CALCIUM CONTROLS FIRING PROPERTIES OF PARVALBUMIN-EXPRESSING SPINAL CORD NEURONS VIA SK CHANNELS

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Abstract

Sensory information from the periphery transits through the dorsal horn of the spinal cord before reaching the brain for interpretation. This sensory relay station processes the incoming information through the coordinated activity of different types of interneurons that will ensure that the appropriate circuits are engaged. Inhibitory parvalbumin interneurons (iPVINs) are necessary for preventing touch inputs from activating pain circuits, and they do so via feedforward inhibition. An important aspect of these neurons is their ability to sustain tonic high firing frequencies without adaptation to produce an "inhibitory blockade" that prevents tactile inputs from reaching pain circuits in the superficial layers of the dorsal horn. After nerve injury, the intrinsic excitability of iPVINs is reduced alongside the expression of the calcium buffering parvalbumin protein. These interneurons express Ca^{2+}-activated small-conductance potassium channel 2 (SK2), whose activation produces spike frequency adaptation. We hypothesize that the tonic firing properties of iPVINs depend on extensive Ca^{2+} buffering during repetitive action potential production to prevent the accumulation of intracellular calcium ($[Ca^{2+}]_i$) and the subsequent activation of SK2 channels that would otherwise cause adaption.

Here, I will test this hypothesis by imposing various $[Ca^{2+}]_i$ in iPVINs and using fluorescence-guided whole-cell recordings from acute spinal slices of PV::Cre;tdTom mice to record their firing properties. This approach was first validated using Ca^{2+} imaging on Fura-2 loaded HEK293 cells to demonstrate a gradual and dose-dependent rise in $[Ca^{2+}]_i$ that plateaus 3-5 minutes after whole-cell configuration. In iPVINs, imposing $[Ca^{2+}]_i$ above 200µM for 5 minutes after performing a whole-cell recording induced adaptation and prevented tonic firing. In contrast, imposing $[Ca^{2+}]_i$ of 50µM did not cause iPVINs to take on an adaptive firing mode, and the neurons continued firing tonically. Applying the SK2 channel blocker Lei-Dab7 in iPVIN with an induced adaptive firing

partially rescued their tonic firing pattern. Our findings indicate that $[Ca^{2+}]_i$ plays a critical role in controlling the electrical properties of iPVINs, likely through the recruitment of SK2 channels.

Résumé

L'information sensorielle de la périphérie transite par la corne dorsale de la moelle épinière avant d'atteindre le cerveau pour être interprétée. Cette station de relai sensoriel traite les informations entrantes grâce à l'activité coordonnée de différents types d'interneurones qui garantiront que les réseaux neuronaux appropriés sont engagés. Les interneurones inhibiteurs exprimant le marqueur parvalbumine (iPVINs) sont nécessaires pour empêcher des entrées tactiles d'activer des circuits de douleur. Un aspect important de ces neurones est leur capacité à maintenir des fréquences de décharge toniques élevées sans adaptation pour produire un « blocus inhibiteur » qui empêche les entrées tactiles d'atteindre les circuits de douleur dans les couches superficielles de la corne dorsale. Après une lésion nerveuse, l'excitabilité intrinsèque des iPVINs est réduite parallèlement à l'expression de la protéine parvalbumine qui tamponnant le calcium. Ces interneurones expriment également le canal potassique de faible conductance activé par le Ca^{2+} (SK2), dont l'activation produit l'adaptation de fréquence de décharge. Nous émettons l'hypothèse que les propriétés de décharge tonique des iPVINs dépendent du tamponnage soutenu de Ca^{2+} qui entre dans le neurone pendant la production de potentiels d'action à haute fréquence afin d'empêcher l'accumulation de calcium intracellulaire ($[Ca^{2+}]_i$) et l'activation subséquente des canaux SK2 qui autrement causeraient l'adaptation.

Dans ce travail, je vais tester cette hypothèse en imposant divers $[Ca^{2+}]_i$ dans les iPVINs et en utilisant des enregistrements en configuration cellule-entière guidées par fluorescence à partir de sections transverses de la moelle de souris PV::Cre;tdTom pour enregistrer leurs propriétés de

décharge. Cette approche a été validée au préalable à l'aide d'imagerie Ca^{2+} sur des cellules HEK293 chargées par Fura-2 pour démontrer une augmentation progressive et dépendante de la dose de $[Ca^{2+}]_i$ qui plafonne 3 à 5 minutes après la transition en configuration cellule-entière. Dans les iPVINs, imposer $[Ca^{2+}]_i$ au-dessus de 200µM pendant 5 minutes induit une adaptation et empêche la décharge tonique des neurones. En revanche, l'imposition de $[Ca^{2+}]_i$ de 50µM n'a pas affecté le mode de décharge tonique. L'application du bloqueur de canal SK2, Lei-Dab7, sur les iPVIN ayant un mode de décharge adaptif a partiellement sauvé leur modèle de décharge tonique. Nos résultats indiquent que $[Ca^{2+}]_i$ joue un rôle essentiel dans le contrôle des propriétés électriques des iPVINs, probablement par le recrutement des canaux SK2.

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Contribution of Authors

All parts of this manuscript were written by Shi Chen Xu. Dr. Reza Sharif-Naeini provided revisions and corrections.

Shi Chen Xu performed all experiments and data analysis. Dr. Loïs Miraucourt contributed recordings for iPVINs. Dr. Lise Rabiller, Alice Gilbert, and Stephanie Mouchbahani-Constance maintained and plated HEK293T cultures for imaging. Xinyue Ma wrote the MATLAB code for the automated whole-cell patch clamp analysis toolkit.

Abbreviations

(i/e)PVIN – (inhibitory/excitatory) parvalbumin interneuron
$[Ca^{2+}]_i$ – intracellular calcium
$[Ca^{2+}]_{ip}$ – intrapipette calcium
AC – adenylate cyclase
AMPA – α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
BDNF – brain-derived neurotrophic factor
BK – large conductance calcium-activated potassium channels
CatS – Cathepsin S
Ca_v – voltage-gated calcium channel
CCI – chronic constriction injury
CCK – cholecystokinin
CGRP – calcitonin gene-related peptide
CNS – central nervous system
CSF1 – colony-stimulating factor
DRG – dorsal root ganglion
eEPSC/eIPSC - evoked excitatory/inhibitory post-synaptic current
eGFP – enhanced green fluorescent protein
ERK – extracellular signal-regulated kinase
fAHP – fast afterhyperpolarization
GABA – γ-Aminobutyric acid
GAD – glutamic acid decarboxylase
Gly – glycine
HCN – hyperpolarization-activated cyclic nucleotide-gated channel
I_h – hyperpolarization-activated current
IPSC – inhibitory post-synaptic current
IR – immunoreactivity

IRF – interferon regulatory factor 5
KCC2 – chloride potassium symporter 5
KO – knock out
K_v – voltage-gated potassium channel
LTMR – low-threshold mechanoreceptor
LTP – long-term potentiation
mAHP – medium afterhyperpolarization
mEPSC/mIPSC – spontaneous miniature excitatory/inhibitory post-synaptic current
Na_v – voltage-gated sodium channel
NK1R – neurokinin 1 receptor
NMDA – N-methyl-D-aspartate
nNOS – neuronal nitric oxide synthase
NO – nitric oxide
NPY – neuropeptide Y
PKB/PKC/PKCG – protein kinase B/C/G
PrP – prion protein
PV – parvalbumin
PVp – parvalbumin protein
-R – receptor
RARα – retinoic acid receptor alpha
SDH – spinal dorsal horn
shRNA – small hairpin RNAs
SK – small conductance calcium-activated potassium channels
SNI – spared nerve injury
SP – substance P
TNFα – tumor necrosis factor alpha
TUNEL – deoxynucleotidyl transferase-mediated biotinylated UTP nick end labeling
VGLUT – vesicular glutamate transporter

Chapter 1: Introduction

1.1 Chronic Pain

1.1.1 Definition

Chronic pain is a debilitating and widespread condition, profoundly impacting individuals and society. While chronic pain usually stems from injury or disease, the development or absence of chronic pain within an injury or disease is highly varied. Thus, the presentation of chronic pain is considered a condition of its own, with clinical practice dedicated to the management of pain separate from underlying injury or disease (Cohen et al., 2021). The complexity of conditions within the chronic pain group poses many challenges for researchers and clinicians alike. Only the latest International Classification of Diseases (ICD-11) gave chronic pain a robust systematic classification, better reflecting its complexities (Smith et al., 2019). All chronic pain classifications share the definition of lasting or reoccurring pain for more than three months (Smith et al., 2019). Chronic pain is further subdivided into chronic primary pain and six chronic secondary pain conditions. Chronic primary pain has a broad definition to encompass the unknown etiology for the contained conditions, in which the only additional diagnosis criteria is the absence of another chronic pain condition. The underlying disease or injury is known for chronic secondary pain conditions and induces chronic pain. Chronic secondary pain is further subdivided into cancer, postsurgical and post-traumatic, neuropathic, headache and orofacial, and visceral pain. The diverse and diffuse conditions under the chronic pain umbrella encompass serious challenges to the treatment of chronic pain.

1.1.2 Impact

Advancements in treatment are dearly needed; chronic pain in the general population is widespread and burdens individuals and society heavily. One out of five adults in Canada and the United States have a chronic pain condition (Campbell et al., 2019; Yong et al., 2022). Despite chronic pain's association with older adults, chronic pain has a prevalence of 11% to 30% in children (King et al., 2011). Regardless of whether the chronic pain is primary or secondary, chronic pain restricts the ability to work and move, compromising the ability to complete routine tasks (Cohen et al., 2021). Nearly half of chronic pain patients have a significant reduction in their ability to work (Yong et al., 2022). Patients often feel they cannot properly engage with their loved ones or daily tasks, leading to frustration, anger, and depression (Cohen et al., 2021). Chronic pain is an invisible condition, with patients often feeling unheard by their peers and clinicians (McManimen et al., 2019). Patients usually have accompanying physical and mental health conditions, which exacerbate and complicate each other leading to a vicious cycle that further demotivates patients from engaging in activity and preventing the effective treatment of chronic pain and comorbid conditions (Campbell et al., 2021; Cohen et al., 2021). Alone, chronic pain causes stress and mental anguish that can precipitate the development of physical and mental health conditions (Campbell et al., 2019). Together, this compounds into a staggering 74.7% comorbidity in patients with chronic pain (Foley et al., 2021). The widespread nature of chronic pain and its ability to exacerbate other health issues leads to staggering costs. Annually, chronic pain costs the United States an estimated $560-$635 billion and Canada $38.2-$40.3 billion per year in healthcare costs and lost productivity (Campbell et al., 2021; Gaskin & Richard, 2012; Smith & Hillner, 2019). In Canada and the United States, chronic pain remains one of the most common causes of seeking medical care (Campbell et al., 2019) and will only continue to build; Health Canada estimates that

with population growth and aging, direct and indirect costs will rise to $52-$55 billion by 2030 (Campbell et al., 2021). Despite shifting clinical practice reflected in the new ICD-11, chronic pain remains poorly understood. With chronic pain's widespread impact on individuals and society, advancements are dearly needed, even incrementally; only a 1% annual reduction in the number of Canadians living with chronic pain could save an estimated $3.5-3.7 billion from 2020 to 2030 (Campbell et al., 2021).

1.2 Neuropathic pain

Of chronic pain patients, 40% experience chronic neuropathic pain due to lesions or disease of the peripheral or central nervous system (van Hecke et al., 2014). For pain clinicians, neuropathic pain is notoriously difficult to treat as no specific pharmacological treatments exist for this condition. The current frontline pharmacological treatments are tricyclic antidepressants, selective-norepinephrine reuptake inhibitors, and gabapentinoids, commonly used in seizure disorders (van Velzen et al., 2020). The most efficacious drug class of tricyclic antidepressants are considered a “dirty drug”, affecting cholinergic, monoamine, and NMDA receptors (Moraczewski & Aedma, 2022). In efficacy trials, a pain reduction of 50% is considered an efficacious result (Sindrup & Jensen, 1999); for the most prescribed drugs, the probability of efficacy is less than 1/3 per patient, with their diffuse action throughout the CNS causes significant side effects (Barohn et al., 2021; Bates et al., 2019; Sindrup & Jensen, 1999). Together, this highlights the challenges and the need for a greater understanding of neuropathic pain.

1.2.1 Central and peripheral neuropathic pain

Neuropathic chronic pain is divided by etiology into central and peripheral neuropathies. There is a shared general schema of both nervous and adjacent immune responses eliciting abnormal

changes to sensory processing that misreads and produces spontaneous pain. They often share the core symptoms of allodynia, where touch-like stimuli elicit a pain-like response; hyperalgesia, where pain responses are amplified; and spontaneous pain, where the sensation of pain occurs even at rest and interferes with sleep patterns (Meacham et al., 2017; Watson & Sandroni, 2016).

Central neuropathic pain develops from a lesion or disease in the brain or spinal cord. The most common causes of central neuropathic pain are stroke, infections, demyelination, trauma, or neoplastic disorders (Watson & Sandroni, 2016). A differentiating factor of central neuropathic pain is its ability to occur weeks to months after the originating injury, presenting a unique challenge for primary care physicians initially seen when the pain develops (Watson & Sandroni, 2016). Often, central neuropathic pain presents with abnormal spasticity, stiffness, and movement in the affected periphery to a greater degree than peripheral neuropathic pain (Watson & Sandroni, 2016). Of the two neuropathic pain aetiologies, central neuropathic pain is rarer in the clinic than its peripheral counterpart. The central nervous system has no sensory component, so central and peripheral neuropathic pain can be hard to distinguish from just pain presentation in the anatomy affected. In addition, central neuropathic pain can have many diffuse presentations based on the origin or location of the resulting insult (Szok et al., 2019; Watson & Sandroni, 2016).

Peripheral neuropathic pain results from injury or disease to the peripheral sensory nervous system. Common peripheral nerve injury causes are metabolic disorders, infections, inflammation, and traumatic injury (Marchettini et al., 2006). While the original insult may be localized, the development of neuropathic pain requires both peripheral and central mechanisms (Meacham et al., 2017). Despite the diversity of causes, the three core symptoms remain common to peripheral neuropathic pain patients. In pain research, these symptoms have been established as correlating to the underlying biological mechanisms of pain processing (Jensen & Finnerup, 2014). Unlike

central neuropathic pain, peripheral neuropathic pain follows a similar developmental trajectory independent of originating insult. Therefore, understanding the biological framework of pain processing is crucial for advancing neuropathic pain treatment.

1.3 Nociceptive Processing

Pain and nociception have distinct definitions. Nociception, the detection of noxious stimuli, is protective by generating both a withdrawal reflex and a complex behavioural response to avoid further stimuli (Latremoliere & Woolf, 2009). This complex behavioural response is part of pain along with the conscious sensory and emotional experience associated with, or resembling, actual or potential tissue damage (Mischkowski et al., 2018). Recognizing pain as a highly dynamic interplay of psychological, social, and biological factors, pain clinicians have developed the now dominant biopsychosocial model of pain to treat patients multifacetedly (Meints & Edwards, 2018). Nociception is only the neural encoding of noxious stimuli; though noxious stimulation leads to pain, pain can exist without stimuli due to pathological nociceptive processing (Mischkowski et al., 2018). By further understanding nociception, we can develop interventions that prevent maladaptive pain and reduce patient needs for biopsychosocial intervention. Here, we will describe nociceptive processing at the periphery and spinal cord. For this review, we will restrict our exploration to cutaneous sensory receptors and their respective spinal circuits.

1.3.1 Primary afferents and the DRG

Primary afferent fibres transmit sensory information from the periphery to the central nervous system. Sensory afferents are pseudo-unipolar neurons; their processes act as a single axon from sensory end organs to the SDH, with the cell body located in the DRG (Middleton et al., 2022). Neurons within the DRG do not form synapses with each other (Krames, 2014; Middleton et al.,

2022). Sensory researchers initially assumed this distinct morphology was to allow for the unmodified transmission of sensory input. We now know that the DRG is critical in modulating sensory processing by neuronal cell bodies and resident immune cells (Kent et al., 2018; Middleton et al., 2022). Surrounding the cell bodies in the DRG are layers of satellite glial cells specific to the DRG and trigeminal ganglia (Hanani, 2005; Hanani, 2010). These glial cells are involved in homeostasis and the immune response of the DRG (Nascimento et al., 2008). Unlike the blood-nerve or blood-brain barrier for respective peripheral and central nervous systems, the satellite glial cells provide little protection against circulating factors (Middleton et al., 2022). As such, the DRG is a unique entry point for peripheral neuropathic pain conditions (Haberberger et al., 2019).

The DRG houses the cell bodies of visceral, cutaneous, and muscle sensory afferents (Middleton et al., 2022). For our purposes, it is helpful to restrict these to cutaneous afferents of the nociceptors, transmitting noxious input; and LTMRs, transmitting innocuous mechanical input. These cutaneous afferents are classified as Aβ-, Aδ- or C-fibers based on their cell body size, speed of conductance, and level of myelination (Abraira & Ginty, 2013; Dubin & Patapoutian, 2010). These are further distinguished within their class by preferred stimuli, their associated cutaneous end organs, and their rate of adaptation to indentation of the skin (Abraira & Ginty, 2013; Middleton et al., 2022). LTMR and nociceptive inputs preferentially terminate at different SDH depths called laminae (Abraira et al., 2017). These laminae, starting from the dorsal surface of the SDH, correspond loosely to the nociceptive recipient zone of lamina I-IIo and the LTMR recipient zone of IIi-V (Abraira et al., 2017). As we will explore further, lamina II, a sparsely myelinated region rich with interneurons, contains crucial circuitry for integrating LTMR and nociceptive inputs during nociceptive processing.

Nociceptors are classically C- and Aδ-fibers, having free nerve endings in the skin and transmitting noxious input to second-order neurons of the SDH (Todd, 2022). Most C-nociceptors are polymodal, playing an important role in sensing noxious thermal, irritant, mechanical, and tissue damage stimuli (Chen et al., 2013). These unmyelinated small-diameter fibres have slow conductance velocities with large receptive fields responsible for encoding pain intensity (Kendroud et al., 2023). Aδ-nociceptors are myelinated small-diameter fibres, having a relatively faster conduction velocity with smaller receptive fields, encoding for sharp pain and its localization (Kendroud et al., 2023). Nociceptive afferents are not only responsible for the transmission of noxious stimuli but a shift towards pronociceptive processing after repeated or intense noxious stimuli called central sensitization (Latremoliere & Woolf, 2009). Peptidergic C- and Aδ-fiber nociceptors release neuropeptides alongside their neurotransmitters that initiate and maintain central sensitization, a crucial feature of neuropathic pain we will explore later (Latremoliere & Woolf, 2009). These peptidergic nociceptors synapse onto pain projection neurons of lamina I and the interneurons of I-IIo (Middleton et al., 2022). Non-peptidergic C- and Aδ-nociceptors preferentially innervate IIo, with sparse terminals to outer laminae (Ferrini et al., 2020; Middleton et al., 2022; Saeed & Ribeiro-da-Silva, 2012). Interneurons modulate the pain projection neuron in response to nociceptive information (Todd, 2022). Altogether, nociceptive afferents transmit noxious stimuli and are essential for central sensitization, a key feature of neuropathic pain.

In contrast to nociceptors, LTMRs are less well characterized due to the historical challenges of identifying subtypes in tissue sections (Abraira & Ginty, 2013). The LTMRs span the Aβ-, Aδ-, and C-fiber classes within which several subtypes exist, distinguished by their distinct end organs in the skin (Abraira & Ginty, 2013). Aδ- and C-LTMRs share their nociceptor counterparts' myelination and conductance properties. The Aβ-fibers have larger diameter fibres with heavy

myelination, giving them a fast conductance velocity (Middleton et al., 2022). Aβ-fibers are largely responsible for non-hairy, or glabrous, skin sensation for discrimination of texture, touch, and shape, reflecting their function with their small receptive fields (Zimmerman et al., 2014). All three classes of LTMRs innervate hair follicles, where they provide discrimination of movement and low-frequency vibrations (Zimmerman et al., 2014). Uniquely, Aβ-fibers directly project via the dorsal column to the second-order neurons of the brainstem dorsal column nuclei, relaying to the thalamus and the somatosensory cortex (Abraira et al., 2017; Johnson & Hsiao, 1992). Initially, this contributed to the "labeled line" model of touch processing, quickly conveying somatotopic inputs for processing in the somatosensory cortex (Johnson & Hsiao, 1992). We now know that only a subset of LTMRs project via the dorsal column, whereas all LTMRs terminate into the SDH (Li et al., 2011). These LTMR inputs to the SDH are functionally separated into innocuous touch processing circuits and those involved in nociception. For nociception, LTMR inputs to laminae II-III are of great importance (Graham & Hughes, 2020). LTMR-innervated inhibitory interneurons provide feedforward inhibition, modulating nociceptive processing and gating LTMR inputs from nociceptive circuits (Abraira et al., 2017). These circuits are further modulated by nociceptor-induced central sensitization (Latremoliere & Woolf, 2009). During central sensitization, LMTR inputs activate excitatory interneurons that poly-synaptically activate pain projection neurons, allowing for protective allodynia in the context of injury (Latremoliere & Woolf, 2009). Altogether, LTMRs modulate nociceptive circuits in normal sensory processing, while central sensitization can drive LTMR inputs to poly-synaptically activate projection neurons.

1.3.2 The SDH and ascending tracts

Nociceptors depend on second-order pain projection neurons to transmit information to the brain (Crawford & Caterina, 2020). Most concentrated in lamina I, these projection neurons comprise

the contralateral anterolateral tract, forming the spinothalamic and spinoreticulothalamic pathways to the brainstem and brain (Crawford & Caterina, 2020; Milligan, 2014). The spinothalamic pathway is important for the sensory discrimination of pain location and intensity (Milligan, 2014). In contrast, the spinoreticulothalamic pathway to forebrain structures is responsible for pain's affective and subjective interpretation (Milligan, 2014). As mentioned, peptidergic nociceptors allow for central sensitization in response to intense noxious stimuli. This is initiated by neuropeptides confined to peptidergic nociceptors, in line with noxious stimulation driving central sensitization (Latremoliere & Woolf, 2009; Todd, 2002). Upon release, these neuropeptides increase the excitability of projection and interneurons with the cognate receptors, a mechanism we will explore later.

While the transmission of pain information ultimately depends on the projection neurons, local interneurons are critical for nociception (Melzack & Wall, 1965; Todd, 2017). Furthermore, nociceptive processing in the SDH depends on both nociceptor and LTMR input on interneurons to modulate pain projection neurons (Todd, 2017). Nearly all interneurons of the SDH are innervated by sensory afferents (Abraira et al., 2017; Todd, 2010). Likewise, most sensory afferents receive feedforward axoaxonic inhibition from interneurons of the SDH (Abraira & Ginty, 2013; Hughes et al., 2012; Stachowski & Dougherty, 2021). A critical framework underlying interneuron circuits is the Gate Control Theory of Pain, proposed by Melzack and Wall (1965) (Figure 1A). Based on clinical observations in healthy and neuropathic subjects, they demonstrated the gating of pain perception by innocuous mechanical input, and the lack thereof in neuropathic patients (Melzack & Wall, 1965). They further proposed that LTMR input to the SDH activates inhibitory interneurons that gate pain projection neurons, whereas nociceptor input inhibits the inhibitory interneuron and opens the gate. Melzack and Wall assigned this critical role

to interneurons of the substantia gelatinosa, or lamina II (Figure 1A). This region is highly populated by interneurons with diverse interconnectivity, receiving both nociceptive and LTMR inputs (Figure 1B) (Melzack & Wall, 1965; Peirs et al., 2015; Sheikh & Dua, 2023; Todd, 2017). This inhibitory gate prevents innocuous and otherwise low-threshold touch inputs from activating pain projection centers, while significant noxious stimuli likely associated with tissue damage are quickly facilitated. Critically, the model includes LTMR inputs to projection neurons forming the underlying biological mechanism of allodynia (Melzack & Wall, 1965). Thus, spinal disinhibition underlies neuropathic pain symptoms in the Gate Control Theory of Pain. We now know that the Gate Control Theory is an oversimplification; all nociceptor inputs and LTMR inputs into the spinal cord are excitatory, and LTMRs do not directly contact pain-projecting neurons in the adult spinal cord (Todd, 2017). Nonetheless, the framework Melzack and Wall proposed remains supported by current evidence of disinhibition during neuropathic pain (Bennett, 2012; Jensen & Finnerup, 2014; Miraucourt et al., 2007). The Gate Control Theory can be further applied to the diverse interneuron populations of the SDH via feedforward pre- and postsynaptic inhibition of sensory afferents and interneurons (Figure 1B) (Todd, 2022). Together, the framework of the Gate Control Theory of Pain provides a succinct explanation of neuropathic pain symptoms: loss of inhibition amplifies pain (hyperalgesia), allows otherwise inhibited nociceptive misinput to excite projection neurons (spontaneous pain), and finally, loss of the gate allowing LTMR polysynaptic pathways to pain projection neurons (allodynia). Here, we will further explore the functional populations of interneurons involved in pain processing.

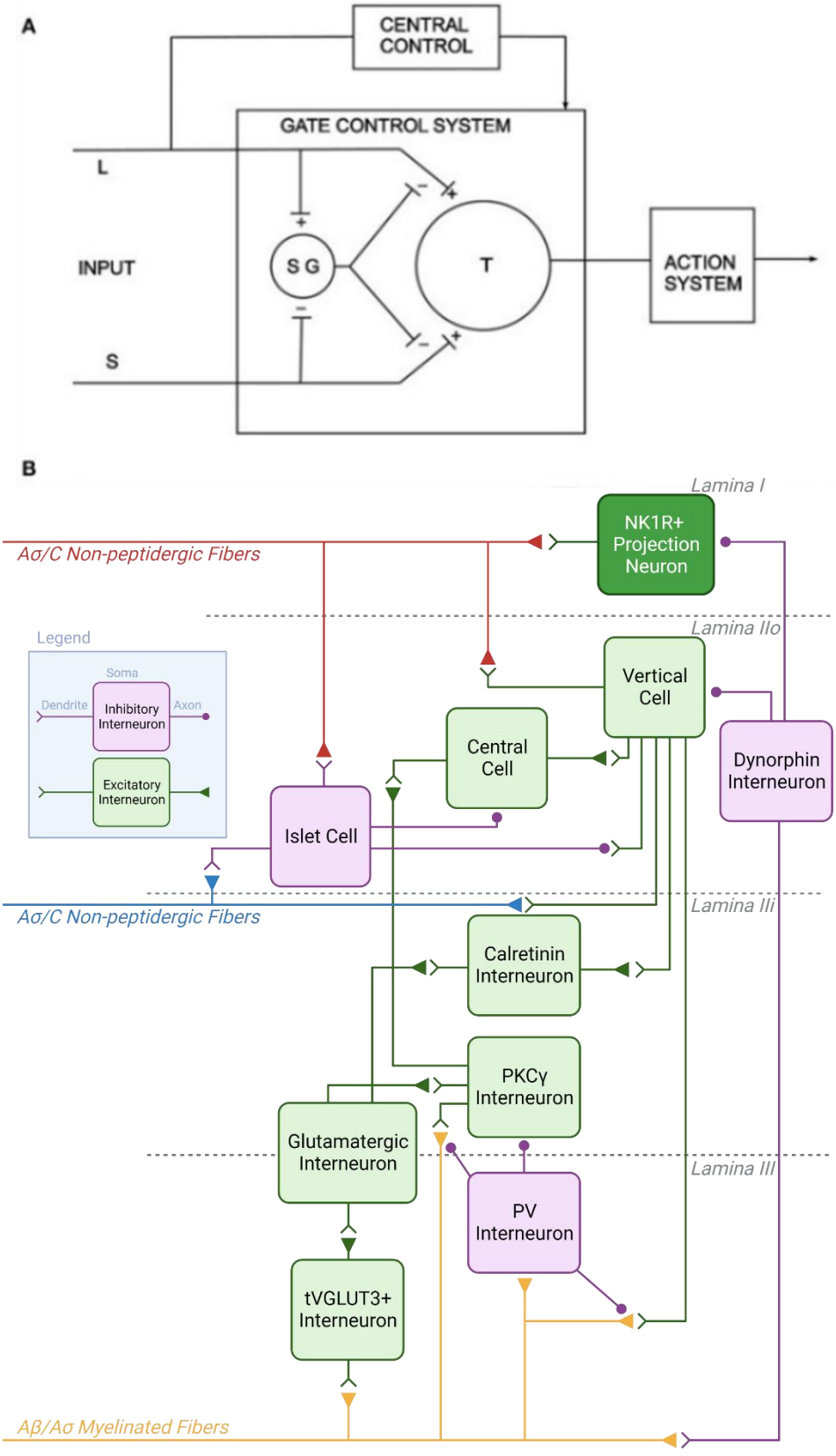

A
CENTRAL CONTROL
GATE CONTROL SYSTEM
L
INPUT
S
S G
T
ACTION SYSTEM
B
Lamina I
Aσ/C Non-peptidergic Fibers
NK1R+ Projection Neuron
Lamina IIo
Legend
Soma
Dendrite
Inhibitory Interneuron
Axon
Excitatory Interneuron
Vertical Cell
Central Cell
Dynorphin Interneuron
Islet Cell
Aσ/C Non-peptidergic Fibers
Lamina IIi
Calretinin Interneuron
PKCγ Interneuron
Glutamatergic Interneuron
Lamina III
PV Interneuron
tVGLUT3+ Interneuron
Aβ/Aσ Myelinated Fibers

Figure 1: A: The Gate Control Theory of Pain. B: Lamina I-III circuit diagram. Created with Biorender.com. Adapted from "An Historical Perspective: The Second Order Neuron in the Pain Pathway", by A. J. Todd (2022), Frontiers in Pain Research (Lausanne, Switzerland). https://doi.org/10.3389/fpain.2022.845211. Copyright 2022, A. J. Todd. Adapted under Creative Commons Attribution License guidelines.

1.2.3 – Descending modulation

Most interneurons in the SDH receive descending modulation from supraspinal centers. Two major descending pathways modulate SDH activity after processing in higher pain centers of the CNS. The serotonergic system from the nucleus raphe magnus terminates into lamina I-II and sparsely in lamina III-IV, affecting interneurons and projection neurons alike (Bardoni, 2019). Activation of serotonin receptors induces more frequent hyperpolarizing currents in inhibitory interneurons and depolarizing currents of excitatory interneurons of the SDH (Abe et al., 2009; Lu & Perl, 2007). Serotonin also modulates synaptic transmission in a similar biphasic matter. Lu and Perl (2007) reported application of serotonin onto SDH slices enhances GABA and glycine release via enhancement of eIPSCs and sIPSCs while preventing Aδ- and C-fiber responses in excitatory interneurons. Likewise, the noradrenergic descending pathway from the midbrain and brainstem structures exerts a primarily inhibitory effect over the SDH (Bahari & Meftahi, 2019). These activate the α2-adrenoceptor coupled to inhibitory G-protein complexes. Activation of α2-adrenoceptor in afferents decreases $[Ca^{2+}]_i$ at the terminal and suppresses glutamate and peptidergic release (Bahari & Meftahi, 2019). Activation of post-synaptic α2-adrenoceptor activates G-proteins and increases K^+ permeability, reducing the excitability of neurons (Bahari & Meftahi, 2019). These two descending pathways provide the basis of tricyclic antidepressants and

selective-noradrenaline reuptake inhibitors, as well as gabapentinoids indirectly, to alleviate neuropathic pain.

1.3.4 – Neurons of the SDH

In the dorsal spinal cord, interneurons are subdivided into excitatory interneurons releasing glutamate and inhibitory interneurons releasing GABA, glycine, or both (Todd, 2017). In addition, ionotropic $GABA_AR$ and GlyR activation is tonic in the dorsal spinal cord, giving the dorsal horn its baseline inhibitory tone (Perez-Sanchez et al., 2017). These two inhibitory neurotransmitters mediate transient and tonic inhibition in the spinal cord on projection neurons and other interneurons (Takazawa & MacDermott, 2010). Together, the activation of transient and tonic inhibition determines the inhibitory balance of the SDH.

The spinal interneurons can be further subdivided into morphological, electrophysiological, neurochemical, and genetic characteristics (Abraira & Ginty, 2013; Abraira et al., 2017; Boyle et al., 2017; Todd, 2017; Todd, 2022). These classifications follow the history and development of their respective techniques and their ability to characterize single-cell properties for classification. Morphological studies of spinal cord interneurons began early in the 20^{th} century, with Ramon y Cajal (1909) describing the cell morphologies in the substantia gelatinosa. These morphological studies continued as technologies progressed, extending from gross morphology to the fine structure of synaptic connections (Todd, 2022). Alongside these advancements came electrophysiology, its development coalescing in the 1980s for single-cell recordings of whole-cell current and voltage (Hamill et al., 1981; Sakmann & Neher, 1984). Likewise, advancements in genetic and molecular profiling allowed for high-throughput single-cell characterizations, further expanding neuronal markers of putative populations (Boyle et al., 2017; Häring et al., 2018;

Polgár et al., 2013). Despite these advances, challenges to setting *bona fide* cell types remain. The proposed morphological and neurochemical cell types likely capture more than one functional population with differing synaptic inputs and outputs (Todd, 2017). Furthermore, differences in identified cell types exist between the studies of mice, rats, cats, and primate sensory processing (Todd, 2022). Here, we will focus on the putative interneuron populations of the mouse nociceptive SDH.

1.3.5 Neurons of lamina I-IIo

The superficial lamina of I-IIo contains projection neurons and interneurons innervated by both nociceptors and limited LTMRs (Peirs et al., 2020). These projection neurons are marked by the expression of NK1R, the SP receptor essential for central sensitization (Latremoliere & Woolf, 2009). Projection neurons tend to have dendrites restricted to lamina I, thus relying strongly on the integration of afferent information by lamina I-III interneurons (Braz et al., 2014). The interneurons of lamina I express calretinin, SP, and CCK, though these are often not quantified and likely make up a small fraction of neurons in the area (Peirs et al., 2020). The role of interneurons in lamina I is still unclear (Peirs et al., 2020; Todd, 2022). In contrast, lamina IIo interneurons play important roles in the modulation of nociceptive input to projection neurons (Comitato & Bardoni, 2021). While nociceptors receive axoaxonic presynaptic inhibition, which is critical in reducing nociceptive input (Malcangio, 2018), functional characterizations of these inhibitory interneurons are largely unknown (Comitato & Bardoni, 2021). These inhibitory interneurons have been neurochemically characterized by a subset of dynorphin/galanin-expressing and nNOS-expressing inhibitory interneurons (Peirs et al., 2020). A possible functional population is a mouse line expressing eGFP under prion-promoter control (PrP-eGFP) that captures subsets of galanin/dynorphin- and nNos-expressing inhibitory interneuron populations

(Ganley et al., 2015; Iwagaki et al., 2013; Todd, 2017). These inhibitory interneurons receive unmyelinated and myelinated primary afferent inputs (Hantman et al., 2004), including peptidergic nociceptor input and A-LTMRs via deep dendritic arbors (Ganley et al., 2015). The nNOS-expressing subset of PrP-eGFP seems to preferentially innervate a specific projection neuron known as giant cells, making up 70% of their inhibitory synaptic input (Polgár et al., 2008). Tashima et al. (2021) demonstrated a functional population receiving Aβ-afferent input and directly inhibiting lamina I projection neurons, defining a population in lamina IIo using NPYP+-AAV, selective for neurons with NPY promoter activity in adults. This seems to be a functional population of the larger NPY-Cre population defined throughout lamina I-III (Todd, 2017); whereas only 20% of NPY-Cre neurons received Aβ-input, nearly all NPYP+-AAV interneurons received Aβ-input (Tashima et al., 2021). Together, this demonstrates the modulation of projection neurons and their nociceptive input in lamina I-IIo by interneurons receiving both LTMR and nociceptor input.

1.3.5 Interneurons of lamina II-III

As the proposed site of the Gate Control Theory of Pain, the characterization of lamina II interneurons has been extensive (Peirs et al., 2020). There are negligible projection neurons here as the site of extensive nociceptive processing by polysynaptic circuits to lamina I. In Melzack and Wall's theory, the innocuous inputs here largely inhibit nociceptive transmission, but a loss of inhibition allows for the engagement of nociceptive circuits via unveiled polysynaptic circuits (Melzack & Wall, 1965). The currently accepted morphological classification of lamina II is that of Grudt and Perl (2002), including vertical, radial, central, and islet cells seen in sagittal slices. These morphologies are thought to loosely reflect their role in sensory processing (Grudt & Perl, 2002; Todd, 2017). The concurrent characterization of electrophysiological, neurochemical, and

genetic markers seems to reflect this schema for excitatory interneurons. However, more and more consideration is being placed on the functional populations within these morphologies. Inhibitory interneurons are less well-characterized by morphologies, falling under islet or outside of Grudt and Perl's (2002) characterization (Todd, 2017). Characterizations of inhibitory interneurons have been relatively successful with the more modern techniques; however, excitatory interneuron populations have been less well-defined (Peirs et al., 2020; Todd, 2017). Thus, we will define excitatory interneurons by morphology and connectivity, while functional excitatory circuits will be explored in the context of putative inhibitory populations. Here, we will discuss these characterizations in the context of morphologies and the functional circuits within lamina II-III.

The vertical cells of lamina II have extensive dendrites into lamina III, receive extensive primary afferent innervation of C-, Aδ- and Aβ-nociceptors and LTMRs, and send axons directly to lamina I projection neurons (Boyle et al., 2019; Lu & Perl, 2005a; Peirs et al., 2020; Todd, 2017). Vertical cells also have extensive axon branches restricted to lamina II, making local interneurons their main synaptic target (Lu & Perl, 2005a; Todd, 2017). Thus, vertical cells are thought to play a crucial role in modulating the activity of projection neurons. Neurochemical definitions poorly define vertical cells; these include an excitatory SOM+ expressing population (Duan et al., 2014), a subset of excitatory interneurons expressing CR (Smith et al., 2015), and some excitatory interneurons with GRP expression implicated in both itch and pain (Polgár et al., 2023). Alongside their extensive afferent input, vertical cells receive extensive input from local interneuron populations, serving their roles as integrators of nociceptive information (Todd, 2017). Vertical cells receive extensive innervation by PrP-defined inhibitory interneurons, providing feedforward inhibition in response to diverse afferent classes along with iPVINs innervation for LTMR-specific

inputs (Boyle et al., 2019; Ganley et al., 2015). Thus, vertical cells are thought to be key integrators of nociceptive information.

Radial and central cell excitatory interneurons are essential for local LTMR and nociceptive processing, with axons restricted to lamina II (Grudt & Perl, 2002; Todd, 2017). Radial cells have relatively short dendrites radiating in all directions within lamina II, whereas central cells have long dendritic branches extending in the sagittal plane (Grudt & Perl, 2002). The two morphologies share neurochemically distinct populations of PKCγ- and CR-expressing excitatory interneurons (Peirs et al., 2020). Additionally, transiently firing central cells in lamina IIo outputting on vertical cells have been substantiated to receive inputs from PKCγ-excitatory interneurons (Lu et al., 2013). Functionally, the excitatory interneurons of laminae II-III likely play distinct roles in nociception. PKCγ-excitatory interneurons of lamina IIo receive mainly nociceptive input and express NK1R, whereas deeper lamina IIi-III PKCγ-excitatory interneurons are selectively innervated by A-LTMRs (Boyle et al., 2019; Miraucourt et al., 2007; Neumann et al., 2008; Todd et al., 2000). Similarly, CR-excitatory interneurons receive nociceptor and LTMR input, innervate vertical cells (Smith et al., 2016), local interneurons (Todd, 2017), and projection neurons (Petitjean et al., 2019). CR-excitatory interneurons likely play a role in basal nociception and receive inhibitory modulation by dynorphin/galanin- and nNOS-expressing inhibitory interneurons, as well as a subset of lamina IIo NPY cells receiving peptidergic nociceptive input (Iwagaki et al., 2016; Peirs et al., 2020). NPY-inhibitory interneurons also seem to play a role here in suppressing acute and neuropathic pain (Boyle et al., 2023). In contrast to the circuitry of basal nociception, the proposed allodynic circuits are ordinarily silent but unveiled under spinal disinhibition and neuropathic pain models (Miraucourt et al., 2007). These circuits are deeper in laminae IIi-III and primarily receive A-LTMR input (Abraira et al., 2017). Here, a significant

diversity of excitatory interneurons exists expressing PV, PKCγ, CR, and GRP, receiving axoaxonic and axosomatic input by PV, NPY, and CR-expressing inhibitory interneurons (Peirs et al., 2020; Todd, 2017). Currently, the role of CR-inhibitory interneurons is unclear, but they likely play a role in local processing as their axons rarely extend past lamina II (Peirs et al., 2020). NPY-expressing inhibitory interneurons make up 25% of inhibitory interneurons in lamina III and are thought to play a critical role in feedforward inhibition, receiving C-LTMR and non-peptidergic nociceptor inputs (Iwagaki et al., 2016). While these circuits are thought to play important roles in gating LTMR information, functionally, they are under-characterized, partly due to the diffuse expression of CR and NPY in both excitatory and inhibitory interneuron populations (Peirs et al., 2020). However, iPVINs seem to play a direct functional role in the inhibition of an allodynic circuit; these interneurons receive A-LMTR inputs exclusively, output nearly half of all axoaxonic inputs of A-LTMRs in lamina II (Hughes et al., 2012), and inhibit the usually silent lamina IIi-III PKCγ-excitatory interneuron population during touch stimulation (Miraucourt et al., 2007; Petitjean et al., 2015). Furthermore, they provide exclusive Aβ-LMTR mediated inhibition of vertical cells (Boyle et al., 2019). This has placed the iPVINs populating lamina IIi-III as essential in setting the mechanical threshold. The PKCγ-excitatory interneuron population further contacts vertical and central transient cells (Boyle et al., 2019; Lu et al., 2013), leading to an NMDAR-dependent excitatory pathway to projection neurons (Miraucourt et al., 2007). The disinhibition of iPVINs seems to underlie mechanical allodynia; chemogenetic activation of PVINs increases the mechanical threshold in nerve-injured mice, and the ablation of PVINs induced nerve-injury symptoms (Petitjean et al., 2015). Thus, the lamina III population iPVINs seem to be a well-defined population of inhibitory interneurons necessary for the gating of LTMR-inputs from nociceptive specific circuits of the superficial lamina. Current research has pointed towards several

mechanisms in which iPVINs reduce their inhibitory output, as we will explore later (Qiu et al., 2022; Zhong et al., 2018). To understand how peripheral injury mediates the changes to SDH circuitry underlying allodynia, we will now explore the spinal mechanisms of neuropathic pain.

1.4 Mechanisms of peripheral neuropathic pain

1.4.1 Overview of SDH mechanisms

For peripheral nerve injury to result in chronic neuropathic pain, the initial insult must result in changes to the sensory processing of the SDH. Central sensitization describes how neuronal properties and circuits shift towards pronociceptive processing (Latremoliere & Woolf, 2009). This mechanism underlies the temporal, spatial, and threshold changes in pain sensation in response to activity, inflammation, and nerve injury (Latremoliere & Woolf, 2009). Whereas peripheral sensitization is confined to their sensory modality, central sensitization uniquely shunts non-nociceptive input into nociceptive pathways (Woolf & Salter, 2000). In response to inflammatory and peptidergic signalling, neurons of the superficial SDH respond with increases in membrane excitability and synaptic strength with decreases in inhibitory tone (Latremoliere & Woolf, 2009). In normal wound healing, this amplification in nociceptive processing allows for injury protection by hypervigilance of the wound area. The initial step of central sensitization begins with the activity of peptidergic nociceptors, which is crucial for initiating and maintaining central sensitization (Latremoliere & Woolf, 2009). Neuropeptide SP binds to NK1R-expressing second-order neurons of the superficial SDH, inducing a long-lasting depolarization that potentiates NMDAR-LTP alongside glutamatergic-AMPAR transmission (Latremoliere & Woolf, 2009). Accordingly, 90% of lamina I projection neurons express NK1R; upon binding with substance P, NK1R couples to phospholipase C, generating intracellular messengers with the result of a long-lasting depolarization current and the potentiation of AMPAR and NMDAR transmission

(Ma & Woolf, 1995; Mantyh et al., 1997; Zieglgänsberger, 2019). The neuropeptide CGRP potentiates the effects of SP, participating in central centralization via postsynaptic CGRP1R and further enhancing the release of BDNF from afferents (Latremoliere & Woolf, 2009; Woolf & Wiesenfeld-Hallin, 1986). BDNF further potentiates central sensitization by upregulating NMDARs (Ding et al., 2015). The release of bradykinin, more associated with inflammation, acts through the G_q-protein coupled receptors of dorsal horn neurons, boosting synaptic strength via activation of PKC, PKA, and ERK (Latremoliere & Woolf, 2009; Ma & Woolf, 1995). These neuromodulators function by volume transmission; neuropeptide receptor affinity is close to the nano-micromolar range compared to the micro-millimolar range of neurotransmitters allowing for the activating cognate receptors of surrounding projection neurons and interneurons in neighbouring laminae (Fuxe et al., 2013). This further drives the release of NO by interneurons, activating a PKG cascade that further potentiates NMDAR-LTP (Hopper et al., 2004). The only difference between protective central sensitization and neuropathic central sensitization is its chronicity; in the neuropathic state central sensitization is maintained long after the originating insult as a result of changes to afferent fibers and immune state. After nerve injury, C-fibers become hyperexcitable and generate spontaneous activity after one day, driving central sensitization (Djouhri et al., 2006; Wu et al., 2001). Once initiated, the spontaneous activity required to maintain central sensitization is significantly less, allowing for its chronicity (Koltzenburg et al., 1992). Furthermore, nerve injury seems to reduce the strength of A-LTMR inputs to the spinal cord, reducing the excitatory drive of inhibitory interneurons (Lu et al., 2013). These excitatory interneurons respond to the disinhibited state by NMDAR-LTP and peptidergic receptor activation to varying degrees, further chronifying central sensitization. Altogether,

peptidergic inputs initiate and drive changes to inhibitory and excitatory balance via NMDAR-LTP induced hyperexcitability of SDH projection neurons and excitatory interneurons.

The immune response in the spinal cord is essential for expressing neuropathic pain and is thought to drive the allodynic symptoms. In response to nerve injury, microglia in the SDH activate rapidly (Ward & West, 2020). Microglia play a critical role in the pathogenesis of peripheral neuropathic pain. However, the precise transmission and activation of microglia by peripheral nerve injury remain elusive (Ward & West, 2020). Colony-stimulating factor 1 (CSF1) is a promising candidate; the cytokine is expressed *de-novo* in nerve-injured sensory neurons and transported to the spinal cord (Guan et al., 2016). Their cognate receptor CSF1R is expressed in spinal microglia, with CSF1 applied intrathecally inducing neuropathic pain symptoms and microglial activation (Guan et al., 2016). Conditional deletion of CSF1 from DRG neurons reduced the increase of microglial count and the development of allodynia in nerve-injured mice (Guan et al., 2016). Furthermore, the CSF1R-DAP12 pathways are upstream of microglial genes crucial for mediating neuropathic pain development, including CatS, CX3CR1, P2X4, Irf8, and Irf5 (Beggs et al., 2012; Guan et al., 2016; Masuda et al., 2014; Masuda et al., 2012). Activation of microglial P2X4R by the release of afferent ATP further drives BDNF expression and release in microglia, contributing to central sensitization (Ferrini & De Koninck, 2013). As we will later explore, BDNF and activated immune cells play a further role in mediating SDH circuitry changes.

1.4.2 Changes to inhibitory and excitatory balance in neuropathic pain

The release of BDNF by microglia and peptidergic afferents drives shifts in the ability of inhibitory interneurons to generate IPSCs in targeted neurons by reducing KCC2 expression. The inhibition produced by activation of the $GABA_AR$ or GlyR relies heavily on the Cl^- balance between cytoplasmic and interstitial Cl^- concentrations (Price et al., 2009). Since the inhibitory current

depends on the influx of Cl^- ions, loss of electrochemical gradient of Cl^- at the membrane reduces Cl^- influx, and severe impairment can lead to Cl^- outflux and excitation in the presence of GABA or glycine (Ferrini et al., 2020). The Cl^- gradient depends on the expression of KCC2, utilizing the K^+ gradient to exchange for Cl^- (Ferrini & De Koninck, 2013). Upon KCC2 antagonist application to neurons, GABA puffs began depolarizing the membrane (Ferrini et al., 2020). This K^+/Cl^- cotransporter changes its expression during development. Indeed, until early postnatal days, KCC2 is expressed at low levels and GABA/Glycine are excitatory, a process required for the proper wiring of the circuits. By postnatal day 15, KCC2 expression begins to rise and establishes the mature Cl^- gradient (Li et al., 2002; Rivera et al., 1999).

Evidence of synaptic remodelling in neuropathic pain is growing with the expression of complement proteins C1q and C3 in interneurons, mediating the engulfment of synapses by microglia (Schafer et al., 2012; Ward & West, 2020). A characterization of microglial-mediated removal of spinal synapses by Yousefpour et al. (2023) found extensive microglial synaptic pruning of inhibitory interneuron terminals. However, the mechanism behind the reported loss of excitatory and primary afferent terminals remains elusive (Yousefpour et al., 2023). Together, both immune and afferent inputs in the spinal cord drive the development of neuropathic pain. As we will explore later, these generate the state of disinhibition of the neuropathic SDH.

As previously described, iPVINs play a critical role in mediating allodynia. Here, we will review changes to interneuron inhibitory output and their relevance in iPVINs. Firstly, we will explore the role and function of iPVIN in the dorsal spinal cord. Secondly, we will examine the possible underlying mechanisms of inhibitory interneuron disinhibition and their relevance for iPVINs.

1.5 iPVINs in neuropathic pain

During nerve injury, changes to the properties of iPVINs contribute to neuropathic pain. In 2015, Petitjean et al. demonstrated the necessity of iPVIN in maintaining the mechanical pain threshold by inducing allodynia in mice where iPVINs were ablated. Conversely, the chemogenetic stimulation of iPVIN in nerve-injured mice rescued allodynia (Petitjean et al., 2015). By segmental spinal disinhibition, Lois et al. (2007) demonstrated that the disinhibited Aβ-PKCγ circuit played a critical role in mechanical allodynia. In turn, the iPVIN plays an essential role in processing touch inputs by preventing the activation of PKCγ interneurons by touch input. Vertical cells also receive inhibition from iPVINs, likely contributing to allodynia as a population that integrates nociceptive and LTMR information (Boyle et al., 2019).

1.5.1 The parvalbumin interneuron family

PVINs are found throughout the central nervous system (Nahar et al., 2021). These interneurons are crucial for maintaining the excitatory and inhibitory balance of cortical, hippocampus, striatal, and spinal circuits (Nahar et al., 2021). While delineations of brain iPVIN function have been substantial, less is known about SDH iPVIN. Nonetheless, these interneurons share common characteristics: fast spiking and feedforward inhibition (Hughes et al., 2012; Nahar et al., 2021). In the dorsal spinal cord, iPVINs account for ~25% of inhibitory interneurons in laminae IIi-III (Abraira et al., 2017). These iPVINs have extensive connectivity to other interneurons of laminae IIi-III and account for almost half of the axoaxonic inputs in the region (Hughes et al., 2012; Todd, 2017). Here, we will explore how iPVINs mediate their role as a feedforward inhibitory gate for innocuous touch inputs.

1.5.2 Inputs and outputs of iPVINs

Classes of low threshold mechanoreceptors inputs of iPVINs include Aδ and Aβ hair-follicle afferents and Aβ-fiber afferents of the glabrous skin (Boyle et al., 2019; Hughes et al., 2012). Inputs via these low threshold mechanoreceptors to excitatory nociceptive pathways are inhibited in a feedforward matter via axoaxonic presynaptic inhibition and axosomatic postsynaptic by iPVINs in lamina III (Gradwell et al., 2022; Hughes et al., 2012; Petitjean et al., 2015; Sullivan & Sdrulla, 2022). Most terminals of iPVINs are axoaxonic, inhibiting myelinated LMTRs that terminate on excitatory interneurons (Boyle et al., 2019). iPVIN terminals further provide axodendritic and axosomatic inhibition of both PV and non-PV interneurons of lamina IIi-III (Gradwell et al., 2022; Hughes et al., 2012; Petitjean et al., 2015; Sullivan & Sdrulla, 2022). A recent study from Gradwell et al. (2022) supports polysynaptic ePVIN circuits to projection neurons, the monosynaptic inhibition of ePVINs by iPVIN, and monosynaptic iPVIN terminals on projection neurons. These diverse connections may represent functional populations within iPVINs; however, their role in neuropathic pain has yet to be substantiated and require further characterization. Functional circuits of iPVINs implicated in neuropathic pain include the excitatory PKCγ interneurons and vertical cells (Boyle et al., 2019; Boyle et al., 2017; Petitjean et al., 2015). Vertical cells reside in lamina IIo with extensive medial dendrites across laminae II-III through the dorsal horn (Maxwell et al., 2007). These cells play a direct role in basal nociceptive processing, receiving monosynaptic excitatory input from all classes of afferent fiber, including Aβ-LTMRs (Boyle et al., 2019; Grudt & Perl, 2002; Lu & Perl, 2005b; Yasaka et al., 2014; Zheng et al., 2010). Boyle et al. (2019) demonstrated that lamina IIi-III iPVINs mediated LTMR-innervated vertical cells' pre- and postsynaptic inhibition. The inhibition of PKCγ-interneurons by iPVINs forms a *bona fide* gate, preventing the transmission of any LTMR input to innervated

PKCγ-interneurons in a feed-forward manner. IPVINs' ablation activates PKCγ-interneurons and causes neuropathic symptoms similar to spinal glycinergic blockade, whereas the chemogenetic activation of iPVINs in the nerve-injured state reduces allodynia (Petitjean et al., 2015). Functionally, iPVINs inhibit extraneous LTMR drive to allow for physiological pain processing, which is crucial in setting the mechanical pain threshold.

1.5.3 Intrinsic excitability of iPVINs

Neurons depend on the electrical properties of the cell membrane to produce and propagate action potentials in response to excitatory inputs. Firing properties are set based on the differential expression of ion channels and exchangers. In naïve mice, lamina IIi-III PVINs display tonic firing phenotypes to a depolarizing current (Boyle et al., 2019; Hughes et al., 2012). This property is thought to be important in maintaining the complete inhibitory blockade of touch-activated nociceptive circuits, a shared characteristic by NPY (Iwagaki et al., 2016) and CR-expressing inhibitory interneurons (Smith et al., 2015) of the same lamina. However, a small subset (~30%) of PVIN are burst firing and are unlikely to be able to maintain inhibitory output alongside Aβ-input (Boyle et al., 2019; Hughes et al., 2012). These characterizations relied on co-expression with PV-Cre, and more recent characterizations found that ~30% of PVIN were excitatory (Gradwell et al., 2022). The previous characterizations of bursting PVINs are likely the significant ePVIN population in the dorsal horn, whereas iPVINs require tonic firing to maintain their inhibitory output. As membrane properties determine spike properties, we will review relevant ion channels for enabling high-frequency firing in iPVINs.

During action potentials, voltage-activated sodium channels (Na_v), voltage-activated potassium channels (K_v), and, to a lesser extent, voltage-activated calcium channels (Ca_V) determine the initiation and shape of action potentials. There are currently few direct characterizations of

channels of iPVINs or inhibitory interneurons in the context of neuropathic pain, as changes to ion channel expression are not significant compared to nerve-injured sensory afferents (Alles & Smith, 2021) or SDH interneurons in models of spinal central neuropathic pain (Hains et al., 2003; Zavvarian et al., 2020). Presumably, SDH iPVINs share ion channel expression like fast-spiking iPVINs of the brain and brainstem structures. In a single nuclei RNA sequencing study of SDH interneurons, Häring et al. (2018) found a cluster of PV-expressing inhibitory interneurons (labelled GABA14), likely corresponding to the iPVIN population. Here, the GABA14 cluster strongly expresses $Na_v1.1$, consistent with expression in fast-spiking iPVINs in the hippocampus (Ogiwara et al., 2007). The K_v3 family is highly expressed in mature fast-spiking neurons (Ichinohe et al., 2004). These are thought to promote fast spiking by allowing for fast repolarization and reactivation of Na_v without affecting the threshold of action potentials (Rudy et al., 1999). In the dorsal horn, $K_v3.1b$ has been found to be co-expressed with GAD67 (Nowak et al., 2011), of which a majority of dorsal horn iPVINs express (Dougherty et al., 2009). $K_v3.1$ is most expressed in the GABA14 cluster out of all characterized by Häring et al. (2018), alongside lesser expression of $K_v3.4$, in line with their tonic firing.

Dorsal spinal cord iPVIN strongly expresses hyperpolarization-activated cyclic nucleotide-gated potassium channel (HCN) 1 and 4, with the corresponding I_h current present in electrophysiological recordings (Boyle et al., 2019; Petitjean et al., 2015). The HCN family regulates resting membrane potential and membrane resistance via Na^+ and K^+ permeability during membrane potentials below -60mV (Shah, 2018). Via its activation during hyperpolarization, these channels depolarize the membrane potential, driving the membrane potential to the threshold at the soma and action potential conductance in axons (Hogan & Poroli, 2008). Studies in cortical fast-spiking iPVIN found that HCN1 significantly contributes to depolarizing resting membrane

potential, with pharmacological blockade hyperpolarizing iPVINs and reducing firing frequency in response to depolarizing current (Yang et al., 2018).

While Ca_V contributes little to action potential properties directly, the activation of Ca_V strongly influences the intrinsic excitability of neurons by calcium-dependent K^+ channels. Of note are the BK and SK channels for their roles in modulating the rapid and sustained production of action potentials. Like K_v channels, BK channels are responsible for the fAHP, which is a voltage-sensitive channel with a large K^+ conductance (100-300pS) (Nardi & Olesen, 2008). BK channels require Ca^{2+} to function at physiological voltages, and their sensitivity to $[Ca^{2+}]_i$ allows BK channels to modulate firing frequency to activity (Contet et al., 2016). BK channels are important for modulating high-frequency firing, rapidly inactivating after repolarization; these channels affect firing frequency via the repolarization of Na_v channels (Gu et al., 2007). Chen et al. (2009) found that intrathecal blockade of BK channels with iberiotoxin significantly reduced nociceptive withdrawal in control and nerve-injured rats, while intrathecal injection of BK activator NS1619 dose-dependently reversed allodynia and hyperalgesia without affecting nociception in control rats. While no change of BK-expression was found in the SDH after nerve injury, there was a loss of BK-IR of the lateral SDH, and a gain in BK-IR near the dorsal root entry zone, likely explaining the behavioural results of ibTX and NS1619 application (Chen et al., 2009). PV-IR and PV-eGFP demonstrate more dense dorsal root entry zone clustering, becoming sparser in the lateral SDH (Hughes et al., 2012). With the evident loss of inhibition and amplification of excitatory tone, BK channels may play differential roles in excitatory interneurons and inhibitory interneurons.

Notably, dorsal spinal cord iPVIN expresses calcium-dependent small-conductance potassium channel (SK) 2 (Qiu et al., 2022). After a volley of firing, SK channels activate and induce a medium afterhyperpolarization (mAHP) of approximately 100ms (Faber & Sah, 2003). This long-

lasting hyperpolarizing current directly prevents continued firing and is a common feature of bursting neurons (Faber & Sah, 2003). The voltage independence of SK channels allows for the slowing of firing frequency in response to Ca^{2+} influx without destabilizing action potential production (Faber & Sah, 2003). Presumably, this accounts for the widespread expression of SK channels throughout the nervous system to broadly alter firing frequency. As SK expression has been demonstrated with single-cell RNA-sequencing and immunohistochemistry (Häring et al., 2018; Qiu et al., 2022), iPVIN needs to prevent SK2 activation for maintaining tonic firing. PVp is a cytosolic Ca^{2+}-binding protein that acts as a slow calcium buffer, likely modulating SK2 activation in iPVIN (Permyakov & Uversky, 2022).

1.6 Changes to iPVINs in neuropathic pain

1.6.1 Inhibitory interneuron apoptosis

Apoptosis of spinal cord inhibitory interneurons after nerve injury remains controversial. Around the 2000s, several studies pointed towards NMDAR excitotoxic death of lamina I-III interneurons after nerve injury (Moore et al., 2002; Zimmermann, 2001). Similarly, TUNEL labelling of cell apoptosis was significantly increased after different types of nerve injury (Azkue et al., 1998; de Novellis et al., 2004; Kawamura et al., 1997; Moore et al., 2002; Whiteside & Munglani, 2001). Further characterization has found increased apoptotic activity as measured by increases to Bax, apoptotic protease-activating factor-1, caspase-3, caspase-9, and cytochrome c (Chen et al., 2020; Fu et al., 2017; Hu et al., 2015; Siniscalco et al., 2007). Interventions based on antiapoptotic methods, including antioxidants, reactive oxygen species scavengers, mitoquinone (coenzyme Q10 antioxidant), and hyperbaric oxygen therapy, report both suppressed pain behaviour and reduced apoptotic activity in the dorsal spinal horn after nerve injury (Chen et al., 2020; Fu et al., 2017; Hu et al., 2015; Siniscalco et al., 2007). However, apoptotic activity does not necessarily

correlate with apoptosis, and the methods used to modulate apoptotic activity likely have widespread effects throughout the animal model. Opponents of inhibitory interneuron apoptosis after nerve injury also have significant evidence, finding apoptotic activity in the DRG but not the spinal cord (Campana & Myers, 2003; Schaeffer et al., 2010). Significant efforts by Polgar et al. (2003, 2004, 2005) attempted to use an unbiased stereological method with NeuN labelling for neuronal nuclei and found no loss of interneurons after nerve injury. They further highlight the controversy over TUNEL-staining of neurons, which was initially believed to be neuron-specific; however, in spinal slices, it was present in Iba1-positive microglia. Despite the controversy of SDH neuron apoptosis, some lines of research still support the role of apoptosis in neuropathic pain animal models. Inquimbert et al. (2018) found significant TUNEL expression in NeuN-positive profiles, and a 25% decrease in dorsal horn neurons after nerve injury. Scholz et al. (2005) demonstrated that a caspase inhibitor prevented nerve injury-induced neuronal loss. However, no direct evidence demonstrates that agents inhibiting apoptotic activities attenuate pain behaviour. If there is significant cell death in the dorsal horn, it may be confined to discrete neuronal populations; Inquimbert et al. (2018) further demonstrate a laminar distribution of cell death in NMDAR-dependent excitotoxicity of lamina I-II, in line with the enhanced synaptic transmission after central sensitization. For iPVINs, apoptosis after nerve injury is unsubstantiated. Characterization of fluorescent interneurons in PV-Cre count before and after injury showed no significant difference in PVIN count (Boyle et al., 2019; Petitjean et al., 2015). Alongside the significant controversy surrounding neuronal apoptosis after nerve injury, nerve injury is unlikely to induce apoptosis in spinal iPVIN significantly.

1.6.2 Reduced afferent input

Lu et al. (2013) demonstrated that Aβ-fiber eEPSPs in glycinergic neurons that synapse on PKCγ interneurons are significantly reduced after nerve injury. Likewise, using GAD67-GFP reporter mice, Leitner et al. (2013) demonstrated that the frequency of mEPSCs on inhibitory interneurons was significantly lower in CCI compared to sham surgery animals. Leitner et al. (2013) further argue that this was due to reduced release probability rather than loss of synaptic sites, based upon the unchanged excitatory dendritic spine density and PSD-95 excitatory terminal marker upon GAD67-GFP neurons. Cao et al. (2022) further report that selective RARα-deletion in spinal PVIN prevented synaptic disinhibition and the development of neuropathic pain. Furthermore, in visual iPVINs, retinal lesions and lack of excitatory drive induce RARα-dependent homeostatic synaptic plasticity, reducing inhibitory output to reduce excitatory input (Zhong et al., 2018). This RARα plasticity may represent a maladaptive overcorrection in synaptic strength in the neuropathic condition with reduced LTMR drive. Yousefour et al. (2023) study on synaptic remodelling in neuropathic pain found a significant reduction in VGLUT1+ myelinated LMTR afferents independent of microglial activity. Both synaptic structure and synaptic strength of LTMR afferents are likely affected after nerve injury. However, Boyle et al. (2019) characterization of Aβ-fiber terminals on iPVINs found no significant reduction after nerve injury. This suggests that reductions in synaptic strength contributing to reduced afferent drive are more likely than reductions in afferent synapses.

1.6.3 Reduced inhibitory synaptic efficiency

Shifts in KCC2 and Cl^- balance seen in neuropathic pain would significantly affect iPVIN-eIPSCs. KCC2 downregulation is crucial in the trajectory and maintenance of neuropathic pain (Kitayama, 2018; Mapplebeck et al., 2019). Using a novel KCC2 activity enhancer, Gagnon et al. (2013)

restored KCC2 membrane expression after SNI, restored impaired Cl^- transport, and rescued neuropathic pain behaviours. KCC2 downregulation in postsynaptic targets of PVINs would significantly reduce eIPSC via the disrupted Cl^- gradient.

Changes to the inhibitory output of iPVIN can underlie disinhibition. One possibility is a reduction in synaptic strength. Cao et al. (2022) demonstrate a reduced inhibitory effect of iPVIN output on PKCγ neurons. Synaptic pruning of iPVIN inhibitory synapses may also contribute to disinhibition. Studies find that microglial C1q, proteases, and TNFα are significantly upregulated in neuropathic pain models and are known to mediate synaptic pruning (Griffin et al., 2007; Kawasaki et al., 2008; Shechter et al., 2009; Yang et al., 2017). Only recently did Yousefpour et al. (2023) substantiate microglial pruning of inhibitory synaptic terminals' role in neuropathic pain, demonstrating that microglial depletion prevented pruning and attenuated neuropathic pain. Supporting this, significant retraction of the iPVIN terminal from PKCγ somas was reported by Petitjean et al. (2015) after CCI. However, the characterization of SNI by Boyle et al. (2019) found no significant retraction from PKCγ dendrites or somas. Differences in the termination of injured sciatic nerve branches between the CCI and SNI models may explain the discrepancy. Together, reduced synapses and synaptic function in inhibitory interneurons are likely responsible for reducing inhibitory tone, though further characterizations are required to resolve their contribution to iPVIN-dependent disinhibition.

1.6.4 Reduced intrinsic excitability

Of the changes to iPVIN after nerve injury, intrinsic excitability remains the most striking. After nerve injury, iPVIN loss their intrinsic excitability and fail to fire tonically in response to depolarizing currents (Boyle et al., 2019; Cao et al., 2022; Petitjean et al., 2015). Furthermore, iPVINs after nerve injury fail to fire alongside stimulation of Aβ-fiber afferents at a physiological

frequency (20Hz), demonstrating a physiologically relevant decrease in intrinsic excitability (Petitjean, unpublished). Qiu et al. (2022) demonstrated in recordings from acute slice dorsal spinal horn iPVINs after CCI that these neurons have reduced excitability that is restored to naïve levels after Lei-Dab7 application, a selective SK2 blocker. Conversely, SK channel activator 1-EBIO significantly reduces the ability of iPVINs from naïve mice to fire tonically (Qiu et al., 2022). As the activity of SK channels depends on free cytosolic Ca^{2+}, changes to iPVINs' ability to manage activity-dependent Ca^{2+} accumulation likely play an important role in their intrinsic excitability.

1.6.5 Calcium and the parvalbumin protein in iPVINs

PVp is a small acidic cytosolic Ca^{2+} binding protein of the EF-hand superfamily (Permyakov & Uversky, 2022). The expression of PVp in the central nervous system is common to fast-spiking neurons of the central nervous system (Nahar et al., 2021; Permyakov & Uversky, 2022). The relationship between PVp expression and intrinsic excitability of neurons strongly depends on each other; inhibition of hippocampal PVINs (Donato et al., 2013) and impaired NMDAR function (Gonzalez-Burgos & Lewis, 2012) reduces PVp expression. In line, neurons depend on tight control and modulation of Ca^{2+} to maintain their precise firing properties (Zündorf & Reiser, 2011). After nerve injury, the $[Ca^{2+}]_i$ of SDH interneurons is significantly increased at rest, implicating impaired calcium regulation after nerve injury (Kawamata & Omote, 1996). This rise in $[Ca^{2+}]_i$ coincides with a marked drop in PVp mRNA expression after nerve injury (Qiu et al., 2022). Single-cell RNA-sequencing and fluorescent *in-situ* hybridization have demonstrated predominant SK2 expression in PVINs (Häring et al., 2018; Qiu et al., 2022). Furthermore, Qiu et al. (2022) demonstrated that shRNA-mediated downregulation of PVp in SDH PVINs induces allodynia. The relationship between PVpand SK channels on firing frequency has been

demonstrated in striatal iPVINs (Orbuz et al., 2013). Together, it suggests that $[Ca^{2+}]_i$ dysfunction likely plays a role in reduced iPVIN excitability via SK channels after nerve injury.

1.7 Hypothesis

Accumulating evidence suggests that changes to intrinsic excitability underlie iPVIN dysfunction after nerve injury. Tight control of $[Ca^{2+}]_i$ is essential for the maintenance of neuronal firing patterns, and $[Ca^{2+}]_i$ dysregulation in the SDH is prominent after nerve injury (Kawamata & Omote, 1996). PVp and SK channels modulate firing frequency in iPVINs of the striatum, and manipulation of intracellular calcium changes the firing patterns of these cells (Bischop et al., 2012; Orduz et al., 2013). Qiu et al. (2022) reported significant SK2-IR in iPVIN, loss of tonic firing, and reduction in iPVIN expression after nerve injury. Thus, we hypothesize that iPVINs depend on extensive Ca^{2+} buffering to prevent $[Ca^{2+}]_i$ accumulation and subsequent activation of SK channels that would otherwise cause adaption. After nerve injury, we hypothesize that iPVINs lose their ability to buffer Ca^{2+} and reduce their intrinsic excitability by activating SK channels. We aim to test this hypothesis by raising the $[Ca^{2+}]_i$ of iPVINs in acute spinal cord slices and recording firing properties with whole-cell electrophysiology.

Specific aims:

1) To determine the extent I can control $[Ca^{2+}]_i$ in iPVINs
2) To compare the firing properties of high $[Ca^{2+}]_i$ to control iPVIN recordings
3) To determine if SK channels mediate changes to firing properties of iPVIN under high $[Ca^{2+}]_i$

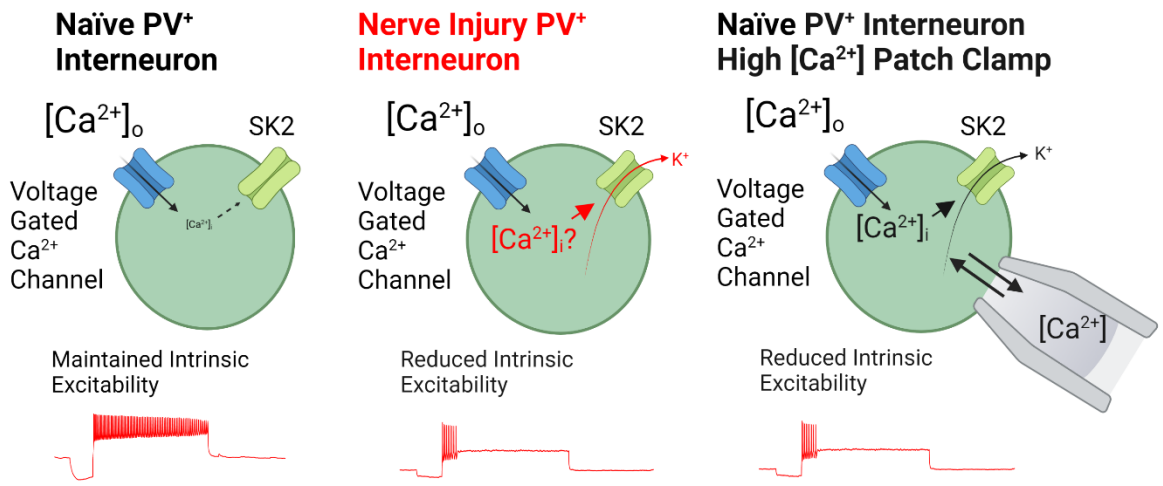


Figure 2: Model hypothesis of the control of intrinsic excitability by $[Ca^{2+}]_i$ and SK2 channels in iPVINs.

Chapter 2: Materials and Methods

2.1 Animals

All experiments were conducted in accordance with the Canadian Council of Animal Care and the McGill University Animal Care Committee. PV-tdTomato mice were used to express the red fluorescent protein tomato in parvalbumin interneurons. PV-tdTomato mice were generated by cross-breeding Ai14 mice [B6.Cg-Gt(ROSA)26Sor$^{tm14(CAG-tdTomato)Hze}$/J, The Jackson Laboratory, RRID:IMSR_JAX:007914] with either mixed genetic background PV-Cre [B6;129P2-Pvalb$^{tm1(cre)Arbr}$/J, The Jackson Laboratory, RRID:IMSR_JAX:008069] or congenic PV-Cre [B6.129P2-Pvalb$^{tm1(cre)Arbr}$/J, The Jackson Laboratory, RRID:IMSR_JAX:017320]. All lines were maintained in-house.

2.2 Acute spinal cord slice preparation and maintenance

Solutions

NMDG-aCSF: 92mM NMDG; 2.5mM KCl, 1.25mM NaH_2PO_4; 30mM $NaHCO_3$, 20mM HEPES; 25mM glucose; 2mM thiourea; 5mM Na-ascorbate; 3mM Na-pyruvate; 0.5mM $CaCl_2$-$2H_2O$; and 10 $MgSO_4$-$7H_2O$; titrated to 7.3-7.4 with 6M HCl. Continuously carbonated.

HEPES-aCSF: 92mM NaCl; 2.5mM KCl; 1.25mM NaH_2PO_4; 30mM $NaHCO_3$; 20mM HEPES; 25 mM glucose; 2mM thiourea; 5mM Na-ascorbate; 3mM Na-pyruvate; 2mM $CaCl_2$-$2H_2O$; 2 mM $MgSO_4$-$7H_2O$; titrated to 7.3-7.4 with 1M NaOH. Continuously carbonated.

Recording-aCSF: 124 NaCl; 2.5 mM KCl; 1.25 mM NaH_2PO_4; 26mM $NaHCO_3$; 15mM glucose. 2 mM $MgSO_4 \cdot 7H_2O$; titrated to 7.3-7.4 with 6M HCl Continuously carbonated.

salt-in solution: 5mL NMDG-aCSF; 180mg NaCl.

Spinal cord dissection and slicing

A glass petri dish and the vibratome buffer dish are filled with ice-cold NMDG-aCSF, and both are kept on ice. 200mL HEPES-aCSF and 50mL NMDG-aCSF are placed in their respective slice incubators and are kept at 37°C in a hot water bath.

PV-tdTomato mice 4-8 weeks post-natal are deeply anesthetized with intraperitoneal avertin. The ribcage is removed to access the heart for transcranial profusion with 10mL 0°C NMDG-ACSF. The spinal column is removed, and the spinal cord is dissected in a glass petri dish with NMDG-aCSF on ice. The spinal cord is mounted ventrally against the cutting face of an agar cube with veterinary glue. The vibratome mount is placed in the vibratome buffer well on ice containing NMDG-aCSF. Transverse slices from L3-L5 300µM thick were cut using a vibratome (model).

Slices were placed in a recovery chamber with 50mL 37°C NMDG-ACSF for 10-15 minutes, depending on age. Na^+ salt-in solution was pipetted into the recovery chamber in increments of 83µL, 83µL, 167µL, 333µL and 667µL upon slice recovery and every 2 minutes or 3 minutes for mice younger or older than 6 weeks, respectively, adapted from Ting et al. (2018). At the end of Na^+ salt-in, slices are transferred to a 37°C HEPES-aCSF holding chamber, removed from the hot bath and allowed to cool to room temperature. Slices were maintained for up to 6 hours in HEPES-aCSF.

2.3 Whole-cell electrophysiology

Internal Solutions

Control ($0Ca^{2+}$*):* 135 mM K-gluconate; 6 mM NaCl; 2 mM $MgCl_2$; 10 mM HEPES; 0.1 mM EGTA; 2 mM MgATP

50µM $[Ca^{2+}]_{ip}$: 135 mM K-gluconate; 6 mM NaCl; 2 mM $MgCl_2$; 10 mM HEPES; 0.1 mM EGTA; 0.1 mM $CaCl_2$; 2 mM MgATP

200µM $[Ca^{2+}]_{ip}$: 135 mM K-gluconate; 6 mM NaCl; 2 mM $MgCl_2$; 10 mM HEPES; 0.2 mM $CaCl_2$; 2 mM MgATP

1.9mM $[Ca^{2+}]_{ip}$: 135 mM K-gluconate; 6 mM NaCl; 2 mM $MgCl_2$; 10 mM HEPES; 2mM $CaCl_2$; 0.2 EGTA; 2 mM MgATP

Free calcium calculated using MAXCHELATOR software by Bers, Patton and Nuccitelli (2010).

Whole-cell Patch Clamp

Slices were transferred to the recording chamber continuously superfused with gravity-fed room temperature recording-ACSF at 1-2mL per minute. Slices were kept under constant recording-

aCSF superfusion. PVIN were identified under fluorescence using a filter set, captured by CoolSnap MYO camera, and displayed with WinFluor software. Patch pipettes with 6-8MΩ resistance were pulled using Stutter Instruments Co., Model P-97, with 1.5mm OD 0.6 ID glass capillaries. The whole-cell recording configuration was first established in voltage clamp holding at -70mV.

Action potential discharge was recorded in current-clamp mode. The holding current was adjusted to reach -70mV per recording. The excitability and action potential discharge was studied by injecting a series of depolarizing step currents into the recorded neuron (Figure 2A), recorded upon opening, 5 minutes, and 10 minutes after whole-cell configuration. Lei-Dab7 (200nM) in recording-aCSF was superfused using a diverter valve in line with the main recording-aCSF. Recordings and analysis were done in pClamp11 Software Suite and in-house analysis software developed by Xinyue Ma.

2.4 Calcium Imaging

Cell line and culture conditions

Human embryonic kidney cells, HEK293T, were grown in Alpha Modification of Eagle's Medium (AMEM 1X) supplemented with 10% fetal bovine serum and 1% penicillin/streptavidin in a humidity-controlled incubator at 5% CO_2.

Fura-2AM Incubation

Pluronic (Sigma-Aldrich F-127) and Fura-2AM were dissolved in DMSO then mixed with bath solution (150mM NaCl, 3mM KCl, 10mM glucose, 2mM $CaCl_2$, and 1mM $MgCl_2$) for 2.5µM Fura-2AM and 0.008% Pluronic acid incubation solution. HEK293T culture plates were washed

twice and incubated for 45 minutes. The plates are rinsed twice with fresh bath solution before imaging, and the bath solution is replaced by a pipetter every 10 minutes.

Calcium Imaging Protocol

Calcium-dependent changes in Fura-2 fluorescence were recorded using a NeuroCCD-SM256 mounted on an imaging system (Redshirt Imaging) mounted to a Zeiss AxioExaminer microscope with filter set 46HE and a 40× water immersion objective. The micropipette is loaded with the $[Ca^{2+}]_{ip}$ internal solution and sealed to the cell. Images were acquired at 0.3s exposure for 340 and 380nm excitation every 1s for 3s before whole-cell configuration and 10s after. Images were produced with Metafluor acquisition software and analyzed in ImageJ by outlining cells and capturing mean fluorescence, with relative Ca^{2+} signal expressed as the 340/380nm excitation ratio.

Chapter 3: Results

3.1 High intrapipette calcium maintains high $[Ca^{2+}]_i$ during whole-cell patch clamp

To validate our whole-cell high Ca^{2+} dialysis methodology, calcium imaging of HEK293 cells loaded with Fura-2 was recorded during patch clamp. Each concentration followed a dose-dependent increase and plateau of $[Ca^{2+}]_i$ after whole-cell configuration. The 2mM Ca^{2+} internal loss of fluorescence at 8 minutes was due to cell lysis, leading to the omission of the condition for patching PVINs. Here, we demonstrate 50µM and 200µM intrapipette $[Ca^{2+}]$ dose-dependently raises the $[Ca^{2+}]_i$ and plateaus at 3-5 minutes, allowing us to make an initial baseline recording at opening to compare to 5 and 10 minutes after elevating $[Ca^{2+}]_i$. We further compare the firing properties of PVINs recorded at opening to validate our approach.

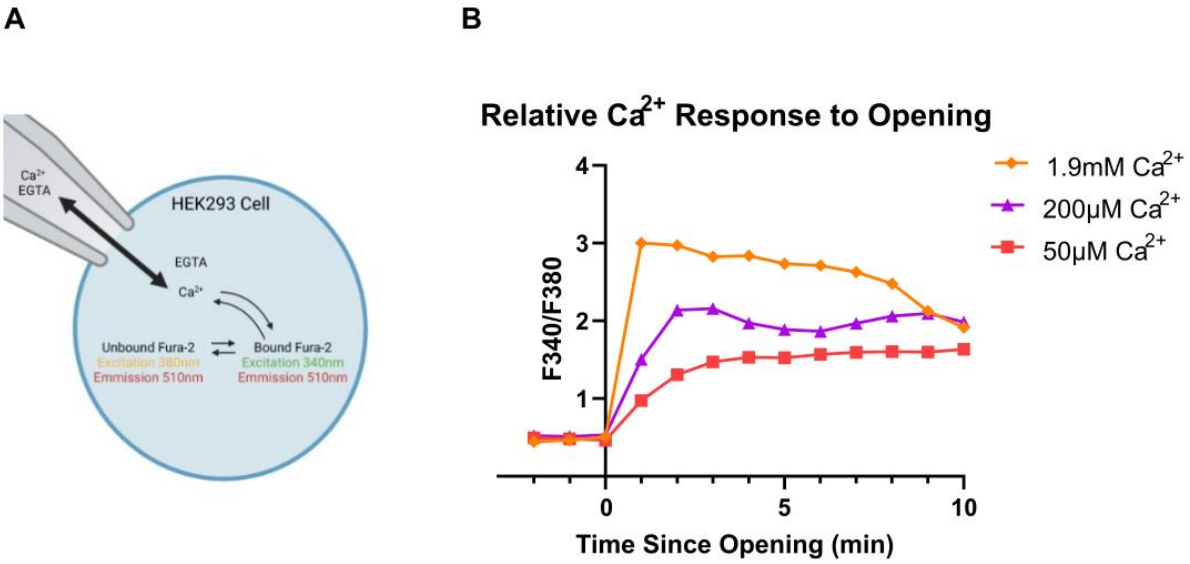


Figure 3: Ca^{2+} Imaging of HEK293T Ca^{2+} dialysis. A: Schematic of whole-cell patching and Fura-2 imaging. B: Relative Ca^{2+} response to opening. Opening at 0 minutes.

3.2 Electrophysiology of iPVINs

The firing pattern to depolarizing current was recorded for each intrapipette condition to determine whether high $[Ca^{2+}]_i$ affects iPVIN properties at opening. Using acute spinal cord slices of PV-Cre;;Ai14 mice, we whole-cell patched fluorescently labelled PVINs. Previous characterizations of fluorescent-guided recordings of the PVIN population show an approximately 30% of PV interneurons fire in bursts instead of tonically (Boyle et al., 2019; Hughes et al., 2012). Recordings from 0 (control), 50µM, and 200µM Ca^{2+} at opening match PVIN electrophysiological properties in literature (Figure 2B). As a similar proportion of ePVINs exists in lamina IIi-III and the crucial function of high-frequency firing in iPVINs, burst firing cells likely correspond to the ePVIN population (Gradwell et al., 2022). Non-tonic PVINs at opening were omitted from the firing pattern time course to select for the iPVIN population.

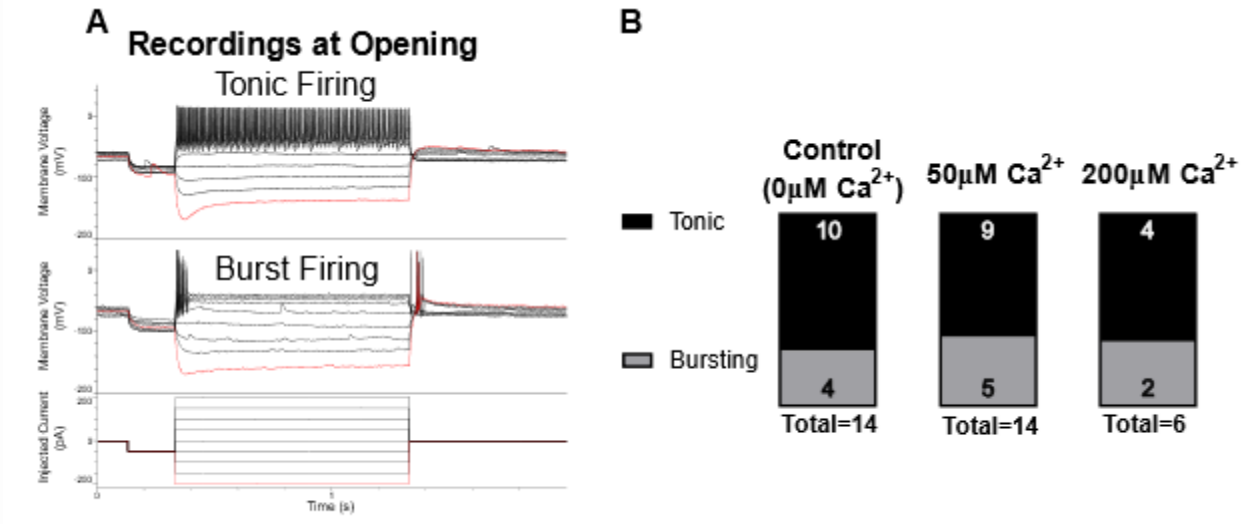


Figure 4: Recorded opening firing properties of SDH PVINs. A: Representative traces of PVIN tonic firing and burst firing to current injection protocol. Red indicates starting sweep of the recording. B: Recorded firing properties at whole-cell opening for different in response to depolarizing current for $[Ca^{2+}]_{ip}$ concentrations. Tonic firing is defined as the ability to maintain firing without gaps to the end of the 1s current injection.

3.3 High intrapipette calcium abolishes tonic firing in iPVIN

To determine whether high $[Ca^{2+}]_i$ affects the firing pattern of iPVINs, we recorded the firing patterns of iPVINs at opening, 5 minutes, and 10 minutes in response to depolarizing current. At 5 minutes and 10 minutes, 200µM Ca^{2+} significantly reduced the ability of iPVINs to fire tonically. This demonstrates that high $[Ca^{2+}]_i$ reduces the ability of iPVINs to fire tonically. However, 50µM Ca^{2+} did not reduce the ability of iPVINs to fire tonically within 10 minutes. Together, this demonstrates that high $[Ca^{2+}]_i$ of 200µM abolishes tonic firing in iPVINs but not 50µM.

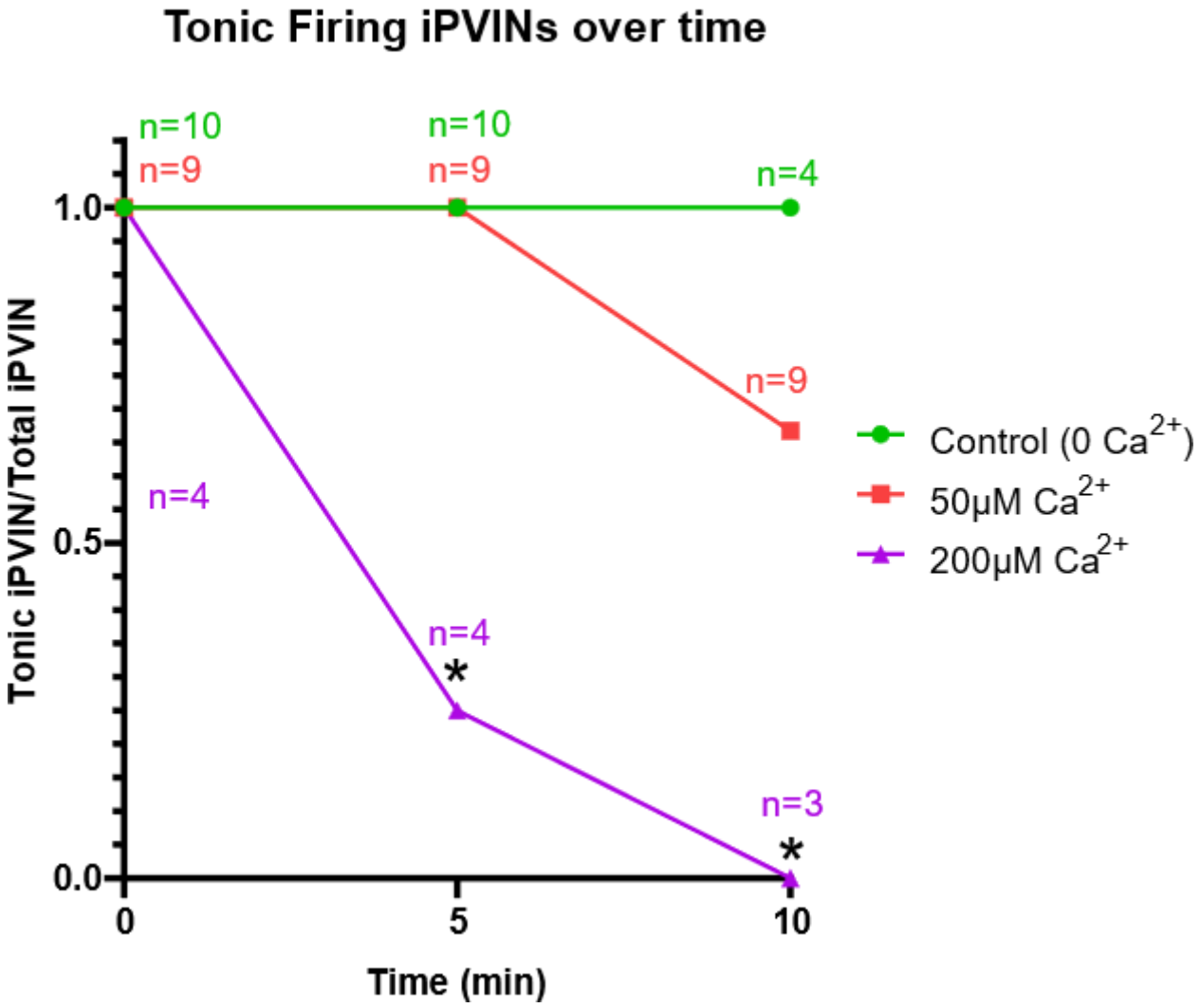


Figure 5: High $[Ca^{2+}]_{ip}$ effect on iPVIN tonic firing. Recordings were initiated at opening, 5 and 10 minutes in single iPVINs with different $[Ca^{2+}]_{ip}$. Tonic firing is the ability to maintain firing without gaps to the end of the 1s current injection. Fisher's exact test.

3.4 50µM $[Ca^{2+}]_i$ increases action potential production at 5 minutes.

To further characterize the firing patterns of recorded iPVINs, we quantified the action potentials elicited for each depolarizing step at the different time points. Unexpectedly, at 5 minutes, the 50µM Ca^{2+} condition significantly potentiated action potential production at higher depolarizing

currents of 150pA and 200pA (Figure 3B). This effect was seen intensely for one 200µM $[Ca^{2+}]_{ip}$ iPVIN. Furthermore, 50µM Ca^{2+} was able to convert two non-tonic cells at opening to tonic firing at 5 minutes and similarly potentiated action potential firing at a higher current (Suppl. Figure 1B & 1D). These results suggest that increasing $[Ca^{2+}]_i$ does not reduce intrinsic excitability dose-dependently.

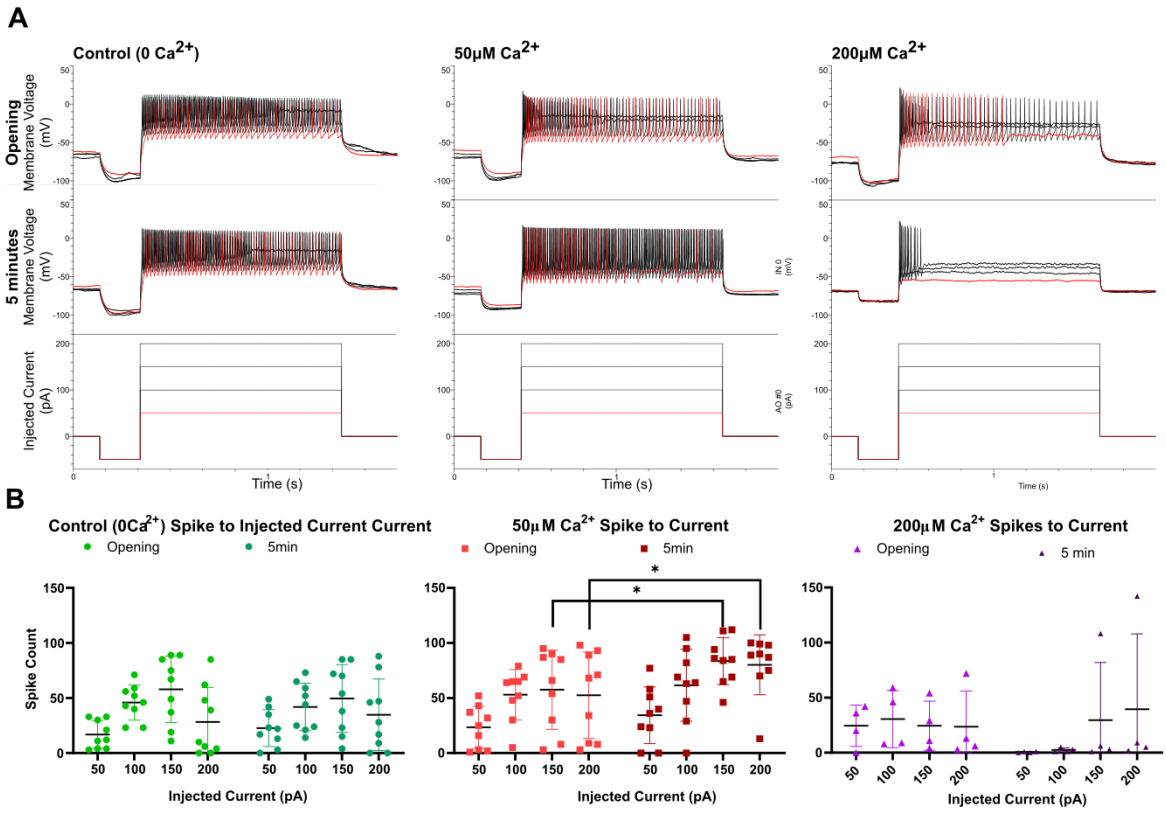


Figure 6: 50µM Ca^{2+} increases action potential production at higher currents. A: Representative traces of recorded membrane voltage and depolarizing currents. B: Quantification of spike count to current injection sweep at opening and 5 minutes for $[Ca^{2+}]_{ip}$ conditions. Two-way ANOVA, Šídák.

3.5 Pharmacological blockade of SK2 partially rescues high $[Ca^{2+}]_i$ induced adaptation

To determine if SK2 channel is responsible for high $[Ca^{2+}]_{ip}$-induced adaption, Lei-Dab7 was superfused into acute slices in a PVIN with 50µM $[Ca^{2+}]_{ip}$-induced adaptation at 20 minutes. Lei-Dab7 blockade of SK2 partially restored action potential count. This demonstrates that SK2 is implicated in high $[Ca^{2+}]_i$ induced adaptation.

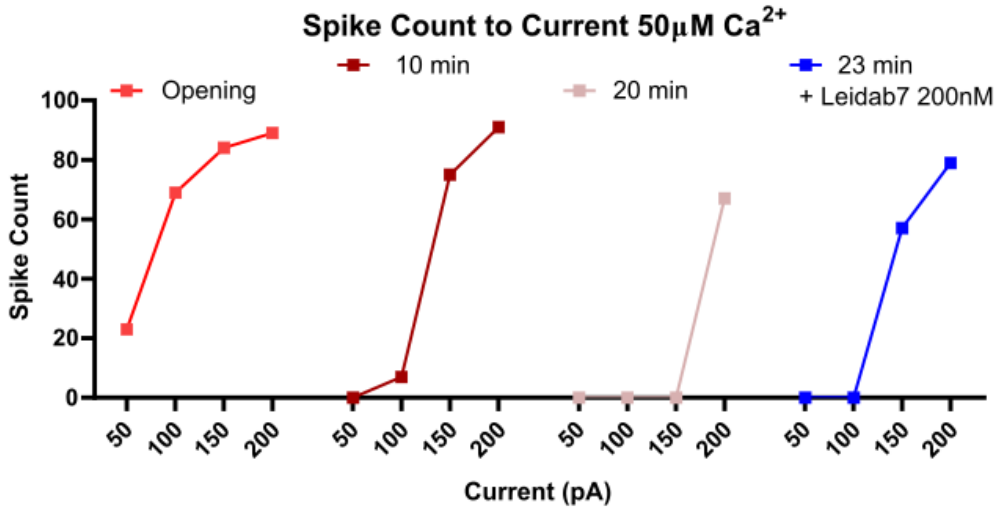


Figure 7: SK2 channel blockade increases spike count after 50µM Ca^{2+}-induced adaptation. Spike count to the injected current of iPVINs at opening, 10 minutes, 20 minutes, and 23 minutes with Lei-Dab7 (200nM) superfusion.

Chapter 4: Discussion

The present study demonstrates the critical nature of intracellular calcium in maintaining the firing properties of SDH iPVINs. In the results, we demonstrate that high intrapipette [Ca^{2+}] increases $[Ca^{2+}]_i$ over 3-5 minutes and plateaus (Figure 3), in line with the recordings upon opening (Figure 4). In line with our hypothesis, a high $[Ca^{2+}]_{ip}$ of 200µM significantly reduces the ability of iPVINs to fire tonically 5 minutes after opening (Figure 5). Furthermore, applying SK2 blocker Lei-dab7 in an adapting iPVIN partially restored action potential production (Figure 7). Together, this suggests that SK2 channels mediate reduced intrinsic excitability caused by high [Ca^{2+}]. In contrast, we observed that 50µM $[Ca^{2+}]_{ip}$ did not significantly reduce the ability of iPVINs to fire tonically and increased action potential production (Figure 6). However, these observation need to be reproduced before being interpreted further. Altogether, these findings suggest that $[Ca^{2+}]_i$ is critical in setting the firing properties of iPVINs. In this section, we will discuss the implications of these findings, their limitations, and future directions from this study.

4.1 High $[Ca^{2+}]_i$ reduces the intrinsic excitability of iPVINs via SK channels

In this study, we demonstrated that high $[Ca^{2+}]_i$ utilizing 200µM Ca^{2+} internal solution significantly reduced tonic firing in iPVINs compared to control at 5 and 10 minutes. These findings align with the findings from our lab by Qiu et al. (2022) characterization of iPVINs and PVp after nerve injury; PVp-RNA expression is significantly reduced, suggesting a reduction in calcium buffering ability. In line with Ca^{2+} dysregulation of SDH interneurons (Kawamata & Omote, 1996) and the role of SK family channels in mediating spike-frequency adaption (Ha & Cheong, 2017), our results further build on how [Ca^{2+}] dysregulation plays a role in disinhibition and allodynia in the spinal cord. The expression of SK channels in naïve SDH iPVINs likely plays a modulatory role as in striatal iPVINs by restricting free Ca^{2+} amplitudes (Bischop et al., 2012;

Orduz et al., 2013). During nerve injury, the reduction of PVp expression could allow for pathologically high $[Ca^{2+}]_i$, strongly activating the SK channel pool and preventing tonic firing.

Interestingly, while both striatal and SDH PVINs have similar firing frequencies in response to depolarizing currents (Boyle et al., 2019; Hughes et al., 2012; Orduz et al., 2013), PV-KO increased PVIN firing frequency in the stratum in contrast to SDH iPVINs (Orduz et al., 2013). Using computer modeling of neuronal firing, Orbuz et al. (2013) demonstrate that this PV-KO induced increase in firing frequency was due to a quicker decay in $[Ca^{2+}]_i$ at the membrane, allowing for quicker depolarizations and shorter inter-spike intervals. In contrast, the silencing of PV in the SDH iPVINs of adult mice significantly reduces the ability of iPVINs to fire tonically (Qiu et al., 2022). The results of Orbuz et al. (2013) global PV-KO may have allowed for compensatory developmental factors, in which the remaining calcium buffering capacity facilitates the clearing of Ca^{2+} at the membrane. During the silencing of PV in the SDH of adult mice, a larger loss of buffering capacity without compensation may allow for large unfiltered Ca^{2+} transients and the accumulation of Ca^{2+} in the cytosol, ultimately resulting in greater activation of the SK pool. By demonstrating that high $[Ca^{2+}]_i$ reduces intrinsic excitability by whole-cell diffusion through the cytosol, this study supports that elevated $[Ca^{2+}]_i$ prevents tonic firing. In an adapting iPVINs 50μM Ca^{2+} recording over 20 minutes, the application of Lei-Dab7 increased action potential production. This result supports the role of SK2 adapting tonic firing in response to high $[Ca^{2+}]_i$, in line with the role of SK channels. In line, the reduction in iPVIN excitability after nerve injury is rescued by the application of Lei-Dab7 (Qiu et al., 2022), suggesting a role for elevated $[Ca^{2+}]_i$. While the Lei-Dab7 application does not complete rescue action potential production as in opening, it is likely that other SK channel families, likely SK3, contribute to adaptation in iPVINs. Unselective blockade SK channel family by apamin may have further rescued action potential

production. Due to time constraints, the Lei-Dab7 pharmacology remains incomplete and requires repeat experiments to further substantiate the SK2 channel and the contribution of other SK channels as the mechanism behind high $[Ca^{2+}]_i$ induced adaptation. Together, these results support our hypothesis that iPVINs depend on low $[Ca^{2+}]_i$ to maintain their tonic firing patterns by preventing the activation of SK2 channels.

4.2 Elevated $[Ca^{2+}]_i$ facilitates action potential production

Unexpectedly, 50µM $[Ca^{2+}]_{ip}$ significantly potentiated action potential production at higher depolarizing currents of 150pA and 200pA, but not 50pA or 100pA. Interestingly, this potentiating effect occurred in the recorded bursting cells (Suppl. Figure 1D), and two were able to fire tonically at 5 minutes before reverting to burst firing (Suppl. Figure 1B). This suggests that ePVINs and iPVINs have similar $[Ca^{2+}]_i$-dependent mechanisms behind their intrinsic excitability.

With the methodology used, SK channels themselves may potentiate action potential production. While the HEK293 Ca^{2+} imaging seems to suggest that cells should equilibrate with $[Ca^{2+}]_{ip}$ around 3-5 minutes (Figure 2B), spinal iPVINs likely have similar high buffering capacities as cortex or hippocampal iPVINs (Eggermann & Jonas, 2011). This facilitatory effect seems to subside at 10 minutes and beyond for 50µM $[Ca^{2+}]_{ip}$ (Figure 4B). This slows Ca^{2+} diffusion through iPVINs and may contribute to this optimal action potential election between maximal and intermediate activity as a pool of membranous SK channels. As more SK channels open, the facilitatory effect of the voltage-independent hyperpolarizing current through assisting with action potential repolarization may be overcome by the opposing effect of hyperpolarization on action potential initiation, as seen by the inhibition of iPVIN's tonic firing in acute slices by superfusion of 1-EBIO, an SK channel activator (Qiu et al., 2022). By using sub-maximal amounts of 1-EBIO, itself not a potent activator compared to more recently identified activators, we could determine if SK channels contribute to

action potential production in naïve iPVINs. With Lei-Dab7 superfusion starting after the initial recording, we could determine if this high $[Ca^{2+}]_i$ facilitation acts through SK channel activation. The whole-cell current clamp configuration may further potentiate this; while firmly established for the recording and manipulating whole-cell characteristics such as firing and ionic conductances, the injection of current at the soma is unphysiological. In whole-cell configuration recordings of inhibitory interneurons to Aβ-fiber stimulation, the eEPSC can reach transiently 200-400pA before rapidly decaying (Betelli et al., 2015). Here, activation of SK channels by EPSC elicited action potentials significantly impacts the cell's ability to depolarize against the hyperpolarizing current. In contrast, whole-cell current-clamp depolarizations continuously inject current until the end of the stimulus. Here, activation of SK channels may enhance repolarization against these injected currents, allowing for the enhanced reactivation of Na_v channels not seen during Aβ-input (Iyer et al., 2017). Another possibility is the activation of HCNs. In response to elevated $[Ca^{2+}]_i$, Ca^{2+}/calmodulin-stimulated adenylyl cyclases AC1 and AC8 activate and produce, critical for long-lasting LTP (Ferguson & Storm, 2004). HCN channels are allosterically activated by cAMP, shifting towards activation for more depolarized currents (Porro et al., 2019). Indeed, iPV-basket cells depend on HCN channels to control action potential production; pharmacological blockade results in adaption and increased failure rates to somatic current pulses, but only at high frequencies (Byczkowicz et al., 2019). This facilitatory effect is mediated by the depolarizing HCN current opposing the hyperpolarizing $Na+/K^+$ ATPase current during intense firing (Kase & Imoto, 2012). Spinal iPVINs express HCN1 and HCN4 (Hughes et al., 2013; Hughes et al., 2012). Furthermore, AC1 is highly expressed alongside PV in Häring et al. (2018) GABA14 cluster. Under our imposed high $[Ca^{2+}]_i$, HCNs are allosterically activated by cAMP production, increasing its depolarizing current and increasing action potential production at the

higher currents and thus higher frequency firing of 50µM $[Ca^{2+}]_{ip}$. With the facilitatory effect with SK channels, the inhibitory contribution of SK channels may overcome this facilitation, leading to the loss of tonic firing. These results demonstrate that the relationship between elevated $[Ca^{2+}]_i$ and decreasing intrinsic excitability is not dose-dependent, and requires further experimentation to delinate. All together, my results show that tight regulation of $[Ca^{2+}]_i$ is critical for maintaining the intrinsic excitability of dorsal horn iPVINs.

4.3 Future Directions

The methodology used in this study depended on the diffusion of calcium from the micropipette to the cytosol. This provides a simple method to probe iPVINs intrinsic excitability while simultaneously equilibrating $[Ca^{2+}]$ over time, allowing for comparison of firing properties at opening to later time points. Intracellular calcium is a ubiquitous signaller (Brini et al., 2014), and our methodology likely triggers cellular cascades and masks calcium-induced calcium release. To establish a connection between calcium influx and SK channel activation more physiologically, we can increase $[Ca^{2+}]$ in the extracellular ACSF and recording from naïve iPVINs; the amplitude of free-Ca^{2+} sharply drops from the membrane due to calcium buffering (Fakler & Adelman, 2008). This method has been successfully used to probe SK channel activity in acute slices of locus coeruleus and the cerebellum (Matschke et al., 2018; Womack et al., 2004), allowing us to circumvent the unquantified diffusion of Ca^{2+} through the whole-cell. A significant limitation placed was the fidelity of single action potential properties for the 10-minute timescale. The large, long-timescale rise of $[Ca^{2+}]_i$ likely induces changes to membrane properties beyond channel activation, contributing to a lack of statistical power in attempted analyses. Without the need to modify $[Ca^{2+}]_{ip}$, the quality of the seal can be maintained while probing the effects of high $[Ca^{2+}]_i$ mediated at the membrane. By switching the $[Ca^{2+}]$ of the milieu, time in whole-cell configuration

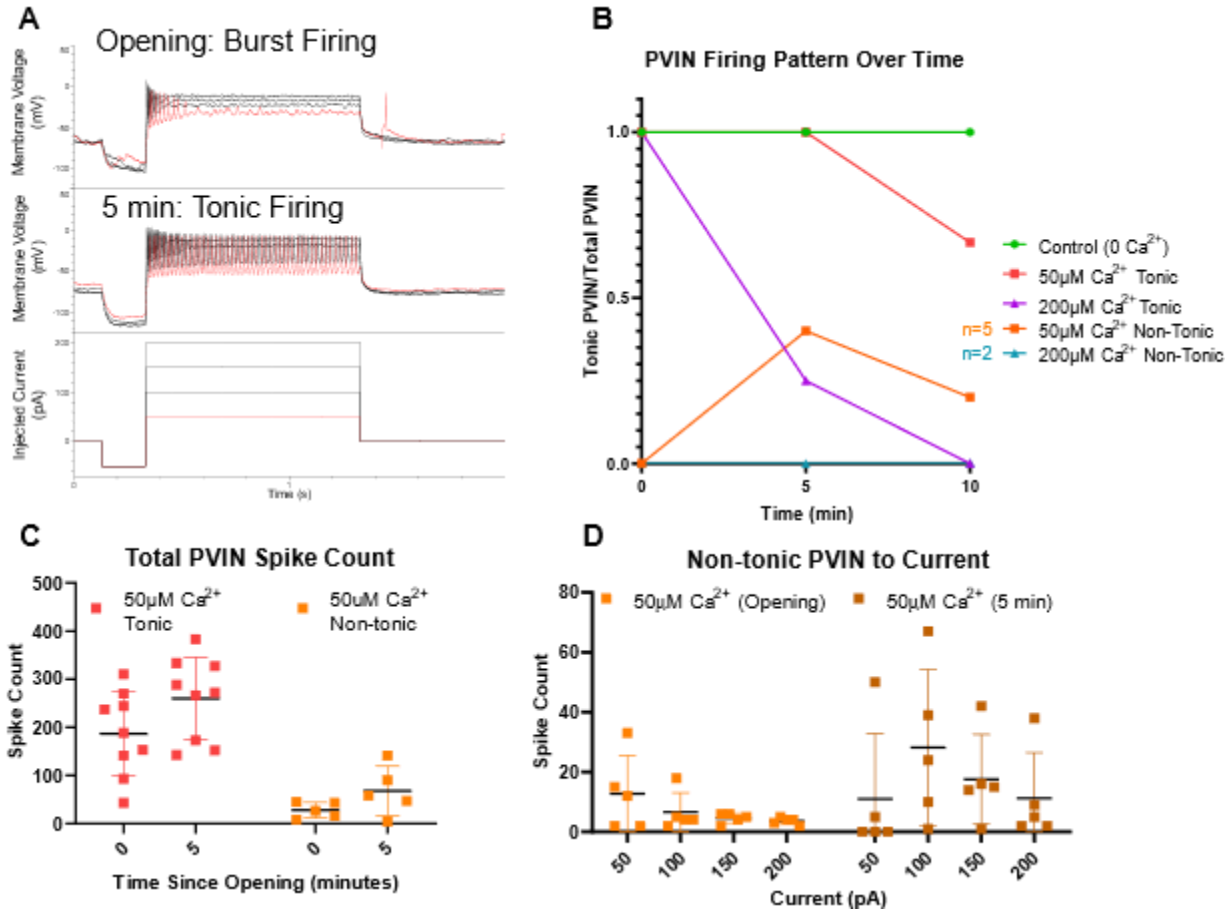
can be further minimized, and determine changes in action potential properties that better reflect membrane contribution of high $[Ca^{2+}]_i$. Furthermore, dorsal root stimulation at the physiological frequency of Aβ-fibers while recording in whole-cell configuration can better determine if these manipulations to $[Ca^{2+}]_i$ would have physiological relevance. Pharmacological interrogation of the SK channel families would further benefit from the smaller timescales required.

The magnitude of calcium influx during repetitive action potential production could be quantified by using low-affinity Calcium Green-5N in the recording micropipette. SK channels are highly sensitive to buffering capacity; in pyramidal hippocampal neurons, the high-affinity calcium indicator Oregon Green BAPTA-1 blocks the SK-mediated mAHP (Scott & Greg, 2013). Furthermore, low-affinity Calcium Green-5N provides faster dissociation kinetics for monitoring fast Ca^{2+} transients and allows for measuring of Ca^{2+} concentrations up to 50μM (Mulligan & MacVicar, 2005; Rajdev & Reynolds, 1993). By generating a calibration curve with Calcium Green-5N using whole-cell patching of PVINs with known $[Ca^{2+}]_{ip}$, we could quantify $[Ca^{2+}]_i$ in naïve and nerve injury iPVINs in response to action potential production. Using this methodology, we can establish differences in calcium processing between nerve-injured and naïve iPVINs. Furthermore, we could identify the calcium-induced calcium release from intracellular stores and their contribution to intrinsic excitability by utilizing calcium imaging. This could further differentiate between the Ca^{2+}-SK mediated changes in intrinsic excitability and other contributing factors, such as internalization that would occur due to longer timescales with elevated $[Ca^{2+}]_i$. More robust characterization of $[Ca^{2+}]_i$'s role in maintaining the intrinsic excitability of iPVINs would allow us to understand mechanisms underlying neuropathic pain.

5 Conclusion

The present study has aimed to advance our understanding of how Ca^{2+} dysregulation affects the ability of iPVINs to produce action potentials. Using 200µM $[Ca^{2+}]_{ip}$ reduces the ability of iPVINs to fire tonically, and in an adapting iPVIN application of SK2 blocker Lei-Dab7 partially restores action potential production. These results support our hypothesis that iPVINs depend on low $[Ca^{2+}]_i$ to prevent the activation of SK2 channels that would otherwise cause adaptative firing. In contrast, 50µM Ca^{2+} did not change the ability of iPVINs to tonically fire and increased action potential production at 5 minutes. These results demonstrate that iPVINs depend on tightly controlling $[Ca^{2+}]_i$ to maintain their naïve firing properties. This thesis provides a direct probe on how $[Ca^{2+}]_i$ affects the excitability of PVINs, contributing to our lab's research in understanding how iPVINs properties change after nerve injury. The role of $[Ca^{2+}]_i$ in interneuron populations remains understudied in light of broad SDH Ca^{2+} dysregulation (Harding et al., 2020; Kawamata & Omote, 1996). Our lab seeks to further identify the role of $[Ca^{2+}]_i$ in iPVINs; efforts by Qiu et al. (2022) have established PVp as necessary for maintaining intrinsic excitability, and Ma et al. (2023) have furthered a computational model of calcium buffering and SK channels for SDH iPVINs. By further understanding how nerve injury induces changes to functional populations of the SDH, we can elucidate targets for selective modulation and develop treatments with greater efficacy and fewer side effects. A greater understanding of the discrete spinal cord populations contributing to neuropathic pain will further elucidate selective spinal cord targets and mechanisms for clinicians and their patients.

Supplementary Figures



Supplemental Figure 1: 50µM Ca^{2+} increases the intrinsic excitability of non-tonic PVINs A: Representative trace of 50µM Ca^{2+} whole-cell recording in non-tonic PVIN. Top: Membrane voltage recorded at opening. Middle: Membrane voltage recorded at 5 minutes. Bottom: Depolarizing current sweeps. Red indicates the first depolarizing sweep in the recording. B: PVIN firing pattern over time for tonic and non-tonic PVINs at 0Ca^{2+}, 50µM Ca^{2+} and 200µM Ca^{2+}. C: Total PVIN spike count for 50µM Ca^{2+} tonic and non-tonic PVINs at opening and 5 minutes. D: Spikes elicited to current injection for 50µM Ca^{2+} non-tonic PVINs at opening and 5 minutes.

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