Molecular determinants of dorsal horn excitability and pain processing in male and female rats and humans

by

Marrium Khan

A thesis presented to the Faculty of Graduate and Postdoctoral Affairs

in partial fulfilment of the requirements for the degree of

Master of Science

in

Neuroscience

Carleton University

Ottawa, Ontario

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Table of Contents

Abstract ........................................................................................................................................... 8

Acknowledgements .......................................................................................................................... 9

Introduction ...................................................................................................................................... 10

1 General Introduction .................................................................................................................. 10

2 Pain .............................................................................................................................................. 10

2.1 Pain Processing in the Spinal Cord ....................................................................................... 10

2.2 Plasticity in the Nociceptive Pathway ..................................................................................... 14

2.3 Pain and the Dorsal Horn ....................................................................................................... 16

2.4 Pre- and post-synaptic membranes ....................................................................................... 18

3 Spinal excitability and role of glutamate receptors .................................................................. 19

4 Molecular Determinants of Pain ............................................................................................... 20

5 Sex differences in pathological pain .......................................................................................... 27

6 Translational Studies in Human Tissues ..................................................................................... 30

Study Paradigm ............................................................................................................................. 31

Methods .......................................................................................................................................... 32

1 Animals ...................................................................................................................................... 32
2 Rat Spinal Cord Isolation ................................................................. 34

3 Human Donor Tissue Isolation .......................................................... 35

4 Protein Extraction and Quantification .................................................. 36

5 Western Blot .................................................................................. 37

6 RNA extraction and cDNA synthesis ................................................. 39

7 Real time quantitative polymerase chain reaction (RT-qPCR) ............... 41

8 Statistical Analysis ........................................................................ 43

Results ........................................................................................................ 45

Discussion .............................................................................................. 61

Conclusion ............................................................................................ 69
List of Tables

Table 1 Human donor tissue data sheet ................................................................. 35
Table 2: cDNA synthesis thermocycler protocol .................................................... 40
Table 3 RT-qPCR reaction protocol .................................................................. 41
Table 4 Typical qPCR 96-well plate template ...................................................... 42
Table 5 Primers used for RT-qPCR experiments in rats ......................................... 42
Table 6 Primers used for RT-qPCR experiments in humans ................................. 43
Table 7 A two-way ANOVA was used to make comparisons between male and female rats and the SDH, DDH and VH regions; followed up with Bonferroni post HOC test. *p<0.05 48
Table 8 A two-way ANOVA was used to make comparisons between the male and females and targets; followed up with Bonferroni post HOC test. *p<0.05 ................................. 50

List of Figures

Figure 1 Schematic representation of Melzack's and Wall's gate theory of pain (image adapted from (Bear et al., 2020)). ........................................................................ 13
Figure 2 Molecular players involved in spinal hyperexcitability .......................... 26
Figure 3 Schematic overview of experimental plan ............................................. 33
Figure 4 Coronal slices of the SDH, DDH and VH sections of the lumbar spinal cord. 34

Figure 5 Gel lane plan for STEP61. 38

Figure 6 Gene expression is conserved across sex, with significant regional changes for a subset of targets. 47

Figure 7 The SDH to DDH localization of genes is conserved across sex in rats with a significantly different preference to SDH localization between select targets. 50

Figure 8 Potential sex differences in select targets with conserved regional expression for human spinal cord. 52

Figure 9 Potential sex difference in the DH/VH ratio localization of genes in humans for select targets, with differential localization patterns between targets. 54

Figure 10 Human and rat genes are differentially expressed in the spinal cord, potentially implicating both species and sex differences in select targets. 56

Figure 11 Western blot PVDF gel image capture of B-actin and STEP61 multiplex at the 700 and 800 channel respectively on the LI-COR Odyssey system for infrared antibody conjugates. 58

Figure 12 STEP61 protein levels are conserved between male and female rat lumbar spinal cord, with decreased protein levels in the ventral horn. 59

Figure 13 The SDH:DDH ratio reveals no sex differences in preferential localization of the STEP61 protein in rats. 60
List of Abbreviations

AMPA  \( \alpha \)-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors
ANOVA  analysis of variance
ATP  adenosine triphosphate
BDNF  brain derived neurotrophic factor
CALCA  calcitonin gene-related polypeptide alpha
CaMKII  calmodulin dependent protein kinase II
CCI  chronic constriction injury
cDNA  complementary DNA
CFA  complete Freund’s adjuvant
CGRP  calcitonin gene-related peptide
CHRNA9  cholinergic receptor nicotinic alpha 9
CIP  congenital insensitivity to pain
CNS  central nervous system
CREB  cyclic adenosine monophosphate–responsive element-binding protein
\( C_t \)  cycle threshold
DAG  diacylglycerol
DDH  deep dorsal horn
DRG  dorsal root ganglion
EPSCs  excitatory postsynaptic current
GABA  \( \gamma \)-aminobutyric acid
GluN1  glutamate receptor subunit 1
GluN2B  glutamate receptor subunit 2B
GlyR  glycine receptor
IASP  International Association for the Study of Pain
IP3  inositol-1,4,5-triphosphate
KARs  kainite receptors
KCC2  potassium-chloride cotransporter 2
mRNA  messenger RNA
NaV1.8  sodium ion channel subtype 1.8
NMDAR  N-methyl-D-aspartate receptor
P2RX3  purinergic receptor 3
P2RX4  purinergic receptor 4
PKA  protein kinase A
PLC-\( \gamma \)  phospholipase c \( \gamma \)
PSD  post-synaptic density
PTKs  protein tyrosine kinases
| PV   | parvalbumin                             |
| PVDF | polyvinylidene difluoride              |
| PWT  | paw withdrawal threshold               |
| RACK1| receptor for activated C kinase 1      |
| RNA  | ribonucleic acid                       |
| RT-qPCR | quantitative reverse transcription polymerase chain reaction |
| SDH  | superficial dorsal horn                |
| SEM  | standard error of the mean             |
| siRNA| small interfering RNA                  |
| STEP61 | striatal-enriched protein tyrosine phosphatase 61 |
| TrkB | tyrosine kinase receptor B             |
| TrkB-FC | recombinant human TrkB/Fc fusion protein (BDNF scavenger) |
Abstract

Chronic pain is a debilitating and progressive healthcare challenge imparting a heavy societal burden on sixty million people worldwide. While females exhibit higher pain prevalence, most fundamental pain research has not been sex-inclusive, and thus sex-specific therapeutics remain unexplored. To address this, we characterized the gene and protein expression of molecular determinants of a canonical dorsal horn hyperexcitability and chronic pain pathway across sex by employing RT-qPCR and Western blot approaches on micro-dissected regions of rat and human spinal cord. Brain derived neurotrophic factor-tyrosine kinase B (BDNF-TrkB) signalling drives disinhibition that couples to increased excitatory N-methyl-D-aspartate receptor (NMDAR) activity only at male dorsal horn synapses. We aimed to investigate whether differences in baseline expression of upstream NMDAR regulators (such as the BDNF receptor, TrkB (Ntrk2); the potassium-chloride cotransporter 2, KCC2 (Slc12a5); FYN kinase (Fyn); and striatal-enriched protein tyrosine phosphatase, STEP61 (PTPN5)) in this pathway account for this sexually dimorphic pain processing across species. We report, for the first time, a conserved baseline gene expression across sex in all targets in 3-4-month-old rats with regional differences in the superficial dorsal horn (pain-processing; SDH) and deep dorsal horn (other somatosensory modalities; DDH) in select targets. Preliminary human data suggest potential sex difference in gene expression of PTPN5, Slc12a5, Grin2B (GluN2B) and Grin1 (GluN1). There also appears to be a species difference for Fyn, Slc12a5 and Grin1. We report a preferential localization to the SDH of Ntrk2, Fyn, Grin2B, and PLCG1 (PLCγ), albeit with no differences in sex. In contrast, inhibitory signalling targets, Slc12a5 and PTPN5 are not as elevated in the SDH. This predisposes these SDH circuits for increased excitability. This study furthers fundamental understanding of spinal mechanisms of pain processing, which may help identify future pain therapeutic targets that are efficacious across sex and species.
Acknowledgements

The passion for learning makes dreams become reality. And a nurturing environment for learning and growth takes one to heights. This degree would not have been possible without my incredibly kind-hearted, knowledgeable, and encouraging supervisor, Dr. Michael Hildebrand. From him, I learnt what a true mentor looks like. I have grown so much from his expertise and genuine advice, for which I am truly grateful.

This research would also not be possible without our wonderful lab manager, Laurence David, whose skills, and dedication to teaching, I value so much. His assistance has been instrumental in this journey. A huge thank you to Annemarie Dedek for extracting the human donor spinal cord tissues and for answering countless emails and just being so contagiously enthusiastic about research!

Thank you to Dr. Shawn Hayley and Dr. Natalina Salmaso. Their unique guidance helped shape me as a researcher through the courses I took with them and then further through being my remarkable thesis committee members. I would also like to express my gratitude to Dr. William Willmore for kindly serving as an internal examiner on my committee, and Dr. Matthew Holahan for chairing my defence. Thanks to Teresa Fortin, Natalie Prowse, and Georgina Lau for helping me troubleshoot experiments and for lending me an ear when things got rough. Thank you to my lab mates Jennifer Armstrong and Jessica Parnell for showing me the ropes of grad school and to Clare-Murray Lawson, Newton Martin, and Katie Griffiths for all the laughs! Thank you to Alex Edwards and Lindsay Hyland for making life as a lab TA enjoyable and inspiring!

A special thank you to my parents, Rashid Ali Khan and Bushra Khan, and my siblings for their affection! I love them all so dearly! Thank you to my parents-in-law, Noor-Jahan Dilawar and Dilawar Muhammad, for their prayers from miles away! A heartfelt thank you to my dear husband, Zaid Muhammad, who deserves greater credit for this research than myself. It would not have been possible without his unwavering support and love. Thank you for cheering me on and believing in me when juggling motherhood and grad school seemed unbearable! And thank you to my little Leena, whose smiles and giggles were all the encouragement a mom could ask for. Above all, I am grateful to God, for all His blessings.
Introduction

1 General Introduction

Chronic pain is a debilitating and progressive healthcare challenge impacting a person’s ability to perform day-to-day functions. Sixty million people worldwide, suffer from chronic pain, out of which 7.6 million are Canadian (Canada, 2021). With the rise in the opioid epidemic, the need for effective treatments beyond the use and abuse of these medications is becoming even more vital.

While females exhibit higher pain prevalence, research has not always been sex inclusive, and thus treatments for pain have typically not taken sex differences into consideration (Macchia, 2022; Zajacova et al., 2021). Hence, it is imperative to bridge this gap in research and delve into potential sex differences that may yield effective therapeutics.

2 Pain

2.1 Pain Processing in the Spinal Cord

The International Association for the Study of Pain (IASP) has referred to pain as an unpleasant sensory and emotional experience associated with actual or potential tissue damage or described in terms of such damage (Raja et al., 2020). In normal physiological conditions, pain exists in the acute form where it benefits an individual to be able to respond to aversive stimuli. A good example of the importance of this protective function are individuals having congenital insensitivity to pain (CIP) due to a mutation in the SNN9A gene. These individuals inadvertently experience regular self-harm through injury simply because they are unable to detect aversive stimuli when it occurs. Consequentially, a significantly lower than average life
expectancy is observed for CIP patients, highlighting the importance of acute pain (Cox et al., 2006).

Sensory stimuli are detected by nociceptors (sensory neurons) in the periphery which are tuned to respond to changes in external stimuli. Nociceptive stimuli can occur in the mechanical, chemical, or thermal modalities, and are generally associated with an injury, surgical procedure, or an illness. Normal nociceptor functioning enables acute pain to serve its protective function with the key feature being the transient nature of such signals. These stimuli elicit several functional and neurochemical modifications in primary sensory neurons within the periphery, the spinal cord and then finally the supraspinal regions of the brain. Processing within these brain regions encode the subjective sensory and emotional experience of pain, which is typically followed by descending motor control to respond appropriately to prevent continued exposure to the painful stimuli (Giesler, 2013).

While pain can be perceived in both emotional as well as physiological modalities (Woolf & Salter, 2000), the spinal cord is a region of great interest for pain because of its integrational function. One mechanism of modulation is through inhibitory neurotransmission. Noxious stimuli can have one of three fates: they may follow the spinothalamic tract to supraspinal regions of the brain; they may immediately follow the reflex arc to direct motor functions; or alternatively they may be silenced instead (Price & Prescott, 2015).

Activation of nociception that evokes pain can be reduced by simultaneous activity in low-threshold mechanoreceptors (Aβ fibers) (Bear et al., 2020; Melzack & Wall, 1965). The phenomenon of noxious stimuli silencing is explained by the gate theory of pain. The gate theory of pain suggests that projection neurons in the substantia gelatinosa (a section of the superficial dorsal horn) that project an axon up the spinothalamic tract are excited by both
myelinated sensory fibers (A\(\delta\) fibers) as well as unmyelinated nociceptive axons (C fibers). This projection neuron is also inhibited by an interneuron. At the same time, this interneuron is both excited by the A\(\alpha\) fibers or A\(\beta\) sensory fibers, and inhibited by the C pain fiber (Bear et al., 2020). Hence, by this arrangement, nociceptive fibers greatly excite projection neurons allowing nociceptive signals to project to the brain. However, innocuous sensory fibers activate the interneuron and suppress nociceptive signals, which reduce the activation they impart on the projection neurons by a negative feedback mechanism (Bear et al., 2020); see Figure 1 for an example of the original gate theory. This phenomenon can be observed in an individual who puts pressure and rubs a papercut they received to reduce the sensation of pain. The mechanical sensory fiber input suppresses the nociceptive signals by inhibiting the projection neuron’s signalling to reach the brain (Melzack & Wall, 1965). Loss of spinal inhibition (disinhibition) is thought to contribute to tactile allodynia, a state in which light, non-noxious mechanical signals can activate the nociceptive network and evoke pain (Hughes & Todd, 2020; Peirs & Seal, 2016).
Figure 1 Schematic representation of Melzack's and Wall's gate theory of pain (image adapted from (Bear et al., 2020)).

The gate theory of pain was first proposed by Ronald Melzack and Patrick Wall in 1965 but the concept is being utilized by researchers to date to understand dorsal horn plasticity. For example, Petitjean et al. found that a subset of inhibitory interneurons which contain the marker parvalbumin (PV), inhibit tactile inputs from activating nociceptive circuits. However, following nerve injury, a reduction of these synapses was seen where subsequent light touch could then elicit pain. This group called these PV-expressing inhibitory neurons as ‘gatekeepers’ of touch-evoked pain (Petitjean et al., 2015).

What happens at the level of the spinal cord integration process that drives two different outcomes, one of pain being appropriately gated by a feed-forward inhibition (Peirs & Seal, 2016) and the other where the gate is atypically left ‘opened’? What roles do molecular players of this circuitry play? And why does acute pain transform into a one that is persistent and
chronic? Hence, to answer these questions, the dorsal horn is taken as a region of interest for this study in investigating physiological and pathological mechanisms of pain.

2.2 Plasticity in the Nociceptive Pathway

By definition, chronic pain is that which persists or recurs for longer than 3 months (Nugraha et al., 2019). Although this definition is clinically useful, it does not provide insight on the mechanism, nor does it indicate when or how the transition from acute to chronic pain occurs. The authors of a recent study argue that transition most likely occurs far earlier than the 3-month period that clinical studies suggest. They propose the idea of the transition to occur at timepoints that near injury or are caused by the injury itself (Price & Ray, 2019). This is plausible, given the nature of cellular plasticity that is often driven by situations such as injury or insult and the microenvironment that is created, consequently.

Nociceptors are remarkably plastic in nature; they possess the ability to alter and increase their excitability upon sustained activation (Latremoliere & Woolf, 2009). Although sustained activation and sensitivity is beneficial post injury to allow resolution of injury, these plastic changes that result in maladaptive sensitization of receptors read pain as a constant state and give rise to a sort of “pain” memory track (Latremoliere & Woolf, 2009; Pace et al., 2018).

In nociceptive pathways, neuroplasticity can be involved at two levels: the peripheral level (peripheral sensitization) where noxious stimuli are transduced; and at the central level (central sensitization) which is involved in signal transmission, modulation, and ultimately, pain perception. The former includes changes that occur at the site of injury where primary afferents receive the stimuli. The latter encompass the plasticity of the spinal cord and the brain.
associated with sensitization processes that modify or enhance nociceptive sensations (Pace et al., 2018)

The transition to chronic pain often includes a structural reorganization in nociceptive pathways (Kuner & Flor, 2017). Numerous studies implicate increased activity of nociceptive primary afferent nerve fibers (Reichling & Levine, 2009), with molecular changes in the pre- and postsynaptic regions of the spinal cord. Synaptic contacts of primary afferents on spinal dorsal horn neurons may undergo structural remodelling which is causally associated with nociceptive hypersensitivity (Kuner & Flor, 2017). Patients of various chronic pain types, including chronic back pain (Smallwood et al., 2013), rheumatoid arthritis (Wartolowska et al., 2012) and post-amputation pain (Preissler et al., 2013) exhibit macroscopic local alterations in grey-matter volume and density in not only the brain, but more recently in the spinal cord as well (Wilcox et al., 2015). Interestingly, these grey-matter changes are reversed in some patients following analgesic interventions, suggesting a cellular and molecular basis for these macroscopic changes (Seminowicz et al., 2011).

Central sensitization and enhanced nociceptive circuit excitability represent manifestations of reduced inhibition of these nociceptive pathways in the dorsal horn of the spinal cord (Latremoliere & Woolf, 2009). While the output of central sensitization appears to be the same–hyperexcitability of neurons– at a molecular level, these can look quite different. A myriad of molecular pathways are implicated in research with roles in altering neuronal function in chronic pain. Although many of these players are a subject of varying focus among researchers, looking at sex differences within their targets has often been neglected (Mogil, 2020). It is thus very important to investigate sex-differences in what the drivers of these plastic changes may be and what players have roles in modulating the striking transition to a detrimental chronically pained state from the advantageous acute one.
2.3 Pain and the Dorsal Horn

The spinal cord is often attributed to being the relay center of nociception to and from the brain (D’Mello & Dickenson, 2008). The human spinal cord consists of 31 spinal segments which are divided into four regions: cervical (C1-C8), thoracic (T1-T12), lumbar (L1-L5), and sacral (S1-S5) with the last segment being the coccygeal cord. There are 31 spinal nerves associated with each segment that innervate a unique area of skin or ‘dermatome’ (Bear et al., 2020). Dermatomes of the different spinal segments allow narrowing down the appropriate spinal cord segment to the affected body region in methods of research and clinical diagnosis. The dermatomes of the lumbar and thoracic cord can hence be mapped to regions often impacted by inflammatory (Saal, 1995) or neuropathic (Forero et al., 2016; Straube et al., 2013) pain processes within the legs and lower body. Furthermore, in rats, the lumbar spinal nerves innervate the hind paws, which are mechanically and thermally stimulated in pain research as a measure of pain tolerance and sensitivity.

Looking at a transverse cross-section of the spinal cord, the dorsal and ventral horns can be visualized throughout the segments. In 1952, Bror Rexed proposed an organizational system of the gray matter of the spinal cord called the Rexed Laminae (Rexed, 1952). This classification was based on the distribution of cells and fiber in layers (laminae) within the gray matter (Rexed, 1952). The Rexed Laminae consist of 10 laminae. Lamina I and lamina II (marginal zone and substantia gelatinosa, respectively) are of key interest in pain processing and make up the superficial dorsal horn (SDH).

Noxious stimuli activate nociceptive dorsal root ganglion (DRG) neurons in the periphery. These sensory stimuli are transduced into electrical action potentials which are then transmitted to lamina I and lamina II of the superficial dorsal horn via myelinated A\(\delta\) fibers
or unmyelinated C fibers. Here, electrical nociceptive signals are converted to chemical signals by the secretion of neurotransmitters and neuropeptides onto second order spinal neurons (Cheng, 2010).

Lamina I consists of a thin layer of cells with small dendrites and nonmyelinated axons. It contains subtypes of projection neurons (spinothalamic and spinobulbar) (Benarroch, 2016) as well as interneurons which arborize locally (Peirs & Seal, 2016). Ninety % of lamina I neurons are interneurons (Todd, 2010). Lamina II is subdivided into lamina IIo (outer) and lamina IIIi (inner), both containing different subtypes of local GABA/glycine inhibitory or glutamatergic excitatory interneurons (Peirs & Seal, 2016). The dense network of neurons in lamina I and IIo respond to noxious stimuli, while lamina IIo also receives some inputs from non-noxious stimuli.

Although overall, neuronal populations in the SDH can either be GABAergic/glycinergic or glutamatergic, further classifications systems are also used for specific neuronal subtypes. These include the neuronal morphology (fusiform, central, vertical, islet cell, and radial), electrophysiological firing properties (tonic, gap-firing, delayed, and single spiking cell), the neuropeptides that cells release (e.g. – somatostatin), and the type of surface receptors (e.g. – NK1, substance P receptors) they possess (Todd, 2010).

The deep dorsal horn (DDH) which spans from lamina IIi to lamina VI receives inputs primarily from non-noxious stimuli-encoding mechanoreceptors (Aβ fibers) and proprioceptors. However, some noxious stimuli from Aδ fibers are also received here. Both projection neurons and inhibitory interneuron populations are found in the DDH.
The vast majority of interneurons in lamina I-III that demonstrate high glycine immunoreactivity are also labelled with GABA-specific antibodies, while there are numerous neurons that are only GABA-immunoreactive. This suggests that most of the inhibitory interneurons in lamina I-III are of GABA-secreting type while some also release glycine (Todd, 2017). Interestingly however, a study on rat DDH tissue reported that interneurons enriched with glycine are particularly numerous in lamina III (Todd & Sullivan, 1990). In fact, glycinergic cells account for around 90% of the inhibitory interneurons in deeper dorsal horn (laminae III-VI) and ventral horn (VH), but only around 20% of those in lamina I-II (Todd, 2017). Although many studies emphasize a loss of GABAergic inhibition function that accounts for loss in pain regulation (Delgado-Lezama et al., 2021; Moore et al., 2002; Polgár & Todd, 2008; W. Sun et al., 2018), evidence also supports that disrupted glycinergic transmission could also contribute to tactile allodynia in neuropathic pain states (Miraucourt et al., 2007).

As there are distinct inhibitory interneuron populations performing specific functions, it is highly likely that these populations have differential roles during physiological and pathological nociception. The differences in interneuron subtype may contribute to modulating the different aspects of somatic sensations in the SDH versus the DDH and hence, understanding the differences in molecular mechanisms in these two regions are essential to understand pain.

2.4 Pre- and post-synaptic membranes

Synaptic function is driven by the function of proteins, whether in the form of receptors, intracellular kinases, or structural proteins. Therefore, the regulation of gene expression is fundamental in the development, modification and functioning of a synapse (Glock et al.,
The degree of gene expression is usually measured by the number of mRNA transcripts synthesized by different cells to direct the synthesis of specific proteins (Bear et al., 2020). Conventionally, mRNA translation occurs in the somatic space near the nuclei, after which post-modified proteins are trafficked to their dedicated site(s) of function such as within dendrites and axon terminals. Thus, proteins that regulate spinal excitability are located at both pre-synaptic and post-synaptic membranes of synaptic terminals, but only the transcripts for post-synaptic proteins are transcribed within the spinal cord.

Most nociceptive signals following the path of primary afferent nerve fibers form synaptic connections with interneurons in the superficial dorsal horn. Since the cell bodies of DRG neurons are localized distal to the spinal cord in the dorsal root ganglion (DRG), experimental tissue using only the dorsal horn does not include mRNA residing within the DRG cell bodies. In contrast, experimental dorsal horn tissue does contain mRNA of neural populations such as interneurons and projection neurons, who have their somas within the dorsal horn. These are important considerations for experimenters to take into account when comparing gene versus protein expression patterns within the spinal cord.

3 Spinal excitability and role of glutamate receptors

The phenomenon of functioning pain relies on the balance of both the excitatory as well as inhibitory feedback in dorsal horn neurons. Glutamate is the primary excitatory neurotransmitter of the central nervous system and hence the spinal cord, and is modulated by its binding to post-synaptic or pre-synaptic glutamate receptors on second order neurons in the dorsal horn (Liu & Salter, 2010). Ionotropic glutamate receptors include three subtypes, namely, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPARs), N-methyl-D-aspartate receptors (NMDARs), and kainite receptors (KARs).
High-frequency inputs of acute noxious stimuli to the dorsal horn result in firing of AMPARs. NMDARs are silenced by a pore-blockage by Mg$^{2+}$ ion. However, upon sustained firing, the pore is released from the Mg$^{2+}$ blockage which leads to nociceptive SDH neurons to increase in their excitability. This frequency-dependent increase in excitability describes the phenomenon of ‘wind-up’ which is required for NMDAR activity (Herrero et al., 2000). Wind-up typically is a reversible phenomenon. However, when NMDARs become sensitized and fire extensively, wind-up takes the form of central sensitization which may transpire to chronic pain as allodynia or hyperalgesia. (Herrero et al., 2000; Latremoliere & Woolf, 2009). A myriad of molecular players are implicated in the pathology of NMDAR hyperexcitability which will be discussed next.

4 Molecular Determinants of Pain

Repeated C-fiber stimulation which induces wind-up reflects an activity-dependent excitability increase in neurons during a nociceptor conditioning paradigm (Latremoliere & Woolf, 2009; Woolf & Thompson, 1991). Wind-up requires very low frequency input and manifests only with repetitive trains of inputs (Moore et al., 2002). In contrast, central sensitization depicts changes that follow inputs that initiate windup and is a process that results in prolonged enhanced responses to pain stimuli, even after the original triggering repetitive input event has resolved (Pace et al., 2018). It has become clear that central sensitization can occur in the absence of wind-up (Herrero et al., 2000; Woolf, 1996) although they share common mechanisms (J. Li et al., 1999), one being that both are NMDAR-dependent.

NMDAR-dependent wind-up and hyperexcitability has been implicated as a necessary prelude to the induction of central sensitization (Herrero et al., 2000). A large body of research has associated NMDAR hyperexcitability in mediating chronic pain (Bliss et al., 2016; Deng et
al., 2019; Kreutzwiser & Tawfic, 2019; X.-H. Li et al., 2019) but the exact mechanisms across sex and species are not fully understood.

The NMDAR is made up of two glutamate-binding GluN2 subunits and two glycine-binding GluN1 subunits. Whereas the GluN1 subunit is encoded by the GRIN1 gene and is present in all NMDARs, the GluN2 subunits can be encoded by either of four genes—GRIN2A through GRIN2D (Hildebrand et al., 2014). The composition of the NMDAR subunit determines its functional properties which may elucidate their distinctive roles in chronic pain pathologies.

The GluN2B-containing NMDARs have shown an increase in excitability in lamina I synapses of a male rodent peripheral nerve injury model of neuropathic pain (Hildebrand et al., 2016). Blocking the GluN2B subunit in the dorsal horn alleviates neuropathic pain hypersensitivity in unsexed mice models of chronic pain, and eliminates the pain entirely in mice lacking Fyn kinase (Abe et al., 2005). Furthermore, a study on male rats reported a downregulation of GluN2B in the rostral anterior cingulate cortex through siRNA attenuates thermal hyperalgesia as well as mechanical allodynia rat models of pain (Xu et al., 2016). GluN2B also plays an important part in polysynaptic NMDAR EPSCs following inhibition demonstrated by the analgesic effect of GluN2B antagonist on neuropathic pain (MacDermott, 2014).

4.1 Pronociceptive effect of BDNF

Several molecular players directly or indirectly interfere with conventional NMDAR-dependent excitability. One pathway that is involved in potentiating the GluN2B-containing NMDARs is mediated through brain derived neurotrophic factor (BDNF) (Hildebrand et al., 2016). BDNF is a neurotrophin initially attributed to its role in sensory neuron maintenance and survival during development (Jones et al., 1994). It also is known for its modulatory
function on synaptic plasticity in the central nervous system (Bramham & Messaoudi, 2005). However, more recently, a large body of emerging research has brought forth the pronociceptive effect of BDNF. BDNF is released during burst firing of primary nociceptor afferents during inflammatory pain (Zhao et al., 2006) and the pain is attenuated by blocking or sequestering BDNF (Lu et al., 2007).

However, in models of neuropathic pain achieved through chronic constriction injury (CCI) of the sciatic nerve in male rodents, microglia instead, release a set of mediators including BDNF (Coull et al., 2005; Lu et al., 2007). Growing evidence supports spinal microglial-released BDNF as being critical in microglia-neuron signaling that modulates aberrant nociceptive processing (Biggs et al., 2010; Trang et al., 2011). While these two possible sources of BDNF release exist, the predominant view from evidence in male rodents favours presynaptic afferents to release this neurotrophin during inflammatory pain, while during neuropathic pain, it is thought be sourced from microglial cells (Beggs & Salter, 2013; Chen et al., 2014).

Moreover, a study investigated whether the long-term action of BDNF on the substantia gelatinosa occurs during CCI and central sensitization (Lu et al., 2007). Exposure of BDNF on unsexed rat fetus culture demonstrated effects reminiscent to CCI models of pain, with decreased synaptic drive to ‘tonic’ neurons and increased synaptic drive to ‘delay’ neurons. These selective and differential action of BDNF on excitatory and inhibitory neurons contributed to a global increase in dorsal horn network excitability as measured by the amplitude of depolarization-induced increases in intracellular Ca$^{2+}$. Such changes and their underlying cellular mechanisms are likely to contribute to CCI-induced central sensitization and hence to the onset of neuropathic pain (Lu et al., 2007).
4.2 Downstream dysregulation through BDNF signaling

The interaction of BDNF with its high-affinity receptor tyrosine receptor kinase B (TrkB) triggers receptor dimerization, autophosphorylation of intracellular tyrosine residues, and the subsequent activation of signaling pathways (Lewin & Barde, 1996; Zhang et al., 2013). Genetically deleting BDNF from NaV1.8-expressing nociceptors in adult male mice attenuated the onset of inflammation-induced thermal hyperalgesia and thermal pain responsiveness. Administering an antisense directed against BDNF or TrkB mRNA resulted in a decrease in both of the aforementioned phenomenon suggesting the role of both BDNF and TrkB in these types of pain (Groth & Aanonsen, 2002). Furthermore, blocking TrkB with TrkB-FC has shown to alleviate neuropathic pain in male rats (Hildebrand et al., 2016). Moreover, transgenic mice with a mutated TrkB receptor administered with TrkB receptor autophosphorylation antagonist, 1NM-PP1, consequently halted TrkB signalling cascades. By signifying an alleviation in CFA-inflammation-associated mechanical and thermal hyperalgesia (Wang et al., 2009), the authors demonstrated the critical role of TrkB signaling in pain.

One signaling cascade that is mediated through BDNF-TrkB signalling works by downregulating the potassium-chloride co-transporter KCC2, which is responsible for maintaining intracellular chloride homeostasis. In mature neurons, KCC2 expels Cl⁻ ions from the cytosol thereby preserving the electrochemical gradient necessary for GABA_A and glycine receptors (GlyRs) to mediate synaptic inhibition (Kaila et al., 2014). In male rats, downregulating KCC2 in lamina I neurons results in a depolarizing shift in the Cl⁻ reversal potential, weakening GABA_A/GlyR-mediated inhibition and feeding into downstream effects that resulted in allodynia (Coull et al., 2003).
The accumulation of Cl− ions in the cytosol of lamina I neurons downregulates the activity of the striatal-enriched protein tyrosine phosphatase STEP61. STEP is an intracellular tyrosine phosphatase that is enriched in the striatum of the brain (Kurup et al., 2017) and is involved in neuronal excitation and synaptic plasticity (Goebel-Goody et al., 2012). It exists as two major isoforms, STEP61 and STEP46, through alternative splicing of a single STEP gene (Ptpn5) (Bult et al., 1996). The 61-kD isoform, STEP61, is widely expressed in the dorsal horn of the spinal cord (Pelkey et al., 2002).

STEP61 in its active form dephosphorylates Fyn, a tyrosine kinase from the Src family of protein tyrosine kinases (PTKs), at pY420 (Ingley, 2008). Fyn typically associates with GluN2B by interacting with the scaffolding protein RACK1 (Thornton et al., 2004), enabling active (phosphorylated) Fyn to phosphorylate and potentiate GluN2B-containing NMDARs to increase neuronal excitability (Tezuka et al. 1999, Sato et al. 2008). Thus, the inhibitory dephosphorylating activity of STEP61 on Fyn consequently halts Fyn’s ability to interact with the GluN2B subunit of NMDARs. STEP61 therefore acts as a molecular brake maintaining the balance of neuron excitability. Indeed, impaired activity of STEP61 promotes phosphorylation of NMDARs in the spinal dorsal horn (Abe et al., 2005; Hildebrand et al., 2016; Yang et al., 2011). Ultimately, a feedforward coupling between KCC2-dependent inhibition (that is mediated through BDNF) and potentiation of NMDARs drives neuronal hyperexcitability in the SDH of male rats and humans (Dedek et al., 2019; Hildebrand et al., 2016).

Another intracellular signalling cascade that is mediated through BDNF-TrkB is the phospholipase C-γ/inositol-1, 4, 5-triphosphate (PLC-γ/IP3) cascade (Garraway & Huie, 2016). PLC-γ is part of the PLC serine/threonine family. Phosphorylation of the TrkB receptor at its Tyr816 residue causes an activation of the PLC-γ pathway, generating IP3 and
diacylglycerol (DAG) (Kusuda et al., 2013). Subsequently, IP3 triggers an influx of Ca$^{2+}$ by release of Ca$^{2+}$ from intracellular stores activating CaMKII (Ca2C/calmodulin dependent protein kinase II), which leads to cyclic adenosine monophosphate–responsive element-binding protein (CREB) phosphorylation and activation. Studies have reported marked phosphorylation of CREB, a constitutively expressed transcription factor, in the dorsal horn following peripheral nerve injury and it has been suggested to play a crucial role in the activity-dependent synaptic plasticity that underlies neuropathic pain (Ma & Quirion, 2001; Miletic et al., 2002). Furthermore, this transcription factor is reported to be involved in transcribing 4000 human genes including those which are related to neuropathic and inflammatory pain and often used as a pain-related neuronal change marker (Descalzi et al., 2012). Thus, activation of these pathways ultimately regulates gene transcription and protein production, thereby modulating cellular physiology.

Downstream effects of protein signaling pathways can also directly or indirectly alter the activity of key Ca$^{2+}$ channels and other Ca$^{2+}$-permeable channels such as NMDARs (R.-R. Ji & Strichartz, 2004) and hence this molecular player may be involved in NMDAR hyperexcitability. However, pain research directly investigating crosstalk between PLC-$\gamma$ and NMDARs is lacking.

An additional molecular player that interacts with CREB is cAMP-dependent protein kinase A (PKA). Phosphorylation of CREB by PKA may lead to long-lasting plasticity (Miletic et al., 2002). PKA is also able to modulate Fyn phosphorylation activity on GluN2B-Y1472 which again feeds into hyperexcitability of NMDARs (Yaka et al. 2003b; Maksumova et al. 2005). One study found that an inhibition of spinal PKA activity impaired GluN2B accumulation at the post-synaptic density (PSD)-enriched fraction in male mice injected with complete Freund’s Adjuvant (CFA) (Yang et al., 2011). There seems to be cross-talk (see
Figure 2) between these molecular players and investigating spinal gene expression and protein translatomes can be very informative for understanding chronic pain development and maintenance.

**Figure 2 Molecular players involved in spinal hyperexcitability**

Image created in Biorender.com. During chronic pain, elevated brain-derived neurotrophic factor (BDNF) released by microglia or primary afferents bind to its high affinity tyrosine kinase B (TrkB) receptor. This results in downregulation of the potassium-chloride co-transporter 2 (KCC2) which is responsible for maintaining an intracellular chloride gradient. As a result, the chloride levels within the cell begin to rise, triggering a downregulation of the striatal-enriched protein tyrosine phosphatase (STEP61), a molecular brake, and hence dampening its inhibitory activity on Fyn kinase. Consequently, Fyn is allowed to irregularly heighten the phosphorylation of the excitatory N-methyl-D-aspartate (NMDA) receptor subunit B (GluN2B), resulting in hyperexcitability of the cell. BDNF-TrkB signaling drives hyperexcitability in males but not females in this mechanism. In another pathway, phospholipase C (PLC-γ) is seen to interact with the TrKB receptor at its Tyr816 reside consequently activating the (PLC-γ/IP3) cascade driving neuropathic pain signal transduction as well as transcription of pain related genes via CREB. In addition, PKA activates CREB transcription activity similarly and has been shown to have modulatory effects on Fyn and consequentially on the GluN2B receptor subunit.
5 Sex differences in pathological pain

It is apparent looking at the above cited literature that historically, research investigating pain mechanisms has primarily used either unsexed animal models or only male animals (Mogil, 2012). The lack of female models of pain is even more so important for understanding pain mechanisms given that females are more susceptible to many chronic pain syndromes and are more likely to report experiences of pain than males (Zajacova et al., 2021). Even when the behavioral phenotypes and clinical features of pain appear similar between males and females, the underlying molecular mechanisms may diverge (Dedek et al., 2022; Mogil, 2012). It is only recently that the scientific community and funding agencies have recognized this unmet need and have set the requirement for female-inclusive research, especially in human studies (Clayton, 2016). Hence, it is imperative to investigate potential sex differences in molecular regulators of pain for a complete understanding of pain mechanisms.

One molecular mechanism that seems to diverge between sexes is the BDNF-TrkB-mediated NMDAR hyperexcitability pathway. In males, BDNF is released from activated microglia through the activation of P2RX4 purinergic receptor which binds ATP (Trang et al., 2012).

In an in vivo CFA hindpaw injection model of inflammatory pain in rats, Dedek et. al found that although both sexes displayed tactile allodynia, the STEP61–pFyn–pGluN2B spinal hyperexcitability pathway was activated only in males, but not in females. In contrast to males, lamina I synaptic NMDAR responses were not potentiated in CFA-injected female rats and ex-vivo application of BDNF on female lamina I neurons failed to trigger this downstream cascade (Dedek et al., 2022). Adaptive immune cells, such as T lymphocytes, have been implicated to initiate pain in females (Beggs et al., 2012).
Furthermore, male but not female BDNF+/− mice presented a greater TrkB phosphorylation in the frontal cortex and striatum (Hill & van den Buuse, 2011). Given the pronociceptive role of BDNF-TrkB signalling, it was surprising to see that studies regarding differential TrkB expression in males and females in the spinal cord are lacking.

Downstream, TrKB-mediated downregulation of KCC2 and consequential shift Cl− reversal potential, results in allodynia in male rats (Coull et al., 2003). Despite evidence supporting BDNF-mediated hypersensitivity to occur in males and not females (Dedek et al., 2022; Hill & van den Buuse, 2011), a more recent study reported no sex differences in KCC2-dependant disinhibition in male and female rat models of neuropathic pain (Mapplebeck et al., 2019). Hence, the molecular mechanisms that underlie SDH hyperexcitability in females remain inconclusive.

Furthermore, what causes sex differences in subsequent STEP61 disinhibition (Azkona et al., 2016) if the KCC2 levels are reportedly not significantly varied? Azkona et al. reported female mice (ages 3-6 months but not older mice) having significantly lower levels of STEP61 protein than male mice in whole lumbar cords. In addition, female STEP knockout mice had a lower paw withdrawal latency than males (Azkona et al., 2016). Therefore, further studies investigating the genetic and proteomic elements of players in this pathway are needed to understand drivers of male and female hyperexcitability.

Further downstream in the BDNF-TrkB signaling pathway, STEP61 associates with Fyn kinase which enhances phosphorylation of GluN2B subunit of NMDARs, driving hyperexcitability in experiments on male rat lumbar dorsal horns (Hildebrand et al., 2016; Yang et al., 2011). Female rats treated with CFA did not demonstrate any significant increase in Fyn kinase in lamina I spinal cord tissue (Dedek et al., 2022), which brings to question, how
exactly do the discrepancies of players of the BDNF-TrkB-mediated hyperexcitability play their part. In addition, these studies have not taken differential expression of the SDH versus DDH into account which perhaps may provide a broader understanding of molecular mechanisms of nociceptive signaling.

There are numerous studies on the effect of sex steroids on pain. Hormones act on the brain through interactions with other hormones and molecular players. Estrogen imparts a positive regulatory effect on BDNF. Administration of 17-β-estradiol, a form of estrogen, to primary cultures of DRG neurons (McRoberts et al., 2007) and to spinal SDH neurons (Zhang et al., 2012) produces a greater increase in NMDAR currents in females compared to males in a dose-dependent manner. Moreover, estradiol modulates hyperalgesia in female rats by increasing NMDAR subunit GluN2B activity in the thoracolumbar spinal cord (Y. Ji et al., 2015).

The estrogen receptor, ERα, is co-expressed with NMDARs in the superficial dorsal horn, the deeper laminae, and lamina X of the spinal cord thus providing an anatomical basis for the role of NMDAR’s in estrogen modulation of pain processing at the level of the dorsal horn (L. Sun et al., 2019). The treatment of estrogen on GluN1 significantly increased its phosphorylation and resulted in estrogen-mediated facilitation of nociceptive processing. Estrogen treatment on ovariectomized rats significantly increased the phosphorylation of spinal GluN1, which was shown to contribute to estrogen-mediated facilitation of nociceptive processing (Tang et al., 2008). Moreover, this facilitation was through altered spinal GluN1 receptor activity via action of PKA signaling in these ovariectomized rats (Tang et al., 2008).

The injection of PKA inhibitors in male Sprague Dawley rats before or after hyperalgesia agents result in attenuated hyperalgesia and inflammatory pain suggesting it is maintained by
persistent PKA activity in male rats only (Aley & Levine, 1999). However, direct implications of PKA in females need further studies.

BDNF triggers another signaling cascade aside from the aforementioned. PLC-γ signaling facilitated by BDNF is exhibited in the dorsal root ganglia of male Sprague Dawley rats undergoing colitis-induced visceral pain (Hashmi et al., 2016). PLC-γ is implicated to be driving neuropathic pain signal transduction in males (Zhou et al., 2021). The functional role of PLC-γ in female-specific pain pathologies, however, is uncertain.

Thus, looking at sex differences of the molecular players that mediate chronic pain can provide insights on underlying mechanisms of pain.

6 Translational Studies in Human Tissues

Historically, distinct pain disorders were conceptualized where their only common feature was persistent pain, but now it is coming into light that separate chronic pain conditions may share conserved pathophysiological mechanisms (Gereau et al., 2014). While rodent studies are an excellent tool in discovering a wide range of physiological and pathological processes and functioning, it is vital for basic science research findings to translate to humans. This is especially important when the research is targeted towards the development of therapeutics. There is an increasingly pressing need for fundamental preclinical studies using human tissue to bridge the gap between foundational understanding of mechanisms from rodent to achieve more effective analgesic approaches for humans.

For example, differences in nociceptor biology exists between humans and rodents and even amongst mice and rats too (Smallwood et al., 2013). Researchers aimed to highlight this gap by using RNAscope and in situ hybridization on male mouse versus human sensory DRGs in
order to assess the distribution of standard nociceptor subpopulation markers across species. They identified that the markers, *CALCA* (calcitonin gene-related polypeptide alpha; CGRP) and *P2RX3* (purinergic receptor P2X3; P2X3R) used to label neuronal populations in the DRG were distinct in mouse, but overlapped in humans and in rats (Smallwood et al., 2013). They also saw similarities but importantly to this context, differences in pain processing and/or drug targets. Currently, there is a lack of fundamental mechanisms of spinal nociceptive processing in human spinal cord. Furthermore, since it is highly likely that certain molecular processes and targets are unique to humans, therapeutic targets may be overlooked due to studies that are exclusively biased to rodents (Smallwood et al., 2013). For instance, the expression of *CHRNA9* mRNA (cholinergic receptor nicotinic alpha 9) is not expressed in mouse DRGs but it is found in around 25.6% of all human sensory neurons (Ray et al., 2018).

The societal impact of chronic pain is large; research in the pain field undertaking variability among species needs an aggressive approach to bridge the gap of deficient pain research to achieve a substantial advancement in pain therapeutics (Gereau et al., 2014).

**Study Paradigm**

Brain derived neurotrophic factor-tyrosine kinase B (BDNF-TrkB) signalling drives disinhibition that couples to increased excitatory N-methyl-D-aspartate receptor (NMDAR) activity at male but not female dorsal horn synapses (Dedek et al., 2022). We aimed to investigate whether differences in baseline expression of upstream NMDAR regulators in this pathway account for this sexually dimorphic pain processing across species. These targets include the genes for BDNF receptor, TrkB (*Ntrk2*); the NMDA receptor subunits GluN1(*Grin1*) and GluN2B (*Grin2B*); the potassium-chloride cotransporter 2, KCC2 (*Slc12a5*); FYN kinase (*Fyn*); protein kinase A catalytic subunit, PKA (*PRKACA*); striatal-
enriched protein tyrosine phosphatase, STEP61 (*PTPN5*); and phospholipase C γ, PLC-γ (*PLCG1*). We hypothesized that differential gene and protein expression of players of the hyperexcitability pathway may contribute to diverging spinal pain pathophysiology in males and females.

We tested this hypothesis by quantifying gene expression of mRNA transcripts using real-time quantitative polymerase chain reaction (RT-qPCR) and STEP61 protein levels using Western blot analysis of spinal cord tissue from micro-dissected adult (3–4 months) male and female Sprague Dawley rats. In addition, to bridge the translational gap between preclinical human and rodent research, we extended gene expression profiling of mRNA transcripts to human spinal cord tissue as well.

**Methods**

1. **Animals**

Experiments were performed on 3–4-month-old male and female Sprague Dawley rats ordered from Charles River Laboratories. The rodents were housed in same-sex pairs and followed a 12-hour day/night cycle. Animals had free access to food and water and were cared for following guidelines provided by the Canadian Council for Animal Care, Carleton University, as well as by the University of Ottawa Heart Institute. During multiple rounds of dissections, rats were sex-matched per set. Furthermore, for all experiments, animals were selected at random in male versus female sets. All experiments (refer Error! Reference source not found.) on male and female rats were run in parallel.
Figure 3 Schematic overview of experimental plan.
Image created in Biorender.com
2 Rat Spinal Cord Isolation

An intraperitoneal injection of 3g/kg urethane (Sigma) was used to anesthetize the Sprague Dawley rats until a deep anesthesia was achieved. Once no response was evoked to a paw pinch test, the rodents were dissected to extract their spinal cords via ventral vertebrectomy. The cords were then immediately placed in a protective ice-cold oxygenated sucrose artificial CSF solution (50 sucrose, 92 mM NaCl, 15 mM D-glucose, 26 mM NaHCO₃, 5 mM KCl, 1.25 mM NaH₂PO₄, 0.5 mM CaCl₂, 7 mM MgSO₄, 1 mM kynurenic acid, bubbled with 5% CO₂/95% O₂). With the aid of a dissecting microscope, the dorsal and ventral roots of the spinal cord were removed followed by isolating the lumbar and thoracic regions identified by the lumbar enlargement. Next, the isolated spinal regions were aligned at the coronal plane with the help of an agarose cube with the ventral side facing the cube. The tissue was then flash frozen using histo-freeze (Fisher Super Friendly Freeze’It) and sliced into three sections: the superficial dorsal horn, the deep dorsal horn and the ventral horn using a scalpel (refer Figure 4 for slice sections). The Atlas of the Spinal Cord was used as a reference (Sengul et al., 2013). The tissues were then placed in cryotubes, flash-frozen with liquid nitrogen and stored in -80°C before protein and RNA extraction.

Figure 4 Coronal slices of the SDH, DDH and VH sections of the lumbar spinal cord.
Image created in Biorender.com
3 Human Donor Tissue Isolation

Human spinal cord tissue was collected from male and female adult (18-70 years old; Table 1) organ donors identified by the Trillium Gift of Life Network. Consent was first obtained by the donor’s family prior to collection. Approval by the Ottawa Health Science Network Research Ethics Board and the Carleton University Research Ethics Board was acquired to collect and conduct experiments with human tissue.

Donors were pre-screened to exclude any blood-borne illnesses such as HIV, hepatitis, and syphilis. Those with serious chronic illnesses such as (cancer, chronic pain, etc.) or with damage to the spinal cord were excluded from the study to prevent any interference from confounding variables.

<table>
<thead>
<tr>
<th>Patient ID</th>
<th>Sex</th>
<th>Age</th>
<th>Region of Spinal Cord</th>
<th>Experiment</th>
</tr>
</thead>
<tbody>
<tr>
<td>P54</td>
<td>Male</td>
<td>Adult</td>
<td>Thoracic</td>
<td>RT-qPCR</td>
</tr>
<tr>
<td>P55</td>
<td>Male</td>
<td>Adult</td>
<td>Thoracic</td>
<td>RT-qPCR</td>
</tr>
<tr>
<td>P56</td>
<td>Female</td>
<td>Adult</td>
<td>Thoracic</td>
<td>RT-qPCR</td>
</tr>
<tr>
<td>P57</td>
<td>Female</td>
<td>Adult</td>
<td>Thoracic</td>
<td>RT-qPCR</td>
</tr>
</tbody>
</table>

Table 1 Human donor tissue data sheet

To preserve the organ donor body and organ viability during surgery, a cooling bed was used to induce hypothermia and the body was perfused with a preservation solution (ex. Custodiol®HTK Solution or Perfadex®Plus). After the organs were removed, the vertebral column was opened to isolate and remove the spinal cord. This was performed within 1-3
hours of aortic cross-clamping or flushing of the body with protective solutions. The dura mater was removed, and thoracic sections of the spinal cord were cut into 6-10mm sections, wrapped carefully in aluminium foil to prevent distortion of the cord, and immediately flash frozen in liquid nitrogen. Samples were then placed in dry-ice for transportation to the lab at Carleton University, Ottawa, Canada, and stored at -80°C prior to slicing.

Next, the isolated spinal regions were aligned coronally with the help of an agarose cube with the ventral side facing the cube. The tissue will then be flash frozen using histo-freeze (Fisher Super Friendly Freeze’It) and sliced into two sections, the dorsal horn and the ventral horn, using a scalpel. The Atlas of the Spinal Cord will be used as a reference for slicing (Sengul et al., 2013). The sectioned tissues will then be placed in cryotubes, flash-frozen in dry ice and then stored in -80°C ready for RNA extraction and subsequent quantitative real-time PCR experiments.

4 Protein Extraction and Quantification

Before extraction, each rat spinal cord sample length was recorded. To each spinal cord tissue sample, 150uL-200uL of Ripa-like extraction buffer (0.1% SDS, 1 mM Na ortho-vanadate in 10 mM tris, with Roche’s Complete Mini EDTA-free tablet, cat# 1836170) was then added and the tissue will be sonicated at 15 power setting. Short pulses were given until the tissues were completely homogenized, ensuring to cool the sonicator probe with ice-cold water between pulses. The tissue was then cooled on ice for 15 minutes before centrifugation for 10 mins at 4°C at 12,000 rpm (13,800 x g).

The supernatant was carefully extracted and placed in fresh tubes, being careful not to disrupt the pellet. The pellet was discarded. Pierce’s BCA Protein Assay Kit (ThermoFisher,
cat#23225) was used to quantify the supernatant containing the protein which was diluted with 0.9% saline in a 1:25 ratio. Samples were loaded in duplicate on a 96-well plate. Blanks and standards (prepared in accordance with the kit) were loaded in duplicate. Appropriate amount of working reagent was prepared using the kit of which 200 µL was added to each well, followed by shaking for 30 seconds at 500 rpm. The well plate was allowed to incubate for 30 minutes at 37 °C in an incubator after which the plate was cooled for 10 minutes at room temperature. The well plate was then read at 562 nm on a Spectramax Pro plate reader. Absorbance readings obtained were the averaged adjusted concentrations and converted to (µg/µL) for each of the duplicate loads. Samples will be stored at -80°C ready for the Western blot.

5 Western Blot

Equal amounts of protein samples were loaded in each well of a Multi-PROTEAN TGX precast gel (Bio-Rad cat# 4561025) which were diluted with 5X Laemmli loading buffer (25% glycerol; 25% β-mercaptoethanol, Bio-Rad cat # 161-0710; 15% SDS, 0.25% Bromophenol blue, Sigma cat# B8026; pH adjusted to 6.8) to obtain a 1X solution. Protein with a working concentration of 30 µg was picked for loading on the polyacrylamide gel. Male and female tissue samples for superficial dorsal horn (SDH), deep dorsal horn (DDH) and ventral horn (VH) were run on a single gel in parallel for the target protein, STEP61.

Samples were run at 200V for around 30 minutes or until the loading dye front reached the bottom of the gel. The outer lanes were loaded with 3uL Laemmli loading buffer and 7 µL of the Precision Plus Protein Dual Color Standards marker (Bio-Rad, cat#1610374) was used.
The gels were equilibrated in transfer buffer for 15 minutes and transferred onto polyvinylidene fluoride (PVDF) membranes (Bio-Rad) at 100V for 60 minutes using the Bio-Rad Mini Trans Blot Cell.

Once the transfer is complete, membranes were allowed to dry for 1 hour on a clean inverted petri dish surface. Membranes were then rewet for 60s in methanol with gentle shaking and stained with 0.1% Fast Green Stain for 5 mins for total protein imaging. They were rinsed twice for 1 min in wash solution (6.7% acetic acid in 30% methanol) and imaged on the Licor Odyssey system on the 700 channel for 2 minutes.

Next, the membranes were rinsed in 10mM Tris Buffered Saline (TBS) 2 times for 2 minutes. The membranes were blocked in LI-COR Intercept TBS Blocking Buffer (LI-COR Biosciences, Cat#927-60001) protein side-up for 1 hour, washed in TBS-T (0.1% Tween-20) 3 times for 5 minutes each, and incubated in the primary antibodies multiplex (Anti-STEP D9H3, Cell Signaling, Cat# 9069S and anti-β-actin, Santa Cruz Biotechnology, Cat# sc-47778) diluted to 1:1000 and 1:2500 respectively according to the manufacturer’s instructions.
Primary incubations were performed overnight at 4°C with gentle shaking. Membranes were then washed in TBS-T (0.1% Tween-20) 3 times for 5 minutes each and incubated in secondary infrared conjugate antibodies (IR800 and IR680, respectively) for 1 hour. Membranes were then washed in TBS-T (0.1% Tween-20) and TBS and then be imaged on the Licor Odyssey Fc system at appropriate wavelength while wet. All densitometric bands were semi-quantified using Image Studio by LI-COR. Boxes of equal size were used to capture each single band signal per gel. Background intensity was subtracted from signal and normalized to the corresponding β-actin band intensity for each sample. This process was repeated systematically for each gel.

6 RNA extraction and cDNA synthesis

Total RNA was extracted using RNAeasy Plus Mini Kit (QIAGEN Hilden, Germany) and QIA shredder tubes. Cryo-preserved spinal cord tissues that was sliced into the SDH, DDH and VH for rats, and DH and VH for humans, were subsequently weighed prior to extraction and a 600 µL Buffer RLT (from the kit) volume was determined to be added to each tissue sample. Each tissue sample was subjected to mechanical grinding to powder in a mortar and pestle with liquid nitrogen and homogenized by pipetting up and down in the buffer. The entire components were then be transferred to a QIA shredder tube and centrifuged at full speed (14,000 rpm) for 3 minutes. The lysate was transferred to a gDNA eliminator spin column provided in the kit to eliminate genomic DNA and was centrifuged at 12,000 rpm for 30 seconds. The column was discarded while flow through was reserved. 600 µL of 70% ethanol was added to the flow through and the sample homogenized. 700 µL of the sample mixture was transferred to a RNeasy Mini spin column, slowly, followed by centrifugation at 12,000 rpm for 15 seconds. The flow through was discarded and the membrane were preserved. To
this, 700 µL RW1 (from kit) buffer was added, and the tube was centrifuged at 12,000 rpm for 15 seconds. Following this, RPE buffer (from kit) was added to the membrane (flow through discarded) and subjected to centrifugation at 12,000 rpm for 15s. RPE buffer was then added to the membrane a second time and was centrifuged again at 12,000 rpm for 2 minutes. The RNeasy spin column was then placed in a new 2ml collection tube and spun at max speed (14,000 rpm) for 1 minute. Next the membrane was transferred to a 1.5 µL Eppendorf and 50 µL of RNase free water was added. The membrane was allowed to sit for 2 minutes before a final spin at 12,000 rpm for 1 minute. The extracted RNA was then stored at -80°C until further use. RNA concentrations were measured with Nanodrop 2000c (Thermofisher Scientific).

cDNA was synthesized using the iScript cDNA Synthesis Kit (Bio-Rad) and the recipes in provided protocol were used adjusted to the concentration of RNA template and the desired total volume to be synthesized. Equal starting RNA (25 ng or 50 ng) was used to synthesize complementary DNA (cDNA) strands to the extracted RNA depending on initial RNA concentrations. A total volume of 100 µL of cDNA was synthesized per sample which was sufficient for 8 target genes. The reactions were run on a thermocycler with the following protocol:

<table>
<thead>
<tr>
<th>Step</th>
<th>Conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Priming</td>
<td>5 min at 25°C</td>
</tr>
<tr>
<td>Reverse transcription</td>
<td>20 min at 46°C</td>
</tr>
<tr>
<td>RT inactivation</td>
<td>1 min at 95°C</td>
</tr>
<tr>
<td>Hold</td>
<td>4°C</td>
</tr>
</tbody>
</table>

Table 2: cDNA synthesis thermocycler protocol
7 Real time quantitative polymerase chain reaction (RT-qPCR)

Real-time quantitative PCR (RT-qPCR) was performed using SsoADVANCED Universal SYBR Green Supermix (Bio-Rad). Master mixes for target genes were prepared in accordance with the manufacturer’s instructions. Eighteen µL of mastermix was loaded in each sample well in a Hard-Shell qPCR 96-well plate (Bio-Rad). Samples were run in duplicates. Refer Table 4 for typical well plate template. For each reaction, 2 µL of the appropriate cDNA was added to the reaction mix with the appropriate gene-specific primers (refer to Table 4 and Table 5). The plate was sealed, gently tapped down and briefly centrifuged. The RT-qPCR reactions were carried out in the CFX Maestro Real Time System (Bio-Rad). All assays were run in parallel with a negative control to ensure no carry over of genomic DNA occurred. Tubulin was used as the housekeeping gene. Each region of the spinal cord (SDH, DDH, and VH) had a corresponding tubulin control per animal. Tubulin levels did not vary across sex and region. Reactions were run as follows, provided in the protocol:

<table>
<thead>
<tr>
<th>Step</th>
<th>Temperature</th>
<th>Time</th>
<th>Number of Cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Activation</td>
<td>95°</td>
<td>2 min</td>
<td>1</td>
</tr>
<tr>
<td>Denaturation</td>
<td>95°</td>
<td>5 sec</td>
<td></td>
</tr>
<tr>
<td>Annealing/extension</td>
<td>60°</td>
<td>30 sec</td>
<td>40</td>
</tr>
<tr>
<td>Melt curve</td>
<td>65-95°</td>
<td>(0.5°)</td>
<td>5 sec/step</td>
</tr>
</tbody>
</table>

Table 3 RT-qPCR reaction protocol
### Table 4 Typical qPCR 96-well plate template.

Samples were run in duplicate. Column 1 contained the negative control for housekeeping gene *TUBA4A*. The following columns contained male and female samples, with one gene target per column. For example, column 8 wells contained male 7 tissue samples with primers for housekeeping gene while row 4 had primers for *NTRK2*. Furthermore, each column ran SDH, DDH and VH tissue sample per sex.

<table>
<thead>
<tr>
<th>Column</th>
<th>Samples</th>
<th>Primers</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>TUBA4A (Tubulin alpha 4a)</td>
<td>PrimePCR™/ SYBR® Green Assay: Tuba4a, Rat, BIO-RAD</td>
</tr>
<tr>
<td>B</td>
<td>NTRK2 (TrkB)</td>
<td>PrimePCR™ SYBR® Green Assay: Ntrk2, Rat, BIO-RAD</td>
</tr>
<tr>
<td>C</td>
<td>SLC12A5 (KCC2)</td>
<td>PrimePCR™ SYBR® Green Assay: Slc12a5, Rat, BIO-RAD</td>
</tr>
<tr>
<td>D</td>
<td>PTPN5 (STEP61)</td>
<td>PrimePCR™ SYBR® Green Assay: Ptpn5, Rat, BIO-RAD</td>
</tr>
<tr>
<td>E</td>
<td>FYN (Fyn)</td>
<td>PrimePCR™ SYBR® Green Assay: Fyn, Rat, BIO-RAD</td>
</tr>
<tr>
<td>F</td>
<td>GRIN2B (GluN2B)</td>
<td>PrimePCR™ SYBR® Green Assay: Grin2b, Rat, BIO-RAD</td>
</tr>
<tr>
<td>G</td>
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<tr>
<td>H</td>
<td>PLCG1 (PLC γ)</td>
<td>PrimePCR™ SYBR® Green Assay: Plcg1, Rat, BIO-RAD</td>
</tr>
<tr>
<td>I</td>
<td>PRKACA (PKA)</td>
<td>PrimePCR™ SYBR® Green Assay: Prkaca, Rat, BIO-RAD</td>
</tr>
</tbody>
</table>

### Table 5 Primers used for RT-qPCR experiments in rats

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer Set</th>
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<tr>
<td>TUBA4A</td>
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<td>Plcg1, Rat, BIO-RAD</td>
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<tr>
<td>PRKACA</td>
<td>Prkaca, Rat, BIO-RAD</td>
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<tr>
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<td><strong>Assay</strong></td>
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<td><strong>PRKACA</strong> (PKA)</td>
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Table 6 Primers used for RT-qPCR experiments in humans

8 Statistical Analysis

All data is presented as mean ± standard error of the mean (SEM). For real-time RT-PCR analysis, the amount of target each gene mRNA (**NtrK2**, **Slc12a5**, **PTPN5**, **Grin1**, **Grin2B**, **Fyn**, **Prkaca**, and **Plcg**) was normalized to the amount of housekeeping/reference gene **TUBA4A** or **TUBB6** mRNA. Relative quantification of the mRNA was calculated using ΔCt method using housekeeping gene as follows:

\[
\text{Ratio (reference/target)} = 2^{\Delta Ct (\text{housekeeping}) - \Delta Ct (\text{target})}
\]

i.e. \[
\text{Ratio (reference/target)} = 2^{\Delta Ct (\text{TUBA4A}) - \Delta Ct (\text{target})}
\]
The Ct (cycle threshold) values were determined as the cycle at which fluorescence from a sample crossed the threshold level beyond background. Melt curve analysis was performed to rule out genomic DNA contamination to check the specificity of the amplification reaction. Presence of multiple peaks would indicate the detection of additional genes or genomic contamination such as introns by the primers. \( \Delta \text{Ct} \) was the difference between the reference housekeeping gene tubulin beta 4A \((\text{TUBA4A})\) in rats and tubulin beta 6 \((\text{TUBB6})\) in humans, and the experimental gene target from which the fold change was then calculated as \(2^{\Delta \text{Ct}} \times 100\).

Tubulin levels did not vary across sex and region of the spinal cord. Data was tested for normality using the Kolmogorov-Smirnov test. A 2-way ANOVA was used to test the statistical significance between region of dorsal horn (SDH, DDH or VH) and sex (male or female) for each gene target. Post-hOC analysis was performed using the Bonferroni test. A \(p<0.05\) will be considered statistically significant.

To test statistical significance of sex differences in Western blots, a 2-way ANOVA was used to test the statistical significance between sexes (male and female) and dorsal horn region (SDH, DDH, VH). A two-sample t-test was used to compare male and female means (two-tailed) for the STEP61 SDH:DDH ratios. Bonferroni post-hoc test followed ANOVAs. \(p<0.05\) will be considered statistically significant. All data was presented as mean ± standard error of the mean (SEM).
Results

**Baseline gene expression in rats is conserved across sex, with regional changes for a subset of targets**

In a Complete Freund’s adjuvant (CFA)-induced *in vivo* model of inflammatory pain, BDNF-TrkB signalling drives disinhibition, coupling to increased excitatory NMDAR activity only at male but not female dorsal horn synapses (Dedek et al., 2022). However, when comparing between vehicle versus CFA administration, the Hildebrand lab only investigated NMDAR responses using electrophysical approaches as well as sex-dependent changes in protein expression. In the present research, the goal was to analyze baseline mRNA transcripts of proteins involved in this molecular mechanism of pathological pain to determine if any potential sex differences may predispose a certain sex to specific molecular mechanism of chronic pain. Canonical players of this pathway included *NTRK2* (TrkB), *SLC12A5* (KCC2), *PTPN5* (STEP61), *FYN* (FYN), *GRIN2B* (GLUN2B), and *GRIN1* (GLUN1). Two additional targets were selected based on their interaction with molecular players of this pathway, namely *PLCG1* (PLC-γ) and *PRKACA* (PKA).

Hence, to quantify gene expression of our target genes at baseline, total mRNA was extracted from sectioned lumbar tissue (superficial dorsal horn, SDH; deep dorsal horn, DDH; and ventral horn, VH) followed by cDNA synthesis and qPCR. By running male and female assays of each rat target in parallel in a single experiment, we were able to systematically differentiate between the cycle number at which gene expression surpassed the background threshold (C_T value) for each sex, per target, in each region of the lumbar spinal cord. Relative expression of genes was determined by normalizing to the housekeeping gene, *TUBA4A*, which was at constant levels across sex and region of spinal cord.
The findings of this research showed that at baseline, gene expression in rats is conserved across sex, with significant regional changes for a subset of targets (Figure 6). Of note, the relative expression of NTRK2 (Figure 6A) and GRIN2B (Figure 6E) are significantly higher in the SDH than the DDH (p < 0.0001 and p = 0.003, respectively). In contrast, PRKACA (Figure 6H) gene expression is significantly higher in the DDH than the SDH in both sexes. We also found decreased gene expression in the VH for most targets (significance of SDH versus VH: p < 0.0001 for NTRK2, p < 0.0001 for SLC12A5, p = 0.015 for PTPN5, p = 0.003 for FYN, p < 0.0001 for GRIN2B, p = 1.0E-4 for GRIN1 and p < 0.0001 for PLCG; significance of DDH versus VH: p = 0.041 for NTRK2, p < 0.0001 for SLC12A5, p < 0.0001 for PTPN5, p = 1.58826E-4 for GRIN2B, p < 0.0001 for GRIN1, p = 0.017 for PLCG and p = 2.41128E-4 for PRKACA).
Figure 6 Gene expression is conserved across sex, with significant regional changes for a subset of targets.

Panel A-H represent relative expression of target genes normalized to housekeeping gene TUBA4A ($2^{ΔCT}$x100) in the superficial dorsal horn (SDH), the deep dorsal horn (DDH) and the ventral horn (VH) of the lumbar spinal cord in adult rats (n=8/sex). The pink bars represent 3–4-month-old female Sprague Dawley rats while the blue bars represent the male rats. Experiments for Panels A–D were run in parallel on a single qPCR 96-well plate for both male and female rat SDH, DDH, and VH samples. Similarly, experiments for panels E–H were run on a single qPCR 96-well plate. All experiments were run in male and female pairs. A two-way ANOVA was used to make comparisons followed up with Bonferroni post HOC test. *p<0.05 (refer Table 7)
The SDH to DDH ratio of baseline gene expression is conserved across sex in rats, with differential localization to the SDH between gene targets.

The SDH of the spinal cord receives and processes noxious inputs while the DDH primarily processes incoming innocuous mechanosensory and proprioceptive inputs in combination with premotor functions. Thereafter, the expression of our gene set in these two functionally separate areas of the dorsal horn was directly examined. The relative role of each target gene expression in the SDH versus the DDH, within individual animals/subjects enabled us to determine whether the relative local abundance of gene expression in one region over the other varied. Furthermore, this allowed investigating whether any sex differences in the relative preferential abundance existed. Fold change values were normalized to $TUBA4A$ ($2^{\Delta CT \times 100}$) and the ratio between SDH and DDH was derived for both male and females for each set of genes. All SDH:DDH ratios were taken from paired sample data after which an
average expression was derived and plotted. A ratio of greater than 1 indicated a preferential localization to the SDH while a ratio less than 1 indicated preferential localization to the DDH.

Overall, SDH:DDH expression ratios were conserved across sex for all gene targets (Figure 7). Values for the SDH:DDH ratios of NTRK2, FYN, GRIN2B, and PLCG1 all indicate a preferential expression in the SDH for these targets. Interestingly, all of these targets are implicated in the increased excitability mechanism of superficial dorsal horn pain processing (Hildebrand et al., 2016). Amongst targets, the SDH:DDH ratio was significantly greater in NTRK2 than SLC12A5 (p = 5.04598E-4), NTRK2 greater than PTPN5 (p = 0.001), GRIN2B greater than GRIN1 (p = 7.42225E-4), GRIN2B greater than PRKACA (p = <0.0001), and PLCG1 greater than PRKACA (p = 0.002).
A two-way ANOVA was used to make comparisons between the male and females and targets; followed up with Bonferroni post HOC test. *p<0.05 (refer Table 8).
Preliminary human spinal cord gene expression results suggest potential sex differences in select targets.

To bridge the translational gap between rodent preclinical results as described above and new treatments for humans, we performed qRT-PCR gene expression analysis of our target genes on human spinal cord tissue generously donated by consenting human donors and their families. Donors had no blood-borne illnesses or chronic conditions. Flash frozen thoracic spinal cord tissue was isolated (n=2/sex) and sectioned into the dorsal horn (DH) and the ventral horn (VH). The superficial dorsal horn (SDH) was not isolated due to its curved conformation in the human dorsal horn which limits accurate and consistent isolation through scalpel dissections. Total RNA was extracted and transcribed to cDNA followed by qPCR analysis to quantify gene expression of molecular players of pain processing and spinal excitability. RT-qPCR primers were specific to the human gene variants.

By running male and female assays of each target in parallel in a single experiment, we were able to systematically differentiate between the cycle number at which gene expression surpassed the background threshold (C_T value) for each sex, in each region of the thoracic spinal cord, per target.

Preliminary data suggests a potential sex difference showing a greater gene expression in females for *PTPN5*, *SLC12A5*, *GRIN2B* and *GRIN1* in both the DH and VH (Figure 8). Furthermore, for most targets, there seems to be a conserved regional expression between the DH and VH across targets. Indeed, this dataset necessitates further human donor samples for statistical power and robust analysis.
Figure 8 Potential sex differences in select targets with conserved regional expression for human spinal cord

Panel A-H represent relative expression of target genes normalized to housekeeping gene TUBB6 ($2^{\Delta CT} \times 100$) in the dorsal horn (DH) and the ventral horn (VH) of the thoracic spinal cord in adult humans ($n=2/sex$). The pink bars represent female human donor tissue while the blue bars represent the male human donor tissue. Experiments for Panels A-D were run in parallel on a single qPCR 96-well plate for both male and female DH and VH samples. Similarly, experiments for panels E-H were run on a single qPCR 96-well plate. All experiments were run in male and female pairs.
Potential sex difference in the DH to VH ratio of baseline gene expression in NTRK2, SLC12A5, PTPN5 and GRIN1, with differential localization to the SDH between gene targets.

Following relative gene expression analysis of human genes, we adapted a similar approach as the rat samples where we had derived the SDH:DDH ratio to identify trends of preferential localized gene expression. For human donor tissue, we instead used the DH:VH ratio of fold change values normalized to \(TUBB6\) \((2^{\Delta CT} \times 100)\) and plotted the results for each specific target gene. \(TUBB6\) levels remained constant across sex and spinal cord region and hence were a good housekeeping control. All DH:VH ratios were taken from paired sample data from each donor, after which an average expression was derived and plotted. A ratio of greater than 1 indicated a preferential localization to the DH while a ratio less than 1 indicated preferential localization to the VH.

A potential sex difference may exist in the genes NTRK2, SLC12A5 and PTPN5 (Figure 9A) in addition to GRIN1 (Figure 9B). SLC12A5 and PTPN5 appear to be localized in the VH for males opposed to a uniform DH-VH distribution in females. GRIN1 seems to be preferentially localized in the DH in females with males to be favouring the VH. On the contrary, NTRK2 seems to have preferential expression to DH in males but to the VH in females. FYN, PLCG1, PRKCA seem to have a fairly uniform distribution in both regions of the dorsal horn.
Specific determinants of excitability appear to be differentially expressed in rat versus human spinal cord mRNA transcripts.

We next were interested in assessing how the rat (n=8/sex) mRNA transcript levels compared to the human (n=2/sex) mRNA preliminary expression profile. To do this, we plotted the $\Delta C_T$ difference in $C_T$ values between our target gene and housekeeping gene.

While the bars generated for the human samples are preliminary, they are indicative of the extent of disparity of expression between rat and human data. Looking at the $\Delta C_T$ value of
\textit{NTRK2} (Figure 10) at 0, it is clear that in both male and female humans, there is a relatively equal expression of \textit{NTRK2} compared to the housekeeping gene (\textit{TUBB6}). On the other hand, rat data shows a $2^3$ or 8X decrease in gene expression compared to the housekeeping gene.

Interestingly, \textit{FYN} displays a $2^1$ or 2X greater expression than the housekeeping gene \textit{TUBB6} in humans. Rats, however, show a remarkable contrast of $2^5$ or 32X decreased gene expression compared to \textit{TUBA4A}, highlighting its considerably lowered expression.

Furthermore, statistical analysis on rat gene expression demonstrates no sex differences in all gene targets. However, there potentially exists a sex difference in \textit{SLC12A5} (KCC2) and \textit{GRIN1} in humans with females having a greater expression in both genes. The graph suggests that in male humans, there may be a 1448X lowered expression of \textit{SLC12A5} transcripts than housekeeping while females only a 45X decrease. Likewise, the \textit{GRIN1} gene transcripts also suggest a decreased expression in male compared to female humans. Overall, there seems to be a conserved gene expression in only \textit{GRIN2B} across both sex and species.
Biochemical analysis of STEP61 protein reveals no sex differences of protein expression in adult rats, while regional differences exist.

STEP61 is a molecular brake that is downregulated to facilitate disinhibition in the BDNF-TrkB mediated hyperexcitability pathway in male but not female rats and humans (Dedek et al., 2019, 2022). As our RT-qPCR results reported no baseline sex difference in STEP61 mRNA transcript levels (PTPN5) in the whole lumbar SDH, DDH as well as the VH spinal cord (Figure 6), we next aimed to verify whether this transcript level conservation translated to equal protein levels between sexes in these specific tissues.

Figure 10 Human and rat genes are differentially expressed in the spinal cord, potentially implicating both species and sex differences in select targets

Graph represents ΔC\textsubscript{T} values (housekeeping C\textsubscript{T}- target C\textsubscript{T}) for target genes in rat lumbar SDH (n=8/sex) and human thoracic DH (n=2/sex) across sex. The darker bars represent human data whilst the lighter bars represent rat. Shades of blue denote males while the pinks denote females. At each cycle of the qPCR, the genes transcribed increase two-fold, exponentially, to the previous cycle. Hence, a C\textsubscript{T} value of 2 would indicate a 2\textsuperscript{2}, or a 4X increase in gene transcript levels.

Biochemical analysis of STEP61 protein reveals no sex differences of protein expression in adult rats, while regional differences exist.
Total protein was extracted from 3–4-month-old male and female rat lumbar spinal sections and subjected to biochemical analysis in a multiplex system. PVDF transfer membranes visualized on the 700 and 800 channel for each of the antibody conjugates demonstrate bands for B-actin, visible at the 43kD length and bands for STEP61, visible at the 61kD length (Figure 11). Clear bands for cytosolic STEP46 isoform were also visible in addition to smaller perhaps truncated forms of the STEP protein. These other bands were also present in the supplier-provided antibody data sheet for Western blot analysis of rat brain extracts, however, no data on spinal cord bands was indicated.
Figure 11 Western blot PVDF gel image capture of B-actin and STEP61 multiplex at the 700 and 800 channel respectively on the LI-COR Odyssey system for infrared antibody conjugates.

Gels were incubated in primary antibodies overnight at 4°C (anti-STEP D9H3 and anti-β-actin, diluted to 1:1,000 and 1:2,500 respectively). Membranes were allowed to incubate in secondary antibodies (IR680 and IR800; both diluted to 1:15,000) at room temperature for 1 hour. The lane plan is as follows: lane 1 was loaded with Precision Plus Protein Dual Color Standards marker (Bio-Rad, cat#1610374); lane 2 was loaded with male superficial dorsal horn protein extract; lane 3 was loaded with male deep dorsal horn protein extract; lane 4 was loaded with male ventral horn protein extract; lane 5 and 6 were left empty; lane 7 was loaded with the marker; lane 8 was loaded with female superficial dorsal horn protein extract; lane 9 was loaded with female deep dorsal horn protein extract; lane 10 was loaded with female ventral horn protein extract. An equal amount of protein was loaded in each well (30 µg).

Statistical analysis of normalized band intensity of STEP61 reveals conserved protein levels across male and female rats with the lowest levels of STEP61 in the ventral horn (refer Figure 12). This trend was similarly observed in STEP61 mRNA (PTPN5) transcripts as well (Figure 6). Moreover, a significant regional difference between the SDH and DDH ($p = 0.00376443$)
as well as between DDH and VH (p = 0.00200837) was observed. Overall, compared to the housekeeping protein, B-actin, STEP61 was very low in abundance (band intensity (normalized) <0.020).

Figure 12 STEP61 protein levels are conserved between male and female rat lumbar spinal cord, with decreased protein levels in the ventral horn.

The graph illustrates normalized (B-actin) band intensity of STEP61 on PVDF membrane gel in the superficial dorsal horn (SDH), deep dorsal horn (DDH) and ventral horn (VH). Densiometric bands captured from the Western blot experiments were semi-quantified on LI-COR Image Studio. Boxes of equal size were used to capture each single band’s signal per gel. Background intensity was subtracted from signal and normalized to the corresponding B-actin band intensity for each sample and plotted. The pink bars represent 3–4-month-old female Sprague Dawley rats while the blue bars represent the male rats. A two-way ANOVA was used to compare means and followed up by a Bonferroni Post HOC analysis. *p<0.05.
When comparing the STEP61 SDH:DDH ratio between sexes (Figure 13), we found that STEP61 had a preferential localization to the SDH compared to the DDH (ratio>1.0) in male, but not female. However, there was no significant difference in protein localization between the two sexes.

**Figure 13** The SDH:DDH ratio reveals no sex differences in preferential localization of the STEP61 protein in rats

The graph illustrates the ratio of superficial dorsal horn divided by the deep dorsal horn (SDH:DDH) of levels of the molecular brake, STEP61, in the lumbar spinal cord of male (blue) and female (pink) adult rats (n=8/sex). All SDH:DDH ratios were taken from paired sample data after which an average expression was derived and plotted. A two-sample t-test was used to compare means (two-tailed). *p<0.05.
Discussion

To address historical shortcomings of past sex-biased research, this study aimed to bridge the translational divide between sex as well as species to broaden our understanding of pain processing and molecular players of spinal hyperexcitability at baseline. We sought to investigate three overarching themes amongst targets: sex differences, regional differences within the spinal cord, and species differences between rats and humans. This study used molecular and biochemical approaches to investigate these themes with the lens on canonical players of the hyperexcitability pathway as well as new potential targets that may interact directly or indirectly with these players.

We hypothesized that there may be a sex difference in target expression at the genetic level that could underlie the divergence of the BDNF-TrkB-mediated hyperexcitability pathway between male and female rats and humans. However, interestingly, through our RT-qPCR analysis of target mRNA transcripts, we report that there is a conserved gene expression at baseline across sex in all our targets (NTRK2, SLC12A5, PTPN5, FYN, GRIN2B, GRIN1, PLCG1, PRKACA) in 3-4-month-old Sprague Dawley rats. We propose that mRNA splicing that generates differing variants of proteins may be one factor that contributes to the sex difference reported by Dedek et al. (Dedek et al., 2022). The varying levels of regulating translation and protein trafficking to synapses could potentially underlie these differences. It is becoming more widely acknowledged that mRNA splicing has a key role in rendering a variety of different proteins, which do not equate to the number of protein-coding genes present within a cell nucleus (Stetefeld & Ruegg, 2005). This became even more so evident after the Human Genome Project where an astoundingly lower abundance of genes were
identified compared to the number of proteins (Lander et al., 2001). Hence, mRNA levels are not conclusive to functional proteins at a given physiological state, be it baseline.

For example, in the current study, we do not see a sex difference in the TrkB gene at baseline (*NTRK2*). This may be unexpected, given the predominant role of TrkB as an initiator ligand-receptor of this pathway in a male but not female inflammatory model of pain (Dedek et al., 2022). It is quite plausible that we would discern a differing outcome if we instead investigated the specific splice variants of TrkB. TrkB.T1 and TrkB.FL are the most abundant isoforms of TrkB expressed in the mammalian nervous system of adults (Tomassoni-Ardori et al., 2019). TrkB.T1 is the alternatively spliced truncated isoform of the full-length (TrkB.FL) isoform. Several studies have implicated distinct signalling of both TrkB.FL and TrkB.T1 (Carim-Todd et al., 2009; Dorsey et al., 2006; Rose et al., 2003). TrkB.T1 is upregulated in the SDH following noxious stimulation, while genetic deletion attenuated hind paw-inflammation-induced thermal hyperalgesia (Renn et al., 2009). Differing levels of levels of translation, trafficking, and co-localization between pathway partners (such as Fyn and GluN2B) could also explain the potential differences in males versus females. This is especially true as the pathway we studied involves changes in protein phosphorylation and function, which may not rely on different levels of transcription at that instance. Notably, the current study does report a significantly elevated regional difference of *NTRK2* in the SDH than the DDH. As noxious stimulation increases BDNF expression within the SDH (Coull et al., 2005), elevated *NTRK2* expression here aligns with its functional role as a BDNF receptor in the noxious pain processing centre of the spinal dorsal horn. It is also interesting to note that TrKB.FL is restricted to neuronal populations whereas TrkB.T1 extends to glial cells as well (Dorsey et al., 2006; Tomassoni-Ardori et al., 2019).
Similarly, gene expression of GluN2B (Grin2B) shows a significantly elevated expression in the SDH than the DDH with no differences in sex. There is increased phosphorylation and total GluN2B (NMDAR subunit) at dorsal horn synapses mostly in male but not female pain models in which is concurrent with an increase in NMDAR synaptic currents (Dedek et al., 2022; Hildebrand et al., 2014, 2016). The current research further validates previous immunohistochemical staining experiments that demonstrate localization of GluN2B receptor subunits to be greater in SDH indicated by colocalization with the CGRP peptide, a marker of nociceptive afferent fibres that highlight the SDH region (lamina I and Ilo) (Temi et al., 2021). Indeed, this finding lines up with literature affirming the functional role of GluN2B in modulating pain in the SDH of the spinal cord compared and its relatively higher abundance there (Mahmoud et al., 2020; Hildebrand et al., 2014; Larsson, 2009).

In contrast, gene expression of PKA (PRKACA) demonstrated a higher expression in the DDH rather than the SDH. PKA phosphorylates cAMP response element-binding protein (CREB) which alters sensory function in the spinal dorsal horn following peripheral nerve injury or inflammation (Moore et al., 2000). Miletic and colleagues revealed a close association between CREB and the expression of thermal hyperalgesia (Miletic et al., 2002). Phosphorylated CREB (p-CREB) is found to increase trafficking of the GluN2B receptor to the post-synaptic density (PSD) in a model of inflammatory pain (Yang et al., 2011). Coinciding with this, Bement and Sluka demonstrated that a larger population of pCREB-expressing cells were present within lamina I of the SDH than the deeper laminae. However, this study only counted cells co-localizing with spinothalamic neuronal cell marker NR-1 (for NMDAR subunit GluN1) due to their role in pain (Bement & Sluka, 2007) and thus may not be an accurate representation of global pCREB expression within the spinal cord dorsal horn.
regions. Spinothalamic tract neurons respond to noxious but not innocuous mechanical stimuli upon activation of the cAMP pathway.

Intuitively, a higher distribution of PRKACA would be expected in the SDH in relation to noxious stimuli processing and NMDAR subunit function, which the current research contradicts. Hence, one would question why there would be a significantly increased gene expression of PRKACA in the DDH in this study. Potentially this may be due to the trafficking of PKA. The catalytic subunit of PKA is known to translocate to the nucleus where it phosphorylates CREB (Bement & Sluka, 2007). As the current study does not take DRG neurons for mRNA extraction, but only spinal cord sections, the nuclei of first-order neurons reside beyond the spinal cord regions that have been processed for experimentation. Hence, a higher expression of the PRKACA gene may thus be accounted to be within the DDH instead.

Furthermore, a caveat of the qRT-PCR technique following total mRNA extraction is that gene expression represents overall expression of all cell types within the region. Different percentage and subtypes of non-neuronal cells between SDH versus DDH may influence this finding as well.

Looking at gene expression across sex, Basu et al. demonstrated through RT-qPCR, that PRKACA (as well as Grin1) gene expression seems to be conserved in both SHAM animals as well as 2 hours and 21 hours following plantar incision (Basu et al., 2021). The current research also reports similar conserved transcript expression across sex at baseline in both targets.

We next analyzed further the SDH:DDH ratios of all gene targets. This enabled us to determine whether target gene expression (regardless of relative expression to the housekeeping gene) demonstrated a preferential expression favouring one region over the other within individual
targets (per sex). Although no sex differences emerged, \textit{NTRK2}, \textit{FYN}, \textit{GRIN2B}, and \textit{PLCG1} all indicated a significant preferential localization to the SDH than the DDH compared to the other targets. As \textit{NTRK2}, \textit{FYN}, and \textit{GRIN2B} are all directly involved in the BDNF-mediated downstream signalling pathway of NMDAR hyperexcitability during pain, preferential localization of these targets to the SDH provides valuable insight into the signalling and functional mechanisms of these genes within the SDH laminae. This contrasts to the inhibitory signalling targets, KCC2 (\textit{SLC12A5}) and STEP61 (\textit{PTPN5}), which aren't as elevated in the SDH. This predisposes these SDH circuits for increased excitability (albeit in both sexes).

Because BDNF is released into the SDH of the spinal dorsal horn, and the PLC\textsubscript{\gamma} pathway begins by the phospholipase interacting with the BDNF receptor (TRKB), preferential localization of this target is complacent to being at the SDH.

Looking into how these genes are expressed in humans was our next objective. Due to limited spinal donor tissue received (n=2/sex), we were not able to reach power for statistical analyses in these human datasets. Furthermore, a further limitation was that human spinal cord tissue was sampled from the thoracic spinal cord whilst the rat was from the lumbar cord. This was due to limited donor availability. However, previous lab research has shown no significant difference between lumbar and thoracic tissue in the context of NMDAR receptor subunit expression and hence, we decided to proceed with preliminary data collection for all targets.

In humans, gene expression seems to be conserved across regions of the spinal cord for most targets with a potential sex difference in \textit{PTPN5}, \textit{SLC12A5}, \textit{GRIN2B} and \textit{GRIN1} in both the DH and VH. Although the data is preliminary, literature supports sex differences in pain processing in male and female humans. Gonadal steroid hormones are considered to contribute to sex differences in pain sensitivity, especially over the varying phases and circulating hormones during the menstrual cycle (Veldhuijzen et al., 2013). Women with recurrent pain
demonstrated changes in plasma estrogen levels that correlated with pain states (Pieretti et al., 2016). Moreover, evidence supports the analgesic effect of androgens in a clinical study comparing male and female rheumatoid arthritis patients with lower testosterone and DHT levels than the controls (Pieretti et al., 2016).

Estradiol also is reported to modulate pain through endogenous opioid neurotransmission in women. A study examining μ-opioid receptor levels at baseline as well as during sustained pain in healthy men and women demonstrated that women had a higher activation level of this endogenous opioid neurotransmission during the pain stressor. Women also showed regional increases in baseline μ-opioid receptor availability, while males did not differ at baseline versus the pain state (Smith et al., 2006). Hence, potential sex differences seen in the current study may be plausible and have associations with the aforementioned.

*PTPN5*, the gene for the molecular brake, STEP61, is a promising target of interest as literature implies sex differences in pain pathology. The Hargreaves test on 3–6-month-old mice revealed females presenting a lowered paw-withdrawal latency and significantly lowered STEP61 protein level than their age-matched males. However, this sex difference disappeared in older mice (Azkona et al., 2016). Our data demonstrate no sex difference in STEP61 protein nor its gene in 3–4-month-old Sprague Dawley rats while experiments by Azkona et al. were conducted in mice. This suggests a species variance perhaps dependant on developmental age. We also divided the spinal lumbar cord into SDH, DDH and VH; which the former authors did not.

A sex difference in the STEP61 protein is also observed in physiological inflammatory pain states in rats and humans. Dedek et al. account for no effect on the STEP61/pFyn/pGluN2B spinal hyperexcitability pathway in 3-4 month-old female rat SDH synaptosomes in a CFA
inflammatory pain model despite a downregulation of STEP61 observed in male rats (Dedek et al., 2019). Literature on male rodents consistently demonstrates STEP61 hypoactivity to activate GluN2B containing NMDARs and ultimately nociception (Li et al., 2015; Suo et al., 2017). Further teasing out is necessary to determine the exact mechanisms and expression levels of this molecular brake. Whether changes in expression are dependent on age, spinal cord region or cellular region such as synaptosomes, needs to be systematically investigated to be able to make concrete statements regarding the particulars of sex differences and justify the emerging data. Considerations such as differential levels of STEP61 trafficking to synapses and/or co-localization with their target proteins that they dephosphorylate (Fyn and GluN2B) also need to be taken to fully comprehend mechanisms that relate to potential sex differences.

Our next aim was to compare human and rat gene expression. Future studies include plans on adding power to human donor sample data when available. However, a limitation when comparing across species arises due to the differing species-specific primers being used. As primers are commercially prepared to target specific gene sequences, primer efficacy can vary not only across species (albeit targeting the same gene), but also across different primer targets within the same species. The current study ensured that the same gene variant in both rats and humans were selected for gene expression and RT-qPCR to control for variability.

In preliminary analyses, the raw $\Delta C_T$ values of rats, adjacent to the respective human target genes, shed light on the vitality of translational work. Our preliminary human data makes clear suggestions on how there exists variation across species, here at the genetic level of modulators of pain. When baselines differ across species, it becomes apparent that a single therapeutic approach cannot be used to treat pain homogeneously.
For instance, we see a striking difference in gene expression of \textit{FYN} between species: Fyn expression was higher than the housekeeping gene in humans, while substantially lower in rats. Furthermore, while all the targets of this study illustrate no sex differences in rats, human genes potentially present a sex difference in \textit{SLC12A5} (KCC2) and \textit{GRIN1} (GluN1). Hence, it is critical to supplement conventional research with this ongoing translational human tissue approach.

Replicating gene expression analysis studies of our targets in an appropriate model of pain would be the very next step in understanding whether sex differences arise that are specific to pathological pain states. Epigenetic changes as well as the microenvironment that presents in pain states may influence gene and protein expression. Investigating protein expression of all targets pre- and post-induction of pain is vital. In addition, identifying the cell types that represent mRNA and protein expression in our models would also enable a more sophisticated understanding of cellular mechanisms of pain. A challenge of this study was accurately sectioning the spinal cord tissue into SDH, DDH and VH regions without overlapping regions; enhanced techniques need to be developed for accurate spinal cord tissue microdissection in the coronal plane, with minimal overlap. Careful assessment in combination with other techniques such as immunohistochemistry, RNAscope, or single-cell sequencing may be used to validate not only accurate slicing but also gene and protein expression of new targets. It would also be interesting to see how the additional targets, PKA and PLC\textsubscript{γ}, interact with the BDNF-TrkB mediated pathway by using sequestering techniques or blockers to assess any alterations in gene and protein expression of other players and ultimately the outward presentation of pain.
Conclusion

The current research demonstrates for the first time a conserved baseline gene expression of molecular players of the BDNF-TrkB mediated hyperexcitability pathway in rats across sex in sectioned and isolated spinal cord regions. Preliminary data suggests potential sex differences in gene expression in select targets in humans only, such as \textit{PTPN5}, \textit{SLC12A5}, \textit{GRIN2B} and \textit{GRIN1}. In both species, most targets present a preferential localization to the SDH, the region responsible for nociceptive processing. However, striking species differences are implicated in individual target gene transcript levels across sex and region such as \textit{NTRK2}, \textit{SLC12A5}, \textit{FYN}, and \textit{PLCG1}. Further translational studies are crucial to deepen the overall understanding of spinal mechanisms in rodents and humans and thus would enable a more accurate development of safe and effective pain therapeutics.
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86


