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Electrophysiological assessment of TrkB signaling in primary sensory neurons following spinal cord injury

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Electrophysiological assessment of TrkB signaling in primary sensory neurons following spinal cord injury

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B.S., Biology, University of Kentucky, 2016

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An abstract of
a dissertation submitted to the Faculty of the
James T. Laney School of Graduate Studies of Emory University
in partial fulfillment of the requirements for the degree of
Doctor of Philosophy in Graduate Division of Biological and Biomedical Sciences
Molecular and Systems Pharmacology
2024

Abstract

Electrophysiological assessment of TrkB signaling in primary sensory neurons following spinal cord injury

By Kyeongran Jang

Chronic neuropathic pain after spinal cord injury (SCI) is debilitating, maladaptive pain that remains refractory to current treatments. While the nociceptive processing underlying pain hypersensitivity is well-studied in the spinal cord, the mechanisms at peripheral sites of pain integration, such as the dorsal root ganglia (DRG), are less explored. Previous studies that evaluated the contribution of brain-derived neurotrophic factor (BDNF) and tropomyosin receptor kinase B (TrkB) in nociceptive plasticity after SCI have not identified a specific mechanism, although both central and peripheral changes have been reported. Using $TrkB^{F616A}$ (F616) mice, our lab recently found that, after SCI, systemic inhibition of TrkB signaling with 1NM-PP1 (1NMP) delayed onset of SCI-induced pain hypersensitivity, implicating maladaptive TrkB signaling in pain development.

To identify a potential peripheral mechanism by which TrkB signaling contributes to pain after SCI, I investigate changes in TrkB signaling that may drive sensory neuron hyperexcitability after SCI. My overall hypothesis was that SCI-induced hyperexcitability of small-diameter TrkB-responsive neurons, presumed to be nociceptors or Aδ-low threshold mechanoreceptors (Aδ-LTMRs), underlies neuropathic pain after SCI. To test my hypothesis and accomplish my research goals, I obtained DRG neurons from two transgenic mouse strains (F616 and TrkB::ChR2 mice) to characterize changes in 1) neuronal excitability and 2) TrkB-mediated signaling in small-diameter nociceptors or Aδ-LTMRs following SCI. Whereas F616 mice enable selective pharmacogenetic inhibition of TrkB in sensory neurons, TrkB::ChR2 mice express yellow fluorescent protein (EYFP), that allow selective, visual targeting of TrkB+ neurons.

Patch-clamp recordings from dissociated thoracic DRG neurons revealed that the TrkB agonist 7,8-dihydroxyflavone (7,8-DHF) induced inward currents in both nociceptors and TrkB+ neurons. However, following SCI, TrkB-mediated responses decreased in nociceptors but increased in Aδ-LTMRs. Furthermore, changes in electrophysiological and firing properties suggested that SCI induced nociceptors to be more excitable but not Aδ-LTMRs. In general, the inhibition of TrkB did not reverse nociceptor hyperexcitability. The results revealed that thoracic contusion SCI leads to alterations in nociceptor hyperexcitability, but these changes are partly independent of TrkB activation in DRG neurons. This demonstrates complex interactions involving TrkB signaling, potentially recruiting normally non-nociceptive TrkB+ sensory neurons into post-injury nociceptive pathway.

This study is the first to delineate changes in peripheral TrkB signaling after SCI, offering new insights into the peripheral drivers underlying SCI-induced neuropathic pain. The current

findings suggest that peripheral BDNF or TrkB signaling is involved in modulating nociceptive pathways after SCI. Further investigation of potential modulators of TrkB activity, such as voltage-gated sodium channels, that changes neuronal excitability and response amplitude is necessary to gain a deeper mechanistic understanding of pain hypersensitivity and its underlying cellular drivers.

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Acknowledgements

This dissertation is a culmination of many years of dedicated work, collaboration, and invaluable guidance. I would like to acknowledge the role of several important individuals in my graduate career without whom the completion of my degree would not have been possible.

I have to first express my gratitude to my mentor, Dr. Sandra M. Garraway, for her expertise and instruction. She has been the most enthusiastic advocate of my work, encouraging me to be confident and excited about my scientific achievements. Thank you for your patience and grace during some of my most challenging moments.

I also express my appreciation for my thesis committee members, Dr. Peter Wenner, Dr. Shannon Gourley, Dr. Yoland Smith, and Dr. Ellen Hess, for their insightful feedback and encouragement at every meeting. Additionally, I extend my sincere gratitude to Dr. Samantha Yeligar, whose kind words, encouragement, and practical advice helped me through difficult times. I also want to thank the Molecular and Systems Pharmacology program for providing a supportive community throughout my graduate career and resources for the career beyond.

I would like to acknowledge my previous mentors who laid the foundation for my scientific career. Dr. David Magnuson at University of Louisville, for introducing me to research in spinal cord injury; Dr. John Gensel at University of Kentucky, for his patient guidance through fundamental laboratory techniques and scientific literature, which set me on the path toward a career in research; and Dr. Kaiwen Kam at Rosalind Franklin University of Medicine and Science, for giving me the freedom to develop confidence as an independent researcher.

On a personal note, I would like to express my deepest love and gratitude to my family:

To my husband – DR. Jacob Mattingly. My appreciation and love for you cannot possibly be expressed completely on this page. I am proud of us for reaching the end of this tunnel together and, come what may, excited to start the next chapter of our lives together. Thank you for appreciating my love for electrophysiology and letting me bounce off experimental, technical, and scientific frustrations, confusions, and enthusiasm. (Calculating dilutions is still difficult.)

My parents – Meejeong Han and Jaeil Jang. Twenty years ago, my mom crossed an ocean with me, where I grew up watching her study physiology, biology, and neuroscience in an unfamiliar language while working to support our family. She is my role model, my best friend, and my biggest inspiration. My dad, whose dry humor, love for deep knowledge, and relentless pursuit of his passion I have inherited, has always offered the best, wisest advice for difficult situations.

My second parents – Lisa and Mike Mattingly, I am truly the luckiest person to be a part of your family. Thank you for always welcoming me with open arms and for the thoughtful gestures that bring joy and comfort. Thank you for the periodic boxes of sweets, trips to the beach, and those five consecutive nights of ice cream. Every moment I spend with you I get to be a child again.

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List of Abbreviations

ACSF Artificial cerebrospinal fluid

AHP: Afterhyperpolarization

AHP_{50}: 50% afterhyperpolarization amplitude recovery back to baseline

AHP_{80}: 50% afterhyperpolarization amplitude recovery back to baseline

AP: Action potential

Akt/PKB Akt kinase / protein kinase B

AMPAR: α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid

ANS: Autonomic Nervous System

ANOVA: analysis of variance

BAC: Bacterial artificial chromosomal

BCA Bicinchoninic acid

BDNF Brain-derived neurotrophic factor

BMS: Basso Mouse Scale

cAMP: cyclic adenosine monophosphate

Cav: T-type calcium channels

CGRP: Calcitonin gene-related peptide

ChR2: Channelrhodopsin-2

C_m: Membrane capacitance

CNS: central nervous system

COX-2: cyclooxygenase-2

DAG: diacylglycerol

DMSO: dimethyl sulfoxide

Dpo: Days post operation

DRG: dorsal root ganglion

ER: Estrogen receptor

ERK: extracellular signal-regulated kinase

EPSP: excitatory postsynaptic potentials

EYFP: Enhanced yellow fluorescent protein

FL: Full length

GABA: gamma-aminobutyric acid

GFAP: glial fibrillary acid protein

HBSS: Hanks' balanced salt solution

HEPES: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HTMR: High threshold mechanoreceptors

IA: Intermediately adapting

IASP: International Association for the Study of Pain

IB4: Isolectin B4

IL: interleukins

IP3: inositol-1, 4, 5-trisphosphate

JNK: c-Jun *N*-terminal kinase

KCC: K^+-Cl^- cotransporter

K-EDTA: Potassium ethylenediaminetetraacetic acid

Kv: Voltage-gated potassium channels

LTMR: Low threshold mechanoreceptors

LTD: Long-term depression

LTP: Long-term potentiation

MAPK: mitogen-activated protein kinase

MCP-1: monocyte chemoattractant protein

mEPSC: miniature excitatory postsynaptic currents

mGluR: metabotropic glutamate receptors

Nav: voltage-gated sodium channel

NGF: Nerve growth factor

NMDAR: N-methyl-D-Aspartate receptor

1NMP: 1NM-PP1; 1-(1, 1-dimethylethyl)-3-(1-naphthalenylmethyl)-1H-pyrazolo[3,4-d]pyrimidin-4-amine

NT3: Neurotrophin-3

NT4/5: Neurotrophin-4/5

PBS: phosphate-buffered saline

PFA: Paraformaldehyde

PI3K: phosphoinositide 3-kinase

PKC: protein kinase C

PNS Peripheral nervous system

PLC: phospholipase C

p75NTR: p75 neurotrophin receptor

RA: Rapidly adapting

R_{in}: Input resistance

RM: Repeated measures

RMP: Resting membrane potential

ROS: Reactive oxygen species

SA: Slowly adapting

SCI: Spinal cord injury

SCDH: spinal cord dorsal horn

SDS-PAGE: Sodium dodecyl-sulfate Polyacrylamide gel electrophoresis

SEM: Standard error of the mean

SGC: Satellite glial cells

siRNA: Small interfering RNA

SP: Substance P

TAM: Tamoxifen

TBST: Tris-buffered saline Tween-20

TH: Tyrosine hydroxylase

TNF-α: tumor necrosis factor- α

TRPV1: transient receptor potential vanilloid 1

Trk: Tropomyosin receptor kinase

TrkB.Fl: Tropomyosin receptor kinase B, Full-length

TrkB.T1: Tropomyosin receptor kinase B, Truncated 1

TTX: Tetrodotoxin

TTX-s: Tetrodotoxin-sensitive

TTX-r: Tetrodotoxin-resistant

Veh: Vehicle

VGLUT: Vesicular glutamate transporter

VGSC Voltage-gated sodium channels

WT: wild type

Chapter 1: Introduction

The central nervous system (CNS) is a highly complex network responsible for processing and transmitting signals between the brain and the spinal cord, and peripheral regions of the body (Sherrington, 1926). This interconnected system, composed of neurons and glia, facilitates integration of diverse stimuli and orchestrates complex responses, while the peripheral nervous system (PNS), which includes sensory and motor components, mediates interactions between the body and the environment.

Over the past century, foundational studies by (Golgi, 1873) and Ramon y Cajal (Ramón, 1987) have greatly advanced our understanding of the nervous system, elucidating key aspects of the neuronal morphology and network. Pain, however, remains a particularly complex aspect of nervous system function due to its multifaceted nature, involving sensory, emotional, and cognitive dimensions (Melzack & Wall, 1965). The intricacy of pain is due to its subjective interpretation and the myriads of pathways and modulatory mechanisms that influence its perception. Pain can be adaptive and maladaptive (Woolf, 2010). One categorization that is commonly used is nociceptive pain versus neuropathic pain. Nociceptive pain results from actual tissue damage or potentially damaging stimuli (Goucke, 2003). On the other hand, neuropathic pain is less localized and caused by damage to the nervous system (Goucke, 2003). Neuropathic pain can occur following nervous system lesion or dysfunction, such as spinal cord injury, and can be sustained by a wide range of factors involving both molecular and cellular changes in the environment, even in the absence of further pain-causing stimuli (Nicholson, 2006). Various models and approaches have been employed to study pain, yet despite significant progress, the specific pathways and molecular components involved in pain modulation remain only partially understood.

Spinal cord injury (SCI) is a traumatic insult to the nervous system that triggers cascades of immune and neuronal responses locally and systemically. Because SCI can lead to debilitating sensorimotor dysfunctions, changes in the CNS have been studied extensively in context of locomotor recovery and spinal cord regeneration. However, chronic neuropathic pain is frequently reported by individuals with SCI (up to 80%), manifesting as allodynia, hyperalgesia, and spontaneous pain, which severely limits daily activities and psychological well-being (Hulsebosch et al., 2009). Despite the high prevalence, the mechanisms driving chronic pain are limited (Decosterd & Woolf, 2000). Bridging this gap of knowledge is critical for developing comprehensive and effective treatment strategies that address both functional impairments and chronic pain.

Emerging evidence suggests that peripheral mechanisms, particularly those involving the dorsal root ganglia (DRG), play a critical role in pain hypersensitivity. Among these peripheral mechanisms, brain-derived neurotrophic factor (BDNF) signaling through its receptor, tropomyosin kinase B (TrkB), has surfaced as a potential key modulator of nociceptor activity. Although BDNF-TrkB signaling is well-characterized within the CNS, its role in pain development and nociceptor sensitization following SCI remains to be studied. This gap in knowledge likely stems from the traditional focus on central pain processes and the technical challenges associated with selectively manipulating neurotrophins and their receptors. Understanding how peripheral TrkB signaling contributes to pain hypersensitivity is critical for developing novel therapeutic strategies that target underlying causes of pain, rather than merely addressing symptoms. Therefore, this dissertation aims to fill this gap by investigating the role of peripheral TrkB signaling in the DRGs, specifically in the context of neuropathic pain after SCI.

1.1 The spinal cord: Introduction

The nervous system is broadly divided into the CNS, which is comprised of the brain and spinal cord, and the PNS, which mediates interactions between the CNS and the rest of the body. The PNS includes sensory neurons that detect environmental stimuli and motor neurons that control muscle and gland functions (Le Pichon & Chesler, 2014; Stifani, 2014). It also encompasses the autonomic nervous system (ANS), responsible for involuntary bodily functions integral to maintaining homeostasis. Dysfunction of the PNS can lead to a wide range of sensory and motor impairments (Lanigan et al., 2021), which will be discussed in depth in Chapter 2, particularly in the context of pain.

1.1.1 The spinal cord: structure and anatomy

The spinal cord is a vital region of the CNS that extends from the lower brain stem, serving as the primary conduit for neural signals between the brain and the periphery. Protected by the vertebral column and encased in meninges (dura, arachnoid, and pia mater; Frostell et al. (2016)), the spinal cord is organized into 5 main segments: cervical, thoracic, lumbar, sacral, or coccygeal (Bican et al., 2013; Krames, 2014). Each segment gives rise to a pair of spinal nerves (Bican et al., 2013) that innervate and receive somatosensory inputs from different parts of the body. Cervical nerves (C1-C8) innervate the neck, shoulders, and arms; thoracic nerves (T1-T12) innervate the trunk and abdominal muscles; and lumbar (L1-L5) and sacral (S1-S5) nerves control lower back and general lower body regions (Bennett et al., 2024). From the spinal nerves, dorsal root ganglion (DRG) emerges from the dorsal root, carrying sensory information received from the various receptors on the peripheral nerve terminal. DRGs are well-known contributors to chronic pain in peripheral processes, such as inflammation and neuropathic pain (Noh et al., 2020).

A cross-section of the spinal cord reveals an “H”-shaped gray matter, which houses the cell bodies of neurons, dendrites, axons, and glia (Harrow-Mortelliti et al., 2019), surrounded by white matter, which contains myelinated axons (Frostell et al., 2016; Harrow-Mortelliti et al., 2019). The gray matter is organized into ten different layers, or laminae, each associated with specific functions. Of particular interest is the spinal cord dorsal horn (SCDH), which plays an important role in processing sensory information, including nociceptive (pain) signals (Petkó & Antal, 2012). The white matter consists of descending or ascending tracts (Cho, 2015), including the spinothalamic tract, which is critical for transmitting pain and other sensory signals to the brain. The role of the spinothalamic tract, particularly in relation to its involvement in both central and peripheral sensitization following SCI, will be further discussed in Chapter 2.

The structure and function of the spinal cord make it highly susceptible to damage from traumatic injuries. Because the spinal cord plays an important role in mediating signal transduction between the central and peripheral nervous systems, location and the severity of SCI can significantly impact sensorimotor outcome in an individual (Alizadeh et al., 2019).

1.1. Spinal cord injury

Spinal cord injury (SCI) results from trauma to the spinal cord, leading to partial or complete loss of motor, sensory, and autonomic functions below the level of the injury. SCI occurs in two main phases (Ahuja et al., 2017; Alizadeh et al., 2019; Anjum et al., 2020; Oyinbo, 2011): primary injury, which results from the initial mechanical trauma; and the secondary injury, which is a cascade of molecular and cellular events that follow. During secondary injury, processes such as ischemia and subsequent tissue death, excitotoxicity, inflammation, and glial scar formation further exacerbate tissue damage and functional loss. Additionally, chronic pain syndromes often emerge as a consequence of central and peripheral sensitization, where enhanced neuronal

excitability and altered pain signaling pathways contribute to persistent pain, potentially due to changes in DRG neurons. Clinically, changes in pain perception, including after SCI, are reported as hyperalgesia, which is heightened sensitivity to pain, and allodynia, which is pain in response to normally non-painful stimuli. The complex interplay between these processes underlies the chronic and complex nature of SCI-related pain and will be discussed more in detail in Chapter 2.

1.1.3 The dorsal root ganglia and nociceptors

The DRG is a cluster of cell bodies derived from primary afferent neurons, positioned at the intersection of the PNS and the CNS. DRG neurons have dual roles: they receive sensory input from the periphery via their peripheral processes and relay this information to the spinal cord through their central processes (Esposito et al., 2019; Lee et al., 1986). The DRGs play a pivotal role in sensory signal transduction, including pain perception, which will be explored in detail in Chapter 2.

Nociceptors, a subset of specialized sensory neurons within the DRG, are responsible for detecting high-threshold mechanical, thermal, and chemical stimuli (Dubin & Patapoutian, 2010). Sensory neurons, including nociceptors, innervate various tissues, such as skin, viscera, muscle, or joint (see reviews by Raja et al. (1988); Willis Jr and Coggeshall (2012); Robinson and Gebhart (2008)). Nociceptors are classified into two primary groups: mechano-sensitive Aδ-myelinated fibers and polymodal unmyelinated C-fibers (Glatte et al., 2019). Under normal conditions, nociceptors only respond to high-threshold, potentially harmful stimuli; however, after an injury, their sensitivity can increase, leading to an amplified pain signaling. Nociceptor sensitization is particularly relevant in the context of SCI, where damage to both the CNS and the PNS disrupts normal pain signaling. Injury-induced changes in nociceptors lower their

activation thresholds and increase their excitability, resulting in heightened pain sensitivity, a phenomenon known as peripheral sensitization (Gold & Gebhart, 2010). Thus, understanding the role of DRGs and nociceptors in pain perception in neuropathic pain development following injury, is a central focus of this dissertation.

1.4 Central and peripheral sensitization

Pain typically serves as an adaptive response to harmful stimuli or as a protective measure against further tissue damage; however, following injury, it can become chronic and maladaptive, triggered by otherwise innocuous or moderate stimuli. As briefly mentioned above, peripheral sensitization occurs when damage or inflammation lowers the threshold for nociceptor activation, resulting in hyperexcitability. The release of inflammatory mediators drives neuronal hyperexcitability, which sensitizes receptors on nociceptor terminals, causing these neurons to fire more frequently and respond to weaker stimuli. This process plays a crucial role in the development of post-SCI neuropathic pain, potentially by altered DRG nociceptor thresholds. Central sensitization, on the other hand, refers to the enhanced responsiveness within the spinal cord and other central pain pathways following repeated or prolonged nociceptor activation. This involves changes in receptor activity and intracellular signaling within the SCDH, where the accumulation of excitatory input leads to amplified pain responses. Even normally innocuous stimuli can be perceived as painful, a condition known as allodynia. After SCI, central sensitization is thought to be sustained by ongoing peripheral input from sensitized nociceptors, leading to persistent pain state (Carlton et al., 2009; Gwak et al., 2006; Wall & Devor, 1981). Additionally, intrinsic changes in central neurons, such as alterations in synaptic plasticity (Latremoliere & Woolf, 2009; Woolf, 1983), the activation of microglia and astrocytes (Zeilhofer, 2008), and enhanced immune responses contribute to the maintenance and

amplification of central sensitization (Donnelly et al., 2020; Kawasaki et al., 2008). The feedback loop between peripheral and central sensitization mechanisms is critical for understanding chronic neuropathic pain. Both central and peripheral sensitization are foundational to the development of neuropathic pain after SCI and will be considered in depth in context of TrkB signaling in following chapters.

1.5 Brain-derived neurotrophic factor and tropomyosin kinase receptor B

Brain-derived neurotrophic factor (BDNF) and its high affinity tropomyosin receptor kinase B (TrkB) play a critical role in neuronal development, survival, and synaptic plasticity in the CNS. TrkB is a transmembrane receptor that undergoes dimerization and autophosphorylation upon binding of BDNF. This triggers multiple signaling cascades that regulate processes like neuronal proliferation, axonal growth, and synaptic strength (Huang and Reichardt, 2001; Numakawa et al., 2010). Central BDNF-TrkB signaling has been extensively studied for its role in promoting long-term potentiation (LTP), learning, and memory in brain regions like the hippocampus (Schuman, 1999). Given its role in promoting adaptive plasticity, BDNF-TrkB signaling has been widely explored for potential therapeutic applications, particularly in promoting functional and locomotor recovery after SCI (Houle & Côté, 2013; Mantilla et al., 2013). However, other studies suggest that BDNF-TrkB signaling also contributes to pro-nociceptive effects, both centrally and peripherally, following injury (Ding et al., 2020; Garraway & Huie, 2016; Lin et al., 2011; Park & Poo, 2013; Thibault et al., 2014; Zhang et al., 2016; Zhou et al., 2011), suggesting a dual role in modulating plasticity.

In the PNS, BDNF is synthesized by sensory neurons in the DRG, and TrkB is also expressed on certain subpopulations of sensory neurons. In these sensory neurons, BDNF-TrkB signaling regulates both normal sensory function and the modulation of pain. Upon peripheral nerve injury

or inflammation, BDNF is anterogradely transported from the DRG somas to peripheral and central terminals to enhance nociceptive signaling, thereby contributing to heightened pain perception (Cho et al., 1998; Ernfors et al., 1993; Fukuoka et al., 2001; Ha et al., 2001; Merighi et al., 2008; Michael et al., 1997; Pezet, Malcangio, Lever, et al., 2002; Zhou et al., 1999). TrkB activation has been linked with hypersensitization of thermal sensory neurons (Shu et al., 1999) and mechanical allodynia (Dhandapani et al., 2018; Hu et al., 2023). While BDNF is not the only endogenous ligand that binds TrkB, neurotrophin (NT)-4 does not significantly contribute to nociceptive or neuropathic pain (Buck et al., 2000; Funakoshi et al., 1993; Heppenstall & Lewin, 2001). Therefore, the current dissertation will focus on BDNF-TrkB signaling and its involvement in pain pathways.

Previous research from our lab revealed a novel finding that maladaptive TrkB signaling is a key driver of mechanical hypersensitivity immediately following SCI (Martin et al., 2022). Based on the results of this study, the primary goal of this dissertation will be to identify the potential site of TrkB activation, with emphasis on periphery, and the specific molecular mechanisms underlying the onset of mechanical hypersensitivity after SCI. Given the evidence of bidirectional plasticity promoted by BDNF-TrkB signaling, changes in BDNF and TrkB expression or signaling in the periphery could influence neuronal mechanisms that drive pain hypersensitivity.

1.6 Summary

This dissertation aims to bridge the gap in our understanding of the peripheral contributors of chronic pain, particularly following SCI. I begin by reviewing prior studies on neuropathic pain, especially in the context of SCI, and examining how changes in peripheral components may contribute to development of chronic pain. Specifically, I discuss changes in the DRGs and,

based on previous findings, suggest that peripherally expressed BDNF and TrkB may be contributing to nociceptor sensitization after SCI (see Figure 1.1 for a graphical summary of various components discussed in the review and the rest of the dissertation). In subsequent chapters, I will assess the contribution of peripheral TrkB in driving nociceptor hyperexcitability after SCI in two different transgenic mouse strains that enable selective targeting of TrkB. I will conclude by providing a comparative electrophysiological analysis of these mouse models, proposing experimental strengths and limitations, and discussing future directions for further studies.

1.7 Figures

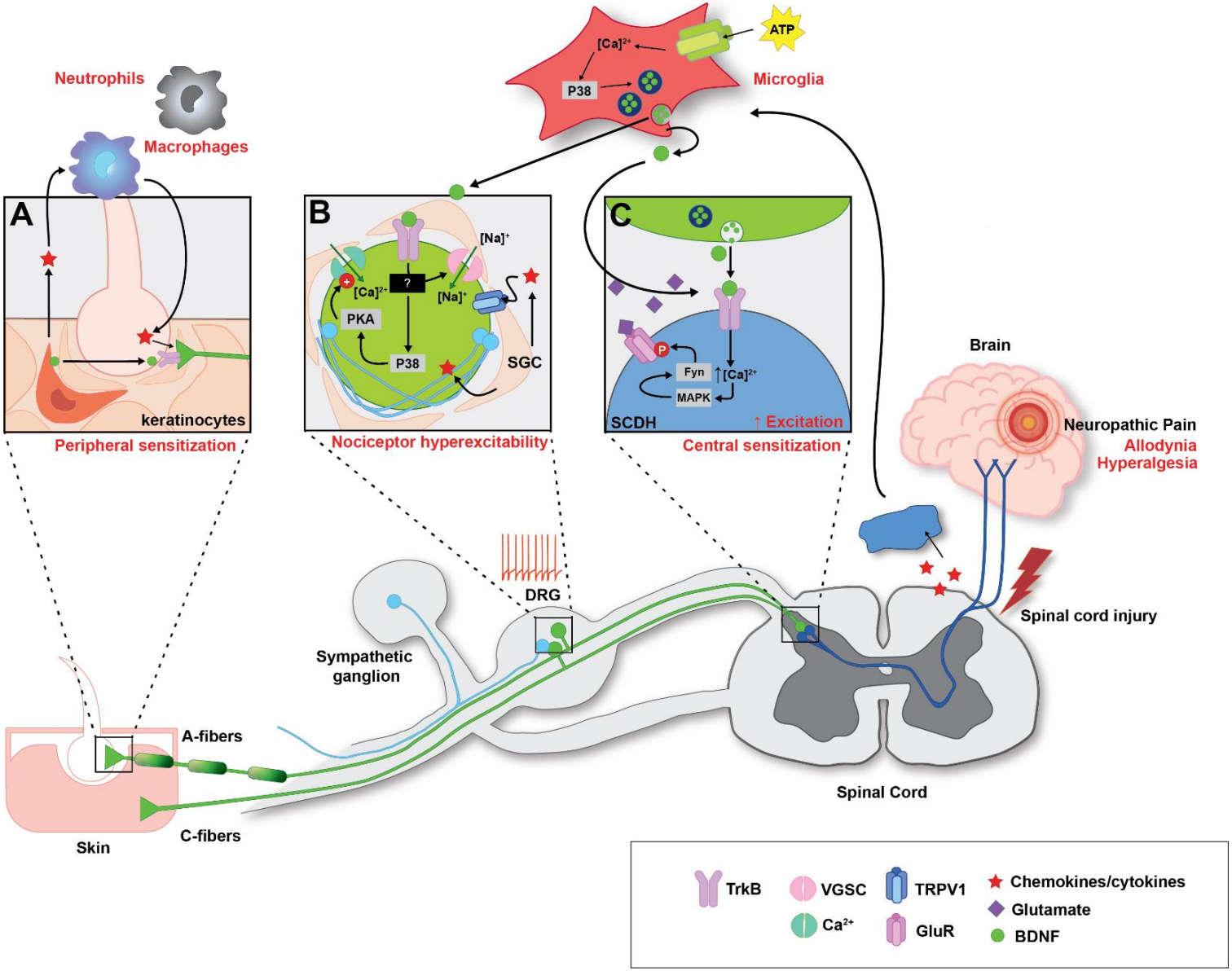

Neutrophils
Macrophages
A
keratinocytes
Peripheral sensitization
B
[Na]+
[Ca]2+
?
[Na]+
PKA
P38
SGC
Nociceptor hyperexcitability
[Ca]2+
P38
Microglia
ATP
C
P
Fyn
↑[Ca]2+
MAPK
SCDH
↑ Excitation
Central sensitization
Brain
Neuropathic Pain
Allodynia
Hyperalgesia
DRG
Spinal cord injury
Sympathetic ganglion
A-fibers
C-fibers
Skin
Spinal Cord
TrkB
VGSC
Ca2+
TRPV1
GluR
Chemokines/cytokines
Glutamate
BDNF

Figure 1.1: Summary of various components in the periphery implicated for dysregulation of nociceptive circuit after SCI with BDNF-TrkB system as an example.

A) Keratinocytes release growth factors (including BDNF) and cytokines to recruit macrophages and neutrophils, which further amplify inflammatory response by secreting more pro-inflammatory cytokines and chemokines (e.g., interleukin [IL]-1β, tumor necrosis factor [TNF]-α). TrkB receptors are expressed on non-nociceptor sensory neurons (e.g., Aδ-LTMRs). During pathological conditions, BDNF derived from immune, epithelial, and Schwann cells can presumably interact with peripherally situated TrkB receptors to functionally alter the nociceptive circuit. ***B)*** BDNF acting through TrkB may participate in nociceptor hyperactivity by subsequent activation of downstream signaling cascades, such as phosphatidylinositol 3-kinase (PI3K) and mitogen-activated protein kinase (MAPK; p38). Studies implicate p38-dependent PKA signaling that stimulates T-type calcium (Ca^{2+}) channels (i.e., Cav3) to regulate T-currents that may contribute to nociceptor hyperfunction. Certain subtypes of voltage-gated sodium channels (VGSCs; tetrodotoxin [TTX]-Resistant Nav 1.9) have been observed to underlie BDNF-TrkB-evoked excitation. Interaction between TrkB and VGSCs has not been clarified, but it may alter influx of sodium to change nociceptor excitability. DRGs also express transient receptor potential vanilloid (TRPV) 1, which is sensitized by cytokines such as TNF-α. Proliferating satellite glial cells (SGCs) surrounding DRGs release cytokines to further activate immune cells and trigger release of microglial BDNF. Sympathetic neurons sprout into DRGs to form Dogiel's arborization, which have been observed in spontaneously firing DRG neurons. Complex interactions between these components lead to changes in nociceptor threshold and behavior, leading to hyperexcitability. ***C)*** Synaptic interactions between primary afferent terminals and dorsal horn neurons lead to central sensitization. Primary afferent terminals release

neurotransmitters and modulators (e.g., glutamate and BDNF) that activate respective receptors on SCDH neurons. Sensitized C-fibers release glutamate and BDNF. BDNF binds to TrkB receptors, which engage downstream intracellular signaling cascades including phospholipase C (PLC), protein kinase C (PKC), and Fyn to increase intracellular Ca^{2+}. Consequently, increased Ca^{2+} increases phosphorylation of GluN2B subunit of N-methyl-D-Aspartate receptor (NMDAR) to facilitate glutamatergic currents. Released glutamate activates NMDA or α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors to activate post-synaptic interneurons.

Chapter 2: A review of dorsal root ganglia and primary sensory neuron plasticity mediating inflammatory and chronic neuropathic pain

This chapter is an adaptation of published work: K Jang, Garraway SM.
A review of dorsal root ganglia and primary sensory neuron plasticity mediating inflammatory and chronic neuropathic pain. Neurobiology of Pain. 2024 Jan 20; 15:100151.
doi: 10.1016/j.ynpai.2024.100151. PMID: 38314104; PMCID: PMC10837099.

2.1 Abstract

Pain is a sensory state resulting from the complex integration of peripheral nociceptive inputs and central processing. Pain can be categorized as adaptive, which is acute and beneficial for healing, or maladaptive, which is often persistent and pathological. Pain is indeed heterogeneous and can manifest as nociceptive, inflammatory, or neuropathic. Neuropathic pain is an example of maladaptive pain that occurs after spinal cord injury (SCI), which triggers a wide range of neural plasticity. The nociceptive processing that underlies pain hypersensitivity is well-studied in the spinal cord. However, recent investigations show maladaptive plasticity that leads to pain, including neuropathic pain after SCI, also exists at peripheral sites, such as the dorsal root ganglia (DRG), which contains the cell bodies of sensory neurons. This review discusses the important role DRGs play in nociceptive processing that underlies inflammatory and neuropathic pain. Specifically, it highlights nociceptor hyperexcitability as critical to increased pain hypersensitivity. Furthermore, it reviews prior literature on glutamate and glutamate receptors, voltage-gated sodium channels (VGSC), and brain-derived neurotrophic factor (BDNF) signaling in the DRG as important contributors to inflammatory and neuropathic pain. While BDNF's role as a bidirectional neuromodulator of spinal plasticity has been previously reviewed, this chapter shifts the focus to peripheral processes, discussing BDNF-TrkB expression on nociceptors, non-nociceptor sensory neurons, and non-neuronal cells as a potential contributor to induction and persistence of pain after SCI. Overall, this chapter presents a comprehensive evaluation of large bodies of work that individually focus on pain, DRG, BDNF, and SCI, to understand their interaction in nociceptive processing.

2.2 Introduction

Pain is a physiological response to injury, defined by the International Association for the Study of Pain (IASP) as ‘an unpleasant sensory and emotional experience associated with, or resembling that associated with, actual or potential tissue damage’ (Pain, 2017). Pain can be categorized as adaptive or maladaptive. Adaptive pain includes inflammatory pain, which is an increase in sensitivity due to an inflammatory response resulting from tissue damage, and nociceptive pain, which is caused by the activation of primary nociceptors located in the peripheral nervous system in somatic (skin, muscle, or bone) or visceral (body organs) tissue. Maladaptive pain typically involves abnormal functioning of the nervous system and is neither protective nor informative. Neuropathic pain, which results from injury to the somatosensory nervous system, is an example of maladaptive pain.

Nociceptive processing underlying adaptive and maladaptive pain involves both peripheral and central mechanisms. In the periphery, post-translational modifications result in peripheral sensitization, where the sensitivity of the peripheral terminals of primary nociceptors is increased (Lewin et al., 1993; Lewin et al., 1994). Meanwhile, central mechanisms involve central sensitization (Woolf, 1983), which refers to an increased excitability of spinal neurons triggered by peripheral noxious input, creating a state in which responses to normal inputs are amplified.

The DRG houses the cell bodies of primary sensory neurons and projects axons to both the peripheral site of injury and the dorsal horn of the spinal cord. Hence, the DRG is a major site of nociceptive processing in both adaptive and maladaptive pain states. Various membrane proteins implicated in the pain pathways are found in DRGs and expressed in nociceptors, include voltage gated ion channels, glutamate receptors and transporters, and G-protein-coupled receptors. DRG neurons also express numerous cytokines and chemokines and their receptors, highlighting the

important role DRGs play in nociception after tissue injury (Miller et al., 2009; White et al., 2007). While much focus has been given to the SCDH, an important central site for the integration of incoming sensory and descending input, less attention has been given to the DRG and peripheral processes that lead to pain hypersensitivity, particularly in the context of neuropathic pain.

SCI leads to significant sensorimotor dysfunction, including chronic neuropathic pain. Extensive research has explored changes in the spinal cord that potentially contribute to the development and maintenance of neuropathic pain. One of the most promising targets for promoting adaptive plasticity and functional recovery after SCI is BDNF due to its role in neuronal growth and development (Barde et al., 1982; Park & Poo, 2013). BDNF, acting through its high affinity receptor Trk B, promotes adaptive plasticity in both uninjured and injured spinal cord. However, BDNF has also been implicated in pain modulation (Merighi et al., 2008; Pezet, Malcangio, & McMahon, 2002) and in central sensitization (Alles et al., 2021; Biggs et al., 2010; Sikandar et al., 2018). Although the expression of BDNF in nociceptors has long been recognized (Kerr et al., 1999; Thompson et al., 1999), more recent studies have shown that BDNF is also expressed in non-nociceptors in the DRG (Rutlin et al., 2014) as well as non-neuronal cells in the periphery (Hahn et al., 2006; Hahn et al., 2005; Wang et al., 2015). Therefore, any exploration into the peripheral processes and the role of the DRG in mediating neuropathic pain development after SCI must also account for BDNF signaling, which is likely modulating the activity of ion channels, glutamate receptors, and cytokines in the DRG.

Given the limited number of studies that have methodically examined DRG plasticity and peripheral BDNF signaling in the development of neuropathic pain after SCI, this work aims to evaluate what is currently known and bring together independent lines of research that focus on

pain, nociceptors and DRG, BDNF and TrkB, and SCI, to develop a comprehensive understanding of how these components work together (see Figure 2.1, Table 2.1 for examples of previous work).

Therefore, the present chapter begins by providing an overview of adaptive and maladaptive pain. It then discusses the DRG as an important peripheral site for nociceptive processing and nociceptor hyperexcitability that leads to both inflammatory and neuropathic pain, particularly after SCI. The chapter also reviews BDNF-TrkB signaling in pain after SCI, with a detailed focus on the role of BDNF-TrkB signaling in pain modulation and plasticity. The focus of the chapter is to discuss cellular and molecular changes in the DRGs and the primary afferent sensory neurons after peripheral inflammatory insults and SCI, assessing how these altered mechanisms may underlie development and maintenance of neuropathic pain.

2.3 Overview of pain

Pain is a complex sensory state that integrates a variety of external pain-causing, or noxious, inputs through both peripheral and central nervous system processing. Pain can be broadly categorized as adaptive or maladaptive and is composed of a perceptive, reflexive component as well as an affective and emotional component (Melzack & Casey, 1968; Raja et al., 2020). Under normal circumstances, pain resulting from inflammation or tissue damage is considered adaptive or protective measure (Millan, 1999; Woolf, 2010). Normal pain is acute and occurs to minimize contact with the noxious mechanical, thermal, or chemical stimuli that are high-threshold and intense (Basbaum et al., 2009), demanding immediate attention and activating motor withdrawal reflexes to prevent further damage (Millan, 1999). In response to injury or tissue damage, specialized peripheral sensory neurons called nociceptors detect nociceptive stimuli and transmit pain information to the initial pain processing site of the CNS, the SCDH. Nociceptive signals

can regulate motor and neural activities through ascending pain pathways (Grau, 2017), resulting in behavioral and cognitive responses, such as reflexive withdrawals and emotional responses like fear or anger. Nociception can be modified by both spinal (Grau, 2017) and peripheral afferent inputs (Garraway et al., 2014; Yang et al., 2014). After initial pain stimuli, nociceptors are further stimulated by the "inflammatory soup" composed of inflammatory mediators like bradykinin, serotonin, prostaglandins, and neurotrophins, which lowers their activation threshold. The release of neuropeptides and other substances trigger an immune response, termed neurogenic inflammation (Loeser & Melzack, 1999), which not only promotes wound healing and protection against potential infections (Millan, 1999), but also lowers the pain-sensing threshold, so that the nociceptors can now detect stimuli with a wider range of intensities, preventing further damage. The decreased activation threshold results in a phenomenon known as pain hypersensitivity (Woolf, 2010), while neurochemical changes in the environment generate peripheral sensitization (Lewin et al., 1993; Lewin et al., 1994).

Nociceptors then transmit the pain information via glutamate release (Petrenko et al., 2003) to the SCDH, where transducer proteins (e.g., transient receptor potential vanilloid 1 (TRPV1) receptors) respond to extrinsic or intrinsic stimuli, such as chemical or heat (Caterina et al., 1999). These signals initiate neurotransmitter release, activating excitatory and inhibitory synaptic transmission mediated by AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid), NMDA (N-methyl D-aspartate), or GABA (γ-aminobutyric acid) receptors (Chery & de Koninck, 1999). Repetitive activation of transducers can lead to autosensitization, where, for example, TRPV1 receptors undergo conformational changes after repeated heat or capsaicin exposure (Caterina et al., 1997). Additionally, increased excitability in the terminal membrane, such as through changes in excitatory postsynaptic potentials that activate NMDA receptors

(Mayer et al., 1984) and voltage-gated calcium channels (Morisset & Nagy, 1999), can lead to a phenomenon known as windup (Woolf & Salter, 2000), amplifying the pain response. These changes occur due to phosphorylation of receptors or ion channels, altering intrinsic functional properties or cell-surface expression of channels or regulatory proteins in dorsal horn or primary sensory neurons (Woolf & Salter, 2000). After transmitting signals to the SCDH, the pain information is subsequently relayed up the spinothalamic tract to the thalamus, where the gain of signal is regulated by the locus coeruleus neurons releasing norepinephrine (Voisin et al., 2005).

Apart from acute, protective pain, some pain outlasts the initial inflammatory, tissue or nerve injury and is not elicited by a particular external stimulus. Maladaptive pain becomes chronic and debilitating and can be expressed as hyperalgesia (i.e., painful stimulus is perceived as more painful) and allodynia (i.e., non-painful stimulus now perceived as painful). Maladaptive pain is neither protective nor adaptive and can occur spontaneously. Transmission of pain is further regulated by descending projections from the brain to the SCDH (Melzack & Wall, 1965), maintaining a baseline level of sensory processing (You et al., 2010). Illness or injury, such as SCI, disrupts this balance, promoting facilitation and maintenance of chronic pain (Ossipov et al., 2014). Neuropathic pain, caused by injury to the nervous system (Millan, 1999; Peirs & Seal, 2016) is an example of maladaptive pain. Figure 2.2 illustrates key differences between nociceptive and neuropathic pain.

Furthermore, somatosensory pain is generally accompanied by cognitive components (Melzack & Casey, 1968; Raja et al., 2020), such as selective attention to pain (Desimone & Duncan, 1995) and cognitive evaluation of the sensation (Kalisch, 2009), which result in emotional and behavioral responses. Both acute and chronic pain can elicit anger, fear, or cognitive effects that are comparable to depressive-like states (Costigan et al., 2009; Woolf & Mannion, 1999), which

are coupled with autonomic and immune responses, including increased anxiety, heart and respiratory rate, and galvanic skin response (Tousignant-Laflamme & Marchand, 2006). Pro-inflammatory cytokines and stress hormone cortisol are released to enhance nociception and further facilitate aversive information in the brain (Chapman et al., 2008; Sommer & Kress, 2004). Finally, behavioral reactions aimed at temporarily reducing pain may actually worsen social and functional disability in the long term (Turk & Flor, 1987; Vlaeyen & Linton, 2000). Despite the prevalence and the severe impact of chronic pain, it is still poorly understood and inadequately managed (Finnerup et al., 2010). A better understanding of the mechanisms underlying chronic pain is essential for developing new therapies to treat chronic neuropathic pain.

2.4 Overview of spinal cord injury

The spinal cord extends from the base of the brain through the vertebral column, transmitting sensory and motor signals between the brain and the body through corticospinal and spinothalamic tracts and the dorsal columns (Bennett et al., 2024). These pathways are responsible for voluntary motor movement, pain and temperature sensation, and proprioception and tactile sensations, respectively (Bennett et al., 2024). Damage to the spinal cord not only affects motor control but also impacts the functions throughout the body. SCI is mechanical damage to the spinal cord that leads to motor deficits below the site of the lesion, autonomic dysfunction, and chronic pain. In the U.S., there are approximately 302,000 individuals living with SCI, with an estimated 18,000 new cases occurring each year (NSCISC, 2023).

The neurologic deficits produced by SCI depend on the location and the degree of the injury. Lesions in the lower thoracic level may result in paraplegia, and cervical level lesions are associated with quadriplegia (Wilson et al., 2012). Most common injury site is C5 (50%)

(Hachem et al., 2017), followed by thoracic (35%) and lumbar (11%) regions. SCI involves mechanical trauma that fractures or dislocates the vertebrae, which compresses, distracts, or transects the spinal cord within. Primary injury occurs immediately from the traumatic impact, usually due to bone fragments or two adjacent vertebrae being pulled apart, directly damaging the ascending and descending pathways in the spinal cord (Figley et al., 2014). Primary injury leads to spinal shock, systemic hypotension, ischemia, ionic imbalances, and neurotransmitter accumulation (Fehlings & Sekhon, 2002). Secondary injury follows within minutes and can last for weeks or months, involving a series of cellular, molecular, and biochemical processes that cause progressive tissue damage around the lesion (Oyinbo, 2011; Rust & Kaiser, 2017). During the sub-acute phase of secondary injury, processes such as apoptosis, demyelination of spared axons, Wallerian degeneration, axon dieback, and glial scar formation around the injury site (Alizadeh et al., 2019) exacerbate the damage, ultimately worsening as the injury progresses to the chronic phase. As the injury recovers, motor functions and reflexes return, pain symptoms also develop (Christensen & Hulsebosch, 1997), including neuropathic pain.

2.4.1 Neuropathic pain after spinal cord injury

Spontaneous functional recovery after SCI involves efforts to regenerate and restore the connectivity in damaged axons, a process that is necessary for functional recovery but can also become maladaptive. Typically, nociceptive pain develops shortly after primary injury due to inflammation in the lesion area (Finnerup, 2013). However, within months after the injury, neuropathic pain develops in 40 - 50% of individuals (Finnerup, 2013). Neuropathic pain is often described as burning, stabbing, and/or shock-like (Finnerup et al., 2001; Siddall et al., 2003). Although the precise mechanisms are not fully elucidated, neuropathic pain after SCI is thought to involve both central and peripheral mechanisms (Bedi et al., 2010; Carlton et al., 2009;

Christensen & Hulsebosch, 1997; Crown et al., 2006; Garraway et al., 2014; Hulsebosch et al., 2009; Yezierski et al., 2004), with many neural substrates implicated in these processes, contributing to the complexity of the condition. Because the underlying mechanisms of neuropathic pain are complex, therapeutic interventions are often limited in their effectiveness. Despite development of diverse treatment strategies, ranging from neurosurgery (Jug et al., 2015; Rath & Balain, 2017) to behavioral (Ilha et al., 2019; Sandrow-Feinberg & Houle, 2015) and pharmacological (Baroncini et al., 2021; Bracken et al., 1997) treatments, chronic neuropathic pain remains largely refractory (Baastrup & Finnerup, 2008). This resistance to treatment is likely linked to complex neural processes and diverse substrates that are implicated, as well as the heterogeneity of SCI and chronic pain etiologies. While many studies have investigated the pathophysiology of SCI, studies specifically addressing the mechanisms of SCI-induced neuropathic pain remain insufficient. Importantly, SCI-induced neuropathic pain is resistant to typical pain-relieving pharmacotherapy, including nonsteroidal anti-inflammatory drugs and opioids, adding the challenge of treating chronic pain (Finnerup et al., 2015).

Neuropathic pain after SCI can be localized to the injury site (“at-level”) or widespread, affecting areas below the injury site (“below-level”) throughout the body. A variety of factors change after SCI that can ultimately contribute to development of neuropathic pain, complicating the identification of underlying mechanisms (Finnerup, 2013). One major cellular change is the onset of central sensitization, a process in which neurons in the spinal cord become hyperexcitable to sensory input. Traditionally, central sensitization was understood to be driven by increased membrane excitability and enhanced synaptic efficacy of spinal neurons in response to sustained or repeated peripheral noxious input (Woolf, 1983), amplifying pain signals and allowing normally non-painful stimuli to be perceived as painful or increasing the sensitivity to

painful stimuli. More recently, evidence has shown that central sensitization can persist even in the absence of ongoing peripheral input, due to long-lasting changes in spinal circuits and the hyperexcited nociceptive neurons (Harte et al., 2018). This hyperexcitability plays a key role in the development and maintenance of chronic pain (Brown & Weaver, 2012).

2.5. Overview of the dorsal root ganglia (DRG)

Pain is transmitted by primary afferent sensory fibers, known as nociceptors, which are either unmyelinated C-fibers or thinly myelinated Aδ-fibers, both of which are activated by high threshold, intense stimuli. The cell bodies of nociceptors, along with other primary sensory neurons, are housed in the DRG. DRGs are highly complex structures situated on either side of the spinal cord that span the length of the spinal column. Each DRG rises from the SCDH as an enlargement of the dorsal root (Krames, 2014). DRGs are typically circular to oval in shape, with diameters ranging from 20 to 150 μm (Esposito et al., 2019; Lee et al., 1986). They are pseudounipolar neurons with an offshoot cell body connected by a single axon that branches into two directions. DRG cell bodies do not synapse onto each other (Krames, 2014), as they are separated by layers of intermittent satellite glial cells (SGCs) (Pannese, 2010) and Schwann cells. The sheaths allow the penetration of neurotransmitters and other molecules into the neuron, rendering the DRG somas susceptible to inflammatory mediators and circulating drugs (Crawford & Caterina, 2020; Esposito et al., 2019). Although previous studies have shown that DRG somas do not directly participate in spike conduction, the soma membrane is still electrically excitable. Action potentials propagating centrally can frequently enter the soma to cause spiking or spontaneous firing (Wall & Devor, 1983). Likewise, spikes can also fail to propagate through the DRG soma, as they can also act as a low-pass filter that selectively impedes high frequency action potential trains (Djouhri et al., 2001; Du et al., 2014; Fang et al.,

2005; Gemes et al., 2013; Lüscher et al., 1994; Stoney Jr, 1985; Stoney, 1990), particularly the T-junction (Du et al., 2017; Hao et al., 2023). The failure in action potential firing has been observed across many species (Ducreux et al., 1993; Gemes et al., 2013; Lüscher et al., 1994; Stoney, 1990)

2.5.1 DRG modulation of sensory information

DRGs have a critical role in modulating peripheral and central sensory processing, such as inflammation and development of neuropathic pain. The offshoot cell body allows continuity of information transmission between the peripheral (somatosensory) end organ (e.g., skin, viscera) and the CNS (spinal cord) through two axons. Though connected to the same cell body, the two axons display distinct electrophysiological, structural, and molecular properties. The peripheral process behaves similarly to a dendrite, by generating the action potential at the terminal, which then propagates towards the stem and the T-junction. The central process receives the signal from the bifurcation and transmits it to the CNS. Some factors that may contribute to pain have been observed in the peripheral axon following inflammation or injury. These include extracellular-signal-regulated kinase (ERK), a kinase downstream of many receptors' activation (Perlson et al., 2005), and the TRPV1 receptor, which responds to noxious heat stimuli in pain-sensing neurons. TRPV1, one of the first receptors associated with sensory transduction (Caterina et al., 1997), is expressed in both peripheral and central terminals and on the cell bodies of sensory neurons (Clark et al., 2018). TRPV1 was shown to be selectively transported to the peripheral axonal branch after inflammation (Ji et al., 2002), implicating its role in the development of pain. The central branch of the DRG enters the spinal cord and terminates in the SCDH (Basbaum et al., 2009; Millan, 1999).

2.5.2 Overview of sensory neurons

Primary sensory neurons are known as the first-order neurons because they receive external sensory stimuli and initiate the transmission process. Upon activation, the first-order neurons conduct action potentials to the SCDH, where the information is passed to second-order neurons through the dorsal column (e.g., mechanosensory, proprioception) or the spinothalamic pathway (e.g., temperature, pain) (Purves D, 2001). DRGs contain the largest proportion of sensory neurons. Sensory neurons are morphologically and functionally classified as Aβ-, Aδ-, or C-fibers, based on the degree of myelination, sensory modality, and action potential conduction velocity (Gardner; Horch et al., 1977). Although these classifications do not fully illustrate the heterogeneity of the sensory neurons in morphology and molecular properties among the sensory neurons and how different sensory neurons can selectively fire action potentials in response to stimuli of varying intensities (Basbaum et al., 2009). Sensory neurons process a variety of sensory modalities such as temperature (thermoreceptors), pain (nociceptors), pressure (mechanoreceptors), and body orientation (proprioceptors). Nociceptors, the smallest of the primary afferents, innervate the skin and deep visceral tissues, detecting thermal, mechanical, and mixed sensory stimuli (Basbaum et al., 2009; Dubin & Patapoutian, 2010; Mendell et al., 1999). C-type sensory neurons, the smallest neurons and most abundant, lack myelination. Aδ- (small to medium) and Aβ- (medium to large) are myelinated processes, resulting in faster conduction velocities of 5 – 100 m/s. Aβ fibers primary serve as low threshold mechanoreceptors (LTMRs) (Lishi Li et al., 2011; Seal et al., 2009) under normal physiological conditions, mediating light, innocuous touch (Lishi Li et al., 2011; Lumpkin & Caterina, 2007). Although most Aδ and C-fibers are thought to function as nociceptors (Abraira & Ginty, 2013), Aδ-LTMRs and C-LTMRs have much lower thresholds than typical nociceptive range (Brown & Iggo, 1967;

Burgess et al., 1968; Iggo & Kornhuber, 1968) and are thought to contribute to directional and gentle, pleasant, light touch used in social interactions (Noble et al., 2022; Olausson et al., 2002). Mechanoreceptors innervate the skin with specialized endings, interacting with cells like hair cells, keratinocytes, and Merkel cells (Lumpkin & Caterina, 2007). LTMRs are further classified as slowly-, intermediately-, or rapidly adapting (SA, IA, and RA) (Burgess et al., 1968; Johnson & Hsiao, 1992), based on their response to stimuli, and by the type of hair follicle they innervate: guard hair, awl/auchene, or zigzag. Each hair follicle type has a unique combination of LTMR endings, showing that each type of hair follicle is a physiologically distinct peripheral mechanosensory end organ (Lishi Li et al., 2011).

2.5.3 Nociceptors

Nociceptors are specialized neurons that exclusively respond to potentially damaging stimuli. Nociceptors include high threshold mechanoreceptors (HTMRs) that detect noxious mechanical stimuli (Abraira & Ginty, 2013), as well as thermal, chemical, and polymodal nociceptors that respond to a wider range of stimuli. Nociceptor classifications are largely based on neurons that innervate the skin, but nociceptors in other tissues, such as visceral organs, behave differently (Millan, 1999; Westlund, 2000), demonstrating the complexity of defining nociceptors based on one feature (i.e., activation threshold, molecular profile). For this reason, the discussion of nociceptors in this work focuses on their role within the DRG and spinal cord.

Nociceptors are composed of four functional components: i) peripheral terminal, which transduces stimuli and initiates action potentials if the stimuli cross the threshold; ii) the axon, which propagates the action potentials towards the central terminal, forming the presynaptic terminal of the first synapse in the CNS sensory pathway (Woolf & Ma, 2007); iii) the cell body, which regulates transcription of neuropeptides, growth factors, and expression of ion channels

and receptors; and iv) the central terminal. Noxious stimuli are received through the free, unencapsulated nerve endings that innervate the walls of arterioles, connective tissue, and skin (Zylka et al., 2005). Nociceptors are broadly classified into two types: Aδ-fibers and C-fibers. Aδ-fibers are small-diameter, thinly myelinated neurons that produce fast onset pain (Djouhri & Lawson, 2004), often described as sharp and pricking (Hladnik et al., 2015). Type I Aδ-fibers respond to mechanical stimuli and high threshold heat (>50 °C), while type II Aδ-fibers are activated by high mechanical threshold and lower temperature threshold, responsible for the initial acute pain response (Hladnik et al., 2015). Most nociceptors are small-diameter unmyelinated C-fibers (Lumpkin & Caterina, 2007), which are polymodal and can detect mechanical, thermal, and chemical stimuli. C-fiber activation results in diffused, dull, burning pain that is poorly tolerated (Hladnik et al., 2015).

Under normal, physiological circumstances, nociceptors are electrically silent and respond exclusively to painful stimuli (Dubin & Patapoutian, 2010; Woolf & Ma, 2007) that surpasses a certain threshold. Once activated, nociceptors transduce high-intensity stimuli, conduct action potentials, and transmit signals to CNS neurons through activation of different receptors and channels on the membrane (Snider & McMahon, 1998). Key ion channels, receptors, and intracellular signaling proteins involved in this process include VGSCs (Nav 1.1-1.9), specifically Nav 1.7, 1.8 and 1.9 (Bennett et al., 2019; Wang et al., 2011), as well as TRP receptors (Caterina et al., 1999), neurofilament peripherin (Bae et al., 2015), and isolectin B4 (IB4; (Bogen et al., 2005). VGSCs and potassium channels determine neuronal excitability, transferring the electrical input from the peripheral nerve terminals to the central neurons in the spinal cord (Woolf & Costigan, 1999). When nociceptors synapse with dorsal horn neurons, they release glutamate and peptides, such as substance P (SP), calcitonin gene-related peptide

(CGRP), and somatostatin, to alter synaptic and efferent signaling (Dubin & Patapoutian, 2010). Glutamate acts primarily on the ionotropic glutamatergic AMPA (Yoshimura & Jessell, 1990) and NMDA (King et al., 1988; Woolf & Salter, 2000) receptors, to mediate fast excitatory postsynaptic potentials (EPSPs). BDNF, released from nociceptors, has also been shown to mediate actions akin to central sensitization (Thompson et al., 1999). Further discussion of VGSCs, glutamate, and BDNF in pain modulation is provided in the following sections.

2.6 Voltage-gated sodium channels in DRG and sensory neuron are critical in pain

While a substantial body of work has examined other ion channels, this chapter focuses on VGSCs, because sodium channels are critical to the action potential initiation and are regulated by various receptors, including receptor tyrosine kinases (D'Arcangelo et al., 1993; Hilborn et al., 1998), such as TrkB. In fact, some subtypes of VGSCs that are exclusively expressed on sensory neurons, have been reported to associate with TrkB for normal function (Blum et al., 2002) and contribute to neuropathic pain (Sun et al., 2022).

2.6.1 Types of voltage-gated sodium channels

VGSCs are generally expressed throughout the body as Nav 1.1-1.9 (Goldin et al., 2000) where they mediate the generation of the rising phase of the action potential (Hille, 1970, 2022), making them critical in regulation of neuronal excitability (Bennett et al., 2019). VGSCs are classified by their response to the channel blocker tetrodotoxin (TTX), as either TTX-sensitive (TTX-s; Nav 1.1-1.7) or -resistant (TTX-r; Nav 1.8 and 1.9) channels (Bossu & Feltz, 1984; Catterall, 1992; Elliott & Elliott, 1993). TTX-s VGSCs activate more rapidly and display faster kinetics (Blair & Bean, 2002). TTX-r VGSCs recover from inactivated state significantly faster than TTX-s currents, almost by tenfold (Cummins & Waxman, 1997; Elliott & Elliott, 1993). At least five VGSC subtypes (Nav 1.1, 1.6, 1.7, 1.8 and 1.9) are highly expressed in DRG neurons,

enabling them to produce both fast-inactivating TTX-s and slow-inactivating TTX-r sodium currents (Rush et al., 1998b). Specifically, TTX-r VGSCs are preferentially expressed in the unmyelinated C and thinly myelinated Aδ sensory neurons (Akopian et al., 1996; Djouhri et al., 2003). However, other DRG neurons also express a combination of TTX-s and TTX-r channels, influencing their electrogenic properties under normal or pathological conditions. The subtypes most associated with nociceptor function include TTX-s Nav 1.7 (Dib-Hajj et al., 2013), and TTX-r Nav 1.8 (Hameed, 2019; Sangameswaran et al., 1996; Watanabe et al., 2014) and 1.9 (Dib-Hajj et al., 2015; Tate et al., 1998), having been identified as mutated or dysfunctional in humans with abnormal pain sensitivity (Dib-Hajj et al., 2005; Faber et al., 2012; Huang et al., 2015). Nav 1.7 is expressed predominantly in small-diameter DRG neurons (Black et al., 2004) and in sympathetic neurons (Black et al., 1996; Toledo-Aral et al., 1997). Nav 1.8 and 1.9 are preferentially expressed in DRG neurons (Djouhri et al., 2003; Fukuoka et al., 2008), but Nav 1.9 is expressed also in myenteric neurons and in free nerve terminals and central terminals in the spinal cord (Dib-Hajj et al., 1998). Nav 1.9 is only expressed on unmyelinated C-fibers, while Nav 1.8 is found in both C- and myelinated A-fibers (Amaya et al., 2000; Decosterd et al., 2002).

Nav 1.7 responds to small depolarizing stimuli close to the neuronal resting membrane potential (Cummins et al., 1998), amplifying accumulated subthreshold signals deployed at nociceptor nerve terminals (Toledo-Aral et al., 1997). Thus, Nav 1.7 determines the gain of nociceptor activation (Rush et al., 2007). Nav 1.7 also affects synaptic transmission by impacting neurotransmitter release (Alexandrou et al., 2016). Hyperfunction of Nav 1.7 has been linked to driving both evoked and spontaneous pain symptoms by increasing neuronal excitability and spontaneous activity (Dib-Hajj et al., 2005). A large portion of nociceptors are dependent on Nav

1.8 and 1.9 for axonal propagation (Klein et al., 2017). Nav 1.8 channels carry most of the sodium current (Blair & Bean, 2002) and recover more quickly from inactivation (Dib-Hajj et al., 1999), thereby contributing to repetitive firing and regulating neuronal excitability. Nav 1.9 is activated near resting membrane potential and produces a large persistent current due to its slow activation and inactivation kinetics (Cummins et al., 1999). Like Nav 1.7, Nav 1.9 is considered a threshold channel that regulates DRG excitability and prolongs the depolarizing response to stimuli rather than contributing to the action potential amplitude (Dib-Hajj et al., 2015; Herzog et al., 2001; Ostman et al., 2008).

2.6.2 VGSCs play a role in mediating injury-induced pain

VGSC expression has been linked to the development of neuropathic pain other studies (Black et al., 1999; Gold et al., 2003; Lai et al., 2002). Nav 1.7, 1.8 and 1.9 have been targeted to relieve symptoms of neuropathic pain. For example, moderate loss of function of Nav 1.8 is associated with reduced pain sensitivity in humans and decreased excitability of DRG neurons in mice (Duan et al., 2016). Ectopic activity in nociceptors and other sensory neurons (Boucher et al., 2000; Liu et al., 2000; Wall & Gutnick, 1974; Wu et al., 2001) contribute to maintaining peripheral neuropathic pain (Devor, 2006; Haroutounian et al., 2014). Intravenous TTX inhibited ectopic activity in damaged rat DRG and SCDH neurons (Omana-Zapata et al., 1997) and reduced neuropathic pain behaviors (Lyu et al., 2000). After injury or inflammation, primary afferent hyperexcitability has been correlated with upregulation of both TTX-s and TTX-r VGSCs on SCDH neurons (Hains et al., 2003; Hains et al., 2005). Studies have also shown increased mRNA and/or protein levels of Nav 1.7, 1.8, and 1.9 in DRG neurons after peripheral inflammation (Black et al., 2004; Coggeshall et al., 2004; Okuse et al., 1997; Strickland et al., 2008). Treating DRG neurons with inflammatory mediators increased the number of active Nav

1.9 channels and heightened neuronal excitability (Binshtok et al., 2008; Maingret et al., 2008), suggesting that Nav 1.9 lowers the threshold and increases the number of action potentials to drive DRG neuron hyperexcitability associated with inflammatory pain. Nav 1.8 expression was reported to increase in patients with neuropathic pain (Joshi et al., 2006), and Nav 1.9 gain-of-function mutations have been identified in individuals with painful peripheral neuropathy (Huang et al., 2014). However, specific changes in VGSC expression after peripheral nerve injury or in experimental neuropathic pain models are less clear. For instance, animal models of neuropathic pain show a reduction in Nav 1.7, 1.8 and 1.9 in the DRGs (Berta et al., 2008; Cummins & Waxman, 1997; Dib-Hajj et al., 1998; Gold et al., 2003; C. H. Kim et al., 2002). Likewise, there was a reduction in Nav 1.8 and Nav 1.9 in the DRGs following peripheral axotomy (Coward et al., 2000) and after lumbar (L) 5 sciatic nerve ligation (Dong et al., 2007). Even though the expression in neighboring uninjured DRGs remained the same, the L5 DRG showed nearly a complete depletion of Nav 1.8 and 1.9. Contrastingly, increased immunoreactivity for Nav 1.8 was observed in tissue samples from animal pain models (Novakovic et al., 1998), likely in uninjured C-fibers (Gold et al., 2003), and patients with persistent pain conditions (Shembalkar et al., 2001). In rodent neuropathic pain models, Nav 1.8-specific inhibitor reversed mechanical allodynia (Jarvis et al., 2007), but selective deletion of Nav 1.8 (Akopian et al., 1999; Kerr et al., 2001) did not prevent development of neuropathic pain. These conflicting outcomes indicate that the injury-related plasticity within the DRG is highly variable, and the contribution of VGSCs may depend on the type of injury and species differences.

2.7 Overview of glutamate and glutamate receptors

Glutamate is one of the most abundant excitatory neurotransmitters in the nervous system and plays a critical role in nociceptive signaling in the periphery (Keast & Stephensen, 2000). Glutamate binds to and activates ionotropic AMPA/kainate and glutamatergic NMDA receptors, and several metabotropic receptors (mGluRs). Glutamate receptors are found throughout the nervous system (Monaghan & Cotman, 1985; Monyer et al., 1994), including primary sensory neurons, and are synthesized in the DRG cell bodies and transported to the nerve terminals in the peripheral end organs, such as the skin, muscles, and joints (Carlton et al., 1995; Ma & Hargreaves, 2000). Primary sensory neurons express and release glutamate from central and peripheral terminals (Bae et al., 2000; deGroot et al., 2000) as well as from their cell bodies (Kung et al., 2013). Likewise, glutamatergic receptors and glutamate transporters have been observed in the cell bodies and terminals of the primary sensory neurons.

All four types of AMPAR subunits (GluA1-4) are expressed on sensory ganglia and central terminals. GluA1 is ubiquitously distributed in both myelinated and unmyelinated peptidergic nociceptors (Sato et al., 1993; Tachibana et al., 1994), while the GluA4 subunit is expressed predominantly on non-peptidergic neurons that are strongly associated with neuropathic pain (Willcockson & Valtschanoff, 2008). AMPARs are also expressed on the central terminals of the primary sensory neurons and in the SCDH, generally in laminae I-II, with varying expression across subtypes (Larsson & Broman, 2011). AMPARs are located in pre-, post-, and extrasynaptic membranes (Bredt & Nicoll, 2003; Malinow & Malenka, 2002). Synaptic AMPARs contribute to nociceptive inputs (Hartmann et al., 2004), and changes to synaptic trafficking and Ca^{2+} permeability are linked to excitotoxicity and persistent inflammatory pain (Choi et al., 2010; Ferguson et al., 2008; Hartmann et al., 2004; Tao, 2010). Extrasynaptic AMPARs move rapidly between the membrane and the intracellular compartments (Bredt &

Nicoll, 2003; Carroll et al., 1999; Petrini et al., 2009) and have been implicated in the maintenance of persistent inflammatory pain (Kopach et al., 2011) and nerve injury-induced pain (Napier et al., 2012).

The NMDA receptor is a ligand-gated ion channel that mediates a major component of excitatory neurotransmission in the CNS. Compared to AMPARs, NMDARs have much slower kinetics due to the requirement for membrane depolarization to release the Mg^{2+} block (Paoletti, 2011). NMDARs consist of combinations of GluN1, GluN2, and GluN3 subunits (Paoletti et al., 2013), with functional receptors typically composed of two GluN1 subunits and either two GluN2 subunits or a combination of GluN2 and GluN3 subunits. GluN2 subunits exist as isoforms GluN2A, 2B, 2C, 2D, and the composition and distribution determine NMDAR function (Salter et al., 2009). NMDARs are found in the sensory neuron cell bodies and almost half of peripheral axons (Li et al., 2004), with differential distribution of subunits in the cell soma and pre-, post-, and extrasynaptic terminals, reflecting the complexity of their role in pain processing. Studies show synaptic inputs in the superficial regions of the SCDH, where nociceptive afferents are integrated with interneurons and descending input from the brain to process pain signals (Todd, 2010), are mainly mediated by GluN2A and GluN2B subunits (Bardoni et al., 2004; Shiokawa et al., 2010).

Glutamate also engages mGluRs, which are family C G-protein-coupled receptors. To date, there are eight subtypes of mGluRs (R1–R8), categorized into three groups based on sequence similarity. Group I, containing mGluR1 and 5, is excitatory, while Groups II and III are inhibitory (Conn & Pin, 1997; Schoepp et al., 1999). All three groups are expressed in DRGs, with mGluR2/3 present in 40-52% of small-diameter neurons (Carlton & Hargett, 2007; Carlton

et al., 2001). These receptors may negatively regulate glutamate release to modulate nociceptive input (Carlton et al., 2001; Palazzo et al., 2014).

2.7.1 Glutamate and glutamate receptors in pain hypersensitivity

While both ionotropic and metabotropic glutamate receptors are implicated in persistent pain states, the role of the ionotropic receptors, primarily the NMDAR, is more thoroughly studied and will be emphasized in this section. NMDARs (Liu et al., 1994; Lu et al., 2003) and AMPARs (Carlton et al., 1995; Coggeshall & Carlton, 1998) are both transported from the cell bodies to the central terminals, where activation by endogenously released glutamate leads to sensitization of spinal cord neurons (Coderre & Melzack, 1992; Dickenson & Sullivan, 1987). Extensive studies have been done on postsynaptic NMDARs in the afferent terminals and their contributions to neuropathic pain, especially in central sensitization (Ji et al., 2003; Woolf & Salter, 2000; Zhang et al., 2016). Specifically, GluN2B-containing NMDARs are involved in development of neuropathic pain following injury (Qu et al., 2009; Suzuki et al., 2001). Consistently, the GluN2B subunit is preferentially localized to the unmyelinated axons that synapse in the superficial regions of the SCDH (Temi et al., 2021). The GluN1 subunit is also implicated in pain. Targeted deletion of GluN1 in the SCDH of adult rats and/or mice attenuated both inflammatory (Garraway et al., 2009) and injury-induced pain (South et al., 2003).

In sensory neurons, NMDARs are found in both small- and large-diameter sensory neurons (Liu et al., 1994; Marvizon et al., 2002; Sato et al., 1993). Under pathological conditions, such as peripheral nerve injury, glutamate expression is increased in the DRG neurons (Kung et al., 2013).After injury or inflammation, endogenous glutamate release activates NMDARs on primary afferent peripheral terminals, resulting in development of pain behaviors (Omote et al., 1998). For example, chronic constriction injury of the sciatic nerve increases glutamate-induced

inward currents in small diameter neurons, as well as neurons that were responsive to NMDA and AMPA (Gong et al., 2014). Peripheral inflammation increased the number of peripheral axons that express glutamate receptors (Du et al., 2006; Du et al., 2003). Exogenous administration of glutamate, NMDA, or AMPA caused an induction of mechanical hyperalgesia in the rat hind paws (Ferreira & Lorenzetti, 1994; Parada et al., 2003). On the other hand, blocking NMDA (Christoph et al., 2005) and AMPA/kainate (Lee et al., 2001) receptors resulted in an attenuation of pain behavior and blockade of nociceptor activity in inflammatory and neuropathic models (Jang et al., 2004). Similarly, in rodent neuropathic pain models, NMDAR antagonists, such as memantine, MK801, and ketamine led to a decrease in mechanical hyperalgesia (Burton et al., 1999) by reduction of spinal cord sensitization. Peripheral inhibition of NMDA, AMPA/kainite receptors similarly reduces pain behavior and nociceptor activity in inflammatory or neuropathic pain models (Christoph et al., 2005; Jang et al., 2004). Exogenously applied glutamate also triggers action potentials in an NMDAR dependent manner (Laursen et al., 2014).

2.7.3 Presynaptic NMDA receptors contribute to pain

Studies show NMDARs are also found at presynaptic nerve terminals in the SCDH (Bardoni et al., 2004; Krebs et al., 1991; Liu et al., 1994; Lu et al., 2003). Nearly a third of SCDH NMDARs are presynaptic, which are translated in DRG neurons and transported to the synaptic terminal, adjacent to the vesicle release sites (Liu et al., 1994). Unlike postsynaptic NMDARs, presynaptic NMDARs can spontaneously release neurotransmitters, such as SP, without the need for neuronal depolarization and removal of the Mg^{2+} block (Dore et al., 2017; Kavalali, 2015). A distinct signaling process involving metabotropic signaling through c-Jun *N*-terminal kinase (JNK) (Abrahamsson et al., 2017) can also trigger neurotransmitter release. Src family kinases, which

phosphorylate and activate NMDARs, have also been implicated in primary afferent presynaptic NMDAR regulation (Marvizón et al., 1997) by increasing presynaptic glutamate release (Madara & Levine, 2008). This persistent increase in glutamate receptor activity can enhance overall synaptic activity. Presynaptic NMDAR activation increases the frequency of miniature excitatory postsynaptic currents (EPSCs) associated with pain hypersensitivity in rodent models of neuropathic pain (S. R. Chen et al., 2014; W. Chen et al., 2014). For example, Xie et al. (2016) found increased mEPSC frequency driven by presynaptic NMDARs in rats with paclitaxel-induced neuropathic pain viaGluN2A phosphorylation instead of typical GluN2B subunits. These results suggest that presynaptic NMDARs are recruited in neuropathic pain conditions to facilitate glutamate release that increases SCDH neuronal excitability (Yan et al., 2013).

NMDARs are also found extrasynaptically and can be activated by glutamate in extracellular compartment (Meur et al., 2007), as well as by astrocytes (Carmignoto & Fellin, 2006) and glia (Nie et al., 2010). Extrasynaptic NMDARs are often distributed in clusters in very specific cell contact areas to act as a point of contact with these processes (Kharazia & Weinberg, 1999; Papouin & Oliet, 2014; Petralia et al., 2010). Although the physiological role of extrasynaptic NMDARs, especially in the context of sensory neurons and nociception is still poorly understood, their expression and contribution to pain signaling after injury or inflammation represent how just a small fraction of variables could change pain signaling after injury or inflammation. Added to this complex landscape of receptors and channels is yet another diverse group of neuromodulators and inflammatory mediators, which we will discuss in more detail below.

2.8 Overview of neurotrophins

Neurotrophins are a family of proteins that are responsible for the development, survival, and differentiation of the neurons (Huang & Reichardt, 2001; Mitre et al., 2017; Numakawa et al., 2010). They play critical roles in regulating axonal and dendritic growth, synaptic structure and plasticity, and neurotransmitter release (Chao, 2003; Huang & Reichardt, 2001). Neurotrophins include nerve growth factor (NGF), neurotrophin 3 (NT-3), neurotrophin 4/5 (NT4/5), and BDNF. Each neurotrophin exerts its actions through cell surface tropomyosin receptor kinases (Trk) and the low affinity, non-selective p75 neurotrophin receptor (p75NTR). NGF preferentially binds TrkA, NT-3 to TrkC, and both BDNF and NT4/5 activate the TrkB receptor (Kaplan & Miller, 2000). Among the neurotrophins, BDNF is the most abundant and widely distributed in the CNS (Pezet, Malcangio, & McMahon, 2002). First identified by Barde et al. (1982), BDNF is encoded by the *bdnf* gene (Leibrock et al., 1989; Timmusk et al., 1995; Timmusk et al., 1993) and is synthesized and released by sensory and motor neurons, as well as peripheral and central immune cells (see reviews by Nijs et al. (2015) and Brigadski and Leßmann (2020)). BDNF is best known for its role in promoting neuronal development and plasticity in the CNS (Ferrini & De Koninck, 2013; Merighi et al., 2008; Nijs et al., 2015). BDNF-TrkB signaling also enhances neuronal excitability by promoting neurotransmitter release (Takei et al., 1997), phosphorylating glutamatergic receptors (Levine et al., 1995), and inducing structural and functional changes in neurons (Abidin et al., 2008; Marty et al., 1997). Despite BDNF's prominent role in early nervous system development, its expression remains high in the adult brain, where it regulates both excitatory and inhibitory synaptic transmission and contributes to activity-dependent plasticity (Tyler et al., 2002; Wardle & Poo, 2003).

BDNF expression is tightly regulated at multiple levels of transcription, translation, and post-translational modifications (Chen et al., 2003; Timmusk et al., 1993). Environmental factors also

modulate BDNF expression, such as exercise (Oliff et al., 1998), antidepressants (Russo-Neustadt et al., 2004), and stress (Lauterborn et al., 1998). The highest level of BDNF is found in the hippocampus (Hofer et al., 1990; Timmusk et al., 1993), where it is essential for learning, memory and LTP (Schuman, 1999). BDNF has been thought to increase the size and complexity of dendritic spines (Alonso et al., 2004; Horch & Katz, 2002) and promote neurogenesis (Lee et al., 2007), resulting in neuroprotection. Thus, BDNF has been studied extensively for its potential therapeutic applications in conditions such as epilepsy (Lin et al., 2020), depression (Castrén et al., 2007), Alzheimer's Disease, and SCI (Fouad et al., 2021). However, clinical use of exogenous BDNF is limited due to the challenges in crossing the blood-brain barrier. As a result, small-molecule TrkB agonists, such as 7,8-dihydroxyflavone (7,8-DHF) (Jang et al., 2010; Ohnishi et al., 2017) have been developed as alternatives.

Pro-BDNF, the inactive precursor of mature BDNF, exerts effects opposite to the mature BDNF by engaging p75NTR, promoting apoptosis and weakening synaptic transmission (Lin et al., 2015; Yamashita et al., 1999; Yang et al., 2009). Pro-BDNF and p75NTR signaling leads to long term depression (LTD) (Rösch et al., 2005; Woo et al., 2005) and acts through sortilin receptors to downregulate synaptic transmission. ProBDNF has been observed to increase with aging (Perovic et al., 2013) and have been implicated in Alzheimer's disease and neuronal death after status epilepticus (Volosin et al., 2008).

After SCI, BDNF has also been studied extensively for its contribution in axon regeneration and functional recovery. BDNF promotes sprouting of spared axons (Henderson et al., 1993; Jin et al., 2002; Kim & Jahng, 2004) increases myelination (Zhao et al., 2017), and supports locomotor recovery (Jung et al., 2016; Leech & Hornby, 2017; Oh et al., 2009). Its neuroprotective role (Crowley et al., 2019; Ji et al., 2020) is further demonstrated by its involvement in exercise-

induced recovery, such as recovery of stepping after cervical hemisection (Gómez-Pinilla et al., 2002; Vaynman et al., 2003; Ying et al., 2008). Notably, serum BDNF in individuals with incomplete SCI was modulated by high-intensity locomotor exercise (Leech & Hornby, 2017). BDNF also promotes learning in spinal cord function after SCI (Boyce et al., 2007; Gómez-Pinilla et al., 2002). Stimulation that impaired locomotion also decreased BDNF expression (Garraway et al., 2011), which was reversed by exogenous application of BDNF (Huie et al., 2012).

2.8.1 Brain-derived neurotrophic factor modulates nociceptive and neuropathic pain

Many studies have also shown the role of BDNF in the development of pain (Kerr et al., 1999; Melemedjian et al., 2013; Slack et al., 2004). In particular, spinal BDNF-TrkB signaling in particular has been linked to pathological mechanisms for neuropathic pain (Cao et al., 2020; Coull et al., 2005; Thibault et al., 2014; Wang et al., 2009) by modulating both presynaptic and postsynaptic neurotransmission to enhance synaptic efficacy in the dorsal horn (Binder & Scharfman, 2004; Cheng et al., 2017; Yoshii & Constantine-Paton, 2010). Moreover, BDNF-TrkB signaling plays an important role in central sensitization (Coull et al., 2005; Lu et al., 2009; Merighi et al., 2008) by persistently increasing the dorsal horn excitability (Dai & Ma, 2014; Kerr et al., 1999; Smith, 2014). During nociceptive signal transduction, BDNF is released from nociceptor terminals and acts as a neuromodulator, enhancing pain signaling. BDNF release occurs not only in the CNS and in the spinal cord (Lin et al., 2011) but also in sensory and motor neurons (Jones et al., 1994; Liu et al., 1995), and in peripheral sites following nerve injury or peripheral inflammation (Narita et al., 2000; Shu et al., 1999). Increased BDNF expression at the level of DRG increases pain transmission and contributes to pain hypersensitivity (Wu et al.,

2021). The increase in the DRGs is correlated with spinal dorsal horn increase (Pezet & McMahon, 2006; Wu et al., 2021).

2.8.2 TrkB receptor

TrkB is a transmembrane receptor with an extracellular ligand binding domain and an intracellular catalytic domain. Binding of BDNF to TrkB induces dimerization of the TrkB heteromer, leading to autophosphorylation of intracellular tyrosine residues. The phosphorylation triggers activation of multiple downstream signaling pathways essential for neuronal survival, differentiation, and the regulation of neuronal structure and function (Bonhoeffer, 1996; Numakawa et al., 2010). The key pathways activated by BDNF-TrkB signaling include mitogen-activated protein kinase (MAPK), phosphoinositide 3-kinase (PI3K), and PLCγ pathways (Huang & Reichardt, 2001; Numakawa et al., 2010; Pezet, Malcangio, Lever, et al., 2002).

Activation of PLCγ pathway promotes synaptic plasticity (Minichiello, 2009) and cell survival (Numakawa et al., 2010) by activating protein kinase C (PKC) via diacylglycerol (DAG) and regulating calcium release from the endoplasmic reticulum through inositol-1, 4, 5-trisphosphate (IP3), driving enhanced neurotransmission and synaptic plasticity (Minichiello, 2009; Numakawa et al., 2010). In the spinal cord, PKC is critical for sensitizing SCDH neurons after injury (Coderre, 1992; Lin et al., 1996; Munro & Derwing, 1994) in neuropathic pain models (Malmberg et al., 1997; Mao et al., 1995; Ohsawa et al., 2001; Ohsawa et al., 2000). In nociceptors, PKC activation induces nociceptor excitation and sensitization in response to bradykinin or histamine *in vitro* (Dray et al., 1992; Mizumura et al., 2000), while *in vivo* studies demonstrated PKC-dependent nociceptive behaviors, such as spontaneous pain (Ferreira et al., 2005). Sensory neuron-specific BDNF was identified as a critical regulator of PKC synthesis and

phosphorylation (Sikandar et al., 2018), which underlies the maintenance of neuropathic pain (Melemedjian et al., 2013).

Activation of PI3K-Akt signaling pathway regulates neurite outgrowth and neuronal survival (Minichiello (Minichiello, 2009) and has been implicated in nociceptive transduction and central sensitization after noxious stimuli (Pezet et al., 2008; Pezet et al., 2005; Zhuang et al., 2004), particularly as a mediator of TRPV1 sensitization (Bonnington & McNaughton, 2003; Sun et al., 2006; Zhu & Oxford, 2007), or modulating levels of microglial receptors (Nasu-Tada et al., 2006; Tsuda, Toyomitsu, et al., 2008; Tsuda et al., 2009; Tsuda, Tozaki-Saitoh, et al., 2008).

MAPK/ERK signaling cascade is essential for protein synthesis and activation of transcription factors (Numakawa et al., 2010), resulting in cellular differentiation and proliferation. MAPKs are also important in regulating neuronal plasticity and inflammatory responses, as well as development of pain hypersensitivity following tissue and nerve injury (Ji et al., 2009), contributing to the induction and maintenance of peripheral and central sensitization in the SCDH neurons (Ji & Woolf, 2001; Ji et al., 1999), microglia (Hains & Waxman, 2006; Tsuda et al., 2004; Zhuang et al., 2005), and astrocytes (Ma & Quirion, 2002; Zhuang et al., 2006). In the periphery, enhanced p38 MAPK and ERK signaling in the lumbar DRGs has been linked to chronic pain (Pezet, Malcangio, & McMahon, 2002; Zhuang et al., 2005). Therefore, BDNF-TrkB signaling involves intricate crosstalk between PLCγ, PI3K, and MAPK pathways, contributing to the modulation of synaptic plasticity, neurotransmission, and neuronal excitability, which underlie pro-nociceptive actions of BDNF.

Four different isoforms of TrkB result from alternative splicing. Full-length TrkB (TrkB.FL) mediates all the physiological functions of BDNF and NT-4/5 signaling (Reichardt, 2006). The most well-known isoforms are truncated at the C-terminal end with some variations: TrkB.T1

(i.e., lacks the tyrosine kinase domain), TrkB.shc (i.e., retains Shc expression in the catalytic domain), and TrkB.T-TK (i.e., retains tyrosine kinase activity; (Luberg et al., 2010). Other N-terminal truncated TrkB isoforms have been identified but have not been studied thoroughly (Luberg et al., 2010). Of the variants, TrkB.T1 is the most abundantly expressed isoform along with TrkB.FL (Cao et al., 2020; Klein et al., 1989; Wu et al., 2013) and are homologous to TrkB.FL in the extracellular domains (Barettino et al., 1999; Middlemas et al., 1991). Although TrkB.T1 is still identified as a tyrosine kinase receptor because it is derived from the same *NtrkB* gene, it lacks the catalytic tyrosine-rich domain containing the Shc and PLCγ binding sites, preventing downstream signaling cascades even with BDNF bound (Patapoutian & Reichardt, 2001). TrkB.T1 expression is widespread in the CNS and changes depending on the developmental timepoints and brain region. The TrkB.T1 protein level is lowest before birth, increases into adulthood (Ohira et al., 1999; Silhol et al., 2005), and continues to increase with aging, in contrast to declining TrkB.FL level (Fryer et al., 1996; Ohira et al., 1999). TrkB.T1 also shows region-specific expression, with shifts in the TrkB.T1/ TrkB.FL ratio reflecting cellular and synaptic pruning, for example, in the cortex (Allendoerfer et al., 1994; Bracken & Turrigiano, 2009), where the timing of the increase and the localization pattern are different between cortical regions (e.g., pyramidal neurons vs. interneurons in the visual cortex (Bracken & Turrigiano, 2009). TrkB.T1 is expressed in both neurons and astrocytes depending on the region (Ohira et al., 2005), but its expression is predominant in astrocytes (Beck et al., 1993; Frisen et al., 1993; Holt et al., 2019), whereas TrkB.FL is expressed predominantly in neurons. TrkB.T1 binds BDNF with high affinity and has been studied under models of neuropathic pain, shown to be upregulated following nervous system injuries (Cao et al., 2020; Matyas et al., 2017; Wu et al., 2013) and in the SCDH during inflammatory pain (Renn et al., 2009). TrkB.T1

primarily acts as an inhibitor of TrkB.FL, as its overexpression impairs the neuroprotective effects of BDNF (De Wit et al., 2006; Haapasalo et al., 2001). Two potential mechanisms have been proposed and evaluated. First, TrkB.T1's role as a dominant-negative receptor by forming a heterodimer with TrkB.FL and inhibiting activation of TrkB.FL signaling (Biffo et al., 1995; Fernette F Eide et al., 1996; Li et al., 1998; Palko et al., 1999). Some hippocampal neurons express both TrkB.T1 and TrkB.FL isoforms together (Armanini et al., 1995) where TrkB.T1 was shown to co-immunoprecipitate with and inhibit BDNF-induced autophosphorylation of TrkB.FL (Haapasalo et al., 2001). Second, TrkB.T1 may also regulate BDNF-TrkB.FL signaling by regulating endogenous BDNF levels. TrkB.T1 expression is predominant in the plasma membrane of regulatory non-neuronal cells, compared to expression of TrkB.FL in neurons (Biffo et al., 1995), which gives strength to TrkB.T1's regulatory role. TrkB.T1 internalizes BDNF to non-neuronal cells when extracellular BDNF is abundant and releases it back via exocytosis, depending on the concentration of BDNF (Alderson et al., 2000). After CNS lesions, TrkB.T1 has been observed to be upregulated to enhance removal of BDNF to prevent axonal regeneration (Beck et al., 1993). In a neuroblastoma model of neuronal development, SH-SY5Y cells expressing TrkB.T1 prevented BDNF-induced neurite outgrowth, but this effect was overturned by increased concentrations of BDNF (Fryer et al., 1996). The sequestration and translocation of BDNF by TrkB.T1 isoform is independent of TrkB.FL, as TrkB.T1 forms a complex with and internalizes BDNF (Alderson et al., 2000; Biffo et al., 1995; Fryer et al., 1997). Once the TrkB.T1-BDNF complex dissociates in a time-dependent manner, BDNF is released back into the extracellular space (Alderson et al., 2000).

2.8.3 BDNF's expression and function in sensory neurons and spinal cord

BDNF is endogenously and constitutively synthesized in primary sensory neurons in the DRG, as demonstrated by observations of mRNA (Wetmore & Olson, 1995) and protein (Barakat-Walter, 1996; Wetmore & Olson, 1995; Yan et al., 1997) expression. Once synthesized, BDNF is anterogradely transported from the DRG somas to the primary afferent terminals (Michael et al., 1997; Zhou & Rush, 1996) and released into the dorsal horn of the spinal cord (Lin et al., 2011; Matayoshi et al., 2005). BDNF is also expressed in Schwann cells (Apfel et al., 1996; Cho et al., 1997; Michael et al., 1997) and epithelial cells (Hahn et al., 2006; Hahn et al., 2005; Wang et al., 2015). The subpopulation of DRG neurons that express BDNF are small-to-medium sized peptidergic cells, presumably nociceptors (Luo et al., 2001; Michael et al., 1997). BDNF is stored in large dense core vesicles of these small cells along with SP and CGRP (Michael et al., 1997; Pezet, Malcangio, & McMahon, 2002), which are released in an activity-dependent manner into the laminae I and II of the spinal cord (Kerr et al., 1999; Lever et al., 2001; Mannion et al., 1999; Qiao et al., 2016; Zhou & Rush, 1996), where SP-containing C-fibers terminate (Michael et al., 1997).

Previous studies have implicated both spinal and peripheral BDNF in pro-nociceptive actions (Ferrini & De Koninck, 2013; Nijs et al., 2015), suggesting its role in central sensitization and pain development (see reviews by Thompson et al. (1999), Garraway and Huie (2016), and Cappoli et al. (2020)). Short bursts of high frequency electrical stimulation of C-fibers and/or NGF treatment (i.e., inflammatory condition) (Lever et al., 2001) or sciatic nerve transection (Lever et al., 2001; Walker et al., 2001) induced the release of BDNF in the dorsal horn. BDNF also facilitated C-fiber evoked synaptic responses lamina II of the spinal cord, which required post-synaptic NMDARs (Garraway et al., 2003). During neuropathic pain, potentiation of NMDARs by BDNF-TrkB signaling in the SCDH (W. Chen et al., 2014) increases activation of

MAPK/ERK signaling pathway (Pezet & McMahon, 2006) and PI3K in spinal microglia (Xu et al., 2019). BDNF is also linked to dysregulation of inhibitory neurotransmission, as spinal nerve ligation reduces BDNF release, resulting in decreased levels of GABA to impair the GABAergic signaling in the SCDH, thus contributing to hyperexcitability and central sensitization (Lever et al., 2003). Together, these observations show the importance of BDNF released from sensory neurons in synaptic and nociceptive plasticity in the spinal cord.

2.8.4 BDNF's contribution to injury-induced pain

BDNF is also implicated in injury-induced pathophysiology, including neuropathic pain. Peripherally, Schwann cell-derived BDNF is upregulated after peripheral nerve axotomy (Michael et al., 1997; Pezet, Malcangio, & McMahon, 2002). BDNF synthesis and anterograde transport from the DRG somas to the terminals is enhanced by peripheral injury (Mannion et al., 1999; Qiao et al., 2016; Zhou & Rush, 1996), and increase in BDNF in the DRGs correlated with development of allodynia in rats with spinal nerve injury (Zhou et al., 2000). Injury can also cause a phenotypic switch in BDNF expressing neurons in the DRG. After spinal nerve ligation, *de novo* synthesis and expression of BDNF is increased in the uninjured small neurons (Mannion et al., 1999) and in injured medium and large neurons, which was also observed after axotomy or peripheral nerve injury (Michael et al., 1999; Zhou et al., 1999). Similarly, after sciatic nerve crush, not only is the intensity of BDNF immunoreactivity increased in the small-diameter DRG neurons, but the number of medium- and large-diameter DRG neurons that express BDNF is also increased (Cho et al., 1998). Antisense and antibody against TrkB decreased mechanical hypersensitivity from repeated tactile stimulation (Mannion et al., 1999), acute heat pain, and thermal hyperalgesia (Groth & Aanonsen, 2002). Beyond the sensory neurons, microglial BDNF has been shown to downregulate the expression of the chloride transporter, K^+-Cl^- cotransporter

(KCC2), thereby disrupting GABAergic-glycinergic-mediated inhibition and produce an overall increase in excitation. Altogether, these studies indicate the involvement of BDNF-TrkB signaling in the DRG and sensory neurons, in inflammatory and injury-induced neuropathic pain.

2.8.5 Peripheral BDNF-TrkB signaling in mechanotransduction and pain

The aforementioned studies support the pro-nociceptive role of BDNF-TrkB signaling in the nociceptive pathways after peripheral and central injury. Because BDNF's functions in the CNS are well established, it is conceivable that BDNF will also mediate adaptive or maladaptive plasticity at peripheral sites. Peripherally, BDNF may regulate pro-nociceptive effects through regulation of synaptic transmission, afferent plasticity, and long-term modification in pain pathways, thereby influencing nociceptive processing both peripherally and centrally. For example, BDNF in sensory neurons was recently shown to be necessary for the transition from acute to chronic inflammatory pain, as well as for some neuropathic states (Sikandar et al., 2018).

Previous studies have shown TrkB expression in different subpopulations of sensory neurons (Ernfors et al., 1993; Foster et al., 1994; Kashiba et al., 1995; Wright & Snider, 1995) and sensory nerve endings innervated by or connected to primary afferent sensory neurons (García-Piqueras et al., 2019; Montaño et al., 2010; Zimmerman et al., 2014). Importantly, BDNF and/or TrkB signaling has been shown to be required for normal mechanosensation (Carroll et al., 1998; L. Li et al., 2011; Rutlin et al., 2014). Specifically, BDNF-TrkB expression and signaling in hairy skin is crucial for development of and normal functioning of the Aδ-LTMRs (L. Li et al., 2011; Rutlin et al., 2014), previously classified as D hair cells (Abraira & Ginty, 2013; Koltzenburg et al., 1997), which transduce directional touch in a BDNF-dependent manner. BDNF is also expressed in a population of myelinated primary afferents, although according to its expression

in these primary afferents makes very limited contributions to pain or itch (Dembo et al., 2018). In addition to neuronal BDNF, BDNF can be released in the periphery from non-neuronal cells, including Merkel cells (Reed-Geaghan et al., 2016), epithelial cells of the skin (Cefis et al., 2020; Rutlin et al., 2014), and peripheral immune cells (see references in Brigadski and Leßmann (2020)). The widespread nature of BDNF expression suggests it can also significantly modify nociceptor and sensory neuron function by actions in the periphery.

2.9 Other events that contribute to inflammatory or injury-induced plasticity of DRG

Inflammation and tissue or neural injury can lead to functional, chemical, or structural modification of all the components of the nociceptive pathway. Following injury or inflammation, primary nociceptors, mainly unmyelinated C-fibers and myelinated Aδ-fibers, display lowered firing thresholds (hypersensitivity) and increased spontaneous firing (hyperexcitability), a phenomenon known as peripheral sensitization. During peripheral sensitization, neuropeptides are released from the peripheral branch of the nociceptor at the injury site or inflamed environment, attracting leukocytes and activating receptors on the nociceptor terminals and/or cell bodies (Basbaum et al., 2009; Gold & Gebhart, 2010). Once recruited, leukocytes differentiate into macrophages to remove and regenerate degenerating axons (Beuche & Friede, 1984; Brown et al., 1991; Hu & McLachlan, 2002; Perry & Brown, 1992). Extending to the CNS, glial activation, characterized by upregulated microglial (e.g., IBA1) and astroglial (e.g., GFAP; glial fibrillary acid protein) markers, and morphological changes can also occur, which have been implicated in pathogenesis of chronic pain.

2.9.1 Effect of peripheral inflammation on DRG plasticity

Inflammatory responses, such as microglial proliferation and macrophage recruitment, are observed in DRGs with peripheral nerve injury (Eckert et al., 1999; Hu & McLachlan, 2002; Lu & Richardson, 1993) or DRG compression (Zhang et al., 1999). SGCs around the DRG soma proliferate (Barron et al., 1990; Gehrmann et al., 1991; Lu & Richardson, 1991) and contribute to generation of ectopic discharges and oscillatory activity (Devor, 2006). Somal ectopic firing is pathological and produces sensory signals even in the absence of pain stimuli (Woolf & Ma, 2007). SGCs also release cytokines that contribute to nerve injury-induced mechanical hypersensitivity (Ji & Strichartz, 2004; Schafers et al., 2003; Svensson et al., 2005; Zelenka et al., 2005). Local immune activation induces prolonged inflammation and increases cytokine production, including interleukins (IL) -1β, -6, -18, monocyte chemoattractant protein (MCP)-1, and growth-related oncogene CXCL1 by 17 folds (Xie et al., 2006). Upregulation of the prototypical cytokines IL-1β and TNF-α is one of the earliest indications of sensory inflammation and increased nociception (Miller et al., 2009). The release of cytokines from immune and other non-neuronal cells (e.g. keratinocytes) can directly interact with the sensory neurons or affect further downstream mediators, including other cytokines, chemokines, prostanoids, neurotrophins, and ATP (Binshtok et al., 2008; Chiu et al., 2012; Gold & Gebhart, 2010; Julius & Basbaum, 2001; Levin et al., 2008; Liu & Ji, 2013; Pezet & McMahon, 2006; White et al., 2005). IL-1β, IL-6 and TNF-α have been linked to DRG neuron excitability (Gadient & Otten, 1996; Gardiner et al., 2002; Inoue et al., 1999; Lee et al., 2004; Li et al., 2005; Nilsson et al., 2005) by sensitizing the TRP channels, leading CGRP release (Obreja et al., 2005; Obreja et al., 2002; Opree & Kress, 2000), or by enhancing TTX-r Na^+ currents (Jin & Gereau, 2006). Consistent with this observation, TNF-α antibodies attenuate the development of thermal

hyperalgesia and mechanical allodynia in models of neuropathic pain (Cunha et al., 2007; Sasaki et al., 2007; Zanella et al., 2008). Increased TRPV1 activation produces increasing inward current with repeated exposure to heat through peripherally released NGF (Zhang et al., 2005), which maintains peripheral sensitization. These changes lead to an increase in substrate for other receptors and amplify and prolong peripheral sensitization, leading to an increase in nociceptor activity.

2.9.2 Effect of SCI on pain and DRG plasticity

Immediately after the initial damage caused by SCI, damaged blood vessels and cellular membranes causes local ischemia and inflammation. The insult leads to calcium and glutamate excitotoxicity, ionic imbalance, reactive oxygen species (ROS) production, and apoptosis, progressively damaging surrounding spinal cord tissue. Microglia release cytokines and recruit macrophages, which infiltrate the damaged spinal cord and remain activated for several weeks (Popovich et al., 1997; Sroga et al., 2003). The primary injury triggers systemic, cellular, and molecular changes that spread the damage to adjacent white and gray matter, leading to secondary injury. Elevated glutamate levels drive NMDA and AMPA receptors to be hyperactive. Hence, glutamate-induced excitotoxicity becomes a leading cause of several secondary mechanisms that further the damage. Amplification of pain responses and spread of pain sensitivity to uninjured regions results in secondary hyperalgesia (Ji et al., 2003).

Nociceptor-specific changes after SCI have yet to be investigated fully, but a few studies have shown that SCI strengthen sensory signals to transform nociceptors to be activated with just low-threshold, usually innocuous stimuli, resulting in neuronal hyperactivity and hyperexcitability. Prolonged C-fiber activity can drive persistent sensitization (Ji et al., 2003). Aberrant axonal sprouting in peptidergic, CGRP-expressing nociceptors (Ackery et al., 2007; Christensen &

Hulsebosch, 1997; Helgren & Goldberger, 1993), as well as small- and medium-sized dissociated DRG neurons (Bedi et al., 2012) has been observed after SCI. Non-peptidergic C-fibers also undergo aberrant sprouting in the SCDH, consistent with injury-induced allodynia (Detloff et al., 2014). SCI can recruit typically non-nociceptive primary afferent neurons to the pain pathway (Torebjork et al., 1992), causing phenotypic changes in myelinated fibers, like Aβ-fibers (Woolf & Salter, 2000), which do not normally drive nociceptive output (Latremoliere & Woolf, 2009). Such functional changes in the primary afferent sensory neurons appear as allodynia.

Previous studies in DRG neurons, particularly nociceptors, have demonstrated that nociceptors become chronically hyperexcitable and display increased spontaneous activity (Bedi et al., 2010; Yang et al., 2014), which is neuronal firing not driven by any sensory input. While spontaneous activity occurring under physiological conditions aids in development of and rehabilitation of the spinal cord (e.g., motor neuron pathfinding, maturation of synapses, axon regrowth), increase in the incidence of spontaneous activity in the pain-transducing neurons after injury could lead to hyperalgesic priming (Reichling & Levine, 2009), so that the nociceptors are now more predisposed to enter a stable hypersensitive state (Walters, 2012). Because primary afferent somas are located distantly from their receptive fields, somal hyperexcitability may emerge to compensate for damage or loss of peripheral sensory branches after injury (Walters, 2012). Nociceptors also display increased afterdischarge (Eller et al., 2022). These aforementioned changes are consistent with the fact that sensory neurons can exhibit lowered thresholds to thermal and mechanical stimuli (Bishop et al., 2010) that indicate their involvement in the generation of pain.

Transcriptional changes occur in the DRG after SCI that are critically associated with the generation of pain (Costigan et al., 2002; Cuevas-Diaz Duran et al., 2023; Perkins et al., 2014;

Wang et al., 2002). For example, changes have been reported in synaptogenesis signaling pathways and networks responsible for inflammatory signaling mechanisms (Yasko et al., 2019). Along these lines, we recently showed that pERK levels are robustly elevated in the adjacent trunk skin after SCI in adult mice that exhibited mechanical pain (Martin et al., 2022). While increased pERK levels might indicate plasticity of sensory neurons, we did not characterize the specific cell types that express pERK. However, a previous study by Dai et al. (2002) showed pERK is elevated in peripheral nerve terminals following noxious stimulation.

2.9.3 Peripheral changes after SCI that contribute to pain

While the neural mechanisms that underlie the emergence of nociceptive and neuropathic pain after SCI have been studied extensively in the spinal cord, the functional, morphological, and molecular changes in the periphery still need investigation. Studies suggest peripheral changes play a role in the induction and maintenance of pain, specifically after SCI. For example, after SCI, some sensory fibers that are normally only responsive to innocuous mechanical or thermal stimuli may be recruited to transmit pain through peripheral sensitization (Latremoliere & Woolf, 2009). Also, sympathetic and sensory neurons show increased interaction after injury through increased sympathetic fiber density and sprouting into the DRGs (García-Poblete et al., 2003; Kinnman & Levine, 1995b; Shinder et al., 1999; Xie et al., 2007). Many neurotransmitters and neuromodulators such as SP and BDNF are synthesized, stored, and released from the central terminals of the sensory neurons in the SCDH in response to the injury. Studies also showed that intense activation of nociceptors shortly after SCI worsens hind paw mechanical hypersensitivity (Garraway et al., 2014; Martin et al., 2019), suggesting that peripheral nociceptor hyperexcitability can exacerbate pain responses after SCI. Although less understood, recent evidence suggests that peripheral TrkB signaling may also underlie pain after SCI. Specifically,

pharmacogenetic and systemic inhibition of TrkB was found to delay the onset of mechanical allodynia after SCI for up to 4 weeks and reversibly attenuate established pain (Martin et al., 2022). This observation is supported by a previous study that reported maladaptive TrkB signaling contributes to neuropathic pain after SCI (Wu et al., 2002). Collectively, these findings suggest that peripheral processes, including nociceptor hyperactivity, play a critical role in the development of pain after SCI.

2.9.4 Other SCI-induced changes: non-nociceptors such as C-LTMRs contribute to pain

Even though nociceptors are essential drivers of pain, non-nociceptors can also undergo plasticity that contributes to pain hypersensitivity. C-LTMRs are small diameter, unmyelinated afferents that innervate the trunk hairy skin and terminate in lamina II of the dorsal horn (L. Li et al., 2011). C-LTMR signals are integrated at the first synapse similarly to other primary afferents, forming synaptic glomeruli (Larsson & Nagi, 2022). They are identified by tyrosine hydroxylase (TH) (L. Li et al., 2011; Lou et al., 2013) and vesicular glutamate transporter (VGLUT) 3 expression (Gras et al., 2002; Seal et al., 2009). C-LTMRs normally encode gentle, pleasant touch contributing to social interactions (Bessou et al., 1971; Iggo, 1960; L. Li et al., 2011; Liljencrantz & Olausson, 2014; Löken et al., 2009; Morrison et al., 2010; Olausson et al., 2002; Zimmerman et al., 2014) and modulate heat pain (Habig et al., 2017). However, under pathological conditions like SCI, C-LTMRs represent a sub-population of cutaneous afferents that may indeed convey pain (Mahns & Nagi, 2013; Seal et al., 2009). For example, VGLUT3-knockout mice showed impaired acute mechanical allodynia in inflammatory and neuropathic pain models (Seal et al., 2009), demonstrating for the first time, that C-LTMRs are involved not only in low-threshold mechanosensation but may also be required for generation of pain response to innocuous mechanical stimuli. Similarly, ablation of Nav1.8-expressing nociceptors

(Abrahamsen et al., 2008) or knocking out T-type Cav3.2 in nociceptors and C-LTMRs also resulted in attenuation of mechanical allodynia (François et al., 2015), suggesting a possible mechanistic overlap between C-LTMRs and nociceptors. In human studies, individuals with SCI exhibited hyperesthesia and allodynia to activation of C-tactile fibers (i.e., human equivalent of C-LTMRs (Löken et al., 2009)) with a gentle brush stroke (Finnerup et al., 2003). Recently, further evidence that C-LTMR afferent plasticity produces pain after SCI was demonstrated using a conditioned place-aversion paradigm. In the study, adult mice with SCI avoided the chamber associated with C-LTMR stimulation (i.e., mechanical and optical stimulation) (Noble et al., 2022). Importantly, these changes took place in parallel to the establishment of hind paw mechanical hypersensitivity, suggesting that C-LTMR afferent plasticity promotes at-level affective pain following SCI, concurrent with below-level pain. Furthermore, stimulation of C-LTMRs also increased pERK levels in TH^+ DRG neurons (presumed C-LTMRs) only after SCI (Noble et al., 2022). C-LTMRs may excite nociceptive lamina I projection neurons through PKCγ-expressing neurons (Artola et al., 2020), which are also required for injury-associated mechanical hypersensitivity (Malmberg et al., 1997). Electrophysiological studies have shown sympathetic neurons directly excite C-LTMRs and increase their sensitivity to mechanical stimuli (Barasi & Lynn, 1986; Roberts & Elardo, 1985; Roberts & Levitt, 1982). Increased sympathetic activity is associated with injury-induced pain (Jänig et al., 1996; Kinnman & Levine, 1995a, 1995b; Ramer et al., 1999), suggesting that C-LTMR interaction with sympathetic neurons may take place after injury that consequently produces maladaptive changes. Despite these observations implicating C-LTMRs in pain, further investigation focusing on the neural mechanisms that enable the functional switch from touch-to-pain encoding is needed.

2.9.5 Sympathetic efferents and sensory neurons interact to produce pain

The organization of the sympathetic nervous system enables it to transmit information from the CNS to target tissues and organs. Sympathetic efferent fibers exit the spinal cord through the ventral roots to synapse with neurons in prevertebral sympathetic ganglia located in the abdomen. Apart from the C-LTMR-sympathetic interaction mentioned in the preceding section, it has long been shown that the sympathetic nervous system contributes to pain in both animal models of neuropathic pain (Kim & Chung, 1991; Levine et al., 1986) and in humans (McDonnell et al., 2011). Minett et al. (2012) showed that Nav 1.7 expression in different sets of sensory and sympathetic neurons underlies distinct types of pain sensation in mice. Specifically, ablation of Nav 1.7 in sensory and sympathetic neurons abolished pain sensation following nerve ligation, clearly demonstrating that the interaction between sympathetic and sensory systems to produce neuropathic pain. Although the exact mechanism underlying the interaction remains unclear, previous studies have investigated potential sympathetic-sensory neuron interactions at nerve terminals, cell bodies, and central terminals (Jänig & Häbler, 2000). Importantly, a prior study by Ren et al. (2005), showed that sympathetic efferent is necessary for C- and Aδ-pain fiber sensitization by capsaicin, further demonstrating that pain afferents are directly modulated by sympathetic activity (also see Lin et al. (2003)). Under normal conditions, sensory neurons and sympathetic nerves primarily interact with blood vessels, but several pain models have shown increased sympathetic fiber density and sprouting into the DRGs, forming “basket” structures known as Dogiel’s arborizations (McLachlan et al., 1993). Dogiel’s arborizations were observed by Ramón y Cajal (García-Poblete et al., 2003), in human neuropathic pain patients (Shinder et al., 1999), axotomized cells (Ma & Bisby, 1999), and locally inflamed (Xie et al., 2006) or compressed (Chien et al., 2005) DRGs though in small numbers. The link between

basket cells and behavioral studies have been conflicting (Xie et al., 2010), but electrophysiological data demonstrate basket cells to be the main source of spontaneous activity (Xie et al., 2011). Basket cells also showed increased nociceptive markers, such as TrkA, CGRP and SP, demonstrating that sprouting sympathetic fibers are probably closely apposed to nociceptive cells.

Sympathetic activity can indirectly interact with sensory pain systems through the neuro-endocrine and immune systems. Notably, sympathetic activity can impact immune functions by producing inflammation, which can then modify sensory systems such as nociceptor activity (see review by Jänig (2014)). Similarly, spontaneously active DRG cells can cause sympathetic sprouting, presumably by the release of neurotrophic factors or cytokines from SGCs or glial cells (Xie et al., 2007; Zhang & Strong, 2008). Norepinephrine and ATP receptors also expressed on SGCs may be activated to release inflammatory neuromodulators, recruiting sympathetic fibers after injury or inflammation (Hanani, 2005; Maruo et al., 2006; Tan et al., 2011). Here, we provide a brief overview of C-LTMRs and sympathetic contribution to pain, although noting that these interactions are quite complex and beyond the scope of this review.

2.9.6 Central sensitization

While this chapter aims to shed much needed light on the role DRG and sensory neuronal plasticity play in pain, it is important to briefly discuss cellular plasticity that occurs centrally, particularly the SCDH where nociceptors terminate. At the central axon terminal of the DRG, the release of neurotransmitters and neuromodulators triggers activation of postsynaptic glutamatergic receptors (i.e., NMDARs, AMPARs), which initiate recruitment of subthreshold synaptic inputs to nociceptive neurons. This process generates a state of potentiation known as the central sensitization (Woolf, 1983), a phenomenon characterized by a C-fiber-mediated

enhancement of synaptic efficacy in the SCDH neurons following peripheral noxious stimuli, tissue injury, or nerve damage (Ji et al., 2003). Increased synaptic transmission decreases pain threshold, increases membrane excitability, and spreads pain sensitivity to areas that were not initially injured, largely due to reduction in inhibitory tone (Ji et al., 2003). Once central sensitization is established, sensory neurons become hyper-responsive to normally subthreshold innocuous stimuli. This heightened sensitivity can persist independently and in the absence of intense, noxious stimuli. For example, SCI can cause neuroinflammation and central sensitization that lead to chronic pain without peripheral insult (Hains & Waxman, 2006), by potentially inducing hyperexcitability of primary sensory neurons (Yang et al., 2014) or triggering the release of inflammatory mediators that can regulate DRG gene expression. Similarly, pain after SCI is worsened by intense activation of nociceptors, further reinforcing central sensitization mechanisms (Ferguson et al., 2006; Garraway et al., 2014; Grau, 2017). Thus, central and peripheral sensitization are likely involved in bi-directional interactions (Ji et al., 2018), working in concert to mediate pain after inflammation or tissue injury, which may underlie the persistence and amplification of pain following SCI.

2.10 Conclusion

This chapter brings together many studies and reviews on mechanisms of pain, SCI, neurotrophins, and nociceptors, highlighting the complex interactions between these systems with the focus on the contribution of the DRGs underlying the individual components. Whereas significant progress has been made in our understanding of nociceptive processing in the SCDH, the initial site of CNS pain processing, there remains a need for continued research emphasizing the critical roles peripheral plasticity and DRGs play in nociception. Continued exploration of DRG plasticity, particularly in the context of peripheral sensitization and recruitment into

nociceptive pathway, will provide deeper insight into how peripheral and central mechanisms interact to drive chronic pain after SCI. Understanding these processes will advance our understanding and efforts of developing more effective therapeutic strategies to manage outcomes for SCI.

2.11 Figures

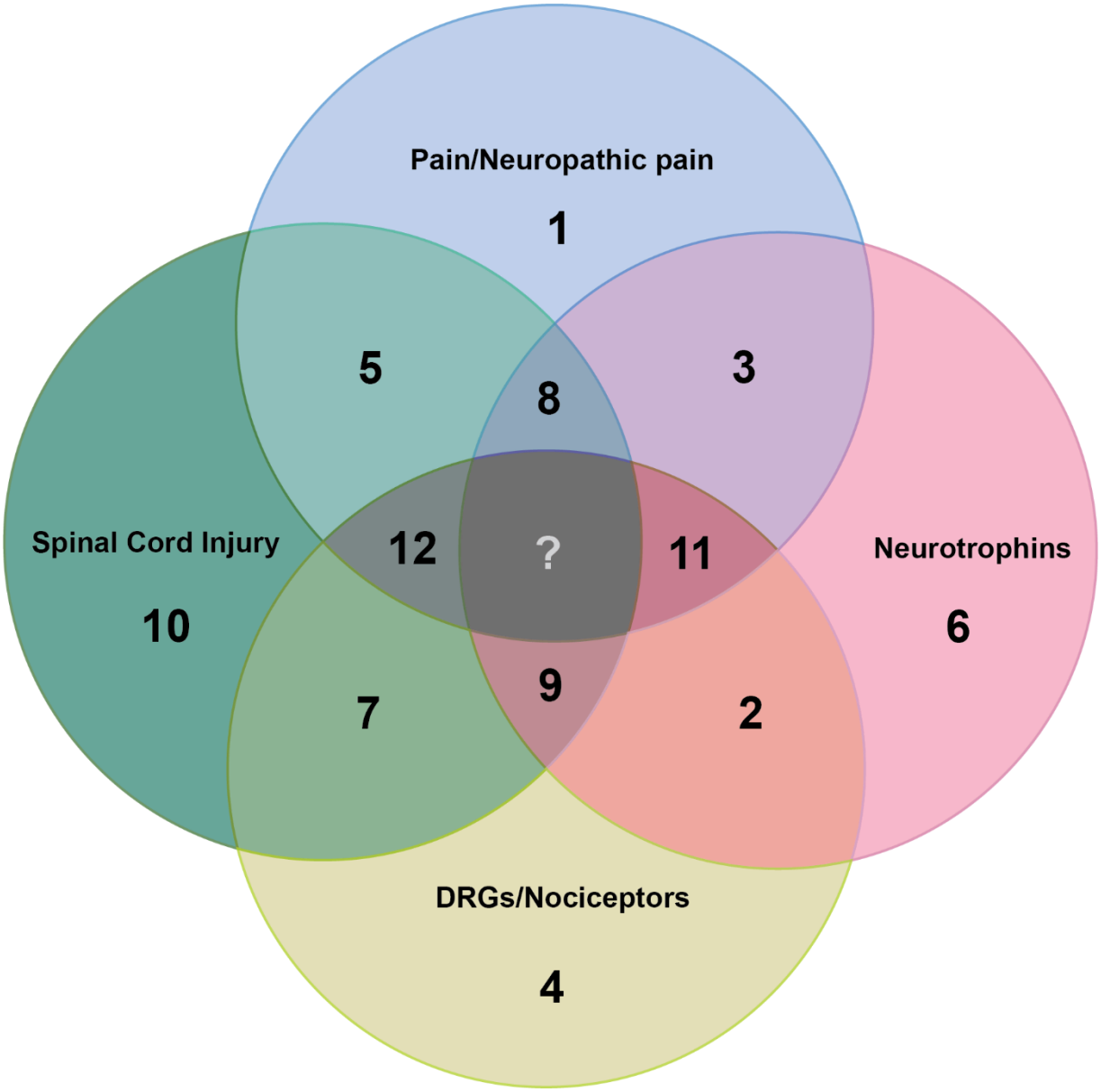


Figure 2.1: Examples of some review literature on pain, SCI, neurotrophins, and nociceptors through the past 30 years.

This figure shows 12 recent review articles related to the field. Each number in the diagram can be linked to an article listed in Table 2.1. Although not demonstrative of the full scope of each topic, these reviews i) show most recent developments in the field or ii) are highly cited in other

work, which implies their impact on driving the direction of other research. It should be noted that while several articles focus on 2 (article #2, 3, 5 and 7) or 3 (article # 8, 9, 11 and 12) topics, none of the articles examines all 4 topics (center space designated by '?'). This demonstrates a lack of reviews that discuss all the topics together to shed light on central as well as peripheral mechanisms including DRG and nociceptor plasticity in pain hypersensitivity, including neuropathic pain after SCI. The gap in perspective shows potential future research opportunities and development of new research questions for the field.

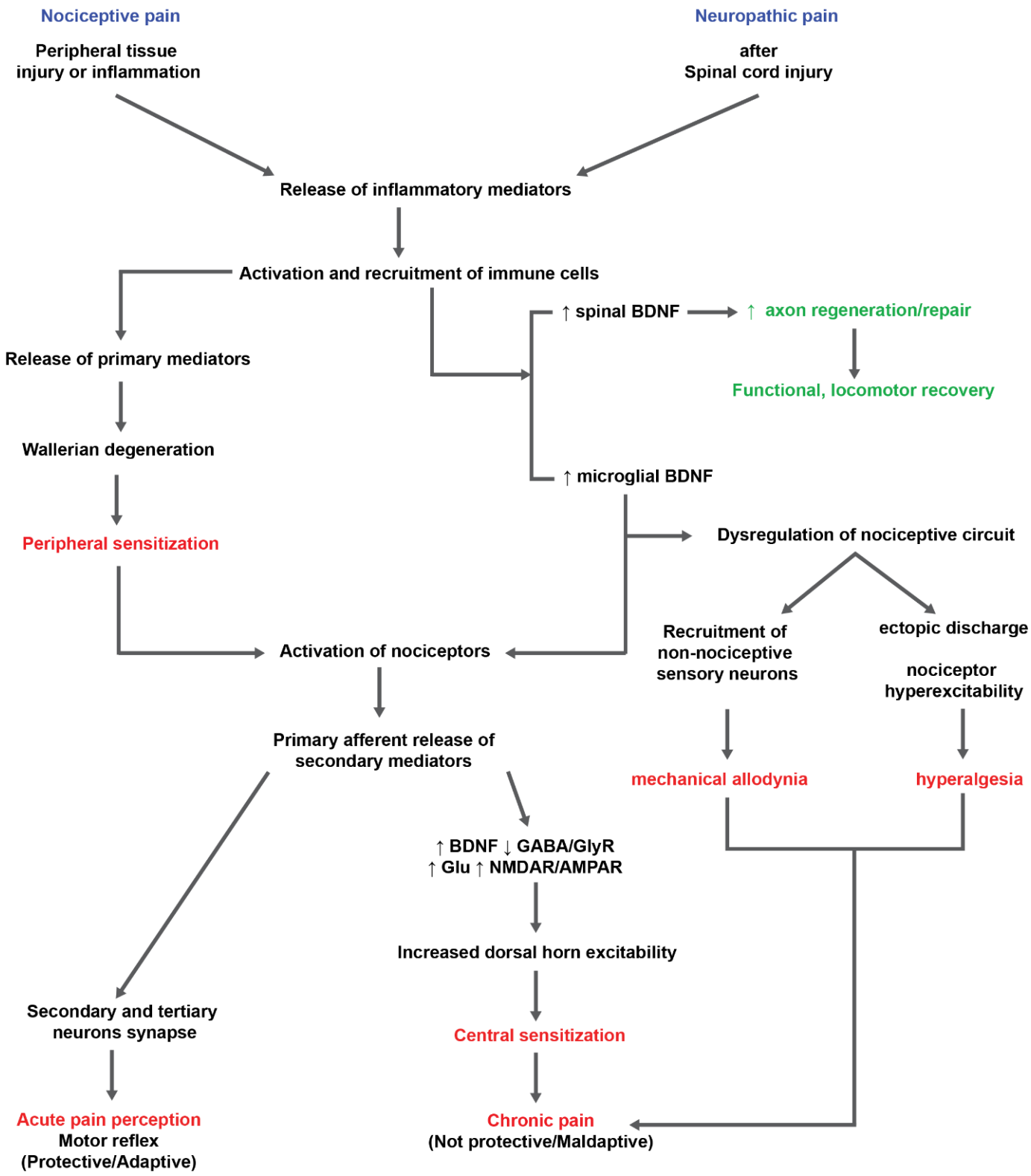

Nociceptive pain
Peripheral tissue injury or inflammation
Neuropathic pain
after Spinal cord injury
Release of inflammatory mediators
Activation and recruitment of immune cells
Release of primary mediators
Wallerian degeneration
Peripheral sensitization
↑ spinal BDNF
↑ axon regeneration/repair
Functional, locomotor recovery
↑ microglial BDNF
Dysregulation of nociceptive circuit
Recruitment of non-nociceptive sensory neurons
ectopic discharge
nociceptor hyperexcitability
mechanical allodynia
hyperalgesia
Activation of nociceptors
Primary afferent release of secondary mediators
↑ BDNF ↓ GABA/GlyR
↑ Glu ↑ NMDAR/AMPAR
Increased dorsal horn excitability
Central sensitization
Chronic pain
(Not protective/Maldaptive)
Secondary and tertiary neurons synapse
Acute pain perception
Motor reflex
(Protective/Adaptive)

Figure 2.2: Comparison of nociceptive and neuropathic pain.

Diagram illustrates an overview of critical mechanisms that lead to development of nociceptive and neuropathic pain after peripheral or central (e.g., SCI) injuries. Some mechanisms overlap, but distinct pathways and modulators involved are noted. Highlighted text indicates negative (red) or positive (green) outcomes of neural plasticity.

#	Reference		Conclusions/summary	Topic
1	Millan (1999)	The induction of pain: an integrative review	Origin and pathophysiological significance of pain from evolutionary perspective	Pain
2	Mendell (2003)	Peripheral neurotrophic factors and pain	Mechanisms underlying sensitization, specifically the substances released and availability of the receptors that contribute to hyperalgesia	Neurotrophic factors Periphery/nociceptors
3	Pezet and McMahon (2006)	Neurotrophins: mediators and modulators of pain	Evidence for the contribution of neurotrophins (NGF, BDNF), the range of conditions that trigger their actions, and the mechanism of action in relation to pain	Neurotrophic factors Pain
4	Woolf and Ma (2007)	Nociceptors: noxious stimulus detectors	Nociceptor components, function, regulation of ion channels/receptors after injury	Nociceptors
5	Yezierski (2009)	SCI pain: Spinal and supraspinal mechanisms	Review of experimental studies focused on the spinal and supraspinal mechanisms with at- and below-level pain after SCI	Pain SCI
6	Numakawa et al. (2010)	BDNF function and intracellular signaling in neurons	Broad overview of the current knowledge concerning BDNF action and associated intracellular signaling in neuronal protection, synaptic function, and morphological change, and understanding the secretion and intracellular dynamics of BDNF	Neurotrophins
7	Walters (2012)	Nociceptors as chronic drivers of pain and hyperreflexia after SCI: an adaptive-maladaptive hyperfunctional state hypothesis	Proposes SCI as trigger for persistent hyperfunctional state in nociceptors that originally evolved as an adaptive response. Focus on uninjured nociceptors altered by SCI and how they contribute to behavioral hypersensitivity.	Nociceptors SCI

8	Garraway and Huie. (2016)	Spinal Plasticity and Behavior: BDNF-Induced Neuromodulation in Uninjured and Injured Spinal Cord	Review of diverse actions of BDNF from recent literatures and comparison of BDNF-induced nociceptive plasticity in naïve and SCI conditions	SCI Pain Neurotrophins
9	Keefe et al. (2017)	Targeting Neurotrophins to Specific Populations of Neurons: NGF, BDNF, and NT-3 and Their Relevance for Treatment of Spinal Cord Injury	Review of neurotrophins NGF, BDNF, and NT-3 and their effects on specific populations of neurons, including nociceptors, after SCI	SCI Neurotrophins Nociceptors
10	Alizadeh et al. (2019)	Traumatic SCI: An overview of pathophysiology, models, and acute injury mechanism	Comprehensive overview of pathophysiology of SCI, neurological outcomes of human SCI, and available experimental model systems that have been used to identify SCI mechanisms	SCI
11	Cao et al. (2020)	Function and Mechanisms of truncated BDNF receptor TrkB.T1 in Neuropathic pain	Review of studies on truncated TrkB.T1 isoform, and its potential contribution to hyperpathic pain through interaction with neurotrophins and change in intracellular calcium levels.	Neuropathic pain Neurotrophins Nociceptors
12	Garraway (2023)	BDNF-Induced plasticity of spinal circuits underlying pain and learning	Review of literature on various types of plasticity that occur in the spinal cord and discussion of BDNF contribution in mediating cellular plasticity that underlies pain processing and spinal learning.	Pain SCI Neurotrophins

Table 2.1

Examples of 12 representative review literatures on pain, SCI, neurotrophins, and/or nociceptors through the past 30 years. Each article can be located as a corresponding number (designated by # column) in Figure 2.1.

Chapter 3: TrkB agonist (7,8-DHF)-induced responses in dorsal root ganglia neurons are decreased after spinal cord injury: implication for peripheral pain mechanisms.

This chapter contains manuscript under review: K Jang, Garraway SM. (2024)
TrkB agonist (7,8-DHF)-induced responses in dorsal root ganglia neurons are decreased after spinal cord injury: implication for peripheral pain mechanisms.
[Manuscript submitted for publication].
Department of Cell Biology, Emory University School of Medicine.

3.1 Abstract

Brain-derived neurotrophic factor (BDNF) and tropomyosin receptor kinase B (TrkB) are known to contribute to both protective and pronociceptive processes. However, their contribution to neuropathic pain after spinal cord injury (SCI) needs further investigation. In a recent study utilizing $TrkB^{F616A}$ mice, it was shown that systemic pharmacogenetic inhibition of TrkB signaling with 1NM-PP1 (1NMP) immediately after SCI delayed the onset of pain hypersensitivity, implicating maladaptive TrkB signaling in pain after SCI. To examine potential neural mechanisms underlying the behavioral outcome, patch-clamp recording was performed in small-diameter dissociated thoracic (T) dorsal root ganglia (DRG) neurons to evaluate TrkB signaling in uninjured mice and after T10 contusion SCI. Bath-applied 7,8-dihydroxyflavone (7,8-DHF), a selective TrkB agonist, induced robust inward currents in neurons from uninjured mice, that was attenuated by 1NMP treatment. Following SCI, 7,8-DHF-induced current was decreased while the latency to the response peak amplitude was increased. Western blot revealed a concomitant decrease in TrkB expression in DRGs adjacent to the spinal lesion. Analyses of cellular and membrane properties showed that SCI increased neuronal excitability, evident by an increase in resting membrane potential and the number of spiking neurons. However, SCI did not increase spontaneous firing in DRG neurons. These results suggest that SCI induced changes in TrkB activation in DRG neurons even though these alterations are likely not contributing to pain hypersensitivity by nociceptor hyperexcitability. Overall, this reveals complex interactions involving TrkB signaling and provides an opportunity to investigate other, presumably peripheral, mechanisms by which TrkB contributes to pain hypersensitivity after SCI.

3.2 Introduction

Neuropathic pain, a pain caused by damage to the nervous system, is a common consequence of spinal cord injury (SCI) (Felix et al., 2022; Siddall & Loeser, 2001). The mechanisms underlying neuropathic pain are complex and shown to involve both central and peripheral mediators [see reviews by (Hulsebosch, 2002; Hulsebosch et al., 2009)]. Brain-derived neurotrophic factor (BDNF), signaling through its high affinity tropomyosin receptor kinase-B (TrkB), serves as an effective growth promoter, including regrowth of damaged axons in the injured spinal cord. However, BDNF is also a potent modulator of pain signaling. BDNF is expressed and synthesized in small-to-medium diameter dorsal root ganglia (DRG) neurons (Kerr et al., 1999; Merighi et al., 2008; Obata & Noguchi, 2006), and its levels are altered in the DRG and spinal cord dorsal horn (SCDH) after tissue or nerve injury [e.g. (Cho et al., 1998; Ernfors et al., 1993; Fukuoka et al., 2001; Ha et al., 2001; Merighi et al., 2008; Michael et al., 1997; Pezet, Malcangio, Lever, et al., 2002; Zhou et al., 1999)]. TrkB is also expressed in DRGs [e.g. (Hougland et al., 2012; Zheng et al., 2019)], and its activation is linked with hypersensitization of thermal sensory neurons (Shu et al., 1999) and mechanical allodynia (Dhandapani et al., 2018; Hu et al., 2023). The duplicity of BDNF-TrkB signaling in both the peripheral and central nervous systems demonstrate the complex involvement of the neurotrophin system and underscores the need for deeper investigation into its mechanistic contribution in post-injury plasticity. Especially after SCI, the role of TrkB activation in generation or maintenance of pain hypersensitivity has not been fully elucidated [see review by (Garraway & Huie, 2016)].

A recent study showed that pharmacogenetic inhibition of TrkB signaling immediately after SCI delayed the onset of mechanical hypersensitivity and improved locomotor recovery in adult $TrkB^{F616A}$ mice (F616) (Martin et al., 2022). Inhibition of TrkB at later time points also

reversibly attenuated mechanical hypersensitivity. F616 mice express TrkB receptors containing a mutation that allows a small molecule, cell-permeable kinase inhibitor, 1NM-PP1 (1NMP), to bind to the ATP-binding pocket (Chen et al., 2005). 1NMP provides a rapid [typically within an hour *in vivo*; Pareja-Cajiao et al. (2020)], robust, and reversible inhibition of TrkB (Chen et al., 2005). Until this study, research had suggested a minimal role of spinal BDNF or TrkB in SCI-induced pain. For example, BDNF and TrkB levels decreased in the injured spinal cord (Garraway et al., 2011; Hajebrahimi et al., 2008; King et al., 2000; Liebl et al., 2001; Strickland et al., 2014) even when mechanical hypersensitivity was evident (Garraway et al., 2011). Moreover, BDNF failed to induce LTP-like facilitation of C-fiber evoked synaptic and glutamatergic responses after SCI (Garraway et al., 2005; S. M. Garraway & L. M. Mendell, 2007), unlike its facilitatory actions in uninjured rats (Garraway et al., 2005). No study has directly assessed whether TrkB signaling in pain after SCI is mediated by spinal TrkB activation or changes in TrkB elsewhere, although in inflammatory and neuropathic pain models, peripheral BDNF was found to be involved in mediating transition from acute to chronic pain (Sikandar et al., 2018). The potential contribution of peripheral BDNF and TrkB to plasticity after SCI has not been fully explored, and the recent observation that maladaptive TrkB signaling contributes to mechanical pain acutely after SCI (Martin et al., 2022) suggests a more complex role for TrkB signaling in nociceptive plasticity. However, the specific neural mechanisms, including the exact site of action, were not determined. Building upon this finding, this study proposes that the engagement of TrkB mechanisms drives changes in excitability of DRG neurons after SCI, postulating that peripheral TrkB mechanisms underlie pain.

DRGs, uniquely positioned at the intersection between the peripheral and central nervous system, play a key role in nociceptive transmission. Bedi et al. (2010) found primary nociceptors to

become chronically hyperexcitable after SCI, consistent with pain hypersensitivity. Here, dissociated DRG neurons obtained from uninjured or SCI F616 mice, treated with 1NMP or vehicle, were evaluated using whole-cell patch clamp electrophysiology. The study investigated neuronal responses to 7,8-dihydroxyflavone (7,8-DHF), a small molecule, selective TrkB agonist that binds TrkB with more specificity than BDNF but triggers the same downstream intracellular signaling cascade (Jang et al., 2010). Furthermore, changes in intrinsic properties of sensory neurons, capsaicin-induced inward currents, and TrkB protein expression in DRGs were assessed. Overall, the results revealed that SCI induced neuronal hyperexcitability that is partly independent of TrkB signaling, and that both TrkB agonist-induced neuronal responses and TrkB expression in DRGs adjacent to the level of injury were reduced after SCI.

3.3 Materials and Methods

3.3.1 Subjects

Experiments were performed in adult male (n = 24) and female (n = 31) $Ntrk2^{F616A}$ (F616) mice that enable selective, yet reversible pharmacogenetic inhibition of TrkB signaling (JAX # 022363) (Chen et al., 2005).They were housed in standard cages in a vivarium on a 12:12-hour light-dark cycle. Animals were fed standard rodent diets *ad libitum*. Experimental procedures were approved by the Animal Care and Use Committee of Emory University and conformed to national standards for the care and use of experimental animals and the American Physiological Society's "Guiding Principles in the Care and Use of Animals". At the time of drug treatment and surgery, all animals were approximately 8-12 weeks of age and weighed 22-26 g (males) and 16-22 g (females). An experimental timeline illustrating when the procedures described below were undertaken is provided in Figure 3.1A.

3.3.2 Drug administration and pharmacological verification

F616 mice were treated with 1-(1, 1-dimethylethyl)-3-(1-naphthalenylmethyl)-1H-pyrazolo[3,4-d]pyrimidin-4-amine (1NM-PP1 or 1NMP; #13330, Cayman Chemical), a small molecule, cell permeable kinase inhibitor to block TrkB signaling systemically and reversibly, such as seen in previous studies (Mantilla et al., 2014; Martin et al., 2022). Consistent with the study by Martin et al. (2022), 1NMP was administered in drinking water at a final concentration of 5 μM (dissolved in dimethyl sulfoxide, DMSO). This treatment paradigm delayed the development of mechanical pain, and electrophysiological and pharmacological evaluation in acutely dissociated DRG neurons obtained from 1NMP- and vehicle (Veh)-treated F616 mice showed effectiveness of 1NMP inhibition. Therefore, this approach ensures consistency in examining cellular mechanisms underlying the observed behavioral attenuation. A cohort of mice were treated with an equal volume of DMSO in drinking water (dilution 1:10,000) and served as Veh-treated control. Mice were provided with 1NMP or Veh-treated water for five days, consuming approximately 30-40 mL, comparable to previous reports.

3.3.3 Surgical procedure

Mice were deeply anesthetized with isoflurane (5% gas; lowered to 2%-3% once stable anesthesia was achieved). A skin incision, followed by a dorsal laminectomy was performed to remove the vertebra over the spinal cord at T9-10. The exposed spinal cord was impacted at T10 under sterile conditions. Mice received midline contusion injuries with an Infinite Horizon Impactor (Precision Systems and Instrumentation, Fairfax Station, VA) with 70 kdyne, zero dwell time impact onto the dorsal surface of the spinal cord, also as previously described (Martin et al., 2022; Noble et al., 2022; Parvin et al., 2021). After the impact, bilateral bruising of the dorsal spinal cord was carefully verified by examination under a dissecting microscope. The

muscle and skin were sutured, and the wound area was treated with a topical triple antibiotic ointment (bacitracin-neomycin-polymyxin B). All mice recovered on a heated pad and were given meloxicam (5 mg/kg, subcutaneously), Baytril (2.5 mg/kg), and saline solution (0.5 mL) intraperitoneally, immediately after surgery for acute pain, infection, and hydration management. Baytril was subsequently given every morning for up to 7 days post operation (dpo) to minimize the risk of urinary tract or bladder infection in SCI animals. Mice bladders were manually expressed twice daily for the duration of experiments. Mice were assessed for impairment of locomotor function at 1 dpo using the Basso Mouse Scale (BMS; Basso et al. (2006)) to ensure the effectiveness of the injury. Only SCI mice scoring 0 or 1 at 1 dpo, which showed little to no locomotion, were included in the study. Uninjured (Uninj in figures) mice, which did not receive laminectomy or contusion, nor subjected to any post-surgical manipulations, served as control. Uninjured and SCI F616 mice were treated with 1NMP or Veh in drinking water as described above. Experiments consisted of the following groups: Uninjured-Veh, Uninjured-1NMP, SCI-Veh, SCI-1NMP. For SCI mice, 1NMP or Veh treatment began on the day of surgery (0 dpo).

3.3.4 Dissociation of DRG neurons

Mice were deeply anesthetized and euthanized with isoflurane. DRGs were extracted from neural segments T4 – lumbar (L) 2 and immediately placed in cold HBSS (#21-022-CV, Corning) grouped by the following segments: T4-T7, T8-T12, and T13-L2. Electrophysiological recordings were primarily made from the T8-T12 cells, which is the region near the site of the injury; however, the separation by region allows for the potential analysis of rostro-caudal differences. The DRGs were then incubated for enzymatic digestion in solution containing Dispase II (2.5 μ/ml; #04942078001, Sigma-Aldrich) and collagenase (200 μ/ml; #LS004176, Worthington Biochemical) in a 37°C water bath for 60 min and were gently inverted 4 – 5 times

once every 15 min. Cells were dissociated 20 – 30 times in neurobasal medium-A (NB-A; #12349-015, Thermo Fisher) with 2% B-27 (#17504-044, Thermo Fisher), 1% penicillin/streptomycin (#17-602E, Lonza Biowhittaker), and 1% Glutamax (#35050061,Thermo Fisher) by trituration through a set of fire-polished glass pipettes in decreasing diameter. Dissociated cells were then centrifuged at 100 rpm for 3 minutes, after which the cells were resuspended and seeded at low density on coverslips coated with laminin (1 mg/ml; #12634-010, Sigma-Aldrich) and poly-L-lysine (0.1 mg/ml; # p1274-100mg, Sigma-Aldrich). Once seeded on 22 x 22 mm coverglass (#10026-140, VWR), the cells were incubated for 30 min. 2 mL NB-A medium was added per well for further incubation (12 mL total). Plates were kept in a 37°C incubator with 5% CO_2 for at least 24 h before electrophysiological recording.

3.3.5 Whole cell recording from dissociated DRG neurons

An inverted microscope (Nikon Eclipse Ti-U) was used to identify small (15 - 30 μm) DRG neurons. Whole-cell patch recordings were made from such cells at room temperature using the conventional patch clamp configuration. Signals were acquired with MultiClamp 700B amplifier (Molecular Devices, Sunnyvale, CA), digitized at 10 kHz (Digidata 1440 A; Molecular Devices), and filtered at 1kHz (Clampex 10.2, Molecular Devices). Patch electrodes with a resistance of 6 – 8 MΩ were pulled from borosilicate micropipettes (World Precision Instruments) using a Flaming/Brown P-97 micropipette puller (Sutter Instrument) and filled with solution containing (in mM) 140 K-gluconate, 11 EGTA, 10 HEPES, 1 $CaCl_2$, 4 Mg-ATP, and 1 Na-GTP (pH 7.4 adjusted with KOH, osmolarity ranged from 290-300 mOsM). Artificial cerebrospinal fluid (aCSF) containing (in mM) 140 NaCl, 3 KCl, 2 $MgCl_2$, 1.8 $CaCl_2$, 10 glucose, 10 HEPES (pH 7.3 adjusted with KOH, ~310-320 mOsM) was oxygenated with 95% O_2 - 5% CO_2 and continuously delivered to the recording chamber. Gentle negative pressure was applied to form a

tight seal (resistance > 1 GΩ) between the electrode tip and the cell membrane. The cell membrane was ruptured by applying gentle pressure to achieve the whole-cell configuration.

Once in whole-cell configuration, the Clampex Membrane Test program (Molecular Devices) was used to determine C_m and membrane resistance, R_m from a holding potential of -70 mV in voltage clamp configuration. While held at -70 mV in voltage-clamp mode, hyperpolarizing (-40 mV) and a series of depolarizing (Δ 20 mV) current steps were undertaken to identify inward current response, and to obtain estimates of several membrane properties. The voltage-gated sodium channel (VGSC) blocker, tetrodotoxin (TTX; 300 nM), was bath applied to a subset of neurons (n=10). Inward current in both voltage and current clamp configuration was blocked by TTX (Figure 3.2B, C). The configuration was then switched to bridge mode (I = 0) and the resting membrane potential (RMP) was recorded. Other electrophysiological properties to determine the excitability of the neurons were recorded using current clamp configuration, at -60 mV, with ascending series of 2 ms long depolarizing pulses until action potential (AP) was evoked. To observe spontaneous firing, neurons were recorded in current clamp gap-free mode over one 1 min period at their RMP.

3.3.6 Assessment of electrophysiological properties

To compare the changes in neuronal activity before and after drug application or injury, various membrane properties were measured (depicted in Figure 3.1B). RMP was noted after a stable patch was established and was carefully monitored for the duration of the experiment. Rheobase, the minimum current required to evoke an AP, and the AP voltage threshold, which measures the voltage at the onset of the AP, were calculated at rest and after drug treatment. The AP and afterhyperpolarization (AHP) were determined from the first trace with an AP spike. AP amplitude was measured between the AP threshold and AP peak. The AHP amplitude was

measured from the peak of the hyperpolarization to the baseline. The AHP duration can be measured in several ways. Here, AHP duration, as milliseconds, was measured at 50% (AHP_{50}) and 80% (AHP_{80}) recovery of the AHP back to baseline, as previously described [e.g. Koerber et al. (1988); Lawson et al. (1996)]. The instantaneous firing frequency describes the rate of AP spike generation over the duration of the current input (2 ms) and was obtained from the first depolarizing step with more than one AP spike. The input resistance (R_{in}) for each cell was obtained from the slope of the second trace in response to a series of hyperpolarizing currents, and the membrane time constant, tau ($\boldsymbol{\tau}$), was determined with a single exponential fitted to the hyperpolarizing step. Cell capacitance was recorded after the whole-cell configuration and back calculated from R_{in} and tau using $R_{in}C_{in} = \boldsymbol{\tau}$.

3.3.7 Preparation and application of drugs

All drugs were delivered by bath perfusion during gap-free voltage clamp recording at -60mV as illustrated in Figure 3.1C. Drugs were first prepared as concentrated stock solution in aCSF and stored at -20 °C. Stock solutions of 7,8-DHF (#D1916, Tokyo Chemical Industry) and capsaicin (#211275, EMD Millipore Corp) were dissolved in 100% DMSO. Drug stock solutions were diluted in aCSF on the day of recording. The final concentration of DMSO was minimal and not expected to influence neuronal activity (Galvao et al., 2014; Zhang et al., 2017). 7,8-DHF was diluted to final concentrations of 50, 100 and 500 nM, which were applied 1 - 2 min after the start of the recording before being washed out for at least 2 min.

For Acute 1NMP application, the neurons were recorded to establish a baseline for response to 7,8-DHF in an identical protocol. After establishing the baseline response to 7,8-DHF, 1 μM 1NMP was superfused, followed by re-application of the same concentrations (50, 100, 500 nM) of 7,8-DHF to observe the acute effects of 1NMP on TrkB-mediated currents. Some cells were

also tested with a TrkB specific inhibitor, ANA-12 (Cazorla et al., 2011). This experiment was undertaken to further confirm that 7,8-DHF-induced inward current is indeed mediated by engaging TrkB. ANA-12 was first dissolved in DMSO at a concentration of 20 mM. Following a baseline response to 7,8-DHF, ANA-12 (1 and 10 μM) was bath applied in the presence of 100 nM 7,8-DHF.

Capsaicin sensitivity is widely considered a nociceptive marker because many nociceptors express the Transient receptor potential vanilloid 1 (TRPV1) (Cardenas et al., 1995; Caterina et al., 1997; Petruska et al., 2000). Here, capsaicin sensitivity was tested in all recorded neurons. In a subset of sensory neurons (n=96), response to capsaicin was first assessed by delivering increasing concentrations of capsaicin (0.3, 1, 3, 10, 30 μM; final concentrations in aCSF diluted from a 3 mM stock solution in DMSO). Consistent with other studies, 3 μM was determined to produce the most reliable response (data not shown). Therefore, in all neurons, after 7,8-DHF was completely washed out by bathing with aCSF for 10 minutes, 3 μM capsaicin was bath applied for 1 minute, followed by another aCSF wash (Figure 3.1C).

3.3.8 Western blot for TrkB expression

At 7 dpo, nine mice (5 SCI and 4 uninjured) were deeply anesthetized with isoflurane and oxygen (as described above), and T4 to L2 DRGs were rapidly removed, and flash frozen in liquid nitrogen. Total protein was extracted using RIPA lysis buffer and quantified using the bicinchoninic acid protein assay. Protein samples were then diluted in the Laemmli sample buffer and stored at -80 °C for western blot. Equal amounts of total protein (30 μg) were assayed through SDS-PAGE using 12% Tris-HCl gels and then transferred to polyvinylidene difluoride membranes (#03010040001, Millipore). Blots were blocked for one hour in 5% blotting grade milk (BioRad Laboratories) in Tris-buffered saline Tween-20 (TBST). The blots were incubated

overnight at 4 °C in primary antibodies: TrkB (1:1000; #AF1494, RRID:AB_2155264; R&D systems) generated in goat and β-tubulin (1:1,000; #05-661, RRID:AB_309885; Upstate Cell Signaling) generated in mouse. The following day, membranes were washed in TBST, then incubated in HRP-conjugated donkey anti-goat (1:10,000; #PA1-28805, RRID:AB_10988865; Thermo Fisher) or goat anti-mouse secondary antibodies (1:2,500; #31430, RRID:AB_228307; Thermo Fisher). The blots were developed with standard enhanced chemiluminescence and imaged with Azure Biosystems c600 Western blot Imaging System. Ratios of the integrated densitometry of each protein of interest to the loading control (β-tubulin) were calculated with AlphaView Software (ProteinSimple), normalized to uninjured controls.

3.3.9 Immunocytochemistry

Coverslips containing dissociated DRG neurons were fixed in 4% PFA at room temperature and then washed briefly in PBS. After washing, culture dishes were permeabilized in 0.1% Triton-X-100 and then blocked in 10% Normal Goat Serum in PBS-Tween 20 at room temperature. Samples were then incubated with primary antibodies (diluted in the blocking solution) overnight at 4 °C. The following primary antibodies were used: mouse anti-NeuN (1:400; #MAB377, RRID:AB_2298772; EMD Millipore), rabbit anti-TrkB (1:2000; #AF1494, RRID:AB_2155264; R and D Systems). Following three 5 min washes in PBS, secondary antibodies were applied for 1 hr at room temperature: ALEXA fluor 488 (1:100; #A-11008, RRID:AB_143165; Invitrogen), 546 (1:250; #A-11035, RRID:AB_2534093; Invitrogen). Samples were mounted onto glass slides with ProLong Gold antifade reagent (#P36934, Invitrogen) followed by image capture under Keyence microscope using Keyence BZ-X710 imaging software.

3.3.10 Statistical analysis

Mice were randomly assigned to each experimental group. All statistical measures and analyses were undertaken with GraphPad Prism 10 (GraphPad Software). While experimenters could not be blinded to SCI versus uninjured groups, experimenters performing behavioral tests and subsequent statistical analyses were blinded to treatment (1NMP vs. Veh). All statistical data in text and figures are presented as mean ± standard error of the mean (SEM) along with the number of samples analyzed (n). Comparison between groups was accomplished using 2-way repeated measures (RM) ANOVA as injury condition/drug treatment as the between-subjects factor. For categorical comparisons, Chi-square (χ^2) test or Fisher's exact test was used. Student's *t*-test were used where only two groups were being compared, and $p < 0.05$ was considered statistically significant. Cells were excluded if they did not exhibit an inward current in response to depolarizing steps, or if their RMP was more depolarized than -40 mV. Outliers were determined using Grubbs' test.

3.4 Results

Forty-six F616 mice (18 males and 28 females) were used for electrophysiology as follows: Uninjured Veh (15), Uninjured-1NMP (8), SCI-Veh (12) and SCI-1NMP (11). An additional nine mice were used for western blot (4 uninjured and 5 SCI). The specific breakdown of animals used in the current study is summarized in Table 1. The contusion at T10 produced hindlimb paralysis in all mice in the study and was confirmed by BMS score (SCI group mean BMS score, 0.1; 1 d after injury). F616 mice express a mutated TrkB that enables selective inhibition of TrkB signaling with the small molecule kinase inhibitor, 1NMP. Mice were treated with 1NMP (or Veh) in drinking water to inhibit TrkB signaling systemically to evaluate contribution of TrkB to neural mechanisms of pain hypersensitivity after SCI.

3.4.1 Morphologies and properties of DRG neurons from uninjured and SCI F616 mice

T4 to L2 DRGs were collected from animals in each group to be dissociated and cultured for 24 h before recording. Dissociated neurons displayed a smooth, clear membrane surface and sometimes other smaller cells attached, possibly satellite glial cells. Most neurons developed neurites with more complex branching the longer they were incubated (Figure 3.2A). Visually, there were no apparent differences in cultured DRG neurons from Uninjured-Veh mice to cultured neurons from other experimental groups (Uninjured-1NMP, SCI-Veh, and SCI-1NMP). Neurons with small diameters (typically < 30 μm) were selected for whole-cell patch-clamp recording as previously described (Davidson et al., 2014). Once a stable recording was established, RMP was determined. Only the neurons that displayed an TTX-sensitive inward current in response to the depolarizing steps (Figure 3.2B) and had a RMP more negative than or -40 mV (≤-40mV) (Figure 3.2F) were evaluated (total n = 124). Recording was undertaken in both voltage-clamp and current-clamp configurations (Figure 3.2B, C) for subsequent analyses of various firing and membrane properties (Figure 3.1B, *right*).

The cells displayed a range of firing properties in response to 20-ms pulses of depolarizing steps ranging from 20-120 pA that could be classified into four categories. Most of the neurons fired one AP spike (single), which was followed by AHP, both of which are characteristic of typical DRG neurons (Peacock et al., 1973). Some neurons exhibited an early train of APs that diminished (phasic), and others fired tonically, exhibiting sustained multiple AP firing pattern throughout the whole depolarizing step once threshold was reached (tonic). These multiple firing patterns are comparable to firing observed in dorsal horn neurons (Hochman et al., 1997). Interestingly, some cells did not fire an AP in response to depolarizing current steps (non-spiking neurons). Notably, these non-spiking cells were included in the neurons that were treated with

TTX and were confirmed to be neurons by the presence of voltage-gated sodium currents. These neurons were also viable, as evidenced by their response to drug treatments. Representative traces of each category are shown in Figure 3.2E. In 81 DRG cells from Veh-treated mice, most neurons fired a single spike (n=30) or did not fire an AP (n=35). Fewer neurons fired phasic (n=6) or tonic spikes (n=10). Similarly, in neurons obtained from 1NMP-treated uninjured mice, 30 of 51 neurons fired once and 14 did not fire an AP, while fewer neurons fired few (n=1) or tonic (n=6) APs. A Chi-square test showed no statistical difference in the distribution of AP firing between the two treatment groups (X^2=7.121, df=3, p=.0681; Figure 3.2D). This result indicates that 1NMP is not altering the ability of cells to reach the threshold for firing APs, and once the threshold is reached, 1NMP does not alter their firing patterns, including the likelihood of neurons to fire repetitively. Indeed, 1NMP treatment did not change basal RMP of the DRG neurons (Veh -50.0 ± .8, 1NMP -51.3 ± .9; Figure 3.2F); however, it significantly increased the rheobase (Veh 203.1 ± 30.6, 1NMP 409.2 ± 48.9; p = .0007; Figure 3.2G), corresponding to the reduction in number of cells that were able to fire repetitively.

3.4.2 TrkB agonist induces inward currents in small-diameter DRG neurons

TrkB activation in the DRG neurons was assessed using the small molecule TrkB agonist, 7,8-DHF (Liu et al., 2016) in voltage clamp at -60 mV. Following a period of stable baseline recording (~ 2 min), 50, 100, and 500 nM of 7,8-DHF was bath applied to DRG neurons for approximately 1 min with washout periods of at least 2 min in between, during which the response returned to baseline. In a total of 95 neurons, 7,8-DHF induced robust inward current response at all concentrations (50 nM, 151.0 ± 23.7 pA, n=30; 100 nM, 249.6 ± 35.4 pA, n=45; 500 nM, 163.0 ± 28.4 pA, n=20; Figure 3.3A, B). The inward current peak amplitudes were compared to those evoked in neurons obtained from mice that were orally administered 1NMP

(Uninjured-1NMP) for systemic inhibition of TrkB. One way ANOVA revealed that systemic 1NMP treatment (Uninjured-1NMP) significantly reduced 7,8-DHF-induced current in DRG neurons ($F_{(5, 162)} = 11.6$, $p < .0001$; Figure 3.3A). In fact, 1NMP reduced the current at all 3 concentrations (50 nM, 32.4 ± 4.3 pA, n=28; 100 nM, 56.5 ± 12.8 pA, n=37; 500 nM 22.9 ± 3.2 pA, n=20, open squares) compared to the corresponding Veh-treated group (filled circles, Figure 3.3A-C). Of the three concentrations of 7,8-DHF, the response evoked with 100 nM 7,8-DHF was the most robust. Thus, the remaining electrophysiological assessments, including results shown in Figure 3.3C and D, were performed with 100 nM 7,8-DHF as the primary concentration.

We assessed the effect of bath-applied 1 μM 1NMP (dissolved in aCSF from a 1mM stock 1NMP) on 7,8-DHF-evoked responses in a subset of neurons (n =31) obtained from Veh-treated uninjured mice (acute-1NMP). Acute 1NMP did not reduce the amplitude of the evoked 7,8-DHF current at all three concentrations, when compared to the Veh-treated population ($F_{(5, 119)} = 1.6$, $p= .179$). However, because concentration-response evaluation was not conducted for acute 1NMP application, 1 μM may be insufficient for effective inhibition. Despite this observation, in 10 cells, we tested the effect of 100 nM 7,8 DHF before and during 1NMP and evaluated their responses using paired analysis. The results revealed that acute 1NMP application significantly reduced the 7,8-DHF-induced inward current from 342.0 ± 104.3 pA (before 1NMP) to 65.32 ± 17.32 pA (during 1NMP; $p=.0256$, Figure 3.3D), confirming that 1NMP treatment attenuates TrkB signaling in the F616 mice. To further confirm that the agonist-induced inward currents observed in sensory neurons were mediated by TrkB, a TrkB specific inhibitor, ANA-12 (Cazorla et al., 2011), was introduced to 5 cells as a secondary method of TrkB inhibition. Concentration-dependent reductions in 100 nM 7,8-DHF-evoked inward currents were observed with 1 and 10

μM ANA-12 (data not shown), with a magnitude of response that was qualitatively comparable to that seen with acutely applied 1NMP. Specifically, 10 μM ANA-12 decreased 7,8-DHF currents from 325.1 ± 99.6 pA to 119.7 ± 60.6.3 pA (63% decrease in amplitude). These results demonstrate that 7,8-DHF is inducing neuronal response through TrkB activation.

3.4.3 Decreased TrkB-mediated inward currents in the DRGs following SCI

The next step in this study was to assess 7,8-DHF-evoked responses in dissociated sensory neurons after SCI. As described above, mice received a T10 contusion injury and were immediately treated with either 1NMP (SCI-1NMP) or vehicle (SCI-Veh) for 5 days (0-5 dpo). T4 - L2 DRG neurons were recorded at sub-acute (5-7 dpo) or chronic (21-28 dpo) phases after SCI (Figure 3.1A). Since no significant differences were found in the inward current amplitude between neurons from the sub-acute and chronic groups, the data were combined to increase statistical power and henceforth referred to as SCI. Surprisingly, compared to the inward currents generated in neurons from Uninjured-Veh mice (249.6 ± 35.9 pA), 100 nM 7,8-DHF evoked a significantly decreased inward current response in SCI-Veh populations (44.5 ± 33.7 pA, p=.0004; Figure 3.4A). In neurons from SCI-1NMP mice, 7,8-DHF evoked an even smaller inward current (34.9 ± 3.2) that was not statistically different to the SCI-Veh group (p=.1183; Figure 3.4B) but different to both uninjured groups. Additionally, 1 μM 1NMP was bath applied acutely to 10 neurons from SCI-Veh group after recording the 100 nM 7,8-DHF-induced inward current. In these neurons, acute 1NMP application caused a reduction in inward current (from 102 ± 31.5 to 50.8 ±10.9 pA), although this decrease was not statistically significant in paired analysis (p=.1034; Figure 3.4C). Unlike the robust reduction observed in the Uninjured group, the response to 1NMP in neurons from injured F616 mice was less pronounced. The lack of

significant 1NMP-induced reduction in SCI neurons may reflect a floor effect in the inward current magnitude following SCI, rather than a lack of TrkB inhibition.

3.4.4 SCI increases latency to peak of TrkB induced current

Inward current amplitude can be influenced by properties and dynamics of the channels mediating the current; thus, potential changes in TrkB channel activation after SCI was investigated by evaluating latency to respond and to peak amplitude of the 7,8-DHF current (Figure 3.1D). Between the Uninjured-Veh (42.5 ± 5.4 s) and SCI-Veh neurons (51.6 ± 4.9 s), latency to respond to bath applied 7,8-DHF did not change (p=.2163, Figure 3.4D). However, SCI (291.5 ± 24.5 s) increased the latency to peak response compared to Uninjured-Veh (207.8 ± 21.6 s; p=.0117, Figure 3.4E). 1NMP treatment significantly decreased latency to peak response after SCI (149.1 ± 13.8, p< .0001) but had no effect on the uninjured group (170.2 ± 19.03, p = .2100; data not shown).

3.4.5 **Small-diameter DRG neurons exhibit differential changes in excitability after SCI**

Passive and active electrophysiological properties were analyzed to investigate potential mechanisms by which SCI or 1NMP alters TrkB-mediated neuronal excitability (Figure 3.1B, right). Thus, electrical properties were monitored and noted after establishment of a stable patch and after each inward current recording in current clamp configuration before and after acute treatments. Although some properties are highlighted in Figure 3.5, the analyzed results for all properties are summarized in Table 3.2. Changes in passive neuronal properties were examined, such as input resistance (R_{in}), membrane time constant (τ_m), and membrane capacitance (C_m), in addition to RMP. Measurements were obtained from an average of 287 cells across all four treatment and injury groups (some cells were excluded by the Grubbs' test for outliers in addition

to not meeting the predetermined criterion for the RMP). 1NMP treatment (-51.3 ± .9 mV) did not change the RMP (-50.0 ± .8 mV, p=.3120; Figure 3.2F). Compared to uninjured-Veh-treated group (229.2 ± 21.5 MΩ, 95.5 ± 8.2 pF), 1NMP significantly decreased R_{in} (143.1 ± 14.6 MΩ; p=.0034) and increased C_m (148.9 ± 16.3 pF; p=.0026) in uninjured cells (Table 3.2). Interestingly, 1NMP increased the rheobase in cells from Uninjured-1NMP animals (from 203.1 ± 30.6 to 409.2 ± 48.9 pA; p=.0007; Figure 3.2G) but decreased it in neurons from SCI mice [261.9 ± 32.2 pA (SCI-Veh) compared to 154.4 ± 18.2 pA (SCI-1NMP); p=.0061, Table 3.2]. The threshold of AP generation, amplitudes, half-widths, and rise slopes of APs and firing frequencies between all groups were measured and compared as well (Figure 3.1B, *right*). APs were evoked by 20 ms depolarizing current injections, and the first AP of the train was used for analysis.

Comparative analyses were also undertaken in neurons obtained from SCI mice. Notably, SCI led to a less negative RMP (from -50.0 ± .8 mV to -48.0 ± .6 mV, p=.0391; Figure 3.5A), and although rheobase also trended upward, it was not statistically significant (p=.2351; Table 3.2). In a total of 92 neurons from SCI-Veh mice, more cells fired at least one spike (62%, n=57) and fewer cells exhibited a non-spiking phenotype in response to current injection (18.5%, n=17) compared to the uninjured group (X^2=14.62, df=3, p=.0022; Figure 3.5B). A few cells fired either phasically (n=9) or tonically (n=9) after SCI. AHP duration was measured at 50% and 80% recovery. SCI decreased AHP_{50} ($p < .003$) but had no effect on AHP_{80}, compared to the uninjured group (Figure 3.5C, D). Lastly, despite the effects of SCI on some firing characteristics of DRG neurons, there was no increase in spontaneous firing after SCI (Figure 3.5E, F), which typically indicates neuronal hyperexcitability.

Because SCI produced significant changes in RMP, neuronal firing properties, and AHP_{50}, additional analyses were performed to assess the effect of 1NMP treatment on these measures. 1NMP treatment in SCI mice (SCI-1NMP) had no effect on RMP or firing properties but returned AHP_{50} values (25.3 ± 1.9 ms) to uninjured levels (27.4 ± 3.2 ms) ($F_{(2, 154)} = 5.8$, $p = .0036$; ANOVA; Figure 3.5C). This effect appears to be specific to 1NMP treatment after SCI, as in the uninjured conditions 1NMP significantly decreased AHP_{50} (refer to Table 3.2). Furthermore, while AHP_{80} was not significantly reduced in neurons from SCI-Veh compared to Uninjured-Veh mice, 1NMP treatment after SCI significantly increased AHP_{80} to values comparable to that seen in neurons from uninjured mice [72.1 ± 7.8 ms compared to 79.1 ± 5.8 ms ($F_{(2, 156)} = 5.1$, $p = .0070$; ANOVA, Figure 3.5D), also see Table 3.2]. Interestingly, although SCI did not change AP amplitude or rheobase compared to neurons from uninjured mice, 1NMP treatment after SCI caused an increase in AP amplitude (94.3 ± 2.2 mV; p= .0303) and decreased rheobase (154.4 ± 18.2; p = .0061; refer to Table 3.2). No other properties were altered by SCI and/or 1NMP treatment. Altogether, these results indicate that SCI-induced changes are not mediated exclusively by TrkB signaling, and furthermore, that neuronal response to TrkB agonist is probably not through altered neuronal excitability.

3.4.6 **Capsaicin induces inward currents in DRG neurons that is decreased after SCI**

Most DRG neurons with small soma diameters (15 - 30 μm) in the DRGs are nociceptors (Fang et al., 2006; Gold et al., 1996; Hagenacker et al., 2005; Körner & Lampert, 2022; Lawson, 2002; Lynn & Carpenter, 1982), and some subpopulations of small-diameter DRG neurons are responsive to TRPV1 agonist, capsaicin. Concentration-response of capsaicin (1-30 μM) was assessed in 29 cells and showed that capsaicin evoked robust inward current responses at all concentrations but showed saturation at 3-10 μM (data not shown), similar to previous study

performed in slice recordings (Liao et al., 2011). Therefore, 3 μM capsaicin was used in all the following experiments as a marker of potential nociceptors (Dirajlal et al., 2003; Le Pichon & Chesler, 2014; Stucky & Lewin, 1999). Large capsaicin currents were recorded in 63 out of 94 cells (Uninjured-Veh, n=32 and SCI-Veh, n=31), consistent with previously reported TRPV1-positive population (Hoffman et al., 2010) (Figure 3.6A). There was no difference in the number of capsaicin-responsiveness neurons between uninjured and SCI groups (Fisher's exact test, two-tailed p=.2710). However, the capsaicin-evoked inward currents were significantly decreased after SCI (p=.0096; Figure 3.6B, C). Though such a reduction after SCI was not expected, the downward change in inward current response of both capsaicin- and 7,8-DHF-induced receptor activation could point to a potential decrease in neuronal responsivity after SCI.

3.4.7 Decrease in TrkB expression in the DRGs around the lesion following SCI

Given the observed differences in the inward current responses in the electrophysiological studies, western blot analyses were performed to assess potential alterations in TrkB expression in the DRGs following SCI. Western blots were processed with DRGs from spinal levels T4 - L2, collected 7 days after injury. Unexpectedly, only the TrkB95 transcript was observed in the study, and its expression was decreased in the DRGs collected from SCI animals (46 ± 15% of control), compared to uninjured animals (100 ± 17%, p= .0469). The results suggest that 7 days following SCI, TrkB protein expression is decreased in the DRGs around the lesion site (Figure 3.7A, B), similar to what is observed in the injured spinal cord (Garraway et al., 2011). The decrease in TrkB protein expression provides a potential mechanism for the observed profound reduction in the 7,8-DHF-induced inward current. Though not quantitated in the current study, cultured plates were stained with antibodies for TrkB and neuronal marker, NeuN, to show colocalization, which

demonstrated that some small-diameter cells did indeed express TrkB and, more importantly, potential differential cell surface expression between uninjured and SCI neurons (Figure 3.7C).

3.5 Discussion

The present study was undertaken to examine the role of TrkB signaling in DRG neurons after SCI, and its potential contribution to pain hypersensitivity. It builds on a prior study that revealed acute maladaptive TrkB signaling contributes to pain after SCI (Martin et al., 2022). While an overwhelming number of studies have implicated BDNF and TrkB in inflammatory pain (reviewed by Garraway and Huie (2016), Garraway (2023)), notable changes that take place after SCI reveal distinct neural processes in SCI-induced neuropathic pain. For example, pain hypersensitivity that develops after SCI despite reduced expression of BDNF and TrkB in the lesioned spinal cord (Garraway et al., 2011), suggests that BDNF-TrkB signaling in the injured spinal cord does not contribute to pain after SCI. More recently, it was reported that SCI increased TrkB expression in the lumbar spinal cord of mice after development of pain although the levels were reduced following TrkB inhibition (Martin et al., 2022). These results indicate the complexity associated with spinal BDNF and TrkB regulation of maladaptive plasticity after SCI. Further, they reveal the possibility that TrkB is involved in mechanisms that contribute to neuropathic pain after SCI that do not reside within the spinal cord, demonstrating the need for additional investigation. Here, 7,8-DHF, the selective TrkB agonist, induced large inward currents in DRG neurons from uninjured mice, which were drastically reduced after SCI. 7,8-DHF-induced currents were also significantly attenuated by treatment with 1NMP, which confirmed that the effects were mediated through TrkB signaling. Though unexpected, the observation that TrkB-mediated effects are reduced after SCI mirrors previously published results showing that BDNF-induced facilitation of afferent-evoked synaptic currents in lamina II

neurons were abolished after SCI (Garraway et al., 2005). Additionally, although SCI did not induce spontaneous firing in DRG neurons as reported by Bedi et al. (2010), it altered several cell membrane properties and increased neuronal excitability, as assessed by other measures. A notable observation in this study is that TrkB protein expression in the corresponding DRGs was decreased by SCI, an observation that is similar to that reported in the lesioned spinal cord after SCI (Garraway et al., 2005; King et al., 2000; Liebl et al., 2001). Together, these results suggest that TrkB signaling is attenuated in both sensory and spinal neurons after SCI.

Sensory neurons detect and transmit peripheral stimuli, and are critical for initiation of somatosensation and pain (Basbaum et al., 2009). Although their role in acute pain is well studied, less emphasis has been placed on understanding the exact role DRG neurons play in neuropathic pain, particularly after SCI, except Bedi et al. (2010), who showed that chronic hyperexcitability of nociceptors critically underlies pain after SCI [see review (Jang & Garraway, 2024)]. Primary afferent plasticity, such as anatomical sprouting in the spinal cord has also been implicated in pain after SCI (Detloff et al., 2016), and the release of BDNF from sensory neurons onto TrkB-expressing spinal cord dorsal horn neurons is critical to inflammatory pain (Kerr et al., 1999). Therefore, TrkB signaling was anticipated to mediate sensory neurons hyperexcitability as a potential underlying mechanism of neuropathic pain. Changes in several neuronal properties demonstrate that SCI produced sensory neuron hyperexcitability. For instance, SCI led to a more depolarized RMP, and more neurons fired an AP once threshold is reached. SCI decreased AHP_{50} in sensory neurons, which was restored by 1NMP treatment to uninjured levels, as was AHP_{80}. AHPs play a role in determining the firing rate of neurons, where shorter AHPs result in higher firing frequencies. Calcium-activated potassium channels (K_{Ca}), which underlie longer duration AHP, are important regulators of neuronal excitability

(Vergara et al., 1998). Hence, this novel observation suggests that neurons are indeed more excitable after SCI, despite not displaying increased spontaneous or tonic firing. A decrease in AHP_{50} in sensory neurons is implicated in pain (Zhu et al., 2017) and supports the notion that sensory neuron hyperexcitability underlies pain after SCI. Importantly, the most substantial effect of 1NMP post-SCI was its ability to return AHP, suggesting that TrkB signaling influences activity in K_{Ca} channels after SCI. Given the complex, albeit unique, processes that underlie TrkB signaling and pain after SCI, some differential outcomes are not surprising. While all measures of neuronal hypersensitivity after SCI are not uniformly reversed by inhibiting TrkB signaling with systemic 1NMP blockade, several conclusions can be drawn.

First, neurons were identified by their sizes and response to capsaicin, thus presumed to be nociceptors. Consequently, TrkB signaling in non-nociceptive DRG neurons was not considered. Notably, the amplitude of capsaicin-induced inward currents was decreased in the recorded neurons after SCI, which suggests that overall sensitivity of small neurons in the DRG is reduced after SCI. While the apparent decrease in neuronal sensitivity is inconsistent with previous observations of TrkB-mediated pain hypersensitivity, these results suggest that TrkB signaling underlying neuropathic pain after SCI may not originate from the nociceptors. Whereas the small-diameter sensory neurons recorded in the current study are presumed to be nociceptors, DRG neurons, and even the small-diameter neurons, display substantial heterogeneity. For instance, nociceptors with cutaneous or viscera origins may have different responses after SCI. Also, according to recent RNA sequencing results, TrkB is expressed across different groups of small DRG neurons, which include C low threshold mechanoreceptors (LTMRs) and peptidergic nociceptors (Zheng et al., 2019), both of which express high levels of *TrpV1*. TrkB is highly expressed in small myelinated, Aδ-LTMRs that are less responsive to capsaicin (Abraira &

Ginty, 2013). An earlier study also reported a substantial increase of BDNF in large-diameter TrkB expressing DRG neurons following axotomy. However, BDNF expression in small neurons were unchanged (Michael et al., 1999), suggesting that TrkB signaling in large DRG neurons might contribute to pain after injury. These observations provide likely scenarios for TrkB to influence nociception by DRG processes that are independent of nociceptors. However, appropriate measures to identify and target TrkB-expressing subpopulations in the DRGs, such as Aδ-LTMRs and large-diameter sensory neurons, would be required to further uncover TrkB's contribution in pain signaling that is not limited to nociceptors.

Second, the locus of TrkB-mediated pain hypersensitivity may be more peripheral than the DRGs. After SCI, BDNF and TrkB (unpublished observation) and phosphorylated extracellular signal-regulated kinase (pERK) (Martin et al., 2022) are increased in the trunk skin at the level of the injury. Because pERK is activated downstream of TrkB, its upregulation could indicate a potential peripheral mechanism for contribution of TrkB signaling in pain. The skin is innervated by heterogeneous populations of sensory neurons, such as D-hairs (L. Li et al., 2011) and Aβ-mechanoreceptors (Michael et al., 1999), which show high sensitivity among cutaneous mechanoreceptors and respond to dynamic stimuli. TrkB expression has been identified in these sensory afferents, and BDNF-TrkB signaling in the skin was shown to be required for normal mechanotransduction (Rutlin et al., 2014). Hence, impaired BDNF-TrkB signaling in the skin could alter the function of these mechanoreceptors, leading to the development of mechanical allodynia. TrkB ablation in the D-hair, Aβ mechanoreceptors, and Aδ mechanoreceptors, reduced neuropathic pain behavior after contusion SCI in rodents (Sliwinski et al., 2023), suggesting that mechanoreceptors are also likely involved in the initiation of neuropathic pain. Therefore, it can be assumed that conversely, overexpression of BDNF and TrkB in the skin leads to increased

pain. Consistent with the notion that peripheral afferents signal pain after SCI, C-LTMRs, which are a subpopulation of cutaneous afferents that normally signal emotional touch, were shown signal pain after SCI (Noble et al., 2022). Additionally, neurotrophins also play an important role in the non-neuronal tissues, such as mast cells (Kawamoto et al., 2002) and endothelial cells (Raychaudhuri et al., 2001). Keratinocytes also release BDNF (Bronzetti et al., 1995), albeit in low amounts (Terracina et al., 2003). Therefore, both afferent plasticity and changes in endogenous BDNF and TrkB in the skin can critically underlie pain after SCI in a manner distinct from the DRGs or the spinal cord.

Clearly, the exact site of maladaptive TrkB signaling that leads to pain is impossible to pinpoint, as 1NMP was administered systemically. While the likelihood of a peripheral site of action is supported, as discussed above, spinal TrkB mechanisms cannot be completely excluded. Despite many reports of TrkB expression being decreased in the lesioned spinal cord, Martin et al. (2022) showed SCI increased TrkB in the lumbar spinal cord, which was attenuated by 1NMP treatment. Interestingly, in the same study, TrkB inhibition effectively blocked hind-paw hypersensitivity but had no effect on at-level aversive pain, suggesting that SCI differentially impacts TrkB signaling in the spinal cord. Similarly, DRGs innervate tissues that are associated with local body segments. DRGs from different vertebral segments have different innervation (e.g., thoracic DRGs innervate the trunk while the lumbar DRGs innervate the lower body; Haberberger et al., 2019; Noseda et al., 2019), morphology (Hasegawa et al., 1996), and composition [i.e., thoracic DRGs had the highest proportion of C-LTMRs; (Jung et al., 2023)] profiles, demonstrating the possibility that changes in TrkB could have regionally distinct effects after SCI. Because this study focused primarily on activity in thoracic DRG neurons adjacent to the injury, assessment of lumbar DRGs could yield different outcomes.

Third, these complex results reveal a potential multifaceted nature of the changes in TrkB expression. While the current study showed a decrease in TrkB expression in the DRG after SCI, conclusions about TrkB synthesis, internalization, or cell surface expression cannot be made. After SCI, there is an increasingly inhibitory environment established around the injury site (Meyer-Franke et al., 1998), including a sustained decrease in external modulators, such as cyclic adenosine monophosphate (cAMP), in the spinal cord and surrounding tissues (Meyer-Franke et al., 1998). cAMP has been identified to regulate TrkB cell surface expression (Boulanger & Poo, 1999; Tartaglia et al., 2001) and phosphorylation (Fawcett & Asher, 1999; Schwab & Bartholdi, 1996; Silver & Miller, 2004), by rapid TrkB surface transport from the pre-existing intracellular source, instead of *de novo* transcription or translation of the receptor mRNA. Given the proximity to the injury, neighboring DRGs are expected to undergo such changes. Thus, a decrease in TrkB transport to the membrane of sensory neurons likely leads to reduced exposure to the agonist. Indeed, studies have shown that even with the robust overall expression levels of TrkB, low cell surface expression in cultured neurons have resulted in low responsiveness to BDNF (Ghosh et al., 2012). Because 7,8-DHF has been shown to mimic BDNF's neurotrophic signaling and TrkB activation, it may also bind similarly to TrkB in cultured dissociated DRG neurons but lead to low responsiveness if cell surface expression of TrkB is decreased after injury. Altered TrkB cell surface expression and regulation of endogenous BDNF could lead to changes in both the TrkB-mediated neuronal responses and overall TrkB expression levels. Although not quantitated in the current study, some differential patterns of fluorescent labeling in TrkB were noted between cultured uninjured and SCI neurons, indicating a potential mechanism. Therefore, an investigation of steady-state levels of TrkB or specific cell surface expression of

TrkB in nociceptors after SCI is needed to further understand the implications of electrophysiological changes reported.

Like BDNF, neurotrophin (NT)-4 binds TrkB with high affinity (Klein et al., 1992). Although NT-4-TrkB signaling is implicated in the survival of different types of sensory neurons [e.g. (Stucky et al., 1998; Zheng et al., 1995)] in the mature system, NT-4 is expressed predominantly by motor neurons in the ventral horn of the spinal cord and does not significantly contribute to nociceptive or neuropathic pain (Buck et al., 2000; Funakoshi et al., 1993; Heppenstall & Lewin, 2001; Yajima et al., 2005). Given the limited role of NT-4 in pain pathways, the current study infers TrkB signaling in DRG neurons are driven by BDNF, which is also altered in the DRG and spinal cord after SCI [e.g. (Hougland et al., 2012)]. Nonetheless, a potential limited role of NT-4 in neuronal excitability after SCI cannot be ignored.

Fourth, while there are no identified endogenous inhibitor of TrkB, truncated TrkB isoform TrkB.T1 is considered a potential inhibitor of the full-length receptor by ligand trapping or acting as a dominant-negative receptor (Biffo et al., 1995; F. F. Eide et al., 1996; Li et al., 1998; Palko et al., 1999). Both spinal and peripheral TrkB are composed of different TrkB isoforms. The two major isoforms include full length TrkB (TrkB.FL) and truncated TrkB (TrkB.T1/T2) forms. Following SCI, truncated forms of TrkB receptor were shown to be upregulated in the spinal cord at the level of the injury (D'Arcangelo et al., 1993; Hilborn et al., 1998), playing an important role in SCI-induced pain through regulation of cell cycle pathways (Wu et al., 2013). Inhibition of TrkB.T1 was also shown to reduce inflammation and improve motor function and symptoms of neuropathic pain after SCI (Matyas et al., 2017). In the DRGs, Lee et al. (1999) reported that mRNA of truncated TrkB receptor increases in inflammatory pain models but not full-length TrkB, and the truncated TrkB increased in the lumbar DRGs in a model of chronic

neuropathic pain (Wei et al., 2021). Although in this study we report changes in the truncated form, SCI can result in changes to the ratio of TrkB.FL and truncated, inactive isoforms in the thoracic DRGs as well to ultimately affect the inward current response and change the overall expression levels.

Lastly, TrkB, as a receptor tyrosine kinase, may alter nociceptor sensitivity after SCI through biochemical pathways that ultimately result in changes in gene expression and synaptic strength, or through coordination with other ion channels such as VGSCs, K_{Ca}, or voltage gated calcium channels (e.g., Cav 3.2). Both SCI and 1NMP treatment after SCI modified AHP duration, suggesting that TrkB interacts with K_{Ca} after SCI although similar changes were not seen in uninjured condition. Also, SCI did not change the onset of 7,8-DHF-induced inward currents which confirms that the responses were most likely mediated by TrkB. However, the changes in the latency to peak and amplitude of inward current may reflect modulatory activity through changes in expression or sensitivity of other ion channels and not merely due to the decreased expression of TrkB. For instance, VGSCs have been investigated thoroughly for their role in setting the AP threshold and maximum firing frequency. VGSCs can also be modulated by receptor tyrosine kinases (D'Arcangelo et al., 1993; Hilborn et al., 1998). Specifically, some subtypes of VGSCs, such as Nav 1.7, 1.8, and 1.9, that are highly or exclusively expressed in DRG neurons (Rush et al., 1998a; Rush et al., 2007) have been found to be functionally associated with TrkB (Blum et al., 2002) as well as neuropathic pain. Therefore, interactions between VGSC (or other channels) and TrkB are expected to be altered following SCI, but further studies are required to ascertain the impact of these interactions on TrkB signaling and pain hypersensitivity after SCI. For example, a pharmacological investigation into changes in

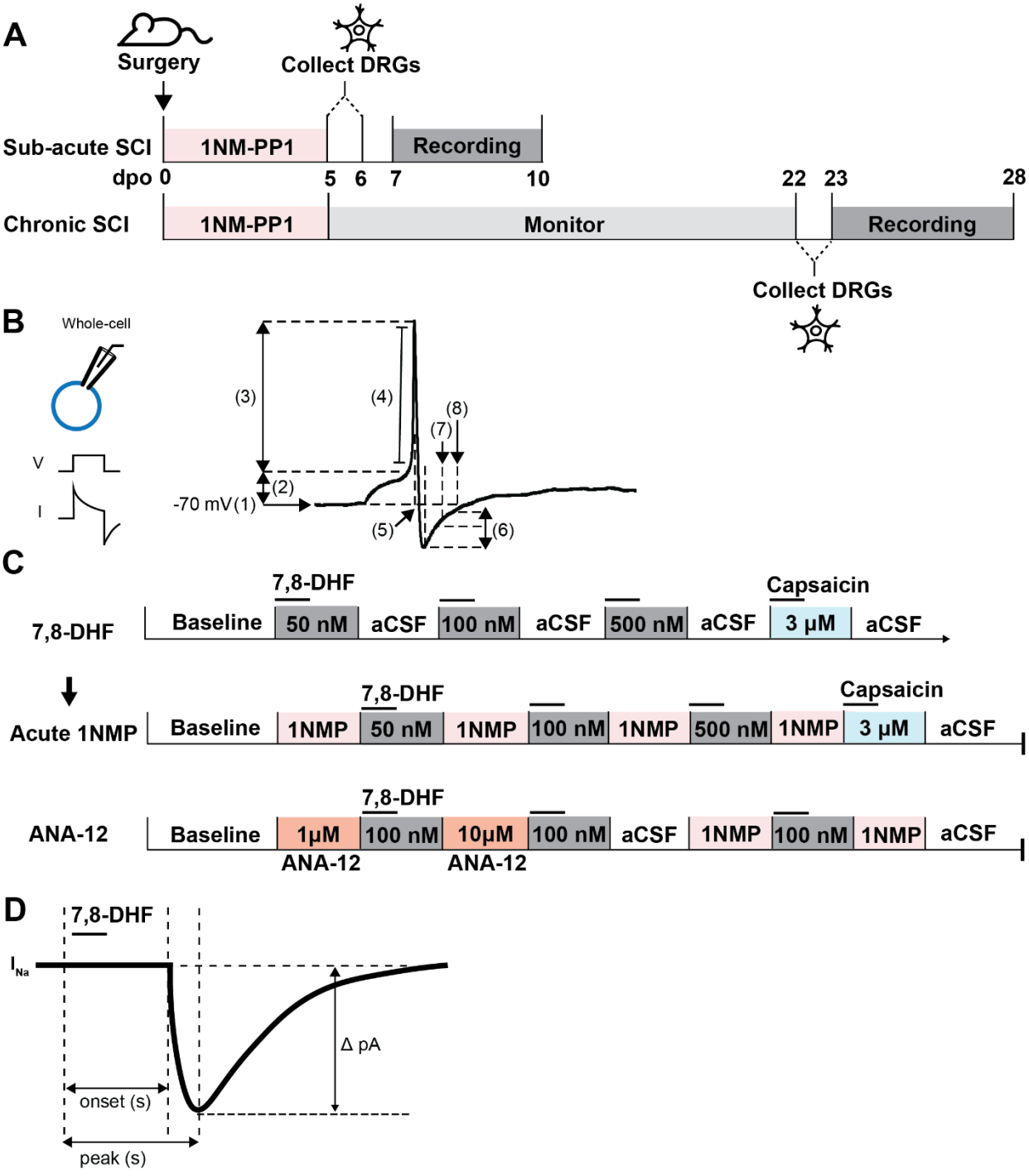
inward current responses with Nav subtype-specific ligands would provide more insight into the role of VGSC in TrkB signaling and plasticity observed in small-diameter neurons after SCI.

Several additional mediators that are not directly addressed in this study can play a role in neuronal hyperexcitability and pain after SCI. For example, as this study was done in SCI and uninjured mice, only SCI mice were treated with meloxicam, the cyclooxygenase-2 inhibitor. Because prior behavioral studies revealed acute treatment with meloxicam immediately after SCI does not abrogate SCI-induced pain hypersensitivity [e.g. (Martin et al., 2022; Noble et al., 2022)], it is not expected that meloxicam will reduce sensory neuron excitability, although the possibility exists. Also, it was previously shown that meloxicam's levels in plasma are drastically reduced by 12 hours after treatment (Chen et al., 2016), further suggesting that its effects in this study are expected to be non-essential. Future studies will more thoroughly examine the myriads of interactions that can influence TrkB signaling in sensory neuron dysfunction or pain hypersensitivity following SCI.

Overall, the current study introduced a mechanistic insight into the role of peripheral TrkB signaling in pain hypersensitivity after SCI, focusing on presumed nociceptive DRG neurons. The results strongly suggest that nociceptor excitability and TrkB signaling are distinctly impacted by SCI, where an increase in neuronal excitability is accompanied by a decrease in TrkB agonist-induced current, probably due to a decrease in TrkB expression in the DRGs. Nonetheless, a notable limitation of our study is that the small-diameter neurons in the electrophysiological recordings are not ascertained to be nociceptors. As stated above, TrkB expression in DRG is not limited to small nociceptors but also includes medium- to large-diameter DRG neurons (Anand et al., 1997; Vega et al., 1994; Widenfalk et al., 1999), as well as touch encoding neurons. To fully elucidate the role of TrkB signaling in DRG neurons in pain

hypersensitivity, future studies that enable specific targeting of TrkB (e.g., transgenic mice that allow visual confirmation of TrkB expression) and nociceptors are needed. Additionally, although these studies were performed in a transgenic mouse that allows selective TrkB inhibition, an important concern is whether the expression of the mutated TrkB in F616 mice fully recapitulates that of native TrkB. This concern is somewhat allayed by a previous study showing similar formalin-induced responses in wild-type and F616 mice (Martin et al., 2022). Yet, additional studies in wild-type mice will be helpful to profile changes in native TrkB signaling after SCI. Lastly, because sensory neurons are sensitive to the dissociation process (Owen & Egerton, 2012), the enzymatic and mechanical dissociation and the 24-h incubation period may have allowed an altered phenotype to be established in neurons, thereby changing their properties. However, because these conditions were consistent across all experimental groups, the observed changes in neuronal responses are more likely induced by SCI and 1NMP treatments rather than an altered phenotype. Despite these limitations, we present novel evidence that suggests SCI-induced maladaptive plasticity in sensory neurons is sensitive to TrkB-mediated procession. Nonetheless, until more studies are done, we conclude that TrkB signaling is implicated in neuropathic pain after SCI but the exact neural mechanism, potentially requiring a collusion of spinal and peripheral plasticity, remains to be determined.

3.6 Figures


A
Surgery
Collect DRGs
Sub-acute SCI
1NM-PP1
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I_Na
Δ pA
onset (s)
peak (s)

Figure 3.1: Illustration of the experimental design and electrophysiology protocol for assessing DRG neuronal response to 7, 8-DHF following SCI and acute and systemic 1NMP treatment

Illustration of the experimental design and assessment of electrophysiological properties.

A) Timeline of experimental procedures for both sub-acute and chronic SCI. For sub-acute SCI group, mice received 1NM-PP1 (1NMP) immediately following SCI surgery (0 days post operation, dpo) for 5 consecutive days. DRGs were collected on dpo 6. Patch-clamp recording began 24 hours after dissociation up to 72 hours. For chronic SCI group, 1NMP was also administered on dpo 0 for 5 days, but the animals were monitored for 3-4 weeks post-injury before DRG collection, dissociation, and electrophysiological recordings. ***B) Left****,* Patch-clamp electrophysiology recording was used to assess neuronal properties in both voltage- and current-clamp configurations. ***Right****,* Key membrane properties were measured: (1) Resting membrane potential (RMP, mV), (2) Action potential (AP) threshold (mV), (3) AP amplitude (mV), (4) AP rise slope (mV/ms), (5) AP half-width (ms), (6) Afterhyperpolarization (AHP) amplitude (mV), (7) AHP duration at 50% recovery (AHP_{50}, ms) and (8) AHP duration at 80% recovery (AHP_{80}, ms). ***C) Top****,* Baseline activity was recorded during superfusion of artificial cerebrospinal fluid (aCSF), before sequential application of increasing concentrations of 7,8-DHF (50, 100, 500 nM), with aCSF washes between each concentration. Capsaicin (3 μM) was applied at the end of each recording to confirm that the recorded neuron is likely a nociceptor. ***Middle****,* For a subset of cells (n=40), the protocol was repeated with the addition of 1 μM 1NMP to assess the effects of acute TrkB blockade *in vitro*. ***Bottom****,* Some cells were treated with two concentrations (1 and 10 μM) of ANA-12, a selective TrkB inhibitor, administered directly into the bath. ***D)*** Quantification of inward currents elicited by 7,8-DHF treatment was done by measuring the

largest difference in amplitude between the baseline and the current response (peak magnitude; Δ pA). Latency to respond and to peak response (s) were analyzed from the time the drug was added, to when the response begins and to the peak of the response, respectively.

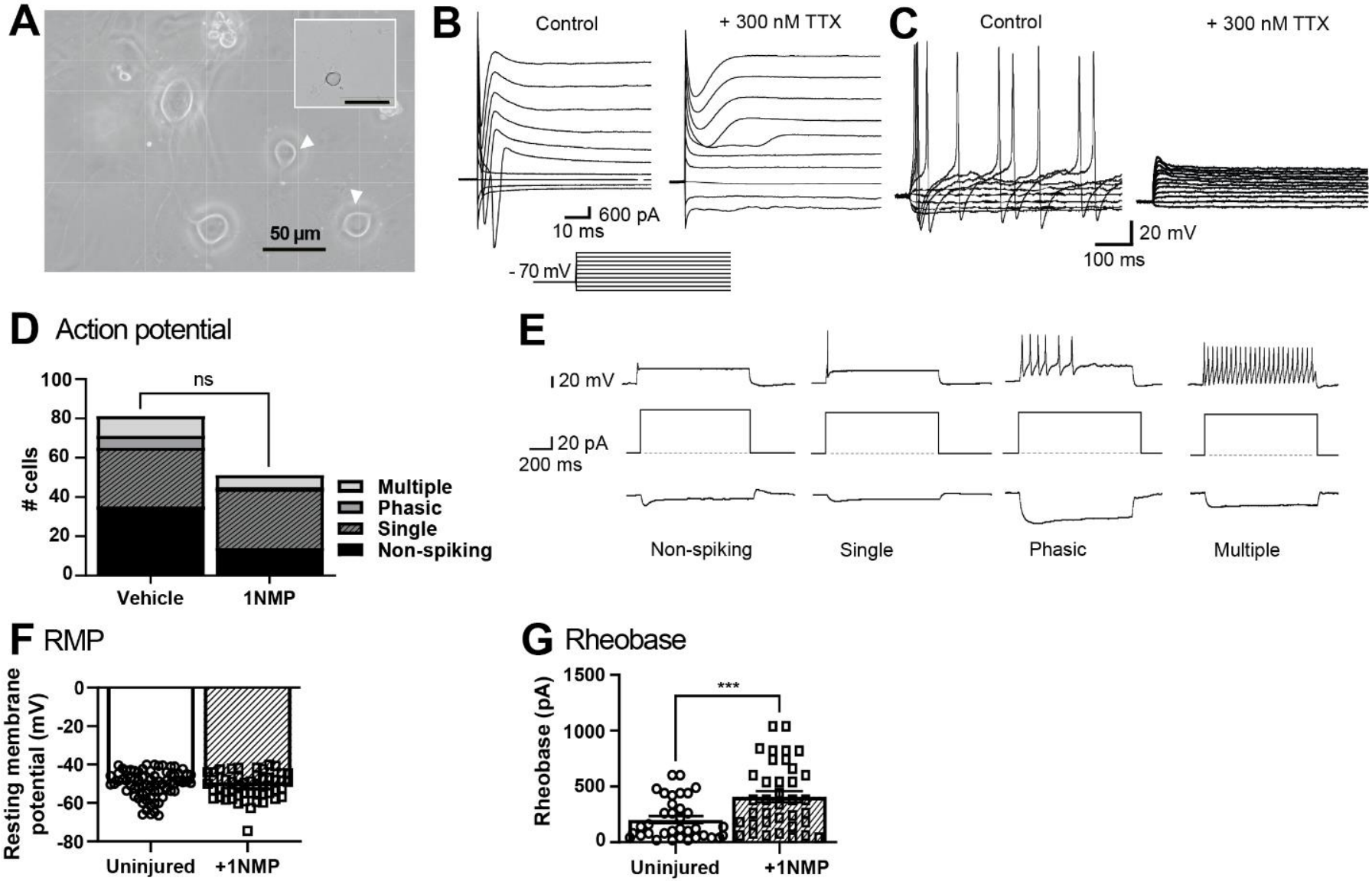
A
50 µm
B
Control
+ 300 nM TTX
600 pA
10 ms
- 70 mV
C
Control
+ 300 nM TTX
20 mV
100 ms
D Action potential
ns
cells
Multiple
Phasic
Single
Non-spiking
Vehicle
1NMP
E
20 mV
20 pA
200 ms
Non-spiking
Single
Phasic
Multiple
F RMP
Resting membrane potential (mV)
Uninjured
+1NMP
G Rheobase
Rheobase (pA)
***
Uninjured
+1NMP

Figure 3.2: Characterization of acutely dissociated DRG neurons.

Characterization of dissociated DRG neurons. ***A)*** Representative brightfield image (40X) showing the morphology of dissociated DRG neurons 24-72 hours after plating and incubation at 37°C. Arrowheads indicate neurons typically selected to patch, in comparison to larger neurons. The inset image shows another cell in the same culture at lower magnification (10X). Scale bars = 50 µm. ***B)*** Representative voltage-clamp recording of large voltage-gated Na^+-mediated inward currents followed by K^+-mediated outward currents in response to 20-ms hyperpolarizing and depolarizing steps. Neurons were held at -70 mV. In the right panel, the addition of 300 nM tetrodotoxin (TTX) blocked sodium mediated inward currents. ***C)*** Representative current-clamp recording showing multiple APs fired in a single neuron in response to a 20-ms depolarizing current injection through the recording electrode. Membrane time constant (**τ**) was determined using single exponential fitted to the first hyperpolarizing step, and input resistance (MΩ) was determined from the second hyperpolarizing step. The right panel shows the effects of TTX, which eliminated the APs. ***D)*** Chi-square analysis of proportion of neurons categorized as non-spiking (veh, n=35; 1NMP, n=14), and single- (veh, n=30; 1NMP, n=30), phasic- (veh, n=6; 1NMP, n=1), and tonic- (veh, n=10; 1NMP, n=6) firing cells from Veh- and 1NMP-treated uninjured (Uninj) mice (X^2=7.121, df=3, p=.0681). ***E)*** Examples of non-spiking, single, phasic, and tonic firing in response to -60 mV depolarizing current injection, with corresponding hyperpolarizing steps depicted underneath. ***F)*** Comparison of resting membrane potential (RMP) and ***(G)*** rheobase between Veh- and 1NMP-treated uninjured populations. Responses of individual neurons (circles, Veh; squares, 1NMP) and sample means (horizontal lines) are both shown. The neuronal RMP did not change after treatment with 1NMP (p=.3120). The rheobase,

defined as the minimum amount of depolarizing current needed to evoke a single AP, significantly increased with 1NMP treatment (***, $p<.001$).

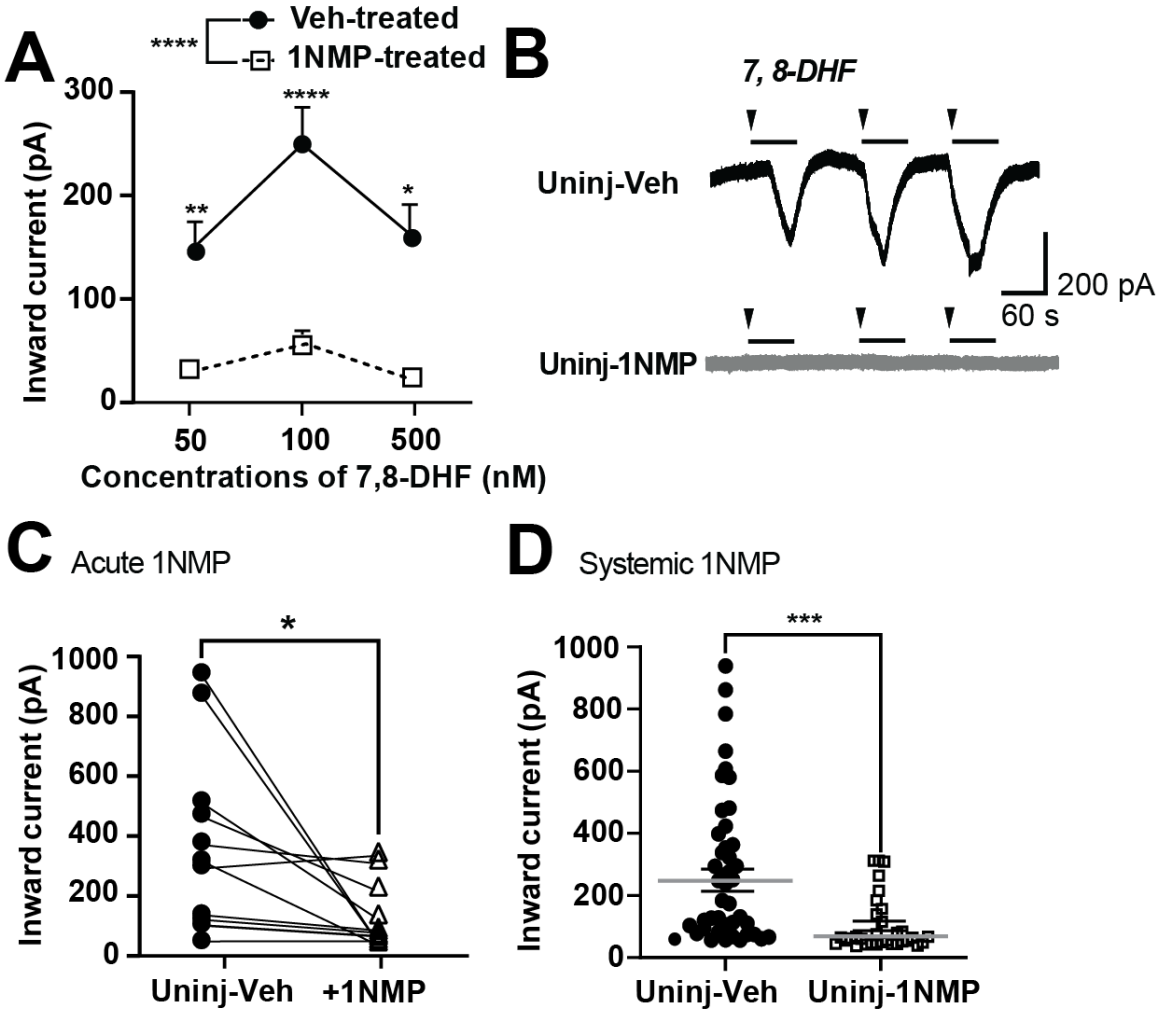
A
Veh-treated
1NMP-treated
****
Inward current (pA)
300
200
100
0
**
****
*
50
100
500
Concentrations of 7,8-DHF (nM)
B
7, 8-DHF
Uninj-Veh
200 pA
60 s
Uninj-1NMP
C
Acute 1NMP
*
Inward current (pA)
1000
800
600
400
200
0
Uninj-Veh
+1NMP
D
Systemic 1NMP
***
Inward current (pA)
1000
800
600
400
200
0
Uninj-Veh
Uninj-1NMP

Figure 3.3: Electrophysiological recordings of small diameter dissociated DRG.

7,8 DHF-induced inward current in DRG neurons. ***A)*** Analysis of the peak magnitude of the inward current elicited by increasing concentrations of 7,8-DHF (50, 100, or 500 nM) in DRG neurons from Uninjured-Veh (filled circle, solid line) and Uninjured-1NMP (open squares, dotted line) mice revealed a significant effect of treatment ($F_{(5, 162)} = 11.6$, $p<.0001$, one-way ANOVA). Post-hoc comparisons showed significant differences between Veh- and 1NMP-treated groups at 50 nM (**, p=.0042), 100 nM (****, p<.0001), and 500 nM (*, p=.0137). ***B)*** Representative traces of whole-cell voltage-clamp recordings (holding potential -60 mV) demonstrate significant reduction in inward current in a neuron from 1NMP-treated animals (*bottom*) compared to a neuron from Uninjured-Veh animals (*top*). Arrowheads indicate the point of drug introduction to the bath, and the bar indicates the duration of 7,8-DHF perfusion into the bath (1 min). ***C)*** Systemic 1NMP treatment (in drinking water) significantly reduced the inward current induced by 100 nM 7,8-DHF compared to current amplitudes in induced in neurons from uninjured animals (***, p=.0001). ***D)*** Paired comparison of inward currents in neurons from uninjured animals subjected to acute 1NMP treatment *in vitro*, revealed a significantly reduced current amplitude during acute 1NMP application (*, $p < 0.05$, paired t-test). Data points represent individual neurons; horizontal lines indicate mean ± SEM. Lines between the two groups in **D** represent pair relationship belonging to the same cell.

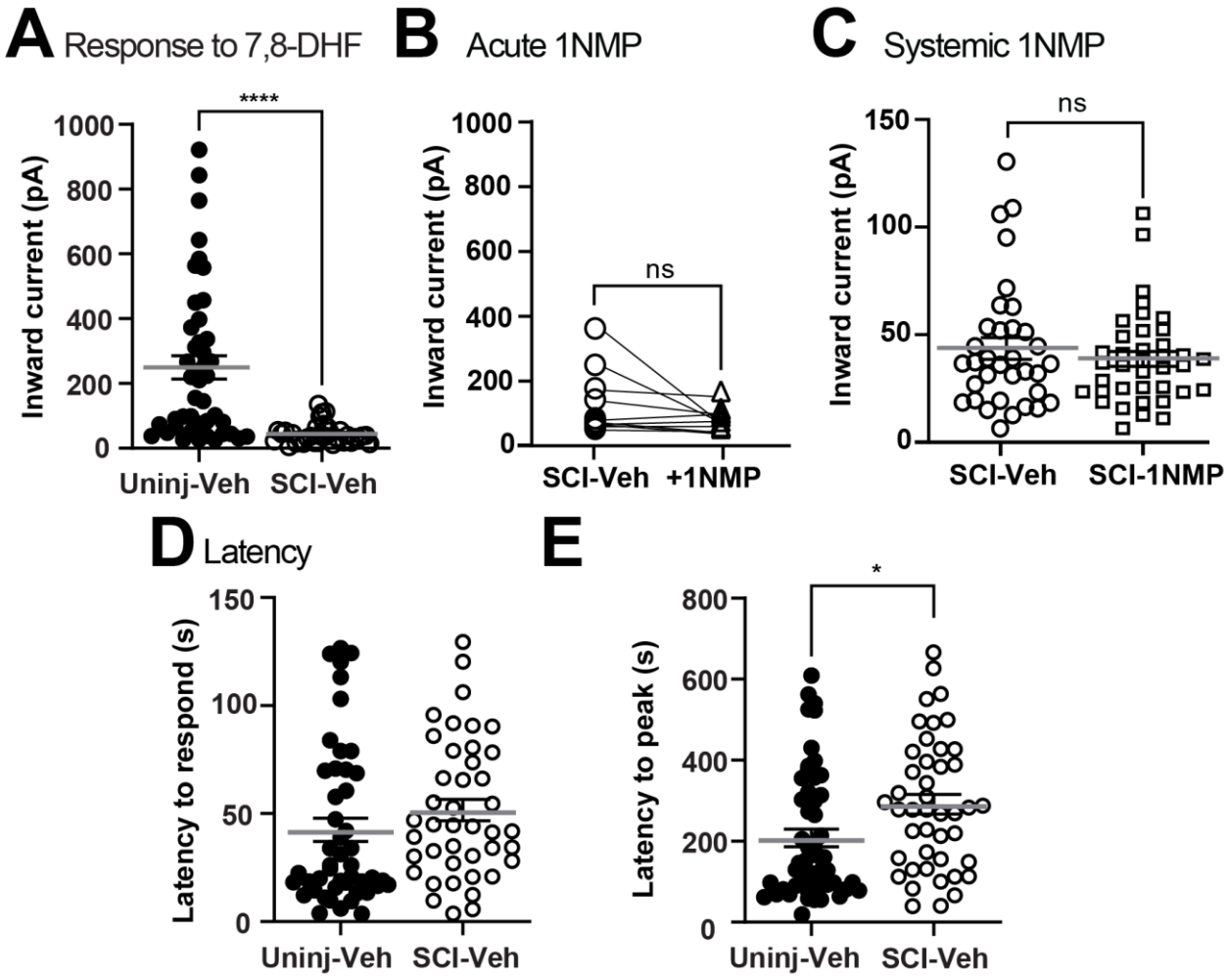
A Response to 7,8-DHF
****
Inward current (pA)
1000
800
600
400
200
0
Uninj-Veh
SCI-Veh
B Acute 1NMP
ns
Inward current (pA)
1000
800
600
400
200
0
SCI-Veh
+1NMP
C Systemic 1NMP
ns
Inward current (pA)
150
100
50
0
SCI-Veh
SCI-1NMP
D Latency
Latency to respond (s)
150
100
50
0
Uninj-Veh
SCI-Veh
E
*
Latency to peak (s)
800
600
400
200
0
Uninj-Veh
SCI-Veh

Figure 3.4: Electrophysiological recordings of small diameter dissociated DRG following SCI.

SCI decreased 7,8-DHF-induced inward current in DRG neurons. ***A)*** Plot of the inward current produced by 100 nM 7,8-DHF in neurons obtained from Veh-treated Uninjured and SCI mice shows SCI significantly reduced 7,8-DHF-induced inward current amplitude (****, p<.0001). ***B)*** Compared to DRG neurons from SCI-Veh animals, systemic 1NMP treatment in SCI subjects had no additional effect on 7,8-DHF-evoked inward currents (p=.1183). ***C)*** Paired comparison of inward currents in neurons from SCI-Veh animals subjected to acute 1NMP treatment *in vitro* showed no difference in 7,8 DHF-evoked current (n=10). ***D, E)*** Latency to onset of 7,8-DHF-induced current remained unchanged (p=.2163), while latency to peak current (peak magnitude) was significantly increased after SCI (*, p=.0117). Data points represent individual neurons; horizontal lines indicate mean ± SEM. Lines between the two groups in **C** represent pair relationship belonging to the same cell.

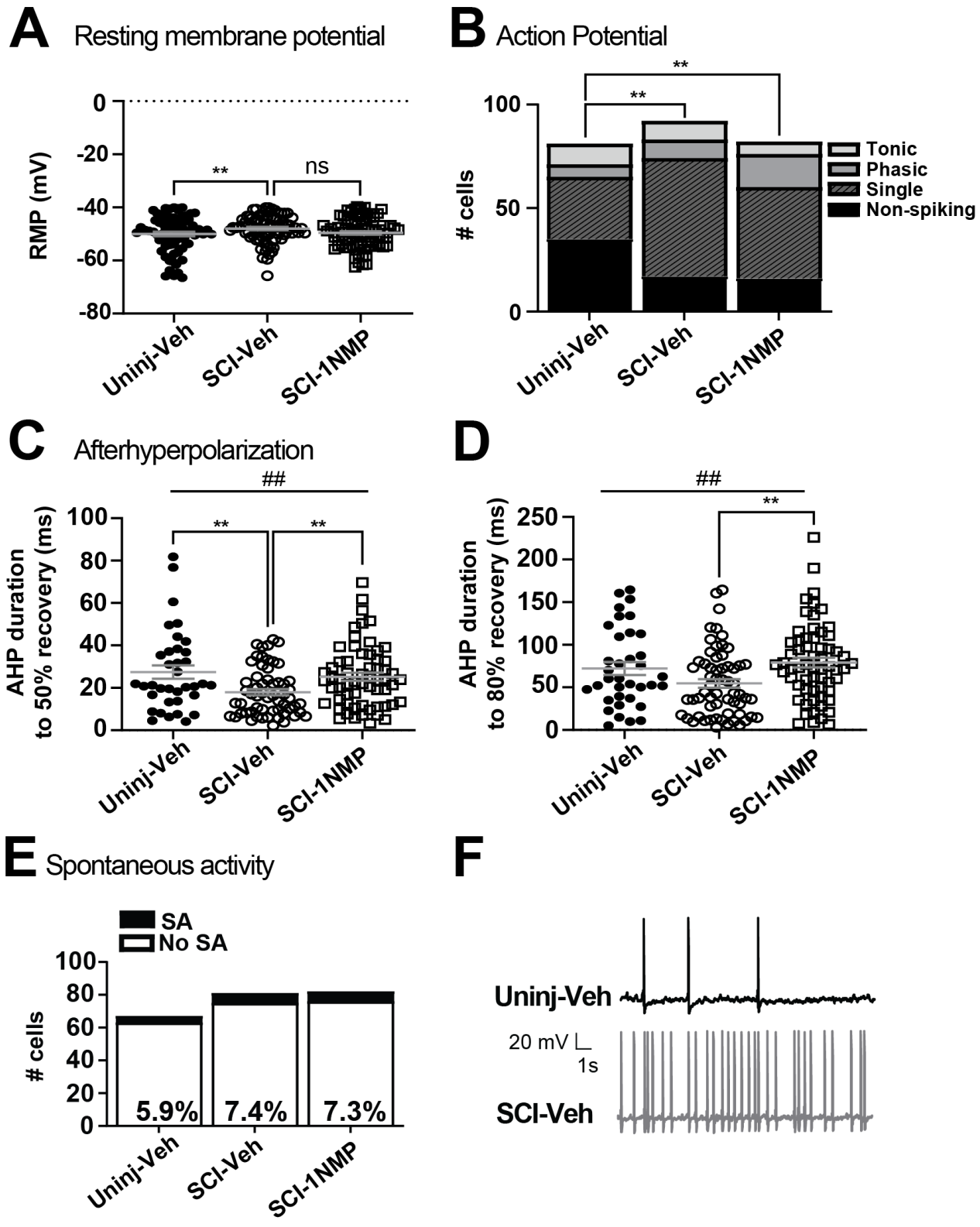
A
Resting membrane potential
RMP (mV)
0
-20
-40
-60
-80
**
ns
Uninj-Veh
SCI-Veh
SCI-1NMP
B
Action Potential
cells
100
50
0
**
**
Tonic
Phasic
Single
Non-spiking
Uninj-Veh
SCI-Veh
SCI-1NMP
C
Afterhyperpolarization
AHP duration to 50% recovery (ms)
100
80
60
40
20
0
##
**
**
Uninj-Veh
SCI-Veh
SCI-1NMP
D
AHP duration to 80% recovery (ms)
250
200
150
100
50
0
##
**
Uninj-Veh
SCI-Veh
SCI-1NMP
E
Spontaneous activity
SA
No SA
cells
100
80
60
40
20
0
5.9%
7.4%
7.3%
Uninj-Veh
SCI-Veh
SCI-1NMP
F
Uninj-Veh
20 mV
1s
SCI-Veh

Figure 3.5: Changes in DRG neuronal response to 7,8-DHF and electrophysiological properties after SCI

Changes in some neuronal properties after SCI. ***A)*** Following SCI, RMP was significantly more depolarized (*****, p=.0391). ***B)*** Chi-square analysis comparing the number of DRG neurons classified as non-spiking, single, phasic and tonic AP firing revealed that SCI significantly increased the proportion of neurons that fire a single AP (X^2=14.62, df=3, p=.0022). 1NMP treatment after SCI failed to return RMP or firing patterns to uninjured-vehicle measures. ***C)*** SCI significantly reduced the AHP_{50} duration (*****, p=.0039) while ***D)*** AHP_{80} was unchanged (p=.1023). 1NMP treatment after SCI significantly increased both AHP_{50} (18 ±1.46 to 25.24 ± 1.86 ms; p=.0029) and AHP_{80} (54.64 ± 5.08 to 79.05 ± 5.71 ms; p=.0018)compared to SCI-Veh, and returning then to values not different to Uninjured-Veh. ***E)*** Bar graphs represent number of cells/total cells recorded and examined for spontaneous activity (SA). Neither SCI nor 1NMP after SCI changed the proportion of DRG neurons displaying SA (7.41% and 7.3%, respectively) compared to neurons obtained from the uninjured vehicle group (5.9%). ***F)*** Representative traces of spontaneous firing in current clamp mode at -60 mV in neurons examined from uninjured (*top*) and SCI (*bottom*) groups. In A, C and D, data points represent individual neurons; horizontal lines indicate mean ± SEM.

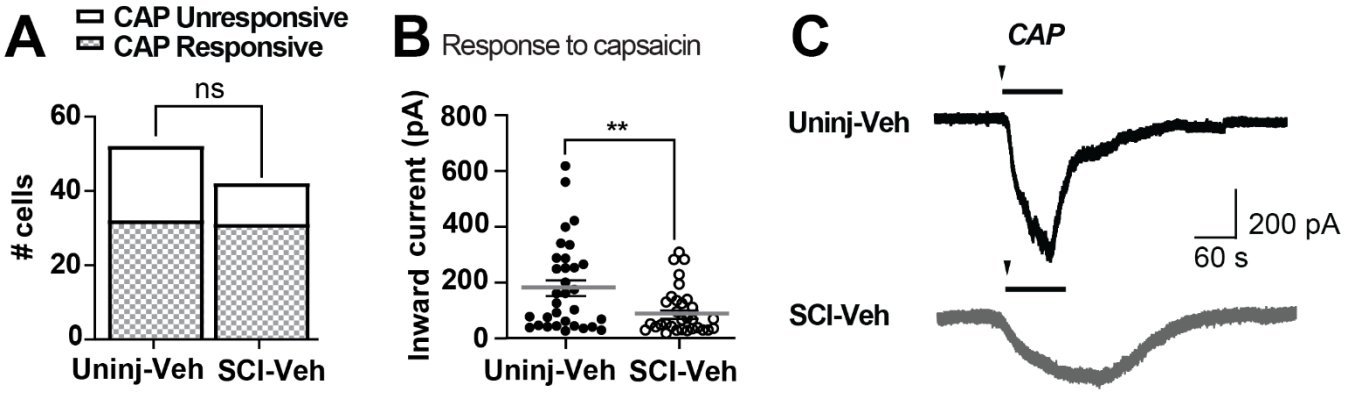

Figure 3.6: Capsaicin-induced inward currents in small DRG neurons also decrease after SCI.

Capsaicin-induced inward currents in DRG neurons that is decreased following SCI. ***A)*** There was no difference in the number of neurons responsive (checkered bar) or unresponsive (white bar) to capsaicin (CAP) between Uninjured-Veh and SCI-Veh groups (Fisher's exact test, two-tailed p=.5494). ***B)*** Comparison of the peak amplitude of CAP-induced inward currents between the two groups revealed a significantly reduced inward currents after SCI (**, p=.0096). ***C)*** Representative traces of the inward currents from voltage-clamp recordings (at -60 mV) elicited by 3μM CAP (1 min) in neurons from the Uninjured-Veh (*top*) and SCI-Veh (*bottom*) groups. Arrowheads indicate drug introduction to the bath, and the bar indicates the duration of CAP perfusion. Data points represent individual neurons; horizontal lines indicate mean ± SEM.

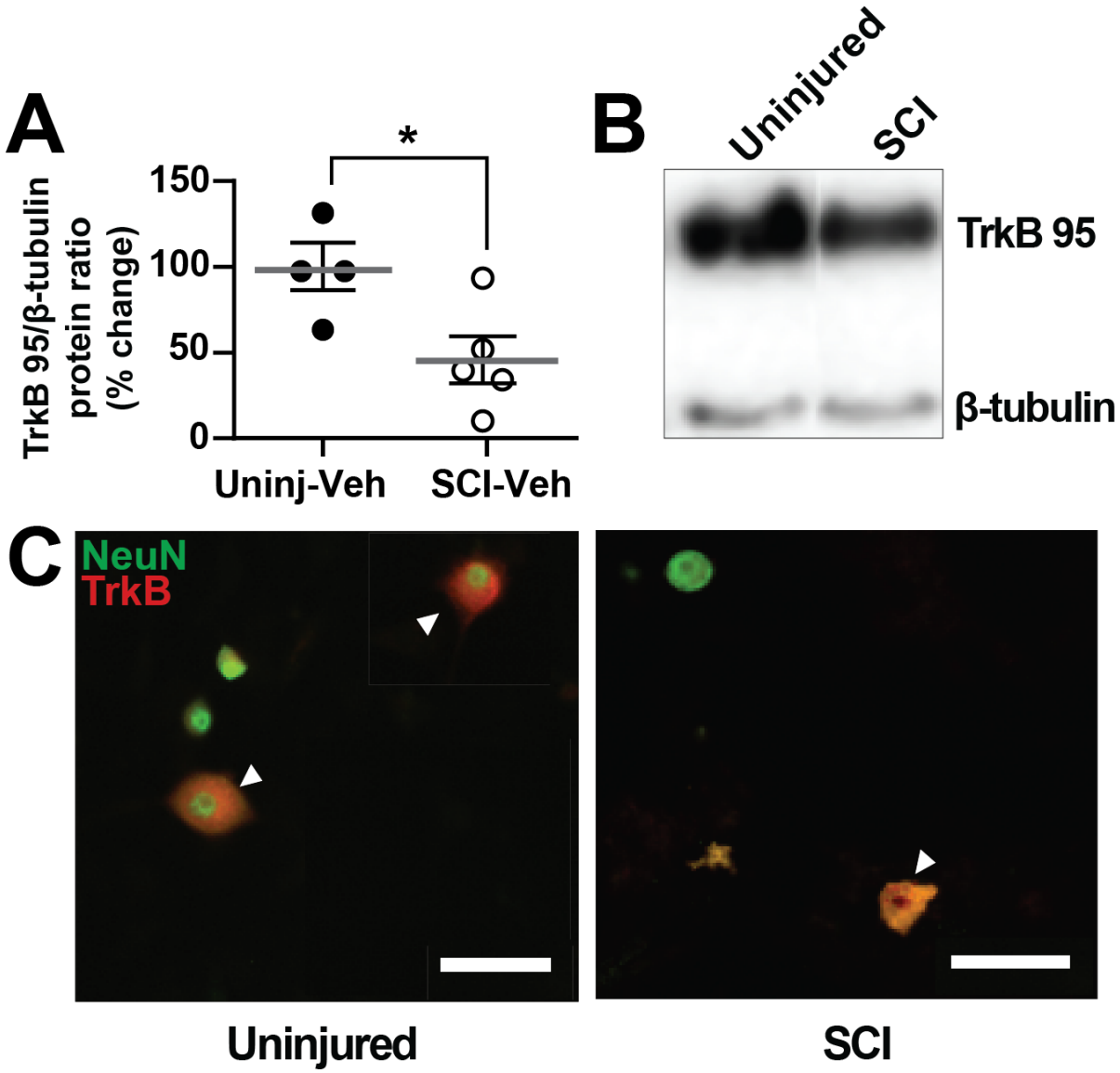

Figure 3.7: DRG expression of TrkB decreases following SCI.

TrkB protein expression in DRG is decreased following SCI. ***A)*** Histogram shows that TrkB expression in DRGs are significantly decreased 5-7 days after SCI (open circles) compared to uninjured condition (black-filled circles) (*, p=.0469). ***B)*** Representative western blot images are shown for TrkB 95 expression in the thoracic DRGs from uninjured and SCI animals (*TrkB145 was not observed*). ***C)*** Double-immunofluorescence demonstrates TrkB expression (red

fluorescence) in NeuN-positive neuron (green fluorescence; merged, yellow fluorescence). Scale bars represent 50 μm. Differential pattern of co-localization (indicated by arrowheads) of TrkB and NeuN shows a potentially altered expression of cell-surface TrkB in uninjured (*left*) versus SCI (*right*) small-diameter DRG neurons.

Condition	**Uninjured**						**SCI**					
Experiments	Electrophysiology				Immunoblotting		Electrophysiology				Immunoblotting	
Treatment	Veh		1NMP				Veh		1NMP			
Sex	Males	Females	Males	Females	Males	Females	Males	Females	Males	Females	Males	Females
n animals	3	12	4	4	3	1	2	10	9	2	3	2
	23				4		23				5	
Total per group	**27**						**28**					

Table 3.1: Summary of Animals used.

Table summarizing total number of animals used in electrophysiological and immunoblotting experiments in each injury and treatment conditions. Veh, vehicle-treated; 1NMP, animals that were treated with 1NMP in drinking water.

Property	Uninjured	1NMP	SCI	1NMP	Uninjured vs SCI		Uninjured vs 1NMP		SCI vs 1NMP	
RMP (mV)	-50.0 ± 0.8 (72)	-51.3 ± 0.9 (52)	-48.0 ± 0.6 (81)	-49.6 ± 0.6 (82)	NS		*	***0.039***	NS	
Rheobase (pA)	203.1 ± 30.6 (35)	409.2 ± 48.9 (37)	261.9 ± 32.2 (64)	154.4 ± 18.2 (56)	***	***0.0007***	NS		**	***0.0061***
R_{in} (MΩ)	229.2 ± 21.5 (62)	143.1 ± 14.6 (42)	253.5 ± 22.7 (70)	316.8 ± 26.4 (81)	**	***0.0034***	NS		NS	
Membrane time constant (ms)	27.1 ± 2.2 (68)	23.5 ± 2.4 (47)	25.6 ± 2.3 (87)	23.0 ± 1.7 (81)	NS		NS		NS	
Capacitance (pF)	95.5 ± 8.2 (57)	148.9 ± 16.3 (47)	79.9 ± 4.7 (74)	77.6 ± 4.8 (77)	**	***0.0026***	NS		NS	
AP threshold at -60 mV	34.1 ± 2.1 (38)	34.8 ± 2.2 (38)	34.6 ± 2.2 (66)	35.2 ± 1.5 (66)	NS		NS		NS	
AP amplitude (mV)	91.1 ± 4.0 (38)	86.7 ± 3.9 (41)	87.4 ± 2.3 (68)	94.3 ± 2.2 (66)	NS		NS		*	***0.0303***
AP halfwidth (ms)	4.6 ± 0.4 (42)	4.7 ± 0.5 (34)	4.9 ± 0.4 (66)	5.0 ± 0.3 (66)	NS		NS		NS	
AP rise slope (mV/ms)	3.1 ± 0.5 (37)	4.4 ± 0.6 (35)	3.1 ± 0.3 (60)	3.0 ± 0.3 (61)	NS		NS		NS	
Instantaneous Firing Frequency	6.1 ± 1.0 (14)	5.7 ± 1.5 (8)	7.4 ± 1.3 (16)	5.9 ± 0.8 (24)	NS		NS		NS	
AHP amplitude (mV)	17.7 ± 1.5 (38)	15.4 ± 1.0 (38)	15.7 ± 1.0 (66)	18.2 ± 1.0 (65)	NS		NS		NS	
AHP_{50} duration (ms)	27.4 ± 3.2 (36)	19.8.1 ± 1.8 (36)	18.0 ± 1.5 (59)	25.2 ± 1.9 (62)	*	***0.0404***	**	***0.0030***	**	***0.0029***
AHP_{80} duration (ms)	72.1 ± 7.6 (36)	68.1 ± 8.6 (37)	54.6 ± 5.1 (61)	79.1 ± 5.8 (62)	NS		NS		**	***0.0018***

Table 3.2: Summary of electrophysiological properties.

Electrophysiological properties of all small dissociated DRG neurons. RMP, resting membrane potential; AP, action potential; AHP, afterhyperpolarization; R_{in}, input resistance. Data are from DRG neurons from both male and female animals in all groups. The table shows means ± SEM, with number of recorded neurons (n) in parentheses. The *p* values are for Student's *t*-test, with significant changes indicated in bold. [NS, not significant]

Chapter 4: Increased excitability in TrkB-expressing primary sensory neurons following spinal cord injury

This chapter includes materials from the manuscript in preparation by
Parvin S, **Jang K**, and Garraway SM. [Unpublished manuscript].
Department of Cell Biology, Emory University School of Medicine.

4.1 Abstract

Previous investigation into the role BDNF and its receptor, TrkB, in nociceptive processes after SCI primarily focused on plasticity within the injured spinal cord. In this study, we aimed to isolate the contribution of peripheral TrkB mechanisms by targeting TrkB-expressing DRG neurons, which include Aδ-low threshold mechanoreceptors (Aδ-LTMRs). Aδ-LTMRs are small myelinated cutaneous afferents that innervate the hairy skin and encode directional touch. They can be identified by their preferential expression of TrkB. In this study, we propose that, like nociceptors, Aδ-LTMRs become hyperexcitable after SCI and contribute to pain signaling.

Here, we used a transgenic mouse, TrkB::ChR2, in which TrkB-expressing cells are reported via enhanced yellow fluorescent protein (EYFP) expression. Patch-clamp recording was performed in EYFP-positive dissociated DRG neurons in uninjured mice and after T10 contusion SCI for assessment of membrane properties, firing characteristics, and responses to TrkB agonist, 7,8-DHF, and capsaicin.

7,8-DHF induced an inward current in neurons that was significantly increased by SCI. This was accompanied by a decreased time to the peak response, which may reflect altered receptor responsiveness following SCI. Capsaicin induced a small inward current that was similar between uninjured and SCI groups, indicating that the enhanced excitability is likely attributed to TrkB-specific signaling changes post-injury. Analyses of intrinsic properties did not support heightened neuronal excitability, and the spontaneous firing was absent following SCI. Taken together, these findings suggest that while SCI increases TrkB responses in Aδ-LTMRs, it does not produce neuronal hyperexcitability that is seen in nociceptors. These interactions observed between TrkB signaling and different populations of DRGs imply heterogeneous, population-specific changes to impact the overall tone of the nociceptive pathway after the injury.

4.2 Introduction

Chronic neuropathic pain is a common consequence of SCI (Felix et al., 2022; Siddall & Loeser, 2001), but limited progress has been made in developing effective treatments due to incomplete understanding of the underlying mechanisms. Both central and peripheral components are implicated in the generation and maintenance of chronic pain (reviews by Hulsebosch (2002) Hulsebosch et al. (2009)). BDNF, signaling through its high affinity receptor TrkB, plays a key role in both adaptive and maladaptive plasticity following SCI. While much research has focused on spinal plasticity, peripheral plasticity, such as changes in nociceptors (Bedi et al., 2010), is increasingly recognized as an important factor in neuropathic pain.

Our previous work using the $TrkB^{F616A}$ (F616) transgenic mouse model revealed that systemic inhibition of TrkB immediately after SCI delayed the onset of mechanical hypersensitivity and promoted functional recovery (Martin et al., 2022). In parallel, DRG neurons from F616 mice displayed decreased inward current response to the small molecule TrkB agonist, 7,8-DHF. Notably, in F616 mice, we primarily targeted the small-diameter neurons, presumed to be nociceptors, and not specifically TrkB-expressing neurons.

Small neurons in the DRGs are not exclusively nociceptors (Zheng et al., 2019). Thus, TrkB sensitivity in DRG neurons could represent both nociceptive and non-nociceptive neurons. For example, a subpopulation of cutaneous afferents known as Aδ-LTMRs uniquely express TrkB (Abraira & Ginty, 2013; Lishi Li et al., 2011) and could be important targets for examining peripheral BDNF or TrkB contributions to pain after SCI. Peripherally, Aδ-LTMRs form lanceolate endings with zigzag and awl/auchene hairs but not guard hairs, where they are recruited by low threshold tactile stimulation and rapid cooling of the skin (Abraira & Ginty,

2013; Lishi Li et al., 2011). Centrally, Aδ-LTMRs enter the spinal cord dorsal horn (SCDH) and terminate in lamina II/III.

In the prior study, the selection of cells was based on nociceptor-like characteristics, led primarily by their small somal diameters and sensitivity to capsaicin. Here, we assess the TrkB signaling in neurons from a transgenic mouse model, TrkB::ChR2, which is designed to report TrkB expression via enhanced yellow fluorescence protein (EYFP) expression. Generated by crossing TrkBCre mice with channelrhodopsin-EYFP reporter mice, the TrkB::ChR2 mice enable selective identification and targeting of TrkB-expressing cutaneous afferents. We interrogated the properties and plasticity of Aδ-LTMRs after SCI to find an increased TrkB agonist-evoked inward current in TrkB-expressing DRG neurons, suggesting that SCI enhances TrkB-mediated neuronal signaling in the periphery.

4.3 Materials and Methods

4.3.1 Subjects - generation of transgenic mice

Experiments were performed in adult male (n = 21) and female (n = 17) TrkB::ChR2 mice for visualization of TrkB expression in cells. To generate these mice, TrkBCreERT2 mice (Jackson Laboratory (JAX) #027214) were crossed with a strain expressing a Cre-dependent channelrhodopsin (ChR)-2-EYFP fusion protein following exposure to Cre recombinase (Ai32(RCL-ChR2(H134R)/EYFP; JAX #024109) to produce TrkB::ChR2 transgenic mice. TrkB::ChR2 mice were treated with tamoxifen dissolved in peanut oil [(TAM), 4 mg/day] subcutaneously administered at the scruff of the neck for 2 days (1 day apart) to induce transgenic expression, typically shortly after weaning, at least 2 weeks before experimentation. The dosing regimen was chosen based on preliminary electrophysiology and immunohistochemistry data that provided confirmation of Cre-driven YFP expression. For

comparison, a cohort of C57BL/6 wild type (WT, 4 males, 5 females; JAX # 000664) mice were also used.

All mice were fed standard rodent diets *ad libitum* and were housed in standard cages in a vivarium on a 12:12-h light-dark cycle. At the time of surgery, mice were approximately 8-12 weeks old and weighed 16–22 g (females) and 22–30 g (males). Experimental procedures were approved by the Animal Care and Use Committee of Emory University and conformed to national standards for the care and use of experimental animals and the American Physiological Society's "Guiding Principles in the Care and Use of Animals".

4.3.2 Surgical procedures

Mice in this study underwent contusion SCI at the spinal thoracic (T) 10 level, as described in Chapter 3. To ensure the effectiveness of the injury, mice were assessed for locomotor impairment at 1 dpo using the Basso Mouse Scale (BMS; Basso et al. (2006)). SCI mice were only included in the study if they recorded BMS scores of 0 or 1 at 1 dpo, displaying limited to no locomotor function. Throughout the experiments, all mice were carefully monitored for signs of infection or distress. Experiments consisted of the following groups: Uninjured, SCI, Uninjured-F616, and Uninjured-WT (Table 4.1).

4.3.3 Behavior assessments

Prior to behavioral testing, all mice were acclimated to the behavioral suite and testing apparatuses for at least 3 days.

Assessment of mechanical hypersensitivity

Mechanical sensitivity was evaluated using the von Frey test pre-surgery (baseline) and at 7 dpo. On testing days, 20-min acclimation period was given to each animal, followed by the

mechanical assessment according to the established up-down method (Chaplan et al., 1994). Calibrated von Frey hairs (NC12775-99, North Coast Medical, Inc., Morgan Hill, CA, USA) were applied from below a metal mesh platform to the hind paws, starting with filament evaluator size 3.22 (target force 0.16 g). Right and left paw withdrawal thresholds were averaged to determine overall mechanical sensitivity. A reduction in von Frey withdrawal threshold values compared to their baseline levels indicated increased mechanical sensitivity.

Assessment of thermal hypersensitivity

Thermal sensitivity was measured using the tail-flick test before surgery (baseline) and at 7 dpo. Mice were loosely restrained, and a heat ramp was applied 4 - 6 cm from the tip of the tail. The time taken for the tail to "flick" (i.e., latency) was recorded (D'Amour & Smith, 1941) at 3 - 5-minute intervals over 3 consecutive tests, after each animal was acclimated to the procedure room and the restraint. A maximum cutoff time of 8 s was used to prevent tissue damage caused by exposure to excessive heat.

4.3.4 Dissociation and whole cell patch-clamp recording of DRG neurons

After 7- 42 d after SCI, TrkB::ChR2 mice were deeply anesthetized and euthanized with isoflurane. DRGs were extracted from vertebral T4 – lumbar (L) 2 and dissociated for electrophysiology as described in Chapter 3. Whole-cell patch-clamp recordings were conducted on EYFP-positive DRG neurons using an inverted microscope (Nikon Eclipse Ti-U) with a fluorescent filter (Nikon T-A2) to visualize EYFP expression (Figure 4.1A). Recordings were also made on small diameter DRG neurons from uninjured WT mice. Whole-cell patch recordings were made at room temperature using the conventional patch clamp configuration. The Clampex Membrane Test program (Molecular Devices) was used to determine membrane capacitance (C_m) and membrane resistance (R_m) from a holding potential of -70 mV in voltage

clamp configuration. While held at -70 mV in voltage-clamp mode, hyperpolarizing (-40 mV) and a series of depolarizing (Δ 20 mV) current steps were undertaken to identify inward current response, and to obtain estimates of several membrane properties, as detailed in chapter 3. Shortly after achieving whole cell configuration, the configuration was then switched to bridge mode (I = 0), and the RMP of the neuron was recorded. Firing properties and other electrophysiological properties (rheobase, AP voltage threshold, AP amplitude, AHP amplitude and duration, and membrane time constant, tau (**τ**)), were calculated as described in Chapter 3, both at baseline and after introduction of the drug.

4.3.5 Preparation and application of drugs

All drugs were applied to the cells by bath perfusion. Drugs were first prepared as concentrated stock solution in aCSF and stored at -20 °C. Stock solutions of 7,8-DHF (#D1916, Tokyo Chemical Industry) and capsaicin (#211275, EMD Millipore Corp) were dissolved in 100% DMSO. Drug stock solutions were diluted in aCSF on the day of recording. The final concentration of DMSO was minimal and not expected to have an effect of neuronal activity (Galvao et al., 2014; Zhang et al., 2017). 7,8-DHF was diluted to final concentration of 100 nM, which was applied 1 min after the start of the recording before being washed out for at least 2 min. Additionally, capsaicin sensitivity was tested in all recorded neurons by addition of 3 μM capsaicin in the bath for 1 minute, followed by another aCSF wash.

4.3.8 Western blot for TrkB expression

T4 to L2 DRGs were rapidly removed from deeply anesthetized TrkB::ChR2 mice (n = 18; 9 SCI, 7 dpo, and 9 uninjured) mice. Total protein was extracted for western blot as detailed in Chapter 3. Blots were blocked in 5% blotting grade milk (BioRad Laboratories; #1706404XTU) in TBST for one hour and were incubated overnight at 4°C in primary: TrkB (1:1000; #AF1494,

RRID:AB_2155264; R&D systems) generated in goat and β-tubulin (1:1,000; #05-661, RRID:AB_309885; Upstate Cell Signaling) generated in mouse. The following day, membranes were washed in TBST, then incubated in HRP-conjugated donkey anti-goat (1:10,000; #PA1-28805, RRID:AB_10988865; Thermo Fisher) or goat anti-mouse secondary antibodies (1:2,500; #31430, RRID:AB_228307; Thermo Fisher). The blots were developed with standard enhanced chemiluminescence and imaged with Azure Biosystems c600 Western blot Imaging System. Ratios of the integrated densitometry of each protein of interest to the loading control (β-tubulin) were calculated with AlphaView Software (ProteinSimple), normalized to uninjured controls.

4.3.9 Statistical Analysis

Mice were randomly assigned to each experimental group. All statistical measures and analyses were undertaken with GraphPad Prism 10 (GraphPad Software). While experimenters could not be blinded to group assignments (SCI vs. uninjured), the subsequent statistical analyses were blinded as much as possible. All data in text and figures are presented as mean ± SEM, and sample sizes (n) are provided for all analyses. Comparisons between groups were accomplished using Student's *t*-test and $p < 0.05$ was considered statistically significant. For categorical comparisons, Chi-square (χ^2) test or Fisher's exact test was used. Cells were excluded if they did not exhibit an inward current in response to depolarizing steps, or if their RMP was more depolarized than -40 mV. Outliers were determined using Grubbs' test.

4.4 Results

Based on the established implication of nociceptor hyperactivity in pain after SCI, we hypothesized that EYFP-positive neurons, which include Aδ-LTMRs, similarly become hyperactive following SCI. To test this, we performed patch-clamp electrophysiology to assess

neural plasticity of TrkB-expressing neurons for their potential contribution to development of pain hypersensitivity.

4.4.1 Electrophysiological properties of EYFP-positive DRG neurons from Uninjured and SCI TrkB::ChR2 mice

We recorded EYFP-positive neurons (Figure 4.1) isolated from 13 uninjured and 11 SCI TrkB::ChR2 mice (4 at 5-7 days and 7 at 28-49 days after SCI). The recordings were biased to ~T8 - T12 DRGs (near the lesion site or uninjured equivalent) though T4 - L2 DRGs were collected. Since there were no significant differences in responses between the neurons from the two SCI groups, data was combined to increase statistical power. A total of 129 neurons were analyzed and included only those with inward Na^+-current responses to depolarizing voltage steps and had an RMP more negative than -40 mV (≤ -40 mV). Most DRG neurons exhibited a single AP followed by a period of AHP, consistent with previous reports (Peacock et al., 1973). In neurons from uninjured animals, 46 of 57 cells (80.7%) fired a single AP, while 10 (17.5%) were non-spiking. In the SCI group, 38 of 67 cells (56.7%) fired a single AP (n=38), while 28 (41.8%) were non-spiking. In both groups, only 1 cell (1.8%) fired tonically. Chi-square analysis of the groups showed that there is a significant difference in the AP firing after SCI [X^2=10.55, df=3, p=.0144; Figure 4.1B), where more neurons did not fire any spikes. Additionally, a significant hyperpolarizing shift in RMP (-58.2 ± 1.1; n=71) was observed following SCI compared to neurons from uninjured (-54.0 ± 0.9; n= 58) [**; p= .0054; Figure 4.1C) animals. Recordings in both voltage-clamp and current-clamp configurations were used to assess various membrane and firing properties (as shown in Table 4.2) that suggested mixed results. Measurements were obtained from a minimum of 67 cells (33 uninjured, 34 SCI), as some cells were excluded via Grubbs' test. Notable changes after SCI included a reduction in cell

capacitance (C_m; 107.1 ± 8.90 pF in uninjured vs. 78.98 ± 4.88 in SCI; Figure 4.1E), decreased AP half-width (5.75s ± 0.596 in uninjured vs. 2.919 ± 0.287 in SCI; Figure 4.1F), and a shorter AHP duration (228.7 ± 24.28 ms in uninjured vs. 149.7 ± 18.03 ms in SCI; Figure 4.1G). However, these results did not converge on heightened neuronal excitability. Next, we assessed spontaneous firing in both groups. In both groups, spontaneous firing was rare, occurring in 7 out of 57 cells in uninjured group and 2 out of 71 cells in the SCI group, the difference which was statistically significant (X^2=4.332, df=1, p= .0374; Figure 4.1H).

4.4.2 Increased TrkB-mediated inward currents in the EYFP-positive DRGs following SCI

To evaluate TrkB-mediated responses, we applied 100 nM 7,8-DHF, a TrkB-specific small molecule agonist (Liu et al., 2016) and recorded the inward currents. Bath application of 7,8-DHF induced an inward current in all EYFP-positive neurons, but the current amplitude was significantly increased after SCI (58.70 ± 8.21 pA) compared to the uninjured group (28.03 ± 4.10 pA) [**, p= .0059; Figure 4.3A, B].

Because the peak amplitude of the 7,8 DHF induced current appeared to be smaller than expected, based on prior observation (Chapter 3), we compared 7,8-DHF evoked responses in small diameter neurons obtained from C57BL/6 wild type (WT) mice. The inward current in neurons from 4 WT mice (23.52 ± 3.17 pA; n = 16 neurons) was similar to that of the EYFP-positive neurons [p=.7327], suggesting that the TrkB activity of the neurons from TrkB::ChR2 mice may be more representative of native TrkB signaling, whereas the neurons from the F616 animals may display exaggerated response. These differences may be due to strain-specific characteristics (data not shown) and are discussed further in Chapter 5.

We also measured the latencies to response and peak response. Following SCI, the onset of TrkB-mediated response increased significantly (85.76 ± 12.67 s) compared to the uninjured

(44.77 ± 5.11 s) [Figure 4.3C; *, p= .0136]. Similarly, SCI increased the latency to peak response (351.0 ± 32.8 s), compared to that in the uninjured group (182.0 ± 29.7 s) [Figure 4.3D; ***, p= .0007]. These results indicate that SCI not only enhances the amplitude of TrkB-mediated currents but also delays the onset and peak of the response, suggesting changes in TrkB activation and kinetics following SCI.

4.4.3 No change in TrkB expression in the DRGs around the lesion following SCI

Western blot analyses were performed to assess potential alterations in DRG TrkB expression following SCI. Unexpectedly, only the TrkB95 transcript was observed in the study. Unlike our previous observation, there was no change in TrkB expression in DRGs of TrkB::CHR2 mice after SCI (82 ± 25%) compared to uninjured mice (100 ± 29 %, p= .659; *data not shown*).

4.4.4 EYFP-positive neurons are categorized by their response to capsaicin.

Small-diameter DRG neurons (15 – 30 μm) tend to be nociceptors (Fang et al., 2006; Gold et al., 1996; Hagenacker et al., 2005; Körner & Lampert, 2022; Lawson, 2002; Lynn & Carpenter, 1982), some of which are also responsive to TRPV1 agonist, capsaicin. If Aδ-LTMRs adapt a nociceptor-like phenotype, they are likely to also respond to capsaicin. Alternatively, TrkB expressing DRG neurons might not be exclusively Aδ-LTMRs. Thus, we examined the response of EYFP-positive neurons to 3 μM capsaicin, as a pharmacological identification of potential nociceptors (Dirajlal et al., 2003; Le Pichon & Chesler, 2014; Stucky & Lewin, 1999). Capsaicin induced an inward current in most neurons, with 85% of cells from the uninjured (21 of 25) and 71% (27 of 38) of cells from the SCI group showing a response (Figure 4.2A). The proportion of capsaicin-responsive cells was consistent with previously reported TRPV1-positive population (Hoffman et al., 2010), and no significant differences were observed in capsaicin responsiveness between the groups (X^2=0.2805, df=1, p= .5296). The amplitude of the capsaicin-induced inward

current was also similar between uninjured (51.6 ±10.7 pA) and SCI (48.1 ± 6.5 pA) populations (Figure 4.2B, C; ns, p=.7732).

4.5 Discussion

The present study investigated the role of TrkB signaling in visually identified EYFP-positive DRG neurons after SCI, focusing on its contribution to neuronal hyperexcitability and pain hypersensitivity. Our findings reveal an enhanced response to TrkB agonist, 7,8-DHF in EYFP-positive Aδ-LTMRs following SCI. In contrast to our previous report where TrkB-mediated currents are decreased in nociceptors after SCI (Chapter 3), the current result suggests that TrkB signaling exerts different outcomes in Aδ-LTMRs, and hence, its contribution to neuronal hypersensitivity is heterogeneous, an effect that might be dependent on the neuronal subtypes. TrkB, the high-affinity receptor for BDNF, is well-known for its dual contribution to both promoting neuronal survival and adaptive plasticity, as well as mediating pro-nociceptive maladaptive plasticity (Garraway & Huie, 2016). In the current study, we observed a significant increase in TrkB-mediated inward currents in DRG neurons after SCI. This is an important and novel observation, as few studies have explored the relationship between peripheral plasticity of TrkB signaling and pain after SCI. Notably, our findings contrast with previous research in the injured spinal cord that indicated a decreased TrkB signaling after SCI (Sandra M Garraway & Lorne M Mendell, 2007). Increased activity in TrkB+ neurons may reflect a shift in the functional role of TrkB signaling in nociceptive pathways after injury, potentially contributing to maladaptive plasticity within the PNS. These distinct outcomes continue to reveal the complexity of TrkB signaling with regards to pain after SCI.

The injury-induced increase in TrkB-mediated inward currents also strongly suggests TrkB's involvement in amplification of nociceptive signaling. Prior research has demonstrated that

BDNF-TrkB signaling, while primarily associated with neuroprotection and neuronal survival, also mediates pro-nociceptive actions within both the peripheral and central nervous systems. For example, it modulates synaptic transmission from C-fibers to SCDH neurons that underlies central sensitization (Pezet & McMahon, 2006), a key mechanism underlying chronic neuropathic pain. Additionally, TrkB activation has been linked with hypersensitization of thermal sensory neurons (Shu et al., 1999) and mechanical allodynia (Dhandapani et al., 2018; Hu et al., 2023). Our findings build on these results by demonstrating that peripheral BDNF-TrkB signaling in sensory neurons is also increased following SCI, highlighting its role beyond central mechanisms.

Recent studies have identified BDNF-TrkB signaling as a critical component in the survival and function of Aδ-LTMRs, which are involved in mechanosensation and directional touch (Lechner & Lewin, 2013), not pain. Additionally, prior studies have suggested an increase in BDNF expression in the skin after SCI, potentially leading to peripheral sensitization of cutaneous afferents, including Aδ-LTMRs (Zhao et al., 2006). Likewise, TrkB expression (unpublished observation) and its downstream kinase, pERK (Martin et al., 2022) are increased in the trunk skin after SCI, further strengthening the notion that peripheral TrkB signaling underlies SCI-induced neuropathic pain. However, the specific contributions or modification of distinct cutaneous afferent fibers, such as Aδ-LTMRs, in chronic neuropathic pain and tactile allodynia after SCI, remains to be further investigated.

In our study, Aδ-LTMRs, which express TrkB, represent a subpopulation of TrkB+ DRG neurons. Following SCI, maladaptive plasticity in BDNF-TrkB signaling may enhance the excitability of Aδ-LTMRs and other TrkB-expressing sensory neurons. The heightened excitability could result in aberrant pain signaling, manifesting as mechanical allodynia or tactile

hypersensitivity. However, in our study, the increased TrkB signaling was not accompanied by heightened neuronal excitability of TrkB+ sensory neurons. These findings raise the possibility that specific subtypes of LTMRs and other non-nociceptive sensory neurons may undergo divergent changes to play a previously unidentified role in chronic neuropathic pain. Furthermore, despite the augmented agonist-induced current after SCI, we observed no significant change in overall expression of TrkB in the thoracic DRGs after SCI, potentially due to the heterogeneity within the DRG where TrkB expression in whole DRGs includes both EYFP-positive and -negative cells.

This study identifies a novel mechanism by which TrkB signaling contributes to pain after SCI that is distinct from previous work suggesting that nociceptor hyperactivity in the form of spontaneous activity is a key driver of pain after SCI (Bedi et al., 2010; Yang et al., 2014). Nonetheless, this observation supports our overall hypothesis that peripheral TrkB signaling underlies pain after SCI.

Despite these significant findings, the study has a few notable limitations. First, the heterogeneity of TrkB-expressing neurons in the DRG prevented delineation of sub-populations, such as nociceptors versus non-nociceptors like Aδ-LTMRs. Surprisingly, most recorded neurons were small-diameter and responsive to capsaicin, a marker widely associated with nociceptive neurons; however, recent deep RNA sequencing data have shown that a small amount of TRPV1 expression can also be found in C- and Aδ-LTMRs (Zheng et al., 2019). Therefore, the current study may have assessed changes in mixed populations of neurons. Further studies can be undertaken to use more specific markers of nociceptor subpopulation or genetic tools that allow better differentiation between these sub-populations to understand their distinct roles in pain hypersensitivity.

Second, following SCI, overall TrkB expression in DRGs remained unchanged, an observation that is inconsistent with increased TrkB expression in the skin (Martin et al., 2022) and the increased TrkB-mediated current in this study. However, unexpectedly, we only captured TrkB95 transcript, representative of the truncated TrkB isoforms. Astrocytes are the primary source of the truncated TrkB isoforms, including TrkB.T1 (Matyas et al., 2017); therefore, unchanged TrkB95 levels are not necessarily indicative of neuronal TrkB expression. While previous studies have linked increases in TrkB and BDNF expression in DRGs to neuropathic pain after tissue or nerve injury (Cho et al., 1998; Ernfors et al., 1993; Fukuoka et al., 2001; Ha et al., 2001), these studies did not specifically focus on neuropathic pain after SCI or the DRGs around the lesion. Therefore, our findings suggest that SCI may alter TrkB signaling without affecting overall TrkB expression levels in the DRGs around the lesion. Additional studies are needed to address neuronal TrkB expression specifically and determine the changes in isoform distribution within the thoracic DRGs following SCI.

Conclusion: Increased TrkB signaling in the PNS emerges as a critical factor in the development of neuropathic pain following SCI. The finding holds significant implications for therapeutic interventions for SCI, many of which aim to improve the functional and locomotor recovery by promoting BDNF-TrkB signaling. While such interventions may facilitate spinal recovery, our study suggests that globally increased BDNF-TrkB signaling could inadvertently increase sensory neuron activity, thereby contributing to the persistence of chronic pain. Therefore, therapeutic strategies must carefully balance the neuroprotective and regenerative benefits of spinal BDNF-TrkB signaling with its potential to drive maladaptive pain responses peripherally.

4.6 Figures

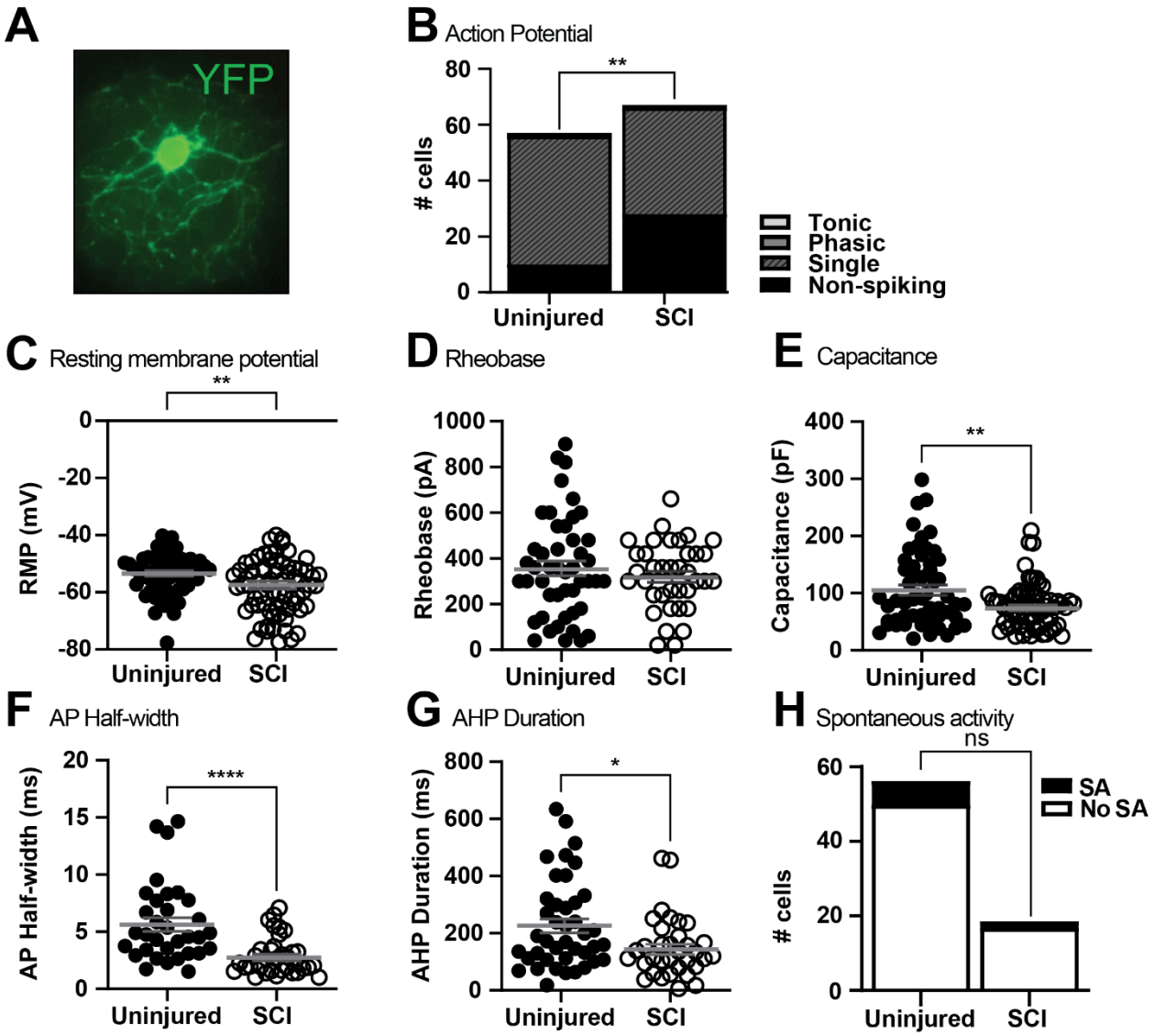

Figure 4.1: Electrophysiological properties of EYFP-positive DRG neurons from uninjured and SCI mice.

A) Representative image showing the morphology of EYFP-positive dissociated DRG neurons 48-72 hours after plating and incubation at 37°C. ***B)*** Proportional representation of DRG neurons classified as non-spiking (Uninjured, n = 10; SCI, n = 28), single-firing (Uninjured, n = 46; SCI,

n = 38), phasic-firing (Uninjured, n = 1; SCI, n = 0), and tonically-firing (Uninjured, n = 0; SCI, n = 1). Chi-square analysis revealed a significant shift in the distribution of firing patterns between uninjured and SCI neurons (X^2 =10.55, df = 3, p=.0144). ***C)*** Resting membrane potential (RMP) was significantly hyperpolarized in neurons from the SCI group compared to uninjured controls (**, p=.0054). ***D)*** Comparison of rheobase (i.e., the minimum current required to evoke an action potential [AP]) showed no significant difference between groups (ns, p=.2423). SCI significantly decreased neuronal ***E)*** capacitance (**, p=.0039), ***F)*** AP half-width (*, p < 0.0001), and ***G)*** afterhyperpolarization (AHP) duration [*, p=.0139], indicating a potential change in neuronal excitability. ***H)*** The number of neurons exhibiting spontaneous activity (SA) did not differ significantly between uninjured and SCI groups (X^2 =.04201, df = 1, p=.8376).

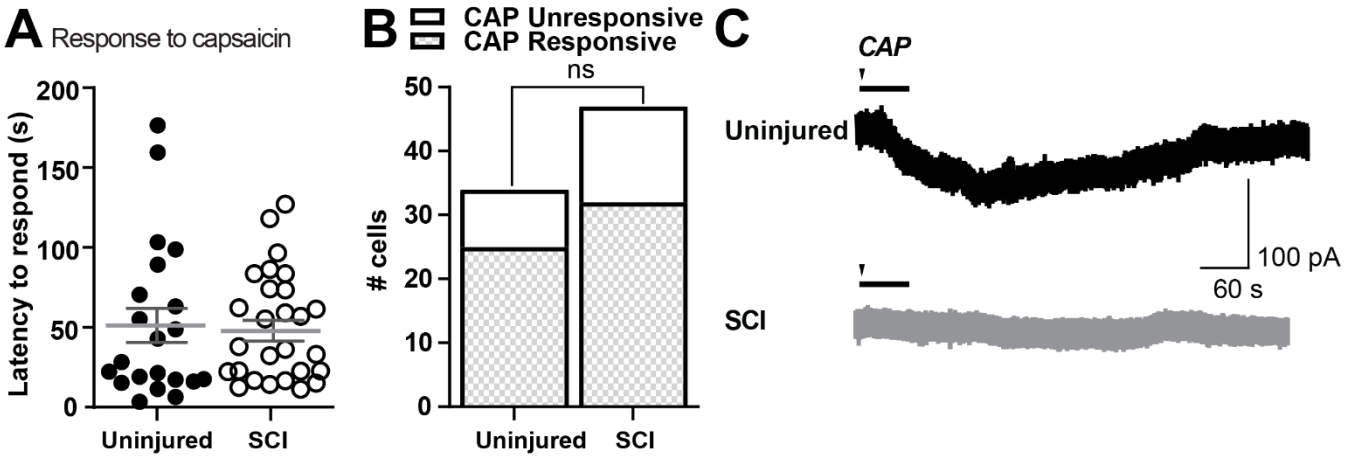

Figure 4.2: Capsaicin-induced inward currents in EYFP-positive DRG neurons decrease after SCI.

A) Number of neurons classified as responsive (light gray checkered bars) or unresponsive (white bars) to 3 μM capsaicin (CAP) stimulation. The proportion of CAP-responsive cells was not significantly different between uninjured and SCI groups (X^2 = .2805, df = 1, p=.5964). ***B)*** Scatter plot showing the magnitude of CAP-induced inward currents in individual neurons from uninjured (filled circles) and SCI (open circles) animals. Bars represent the mean ± SEM (white bar for uninjured and gray bar for SCI). The magnitude of CAP-induced inward currents did not change after SCI (ns, p=.7732). ***C)*** Representative whole-cell voltage-clamp traces (holding potential: -70 mV) illustrating inward currents evoked by 3 μM CAP in neurons from the uninjured (*top*) and SCI (*bottom*) groups. Arrows indicate the time of CAP application, and the solid black bar denotes the duration of CAP perfusion (1 minute).

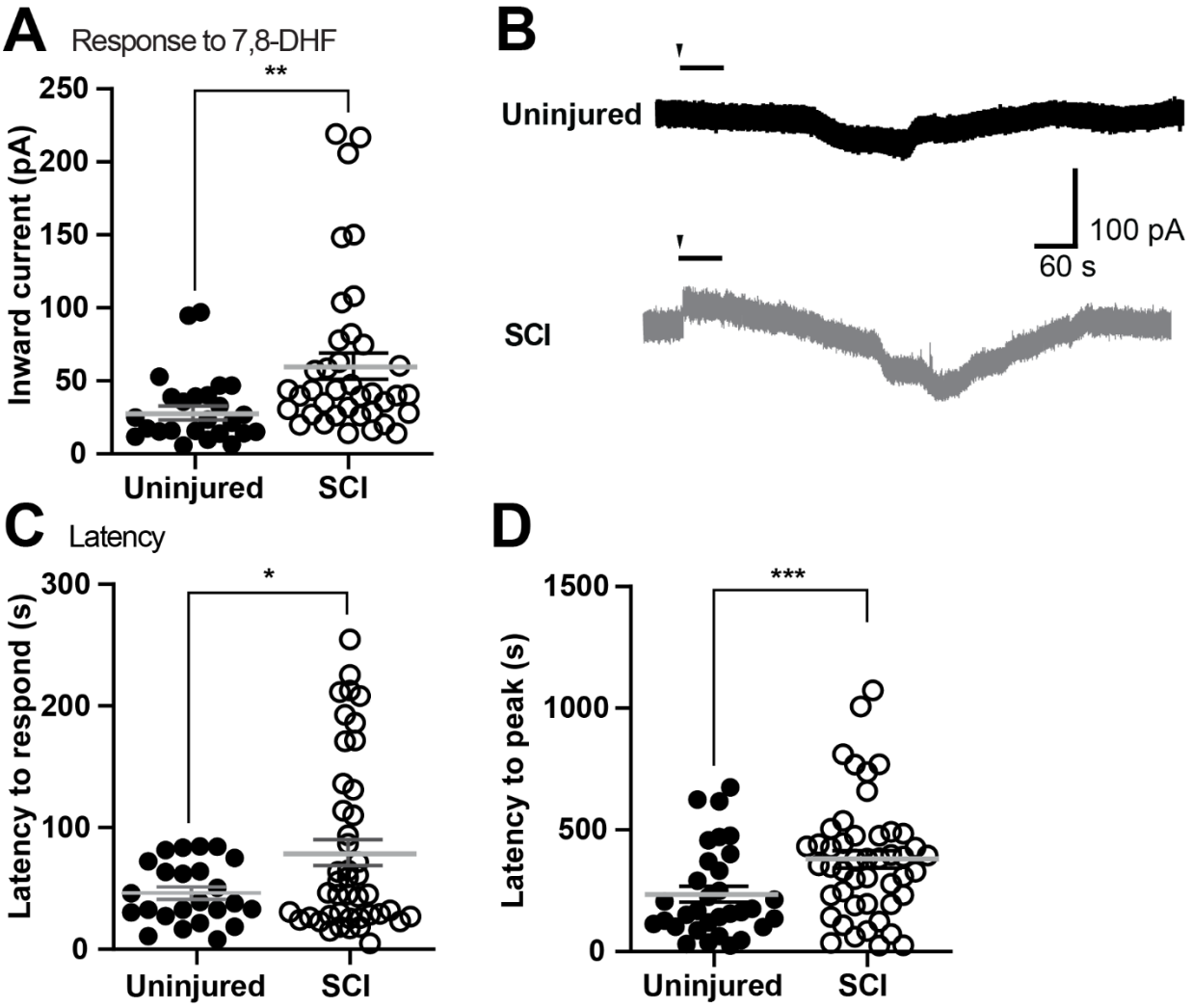

Figure 4.3: Changes in TrkB-expressing DRG neuronal response to 7,8-DHF and electrophysiological properties after SCI.

A) Plot showing the amplitude of inward currents induced by 100 nM 7,8-DHF in EYFP-positive DRG neurons from uninjured (n = 25; filled circles) and SCI (n = 38; open circles) mice. SCI significantly increased the amplitude of 7,8-DHF-induced inward currents compared to uninjured controls (**, p=.0083). ***B)*** Representative traces of 7,8-DHF-induced inward currents recorded in neurons from the uninjured (*top*) and SCI (*bottom*) groups. Arrows indicate the time of 7,8-DHF application, and the bar represents the duration of the drug perfusion (1 minute). ***C)*** Latency to

onset of 7,8-DHF-induced inward currents was significantly increased after SCI (n=36), compared to the neurons from the uninjured group (n=24; *, p=.0136), and significantly prolonged ***D)*** the latency to peak of the 7,8-DHF-induced inward current (n = 36) compared to the uninjured group (n = 24; *, p=.0007).

Condition	**Uninjured**				**SCI**			
Experiments	Electrophysiology		Immunoblotting		Electrophysiology		Immunoblotting	
Sex	Males	Females	Males	Females	Males	Females	Males	Females
n animals	5	8	6	3	4	5	6	3
Total per group	**18**				**16**			

Table 4.1: Summary of animals used in the experiment.

Detailed account of number of animals used in different strains of mice included in the study, and the total number of animals used. Each strain is further broken down into number of male and female mice, and type of experiments.

Property	Uninjured	SCI	Uninjured vs SCI	
RMP (mV)	-53.99 ± 0.94 (58)	-58.23 ± 1.11 (71)	***0.0054***	**
Rheobase (pA)	379.0 ± 36.6 (48)	323.8 ± 24.3 (37)		NS
R_{in} (MΩ)	222.8 ± 20.8 (51)	255.8 ± 19.7 (66)		NS
Membrane time constant (ms)	25.33 ± 2.40 (55)	21.21 ± 1.76 (67)		NS
Capacitance (pF)	107.1 ± 8.9 (55)	78.38 ± 4.88 (65)	***0.0039***	**
AP threshold at -60 mV	41.82 ± 2.99 (48)	37.34 ± 1.47 (36)		NS
AP amplitude (mV)	78.04 ± 4.28 (48)	80.76 ± 2.77 (37)		NS
AP halfwidth (ms)	5.754 ± 0.59 (33)	2.919 ± 0.29 (34)	***<0.0001***	****
AP rise slope (mV/ms)	6.424 ± 0.62 (46)	6.365 ± 0.57 (37)		NS
Instantaneous Firing Frequency	10.60 ± 1.12 (44)	10.43 ± 0.59 (34)		NS
AHP amplitude (mV)	228.7 ± 24.3 (41)	149.7 ± 18.0 (34)	***0.0139***	*

Table 4.2: Summary of electrophysiological properties from uninjured or SCI TrkB::ChR2 mice.

Electrophysiological properties of all small dissociated DRG neurons. RMP, resting membrane potential; AP, action potential; AHP, afterhyperpolarization; R_{in}, input resistance. Data are from DRG neurons from both male and female animals in all groups. The table shows means ± SEM, with number of recorded neurons (n) in parentheses. The *p* values are for Student's *t*-test, with significant changes indicated in bold. [NS, not significant]

Chapter 5: Comparative outcomes in TrkBF616 and TrkB::ChR2 mouse models: Advancing insights into peripheral TrkB signaling in SCI-Induced pain hypersensitivity

This chapter contains manuscript under review: K Jang, Garraway SM. *Two TrkB mice strains show different responses to the small molecule agonist, 7,8-dihydroxyflavone.* [Unpublished manuscript]. Department of Cell Biology, Emory University School of Medicine.

5.1 Abstract

This study compares SCI and TrkB targeted outcomes in F616 (chapter 3) and TrkB::ChR2 (chapter 4) transgenic mouse models that investigated the mechanistic role of peripheral TrkB signaling underlying the development of neuropathic pain following SCI. The electrophysiological studies revealed that DRG neurons from F616 mice exhibited larger inward currents in response to the TrkB agonist 7,8-DHF compared to the EYFP-positive neurons (likely Aδ-LTMRs) from the TrkB::ChR2 mice. This baseline difference in TrkB responsiveness led to contrasting results after SCI where TrkB currents in neurons from F616 mice were decreased but increased in EYFP-positive neurons. Further comparative characterization was needed to uncover potential strain-dependent factors that underlie the observed differences and provide further insight into interpretation of the previous data. The results revealed that the DRG neurons from F616 mice predominantly exhibited single- or no AP firing, whereas most EYFP-positive neurons fired repetitively, indicating intrinsic differences in neuronal excitability. Interestingly, F616 neurons exhibited a more depolarized RMP, a lower rheobase, and higher AP amplitude, which suggested higher excitability at rest. These discrepancies may be due to potential off-target effects of distinct transgene expressions underlying generation of these two models. Additionally, prior patch-clamp experiments targeted two distinct subpopulations of neurons, albeit with some overlap. Thus, the differences may also stem from subpopulation-specific characteristics owing to the heterogeneity of DRG neurons. This comparison demonstrates the need for careful consideration and the use of appropriate controls to establish baseline characteristics and that, TrkB::ChR2 model more accurately reflect native TrkB function in nociception for isolating the role of peripheral TrkB,

5.2 Introduction

The primary objective of this dissertation is to investigate the contribution of peripheral TrkB signaling in pain after SCI. The studies build upon a previous investigation that demonstrated that pharmacogenetic inhibition of TrkB in F616 mice delayed the onset of mechanical hypersensitivity and promoted locomotor recovery after SCI (Martin et al., 2022). This study was the first to identify TrkB signaling as a critical factor to pain after SCI. Building on this initial discovery, the subsequent studies employed two distinct mouse models to explore TrkB signaling in DRG neurons as an underlying contributor to neuropathic pain. I hypothesized that SCI induces nociceptor hyperexcitability (Bedi et al., 2010; Yang et al., 2014) that will be mitigated by inhibition of TrkB signaling. In this chapter, I summarize key findings from Chapters 3 and 4, highlighting both the overlapping results and fundamental differences between the two studies.

In Chapter 3, I investigated the changes in TrkB signaling in small-diameter DRG neurons after SCI and the implication for neuropathic pain. Using the F616 mice, which express mutated TrkB receptors that allow systemic pharmacogenetic inhibition of TrkB, I assessed TrkB-mediated signaling in small-diameter DRG neurons by bath-application of the selective TrkB agonist, 7,8-DHF. The key research questions addressed in Chapter 3 were: i) Does SCI change TrkB signaling or activation kinetics in small-diameter DRG neurons that are likely nociceptors, and ii) Does SCI induce heightened neuronal excitability that is TrkB-dependent?

The results revealed that treatment with 1NMP drastically reduced 7,8-DHF-induced inward currents at both systemic and single-cell level, confirming that the responses are indeed mediated by TrkB. Interestingly, TrkB-mediated responses in DRG neurons were significantly reduced after SCI as well, a change which was not restored by treatment with 1NMP. Furthermore, SCI led to increased neuronal excitability, but it was not accompanied by increased spontaneous

firing, and 1NMP treatment in SCI animals did not totally reverse these changes. These results confirmed previous observations of increased nociceptor activity after SCI (Bedi et al., 2010); however, the increase seemed to be independent of changes in TrkB signaling. Western blots also demonstrated a corresponding decrease in TrkB expression in thoracic DRGs, similarly to what has been observed in the spinal cord and which may underlie a decrease in response. Additionally, capsaicin sensitivity was confirmed in 50-70% of the recorded small-diameter neurons, confirming that most recorded neurons are likely nociceptors. However, because the effect of the TrkB inhibitor 1NMP was systemic, the exact site of its inhibitory effects, whether spinal or peripheral TrkB, remained inconclusive.

In Chapter 4, I assessed neuronal responses in TrkB-expressing small-diameter DRG neurons following SCI by employing transgenic mice, TrkB::ChR2, generated from crossing TrkBCreERT2 mice with ChR2-EYFP mice. The selective expression of EYFP in TrkB-expressing neurons allowed specific visualization and targeting of TrkB+ DRG neurons, such as Aδ-LTMRs, during electrophysiological recordings. The key research questions addressed in Chapter 4 were: i) Does SCI change the response to TrkB agonist 7,8-DHF in EYFP-positive DRG neurons, which include both nociceptors and Aδ-LTMRs? and ii) Does SCI alter the properties of EYFP-positive DRG neurons?

The results revealed that following SCI, TrkB-mediated inward currents were significantly increased in the EYPF-positive DRG neurons. However, despite the increase in TrkB responses, analysis of membrane and firing properties did not suggest heightened neuronal excitability, and a corresponding increase in spontaneous activity was absent in the DRG neurons. Furthermore, while TrkB+ neurons in the DRG are known to be Aδ-LTMRs, 85% of neurons from the uninjured group and 71% of neurons from the SCI group responded to capsaicin, demonstrating

that the majority of the recorded neurons show nociceptor-like characteristics, suggesting a mixture of Aδ-LTMRs and TRPV1-expressing subpopulations. Overall, these findings further strengthened the previous results that SCI alters TrkB signaling in the peripheral TrkB, and the changes in the TrkB signaling are independent of heightened excitability in sensory neurons.

Notably, whole-cell patch-clamp electrophysiological analyses revealed an unexpected difference in the baseline inward current responses to the 7,8-DHF between the DRG neurons from the F616 and the TrkB::ChR2 mice. This difference prompted a detailed characterization and exploration of additional electrophysiological and behavioral differences between the F616 and TrkB::ChR2 mice.

5.3 Comparison of results obtained from F616 and TrkB::ChR2 mice

46 mice (18 males and 28 females) were used for electrophysiology: TrkB::ChR2 (n =15), F616 (n =15), and WT (n = 5). An additional 21 mice were used for western blot analysis (5 TrkB::ChR2, 12 F616, 4 WT). The breakdown of animals used in each experimental group is summarized in Table 5.1.

5.3.1 Electrophysiological characteristics of TrkBF616 and TrkB::ChR2 mice

Presumed nociceptors from the F616 DRGs were identified by the diameter of the cells (15- 30 μm), while TrkB-expressing ChR2 cells were primarily identified by the presence of EYFP expression, in addition to the cell diameter (on average < 30 μm).

Cells from both groups exhibited AP firing at different frequencies, and in all cases, APs were followed by AHP, characteristic of DRG neurons (Peacock et al., 1973). Four different categories of AP firing were observed: 1) cells that did not fire any APs (non-spiking), 2) single-spiking, 3) phasic-firing, exhibiting an initial train of multiple APs followed by a sustained depolarization,

and 4) tonic-firing, consisting of sustained trains of AP firing pattern that lasted until the end of the stimulus (Figure 5.1A). All neurons displayed a TTX-sensitive inward current response to the depolarizing steps. In the 81 cells analyzed from F616 mice, most neurons fired a single spike (n = 30, 37%) or did not fire at all (n = 35, 43%), with fewer neurons displaying phasic (n = 6, 7.4%) or tonic responses (n = 10, 12.3%). In comparison, EYFP-positive cells, only 10 out of 57 cells (17.5%) fired a single spike, and only 1 cell fired tonically (1.7%), but over 80% of the cells exhibited phasic firing (n = 46). A Chi-square test confirmed a significant difference in spike frequency adaptation between the two groups (X^2=81.42, df=3, p<.0001; Figure 5.1A), indicating intrinsic differences in the ability to depolarize and fire repetitively.

5.3.2 TrkB^{F616A} neurons are more excitable than EYFP-positive TrkB::ChR2 neurons

Measurements were collected from an average of 160 cells across all three groups. Despite a higher number of TrkB::ChR2 neurons fired single or more repetitive spikes, they displayed a more hyperpolarized RMP (-54.0 ± 0.9 mV versus [F616] -50.0 ± 0.8 mV) and a larger rheobase (379.0 ± 36.6 pA) compared to F616 neurons (203.1 ± 30.6 pA, p=.0008) (Figure 5.2B, C). EYFP-positive neurons also had a higher voltage threshold (41.8 ± 3.0 mV) compared to F616 (34.1 ± 2.1 mV) [*, p=.0480; Figure 5.1D]. Other membrane properties and firing characteristics were examined and compared between the groups, which showed that, in general, neurons from TrkB::ChR2 mice were more aligned with those from WT mice, whereas the neurons from F616 mice showed higher overall excitability at rest (see Table 5.2). However, more neurons from TrkB::ChR2 and WT mice, upon reaching the threshold, were likely to fire one or more AP spikes than the F616 group.

5.3.3 TrkB^{F616A} neurons produce greater inward current responses to 7,8-DHF compared to EYFP-positive TrkB::ChR2 neurons even though the channel kinetics are the same

Next, we compared TrkB activation in the DRG neurons following application of the TrkB agonist 7,8-DHF. In all 86 neurons included in the analysis, 7,8-DHF induced robust inward currents in both F616 and EYFP-positive DRG neurons. However, small-diameter neurons from F616 mice produced significantly larger inward currents (n=45; 244.6 ± 35.4 pA) compared to EYFP-positive neurons (n=25; 30.69 ± 4.75 pA) [Figure 5.2A, B, p<.0001]. Further comparison to the neurons from WT mice (n=16; 23.52 ± 3.17 pA) revealed to be more similar to that of the EYFP-positive neurons [ns, p=.2749], suggesting that the TrkB activity of the TrkB::ChR2 neurons may be more representative of native TrkB signaling, whereas the exaggerated response in the neurons from the F616 mice (Figure 5.2A, p=.0005) may be due to strain-specific characteristics.

Latency analysis showed no differences in latency to respond to 7,8-DHF between F616 (43.13 ± 6.25 s; n = 39) and EYFP-positive (46.06 ± 5.16 s; n = 23) neurons (Figure 5.2C; p=.7481). Similarly, there was no significant difference in latency to peak response between F616 (204.2 ± 23.8 s; n = 43) and EYFP-positive (180.6 ± 31 s; n = 23) neurons (Figure 5.2D; p=.5542). One way ANOVA including WT cells further revealed no difference in latency to respond (55.38 ± 11.41 s; $F_{(2, 74)} = .6101$, p=.5460) or to peak (148.0 ± 20.7 s; $F_{(2, 79)} = .9300$, p=.3988), which suggested that the differences in the inward current response between the two mouse models are probably not due to differential activation of the TrkB receptor but rather reflect intrinsic differences in the neurons themselves.

5.3.4 Capsaicin

Small-diameter DRG neurons (15 – 30 μm) tend to be nociceptors (Fang et al., 2006; Gold et al., 1996; Hagenacker et al., 2005; Körner & Lampert, 2022; Lawson, 2002; Lynn & Carpenter, 1982), some of which express TRPV1 and are responsive to capsaicin. 3 μM capsaicin was used

in all experiments as a pharmacological identification of potential nociceptors (Dirajlal et al., 2003; Le Pichon & Chesler, 2014; Stucky & Lewin, 1999), as some subpopulations of TrkB- and TRPV1-expressing neurons have been shown to overlap in the DRGs (Zheng et al., 2019). Thus, capsaicin was expected to induce inward currents in some proportion of cells from all three groups. Capsaicin elicited robust inward currents in 21 of 25 (84%) of EYFP-positive neurons, with an average current magnitude of 51.59 ± 10.72 pA (Figure 5.2E, F). The proportion of capsaicin-responsive cells was similar to previously reported TRPV1-positive population (Hoffman et al., 2010). In F616 mice, significantly fewer cells responded to capsaicin (32 out of 52 cells, 62%, X^2=3.970, df=1, p<.0463; Figure 5.2E), but those that did respond exhibited significantly larger inward currents (162.4 ± 26.72 pA, Figure 5.2F, G), which was similar to the response magnitude observed in the neurons from the WT mice (273.0 ± 105.1 pA, 16 out of 20 cells) [ns, p=.1723; Figure 5.2 F]. Interestingly, both neurons from the F616 and WT mice displayed a bimodally distributed magnitudes of response to capsaicin, where some cells responded robustly and others almost similarly to the magnitude seen in the EYFP-positive neurons. The response to capsaicin suggested that, in all three groups, a mix of distinct neuronal populations were targeted.

5.3.5 Differences in the TrkB expression in the DRGs

Western blot analysis was performed to compare TrkB protein expression in the DRGs from T4 – L2 DRGs collected from all animals. Surprisingly, only the TrkB95 transcript was detected in all DRGs, with no significant difference in TrkB expression between F616 (85.05 ± 16.02 %) and EYFP-positive (111.5 ± 31.77 %; p=.4539) DRGs when normalized to expression in the WT (100 ± 20.59 %) DRGs [$F_{(2, 18)}$=1.726; p=.7200] (Figure 5.2 H,I). The lack of difference in global

TrkB protein expression is expected, since the difference in the inward current responses is more representative at the single cell level.

5.3.6 Pain behaviors

Lastly, reflexive mechanical and thermal pain behaviors were assessed in both male and female mice using von Frey and tail flick assessments. No differences in hind paw mechanical withdrawal thresholds was observed between F616 (2.6 ± 0.2 g) and TrkB::ChR2 (2.4 ± 0.1 g) mice (p=.5118; Figure 5.3A). However, TrkB::ChR2 animals showed longer latency to flick their tails from the heat (5.3 ± 1.1 s), indicative of hypoalgesia, compared to the F616 animals (1.7 ± 0.2 s) [Figure 5.3B; p= .0026].

5.4 Discussion of strain differences

In this section, I discuss the cellular properties of two distinct transgenic mouse lines targeting TrkB activity and highlight how the choice of model influences the interpretation of experimental results.

The F616 mice were generated by inserting a phenylalanine-to-alanine (F-to-A) mutation in the ATP-binding pocket of the TrkB receptor, rendering it susceptible to selective inhibition by PP1 derivatives, such as 1NMP (Chen et al., 2005). This pharmacogenetic modification allowing for rapid and reversible inhibition of TrkB signaling without disrupting the normal TrkB function (Chen et al., 2005; Greising et al., 2017; Johnson et al., 2008), offering specificity of genetic manipulation, temporal control, and reversibility without the lethal prenatal effects seen in TrkB-null mice (Klein et al., 1993; Linnarsson et al., 1997). Originally designed for optogenetic manipulation, TrkB::ChR2 mice offer cell-type specific fluorescence reporter inducible by TAM injection. These mice facilitate the selective visual identification and targeting of TrkB-

expressing DRG neurons during patch-clamp electrophysiology recordings, providing unique insights into the functional role of TrkB signaling at the cellular level.

The electrophysiological analyses revealed significantly larger inward current responses to the TrkB agonist 7,8-DHF in the DRG neurons from F616 mice compared to the responses observed in the EYFP-positive neurons from TrkB::ChR2 mice. This enhanced response in F616 neurons was accompanied by increased excitability, indicated by a more depolarized RMP and higher AP amplitudes, even though global TrkB protein levels in the thoracic DRGs remained similar between the two models. Several factors potentially underlie the differences between the two strains that should be considered.

Transgenic mice, including knock-out or knock-in mice (Palmiter & Brinster, 1986; Saunders, 2020), are engineered by inserting an exogenous DNA cassette into the genome. Although the transgene expression technology has improved, random integration of the transgene can still occur to disrupt coding genes or their expression (Cain-Hom et al., 2017; Dubose et al., 2013; Goodwin et al., 2019; Meisler, 1992), leading to unintended variations in transgene expression, such as multi-copy insertions or gene silencing (Gödecke et al., 2017; Gurumurthy & Lloyd, 2019; Gurumurthy et al., 2021). As such, the differences observed between the F616 and TrkB::ChR2 mice may be due to the off-target effects of genetic modification.

Differences between mouse strains have been observed in other models (De Giorgio et al., 2019; Rathnasinghe et al., 2020) and well documented (Owen et al., 1997), as distinct methods of genetic modification and generation of transgenic mice can result in phenotypic, behavioral, and cellular variability.

In the F616 mice, the transgene was delivered using bacterial artificial chromosomal (BAC) vector that includes all regulatory elements required for cell-type specific gene expression (Yang et al., 1997). However, BAC vectors can still integrate randomly into the genome, leading to mosaic expression, where some cells carry the intended modification while others exhibit the off-target insertions (Fischer et al., 2019; Jin et al., 2019; Pravtcheva et al., 1994; Zuo et al., 2019). The high frequency of TrkB-expressing neurons in F616 DRGs during our experiments (i.e., nearly all responded to 7,8-DHF) suggests that some small-diameter neurons, which typically do not express TrkB, may have acquired unintended TrkB expression and/or unusually high cell surface TrkB expression. Additionally, the inserted transgene expression may have led to overexpression of full length, active isoform of TrkB receptors on F616 sensory neurons where, in WT cells, inactive, truncated TrkB isoform may have normally been expressed. Because the protein assay only captured the truncated TrkB expression in all three mouse models, it is possible that F616 neurons had a higher expression of the full-length isoform, TrkB145.

In TrkB::ChR2 mice, the presence of Cre or ChR2 in TrkB::ChR2 mice may also modulate the intrinsic neuronal properties. While Cre expression is generally controlled by cell-specific promoters, it can sometimes 'leak,' leading to Cre activity in unintended cell population (Becher et al., 2018; Becher et al., 2019; Reizis, 2019; Schmidt-Supprian & Rajewsky, 2007; Stifter & Greter, 2020; Van Hove et al., 2020) and substantial changes in functional effects (Müller-Komorowska et al., 2020), which suggests that the small responses to 7,8-DHF in some EYPF-positive cells may not be mediated by TrkB. Furthermore, at high levels, Cre recombinase itself can induce genotoxicity (Rossi et al., 2023; Schmidt-Supprian & Rajewsky, 2007), as persistent high Cre presence has been shown to interfere with growth and cause chromosomal abnormalities in certain cells (Higashi et al., 2009; Loonstra et al., 2001; Schmidt et al., 2000).

However, TrkB::ChR2 mice exhibited no notable behavioral abnormalities, and their pain responses were similar to that seen in WT mice (*Parvin et al. in preparation*). Interestingly, Cre expression in other conditional transgenic mouse models has been associated with altered seizure susceptibility (Kim et al., 2013) and unusual epileptiform events were observed in some of the Cre mice that were not manifested in behaviors and typically absent in commonly studied region (Chen et al., 2018; Steinmetz et al., 2017). Even though these studies are not necessarily evaluating neuronal properties, epileptiform discharges consist of synchronized firing of neurons (Traub & Whittington, 2009) and could reflect the direct influence of Cre recombinase on intrinsic neuronal properties, as it may in TrkB::ChR2 mice.

Additionally, TrkB::ChR2 mice express channelrhodopsin-2 (ChR2), a light-gated cation channel (Boyden et al., 2005; Nagel et al., 2003; Zhang et al., 2006), that may also influence neuronal properties. ChR2 functions by regulating the membrane potential of excitable cells, depolarizing the membrane (Boyden et al., 2005; Nagel et al., 2003) and triggering an AP when illuminated by light (Boyden et al., 2005). Even without light activation, ChR2 can alter membrane conductance and capacitance. For example, in a patch-clamp study done in HEK293 cells, the membrane capacitance of ChR2-expressing cells was significantly higher (about 30%) than that of the control cells (Zimmermann et al., 2008). Other studies have found ChR2 to underlie transient changes in neuronal excitability, such as AP firing and intrinsic inward current (Octeau et al., 2019), or in the native electrophysiological properties, voltage threshold for AP, and the amplitude of the voltage-gated sodium current (Meng et al., 2019) of certain populations of neurons. Higher ChR2 expression in the mouse model has been suggested to disrupt expression, trafficking, or insertion of other ion channels into the membrane (Coetzee et al., 1999; Leão et al., 2005; Lin, 2011; Trimmer, 2015), which could also alter other ion channels that are

responsible for the neuronal response to synaptic inputs and overall excitability and cellular homeostasis.

Thirdly, TrkB::ChR2 transgenic mouse model requires administration of TAM, a selective estrogen receptor (ER) modulator, for Cre activation. The system allows precise temporally controlled gene deletion (Feil et al., 1996) and is often assumed to be inert in the context of transgenic models. However, estrogen receptor signaling is widely interconnected with many cellular signaling pathways, and TAM has been shown to have variable tissue-specific effects and unexpected interactions (McAndrew & Finn, 2020). In the current study, we also compared the results to the WT mice to exclude TAM as a potential contributor. Both exhibited similar inward current responses but a TrkB::ChR2 neurons displayed more subdued neuronal excitability; however, the effect cannot be solely linked to TAM treatment, as some neuronal properties of WT cells were also significantly different from the F616 neurons. Furthermore, previous literature in the CNS demonstrated that TAM did not have a measurable impact on adult neurogenesis or motor, affective, or learning behavior (Rotheneichner et al., 2017), indicating that TAM is not likely to be underlying the intrinsic differences of different transgenic mouse models.

While both models allow manipulation and isolation of TrkB function, it is important to also acknowledge that the two patch-clamp experiments examined two distinct subpopulations of neurons with some overlap: the F616 experiments targeted small-diameter neurons, most of which are unmyelinated C-fibers and do not necessarily express TrkB, whereas TrkB::ChR2 experiments targeted TrkB-expressing neurons, which include cutaneous non-nociceptors, such as Aδ-LTMRs (L. Li et al., 2011). The distinction could further explain the differences in excitability observed in the two models.

Genetic manipulation of mouse genome is a critical tool for understanding and isolating functional and molecular mechanisms underlying physiological processes. Both F616 and TrkB::ChR2 mice are valuable for dissecting TrkB-related mechanisms in ways previous neurotrophin or Trk models could not achieve. However, different transgenic mouse models can be accompanied by potential off-target effects, necessitating careful consideration and the use of appropriate controls to establish baseline characteristics. Considering these factors, the results of the current comparative analysis suggest that the TrkB::ChR2 model is more suitable for isolating TrkB contribution, due to its selectivity and population-specificity in the DRG sensory neurons. These results provide a foundation for future studies aimed at clarifying TrkB signaling underlying neuropathic pain.

5.5 Figures

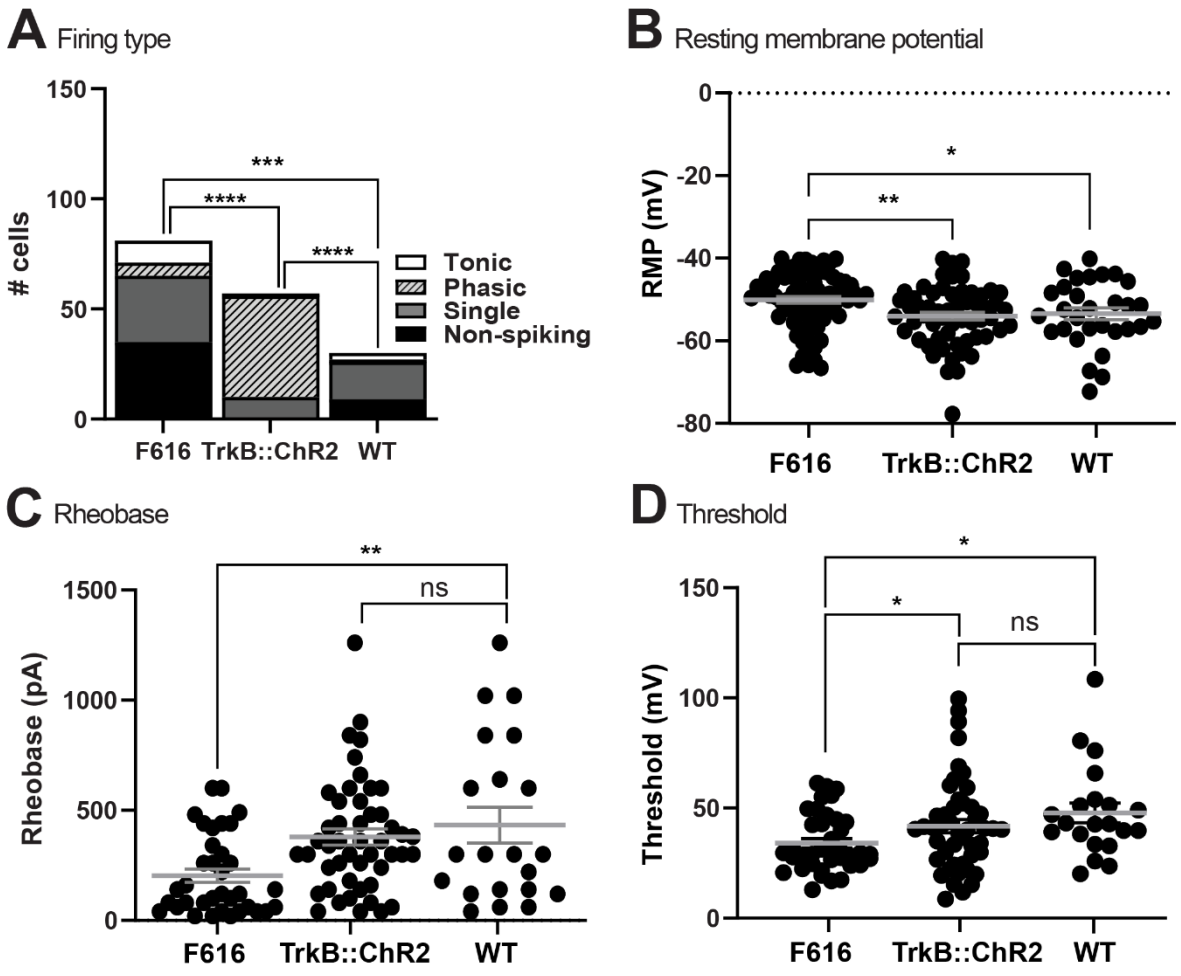

Figure 5.1: Comparison of firing and electrophysiological properties between F616 and TrkB::ChR2 DRG neurons

A) Distribution of AP firing types in DRG neurons from F616 and TrkB::ChR2 mice. Chi-square analysis of proportion of neurons categorized as non-spiking (F616, n=35; TrkB::ChR2, n = 0), single (F616, n = 30; TrkB::ChR2, n = 10), phasic (F616, n=6; TrkB::ChR2, n = 46), and tonic (F616, n = 10; TrkB::ChR2, n = 1) -firing cells from F616 and TrkB::ChR2 mouse models showed a significant difference between the two populations (X^2=81.42, df=3, ****, p<.0001). EYFP-positive neurons displayed a higher proportion of phasic firing cells, compared to F616

neurons. Similarly, compared with neurons from the WT cohort, Chi-square analysis still showed a significant difference in spike adaptation in all three mouse models. ***B)*** RMP of DRG neurons from F616. TrkB::ChR2, and WT mice. Neurons from TrkB::ChR2 and WT mice are significantly more hyperpolarized at rest compared to those from F616 group(**, p=.0017). ***C)*** Rheobase, the minimum current required to elicit an AP, is significantly higher in neurons from TrkB::ChR2 and WT mice compared to the neurons from F616 (***, p=.0008), indicating that EYFP-positive neurons require more depolarizing current to reach threshold. ***D)*** AP voltage threshold is also significantly higher in neurons from TrkB::ChR2 and WT mice compared to the F616 group (*, p=.0480), suggesting that a greater depolarization is required to trigger an AP in the DRG neurons from the TrkB::ChR2 and WT mice. Data points represent individual neurons; horizontal lines represent means ± SEM.

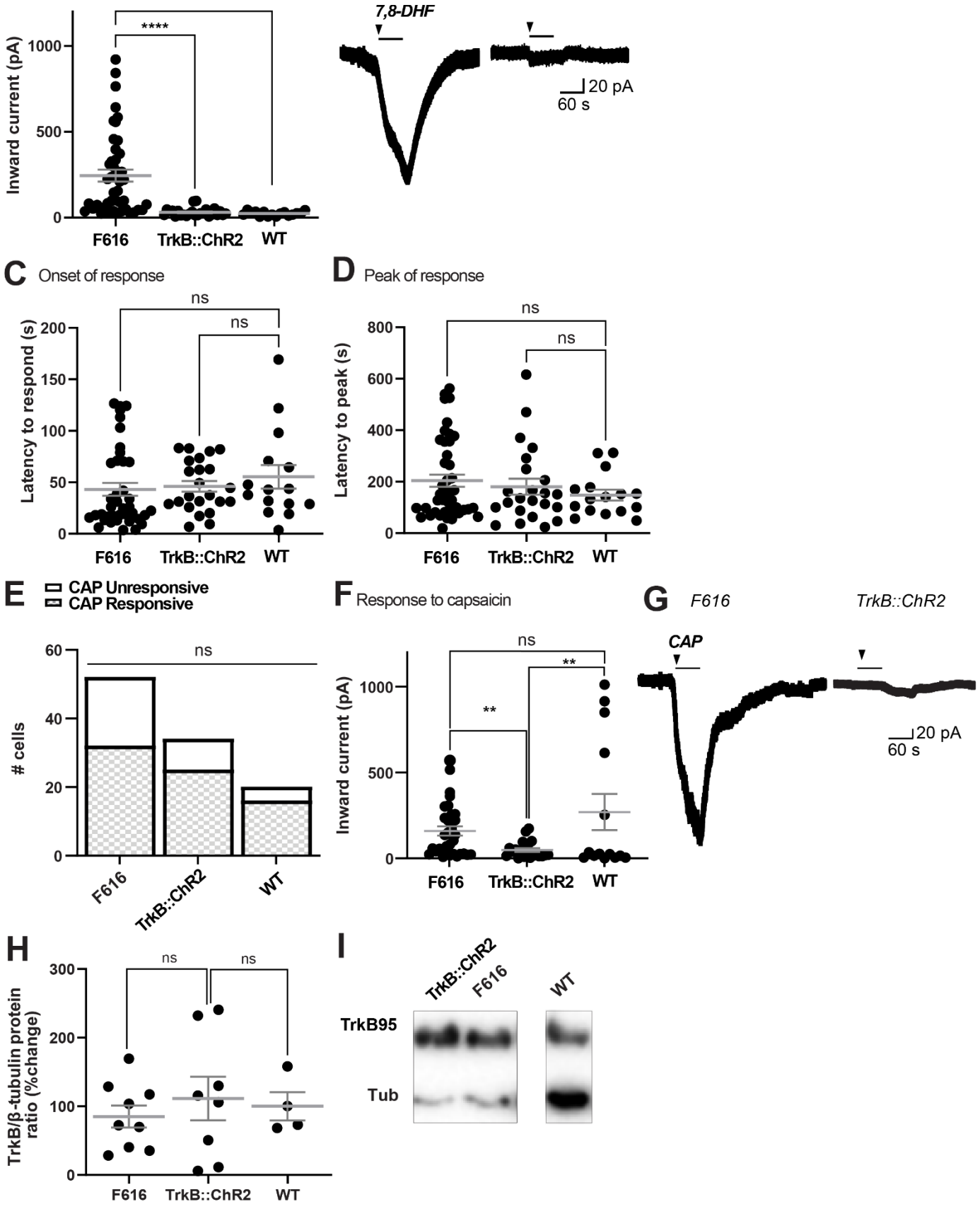
Inward current (pA)
1000
500
0
****
F616
TrkB::ChR2
WT
7,8-DHF
20 pA
60 s
C
Onset of response
Latency to respond (s)
200
150
100
50
0
ns
ns
F616
TrkB::ChR2
WT
D
Peak of response
Latency to peak (s)
800
600
400
200
0
ns
ns
F616
TrkB::ChR2
WT
E
CAP Unresponsive
CAP Responsive
cells
60
40
20
0
ns
F616
TrkB::ChR2
WT
F
Response to capsaicin
Inward current (pA)
1000
500
0
ns
**
**
F616
TrkB::ChR2
WT
G
F616
TrkB::ChR2
CAP
20 pA
60 s
H
TrkB/β-tubulin protein ratio (%change)
300
200
100
0
ns
ns
F616
TrkB::ChR2
WT
I
TrkB::ChR2
F616
WT
TrkB95
Tub

Figure 5.2: Comparison of 7,8-DHF and capsaicin-induced responses between F616 and TrkB::ChR2 DRG neurons.

A) Inward current amplitude in response to 100 nM 7,8-DHF is significantly greater in the neurons from F616 mice (n = 45) compared to EYFP-positive neurons (n = 25; ****, p<.0001) from TrkB::ChR2 mice. ***B)*** Representative traces of 7,8-DHF-induced inward currents from F616 (*left*) and EYFP-positive TrkB::ChR2 (*right*) DRG neurons. Arrows indicate the introduction of 7,8-DHF into the bath. Bar represents the duration of the drug application (1 min). ***C)*** Latency to onset of 7,8-DHF-induced inward currents was not significantly different between the neurons from the F616 (n=39) and TrkB::ChR2 (n=23; ns, p=.7481) mice, nor was ***D)*** the latency to peak of the 7,8-DHF-induced inward current (F616, n=43; TrkB::ChR2, n=23) [ns, p=.5542]. When compared to neurons from the WT mice, no group showed significant difference ($F_{(2, 74)}$ = .6173, p=.5460, one-way ANOVA) ***E)*** The number of neurons responsive (light gray checkered bar) or unresponsive (white bar) to 3 μM CAP. The proportion of cells that are capsaicin-responsive is the same between the neurons from the F616, TrkB::ChR2, and WT animals (Fisher's exact test, two-tailed p=.3511). ***F)*** Plot of capsaicin-induced inward currents in response to 3 μM capsaicin (CAP) is significantly greater in DRG neurons from the F616 (n = 31) mice compared to EYFP-positive neurons (n=21; **,p=.0024) but not to neurons from the WT mice (n=14; ns, p=.1723). Data points represent individual neurons; horizontal lines represent means ± SEM. ***G)*** Representative traces of the inward currents from whole-cell voltage-clamp recordings (holding potential -70 mV) elicited by 3 μM CAP in neurons from the F616 (*left*) and in TrkB::ChR2 animals (*right*). Arrows indicate drug introduction to the bath, and the bar indicates the duration of CAP perfusion into the bath (1 min). ***H)*** Histogram shows no differences in TrkB95 expression in thoracic DRGs from F616 (n = 9), TrkB::ChR2 (n = 8), and

WT (n = 4) mice (ns, p=.7200). ***I)*** Representative western blot lanes are shown for TrkB95 transcripts from TrkB::ChR2, F616, and WT mice, respectively.

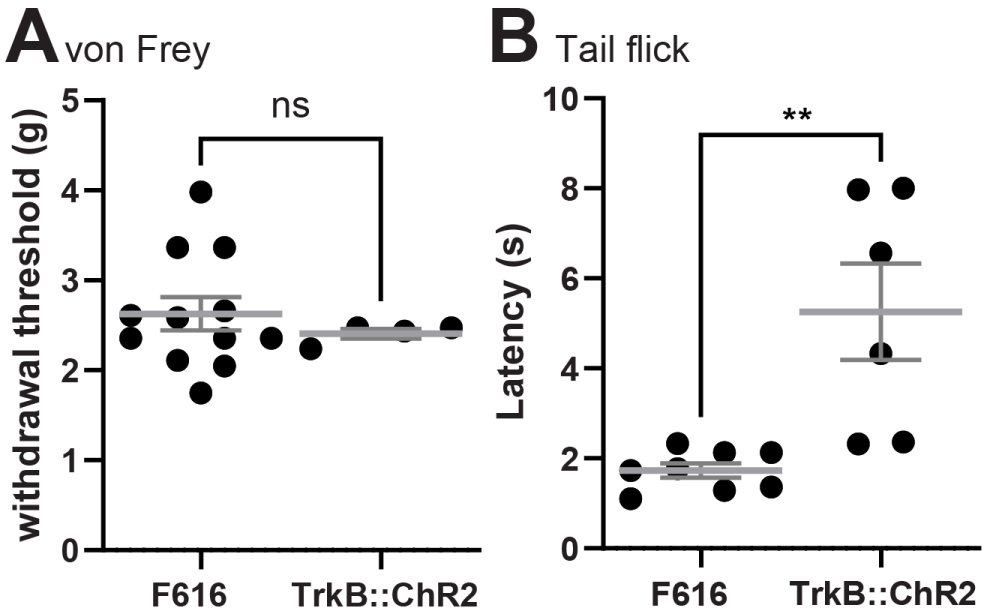

Figure 5.3: Comparison of behavioral pain sensitivity between F616 and TrkB::ChR2 mice. ***A)*** Mechanical sensitivity was assessed by von Frey filament testing. No significant differences were observed in the withdrawal threshold between F616 (n = 12, 2.628 ± .186 g) and TrkB::ChR2 mice (n = 4, 2.406 ± .056 g). ***B)*** Thermal hypersensitivity was measured by the tail-flick test. TrkB::ChR2 mice (n = 6; 5.257 ± 1.072 s) exhibited a significantly increased tail-flick latency compared to F616 mice (n = 8; 1.730 ± .159 s; **, p=.0026), indicating reduced sensitivity to noxious thermal stimuli. Data points represent individual animals; horizontal lines represent means ± SEM.

Strain	**TrkB::ChR2**				**F616**				**WT**			
Experiments	Electrophysiology		Immunoblotting		Electrophysiology		Immunoblotting		Electrophysiology		Immunoblotting	
Sex	Males	Females	Males	Females	Males	Females	Males	Females	Males	Females	Males	Females
n animals	7	8	4	1	3	12	6	6	2	3	2	2
Total	**20**				**27**				**9**			

Table 5.1: Summary of animals used in the experiment.

Detailed account of number of animals used in different strains of mice included in the study, and the total number of animals used.

Each strain is further detailed with number of male and female mice, and type of experiments.

Property	F616	TrkB::ChR2	WT	F616 vs. TrkB::ChR2		F616 vs. WT		TrkB::ChR2 vs. WT		One way ANOVA	
RMP (mV)	-50.04 ± 0.81 (72)	-53.99 ± 0.94 (58)	-53.40 ± 1.44 (30)	***0.0017***	**	***0.0337***	*		NS	***0.005***	**
Rheobase (pA)	203.1 ± 30.6 (35)	379.0 ± 36.6 (48)	433.3 ± 81.4 (21)	***0.0008***	***	***0.003***	**		NS	***0.002***	**
R_{in} (MΩ)	221.4 ± 20.3 (61)	222.8 ± 20.8 (51)	452.5 ± 65.2 (30)		NS	***<0.0001***	****	***0.0001***	***	***<0.0001***	****
Membrane time constant (ms)	27.12 ± 2.18 (68)	25.33 ± 2.40 (55)	45.31 ± 5.81 (30)		NS	***0.0005***	***	***0.0004***	***	***0.0001***	***
Capacitance (pF)	95.49 ± 8.16 (57)	107.1 ± 8.9 (55)	105.3 ± 11.3 (27)		NS		NS		NS		NS
AP threshold at -60 mV	34.07 ± 2.13 (38)	41.82 ± 2.99 (48)	47.86 ± 4.51 (21)	***0.048***	*	***0.0027***	**		NS	***0.0189***	*
AP amplitude (mV)	91.08 ± 4.02 (38)	78.04 ± 4.28 (48)	104.4 ± 4.9 (21)	***0.0324***	*	***0.0459***	*	***0.0005***	***	***0.0009***	***
AP halfwidth (ms)	4.620 ± 0.356 (34)	5.754 ± 0.59 (33)	5.469 ± 0.730 (20)		NS		NS		NS		NS
AP rise slope (mV/ms)	3.102 ± 0.519 (37)	6.365 ± 0.57 (37)	3.693 ± 0.502 (18)	***0.0002***	***		NS	***0.0116***	*	***0.0001***	***
Instantaneous Firing Frequency	17.69 ± 1.46 (38)	10.83 ± 1.071 (44)	22.36 ± 1.42 (21)	***0.0002***	***	***0.0411***	*	***<0.0001***	****	***<0.0001***	****
AHP amplitude (mV)	245.3 ± 25.7 (38)	228.7 ± 24.3 (41)	362.8 ± 31.4 (21)		NS	***0.0067***	**	***0.0017***	**	***0.0048***	**

Table 5.2: Summary of electrophysiological properties from $TrkB^{F616}$, TrkB::ChR2, and WT mice.

Data are from DRG neurons from both male and female animals in all groups. Electrophysiological properties of all small dissociated DRG neurons. RMP, resting membrane potential; AP, action potential; AHP, afterhyperpolarization; R_{in}, input resistance. Data are

from DRG neurons from both male and female animals in all groups. The table shows means ± SEM, with number of recorded neurons (n) in parentheses. The *p* values are for Student's *t*-test, with significant changes indicated in bold. [NS, not significant]

Strain	F616	TrkB::ChR2	WT	F616 vs. TrkB::ChR2	F616 vs. WT			TrkB::ChR2 vs. WT	
Inward Current (7,8-DHF)	244.6 ± 35.4 (45)	30.69 ± 4.75 (25)	23.5 ± 3.2 (16)	**< *.0001***	***	***0.0005***	***		NS
Latency to Response	43.13 ± 6.25 (39)	46.06 ± 5.16 (23)	55.4 ± 11.4 (16)		NS		NS		NS
Latency to Peak	204.2 ± 23.8 (43)	180.6 ± 31 (23)	148.0 ± 20.7 (16)		NS		NS		NS
Inward Current (Capsaicin)	149.2 ± 24.0 (31)	51.59 ± 10.72 (21)	274.5 ± 102.1 (14)	***0.0024***	**		NS	***0.0119***	*

Table 5.3: Summary of 7, 8-DHF- and capsaicin-induced inward current amplitudes and latencies to response onset and peak in F616, TrkB::ChR2, and WT DRG neurons.

Table summarizing the results of whole-cell patch clamp electrophysiology recordings with 7,8-DHF in DRG neurons from each strain of mice: $TrkB^{F616A}$ (F616), TrkB::ChR2 (EYFP-positive cells), and WT. Data collected from DRG neurons from both male and female animals in all groups. The table shows means ± SEM, with number of recorded neurons (n) in parentheses. The *p* values are for *t* test after establishing overall significance with one way ANOVA, with significant changes indicated in bold. [NS, not significant]

Chapter 6: Discussion, Future Directions, and Broader Implications

6.1 Summary

The overall objective of this thesis was an examination of TrkB signaling in small diameter DRG neurons to identify a potential neural mechanism of neuropathic pain after SCI. I hypothesized that SCI induces neuronal hyperexcitability in a TrkB-dependent manner, and therefore, inhibition of TrkB will attenuate sensory neuron hyperexcitability and prevent pain.

Chapter 1 established the framework for understanding pain following SCI, focusing on the spinal cord and the DRG, the pathophysiology of SCI and SCI-induced pain, and the phenomena of central and peripheral sensitization. This chapter highlighted the importance of studying peripheral TrkB signaling in the context of neuropathic pain, emphasizing the pro-nociceptive actions of BDNF-TrkB signaling. Beyond the extensive research on central mechanisms of pain in the field, this chapter provided the rationale for the need to expand our focus to the peripheral contributors, such as DRG nociceptors, in the development of chronic pain after SCI.

Chapter 2 reviewed DRG and primary sensory neuron plasticity in inflammatory and neuropathic pain, especially after SCI. I began by distinguishing between adaptive pain, which is protective and facilitates healing, and maladaptive pain, such as neuropathic pain, which persists beyond the initial injury or potential harm and becomes chronic. DRGs are emphasized as key sites of maladaptive plasticity, especially in the development of SCI-induced neuropathic pain. This chapter explored the various cellular mechanisms and molecular mechanisms underlying pain, focusing on the roles of VGSCs, glutamate receptors, and BDNF-TrkB signaling in mediating pain at the DRG level. Notably, I highlighted the dual role of BDNF-TrkB activity in modulating spinal plasticity and driving pro-nociceptive process, and emphasized peripheral BDNF-TrkB expression as key drivers of neuropathic pain post-SCI.

Additionally, the chapter also addressed a significant gap in understanding the pathophysiology of pain after SCI by emphasizing the need for research into the peripheral changes, such as nociceptor hyperexcitability and phenotypic switch, that contribute to persistent pain. By integrating accumulated research on molecular, cellular, and systemic changes in the DRG and their interactions with central mechanisms, the chapter proposed a holistic systems view of pain processing beyond the spinal cord. This comprehensive analysis not only enhances our understanding of pain mechanisms but also identifies potential therapeutic targets chronic and neuropathic pain and underscores the importance of continued research in this field.

In Chapter 3, I explored the role of TrkB signaling in small-diameter DRG neurons following SCI using F616 mice, which allow systemic inhibition of TrkB activity. Selective small-molecule TrkB agonist, 7,8-DHF, was administered to the dissociated DRG neurons in whole-cell patch-clamp experiments to assess neuronal responses, changes in neuronal excitability, and TrkB activation kinetics. The findings revealed a significant reduction in TrkB-mediated responses and decreased TrkB expression in thoracic DRGs after SCI, although SCI induced neuronal hyperexcitability in presumed nociceptors. These results showed that altered peripheral TrkB signaling after SCI does not drive nociceptor hyperexcitability. However, SCI did alter TrkB-mediated responses, indicating that TrkB is still contributing to post-injury changes, potentially promoting development of pain hypersensitivity through different mechanisms. Additionally, because the effect of 1NMP, used to block TrkB signaling, was systemic, whether spinal or peripheral TrkB was mediating the observed effects remained inconclusive.

In Chapter 4, I employed a transgenic mouse model, TrkB::ChR2, to selectively identify TrkB-expressing DRG neurons, which includes both nociceptors and Aδ-LTMRs, during electrophysiological recordings, by EYFP expression. Following the results presented in Chapter

3, this experiment aimed to specifically discern the contribution of peripheral TrkB expressed on DRG neurons. Unlike the results from Chapter 3, the inward currents in the EYFP-positive cells showed a significant increase in TrkB agonist-induced inward currents after SCI; however, other neuronal properties did not agree on changes in neuronal excitability, and, similarly to the results in Chapter 3, there was no corresponding increase in spontaneous activity. These findings suggested that SCI induces changes in TrkB signaling in TrkB-expressing subpopulations of DRG neurons that are typically not involved in pain transmission. Therefore, peripheral TrkB signaling may contribute to pain hypersensitivity after SCI but likely through a more complex mechanism that involves non-nociceptive subtypes of sensory neurons.

Chapter 5 provided a comprehensive comparison between the F616 and TrkB::ChR2 transgenic mouse models used in the studies. The chapter briefly summarized key findings from Chapters 3 and 4, highlighting that, while both experiments demonstrated altered TrkB-mediated responses after SCI, significant discrepancy of baseline responses to 7,8-DHF existed in the reported electrophysiological outcomes. Specifically, DRG neurons from the F616 mice exhibited larger inward currents in response to 7,8-DHF and higher neuronal excitability at rest compared to the neurons from the TrkB::ChR2 mice. When both groups were compared to the WT group, the neurons from TrkB::ChR2 mice behaved more similarly to cells from WT mice. Several factors, primarily revolving distinct genetic modifications in each mouse model, were speculated to underlie these differences that may result in unintended off-target effects that influence neuronal properties.

6.2 Discussion

This dissertation investigates the contribution of TrkB signaling to the development of neuropathic pain following SCI. Despite extensive research, the exact molecular mechanisms

underlying SCI-induced neuropathic pain remain unclear. Building on previous findings that maladaptive TrkB signaling contributes to pain after SCI, this research was driven by the need to identify the critical neural mechanism and determine the specific site of TrkB's action. To investigate our hypothesis that TrkB signaling drives hyperexcitability of small-diameter, nociceptive DRG neurons after SCI, I used two transgenic mouse models that enabled selective identification and manipulation of TrkB. As central mechanisms involved in the nociceptive processes have been well-studied (Carlton et al., 2009; Grau, 2017; Hulsebosch et al., 2009), my goal was to explore changes in the peripheral TrkB signaling following injury and its effect on pain mechanisms. As previous results have shown nociceptor hyperexcitability to be an important peripheral mechanism of pain after SCI (Bedi et al., 2010), my initial expectation was that SCI would generate increased neuronal excitability and enhanced TrkB activation. Alternatively, I expected that TrkB inhibition would attenuate neuronal hyperexcitability.

6.2.1 Significant findings

Surprisingly, the results from the F616 mice revealed reduced TrkB-mediated responses in small-diameter DRG neurons and corresponding decrease in TrkB expression in the thoracic DRGs. Although SCI induced heightened neuronal excitability, previously observed spontaneous activity was notably absent, suggesting TrkB expressed in these neurons may not promote pain hypersensitivity via increased neuronal excitability.

Subsequent use of TrkB::ChR2 mice, which allowed a more selective identification and targeting of TrkB-expressing DRG neurons (Aδ-LTMRs), revealed contrasting results from the previous study. SCI led to a significant increase in TrkB agonist-induced inward currents. Furthermore, SCI did not increase neuronal excitability, and, similarly to the previous finding, an increase in spontaneous activity was not observed. These results indicated that peripheral TrkB contributes

to sensitization of specific subtypes of sensory neurons, but not through nociceptor hyperexcitability. However, TrkB may be involved in altering the activation threshold in response to the cellular and molecular changes induced by SCI.

Notably, the two experiments targeted two distinct subpopulations of neurons, albeit with some overlap. In DRG neurons from the F616 animals, nociceptors were targeted with small soma diameter, which was confirmed with capsaicin response, whereas TrkB-expressing sensory neurons were targeted in TrkB::ChR2 group. TrkB is expressed predominantly by Aδ-LTMRs, which are also small but normally involved in innocuous and directional touch, not nociception (L. Li et al., 2011). The differences between the groups indicated potential subpopulation-specific variables to drastically alter the response to 7,8-DHF.

The contrasting findings seem to suggest a shift in the locus of TrkB's contribution to pain hypersensitivity after SCI. Rather than acting within nociceptors, TrkB expression on normally non-nociceptive DRG neurons or the nerve endings innervating the skin may become more involved. Ongoing study in our lab observed increases in BDNF and TrkB levels in the skin after SCI (*Parvin et al., in preparation*), and others have reported evidence of specific BDNF-TrkB functions in cutaneous afferents (Carroll et al., 1998; González-Martínez et al., 2004; Perez-Pinera et al., 2008). Maladaptive plasticity in these neurons, resulting from altered BDNF and TrkB signaling in the periphery after SCI could be a critical process that leads to pain hypersensitivity. Such changes would suggest that cutaneous afferents to adapt a nociceptive phenotype that leads to pain after SCI. Altogether, these results emphasize the need to consider the heterogeneity of DRG neurons and the potential for phenotypic and functional plasticity of different neuronal subpopulations that are recruited to the nociceptive pathway as a result of injury.

6.2.2 Experimental approach and potential pitfalls

Challenges of using multiple transgenic mouse models

The use of two different transgenic mouse models provided valuable, nuanced perspectives on the contribution of TrkB signaling, as the results of these studies highlight the complexity of both peripheral TrkB signaling and the impact of SCI in the development of neuropathic pain. The F616 mice allowed systemic pharmacological inhibition of TrkB, providing insights into the systemic contribution as well as confirming specificity of TrkB-mediated responses at single-cell level. TrkB::ChR2 mice allowed selective targeting of TrkB-expressing neurons, offering a more precise assessment of TrkB's role in specific neuronal subpopulations. However, the use of two distinct animal models also presented potential challenges that require careful consideration. Notably, the genetic modification and factors required for transgene expression can have unintended influences on experimental outcomes. For example, the TrkB::ChR2 mice express Cre recombinase and ChR2, and require TAM administration for transgene induction, all of which have been shown to modulate intrinsic neuronal properties.

Although Cre expression is typically controlled by cell-specific promoters, unintended activity can occur (Becher et al., 2018; Becher et al., 2019; Reizis, 2019; Schmidt-Supprian & Rajewsky, 2007; Stifter & Greter, 2020; Van Hove et al., 2020) that can potentially influence functional responses (Müller-Komorowska et al., 2020). High levels of Cre can also induce genotoxicity (Rossi et al., 2023; Schmidt-Supprian & Rajewsky, 2007), interfere with growth, and lead to chromosomal abnormalities in certain cells (Higashi et al., 2009; Loonstra et al., 2001; Schmidt et al., 2000). Although TrkB::ChR2 mice showed no behavioral abnormalities (even when compared to WT mice), the link between Cre expression and altered neuronal firing cannot be ignored.

ChR2 can modulate membrane potential and neuronal excitability, changing membrane conductance and capacitance (Boyden et al., 2005; Nagel et al., 2003), AP firing and intrinsic inward current (Meng et al., 2019; Octeau et al., 2019), and voltage threshold for AP (Meng et al., 2019). Higher ChR2 expression can also disrupt membrane ion channels (Coetzee et al., 1999; Leão et al., 2005; Lin, 2011; Trimmer, 2015), responsible for the neuronal response to synaptic inputs and overall excitability and cellular homeostasis.

TAM is a selective ER modulator with a biochemical structure similar to estradiol. It is commonly used to induce Cre-mediated gene recombination in the Cre-lox system and often assumed to be inert in this context, leading to a lack of investigations into its direct effect. In CreERT2, Cre is fused to the ERT2 that selectively binds TAM but not endogenous estrogens. Upon TAM administration, it binds to the ER, leading to translocation of CreER complex to the cytoplasm. The system allows precise temporally controlled gene deletion (Feil et al., 1996). TAM also acts at the endogenous ERα, and cholesterol epoxide hydrolase, an enzyme complex involved in cholesterol biosynthesis (de Medina et al., 2010). TAM's various binding sites indicate the need to evaluate the physiological effects of TAM-inducible gene control and careful consideration of these effects should be given before using the model. Some studies have also found sex-dependent effects of TAM, including but not limited to reproductive hormone systems (e.g., on testes and endocrine function (Patel et al., 2017), browning of adipose tissue (Zhao et al., 2020), and fibrosis in the kidneys (Falke et al., 2017)). However, in the CNS, no significant, long-term sex-dependent effects of TAM was found at cellular and transcriptomic levels (Chucair-Elliott et al., 2019; Rotheneichner et al., 2017) or on behavioral levels (Rotheneichner et al., 2017).

Comparison of neuronal properties of TrkB+ neurons and baseline behavioral and immunoblotting assessments from TrkB::ChR2 mice to WT counterparts in Chapter 4 and to F616 and WT mice in Chapter 5 demonstrated that these factors are likely not contributing to the differences between the two transgenic mouse strains.

Across these experiments, I observed that F616 mice exhibited larger inward currents in response to the 7,8-DHF compared to the TrkB+ neurons from TrkB::ChR2 mice. These baseline differences led to divergent results after SCI and potentially obscured important changes in TrkB signaling. Given these observations, the two transgenic mouse models were carefully compared to uncover strain-specific differences.

As mentioned earlier, the F616 studies targeted nociceptors based on cell soma diameter and capsaicin response, but TrkB-expressing sensory neurons are predominantly non-nociceptive. However, because most cells recorded were still small in diameter and responsive to capsaicin, a significant overlap in targeted populations is assumed between the two experiments. Nonetheless, subpopulation-specific variabilities seemed likely to underlie the differences observed. Indeed, comparing the present results to previous studies on C- and A-fibers from rodent DRGs show that C-fibers tend to be more excitable than A-fibers, displaying more depolarized RMP, lower AP threshold, and higher AP and AHP amplitudes. In contrast, A-fibers had a higher proportion of tonically firing cells, which parallels our findings as presented in Chapter 5 (Gemes et al., 2013; Harper & Lawson, 1985; Hogan & Poroli, 2008; Mizuta et al., 2012; Viatchenko-Karpinski & Gu, 2016; Villiere & McLachlan, 1996; Waddell & Lawson, 1990). Furthermore, more recent RNA sequencing results have shown both *Ntrk2* and *Trpv1*, genes for TrkB and TRPV1 receptors, respectively, to be more complex than previously established. TrkB expression was observed across diverse subpopulations of small DRG neurons,

including C-LTMRs and peptidergic nociceptors (Zheng et al., 2019), both of which also express high levels of *Trpv1*, the gene for capsaicin receptor. Aδ-LTMRs express high levels of TrkB but have been thought to be less responsive to capsaicin (Abraira & Ginty, 2013). However, the deep sequencing study identified a small amount of *Trpv1* expression on Aδ-LTMRs as well, which is consistent with the small capsaicin-induced currents observed in presumed Aδ-LTMRs. These studies demonstrate that, despite the attempt at selectively targeting TrkB-expressing neurons, the present results may be from mixture of several different subpopulations of DRG neurons.

These considerations demonstrate the importance of selecting appropriate transgenic models for studying TrkB signaling and the use of proper experimental controls when interpreting the data. The TrkB::ChR2 model, with its selective identification of TrkB-expressing neurons, may offer more specific insights into TrkB's role in sensory and nociceptive processing in context of pain hypersensitivity, while F616 model may be more useful for reversible inhibition of TrkB activity. In both models, an improved method to isolate TrkB contribution is crucial, given the complex expression and activity profile of TrkB in the periphery.

Sex-differences

SCI research predominantly focuses on male animals, as approximately 80% of SCI patients are male, with young males having a 20-fold higher incidence of SCI compared to young females (Shank et al., 2019). However, mounting evidence suggests that the pathophysiology and functional, locomotor recovery after SCI may be sex-dependent (Datto et al., 2015; Farooque et al., 2006; Hauben et al., 2002; Stewart et al., 2020; Walker et al., 2019), emphasizing a need to include both female and male subjects in studying SCI-related phenomena.

SCI disrupts reproductive hormone cycles in females, causing abnormal cycling or arrest in a single stage (Hubscher et al., 2006; Shunmugavel et al., 2012). Moreover, women generally

exhibit lower pain thresholds, greater sensitivity, and higher frequency, intensity, and duration of pain compared to men (Berkley, 1997; Fillingim et al., 1999; Greenspan et al., 2007; Hurley & Adams, 2008; Mogil, 2012; Wiesenfeld-Hallin, 2005). This heightened pain susceptibility makes women generally more prone to chronic pain (Mogil, 2012; Munce & Stewart, 2007; Navratilova et al., 2021; Rusman et al., 2018). Expanding research on pain and pain pathology have revealed significant sex differences at the molecular level, from gene expression profiles to behaviors in pain sensitization (Stephens et al., 2019). Neuropathic pain in male and female rodents also appears to involve distinct cellular mechanisms (Inyang et al., 2019; Lopes et al., 2017; Sorge et al., 2015; Taves et al., 2016), with studies showing that male rats develop both mechanical and thermal hypersensitivity, while female rats only exhibit thermal hyperalgesia (Berkley, 1997; Gaudet et al., 2017) after SCI.

To avoid bias toward male subjects and address sex as a biological variable, both male and female mice were incorporated in a balanced manner across all experiments in this dissertation, or, when needed, more females were included, as recommended by Greenspan et al. (2007). This approach mitigates the overrepresentation of male populations in SCI and pain research and ensures that sex-specific differences in SCI-induced pain processing are adequately explored and interpreted. Given the growing recognition of sex-dependent mechanisms in both immune and neuronal changes after SCI, inclusion of both sexes in experimental design is essential for a comprehensive understanding of SCI and its associated pain pathologies. However, we did not observe sex-related outcomes in this study.

Dissociated DRG neurons

Another key strength in the experimental approach was the use of dissociated DRG neurons for whole-cell patch-clamp electrophysiology, which is well-established to be useful for direct

measurement of intrinsic neuronal properties, mechanisms of sensory transduction, and pharmacological manipulations (Cano-Jaimez et al., 2020; Malin et al., 2007; Owen & Egerton, 2012), free from confounding influences of synaptic inputs or network activity. Dissociated DRG neurons retain the capacity to respond to chemical (Jordt et al., 2004; Peier et al., 2002), thermal (Reid & Flonta, 2001), and mechanical (McCarter et al., 1999) stimuli in culture. Because sensory ganglia have high consistency in composition between animals (Malin et al., 2007), primary sensory neuron cultures allow specific targeting of certain subpopulations, such as nociceptors.

Conversely, the isolated cultured neurons may not fully represent the *in vivo* conditions, as the dissociation process itself may alter neuronal physiology. For example, cell isolation requires axotomy of both central and peripheral processes, suggesting that these neurons resemble injured or regenerating sensory neurons *in vivo* (Malin et al., 2007). However, dissociated sensory neurons, especially from genetically modified mice, remain one of the most powerful experimental tools (Malin et al., 2007).

Meloxicam

Meloxicam is a non-steroidal anti-inflammatory drug that inhibits COX-2 (Engelhardt et al., 1996). NSAIDs, such as meloxicam, are beneficial for SCI research because they are an effective pain reliever for mild to moderate pain and can be administered only once a day after surgical procedures (Bourque et al., 2010; Brennan et al., 2009). While effective in reducing pain and inflammation, its role in reducing basal inflammation levels could potentially confound experimental outcomes related to neuronal responses. However, studies done by Ramesh et al. (2015) show that meloxicam had no effects in cultured DRG neurons on levels of inflammatory mediators, lesions, and cell apoptosis compared to the controls or other commonly used

analgesia, such as dexamethasone (Ramesh et al., 2015). One strategy to eliminate the potential meloxicam effect is to replicate additional electrophysiological assessments on uninjured and/or sham-operated (i.e., surgical procedure without impact to the spinal cord) that receive meloxicam. The comparison would demonstrate whether meloxicam treatment affects electrophysiological parameters assessed in this dissertation.

6.3 Future Directions

This thesis provides a novel insight into peripheral TrkB signaling in neuropathic pain after SCI. In general, the results suggest that TrkB signaling that leads to pain appears to involve mechanisms in sensory neurons that are not limited to neuronal hypersensitivity and do not involve increases in spontaneous firing. In this section, I discuss prospective approaches that could be instrumental in resolving the role of TrkB signaling in neuropathic pain after SCI.

6.3.1 Changes in voltage-gated sodium channels (VGSCs)

TrkB signaling may influence neuronal excitability indirectly by modulating other ion channels, such as VGSCs. Based on their critical influence on neuronal excitability and AP generation, VGSCs in DRG neurons have been extensively investigated for their potential roles in abnormal spontaneous activity underlying neuropathic pain. VGSC function can be modulated by other receptors through intracellular signaling cascades, such as PKA and PKC, which alter channel kinetics and expression (Gold et al., 1998). BDNF and other neurotrophins also alter VGSC expression (Kafitz et al., 1999), and pro-inflammatory cytokines, such as TNFα, increases TTX-s and TTX-r currents in the DRG neurons (He et al., 2010; Jin & Gereau, 2006), possibly through MAPK/ERK signaling pathways (Stamboulian et al., 2010). Certain subtypes of VGSCs are physically and functionally associated with TrkB receptors (Blum et al., 2002), suggesting an intricate interaction between the two channels.

Categorized based on their sensitivity to the channel blocker, TTX, at least nine subtypes of VGSCs have been cloned and identified (Nav1.1-1.9) (Wang et al., 2011): TTX-sensitive (TTX-s) Nav1.1-1.7 or -resistant (TTX-r) Nav1.8 and 1.9, as mentioned previously in this work [Chapter 2; (Bossu & Feltz, 1984; Catterall, 1992; Elliott & Elliott, 1993)]. Certain subtypes of VGSCs are exclusively expressed on sensory neurons and are essential for normal nociceptive function.

Following SCI, upregulation of VGSCs in spinal cord dorsal horn (SCDH) neurons has been correlated with primary afferent hyperexcitability (Hains et al., 2003; Hains et al., 2005), and Nav 1.7, 1.8, and 1.9 are implicated in nociceptor function (Dib-Hajj et al., 2005; Faber et al., 2012; Huang et al., 2015). However, specific changes in VGSCs or VGSC modulation of other receptors after SCI are less clear. Therefore, I conducted preliminary experiments to assess the effect of VGSC inhibition on TrkB activity using whole-cell patch clamp electrophysiology and TrkB agonist, 7,8-DHF, to induce TrkB-mediated response. I hypothesized that the inhibition of VGSCs would decrease the inward current induced by 7,8-DHF, and after SCI, the reduction would be greater. Thus, I bath-applied TTX or A-803467 to inhibit Nav1.7 and Nav1.8 activity, respectively. Unexpectedly, VGSC inhibition significantly amplified TrkB agonist-evoked inward currents in uninjured neurons, an effect that was absent following SCI.

This preliminary study reveals a novel interaction between VGSCs and TrkB in DRG neurons. Specifically, the results demonstrated the effect of VGSC inhibition on TrkB activity, rather than directly measuring changes in VGSC expression or function after SCI. Therefore, the observed changes in TrkB-mediated currents may be due to changes in TrkB receptor function or signaling pathways as a result of VGSC modulation, rather than changes in VGSCs themselves after SCI, or reflection of other components involved between VGSCs and the TrkB receptor. This raises

the possibility that yet more ion channels and receptors, such as voltage-gated potassium channels (Kv) or calcium channels (Cav), may interact differently with TrkB directly or compensate for VGSC inhibition.

Kv channels, such as Kv1.2 and 1.3, are critical in establishing membrane potential and regulating neuronal excitability (Pongs, 2008). Kv1.2 is particularly abundant in the DRG (Yang et al., 2004) and may be expressed in small (Ishikawa et al., 1999) and large (Rasband et al., 2001) DRG neurons. Peripheral nerve injury (Ishikawa et al., 1999; D. S. Kim et al., 2002; Rasband et al., 2001; Yang et al., 2004) and axotomy (Fan et al., 2014) have been shown to reduce expression of Kv1.2 in a time-dependent manner, suggesting a potential role of Kv1.2 in neuropathic pain.

Calcium channels, such as Cav2.2 and Cav3.2 are both implicated in contributing to pain signaling. Cav3.2 facilitates neurotransmitter release from nociceptive neurons following SCI (Ikeda et al., 2003; Jacus et al., 2012) and lower the threshold for AP generation (Talley et al., 1999; Todorovic & Jevtovic-Todorovic, 2013). Cav3.2 is found in all small-diameter and subpopulation of medium-diameter DRGs (Jagodic et al., 2007), and its enhancement has been linked to neuropathic and inflammatory pain (García-Caballero et al., 2014). Cav3.2 block suppresses both mechanical and thermal allodynia (Harding et al., 2021; Matthews & Dickenson, 2001), indicating their involvement in neuropathic pain.

Our data suggests that SCI leads to modulation of TrkB signaling by VGSCs that is distinct from normal conditions, possibly through compensatory mechanisms involving other ion channels or intracellular signaling pathways. However, the exact nature of changes in VGSCs remains unclear, especially after SCI, necessitating further investigations. Additional studies should focus on 1) direct assessment of VGSC expression and function after SCI, utilizing VGSC knockdown

or knockout models (e.g., siRNA) and 2) evaluation of molecular interaction between VGSCs and TrkB by employing co-immunoprecipitation and proximity ligation assays to detect potential physical interaction between VGSCs and TrkB receptors in DRGs.

Understanding the exact nature of VGSC alterations post-SCI and mechanisms of interaction between VGSCs and TrkB could provide new insights into pathophysiology of SCI-induced neuropathic pain. Ultimately, this knowledge may inform the development of targeted therapies aimed at modulating VGSC-TrkB interactions to alleviate neuropathic pain syndromes.

6.3.2 Changes in TrkB isoforms after SCI

Our findings also raise the possibility that changes in the balance between TrkB.FL and TrkB.T1 after SCI might alter TrkB signaling dynamics. Changes in the ratio of these isoforms can influence the outcome of TrkB activation, as truncated isoform binds TrkB agonists but does not trigger the same response, and therefore can affect overall neuronal excitability.

6.3.2 Pharmacological inhibition of native TrkB receptors

The current studies undertook a targeted examination of TrkB signaling underlying pain after SCI by employing two transgenic mouse models. Despite the mechanistic strengths of this study, its translational potential is limited. For future studies, we propose targeting native TrkB receptors and examining TrkB siganling in WT mice, specifically, using clinically relevant pharmacological TrkB ligands, such as ANA-12 (Cazorla et al., 2011) or cyclotraxin-B (Cazorla et al., 2010). In the absence of genetic manipulations, we will determine changes in electrophysiological and firing properties of sensory neurons in uninjured and SCI mice using pharmacological tools that target TrkB. It is experimentally and therapeutically advantageous to determine whether the inhibition of native TrkB activation also produces comparable beneficial

outcomes after SCI to the current transgenic models, and critically, if the cellular and molecular mechanisms underlying the outcomes are shared.

6.4 Broader implications for the peripheral changes and role of peripheral TrkB in neuropathic pain after SCI

Significant advances have been made in our understanding of the neurobiology of pain. Adaptive pain, such as nociceptive pain, involves peripheral and central mechanisms. Neuropathic pain, although more complex and less understood, also appears to require plasticity at central and peripheral sites. Similarly, our comprehension of functional plasticity after SCI has greatly improved through clinical and experimental evidence. While early research focused on neuronal survival, regeneration, and functional recovery of the damaged spinal cord, more recent investigations have demonstrated that both adaptive and maladaptive plasticity extend beyond the spinal cord to peripheral sites.

The DRGs are uniquely positioned at the intersection of the periphery and SCDH, and their anatomical accessibility makes them important targets for treatment and therapies of neuropathic pain. Additionally, DRGs house the cell bodies of nociceptors, which are primary mediators of pain under normal and pathological conditions. The present work highlighted critical features of the DRG, commonly expressed pain substrates, and DRG plasticity in both inflammatory pain and neuropathic pain after SCI. Furthermore, the role DRGs play in inflammatory and neuropathic pain states was discussed by focusing on the changes in nociceptor cell soma, terminals, and adjacent non-neuronal cells, such as microglia or skin cells (see Figure 1.1 for summary of the peripheral processes potentially involved in injury-induced pain).

Despite remarkable progress in identifying the neuroanatomy and molecular mechanisms of peripheral pain transduction and diversity of sensory neurons involved, understanding how individual components of the nociceptive circuits interact under pathological conditions remains a significant challenge. For example, while sensory neuron hyperexcitability is thought to underlie chronic pain after SCI, the exact mechanisms leading to neuronal hyperexcitability are unknown. In examining the multitude of changes that occur after SCI, specifically those that may contribute to pain, BDNF has received a lot of attention as a bidirectional neuromodulator of spinal plasticity (Garraway & Huie, 2016). BDNF contributes to multifaceted effects on the spinal cord after SCI and plays a role in spinal learning circuits (Garraway, 2023; Garraway & Huie, 2016). However, as most studies looking at the relationship between SCI and BDNF-TrkB signaling have been centered around spinal cord and the brain, this work not only aimed to replicate the previous findings in the periphery but also sought to identify the specific mechanistic drivers of hyperexcitability in sensory neurons. The results of the experiments in TrkB-specific transgenic mice show that peripherally expressed BDNF and TrkB are potential contributors to induction and persistence of pain after SCI.

Conclusions

Further research is needed to explore the complex mechanisms of pain, both under normal physiological conditions and in the context of injury-induced maladaptive pain, such as neuropathic pain following SCI. The data presented in this dissertation indicates that peripheral TrkB signaling plays a bidirectional role and is closely interconnected with spinally expressed BDNF and TrkB. Importantly, the findings from TrkB+ neurons reveal a previously unrecognized role of peripheral TrkB in driving maladaptive plasticity. This plasticity enables normally non-nociceptive cutaneous afferents to acquire a nociceptive phenotype or become

functionally recruited into the nociceptive network. Although more extensive studies are required to identify the precise molecular components involved, the work presented in this dissertation advances the potential for pharmacologically targeting and selectively modulating this pathway. Such interventions could enable simultaneous functional recovery after SCI and prevent chronic pain.

Appendix

Additional publications (not included in this dissertation) to which the author has contributed

1. Martin KK, Noble DJ, Parvin S, **Jang K**, Garraway SM. “Pharmacogenetic inhibition of TrkB signaling in adult mice attenuates mechanical hypersensitivity and improves locomotor function after spinal cord injury”. Front Cell Neurosci. 2022 Sep 26;16:987236. doi: 10.3389/fncel.2022.987236.

2. Lin J, Rivadeneira AP, Ye Y, Ryu C, Parvin S, **Jang K**, Garraway SM, Choi I. Sodium Bicarbonate Decreases Alcohol Consumption in Mice. Int J. Mol. Sci. 2024 May; 25(9):5006. doi:10.3390/ijms25095006

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