D in SDH and DDH in male rats.** (A) Representative confocal image showing the immunostaining for GluN2D. GluN2D immuno-positive cells are present throughout the dorsal horn, with prevalent diffuse distribution in the SDH and more punctate distribution in DDH. Scale bar: 200 µm. (B) Quantitative statistical analysis of mean O.D./BG for GluN2D NMDAR subunit, indicating a significant high localization of GluN2D in SDH. *p<0.05
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3.1.2. GluN2B is highly expressed in the medial SDH in male rats

It has recently been found that afferent-evoked excitability can vary across the medial-to-lateral axis of the SDH (Mizuno et al., 2019). To evaluate whether GluN2 subunit expression differs across medial to lateral portions of the SDH, we measured the subunit staining O.D. in small regions within the medial, central and lateral areas of the SDH (Fig. 4A). While we observed no consistent differences in qualitative staining patterns and quantified O.D. values for both GluN2A (Fig. 4A,D) and GluN2D (Fig. 4C,F) across medial, central, and lateral SDH regions (see also Suppl. Figs. 1 and 5), GluN2B had a visibly stronger immunoreactivity signal in the medial SDH, which progressively declined in the central and lateral SDH regions (Fig. 4B and Suppl. Fig. 3). Quantification revealed a consistent, large, but non-significant reduction in mean GluN2B O.D. values from the medial to central to lateral SDH (F(2,3) = 5.53, p = 0.099, n= 5, Fig. 4E). However, when we compared the medial versus lateral O.D. ratio between GluN2A, GluN2B and GluN2D subunits, we found a significantly elevated ratio for GluN2B compared to both GluN2A and GluN2D (F(2,13) = 9.11, p = 0.003, n= 6, n=5, n=5; post-hoc Tukey test GluN2B vs GluN2A: p=0.004; GluN2B vs GluN2D: p=0.011, Fig. 5B). We therefore conclude that while GluN2A and GluN2D are uniformly distributed across the medial-lateral axis of the SDH, the GluN2B subunit is preferentially localized to the medial SDH in juvenile male rats.
Fig. 4: GluN2A, GluN2B and GluN2D in Medial, Central and Lateral SDH of male rats. Representative confocal images showing the immunostaining for GluN2A (A), GluN2B (B) and GluN2D (C). Fluorescence was quantified in visually selected oval regions positioned within the CGRP-positive SDH, including in the lateral, central, and medial portions of the SDH, as shown. The immunoreactivity for each GluN2 subunit is similar across the mediolateral SDH axis in (A) and (C); higher immunoreactivity was detected for GluN2B in the medial division of SDH (B). Scale bar: 200 µm. (D-F) Quantitative statistical analysis of the mean O.D./BG for GluN2A (D), GluN2B (E) and GluN2D (F) NMDAR subunit in the medial, central and lateral SDH. *p<0.05
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**Fig. 5:** Relative O.D. ratios of GluN2A, GluN2B and GluN2D in male rats. (A) Quantitative statistical analysis showing that the SDH/DDH O.D. ratio relative to each subunit is not statistically different. (B) Quantitative statistical analysis showing the medial/lateral O.D. ratio for each receptor subunit, indicating a significant difference in the medial SDH expression for GluN2B compared to both GluN2A and GluN2D. *p<0.05

### 3.2. GluN2A, GluN2B and GluN2D in females

#### 3.2.1. GluN2B is expressed predominantly in the SDH laminae of female rats

To investigate whether the relative distribution of GluN2 - NMDAR subunits across the dorsal horn is conserved between sexes, we performed the same immunohistochemistry experiments as conducted on male rats on fixed L4/L5 spinal sections of juvenile female rats. Overall, the qualitative GluN2 staining patterns observed in females was similar to that observed in males. As for males, GluN2A was diffusely distributed across the SDH and DDH (Fig. 6A, Suppl. Fig.2), GluN2B expression was punctate, with a strong signal in cell bodies across SDH and DDH laminae (Fig. 6B; Suppl. Fig.4), and GluN2D was similar to GluN2B, with less intense, but still widespread punctate expression in the SDH and DDH laminae (Fig. 6C and Suppl. Fig.6). However, unlike males, there was not as robust of a visible differential expression
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for GluN2B and GluN2D in the SDH compared to the DDH in female spinal cord, and no visible
difference for GluN2A. Quantitative analysis indicated that only GluN2B is significantly higher
in the SDH compared to the DDH for females (p= 0.020, n=6; Fig. 6E). GluN2A and GluN2D
expression between the SDH and DDH did not significantly differ (p= 0.35, n=6; p=0.21, n=5
respectively; Fig. 6 D, F). To assess whether there was differential expression of one receptor
subunit over the others in the SDH, we next compared the relative SDH vs. DDH ratios for
GluN2A, GluN2B and GluN2D. The relative SDH vs. DDH ratio resulted was not significantly
different across GluN2 subtypes (p= 0.085; n=6, n=5, n=6 respectively; Fig. 7D), but the highest
ratio was observed for GluN2B.

3.2.2. GluN2-NMDAR subunits are equally distributed from medial to lateral SDH in females

To evaluate whether GluN2 NMDAR subunit expression differs across the
medial to lateral axis of the SDH, we measured the O.D. of GluN2A, GluN2B and GluN2D in
the medial, central and lateral regions of the SDH in female rats. As in males, GluN2B staining
intensity appeared to be slightly higher in the medial SDH than central and lateral regions, while
GluN2D staining localization was more variable across this axis between animals. However,
none of the receptor subtypes showed a significant differential distribution from the medial to
lateral SDH (p= 0.45, n=6; p= 0.15, n=6; p= 0.36, n=5; Fig. 7A, 7B, 7C). Thus, in contrast to
males, the relative medial vs. lateral O.D. ratio for GluN2A, GluN2B and GluN2D did not
significantly differ (p= 0.15; n=6, n=6, n=5 respectively; Fig.7E). We therefore conclude that in
females there is not differential expression of any specific GluN2 subtype across the medial to
lateral axis of the SDH.
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Fig. 6: GluN2 subunits in SDH and DDH of female rats. Representative confocal images illustrating the immunostaining for (A) GluN2A, (B) GluN2B and (C) GluN2D. (A) GluN2A immunofluorescence is intense and broadly detected across the SDH and DDH laminae; (B) GluN2B immunoreactivity is punctate along the SDH and DDH grey matter, with a preferential expression in the SDH; (D) GluN2D immunoreactive cells are widely diffuse across the grey matter lamina of the SDH and DDH with a punctate pattern resembling GluN2B immunofluorescence. Scale bar: 200 μm. D-F) Quantitative analysis of the O.D./BG for (D) GluN2A, (E) GluN2B and (F) GluN2D NMDAR subunits in the SDH and DDH. Only GluN2B fluorescence was significantly higher in the SDH vs DDH. *p<0.05
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Fig. 7: GluN2A, GluN2B, GluN2D ratios across the dorsal horn and the mediolateral axis within the SDH in female rats. A-C) Quantitative statistical analysis showing the O.D./BG of GluN2A (A), GluN2B (B) and GluN2D (C) subunits in the medial vs central vs lateral regions of the SDH. The panels below show the quantitative statistical analysis of the OD ratios for SDH/DDH (D) and the medial/lateral ratios (E). None of the parameters measured have any significant differences. *p<0.05

3.3. GluN2A, GluN2B and GluN2D in males versus females

3.3.1. GluN2 - NMDAR subunit expression is partially conserved among males and females

To investigate whether the relative expression of GluN2A, GluN2B and GluN2D subunits across the dorsal horn is sexual dimorphic, we first compared the SDH vs. DDH staining intensity for each receptor subunit between males and females. We observed that the relative immunoreactivity for GluN2A, GluN2B and GluN2D appeared to be similar between the
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male and female SDH. In support of this, statistical analyses revealed no significant difference between sexes for the SDH vs. DDH ratio of GluN2A, GluN2B, and GluN2D (p= 0.29, p= 0.88, p= 0.35 respectively; Fig. 8A, B, D).

In contrast to our hypothesis, when we looked more specifically to the medial-lateral axis of the SDH and compared the relative fluorescence between males and females for each subunit, we surprisingly observed that the specific intense GluN2B immunoreactivity localized to the medial SDH was found in males only (Fig., 9A and B, see Suppl. Fig. 3 and 4). Subsequent statistical analysis showed that the medial vs. lateral SDH ratio for expression of GluN2B was significantly greater in males compared to females (p= 0.008, n=5 and n=6 respectively; Fig. 9D). In contrast, the GluN2A and GluN2B immunoreactivity was equally distributed across the medial to lateral SDH for both males and females (p= 0.46, n=6; p=0.94, n=5 respectively; Fig. 9C and 9E).

Fig. 8: Comparison of GluN2 subunits expression SDH/DDH ratios between males and females. A-C)
Quantitative statistical analysis showing the SDH/DDH OD ratio for GluN2A (A), GluN2B (B) and GluN2D (C) which no significant difference between sexes. *p<0.05
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Fig. 9: Comparison of GluN2 subunits expression medial/lateral ratios between males and females.
Representative confocal images for GluN2B (A) in males and (B) in females. GluN2B immunoreactivity is more intense in the medial division of the SDH of males compared to females. Scale bar: 200 µm. C-E) Quantitative statistical analysis of the mean medial/lateral O.D./BG for all the three subunits in males versus females, including for GluN2A (C), GluN2B (D) and GluN2D (E). Only the medial/lateral ratio for GluN2B was statistically different in males compared to females. *p<0.05

3.4. Development of human spinal cord immunohistochemistry assays

We have conducted preliminary immunostaining experiments on human spinal cord tissues to establish an optimal human tissue staining assay in order to study the GluN2-containing NMDAR subunits in humans (Fig. 10). To accomplish this, we have successfully adapted the rat protocol conditions to humans. These experiments however have shown the presence of some background autofluorescence, which is however more limited to the white
matter portion of the dorsal horn. The mild autofluorescence observed may be due to accumulation of intracellular proteins or pigments such as the lipofuscin (Jung et al., 2007). Deposits of lipofuscin, an autofluorescent protein, are commonly observed in tissues and cells of older individuals as a physiological phenomenon depending on the oxidative stress (Jung et al., 2007; König et al., 2017). In future experiments we will establish further refinements to the human spinal cord immunohistochemistry assay to remove autofluorescent background and then subsequently quantify the relative localization of GluN2 NMDAR subtypes across the human dorsal horn. These ongoing investigations into GluN2 subunit expression in human spinal cord will then be compared to our rodent results to assess the conservation of GluN2 isoform distribution between species.
Fig. 10: Human immunohistochemistry preliminary assay. Representative confocal images showing in (A), left, and (B), left, the CGRP+ region that identifies the SDH; in (A), middle, the immunostaining for the GluN2A subunit; in (B), middle, the CTR (= control). (A) and (B), right, show the red (CGRP) and yellow (GluN2A) stainings combined in presence or absence of GluN2A primary Ab, respectively. Note the presence of GluN2A immunoreactivity in the human dorsal horn which partly overlap the CGRP+ region (A, middle and right); however, the CTR (B, middle and right) shows a mild autofluorescence BG, which is mainly visible outside the grey matter area. Scale bar: 200 µm.
4. Discussion

In this study we used an immunohistochemistry approach with pepsin-antigen retrieval technique to investigate the basal physiological expression of three main NMDAR receptor subunits belonging to the GluN2 subfamily: GluN2A, GluN2B and GluN2D. In particular, we explored the relative GluN2 subtype localization in the dorsal horn of the lumbar segment of the spinal cord of male and female juvenile (p21) rats.

One major finding of the present research was that GluN2-NMDAR subunit expression patterns varied across the dorsal horn, with specialized receptor combinations in the SDH, the dorsal horn region dedicated to processing pain inputs. Furthermore, this differential expression within the SDH is subject to sex-specific variability. Specifically, we found that of the all GluN2 receptor subunits, GluN2B and GluN2D are the isoforms most preferentially localized to the SDH in male rats. Interestingly, female rats displayed a relative dominance of the only GluN2B subunit over the other two subunit isoforms in the SDH, while GluN2A and GluN2D were uniformly widespread in the SDH versus DDH laminae.

The dominant expression of GluN2B and GluN2D in the lumbar SDH laminae of males is indicative that these two subunits may be the major isoforms that contribute to the formation of functional NMDAR complexes and we can speculate that GluN2B- and GluN2D- containing NMDARs would be primarily involved in processing nociceptive signals in males. The results also suggest that the excitability of neurons in pain states may be dependent to an increased activity of NMDARs containing GluN2B in both males and females and GluN2D more selectively in males. Supportive evidence on the preferential involvement of GluN2B and GluN2D subunits in male SDH signalling originate from previous electrophysiology experiments showing that GluN2B and GluN2D are the prevalent subunits mediating NMDAR responses in
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lamina I SDH synapses of adult male rats, while GluN2A contributed less to NMDAR synaptic responses (Hildebrand et al., 2014). Further evidence confirmed the recruitment and activation of GluN2B subunits as key mediators of spinal hyperexcitability in chronic pain after nerve injury in males (Qu et al., 2009). Despite the fact that the functional studies mentioned above were conducted in adult rodents and the present study concerns juvenile rats, we can still speculate the existence of similar functional NMDAR responses in juveniles. Indeed, our lab has recently reported the absence of a developmental switch in subunit composition across early postnatal development. Specifically, in contrast to what occurs in the brain, where there is a developmental switch between NMDAR subunit isoforms such that GluN2B is substituted by GluN2A into adulthood, in the SDH of the spinal cord this early developmental switch does not occur so that GluN2B remains the major NMDAR subtype into adulthood (Hildebrand et al., 2014; Mahmoud et al., 2020). One explanation for this apparent lack of developmental switch in the spinal cord was provided by the work of Zhang and colleagues (Zhang et al., 2020), who have demonstrated that the process of ubiquitination is responsible for the removal of GluN2-containing NMDAR subunits from mature synapses and maintains GluN2B at low levels in adults. The persistence of GluN2B at adult SDH synapses could therefore be due to an altered mechanism of ubiquitination that would set the basis for the subsequent development of pain states (Zhang et al., 2020).

The above studies however have not elucidated whether GluN2B and GluN2D form homo- or hetero-tetrameric NMDAR complexes. Distinguishing whether GluN2B/2B and GluN2D/2D diheteromeric receptors versus GluN2B/2D triheteromeric receptors contribute to spinal synaptic responses will shed further light on how differential composition in GluN2 subunits alters nociception. Future studies are required to investigate this aspect by employing
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western blot analysis followed by immunoprecipitation to evaluate the amount of protein relative to each subunit and their reciprocal interactions.

Another major striking finding of our study was that for the first time, we have identified the presence of an asymmetry in the relative expression of GluN2-NMDAR subunits along the mediolateral SDH axis. The literature to date has focused on differences between SDH and DDH; however, our findings reveal that the medial and lateral divisions of the SDH are not uniform, with GluN2B more prevalent in the medial portion. Surprisingly, we observed that this phenomenon is sex-specific: GluN2B appears to be predominately expressed in the medial division of the SDH only in males, while females do not present any asymmetry in the synaptic organization of the SDH. In agreement with these findings, it has recently been observed that the photostimulation of mouse lamina I neurons aimed at investigating the connectivity of interneurons within the SDH produced a greater excitability of neurons and synaptic input of medial compared to lateral interneurons which correlated with a dendritic asymmetry towards the medial SDH (Kosugi et al., 2013). Moreover, the medial division of the SDH in male rats (2-3 weeks old) responded to focal stimulation in a more pronounced way than the lateral division of the SDH (Mizuno et al., 2019). Based on evidence involving postsynaptic receptor antagonists, the authors propose that this mediolateral discrepancy is intrinsic to the SDH rather than depending on asymmetry of peripheral input to SDH. Our results may therefore signify the presence of localized and specialized subregions of altered postsynaptic receptor identity within the SDH that process nociceptive inputs differently. The mediolateral asymmetry should be taken into account in future investigations on nociceptive mechanisms within the SDH, as such divergence in GluN2 subunit expression could indicate the presence of specialized postsynaptic circuits that differentially process nociceptive inputs across the SDH.
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Due to the lack of studies of NMDAR expression and function in females, there is no evidence on how the mediolateral differential expression of GluN2 subunits, in particular, GluN2B would contribute and influence the neurobiology of pain processing in males versus females. The study of specific neuronal subpopulations and connectivity in the medial portion of the dorsal horn in both sexes would clarify this issue.

The differences that we observed in NMDAR localization between males and females may be ascribed to the action exerted by gonadal hormones, in particular estrogens and testosterone, which act as modulators of synaptic composition and plasticity. As steroid hormones have usually genomic effects, it is plausible that an alteration in gene expression mediated by hormones would result in an alteration in GluN2B expression. Estrogens may promote the upregulation of the GluN2B subunit observed in baseline conditions in males in a regional-specific manner within the SDH, with a prevalence towards the medial portion of the SDH. Estrogen receptor α (ERα) and estrogen receptor β (ERβ) are in fact expressed in lamina I and II of dorsal horn neurons since early embryogenesis (Fan et al., 2007) in rats of both sexes. Therefore, 17-β-estradiol (E2), through the binding to these receptors, when it translocates into the nucleus may facilitate the transcription of GluN2(B) NMDAR genes. As ERα and ERβ are expressed also in glial cells (Platania et al., 2003), the modulation of gene transcription within the neuron may occur indirectly through glia-neuron communication as well. However, it should be noted that there is evidence that when E2 activates ERα it produces hypoalgesic effects, whereas when E2 binds and activates ERβ it mediates a pro-nociceptive role (Coulombe et al., 2011).

The differential contribution of NMDA receptor subunits across the dorsal horn between sexes may also originate from intrinsic genetic differences existing between males and females.
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on specific loci of the sex chromosomes. Moreover, single nucleotide polymorphisms (SNPs) may be responsible of variations in susceptibility to pain in one sex compared to the other.

Similarly, epigenetic factors driven by differential sex-specific experiences may promote changes in dorsal horn NMDAR subunit expression and, therefore, in the subunit composition of NMDARs. Early exposure to pain, for instance, may contribute to the formation of a 'memory' of pain, including LTP and synaptic strengthening, which may trigger permanent variations in subunit expression and in pain sensitivity (Bonin & De Koninck, 2014).

The underlying molecular mechanism that accounts for the differences in the mediolateral specificity between sexes remains to be explored. Future studies should be conducted to elucidate how the alteration on the hormonal steroid levels may affect subunit composition of NMDARs in the medial and lateral divisions of the SDH in males and females and its functional significance. Furthermore, investigations should take into account how the surge of steroid hormones during puberty and adolescence would affect the expression differences between GluN2 subunits across sexes. It will be interesting to gain insight on whether our observed differences in GluN2 subunits, particularly GluN2B and GluN2D, found in pre-pubescent rats would increase or decrease after puberty within the dorsal horn and across the SDH mediolateral axis.

It has been shown that the enzyme aromatase that catalyzes the aromatization of androgens (e.g. testosterone) into estrogens (e.g. estradiol), is expressed at all levels of the spinal cord in birds and mammals (Evrard et al., 2000; Tran et al., 2017). In particular, among birds, quails have shown a wide distribution of aromatase expression across laminae I-III (Evrard et al., 2000); a similar distribution was also observed in rodents (Tran et al., 2017) with an interesting prevalence of aromatase in the medial lamina V and in lamina I. Given the expression of
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aromatase in the dorsal horn of the spinal cord and that scientific evidence converges on the antinociceptive/analgesic role of testosterone, we may speculate that pain signaling in males would be mediated by the action of the aromatase which would convert the testosterone into estradiol. The resultant increased spinal levels of estrogens in males could thus induce an increased expression, trafficking and potentiation of GluN2B-containing NMDARs at SDH synapses.

The relevance of the aromatization has been shown in adult male quails where the inhibition of aromatase by the drug vorozole caused a rapid inhibition of estrogens dependent on local aromatization (non-genomic) followed by a rapid reduction of thermal pain (Evrard & Balthazart, 2003, 2004). Future investigations will be needed in rodents to investigate if this hypothesis is supported by mammalian studies. Moreover, in supraspinal regions such as the anterior cingulate cortex, the acute exposure to estrogens enhanced glutamatergic excitatory post-synaptic currents through their action on NMDARs (Xiao et al., 2013). The induction of LTP on NMDARs required specifically the presence of the GluN2B subunit (Xiao et al., 2013). Similarly, experimentally-induced inflammatory pain in ovariectomized female rats has shown that GluN2B -NMDARs are involved in estrogen modulation (by increasing GluN2B-NMDAR activity) of visceral pain in the thoracolumbar segment of spinal cord (Ji et al., 2015; Ji et al., 2018).

Since we detected the presence of GluN2B also in the soma of cells and outside the grey matter area marked by the CGRP (such as above and around the central canal), it is possible that part of the GluN2B immunoreactivity that we reported is located in glial populations. Increasing evidence is in fact showing the relevance of glial cells in modulating and mediating the overall excitability of neurons through the expression of BDNF, which acts on neurons, and alters their
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excitability. Indeed, NMDAR subunits have been identified in brain and spinal cord glial cells of rodents (Ziak et al., 1998) and in glial cells of the human brain (Conti et al., 1999). Further investigation using specific markers for neurons and glia is needed to uncover the contribution and functional significance of cell types differences between sexes. In recent years, transcriptome analysis through single-cell sequencing has been becoming a powerful tool that allows researchers to identify and characterize the molecular (and associated functional) profile of neuronal populations within the spinal cord. For instance, Sathyamurthy et al. (2018) identified in the adult mouse spinal cord through single-nuclear sequencing- 43 functionally active different neuronal subpopulations, including a larger number of distinct, non-overlapping, neuronal populations in the dorsal horn compared to the ventral horn (Sathyamurthy et al., 2018). Future single cell sequencing studies could therefore investigate whether divergent expression of specific GluN2 subtypes between specific dorsal horn neuron subpopulations underlies the differential expression of these isoforms in the DDH versus SDH as well as across the SDH mediolateral axis.

In conclusion, with the present study we found that GluN2B is the dominant isoform expressed in basal condition in the SDH of both male and female rats while GluN2D contributes to NMDAR composition primarily in males. Furthermore, our results revealed for the first time an asymmetry towards the medial SDH in the expression of the GluN2B subunit exclusively in males. This differential NMDAR subunit expression asymmetry that may contribute to functionally unique nociceptive circuits and processing mechanisms across the SDH in males versus females.
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6. Supplemental Figures

Suppl. Fig. 1: Representative immunostaining showing GluN2A immunoreactivity in male juvenile rats. Scale bar: 200 µm
Suppl. Fig. 2: Representative immunostaining showing GluN2A immunoreactivity in female juvenile rats.
Scale bar: 200 µm
Suppl. Fig. 3: Representative immunostaining showing GluN2B immunoreactivity in male juvenile rats. Scale bar: 200 µm
Suppl. Fig. 4: Representative immunostaining showing GluN2B immunoreactivity in female juvenile rats.
Scale bar: 200 μm
Suppl. Fig. 5: Representative immunostaining showing GluN2D immunoreactivity in male juvenile rats. Scale bar: 200 µm
Suppl. Fig. 6: Representative immunostaining showing GluN2D immunoreactivity in female juvenile rats. Scale bar: 200 µm