Comparison of NMDA receptor subunit RNA expression in the spinal dorsal horn across species (mice, rats, humans), sex, laminar location and neuronal subtypes using single cell RNA sequencing data and qRT-PCR

by

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Abstract

The hyperexcitability and central sensitization of spinal pain processing circuits is mediated by N-methyl-d-aspartate receptors (NMDARs) in the dorsal horn. Distinct circuits of neuronal subtypes in the superficial dorsal horn (SDH) versus the deep dorsal horn (DDH) primarily integrate pain and other somatosensory signals, respectively. Unique combinations of NMDAR subunits (GluN1/GRIN1, GluN2A – D/GRIN2A – D, and GluN3A – B/GRIN3A – B) confer different functional NMDAR properties and have differing effects on synaptic plasticity. In this study, we investigated NMDAR subunit expression across sex, species, spinal region, and neuronal subtypes using single cell/nucleus RNA sequencing data from the mouse and human spinal cord and performing qRT-PCR on adult male and female rat and human spinal cord tissue. We found high GRIN1, GRIN2A, GRIN2B, and GRIN3A expression and moderate GRIN2D expression in the SDH. Unlike in the rat dorsal horn, GRIN2C is highly expressed in the human dorsal horn. Both GRIN2C and GRIN2D are more highly expressed in inhibitory than excitatory neurons of the dorsal horn, but to a greater extent humans than in mice. Grin3a is more highly expressed in excitatory than inhibitory neurons of the mouse SDH. Compared to the other NMDAR subunits, GRIN2A and GRIN3A have variable expression across neuronal subtypes, but could play different roles in synaptic plasticity. Taken together, our results suggest that select NMDAR subunit genes play prominent and specialized roles in SDH somatosensory processing and different neuronal subtypes may have different patterns of GRIN gene expression.
Introduction

Uncovering personalized and effective therapeutic targets for managing pain symptoms requires translational studies that investigate the cellular and molecular underpinnings of pain processing in the rodent and human spinal cord. Pain is an unpleasant sensory and emotional experience that is associated with or resembles that associated with actual or potential tissue damage (Raja et al., 2020). According to the International Classification of Diseases 11th edition (ICD-11), pain is said to be chronic if it persists or recurs for longer than 3 months. ICD-11 distinguishes chronic primary pain as a separate disease and 6 chronic secondary pain categories for pain that is a symptom of an underlying disease. Pain is said to be acute when it has a duration of less than 3 months and there is no other specification that can be ascribed to it (Barke et al., 2022; Korwisi et al., 2022; World Health Organization, 2022). Behavioral increases in pain sensation are commonly described in terms of hyperalgesia, when noxious or pain-inducing stimuli induce a larger pain response, and allodynia, when normally innocuous stimuli become painful (Jensen and Finnerup, 2014; Yam et al., 2018). Pain sensation can be divided into different modalities that span emotional, cultural, and social contexts but that also include nociception. Nociception is the cellular and molecular mechanisms that govern the neuronal excitability and dysregulation of normal pain processing (Dubin and Patapoutian, 2010).
Figure 1. Simplified spinal nociception pathway. Noxious stimuli are initially detected in free nerve endings in the periphery (Figure 1). The periphery includes the dermis of the skin and visceral organs such as the stomach, bones, and muscle. Activated free nerve endings send action potentials along its axon, which passes the cell body in the dorsal root ganglion, and synapses in the dorsal horn of the spinal cord (Figure 1). The dorsal horn contains a collection of interconnected neurons that process incoming noxious, thermal and mechanical stimuli (Watson, 1981; Millan, 1999; Koutsikou et al., 2007). The dorsal horn is divided into six different laminae titled lamina I – VI (Rexed, 1952). It can be further subdivided into the superficial dorsal horn (SDH), which spans lamina I to lamina II outer (Ilo), and the deep dorsal horn (DDH), which spans from lamina II inner (Ili) to lamina VI (Todd, 2010). While the DDH mostly processes somatosensory modalities such as touch, itch, and proprioception, the majority of the nociceptive processing occurs in the SDH (Lai et al., 2016; Takeoka and Arber, 2019; Wu et al.,
2021). On the other hand, the ventral horn spans lamina VII – IX and is primarily involved with motor output.

**Heterogenous neuronal subtypes and neuronal circuitry in the dorsal horn**

Integration of signals from excitatory and inhibitory interneurons in the SDH and DDH dictates whether or not secondary afferent projection neurons send a signal to the brain for further processing by the somatosensory and limbic systems (Mercer Lindsay et al., 2021). Initial attempts to classify dorsal horn interneurons into subtypes began with 4 main morphological groups: central cells, radial cells, vertical cells, and islet cells. Due to the inability to associate individual sensory components to individual morphological groups, neurochemical and protein markers were used instead as the main descriptor for interneuron subtypes. Each subtype is further detailed using other properties such as function, location, synaptic connections, morphology, electrophysiological properties, protein expression, gene expression, as well as the outcomes of physiological or pathological interventions (Todd, 2010; Peirs and Seal, 2016; Duan et al., 2018). For instance, somatostatin-expressing (SOM⁺) interneurons are primarily excitatory interneurons with varying cellular morphology. One population of SOM⁺ interneurons in laminae IIo – III receives direct input from primary afferents carrying mechanical pain stimuli. Meanwhile, another SOM⁺ population in laminae IIIi – III in the DDH receives indirect input from thermal-receptive primary afferents. Ablation of SOM⁺ neurons leads to a reduction in mechanical pain and thermal processing in the spinal cord (Koch et al., 2018).
Another example of an interneuron subtype characterized by a protein marker that can be associated to a distinct sensory outcome, is a class of inhibitory parvalbumin protein expressing (PV\(^+\)) interneurons. PV\(^+\) interneurons, found in laminae IIi – III of the DDH, enhance hyperexcitability and attenuate mechanical allodynia when activated, but increase mechanical allodynia when inhibited (Qiu et al., 2022). After nerve injury, PV\(^+\) interneurons form less synaptic connections to neighboring excitatory interneurons and express less parvalbumin protein, which taken together leads to increased mechanical allodynia (Qiu et al., 2022). PV\(^+\) interneurons have been described as gate keepers of distinct sensory inputs since ablation of PV\(^+\) interneurons results in disinhibition of excitatory protein kinase C type \(\gamma\)-expressing (PKC\(\gamma^+\)) interneurons and subsequent development of mechanical allodynia (Petitjean et al., 2015).

Other excitatory neuron subtypes in the dorsal horn include cholecystokininin-expressing (CCK\(^-\)), Calretinin/Calbindin 2-expressing (CR\(^+/\)Calb2\(^-\)), NK1R\(^+\), PKC\(\gamma^+\), and vGluT3\(^+\) neuron subtypes. CCK\(^-\) interneurons are located in laminae I to III but mostly in lamina III and may be implicated in light touch and mechanical allodynia (Xu et al., 1993; Gutierrez-Mecinas et al., 2019a, 2019b; Nakagawa et al., 2020; Peirs et al., 2021). CR\(^+/\)Calb2\(^+\) are mostly in laminae I – II and modulate mechanical and thermal pain thresholds (Anelli and Heckman, 2005; Petitjean et al., 2019; Smith et al., 2019). NK1R\(^+\) neurons are typically regarded as projection neurons and influence neuropathic pain, inflammatory pain and mechanical allodynia (Hökfelt et al., 1975; Lawson et al., 1997; Naim et al., 1997; Polgár et al., 2007). PKC\(\gamma^+\) interneurons have been found in laminae I, IIi, and III. They are primarily excitatory with smaller inhibitory subgroups being...
immunoreactive to GABA (Polgár et al., 1999). Removal of PKCγ in rodent models either by knockout or pharmacological intervention leads to severely reduced allodynia and neuropathic pain after nerve injury. Therefore, it is likely that PKCγ⁺ interneurons play a major role in mechanical allodynia (Koch et al., 2018). vGlut3⁺ interneurons in laminae II – III synapse with other CR⁺/Calb2⁺, PKCγ⁺, and lamina I NK1R⁺ interneurons. Activation of vGlut3⁺ interneurons leads to increased mechanical hypersensitivity and allodynia. vGlut3⁺ interneurons also play a large role in visceral (internal organ) pain processing (Brumovsky, 2013; Qi et al., 2022).

Most dorsal horn interneurons are excitatory rather than inhibitory (Prescott and Koninck, 2002; Santos et al., 2007; Russ et al., 2021). However, there are inhibitory interneuron subtypes in the dorsal horn that respond to pain and other somatosensory modalities. Basic helix-loop-helix domain containing, class B5-expressing (Bhlhb5⁺), dynorphin⁺, and galanin⁺ inhibitory interneurons are mostly localized to laminae I – II, which is mostly the SDH (Koch et al., 2018). Despite being in the SDH, ablation of Bhlhb5⁺ interneurons mostly leads to elevated itch processing but also leads to elevated responses to formalin, a chemical commonly used in pain rodent models (Ross et al., 2010; López-Cano et al., 2017). In relation to SDH pain processing, ablating dynorphin⁺ interneurons results in spontaneous allodynia. Galanin⁺ interneurons are activated during noxious stimulation (Sardella et al., 2011). On the other hand, neuropeptide Y⁺ (NPY⁺) and transient receptor potential cation channel subfamily V member 1⁺ (TrpV1⁺) inhibitory interneurons are also located in laminae I – II with most NPY neurons located
in laminae III – V; and are responsible for gating pain hypersensitivity and mechanical allodynia (Zhou et al., 2009; Polgár et al., 2011; Koch et al., 2018; Acton et al., 2019).

Many recent studies on dorsal horn neuron subtypes have made use of recent single cell RNA sequencing studies. Single cell RNA sequencing is a novel exploratory method for sequencing the RNA of individual cells in a tissue, then grouping the cells based on similar RNA expression patterns (see section below). Interestingly, recent studies using protein biomarkers to characterize dorsal horn neuron subtypes have found high correlates between distinct single cell RNA sequencing clusters and distinct protein marker groups. One benefit of using single cell RNA sequencing to explore cell types is the ability to define them by more than one marker. For example, projection neurons are known to express the neurokinin 1 receptor (NK1R), which is activated by substance P-releasing primary afferent sensory neurons, and thus NK1R immunoreactivity has traditionally been used as a marker of SDH projection neurons (Hökfelt et al., 1975; Lawson et al., 1997). However, the NK1R protein has more recently been found to be expressed in interneurons involved with itch processing in both mice and humans, limiting its utility as a specific cell type marker (Sheahan et al., 2020). Furthermore, most interneuron subtypes classified by an individual marker have a smaller subgroup that express other markers. For example, SOM is expressed by ~23% of vGluT3+ interneurons and 27% of CR+/Calb2+ interneurons (Koch et al., 2018).

Similarly, subsets of PKCy immunoreactive neurons that are also immunoreactive to the neurotransmitters SOM, GABA and glycine as well as the receptors mu opioid receptor,
or NK1R (Polgár et al., 1999). Using multiple markers for subtypes could uncover specific subpopulations of nociceptive interneurons with more precise roles.

In summation, neurocircuitry in the dorsal horn is heterogenous and complex and each neuronal subtype has unique properties and protein/gene expression that can affect somatosensory signal transmission and modulate future pain responses.

Central sensitization and the role of NMDARs in the excitability of nociceptive circuits

Central sensitization is a phenomenon that occurs in the spinal cord dorsal horn and can be described as an activity-dependent hypersensitivity to mechanical, thermal and nociceptive stimuli that arises post-injury (Woolf, 1983; Wall and Woolf, 1984). The hypersensitivity is caused by a reduction in the nociceptive threshold of dorsal horn neurons in response to repetitive or intense stimulation to point where there is an increase in the amplitude of responses that persists after the triggering event has resolved (Woolf, 1983; Koltzenburg et al., 1992). Windup is a transient increase in neuronal excitability caused by multiple temporally summated low threshold stimuli and requires NMDARs on lamina I neurons. Windup can cause central sensitization but is not always needed for central sensitization to occur (Woolf, 1996; Li et al., 1999; Mendell, 2022). It is hypothesized that the reduction in the pain threshold is driven by molecular mechanisms at the synapse between primary afferents and dorsal horn neurons (Woolf and Salter, 2000; Ji et al., 2003; Latremoliere and Woolf, 2009). Dorsal horn neurons express a collection of receptors and channels with converging cellular cascades that lead to activity dependent post-translational modifications, changes in gene expression
and ultimately changes in synaptic plasticity. Two key examples of these receptors includes α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPARs) and NMDARs (Mannion et al., 1999; Latremoliere and Woolf, 2009).

Woolf and Salter (2000) proposed two phases to central sensitization. In the initial phase, phosphorylation of AMPARs and NMDARs by kinases such as PKA, PKC, or Src leads to increased excitability of SDH circuits. The second longer lasting phase is driven by an increase in intracellular Ca\(^{2+}\) concentration followed by changes in gene expression and synaptic remodeling of SDH circuits. At most CNS synapses, glutamate neurotransmission involves AMPAR-mediated influx of Na\(^{+}\) followed by a NMDAR-mediated influx of both Na\(^{+}\) and Ca\(^{2+}\) after the removal of the Mg\(^{2+}\) blockade of the NMDAR ion channel. Once the Mg\(^{2+}\) blockade has been removed, NMDARs become major drivers of the increase in intracellular Ca\(^{2+}\) that is observed in activity dependent central sensitization (Woolf and Thompson, 1991; Qing-Ping and Woolf, 1995; Latremoliere and Woolf, 2009). Distinct subtypes of NMDARs are composed of different combinations of NMDAR subunits that affect the function, regulation and dysregulation of the receptor. Thus, different NMDAR subtypes play a critical role in SDH nociceptive processing.

**GluN1, GluN2A – D, and GluN3A – B: characteristics and function**

There are 7 different NMDAR subunits: GluN1, GluN2A, GluN2B, GluN2C, GluN2D, GluN3A, and GluN3B. According to Ensembl's UniProt database, each receptor is often called by different names in the scientific literature. The most common historical naming convention was NR1, NR2A – D, and NR3A – B, followed by NMDA1 and
NMDA2A – D, hNR2A – hNR2D. Each NMDAR subunit is associated with its own gene, now described as GluN1, GluN2A – D, and GluN3A – B proteins being transcribed and translated from Grin1, Grin2a – d, and Grin3a – b in rodents and GRIN1, GRIN2A – D, and GRIN3A – B in humans.

Activation of NMDARs involves the binding of both glycine and glutamate. Canonical NMDARs are composed of 2 glycine or D-serine-binding GluN1 subunits and 2 glutamate-binding GluN2 subunits. However, 2 GluN1 subunits may also combine with 2 glycine binding GluN3A subunits or glutamate binding GluN3B subunits. NMDARs with 2 of the same GluN2 or GluN3 subunits are termed di-heteromers such as GluN1/GluN2A or GluN1/GluN2B. Tri-heteromers such as GluN1/GluN2A/GluN2B have 2 different GluN2/3 subunits incorporated.

NMDAR functional properties include deactivation kinetics, Mg$^{2+}$ sensitivity, Ca$^{2+}$ permeability, and single-channel conductance, which all depend on the specific subunits contained (Glasgow et al., 2015). The properties of di-heteromeric NMDARs depends on GluN1 and the other subunit. The properties of tri-heteromeric NMDARs are conferred by the combination but not the average of the properties of each subunit involved (Hansen et al., 2018). GluN2A-containing NMDARs have a much faster decay rate in recordings of excitatory postsynaptic current than GluN2B. GluN2D has the slowest decay kinetics lasting up to 1000 ms (Hansen et al., 2018).

Each NMDAR protein subunit has an N-terminal domain, agonist-binding domain, pore-forming domain, and C-terminal domain that contributes to its unique functional
properties (Shipton and Paulsen, 2014). For example, GluN2A-containing NMDARs can be inhibited by Zn$^{2+}$ due to a highly selective allosteric binding site the N-terminal domain of GluN2A (Fayyazuddin et al., 2000). The agonist binding domain refers to the allosteric site where glutamate, glycine or D-serine bind. The C-terminal domain contains binding sites for many post-translational modifiers and regulators. For instance, the GluN2A C-terminal domain has been shown to selectively interact with Src kinases at specific amino acid sites. However, the GluN2B C-terminal domain interacts with Fyn kinase instead. The selectivity in subunit-kinase interactions alludes to differential GluN2 subtype involvement in cellular mechanisms and differential regulatory responses to specific cellular pathways (Yang et al., 2012; Pérez-Otaño et al., 2016; Hansen et al., 2018).

GluN3 subunits have interesting properties despite being understudied. Most functional NMDARs at CNS synapses are presumed to contain GluN2 subunits. It was previously thought that GluN3A/Grin3a played a role in postnatal synaptic pruning during early development but decreased in expression before adulthood (Pérez-Otaño et al., 2016; Crawley et al., 2022). However, recent studies and databases have shown that Grin3a expression persists into adulthood. Conventional NMDARs do not consist of glycine-binding GluN1/GluN3A subtypes since glutamate binding is absent. However, GluN1/GluN2/GluN3 NMDAR subtypes that respond to glutamate have been identified in neurons and glia in the brain (Pérez-Otaño et al., 2016; Grand et al., 2018; Conde-Dusman et al., 2021; Crawley et al., 2022). The majority of GluN3 containing NMDARs
are also found at perisynaptic and extrasynaptic sites due to their lack of C-terminal domains interacting with post-synaptic density proteins (Crawley et al., 2022).

**NMDAR subunit mRNA/protein expression and their contribution to NMDARs in the spinal cord**

Complementary studies have used a variety of techniques to determine the gene expression, protein expression and receptor contributions of NMDAR subunits in the spinal cord. Studies on adult rodents support the high widespread expression of GluN1/GRIN1 (Tolle et al., 1993; Yung, 1998; Stegenga and Kalb, 2001; Nagy et al., 2004; de Geus et al., 2020; Das Gupta et al., 2021). However, protein, gene and functional studies on GluN2A/GRIN2A and GluN2D/GRIN2D expression in adult rodents show more inconsistency. For instance, all protein and gene studies found low or no GluN2A/GRIN2A expression in lamina II, but one whole-cell electrophysiology study found significant contributions of GluN2A to NMDAR-mediated currents (Tolle et al., 1993; Watanabe et al., 1994; Yung, 1998; Shibata et al., 1999; Stegenga and Kalb, 2001; Nagy et al., 2004; Shiokawa et al., 2010). Furthermore, in lamina I, GluN2A/GIRN2A expression has been reported to be high, moderate, low or not present in adult rodents across different studies (Tolle et al., 1993; Yung, 1998; Stegenga and Kalb, 2001; Nagy et al., 2004; de Geus et al., 2020; Das Gupta et al., 2021). Although reports on GluN2D/GRIN2D expression is very inconsistent, two studies suggest that GluN2D is higher in the male than female lamina II and GluN2D-containing NMDARs are preferentially found on inhibitory neuron subtypes (Shiokawa et al., 2010; Temi et al.,
Similarly, actively sequenced GRIN2D transcripts were found to be present more present in inhibitory SDH neurons (Das Gupta et al., 2021).

On the contrary, most adult rodent studies report high GluN2B/GRIN2B expression and NMDAR contribution in both lamina I and II (Watanabe et al., 1994; Yung, 1998, 1998; Nagy et al., 2004, 2004; Shiokawa et al., 2010; Hildebrand et al., 2014; Das Gupta et al., 2021; Temi et al., 2021). High GluN2B expression aligns with evidence of its role in synaptic plasticity, central sensitization, pain disinhibition and NMDAR regulation (Woolf and Salter, 2000; Zhang et al., 2016; France et al., 2017; Dedek et al., 2022). All studies support the presence of little to no GluN2C/GRIN2C expression in the SDH (Tolle et al., 1993; Watanabe et al., 1994; Yung, 1998; Shibata et al., 1999; Stegenga and Kalb, 2001; Das Gupta et al., 2021). In the brain, however, GluN2C/GRIN2C is highly expressed in a subset of astrocytes and a subset of neurons in the cortex and cerebellum (Ravikrishnan et al., 2018; Batiuk et al., 2020; Sjöstedt et al., 2020; Kronschläger et al., 2021).

There are fewer studies looking at GluN3B/GRIN3B and GluN3A/GRIN3A. Studies that investigated GluN3B/GRIN3B in the spinal cord found that it has little to no expression in the dorsal horn but it is expressed in motor neurons of the ventral horn (Prithviraj and Inglis, 2008; Wee et al., 2008; Kim et al., 2013). Despite being under studied, GRIN3A is reported to be highly expressed in the dorsal and ventral horn with possible selective expression in excitatory neuronal subtypes (Ciabarra et al., 1995; Nilsson et al., 2007; Guo et al., 2012; Das Gupta et al., 2021).
In situ hybridization rodent studies looking at GRIN2 and GRIN3A RNA expression across development reveal interesting patterns despite having opposing results. One study found that GRIN1, GRIN2A, GRIN2B, and GRIN2C have low expression that decreases into adulthood with no GRIN2D overall (Stegenga and Kalb, 2001). Another study found high and consistent expression of GRIN1, increasing expression of GRIN2A, decreasing expression of GRIN2B, no expression of GRIN2C, and generally low expression of GRIN2D (Watanabe et al., 1994). Interestingly, in both studies each GRIN2 gene showed specific patterns of localization to the dorsal or ventral horn (Watanabe et al., 1994; Stegenga and Kalb, 2001). For instance, GRIN2A and GRIN2B RNA expression became more localized to an upper band in the dorsal horn of adult rodents (Watanabe et al., 1994; Stegenga and Kalb, 2001). Whereas, GRIN2D expression increased in the ventral horn transiently then localized to an upper band in the dorsal horn (Watanabe et al., 1994). GRIN3A expression was found to be localized to the SDH across early development and demonstrated distinct co-localization with certain developmental markers of glutamatergic cells in the SDH (Guo et al., 2012). Preferential localization of GRIN RNA could reflect unique demand for NMDAR subunit transcripts within specific cell types in the dorsal horn.

Single cell RNA sequencing datasets across species and neuronal subtypes

Transcription to mRNA occurs in the nucleus, but translation into functional proteins mostly occurs in the cytoplasm before being trafficked to synaptic sites (Rangaraju et al., 2017). The major ingredient to produce functional proteins is mRNA, which gives us a sense of what the cell is currently producing. Several techniques are
used to investigate the transcriptome. *In situ* hybridization involves relative abundance of a few target genes of interest and their spatial location in a tissue. DNA microarray is used to compare a few genes across disease states. Reverse transcription quantitative polymerase chain reaction (RT-qPCR) is used to quantify the amount of mRNA of known sequence in a tissue (Rao et al., 2019).

While each method has its strengths and weaknesses, one dimension that is recently being explored is the heterogeneity of RNA expression across both common and rare cell types. RT-qPCR produces an average expression value for the entire tissue and does not yield direct information about the specific expression of individual cell types (Mu et al., 2019). Single cell RNA sequencing is a novel technology initially developed by Tang et al. (2009) that quantifies the RNA of individual cells in a tissue. The process begins with homogenization and dissociation to separate cells followed by RNA extraction. Then, single stranded RNA is converted to double stranded complementary DNA (cDNA) and quantified using RT-qPCR (Tang et al., 2009, 2010). Dissociation of neurons, glia, adipocytes and other complex tissue types can cause cell rupture, which can induce intracellular stressful events, cause spillage of RNA, and lead to inaccurate readings of the amount of RNA present (Neuschulz et al., 2022). To address this issue, some modern commercial single cell RNA sequencer machines dissociate cells using microfluidics technology, which harnesses the properties of water and oil to encapsulate an individual cell into a single droplet. Each droplet is then sequenced separately (Zhou et al., 2021). Furthermore, instead of using the entire cell,
the cell nucleus of complex cell types can be first isolated and then sequenced by single nucleus RNA sequencing (Grindberg et al., 2013).

Early single nucleus RNA sequencing techniques found that most of the gene transcripts in mouse dentate gyrus nuclei and cells had similar expression levels despite there being lower cDNA product in general (Grindberg et al., 2013). The RNA detected from sequencing nuclei may not only include intron-aligning reads from mature mRNA. This is opposed to single cell RNA sequencing, which filters out intron-aligning reads and only works with exon-aligning reads (Grindberg et al., 2013; Ding et al., 2020). Single nucleus RNA sequencing has been adapted and modified by an ever-growing list of studies to address tissue-specific and experiment-specific demands (Rosenberg et al., 2018; Sathyamurthy et al., 2018; Wu et al., 2019; Slyper et al., 2020; Bakken et al., 2021; Basile et al., 2021; Andrews et al., 2022; Yadav et al., 2023). Some studies have successfully coalesced single cell and single nucleus RNA sequencing data from different sequencing platforms to investigate technical, species, sex, developmental, and multimodal related research questions (Grindberg et al., 2013; Ding et al., 2020; Bakken et al., 2021; Russ et al., 2021).

There has been an exponential emergence of spinal cord single cell/nucleus RNA sequencing studies with data across development, species, sex, or other significant translational variables. There are several studies that performed single cell/nucleus RNA sequencing on spinal cord tissue from mice in early development: embryonic day 9.5 – 13.5, postnatal day 0 (P0), P4, P2, and P11 (Hayashi et al., 2018; Rosenberg et al., 2018; Baek et al., 2019; Delile et al., 2019). Adolescent mouse spinal cord single cell/nucleus
RNA sequencing studies are also available spanning postnatal days 12 – 30 (Häring et al., 2018; Zeisel et al., 2018). Four studies have done single nucleus RNA sequencing on the adult mouse spinal cord at baseline and after various experimental interventions (Sathyamurthy et al., 2018; Alkaslasi et al., 2021; Blum et al., 2021; Matson et al., 2022). Six of the studies discussed above have been integrated into a meta-analysis that characterized individual neuronal and non-neuronal cell types using the combined information from each study (Russ et al., 2021). Other mouse spinal cord single cell/nucleus transcriptomic studies looked at the effects of specific physiological or pathological interventions on cell type specific RNA expression (Fisher et al., 2022; Matson et al., 2022; Sun et al., 2022). Three studies and preprints have performed single cell/nucleus RNA sequencing on adult human donor spinal cord tissue (Zhang et al., 2021; Tansley et al., 2022; Yadav et al., 2023). One study identified sex-specific differences in the microglial transcriptome after nerve injury in both mice and humans (Tansley et al., 2022). Adult macaque and pig spinal cord single cell RNA sequencing studies have also been developed (Li et al., 2021; Arokiaraj et al., 2022). There are single cell/nucleus RNA sequencing studies that sequenced mouse and human dorsal root ganglion cells, which are the cell bodies of primary afferents (Usoskin et al., 2015; Ray et al., 2018; Tran et al., 2020; Nguyen et al., 2021; Tavares-Ferreira et al., 2022; Zhang et al., 2022).

Human single cell transcriptomic studies have massive translational value by facilitating cross-species comparison of spinal pain mechanisms and the distinct cell types involved. However, significant barriers to using single cell transcriptomics for
identifying translatable pain mechanisms exist. To date, there are no rat spinal cord single cell RNA sequencing studies despite a lot of pain studies being conducted on rats. There also needs to be more sex-specific comparisons as well as gene target specific comparisons.

**Sexually conserved and divergent molecular mechanisms of spinal pain processing**

Clinical and behavioral studies highlight that women report greater pain sensitivity, lower tolerance to pain and higher prevalence of pain disorders such as headaches, migraines, low-back pain, neck pain and orofacial pain. Women are also more sensitive to experimentally induced pain stimuli (Bartley and Fillingim, 2013; Mogil, 2020; Templeton, 2020). Multiple lines of evidence in rodents, non-human primates and humans suggest that primary afferents express sexually dimorphic protein/RNA expression that can affect cell signaling pathways and synaptic plasticity (Papka and Storey-Workley, 2002; Chaban and Micevych, 2005; Zhong et al., 2010; Tran et al., 2020; Tavares-Ferreira et al., 2022). Other evidence suggests the type of immune cells activated by painful stimuli and the chemical cascades that follow painful stimuli affect dorsal horn nociception through sex specific pathways. For instance, peripheral nerve injury in male rats causes proliferation of microglia to the dorsal horn and leads to pain hypersensitivity (Eriksson et al., 1993; Echeverry et al., 2008). In females, peripheral nerve injury induces the proliferation of T lymphocytes instead (Sorge et al., 2015). High mobility group box 1 (HMGB1) protein is an inflammatory molecule that induces pain when released endogenously or injected experimentally into the spinal cord. In male
mice, HMGB1-induced pain causes a larger increase in cytokine and chemokine production than in female mice. However, microglial activation occurs in both male and female HMGB1 pain-induced mice (Agalave et al., 2021). Therefore, some parts of nociception are conserved between male and female and others diverge.

Most of the above-described studies on dorsal horn NMDAR subunit expression have used only male rodents without investigating whether the same mechanisms are present in females. Only a few studies used female rodents, and even fewer studies looked at both (de Geus et al., 2020). Based on these limited sex-inclusive rodent studies, GluN1 and GluN2B protein/RNA expression is very high in both male and female SDH (Tolle et al., 1993; Yung, 1998; Shibata et al., 1999; Stegenga and Kalb, 2001; Nagy et al., 2004; Shiokawa et al., 2010; Temi et al., 2021). Meanwhile, GluN2D may be more highly expressed in males (Temi et al., 2021). A lack of sex-specific NMDAR expression studies on the human dorsal horn presents a translational divide and impedes the development of efficacious therapies. Still, some translatable pain mechanisms have begun to be unraveled. In one study, experimentally induced mechanical allodynia caused phosphorylation and increased activity of GluN2B NMDARs in the dorsal horn of rat and human males but not females (Dedek et al., 2022).

**Aims and techniques**

In the present study, we aimed to explore NMDAR subunit gene expression across species, sex, region, and neuronal subtypes. We used mouse and human single cell/nucleus RNA sequencing data to analyze NMDAR subunit gene expression across different neuronal subtypes. Using single cell/nucleus RNA sequencing data gives us the
potential to pool gene expression across multiple different cell types, which is an added value approach to previous *in situ* hybridization, bulk RNA sequencing and qRT-PCR studies on *GRIN* gene expression. Additional metadata from the original single cell/nucleus RNA sequencing studies was used to make comparisons across region. The single cell RNA sequencing data could not be separated by sex, so we performed qRT-PCR on the male and female rat SDH, DDH, and VH and human DH and VH. We used a rat model instead of a mouse model for the qRT-PCR analysis because the rat model is frequently used in pain research. Using the rat model also allows investigation into conserved or diverged trends of *GRIN* gene expression in the spinal cord across three species: mice, rats, and humans.
Methods

Source of mouse and human single cell RNA sequencing data

Fully clustered *Mus musculus* (mouse) and human single cell RNA sequencing data were shared with us by Dr. Ariel Levine and Dr. Kaya Matson from the National Institute of Neurological Disorders and Stroke in Maryland, United States.
Mouse single cell RNA sequencing data information and analysis

Table 1. The region assignments are based on the estimated laminar location of the cells in each cluster. The laminar estimates were adapted from Russ et al. (2021). The lamina in parentheses indicate a lower number of cells from that lamina in the cluster. MN stands for Motoneuron, PGC stands for preganglionic cells, and Excit and Inhib containing clusters are excitatory and inhibitory neurons respectively (table bellow).

<table>
<thead>
<tr>
<th>Cluster</th>
<th>Lamina</th>
<th>SDH</th>
<th>DDH</th>
<th>VH</th>
</tr>
</thead>
<tbody>
<tr>
<td>MN-alpha</td>
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<tr>
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</tr>
<tr>
<td>PGC</td>
<td>7-IML</td>
<td></td>
<td>●</td>
<td></td>
</tr>
<tr>
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<td>●</td>
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<td>1/2o/2i</td>
<td>●</td>
<td>●</td>
<td></td>
</tr>
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<td>1/2o/2i</td>
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<td>●</td>
<td></td>
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<td>2i/3</td>
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<td></td>
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<td>1/2</td>
<td></td>
<td>●</td>
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<td>Excit-9</td>
<td>1/2/3</td>
<td>●</td>
<td>●</td>
<td></td>
</tr>
<tr>
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<td>1/2</td>
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<td>2i/3</td>
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<td></td>
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<tr>
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<td>1/2o</td>
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</tr>
<tr>
<td>Inhib-1</td>
<td>3 (1/2o/2i/4)</td>
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<td>●</td>
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<table>
<thead>
<tr>
<th>Cluster</th>
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<th>DDH</th>
<th>VH</th>
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<tr>
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<td>●</td>
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<td>Inhib-12</td>
<td>1/2o/4</td>
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<td>●</td>
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<tr>
<td>Inhib-13</td>
<td>1/2o/2i</td>
<td>•</td>
<td>●</td>
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</tr>
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<td>4</td>
<td>•</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Excit-21</td>
<td>4/lat 5</td>
<td></td>
<td>●</td>
<td></td>
</tr>
<tr>
<td>Excit-22</td>
<td>4/5/6</td>
<td>●</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Excit-23</td>
<td>4/med 5</td>
<td>●</td>
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<tr>
<td>Excit-24</td>
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<td>●</td>
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</tr>
<tr>
<td>Excit-25</td>
<td>4/5/6</td>
<td>●</td>
<td></td>
<td></td>
</tr>
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<td>4</td>
<td>•</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Inhib-16</td>
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</tr>
<tr>
<td>Inhib-18</td>
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<td>•</td>
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<td></td>
</tr>
<tr>
<td>Inhib-19</td>
<td>med 5</td>
<td>•</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Inhib-20</td>
<td>5/6</td>
<td>•</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Inhib-21</td>
<td>4/med 5</td>
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</tr>
<tr>
<td>Excit-31</td>
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<td>Excit-32</td>
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<td>Excit-36</td>
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<td>Excit-37</td>
<td>7</td>
<td>•</td>
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<td>Inhib-22</td>
<td>7</td>
<td>•</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Inhib-23</td>
<td>7/8</td>
<td>•</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Inhib-24</td>
<td>7</td>
<td>•</td>
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<td></td>
</tr>
<tr>
<td>Inhib-25</td>
<td>7/8</td>
<td>•</td>
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<td></td>
</tr>
<tr>
<td>Inhib-26</td>
<td>ventral 7</td>
<td>•</td>
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</tr>
<tr>
<td>Inhib-27</td>
<td>7</td>
<td>•</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
The mouse single cell RNA sequencing data originated from a meta-analysis of six individual studies that performed single cell/nuclei RNA sequencing on mouse lumbar spinal cord tissue. Of the six studies Hayashi et al. (2018), Baek et al. (2019), Häring et al. (2018) and Zeisel et al. (2018) performed single cell RNA sequencing on male and female mice at postnatal day 0 (P0), P4, P21 – P28 and P12 – P30 respectively. Rosenberg et al. (2018) and Sathyamurthy et al. (2018) performed single nucleus RNA sequencing on P2 – P11 mice (sex unspecified) and P56 – P84 male and female mice respectively. Using the laminar location estimate adapted from Russ et al. (2021), we subset the clusters into SDH or DDH using the following criteria. SDH category included the clusters from laminae I, II, and/or IIo. The DDH category included clusters from laminae II, III, and/or III – VI. The clusters and their assignments are included in Table 1. The laminar locations in the parentheses indicate that there is a smaller percentage of cells from this lamina in the cluster and, therefore, did not affect the assignment criteria.

**Human single cell RNA sequencing data information and analysis**

The human spinal cord single cell RNA sequencing data originated from a study by Yadav et al. (2023) that performed single nucleus RNA sequencing on highly viable human donor lumbar spinal cord tissue from four male and three female donors aged at approximately 20 – 80 years. The data set was subset to the dorsal horn neuronal clusters and categorized under excitatory or inhibitory neurons.
Data analysis for single cell RNA sequencing datasets

Seurat is a R library that is specifically built to handle different kinds of single cell genomics data. It is continually updated and developed and contains most of the important pipelines for quality control, clustering, and data integration. Seurat was used to access and subset the data to the genes and cells of interest. A modular cell grouping pipeline was developed by Newton Martin, Justin Bellavance and Simon VanDerLoo to easily assign and group clusters across new or already encoded categories from the metadata. The pipeline algorithmically handles data structure and calculation challenges that occur with overlapping groups – where one cell type may belong to more than one group. The pipeline was uploaded to GitHub and titled (GScpter [gee-sep-ter]: https://github.com/NewtontheNeuron/GScpter).

Average expression and percent expressed were the two main metrics used to interpret the single cell RNA sequencing data. In this study, average expression was calculated using the average counts per million (CPM) by dividing each count by the total number of counts, multiplying by one million, and then taking the average CPM across all the cells in a group. Percent expressed in GScpter was calculated for each gene by dividing the number of cells that have greater than 0 RNA counts by the total number of cells in the group, and then multiplying by 100.

Neurons were grouped into the original cluster groupings from Russ et al. (2021) and Yadav et al. (2023) and were subsequently grouped further across the following smaller categories: excitatory or inhibitory, SDH or DDH, and DH overall. Average expression and percent expressed of $GRIN1$, $GRIN2A$, $GRIN2B$, $GRIN2C$, $GRIN2D$, $GRIN2G$, $GRIN2H$, $GRIN2L$, and $GRIN2M$. 
GRIN3A, and GRIN3B were displayed using a dot plot for the grouping using the original clusters and the grouping using the smaller categories. Percent expressed was represented by the size of each dot. Average expression was represented by the color of the dot. To map the average expression on a color scale, the log\textsubscript{10} of one plus the average CPM was placed on a z-score as previously done in Yadav et al. (2023).

**Animals used in the RT-qPCR experiments**

Male (n = 8) and female (n = 8) Sprague Dawley *Rattus norvegicus* rats aged 3 – 4 months were ordered from Charles River Laboratories. Same sex pairs were housed on a 12 h day/night cycle with *ad libitum* food and water and cared for following the guidelines of the Canadian Council for Animal Care and Carleton University. The rats were sacrificed after reaching adulthood for spinal cord extraction and dissection.

**Protocol for spinal cord dissection**

Rats were anesthetized using an intraperitoneal injection of 97% urethane (Acros Organics, Janssen Pharmaceutica, Geel, Belgium). Shortly after deep anesthetization, the spinal cord was removed by vertebral corpectomy and placed under an oxygenated sucrose protective solution containing 50 mM sucrose, 92 mM NaCl, 15 mM D-glucose, 26 mM NaHCO\textsubscript{3}, 5 mM KCl, 1.25 mM NaH\textsubscript{2}PO\textsubscript{4}, 0.5 mM CaCl\textsubscript{2}, 7 mM MgSO\textsubscript{4}, 1 mM kynurenic acid, and bubbled with 5% CO\textsubscript{2}/95% O\textsubscript{2}. The dura matter, dorsal roots and ventral roots were removed, and the lumbar spinal cord was isolated. The lumbar section was spray frozen and divided into the superficial dorsal horn (SDH), deep dorsal horn (DDH) and ventral horn (VH). The SDH were contain the most dorsal 1/3 of the dorsal horn. The DDH will contain the most ventral 2/3’s of the dorsal horn, and the VH
will contain the ventral half of the spinal cord. The tissue was stored separately in dry ice before long term storage in a -80°C freezer.

**Human spinal cord isolation and preparation**

Spinal cord tissue was collected from adult male and female neurological determination of death organ donors that were identified by the Trillium Gift of Life Network. Each donor candidate was screened for communicable diseases such as HIV/AIDS or syphilis and conditions that could affect tissue quality such as morbid obesity. We were single blinded to the sex of each donor until after the experiment was performed. Ethical approval for the collection of and experimentation on human spinal cord tissue was obtained from the Ottawa Health Science Network Research Ethics Board. Before surgical removal of transplant organs, the organ donor was induced to hypothermia and perfused with a high magnesium protective solution (histidine-tryptophane-ketoglutarate, Celsior or Belzer UW). After the removal of the transplant organs, the spinal cord was removed by vertebral corpectomy, within 3 hours of aortic cross clamp. The thoracic and lumbar spinal regions were removed and stored separately in an ice-cold oxygenated saline. The thoracic spinal region was flash frozen in liquid nitrogen. Then each thoracic section was divided into the dorsal horn (DH) and ventral horn (VH). Each spinal region was stored in a -80°C freezer for use in RT-qPCR experiments.

**Protocol for RNA extraction**

Total RNA extraction was carried out using the QIAGEN RNeasy® Plus Mini Kit Markham, Ontario, Canada. All buffers used were provided in the kit. Each tissue sample
was mechanically ground in liquid nitrogen and homogenized with 600 µL of RLT buffer. The resulting solution was transferred to QIA shredder spin columns and spun at 14,000 rpm for 3 mins at 20°C. The lysate was transferred to a gDNA Eliminator spin column and spun at 12,000 rpm for 30 sec at 20°C. 600 µL of 70% ethanol was added to precipitate the lysate. 700 µL of the solution was transferred to a spin column and centrifuged at 12,000 rpm for 15 sec at 20°C. The flow through was discarded and 700 µL of RW1 buffer was added to the filtrate membrane. The resulting mixture was centrifuged at 12,000 rpm for 15 sec at 20°C. The filtrate was mixed with 700 µL of RPE buffer and the centrifuged at 12,000 rpm for 15 sec at 20°C. 700 µL RPE buffer was added to the filtered membrane and the mixture was spun at 12,000 rpm for 2 min at 20°C. The spin column was placed in a new collection tube and then centrifuged at 14,000 rpm for 1 min at 20°C. The spin column was transported to a new tube. 50 µL of RNAse free water was added to the spin column and left to sit for 2 min to ensure the membrane is completely wet. The mixture was centrifuged at 12,000 rpm for 1 min at 20°C. 1 – 2 µL of each sample was placed on the Thermo Fisher Scientific NanoDrop 1000 Spectrophotometer to record final RNA concentrations. The extracted total RNA was stored at -80°C for subsequent use.

**Protocol for cDNA synthesis**

We followed the Bio-Rad iScript™ cDNA Synthesis Kit protocol. To produce the cDNA needed for each experiment, we used equal amounts of 50 ng/µL RNA from each rat and human tissue sample combined with manufacturer specified ratios of Bio-Rad 5x iScript™ Reaction Mix, Bio-Rad iScript™ reverse transcriptase, and nuclease free water.
A negative control mixture was created by adding more nuclease free water in lieu of sample RNA. The resulting mixture was placed in the Bio-Rad Thermal Cycler™ and ran through the following cycles: 5 min of priming at 25°C, 20 min of reverse transcription at 46°C, and a 1 min reverse transcriptase inactivation phase at 95°C. The final product containing cDNA was stored at -20°C for later qPCR.

**Protocol for qPCR**

**Table 2. Unique assay IDs for all the primers used.** All primers for rat and human TUBA4A, GRIN1, GRIN2A, GRIN2B, GRIN2C, GRIN2D, GRIN3A, and GRIN3B were ordered from Bio-Rad. The only exception was for human GRIN3B. A second human GRIN3B primer assay was ordered from Integrated DNA Technologies (IDT) because the Bio-Rad primer assay had very low detection.

<table>
<thead>
<tr>
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<th>Human ID</th>
</tr>
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<td>Bio-Rad: qHsaCED0003359, IDT: Hs.PT.58.2896469</td>
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</table>

The cDNA collected from the 50 ng/μL RNA samples were mixed with duplicate assays containing SsoAdvanced™ Universal SYBR® Green Supermix, Nuclease free water, and GRIN gene primers according to Bio-Rad specifications. Primers for the housekeeping gene Tuba4a in *R. norvegicus* and TUBA4A in *H. sapiens* were used to normalize target genes for the rat and human assays respectively (Table 2). Pre-validated primers for rat Grin1, Grin2a, Grin2b, Grin2c, Grin2d, Grin3a, and Grin3b and
human *GRIN1, GRIN2A, GRIN2B, GRIN2C, GRIN2D, GRIN3A*, and *GRIN3B* were ordered from Bio-Rad (Table 2). The Bio-rad human *GRIN3B* primer showed very low template detection, which was confirmed by using another pre-validated primer assay from Integrated DNA Technologies (IDT) (Table 2). To rule out the possibility of sample contamination by human genomic DNA we treated the RNA samples with genomic DNase before proceeding. We also performed melting curve analyses for both GRIN3B primers and found that *GRIN3B* had very low detection, but we used IDT’s primer since it was more reliable than Bio-Rad’s.

With several runs, each rat and human assay was placed in the Bio-Rad CSFX Connect™ Real-Time System qPCR machine coupled with the Bio-Rad CFX Maestro software version 2.2. The following thermal cycles were used: polymerase denaturation and DNA denaturation for 30 sec at 95°C, denaturation for 15 sec at 95°C, annealing/extension, and plate readout for 40 repetitions of 15 sec at 60°C, and melt curve analysis for 5 sec per step at a 0.5°C increment from 65 – 90°C. After each run, quality control analysis was performed and then the cycle threshold (*C*<sub>T</sub>) value at 10<sup>2</sup> of log<sub>2</sub> scaled RFU was obtained.

**qRT-PCR statistical and data analyses**

We used the Livak 2<sup>-ΔΔCT</sup> method for the rat and human data analysis with *TUBA4A* as the reference gene and the geometric mean of all normalized Δ*C*<sub>T</sub> as a calibrate. First, the duplicate *C*<sub>T</sub> values for each assay were averaged. To normalize the *C*<sub>T</sub> values and obtain the Δ*C*<sub>T</sub> values, the average *C*<sub>T</sub> value for each assay was subtracted by the associated average *C*<sub>T</sub> value for *TUBA4A*. Next, the ΔΔ*C*<sub>T</sub> values for
each assay were obtained by subtracting each $\Delta C_T$ value by the geometric mean of all $\Delta C_T$ values. Then 2 was raised to the $-\Delta \Delta C_T$, resulting in the $2^{-\Delta \Delta CT}$ values. For the rat qRT-PCR data, the $\Delta \Delta C_T$ values were statistically tested using a two-way ANOVA on a linear mixed effects model and Tukey HSD for post hoc comparisons. The human qRT-PCR data was not statistically analysed due to it having an insufficient sample size.

For the rat qRT-PCR data, the SDH to DDH ratio for all GRIN genes and the SDH to VH ratio for Grin3a and Grin3b were also calculated for each observation. The region-to-region ratios were calculated as a ratio of the $2^{\Delta \Delta CT}$ values. The ratios were first analyzed using a two-way ANOVA on a linear mixed effects model to identify main effects or interactions between sex and target. Then, the ratios were analyzed using a one-sample two-sided t-test to test the difference between the sample distribution and a theoretical distribution with a mean of one. The significance value was set to 0.05 and the p-values were corrected with Bonferroni corrections.
Results

GScpter: a modular low-code pipeline to analyze target-specific single cell RNA sequencing gene expression across user-defined cluster groups

In single cell RNA sequencing data thousands of cells are clustered according to their similar patterns of gene expression. Many derivative techniques of single cell RNA sequencing and analysis pipelines or algorithms have been developed to address specific research needs (Stuart and Satija, 2019; Zhou et al., 2021; Jovic et al., 2022). To meet pipeline and algorithmic needs, researchers have leveraged the flexibility of computer language programming. Since other research projects could potentially benefit from these tools they are often publicly and freely available on git repository hosting databases such as GitHub.

The current investigation required a tool that could generate publication-ready dot plots with the average- and percent-expression of GRIN genes across different levels of overlapping groups of cell clusters. Hence, we developed a modular pipeline titled GScpter and pronounced gee-sep-ter with the potential to generate the required dot plots, manage cluster grouping, and facilitate further data science or data visualization, which could then be applied to any gene set of interest (Figure 2). It was designed to be low code with all the algorithmic intense attributes occurring in the background. Development on a user interface has begun and may be completed at a later stage.
Figure 2. GScpter: Modular low-code analysis pipeline that subsets and groups single cell RNA sequencing data clusters to investigate the gene expression of target genes of interest. GScpter is pronounce as gee-sep-ter. A) A user-generated configuration (config) file contains information on how to group the clusters, the name of the user-defined groups, and the names of the genes of interest. B) A fully clustered Seurat R object containing the single cell RNA sequencing dataset containing the count matrix and metadata. C) Information from the config file and the metadata are used to subset the count matrix to the cells and genes of interest. The subset count matrix is combined with the metadata as well as user-defined groups from the config file to create a tidy R data table called the “cell roster”. H) A sample of a cell roster is shown in the table. Each row contains the gene expression of one gene in one cell as well as any associated metadata or user-defined categories. D and E) The counts of the cells in the cell roster can be grouped by gene and cluster to yield a regular or full dot plot or several pooled dot plots where the average expression and percent expressed are calculated for different configurable grouping levels. F) The cell roster can be used to perform other exploratory data science or generate other types of graphs such as heat maps or violin plots. E and H) The overlapping groups expander algorithm allows each cluster to be in multiple user-defined groups as demonstrated in the top two rows of H without breaking the tidy format of the cell roster. G) For the dot plots generated in D and E, average expression and percent expressed is calculated and scaled separately for each “pool by” level. The graphing and output functions are provided by GScpter to be as polished as shown by the figures displayed in this study, with the potential for user modifications.
The main inputs for the GScpter pipeline include a configuration (config) file and a fully clustered single cell RNA sequencing data Seurat R object (Figure 2A and 2B). The information stored in the config file includes the genes of interest, group names, and the clusters placed in each group. The information extracted from the Seurat object includes the count matrix, which contains the count data for each gene in every cell, and the metadata, which stores additional information about each cell. In step 1, the config file information along with the metadata are used to subset the count matrix. The resulting count matrix, the additional metadata, and any user generated groups are combined and transposed into the tidy data science friendly R data table. Each row of the data table contains the RNA counts of a single gene in a single cell and all the metadata associated with that cell (Figure 2H).

The R data table is called the cell roster (Figure 2C). Using the cell roster, the user can branch into three different directions to: generate the full dot plot (Figure 2D), generate multiple levels of pooled dot plots (Figure 2E), or perform further data science (Figure 2F). The user can generate full (regular) dot plots that show the average expression and percent expressed of each gene of interest across each individual cell cluster. The pooled dot plot shows the gene expression at different levels of cell grouping over the metadata available. For instance, the user can group by gene to obtain the GRIN gene expression of all cells combined, or group by both gene and region to obtain the combined gene expression of cells in the SDH and cells in the DDH separately. Some cells are categorized in both the SDH and DDH (Table 1). When groups overlap in the cell roster, they are nested as a list containing each category. For
example, the first row in Figure 2H is categorized as being part of both the SDH and DDH. Using a nested data structure ensures that the data follows the R Tidyverse tidy format but can be expanded if needed to group across the overlapping variable(s) as shown in Figure 2E. To perform further data science or generate other visualizations such as heatmaps or violin plots, the cell roster is formatted using the R Tidyverse tidy format and can seamlessly integrate into common R data science pipelines. When the user requires grouping across overlapping groups, the functions for working with the nested data structure in Figure 2H are provided within GScepter.

After the pooling parameters have been designated, the average expression, percent expressed, and the scaled average expression are calculated (Figure 2G). The user can switch between using a z-score scale, a logarithm base 10 scale ($\log_{10}x$), a counts per million (CPM) scale, a natural logarithm of one plus the value scale ($\ln(x + 1)$), or a z-score on a logarithm base 10 of one plus the CPM value ($\log_{10}(\text{CPM} + 1)$), which was used in this investigation. The resulting average expression and percent expressed values are plotted using modular dot plot functions that use ggplot2, a commonly used grammar of graphics R library, with formatted output plots as shown in this investigation. However, the user can modify the graphic parameters as well by using ggplot2 functions.

**GScepter: Use cases outside of spinal cord and neuronal GRIN gene expression**

As proof of the utility of the GScepter pipeline, I have used the above analyses and plotting approaches for a variety of gene targets other than the GRIN genes. For
example, GSctper was used to generate full and pooled dot plots for the expression of CNR1 and CNR2 in the mouse and human spinal cord dorsal horn. CNR1 and CNR2 genes code for the cannabinoid receptors CB1 and CB2 and are being included in an upcoming publication (Parnell et al., 2021). GSctper was used for a preliminary investigation of serotonin (5-HT) receptor subtypes in the mouse and human spinal cord dorsal horn. The results for the 5-HT receptor subtypes highlighted important molecular targets that guided ongoing genetic and protein experiments. GSctper was used to compare single cell RNA sequencing data with existing genomic, proteomic, and functional data. GSctper was used to investigate other ionotropic glutamate receptor genes such as the kainite receptor and the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor as well as genes related to the key molecular players in the pain disinhibition pathway such as brain-derived neurotrophic factor (BDNF) and potassium chloride cotransporter (KCC2). It was also used to look at genes of interest for research collaborators outside of our lab such as Becn1, Cacna1h – i, and Phox2a – b. In addition to the spinal cord data, the pipeline was tested on a publicly available brain single cell RNA sequencing dataset from the Allen Brain Institute.

**Investigation of GRIN gene expression across neuronal subtype**

We used two single cell/nucleus RNA sequencing datasets to examine GRIN gene expression across neuronal subpopulations of the dorsal horn. The dataset from Russ et al. (2021) is a meta-analysis that integrated 6 different single cell/nucleus RNA sequencing mouse spinal cord datasets. The dataset from Yadav et al. (2023) contains single nucleus RNA sequencing performed on 7 highly viable organ donor spinal cord
tissue. We used GScepter to filter both datasets to the genes \textit{GRIN1}, \textit{GRIN2A}, \textit{GRIN2B}, \textit{GRIN2C}, \textit{GRIN2D}, \textit{GRIN3A}, and \textit{GRIN3B} and the dorsal horn neuronal cells shown in Figure 3 and 4.
Figure 3. Single cell/nucleus RNA sequencing data showing GRIN gene expression across dorsal horn neuronal subpopulations in the mouse and human spinal cord. The average expression and the percent expressed of GRIN genes is displayed for dorsal horn neuronal subpopulations (A and B) and dorsal horn neurons overall (C and D). A and C) The mouse data is from Russ et al. (2021). B and D) The human data is from Yadav et al. (2023). The average
expression is represented in the color scale spanning a z-score of $\log_{10}(\text{CPM} + 1)$ counts, where CPM is counts per million. Percent expressed is represented by the size of each dot. A and B) The neuronal subtypes marked with an "E" are excitatory neuronal subtypes and those marked with an "I" are inhibitory subtypes. The number associated with each subtype corresponds to the same numbered clusters in Russ et al. (2021) and Yadav et al. (2023) (ex. ED3 = Ex-Dorsal-3 or E4 = Excit-4). The colored blocks underneath the neuronal clusters in (A) indicate whether it was categorized to consist of cells from the SDH, DDH, or both based on information from Russ et al. (2021) (see Table 1). The mouse and human icons were made with BioRender.
Figure 4. Single cell/nucleus RNA sequencing pooled expression of mouse and human excitatory and inhibitory neurons. A) Mouse GRIN gene expression in excitatory and inhibitory dorsal horn neurons. B) Human GRIN gene expression in excitatory and inhibitory dorsal horn neurons. C) Mouse GRIN gene expression in SDH excitatory and SDH inhibitory neurons. The average expression is represented as the color scale spanning a z-score of log_{10}(CPM + 1) counts, where CPM is counts per million. Percent expressed is represented by the size of each dot. The mouse and human icons were made with BioRender.
Single cell RNA sequencing reveals highest *GRIN2B*, variable *GRIN2A*, robust *GRIN3A*, and moderate *GRIN2D* expression in dorsal horn neurons, which is conserved between mice and humans.

From the average expression and percent expressed we identified conserved *GRIN* gene expression trends across mouse and human neuronal subtypes of the dorsal horn (Figure 3). The average expression and percent expressed of *GRIN1* is consistent across excitatory and inhibitory subtypes and in both species (Figure 3A and 3B). Interestingly, *GRIN2B* average expression was higher than the constitutive *GRIN1* subunit, with consistent *GRIN2B* expression across cell types in both species (Figure 3A and 3B).

*GRIN2A* expression was also robust. However, unlike *GRIN1* and *GRIN2B*, the percent expressed of *GRIN2A* varied widely across mouse and human dorsal horn neuronal subpopulations (Figure 3A and 3B). For example, mouse neuronal subtypes E6 and E8 are both excitatory but have largely different percent expression of *Grin2a* (Figure 3A). The similar is true for mouse inhibitory subtypes I1 and I10 (Figure 3A). Like the mouse data, the human dorsal horn neuronal subtype percent expressed and average expression of *GRIN2A* is much higher in ED1 than ED6 (Figure 3B). However, the human dorsal horn inhibitory neuronal subtypes have less qualitative variation than inhibitory neuronal subtypes from the mouse data (Figure 3).

*GRIN3A* expression is high across all neuronal subtypes in both the mouse and human dorsal horn (Figure 3A and 3B). The *GRIN3A* average expression and percent expressed vary in the human dorsal horn neuronal subtypes (Figure 3B). For example,
ED1 and ED10 have higher values than ED2 and ED3. ID1 and ID8 have much higher GRIN3A expression than ID5 and ID9 (Figure 3B). GRIN3A expression in the human dorsal horn has a much higher variation of average expression and percent expressed than mouse dorsal horn neurons. In the mouse dorsal horn neurons, Grin3a expression varies much less in the excitatory neuronal subtypes (E1 – E35, Figure 3A) than in the inhibitory neuronal subtypes (I1 – I20, Figure 3A). GRIN2D expression is modest in both the mouse and human dorsal horn, with a much higher percent expressed in human neuronal populations (Figure 3A and 3B). Consistent with previous literature, GRIN2C and GRIN3B have very low overall percent expressed and average expression (Figure 3A and 3B) (Tolle et al., 1993; Watanabe et al., 1994; Yung, 1998; Shibata et al., 1999; Stegenga and Kalb, 2001; Wee et al., 2008; Das Gupta et al., 2021). GRIN2C has moderate average expression and percent expressed in a few inhibitory mouse and human neuronal clusters (Figure 3A and 3B). The clusters with moderate expression of GRIN2C in the human dorsal horn have higher percent expressed than those from the mouse dorsal horn (Figure 3A and 3B).

From the pooled GRIN gene expression across all dorsal horn neurons combined, the order of GRIN gene expression in both mouse and human dorsal horn neurons from most highly expressed to least expressed/undetected is GRIN2B > GRIN2A > GRIN1 > GRIN3A > GRIN2D >> GRIN2C > GRIN3B (Figure 3C and 3D). The average expression is nearly identical between the two species. The percent expressed of each GRIN gene is mostly higher in the human compared to the mouse dorsal horn neurons. The percent expressed of GRIN3A is equivalent in mouse and human dorsal
horn neurons. The percent expressed of *GRIN2D* is almost as high as *GRIN3A* for humans only.

**Mouse SDH excitatory neurons have higher *GRIN3A* expression than inhibitory neurons**

The order of *GRIN* gene expression remains the same after pooling across all dorsal horn neurons (Figure 3C and 3D), excitatory vs. inhibitory neurons in the mouse or human dorsal horn (Figure 4A and 4B), or mouse SDH excitatory and inhibitory neurons (Figure 4C). *GRIN3A* average expression and percent expressed in mice is higher in mouse excitatory dorsal horn neurons but not in human dorsal horn excitatory neurons (Figure 4A and 4B). The greater expression of *Grin3a* in excitatory neurons than inhibitory neurons is more pronounced in the mouse SDH (Figure 4C). Notably, the *Grin2a* average expression and percent expressed is more pronounced in the DH than the SDH alone for both excitatory and inhibitory neurons (Figure 4A and 4C). Additionally, in the SDH alone, the percent expressed of *Grin3a* in inhibitory neurons (Figure 4C) is much smaller than in DH inhibitory neurons (Figure 4A). This suggests that *GRIN3A* is preferentially expressed in mouse but not human SDH excitatory neurons.

**Human *GRIN2C* and *GRIN2D* expression higher in inhibitory dorsal horn neurons but not as high in mice**

*Grin2d* has a higher percent expressed in mouse SDH inhibitory neurons than SDH excitatory neurons (Figure 4C). Although the difference is small, it supports
functional evidence of prominent contribution of GluN2D to mouse inhibitory neuronal NMDARs (Shiokawa et al., 2010). Human inhibitory dorsal horn neurons also have a larger percent expressed of GRIN2D than human excitatory dorsal horn neurons (Figure 4A – C), but the effect is much greater than in humans. The average expression of GRIN2D in human inhibitory dorsal horn neurons is also higher than excitatory dorsal horn neurons (Figure 4B). GRIN2C may also have a higher percent expression in inhibitory than in excitatory interneurons of the human dorsal horn (Figure 3B and 4B). The difference is less pronounced with the mouse single cell RNA sequencing data (Figure 3A and 4A).
Investigation of GRIN gene expression across sex

A

B

C

D

GRIN1 GRIN2A GRIN2B GRIN2C GRIN2D GRIN3A GRIN3B
Figure 5. qRT-PCR data showing the relative expression ratio and the region-to-region expression ratio of GRIN1, GRIN2A, GRIN2B, GRIN2C, GRIN2D, GRIN3A, and GRIN3B across the rat and human spinal cord. A – C) Adult rat male (n = 8) and female (n = 8) relative expression ratio and region-to-region expression ratio. The rat spinal cord tissue was divided across the SDH, DDH, and VH of the lumbar region. D) Adult human male (n = 2) and female (n = 2) relative expression ratio. The human spinal cord tissue expression is divided across the DH and VH of the thoracic region. A and D) The relative expression ratios are calculated using the Livak $2^{\Delta\Delta Ct}$ method with β-tubulin subtype 4a as the reference gene and the geometric mean of all ΔCt values as the calibrate. The individual $2^{\Delta\Delta Ct}$ values are the points. A) The dark blue bars are from male rats, meanwhile the bright blue colors are from female rats. D) The darker bar is for the DH spinal cord and the lighter colored bar is for the VH. B and C) The region-to-region expression ratios were calculated by dividing the $2^{\Delta\Delta Ct}$ values of the SDH by the $2^{\Delta\Delta Ct}$ values of either the DDH (B) or the VH (C). The SDH:DDH ratio is shown for Grin1, Grin2a, Grin2b, Grin2c, Grin2d, Grin3a, and Grin3b. The SDH:VH ratio is shown for Grin3a and Grin3b. The dotted line indicates a theoretical ratio of 1 where the expression in the SDH is equivalent to the expression in the DDH/VH (B and C). The darker bars are from male rats and the lighter bars are from female rats. The individual region-to-region ratios for each sample are represented as the points (B and C). A – C) The rat data was analyzed using a two-way ANOVA on a linear mixed effects model. A) The main effect for region was significant after testing the relative expression ratios ($A, \chi^2 = 18.9, df = 2, p = 7.73 \times 10^{-5}$). There was no main effect of sex. Cross-regional Tukey HSD post hoc comparisons were performed on the relative expression ratios with a significance level of $p < 0.05$. The statistically significant post hoc comparisons are indicated by the brackets. B and C) The main effect of target was significant after testing both region-to-region ratios (B, $\chi^2 = 21.2, df = 6, p = 1.66 \times 10^{-2}$; C, $\chi^2 = 36.5, df = 1, p = 1.53 \times 10^{-9}$). A one sample two-sided t-test
compared the SDH:DDH and the SDH:VH ratios to the theoretical distribution with a mean of 1. The significance level was 0.05 with Bonferroni p-value correction. The stars above each gene in B and C indicate significance. A – C) The stars indicate the size of the p-value (* = p > 1.00 x 10^{-2}, ** = p > 1.00 x 10^{-3}, *** = p > 1.00 x 10^{-4}, **** = p > 1.00 x 10^{-5}, ***** = p > 1.00 x 10^{-8}). The statistical analysis for the relative expression ratios from the human spinal cord qRT-PCR data was not performed due to insufficient sample size. The rat and human icons were made with BioRender.
qRT-PCR in rat dorsal horn reveals high expression of Grin1 followed by Grin2a, Grin2b, Grin3a, and Grin2d which is conserved across sex

Most of the existing functional and pharmacological evidence on NMDAR subtypes involves investigations on male rats (de Geus et al., 2020; Mogil, 2020). To enable statistical comparisons across sex and region in the rat spinal cord we performed qRT-PCR on GRIN gene expression in the male (n = 8) and female (n = 8) rat lumbar spinal cord (Figure 5A). The rat lumbar spinal cord was mechanically separated into the SDH, DDH and VH to identify the relative GRIN gene expression in each region. Like the mouse and human single cell/nucleus RNA sequencing findings Grin1, Grin2a, Grin2b, and Grin3a are highly expressed in both male and female rats, with moderate Grin2d expression (Figure 5A). However, the order is slightly different, with Grin1 having the highest expression followed by Grin2a, Grin2b, Grin3a >> Grin2d, and then either Grin3b or Grin2c (Figure 5A).

The rat spinal cord data was statistically tested using a two-way ANOVA on a linear mixed effects model with the region and sex as dependent variables, the biological sample as a non-independent random variable, and the gene target as an independent blocking variable. Only the main effect for region was statistically significant (Figure 5A, \(X^2 = 18.9, \text{df} = 2, p = 7.73 \times 10^{-5}\)). The main effect for sex was not significant. Further cross-regional comparisons within each gene were done using Tukey HSD with a significance level of \(p < 0.05\). The only statistically significant difference between the SDH and DDH was for Grin3b.
SDH:DDH cross-regional expression ratio is conserved across sex and divulges prominent expression of Grin2b and Grin3a in the SDH

To semi-quantify the magnitude of the genes’ involvement in pain processing spinal circuits, we calculated the ratio of expression in the SDH to expression in the DDH for all GRIN genes (Figure 5B and 5C). The SDH:DDH ratios were first tested using a two-way ANOVA on a linear mixed effects model with target and sex as dependent variables and biological sample as a non-independent random variable. Only the main effect of target was statistically significant ($X^2 = 21.2$, $df = 6$, $p = 1.66 \times 10^{-2}$). To compare the SDH:DDH ratios to a theoretical distribution with a mean of one, the ratios were tested using a one-sample two-sided t-test with Bonferroni corrections and a significance level of $p < 0.05$. The ratio for Grin2b was statistically different and greater than one, which suggests that Grin2b is primarily expressed in the SDH compared to the DDH – an effect that is consistent with past publications (Temi et al., 2021) (Figure 5B). The ratio for Grin3a and Grin3b were statistically different from one with Grin3a being greater than one and Grin3b being less than one. This suggests that Grin3a expression is greater in the SDH than the DDH, but Grin3b is more expressed in the DDH than the SDH (Figure 5B). Greater expression of Grin3a and Grin3b in the SDH or DDH is consistent with previous protein and genetic studies (Ciabarra et al., 1995; Nilsson et al., 2007; Wee et al., 2008; Das Gupta et al., 2021). Although not statistically significant, the mean SDH:DDH expression ratio of Grin2b in male rats is slightly higher than in female rats (Figure 5B), which could suggest more pronounced expression of Grin2b in the male rat SDH over the DDH than in female rats.
SDH:VH cross-regional expression ratio is conserved across sex and highlights prominent expression of *Grin3a* in the SDH and *Grin3b* in the VH

*Grin3a* and *Grin3b* are understudied NMDAR subunit genes. Previous evidence suggests that there is widespread GluN3A/*Grin3a* expression across the spinal cord that is preferentially localized to the dorsal horn, and preferential GluN3B/*Grin3b* expression in motor neurons of the ventral horn, with low GluN3B/*Grin3b* expression in the SDH (Chatterton et al., 2002; Wee et al., 2008; Das Gupta et al., 2021). To investigate the expression of *Grin3a* and *Grin3b* in the primary pain processing spinal region vs. primary motor output spinal region, we calculated the SDH:VH ratio. A two-way ANOVA on a linear mixed effects model was used to test for main effects and interactions across the SDH:VH ratios. Target and sex were dependent variables and biological sample was the non-independent random variable. Only the main effect of target was statistically significant (Figure 5C, $X^2 = 36.5$, df = 1, $p = 1.53 \times 10^{-9}$). A one-sample two-sided t-test testing difference from a theoretical distribution with a mean of one showed that *Grin3a* expression is greater in the SDH than the VH, but *Grin3b* expression is greater in the VH than the SDH (Figure 5C).
 Preliminary human thoracic dorsal horn qRT-PCR shows high $\text{GRIN1}$ and $\text{GRIN2C}$ expression with minute differences in expression between other $\text{GRIN}$ genes.

Table 3. Mean $\text{GRIN}$ gene expression of qRT-PCR data from the male and female human thoracic spinal cord dorsal horn

<table>
<thead>
<tr>
<th>Target</th>
<th>Region</th>
<th>Mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\text{GRIN1}$</td>
<td>DH</td>
<td>0.0296</td>
</tr>
<tr>
<td>$\text{GRIN2C}$</td>
<td>DH</td>
<td>0.0218</td>
</tr>
<tr>
<td>$\text{GRIN2A}$</td>
<td>DH</td>
<td>0.0129</td>
</tr>
<tr>
<td>$\text{GRIN3A}$</td>
<td>DH</td>
<td>0.0122</td>
</tr>
<tr>
<td>$\text{GRIN2B}$</td>
<td>DH</td>
<td>0.0110</td>
</tr>
<tr>
<td>$\text{GRIN2D}$</td>
<td>DH</td>
<td>0.00997</td>
</tr>
<tr>
<td>$\text{GRIN3B}$</td>
<td>DH</td>
<td>0.00173</td>
</tr>
</tbody>
</table>

To identify species differences in $\text{GRIN}$ gene expression across the dorsal horn we also performed qRT-PCR on the male ($n = 2$) and female ($n = 2$) human donor thoracic spinal cord dorsal and ventral horn. The human tissue was separated as DH vs. VH only, due to the different shape of the DH preventing easy mechanical separation of SDH versus DDH regions. Unlike rat spinal cord qPCR results and mouse and human single cell RNA sequencing data, the order of the expression ratios for the human dorsal horn qRT-PCR data was $\text{GRIN1} > \text{GRIN2C} >> \text{GRIN2A} > \text{GRIN3A} > \text{GRIN2B} > \text{GRIN2D} >> \text{GRIN3B}$ (Figure 5D and Table 3). In contrast to rats, the differences between the expression ratios for each gene is minute (Table 3). Also, the expression ratios in the VH are larger than those in the DH whereas the opposite is true for rat spinal cord expression ratios (Figure 5A and 5D). $\text{GRIN2C}$ has mostly been found in cerebellar neurons and certain glial cells (Schwartz et al., 2012; Chipman et al., 2021). However,
we found surprisingly very high GRIN2C expression in the human dorsal horn that was comparable to GRIN1 dorsal horn expression (Table 3). This was not seen in the rodent data (Figure 3 – 5). Due to insufficient sample size the human spinal cord data could not be tested statistically and further experiments are required to test whether these apparent differences may be due to differences in spinal cord segments (lumbar versus thoracic), species, or the methods of tissue division.


Discussion

This investigation used single cell RNA sequencing data and qRT-PCR to look at GRIN gene expression as it relates to primary pain processing center in the spinal cord across species, sex, region, and neuronal subtypes. Unlike the single cell RNA sequencing data, the qRT-PCR data shows that GRIN1 expression is the largest expressed GRIN gene, which highlights key differences between the techniques. GRIN2D and GRIN2C expression is more highly expressed in inhibitory than excitatory dorsal horn neurons. The magnitude of the cell type difference of GRIN2C and GRIN2D expression is larger in humans than in rodents. GRIN3A is more highly expressed in excitatory than inhibitory neurons of the mouse SDH. This effect is not seen in the human dorsal horn. We found that GRIN2B and GRIN3A are more highly expressed in the SDH of male and female rats. This indicates that GRIN2B and GRIN3A may play an important role in SDH somatosensory processing. The high expression of GRIN2B in the SDH compared to the DDH is more pronounced in male than female rats. Our data points toward complex and multimodal expression patterns of GRIN genes that differ across technique, species, cell type, and region, but is mostly conserved across sex. The specific expression patterns of NMDAR subunit mRNA across dorsal horn neurons suggests that they may have different roles in the plasticity of pain processing circuits.

The dissimilarity of GRIN1 expression between the single cell RNA sequencing data and qRT-PCR data could be due to its widespread expression across other dorsal horn cells. Since two GluN1 subunits are a necessary part of functional NMDARs, GRIN1 expression is likely to be much larger than the other GRIN genes. A study analyzing the
translatome of the \textit{GRIN} genes in the mouse SDH found that \textit{GRIN1} expression was the most highly translated \textit{GRIN} gene (Das Gupta et al., 2021). The mouse \textit{GRIN1} translatome evidence along with the \textit{GRIN1} rat and human qRT-PCR evidence suggests that the discrepancy in \textit{GRIN1} expression could be a technical difference rather than a species (mouse vs. rat) difference. There are many factors that may contribute to the discrepancy between the single cell RNA sequencing and qRT-PCR results. First, sequencing data from single nuclei is present in both the mouse and human datasets. Single nucleus RNA sequencing data is said to contain 20 – 50% less transcript than data from single cell RNA sequencing (Bakken et al., 2018; Armand et al., 2021). Most of the cells/nuclei in the mouse dataset originated from studies that sequenced single nuclei, but it included 4 studies that sequenced single cells. The pattern of \textit{GRIN1} expression in the mouse and human dorsal horn is similar, which could suggest that there is some congruence between cellular and nuclear \textit{GRIN1} expression. qRT-PCR was performed on the whole tissue, which includes the nuclei, cytosol, dendrites, glial cells, grey matter, and white matter. Previous spatial transcriptomics and genomics studies have shown that the majority of CNS GluN1 and \textit{GRIN1} expression is in the grey matter (Yung, 1998; Atoji and Sarkar, 2019; de Geus et al., 2020; Yadav et al., 2023). Evidence from rodent hippocampal neurons demonstrates enriched expression of \textit{GRIN1} in the soma as opposed to the neuropil (dendrites and axons) (Glock et al., 2021). The enriched expression of \textit{GRIN1} in the soma could suggest that the discrepancy between the two techniques could be most attributable to non-neuronal cells and/or cytosolic expression.
The human inhibitory dorsal horn neurons express *GRIN2C* more profoundly than rodent inhibitory dorsal horn neurons express *Grin2c*. Compared to rodents, human whole dorsal horn tissue expression of *GRIN2C* is much higher than most other *GRIN* genes. *GluN2C/GRIN2C* has been identified in cortical parvalbumin+ interneurons and astrocytes as well as cerebellar oligodendrocytes and neurons (Xi et al., 2009; Burzomato et al., 2010; Schwartz et al., 2012, 2012; Paoletti et al., 2013; Ravikrishnan et al., 2018; Sjöstedt et al., 2020). One study found *GluN1/GluN2C* diheteromers and *GluN1/GluN2C/GluN3A* triheteromers with low Mg$^{2+}$ blockade in oligodendrocytes of the cerebellum (Burzomato et al., 2010). In rats, Hildebrand and colleagues (2014) used the molecule DQP-1105 and found a large component of DQP-sensitive mEPSCs with long decay time constants, which is characteristic of *GluN2D*-containing NMDARs. Since DQP-1105 can potentially block both *GluN2D* and *GluN2C*, it is possible that a small subset of rat dorsal horn inhibitory interneurons contains functional *GluN2C*-containing NMDARs. That subset of *GRIN2C/GluN2C* expressing inhibitory interneurons may be much larger in the human than the rat dorsal horn. Future studies could investigate *GluN2C* protein expression, *GRIN2C* spatial transcriptomic expression, as well as *GluN2C*’s functional contribution to inhibitory interneurons, astrocytes, and oligodendrocytes of the human spinal cord dorsal horn.

Like *GRIN2C*, *GRIN2D* is more highly expressed in the human than mouse dorsal horn inhibitory interneurons. Unlike *GRIN2C*, *GRIN2D* expression in mice is not limited to a small subset of inhibitory or excitatory neuronal subtypes, suggesting a consistent contribution to dorsal horn excitability. The whole tissue qRT-PCR findings on *GRIN2D*
did not reveal any easily identifiable qualitative differences between rats and humans. The magnitude of the GRIN expression patterns in the human thoracic spinal cord could be smaller than in rat lumbar spinal cord. Although further investigation involving the human lumbar spinal cord dorsal horn will be necessary to confirm this hypothesis. Evidence in the CNS suggests that Grin2d/GluN2D expression is preferentially expressed in inhibitory interneurons but not very much in astrocytes and oligodendrocytes (Xi et al., 2009; Shiokawa et al., 2010). Evidence from rodent hippocampal neurons demonstrates enriched expression of Grin2d in the neuropil (dendrites and axons) (Glock et al., 2021). It is possible that localized translation of GRIN2D plays an important and understudied role in synaptic plasticity especially since local translation can differ across cell type (Perez et al., 2021). Nevertheless, GluN2D studies in the brain and SDH suggest that Grin2d may be preferentially expressed in dorsal horn inhibitory interneurons (Shiokawa et al., 2010; Hanson et al., 2019). However, the data from this investigation suggests that either GRIN2D is more enriched in human inhibitory dorsal horn neurons than rat inhibitory dorsal horn neurons, or GRIN2D is more enriched in human inhibitory nuclei over the somata. Future experiments could investigate the localized translation of GRIN2D in the rodent and human dorsal horn as well as the significance of inhibitory pain processing neurons that carry slow decaying NMDAR subtypes.

GRIN3A may be more highly expressed in excitatory neurons of the rodent SDH but more work is needed to determine if this is true in humans. Previous studies show that Grin3a/GluN3A is more highly expressed in excitatory neurons of the rodent brain
and spinal cord and that GRIN3A is involved in specific excitatory neuronal developmental pathways in the spinal cord (Ciabarra et al., 1995; Chatterton et al., 2002; Guo et al., 2012). In the adult human spinal cord, the protein levels of GluN3A were low (Nilsson et al., 2007). In contrast, the single cell RNA sequencing and qRT-PCR findings suggest that GRIN3A is highly expressed in both adult human dorsal horn neurons and dorsal horn tissue overall. Curiously, Yadav et al. (2023) used genome wide association studies to identify genes that were enriched in the human or mouse single cell RNA sequencing data, and GRIN3A was found to be enriched in several mouse excitatory neuronal subtypes. Hence, GluN3A/GRIN3A may play a diminished role in adult human excitatory dorsal horn neuronal excitability, but it may still play a role in the development of adult human dorsal horn excitatory neurons. Nevertheless, the high expression of GRIN3A in this investigation merits further studies into its role in the development and excitability of rodent and human excitatory dorsal horn neurons. Since GluN3A/GRIN3A as well as functional GluN3A-containing NMDARs have been found on oligodendrocytes and astrocytes of the CNS, it is difficult to suggest whether the qRT-PCR expression of GRIN3A in this investigation is primarily due to neuronal expression (Paoletti and Neyton, 2007; Piña-Crespo et al., 2010). Future investigations should examine the GluN3A protein expression in the SDH of rodents and humans and then perform co-localization staining with known excitatory neuronal markers. Other cell type specific experiments such as laser capture qPCR (Pietersen et al., 2011; Chang et al., 2021), could help tease apart GRIN3A expression in NK1+ neurons, excitatory and inhibitory interneurons, or glial cell types in the dorsal horn of both rodents and humans.
On the other hand, in line with *GRIN2B*’s greater expression in the rat SDH over the DDH, multiple lines of evidence support predominant expression of GluN2B at adult SDH synapses (Shiokawa et al., 2010; Hildebrand et al., 2014; Temi et al., 2021; Armstrong et al., 2022). Armstrong et al. (2022) found that GluN2A, GluN2B, and GluN2D were preferentially localized to the SDH in both rats and humans. For our qRT-PCR experiments, the SDH and DDH were separated using rough anatomical and not neurochemical landmarks, which could have diluted the differences between the regions. Since our qRT-PCR experiments processed both grey and white matter, it is also possible that a minute expression of *GRIN* genes but widespread expression of the housekeeping gene in the white matter could dilute the concentration of the true expression value. The mouse and human single cell data suggest that the housekeeping gene used here is enriched in oligodendrocytes and motoneuron cells and nuclei but most of the neuronal cells and nuclei had near zero percent expressed (Russ et al., 2021; Yadav et al., 2023). That is in the cells/nuclei not dendrites and axons. *Tubb4a* is enriched in the neuropil of hippocampal cells but at a lower ratio than the majority of the other neuropil enriched genes (Glock et al., 2021). The somatic expression and neuropil expression of *Tubb4a* were statistically different but still very close (Glock et al., 2021). The spinal cord grey matter contains dendrites, axons, and glial processes in addition to cell somas. Spatial transcriptomics in the human lumbar spinal cord shows a widespread and near-even distribution of *TUBB4A* expression across the spinal cord grey and white matter with slightly different distribution across region (Yadav et al., 2023). The preferential *GRIN2* gene expression in the SDH over the DDH could be
tested with spatial transcriptomic techniques such as RNAscope™ or the existing Visium human lumbosacral spinal cord dataset from Yadav et al. (2023).

The more pronounced male \textit{GRIN2B} expression in the SDH compared to the DDH in this investigation is similar to previous observations made relating to GluN2B protein localization in rats (Armstrong et al., 2022). Sex-specific GluN2B-mediated mechanisms of neuropathic pain in adult rats and humans have been identified (Dedek et al., 2022). Furthermore, GluN2B expression is more prominent in the medial SDH of postnatal male but not female rats (Temi et al., 2021). This implies that \textit{GRIN2B}/GluN2B may play an enhanced role in the male pain processing pathway across development. In this investigation, we could not test for mediolateral differences in \textit{GRIN} gene expression in the SDH. However, identifying genes with medial, central, and lateral expression patterns in the dorsal horn could allow for mediolateral single cell RNA sequencing analysis in the future. Future investigations could scrape spatial transcriptomic data for potential marker genes. Nevertheless, emerging approaches using RNAscope™ technology show promising parallels with existing GluN2A, GluN2B, and GluN2D protein studies.

The variability or consistent expression of \textit{GRIN2A}, \textit{GRIN3A}, \textit{GRIN2B} across mouse and human dorsal horn neuronal subtypes suggests that each NMDAR subunit may play a different role in DH synaptic plasticity. Each neuronal cluster from the mouse dataset contained cells from different developmental time points. Even though, most of the cells in the entire mouse dataset originated from adult mice (Russ et al., 2021), looking at what percentage of cells in a cluster came from adult mice could help to
roughly discern the maturity of each cell cluster. That type of analysis would be valuable for future developmental studies. The consistency of GRIN2B across all neuronal subtypes implies that within neurons GRIN2B could be constitutently expressed, providing a default subunit for any newly synthesized NMDARs. The variable expression of GRIN2A and GRIN3A could be characteristic to each cell-type, mediolateral location, upstream or downstream signaling pathways, protein turnover rate, receptor trafficking, endoplasmic reticulum retention signals or spatiotemporal regulation, to name a few factors (Bard et al., 2010; Liu and Salter, 2010; MacDermott, 2014; Wee et al., 2016; Yang et al., 2018; Zhang et al., 2020; Booker et al., 2021; Temi et al., 2021; Dedek and Hildebrand, 2022; Li et al., 2022; Pitcher et al., 2023). Evidence from the rodent brain suggests that GluN2A and GluN3A have different roles in synaptic plasticity, whereby GluN2A maintains mature brain synapses while GluN3A refines immature synapses. For example, in the brain, while GluN2A is credited as a characteristic of mature synapses, experiments involving GRIN3A/GluN3A have identified roles in reducing dendritic spine density, reducing NMDAR-mediated currents, resulting in more morphologically immature synapses, and reduced LTP (Paoletti et al., 2013; Hildebrand et al., 2014; Pérez-Otaño et al., 2016; Pitcher et al., 2023). It is possible that GluN3A-containing NMDARs could be linked to mechanisms that regulate the synaptic maturity of excitatory synapses in the SDH. Another factor that could affect synaptic plasticity is the presence of GluN2A, GluN2B, GluN2D, or GluN3A in extrasynaptic or presynaptic NMDARs, which has been identified in several studies across the CNS (Momiyama, 2000; Harney et al., 2008; Pérez-Otaño et al., 2016; Dedek and Hildebrand, 2022). Future studies could
investigate potential GluN3A-mediated down- or up-stream mechanisms that attenuate the expression of mature GluN2A, GluN2B, or GluN2D containing NMDARs in the SDH as well as NMDAR subunit expression at extrasynaptic and presynaptic sites to understand their role in SDH plasticity.

GScpter is a versatile tool that enables users with limited coding knowledge to get experience with both single cell RNA sequencing data and computer language programming. It also allows users to examine and compare the gene expression of specific genes of interest. Most basic science research questions focus on a particular set of relevant genes to observe differences in expression between disease states, coarse cell types and more. On the other hand, single cell genomic research creates a database of cells, genes, and additional information, which can then be used to dive deeper into a select subset of genes that stand out the most. For example, the study for the mouse dataset here by Russ and colleagues (2021) highlighted important gene expression differences across cell types and performed further in-depth analysis to create an atlas of the mouse spinal cord. The authors compiled their atlas into SeekSeq, an online tool that gives basic information and dot plots for any gene of interest.

Single cell/nucleus RNA sequencing studies generate incredible amounts of data that are useful for a variety of research questions about a particular set of genes. Multilayered cell grouping is also useful for cross-technique comparisons as demonstrated here with qRT-PCR, and for global conclusions about coarse and small groups. The inclusion of overlapping grouping (or tree-like categories) as a feature of GScpter, facilitates investigation across groups that are not perfectly separable into one
category or another. For example, the laminar location estimates of the clusters in Russ et al. (2021) were used to categorize neuronal clusters into SDH and DDH, yet some clusters had to be categorized into both. Another example includes the ability to assign new groups to look at all SDH excitatory versus SDH inhibitory neurons. The low-code requirements of GScpter enables users without prior computational experience to subset single cell/nucleus RNA sequencing data to the relevant set of genes, cells, and meta data and then either produce dot plots at different grouping levels or perform their own project-specific data science. Future improvements to GScpter could enable users to perform differential gene expression analysis on multiple group layers and improve its integrations with Seurat data structure and functions.

In conclusion, single cell RNA sequencing data and qRT-PCR data on the rodent and human dorsal horn reveals important technique, species, cell type, and regional expression differences that could improve our understanding of spinal pain processing and spinal pain circuit plasticity. \textit{GRIN1} qRT-PCR expression is much larger than the other \textit{GRIN} genes, which could reflect its potential expression in glia or neuronal cytosol. A few human inhibitory interneurons have high \textit{GRIN2C} expression. \textit{GRIN2C} may also be expressed in astrocytes and oligodendrocytes, especially in the human over the rodent dorsal horn. Meanwhile, \textit{GRIN2D} expression is consistent across mouse and human dorsal horn subtypes with higher expression in inhibitory interneurons, but a more pronounced cell type difference in humans than in mice. In mice, \textit{Grin3a} is preferentially expressed in SDH excitatory neurons, but that effect is not present in the human dorsal horn and could reveal potential species-specific roles for GluN3A in SDH
somatosensory processing. The higher relative expression of GRIN2B in the rat SDH along with its consistently high expression across mouse and human neuronal subtypes suggests that GluN2B could be a default subunit in SDH neurons. The variable expression of GRIN2A and GRIN3A invites deeper investigations into their opposing roles in SDH synaptic plasticity and their impact on nociception and behavior. Taken together, the expression of NMDAR subunit genes across species, sex, region, and neuronal subtypes reveals interesting species differences and raises questions that highlight their potential expression in non-neuronal cells and neural processes. This could help deepen canonical understanding of the plasticity of pain processing circuits in the SDH.
References


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