Spinal Cord Structural Plasticity in Neuropathic Pain

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Abstract

Neuropathic pain is a complex chronic pain disorder which is caused by damage to the somatosensory nervous system. Clinically, it is characterized by spontaneous or ongoing pain, abnormal sensations, and amplified evoked pain responses after noxious or non-noxious stimuli. Pharmacological management of neuropathic pain remains a great challenge as existing therapies have low levels of analgesic efficacy which are commonly associated with deleterious side effects. Furthermore, treatment strategies need to progress from only suppressing sensory symptoms to disease-modifying efficacy by targeting the underlying pathological mechanisms. To move towards this goal, a comprehensive understanding of the fundamental changes driving the chronification of neuropathic pain is required.

The dorsal horn of the spinal cord is the first area of the central nervous system (CNS) that receives primary sensory information including diverse forms of innocuous and nociceptive inputs. The local dorsal horn neural circuits integrate and modulate sensory inputs before they are forwarded to the brain. Emerging evidence suggests that after peripheral nerve injury, changes intrinsic to the dorsal horn, such as disinhibition, develop and maintain pain that is independent of the peripheral input. The work presented in this thesis is focused on the role of microglia, the resident CNS immune cells, and the complement system in nerve injury driven maladaptive changes in dorsal horn circuits. The overall hypothesis is that nerve injury-activated microglia mediate structural changes in dorsal horn synaptic circuitry. These changes result in loss of dorsal horn inhibition which contributes to the maintenance of neuropathic pain.

In pre-clinical models of neuropathic pain, dorsal horn synapse densities were observed to be selectively altered by nerve injury, with a significant loss of inhibitory synapses and a subset of excitatory synapses formed by touch coding-primary afferents. Furthermore, we showed that nerve injury activated microglia were responsible for loss of inhibitory synapses and mediate a significant reorganization of the dorsal horn excitatory synaptic network. These actions of microglia were mediated by the complement synapse pruning pathway in which C1q, the initiating protein in the classical complement cascade, was deposited on vulnerable dorsal horn synapses, to flag them for removal by microglia. Pharmacological inhibition of C1q protected the

spinal cord circuitry by disrupting synapse engulfment and ameliorated pain-related behaviour at chronic stages of the pathology.

Using an imaging-based approach, we further showed that nerve injury results in temporal and cell specific changes in membrane chloride potassium co-transporter, KCC2, expression and inhibitory inputs on dorsal horn neuronal cell bodies.

Collectively these results highlight remarkable dorsal horn structural plasticity in the neuropathic pain state. Moreover, indicating a role for complement-dependent synaptic pruning pathway and microglia in mediating these pathological changes. This knowledge provides an opportunity for developing novel therapeutic strategies that target complement proteins to mitigate dorsal horn synapse loss and prevent the progression of chronic neuropathic pain.

Résumé

La douleur neuropathique est une douleur chronique complexe engendrée par une lésion du système nerveux somatosensoriel. Le traitement pharmacologique de la douleur neuropathique reste compliqué car les thérapies actuelles n'offrent généralement qu'une analgésie très modeste à laquelle s'ajoutent souvent des effets secondaires indésirables. Il est donc nécessaire de modifier ces approches thérapeutiques qui tentent uniquement d'alléger les symptômes afin de développer des stratégies plus approfondies, visant directement les mécanismes pathophysiologiques sous-jacents de la douleur neuropathique. Pour ce faire, il est primordial d'approfondir nos connaissances des changements physiologiques menant au développement de la douleur neuropathique chronique.

La corne dorsale de la moelle épinière constitue le premier site d'arrivée d'une multitude d'informations sensorielles, qu'elles soient nociceptives ou inoffensives. De nouveaux rapports suggèrent qu'après une lésion d'un nerf périphérique, des changements intrinsèques à la corne dorsale se développent et maintiennent la douleur indépendamment des apports sensoriels périphériques. Le travail présenté dans cette thèse se concentre sur le rôle des cellules de la microglie, et du système du complément dans le développement de changements structurels mésadaptés des circuits neuronaux de la corne dorsale, engendrés par la lésion d'un nerf périphérique. Notre hypothèse principale soutient que les cellules de la microglie activées par une lésion nerveuse entrainent des changements structurels à la circuiterie synaptique de la corne dorsale. Ces modifications mènent à une perte de l'inhibition au sein de la corne dorsale, maintenant ainsi la douleur neuropathique de façon chronique.

Nos recherches ont révélé que la densité synaptique de la corne dorsale est sélectivement modifiée dans des modèles précliniques de douleur neuropathique. En effet, nous avons observé une perte significative des synapses inhibitrices et d'une sous-population de synapses excitatrices correspondant aux 'afférents primaires codant pour le toucher. De plus, nous avons démontré que les cellules de la microglie activés par une lésion nerveuse sont responsables de cette perte de synapses inhibitrices et entrainent ainsi une réorganisation significative du réseau synaptique excitateur de la corne dorsale. Cette activité microgliale est fonction du processus d'élagage

synaptique entrainé par le système du complément. Au sein de cette cascade enzymatique, la protéine d'initiation C1q est déposée sur les synapses vulnérables de la corne dorsale afin de les identifier comme cibles d'élagage synaptique. L'inhibition pharmacologique de C1q nous a permis de protéger les circuits neuronaux de la moelle épinière en interrompant l'engouffrement synaptique par la microglie et a mené à une amélioration de la douleur neuropathique pendant la phase chronique de la pathologie.

Afin d'étudier la dynamique des changements structurels propres à chaque type cellulaire, nous avons utilisé une approche utilisant l'imagerie qui nous a permis de quantifier simultanément les synapses inhibitrices et le symporteur de potassium KCC2. Ceci nous a permis de démontrer qu'une lésion nerveuse entraine un changement d'expression membranaire de KCC2 et des stimulations inhibitrices vers les neurones de la corne dorsale. Ces changements sont spécifiques à certaines cellules et ont lieu à un moment bien précis du développement de la douleur neuropathique.

L'ensemble des résultats que nous présentons ici met en valeur l'importante plasticité structurelle de la corne dorsale de la moelle épinière dans le contexte de la douleur neuropathique et démontre le rôle de médiateur de la cascade du complément et de la microglie dans le développement de ces changements pathologiques.

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Contribution to original knowledge

The results contained in this thesis demonstrate a novel mechanism underlying the pathogenesis of altered sensory transmission in neuropathic pain, which is the selective structural remodelling of synaptic connectivity in the dorsal spinal cord by microglia. Original findings contribute to the knowledge base and introduce new targets for treating neuropathic pain. The findings presented in this thesis resulted in the following three manuscripts:

Manuscript 1:

Yousefpour N, Locke S, Wang C, Deamond H, Marques L, St-Louis M, Ouellette J, Khoutorsky A, De Koninck Y, Ribeiro-da-Silva A. Microglia mediate loss of spinal cord inhibitory synapses in neuropathic pain. **Cell Reports**, 2021, *In Revision. Published online as a Cell Press Sneak Peek at https://dx.doi.org/10.2139/ssrn.3710746.*

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Manuscript 3:

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Contribution of Authors

The following statements describe the responsibilities of all the authors of the above co-authored manuscripts:

Dr. A. Ribeiro-da-Silva: Principal investigator of all the projects and overseeing the project as a whole and manuscript editing.

N. Yousefpour: Investigator and the main intellectual influence of all the projects reported in this thesis. Designed and planned all the experiments (excepting any specific experiments mentioned below), analysed the data, prepared the figures, and wrote the first versions of all the manuscripts.

Dr. S. Locke: Intellectual influence on all the projects. Helped with designing experiments and optimization of image analysis techniques. Edited the manuscripts and performed minocycline injections (manuscript 1).

S. Tansley: Designed and performed all the behavior experiments for C1q neutralizing antibody treatments (manuscript 2).

M. St-Louis: Assisted with technical problems and optimization of experiments (manuscripts 1 and 2)

H. Deamond: Performed intrathecal injections of PLX3397 (manuscript 1) and helped with immunohistochemistry experiments (manuscript 2) and proofread manuscripts (manuscripts 1 and 2).

B. Sharif: Performed parts of neuropathic pain surgeries (manuscript 2).

C. Wang: Performed a portion of engulfment and synapse quantifications (manuscripts 1 and 2).

Dr. M. Parisien: Performed genetic data analysis (manuscript 2).

J. Ouellette: Assisted with optimization of the electron microscopy experiments and imaging (manuscript 1).

J.S. Austin: Performed parts of neuropathic pain surgeries (manuscript 2).

V. Bourassa: Processed tissue for histology (manuscript 2).

M. Foret: Associated with histology experiments and proof

read the manuscript (manuscript 3)

V. Cabana: Processed tissue for histology (manuscript 2).

K. Lister: Assisted with tissue extraction (manuscript 2).

Dr. A. Khoutorsky: Supported mouse experiments and super-resolution imaging experiments as well as edited the manuscript (manuscripts 2 and 3).

Dr. Y. De Koninck: Intellectual influence and manuscript editing (manuscripts 1 and 2).

Dr. L. Diatchenko: Intellectual influence and manuscript editing (manuscript 2).

Dr. J. Mogil: Technical support and manuscript editing (manuscript 2).

Dr. P. Séguéla: Technical support and manuscript editing (manuscript 2).

Dr. A.C. Cuello: Technical support and manuscript editing (manuscript 3).

Dr. Y. Andrews-Zwilling: Experimental design (manuscript 2).

Dr. V. Matur: Experimental design (manuscript 2).

Dr. T. Yednock: Experimental design (manuscript 2).

Chapter 1

General Introduction

1.1 Pain

Pain is an alarming sensory and emotional experience, a warning sign that evolved to protect our species from potential or actual harm. From a neurobiological point of view, what we describe as pain can be classified into two subtypes: physiological, and pathological pain (Woolf, 2010b). Nociceptive pain, a type of physiological pain, is related to sensing noxious stimuli such as something too hot, or too cold. The instant unpleasantness of this sensation commonly results in immediate attention and a protective action such as a withdrawal reflex (Costigan et al., 2009). Throughout postnatal development, our sensory system is hardwired and perfectly tuned to determine what is causing nociceptive pain so that we can avoid such actions in the future (Fitzgerald, 2005, Koch and Fitzgerald, 2013). Another form of physiological pain is commonly associated with tissue damage such as a sprained ankle or sun burn. In this case, the sensory sensitivity is heightened, and pain enables the repair of the injured body part by preventing movements and physical contact that could potentially exacerbate tissue damage (Costigan et al., 2009). While physiological pain is mediated through normal activity of the somatosensory system to protect the body, maladaptive and pathological pain results from aberrant functioning of this system. In this case, pain is not proportionally associated with an injury and instead, it is a disease state of the nervous system (Costigan et al., 2009). Neuropathic pain is a form of pathological pain that can occur after damage to the nervous system and persists chronically (Baron et al., 2010). This body of work is primarily focused on investigating the mechanisms that contribute to this form of pain pathology.

1.1.1 Neuropathic pain

Similar to how frontal lobe damage can cause a permanent personality change, damage to the somatosensory nervous system can trigger chronic pathological changes that may lead to neuropathic pain (Harlow, 1999, Jensen and Finnerup, 2014). During the prenatal and postnatal periods, the somatosensory nervous system develops into a sophisticated neural network that is tuned by early-life sensory experiences to detect, compute, and perceive sensory information such as pain and touch accurately and reliably (Koch and Fitzgerald, 2013). It is conceivable that any damage to this well-adjusted and interactive system can lead to a disconnect between the nature of sensory stimuli and their perception resulting in an abnormal sensory experience.

Deficits in correct computation of sensory information explains why unlike other chronic pain disorders, neuropathic pain symptoms are not limited to exaggerated and prolonged pain, but also include other odd symptoms such as numbness, shooting electrical attacks, burning, or tingling sensations (Jensen and Finnerup, 2014). Because neuropathic pain is caused by a malfunction of the somatosensory system itself, traditional analgesics, which rely on normal operation of this system, are mostly ineffective (Woolf, 2020, Finnerup et al., 2015). The first-line pharmacological treatment options for neuropathic pain are tricyclic antidepressants, dual reuptake inhibitors of serotonin and norepinephrine, calcium channel α_2-δ ligands (i.e., gabapentin and pregabalin), and topical lidocaine (Dworkin et al., 2010). Despite the incomplete efficacy and treatment-limiting toxicities in current neuropathic pain medications, the mechanism of action of these treatments suggests that modulation of the maladaptive changes within the somatosensory nervous system is an effective therapeutic avenue for neuropathic pain (Yekkirala et al., 2017, Sindrup et al., 2005, Dickenson et al., 2002, Woodcock et al., 2007, Chen et al., 2018b, Rose and Kam, 2002).

Basic science research in the pain field is focused on understanding the underlying mechanisms of neuropathic pain to identify new targets for treating and ideally preventing neuropathic pain. Animal models of neuropathic pain have been our main tool for studying mechanisms of disease and exploring new therapeutic options in the modern era of pain research.

1.1.1.1 Animal models of neuropathic pain

The goal of developing rodent models of neuropathic pain is to mimic the human disease sufficiently enough to explore pathophysiological mechanisms, and screen for putative therapeutic interventions. Apart from the relatively small fraction of neuropathic pain cases that result from an identifiable central or peripheral nerve damage, other cases of this disorder are either idiopathic or are associated with complex disorders such as diabetes, cancers, viral infections, etc (Baron et al., 2010). Although animal models for central pain disorders and disease induced-neuropathic pain exist, the most used class of neuropathic pain models is a peripheral mononeuropathy which involves traumatic injury to a single neve, most commonly the sciatic nerve (Mogil, 2009). Wall and co-workers in the 1970s made the first attempt to produce a

chronic neuropathic pain model by transecting the sciatic nerve (Wall and Gutnick, 1974). Although a complete sciatic nerve transection was not a sustainable pain model, it revealed the unique pathophysiological properties of a damaged nerve and led to other nerve injury models in animals as surrogates for neuropathic pain. Constriction chronic nerve injury (CCI) and spared nerve injury (SNI) models are among the notable examples of a sciatic mononeuropathy model (Bennett et al., 2003, Decosterd and Woolf, 2000, Shields et al., 2003). In these models, the sciatic nerve undergoes loose constriction (CCI), or partial transection (SNI). The popularity of these models is largely because they are reproducible in rats and mice and generate robust neuropathic-like symptoms which can be quantified by nociceptive behavior tests. Moreover, as clinical research indicates, a lesion of the afferent pathways is necessary for the development of neuropathic pain, therefore, these models are logical for elucidating fundamental mechanisms in nociception and pain (Orstavik et al., 2003, Orstavik and Jorum, 2010). However, given the difficulties in interpretation of pain-related behavior in animals, and complications related to interspecies biological differences, it is impossible to evaluate to what extent the human disease with all its sensory and emotional aspects is reproduced in these animal models. Recent recognition of these apparent limitations in current preclinical pain models can be found in reviews by Mogil's group (Mogil, 2019, Mogil et al., 2010). Such limitations highlight the need for improving the current animal pain models to capture diverse biopsychosocial elements of human pain such as sex, genotype, and social communication.

1.2 Somatosensory nervous system

1.2.1 Introduction

As a part of the sensory nervous system, the somatosensory system is responsible for the perception of a wide range of sensory modalities such as pain, temperature, touch, and movement (Nelson, 2001). The first step towards perception of different sensory information is activation of primary afferents through specialized sensory receptors expressed in their peripheral terminals or in the associated sensory organs. The adequate intensity of sensory stimulus activates primary afferents and generates action potentials which represent the sensory input that is conveyed to the dorsal horn of the spinal cord (or the trigeminal nuclei of the brainstem for trigeminal sensory neurons). Neuronal networks in the spinal cord receive,

integrate, and process sensory input before it is relayed to the higher CNS regions. The sensory input that reaches the somatosensory cortex then produces the sensory perception associated with the initial stimulus (Wall and Dubner, 1972). Landmark discoveries in recent years have unravelled the tremendous amount of modulation and integration of sensory signals occurring at different parts of the central nervous system (CNS) including the dorsal horn of the spinal cord (Sharif et al., 2020, Kim et al., 2016b, Boyle et al., 2019, Feldman and Brecht, 2005, Prescott et al., 2014). These findings challenge the classical ideas that sensory modalities are coded by anatomically and physiologically discrete pathways that faithfully convey particular modalities of sensory information from the periphery to the somatosensory cortex (Ma, 2010). These emerging findings not only inform us about the enormous capacity of our somatosensory system for perceiving our environment but provides clues about how damage to this complex system can potentially trigger variable outcomes that may explain the heterogeneity of symptoms observed in neuropathic pain patients. The following is a brief review of the current state of knowledge about the healthy somatosensory nervous system with a focus on primary afferents and the spinal cord which are more relevant to the work presented in this thesis.

1.2.2 Peripheral somatosensory nervous system: from the skin to the spinal cord

Newly born sensory neurons delaminate from the dorsal neural tube or ectodermal placodes (head) and migrate along a ventral pathway to produce sensory cells in the dorsal root ganglion (DRG) for the body or trigeminal ganglion for most of the head region including the face. After crossing (CNS/PNS) transition zones, these cells commit to a sensory and neuronal fate and then progress through a series of specialization steps controlled by dedicated gene programs and environmental cues (i.e., axonal guidance molecules and neurotrophic signals) resulting in four broad sensory subtypes of primary afferents which are nociceptive, thermoceptive, mechanoreceptive and proprioceptive neurons (Marmigere and Ernfors, 2007). In the adult nervous system, primary afferents are pseudo-unipolar neurons that have both a peripheral and central axonal branch that innervates their target organ and the spinal cord, respectively. PNS glia, satellite cells, and Schwann cells surround cell bodies of primary afferents and their axons, respectively. Although satellite cell functions are largely unknown, based on their molecular characteristics (expression of ion channels, gap junctions, and different species of

purinoreceptors), they are thought to be involved in regulating neuronal function by sensing neighboring neuronal activity and modulating external chemical environment of DRG (Hanani and Spray, 2020, Vit et al., 2008, Spray et al., 2019). On the other hand, myelinating Schwann cells form an electrical insulator around large peripheral axons that allows rapid signal transmission, while non-myelinating Schwann cells are largely associated with small-diameter axons and are known to be involved in sensory transduction (Abdo et al., 2019). In the last decade, powerful tools and technologies revealed a remarkable genetic and functional diversity of primary afferents as well as their complex interactions with peripheral sensory organs (Li et al., 2011, Usoskin et al., 2015, Abraira and Ginty, 2013).

1.2.2.1 General classification of primary afferents

There were several early attempts to classify primary afferents. One anatomical study that stands out came from Lawson & Waddell in 1991, who divided these cells into two morphologically defined neuronal subtypes: the large light and the small dark neurons (Lawson and Waddell, 1991). Other studies put forward other classifications based on different criteria such as the primary afferent's peripheral target organ (for example, skin, vessels, and internal organs), conduction velocity (which is a function of their size and degree of myelination), response properties (including sensory modality, tuning properties, tonic firing rates, receptive field sizes, and the intensity of stimulus necessary to activate them), and neurochemical phenotype (such as peptide expression)(Todd, 2010, Abraira and Ginty, 2013). Although these classifications contributed largely to our current understanding of the peripheral nervous system, they failed to fully explain the functional heterogeneity among primary afferents. Recent advances in single-cell transcriptomics allowed

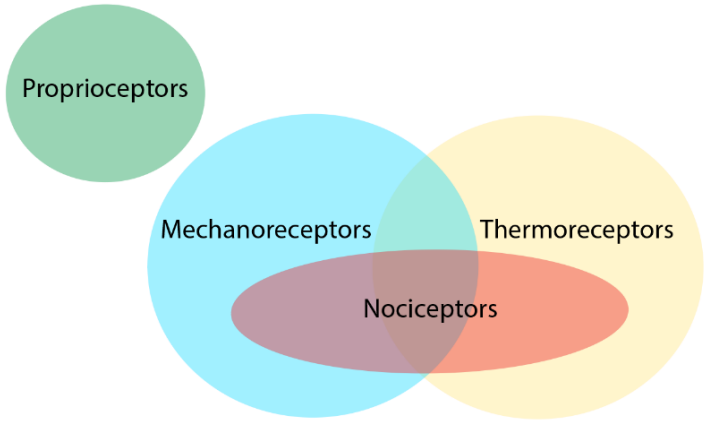

Figure 1 Classification of primary afferents

Venn diagram of different classes of primary afferents encoding somatosensory information showing different subtypes of modality-specific and polymodal primary afferents.

unbiased classification of DRG neurons based on their gene expression profiles (Usoskin et al., 2015). The latest studies of this nature revealed 18 molecularly and functionally distinct classes of neuronal types that can explain the genetic heterogeneity of modality-specific and polymodal primary afferents coding for different types of sensory information (Zeisel et al., 2018). Inspired from the classic and the most recent classifications of primary afferents, here, we divided DRG cells based on the nature of the stimuli that each afferent type transduces, into four major groups: nociceptors, thermoreceptors, mechanoreceptors, and proprioceptors (Figure 1). Within each group, we will briefly introduce geneticly distict, modality-specific, and polymodal subtypes.

1.2.2.1.1 Nociceptors

Originally described by Charles Sherrington, nociceptors are a specialized class of primary afferents that respond to potentially damaging stimuli (Sherrington, 1903, Sherrington, 1952). These DRG neurons are physiologically defined as high-threshold afferents because they are optimally activated at extremes in temperature, pressure, and through injury-related chemicals. The majority of known nociceptors are unmyelinated C or thinly myelinated Aδ that innervate the superficial layers (laminae I-II) of the dorsal spinal cord (Figure 2)(Todd, 2010).

On the peripheral front, nociceptors that code temperature have free nerve endings, whereas those that are sensitive to mechanical stimuli are associated with modified cutaneous Schwann cells (Abdo et al., 2019). A fraction of nociceptors, known as peptidergic afferents, have vesicles containing neuropeptides such as substance P and calcitonin gene related peptide (CGRP) that are released upon stimulation to cause physiological effects in the surrounding tissue known as ‘neuroinflammation’ (Julius and Basbaum, 2001). Based on their gene expression profile, peptidergic afferents can be classified into 5 subtypes (PEP1.1, PEP1.2, PEP1.3, PEP1.4, and PEP2). The other major class of nociceptors are non-peptidergic afferents which are genetically divided into 3 classes (NP1, NP2, and NP3). While NP1 neurons are shown to be important for noxious mechanical sensitivity, NP2, and NP3 are involved in non-noxious warm sensitivity and itch (Zeisel et al., 2018). Contrary to the modality specificity view (Zhang et al., 2013), emerging studies suggest that the majority of nociceptors are polymodal and activated by several classes of noxious stimuli (i.e., heat, mechanical and chemical stimuli), while a fewer number of subsets are

more specialized in their responses (Wooten et al., 2014, Julius, 2013, Wang et al., 2018) (Figure 1).

1.2.2.1.2 Thermoreceptors

Small changes in skin temperature are detected by thermoreceptors. These are C or Aδ fibers which have free nerve endings embedded in the skin and their central terminals projecting to the most superficial layers of the dorsal horn (LI) (Figure 2). Functionally, they can be divided into low- and high-threshold (nociceptive) afferents which are sensitive to different ranges of temperature changes across their receptive field. Besides their sensory role, low-threshold thermoreceptors constantly provide information about changes in body temperature and contribute to the homeostatic mechanisms which keep the body at an optimal working temperature. On the other hand, high-threshold thermoreceptors are often silent and only fire in the presence of potentially noxious thermal stimuli.

Small low-threshold cold-sensing neurons are primarily Aδ fibers (although cold-specific C fibers exist) and can be broadly identified by the expression of the putative cold sensor, the Trpm8 channel (McKemy et al., 2002, Bautista et al., 2007, Darian-Smith et al., 1973). These cold-sensing neurons are further divided into 3 modality-specific subtypes based on their gene expression profile (i.e., Trpm8.1, Trpm8.2, and Trpm8.3)(Zeisel et al., 2018). On average, they respond to a temperature around 15°C and show a constant level of activity below that temperature. At extreme temperatures (<0°C) (Simone and Kajander, 1997).

Conversely, sensation of warmth and heat has been classically attributed to C fibers. The low-threshold warm receptors signal temperatures between 30-45 °C, and high-threshold receptors respond mainly to temperatures higher than 45 °C (Schepers and Ringkamp, 2010). Genetically, two main classes of non-peptidergic primary afferents that code for non-noxious warmth sensation are characterized (i.e., NP2, and NP3) (Zeisel et al., 2018). NP3 neurons are also the only DRG neuron type that expresses Trpm2, an ion channel required for sensitivity to warmth, and therefore predicted to be involved in mediating ambient warm sensitivity (Tan and McNaughton, 2016). NP3 is also thought to have a role in transducing noxious pain (Morton et al., 1989). Additionally, noxious heat is coded by all peptidergic nociceptors which express heat-

sensitive Trp channel, TrpV1, which is known for its role in transducing heat hypersensitivity (Jordt and Julius, 2002).

1.2.2.1.3 Mechanoreceptors

At approximately the eighth week of gestation, the human fetus gains the ability to experience touch, the first sense that becomes functional during the embryonic development (Hooker, 1952). Different flavors of the sense of touch are coded by mechanoreceptors which innervate the deeper layers of the dorsal horn (LII-III) (Figure 2). These sensory neurons are classified as highly myelinated Aβ, thinly myelinated Aδ, or unmyelinated C fibers (Todd, 2010). The degree of myelination affects the axonal conduction velocities of these afferents and consequently, the transduction speed of sensory information which determines at what sequence sensory information reaches the CNS in an event that all these fibers are activated simultaneously. Cutaneous Aβ, Aδ, and C Low-threshold mechanoreceptors (LTMRs) are activated by weak, innocuous mechanical force applied to the skin and are referred to as Aβ-LTMRs, Aδ-LTMRs, and C-LTMRs, respectively (Abraira and Ginty, 2013). Genetically, Aβ-LTMRs can be divided in two broad classes of molecularly and functionally distinct neurons (i.e., NF2, NF3)(Zeisel et al., 2018). The NF2 neurons are rapidly adapting mechanoreceptors terminating as Meissner corpuscles in the skin, and as longitudinal lanceolate endings in hair follicles. NF3 neurons are slow adapting mechanoreceptors innervate in epithelial mechanoreceptive cells known as Merkel cells. Aδ-LTMRs are a genetically distinct class of LTMRs (NF1) classically known as direction-selective hairy skin mechanoreceptors because of their association with hair follicles (Abraira et al., 2017, Abraira and Ginty, 2013). Another genetically distinct class of LTMRs are known as C-LTMRs. These neurons express Slc17a8 (the gene encoding vesicular glutamate transporter 3) and contribute to the affective aspect of pleasant touch elicited by low mechanical forces (Seal et al., 2009).

High-threshold mechanoreceptors (HTMRs) are nociceptors that express mechanosensitive ion-channels such as Piezo2 along with molecular markers of nociceptors. These primary afferents primarily belong to the NP1 class of non-peptidergic nociceptors that express the Mas-related G protein-coupled receptor type D protein (Mrgprd), some sub-classes of polymodal peptidergic

fibers, and Aδ fibers (Ranade et al., 2014, Cavanaugh et al., 2009, Dussor et al., 2008). Although there is physiological evidence for the presence of A-HTMRs, not much known about their molecular properties (Burgess and Perl, 1967, Arcourt et al., 2017).

The central termination of mechanoreceptors within the laminar structure of the dorsal horn are loosely related to their functional class, with C-HTMRs fibers generally innervating the outermost laminae. C-LTMRs and Aδ-LTMRs terminate in lamina II and myelinated Aβ-LTMRs innervate deep dorsal horn laminae (Abraira et al., 2017) (Figure 2).

1.2.2.1.4 Proprioreceptors

While nociception, thermoception, and mechanoception allow us to perceive and react to stimuli originating outside and inside of the body, proprioceptive functions mediate the control of body position and balance. Proprioceptive neurons are a unique neuronal class of Aβ afferents that are functionally divided into 3 types (i.e., 1a, II, and 1b afferents innervating the muscle spindles and Golgi tendon organs) and are genetically represented by one proprioceptive neuron type, NF4 (Zeisel et al., 2018, Proske and Gandevia, 2012). Proprioceptive neurons in the DRG provide feedback information about muscle tension as well as joint position which allows for planning of movements. These neurons terminate in deeper layers of dorsal horn as well as in the ventral horn (Figure 2).

1.2.2.2 VGLUTs in peripheral neurons

Primary afferents are exclusively excitatory neurons and therefore express vesicular glutamate transporters (VGLUTs) in their central terminals. In the adult mammalian DRG, VGLUT1, VGLUT2, and VGLUT3 are expressed in A fibers, C fibers, and C-LTMRs, respectively (Brumovsky, 2013). This non-overlapping and modality-specific expression pattern of VGLUTs allows clear identification of central terminals of A fibers and C-LTMRs in the spinal cord. However, VGLUT2 is less useful for identification of C fiber terminals as it is also expressed by spinal excitatory interneurons.

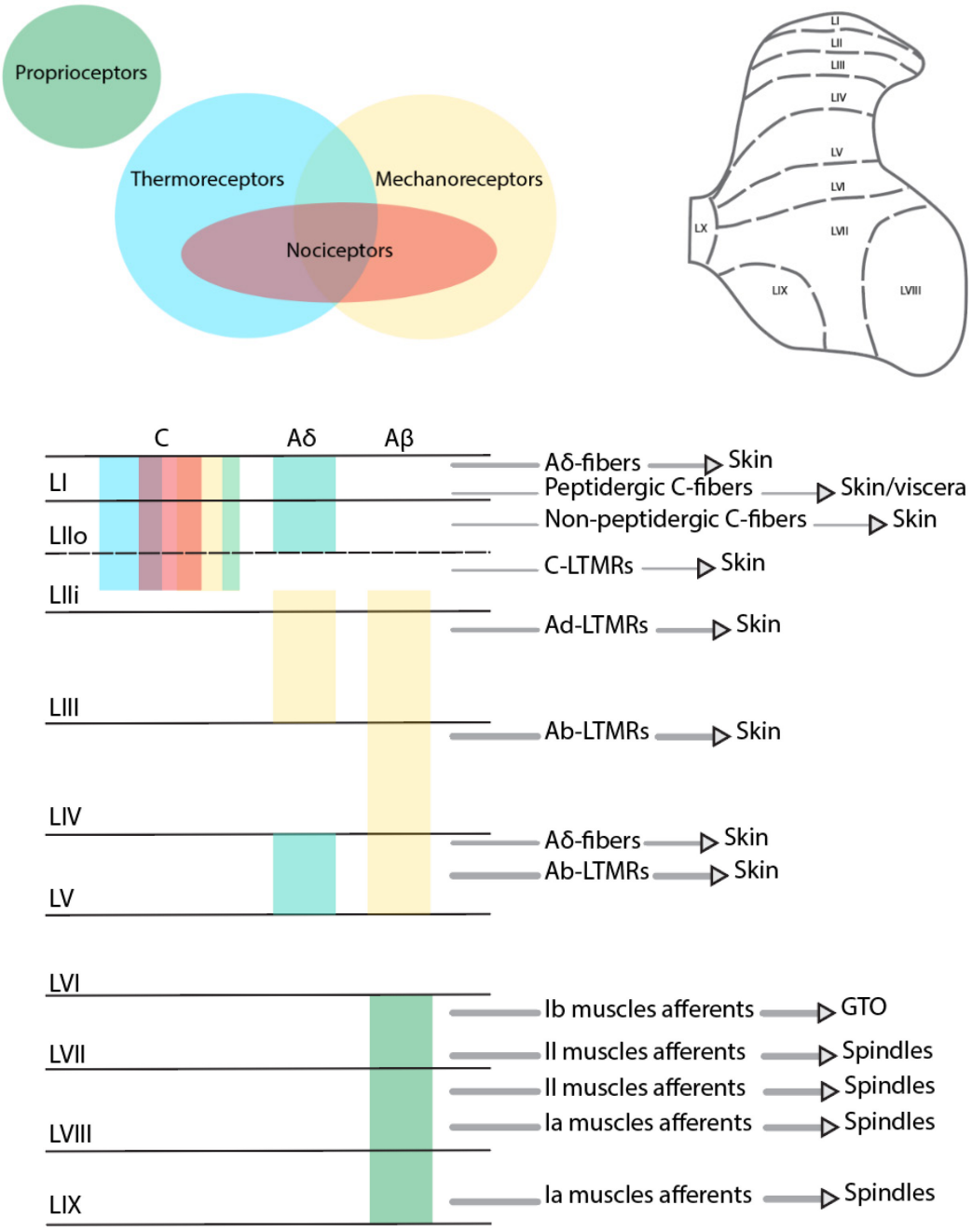

Figure 2 Central organization of different classes of primary afferents.

Simplified schematic representation of central termination patterns for different classes of primary afferents in the layers of the spinal cord. Primary afferents are categorized as Aβ, Aδ, and C, and can be further divided according to their responsiveness to different modalities. The spinal gray matter is divided into ten laminae (shown in the top left schematic). Different types of afferents (myelinated fibers were represented with thicker lines) encoding various modalities terminate in different laminae. Colors correspond to spatial recruitment of thermoreceptors (yellow), nociceptors (red), mechanoreceptors (blue) and proprioceptors (green).

1.2.3 Central somatosensory nervous system: from the spinal cord to the brain

Primary afferents convey a colourful range of sensory information which is integrated and processed at different levels of the spinal dorsal horn, brainstem, and cortex. There seem to be multiple anatomically distinct ascending and modulatory pathways for central transmission of different sensory modalities (Todd, 2010, Abraira and Ginty, 2013). Except for a subset of A-LTMRs that have direct access to the brainstem, other primary afferents feed sensory information to spinal cord projection neurons either directly or through spinal cord local circuitry (Brown, 2012). Like primary afferents, projection neurons, can be modality specific or polymodal. The majority of nociceptive projection neurons are in LI and receive monosynaptic inputs from Aδ and C fiber nociceptors (Choi et al., 2020, Todd, 2010, Luz et al., 2015). There are other projection neurons located in LI as well as in the deep dorsal horn which typically respond to both nociceptive and non-nociceptive inputs and therefore belong to the class of wide-dynamic-range (WDR) neurons (Wercberger and Basbaum, 2019, Lavertu et al., 2014). Non-nociceptive neurons exist in both LI and deeper laminae but little is known about their specific afferent inputs.

Non-nociceptive projections ascend through the dorsolateral white matter and synapse onto the lateral cervical nucleus (LCN) located at cervical levels C1–C3. From there, LCN second order neurons synapse onto targets in the dorsal column nuclei (DCN). The sensory input is then sent to the thalamus and projected to the somatosensory cortex. On the other hand, LI nociceptive projections ascend in the ventrolateral white matter and synapse onto several areas in the brain and brain stem. A fraction of nociceptive projection neurons transmits information to the somatosensory cortex via the thalamus to code information about the location and intensity of the noxious stimulus. Other nociceptive projection neurons recruit cingulate and insular cortical areas via connections in the brainstem and amygdala to code for the affective component of the pain experience (Browne et al., 2021, Brown, 2012).

Activation of nociceptive projection neurons can stimulate the descending pain modulating system which is composed of multiple parallel pathways. LI nociceptive projection neurons stimulate descending feedback systems that control the nociceptive output from the spinal cord through engaging the rostral ventral medulla (RVM) and the mid-brain periaqueductal gray

(PAG). These mid-brain structures also receive inputs originating from higher structures in the anterior cingulate gyrus (part of cortex), amygdala, and hypothalamus. Inhibitory control through PAG-RVM acts directly on the dorsal horn of the spinal cord and is mediated by serotonin, norepinephrine, and endogenous opioids. A second major descending pathway originates from locus coeruleus and adjacent regions of the pons and is primarily norepinephrinergic. Additionally, there are GABAergic/glycinergic projections from the ventromedial medulla that arborise throughout the dorsal horn (Brown, 2012, Abraira and Ginty, 2013, Todd, 2010, Basbaum et al., 2009, Wercberger and Basbaum, 2019). The balance between ascending and descending modulatory circuits is critical for normal transmission and regulation of sensory information

Finally, at the cortical level, a wide host of variables including the behavioral and environmental context, as well as competing sensory inputs affect the sensory perception. Specifically, for pain experience, other factors including emotional and motivational pain states, self-versus external induced pain, and controllability contribute to the intensity and the quality of pain perception the patient is experiencing which can vary between individuals with comparable amount of tissue damage they have sustained (Lumley et al., 2011).

1.2.4 Dorsal horn neural circuitry: beyond a relay station

The output of spinal projection neurons is a function of the inputs these neurons receive from primary afferents, and spinal cord local circuitry which is composed of locally projecting interneurons linked by a highly complex set of synaptic connections. Dorsal horn interneurons integrate, and process sensory information delivered by primary afferents and descending pathways. To understand what information processing at the level of the spinal cord entails, it is essential to know about the dorsal horn interneuron types, interconnections, and their relationships with projection neurons, and primary afferents. There have been numerous attempts to classify dorsal horn interneurons into discrete populations. Fundamentally, these interneurons are either inhibitory (GABAergic and/or glycinergic), or excitatory (glutamergic). Interneurons can be classified based on their morphology (e.g., islet cells, central, radial, and vertical), physiology (e.g., tonic, delayed, initial bursting, and single spiking action potential firing

in response to current injection), the expression of neurochemical markers (e.g., parvalbumin, PKCγ, substance P, among others), and genetic profile (Zeisel et al., 2018, Batti et al., 2016, Todd, 2010, Abraira et al., 2017).

Unfortunately, there is no integrated neuroanatomical and electrophysiological blueprint for spinal cord circuits and our knowledge is limited to small, isolated microcircuits. Much of what we know about the physiology, anatomy, and connectivity of dorsal horn interneurons is from the seminal studies of Perl and colleagues (Grudt and Perl, 2002, Lu and Perl, 2005, Narikawa et al., 2000, Lu and Perl, 2003, Zheng et al., 2010, Yasaka et al., 2007). They performed simultaneous whole cell patch clamp recordings in LII of spinal cord slices and identified synaptically connected interneurons as well as synaptic inputs from identified primary afferents (Figure 3).

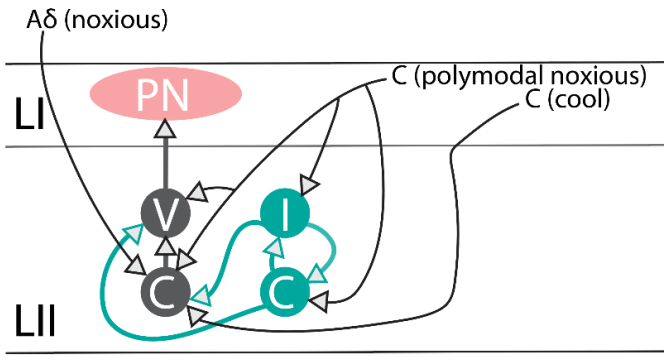

Figure 3 Functional connectivity in the superficial dorsal

LI projection neurons, excitatory neurons, and inhibitory neurons are depicted in red, black, and green, respectively. [Diagram based on data from the groups of Perl and Yoshimura]

Based on the studies by Lu and Perl, LI projection neurons receive input from deeper layers of the dorsal horn through excitatory vertical cells. These cells receive excitatory input from C and Aδ nociceptors and are inhibited through central and islet inhibitory interneurons. These inhibitory interneurons are activated by the same C nociceptors that activate excitatory vertical cells. These inhibitory cells not only control the flow of information through excitatory interneurons, but they also modulate each other's activity. The type of inhibition presented in this model is known as feed-forward inhibition which is a form of lateral inhibition. Feed-forward inhibition typically occurs when excitatory neurons (primary afferents in this context) excite inhibitory cells, which then inhibit a group of postsynaptic excitatory neurons excluding the initial excitatory neurons. Lateral inhibition increases sensitivity to spatially varying stimuli and results in activation of higher order neurons only when a strong and consistent signal is received

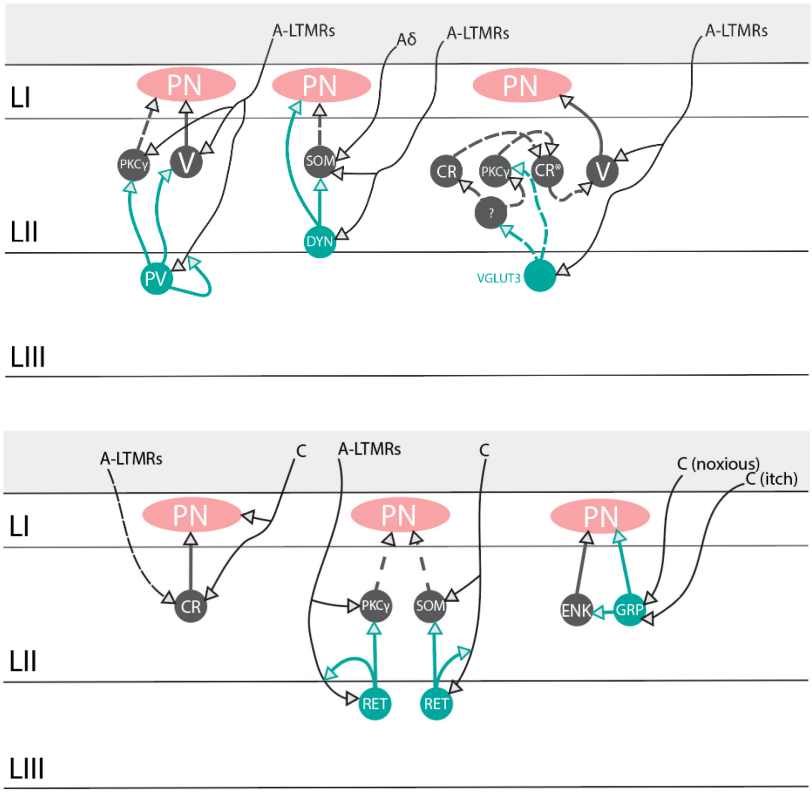

Figure 4 Microcircuits of the dorsal horn of the spinal cord.

Molecularly defined dorsal horn interneurons in the superficial dorsal horn. Circuit diagrams showing the proposed connectivity of molecularly defined classes of dorsal horn interneurons. LI projection neuron, excitatory neurons, and inhibitory neurons are depicted in red, black, and green, respectively. Dashed lines indicate that the connection was not directly demonstrated. Abbreviations: PN, projection neurons; V, vertical cells; PKCγ, protein kinase C gamma; SOM, somatostatin; CR, calretinin/Calb2; CR*, presumably calretinin/Calb2; DYN, dynorphin; RET, receptor tyrosine kinase Ret; ENK, enkephalin, GRP, gastrin-releasing peptide; A-LTMR, A-low-threshold mechanoreceptor.

(Zeilhofer et al., 2012). Inhibitory neurons can also participate in presynaptic inhibition through axo-axonic synapses with terminals of primary afferents (Ribeiro-da-Silva and Coimbra, 1982, Hughes et al., 2012, Zimmerman et al., 2019). The existence of such connections is well established for non-peptidergic C terminals and low-threshold primary sensory axon terminals. Inhibition on large-diameter afferents and small-diameter afferents relies on $GABA_A$ and NMDA receptors, respectively. Genetic deletion of these receptors in primary afferents causes mechanical hypersensitivity (Zimmerman et al., 2019). Given their key position in sensory transmission, it is likely that functional and structural alterations of axo-axonic inhibition on primary afferents terminals are implicated in neuropathic pain.

In the past few decades, many efforts have been made to build on the studies by Lu and Perl. Taking advantage of the mouse molecular genetics, neurochemically distinct classes of inhibitory and excitatory interneurons were identified and their connectivity, and roles in the sensory networks were explored (Figure 4) (Lu et al., 2013, Petitjean et al., 2015, Boyle et al., 2019, Duan et al., 2014, Peirs and Seal, 2016, Petitjean et al., 2019, Cui et al., 2016, Sun et al., 2017). However,

despite all these advances, our understanding of the functional motifs that build the dorsal horn circuitry structure has not gone beyond what was mapped by Lu and Perl (Figure 3). Therefore, we are still a long way from understanding exactly how superficial spinal circuits process sensory information. With respect to deeper dorsal horn circuits even more remains unknown due to technical challenges.

1.2.4.1 Dorsal horn glial cells: No longer underrated

Non-neuronal cells, named neuroglial cells or simply glia, account for more than half of cells in the mammalian CNS and regulate many aspects of CNS function including development, homeostasis, and plasticity. Like the rest of the CNS, the spinal dorsal horn hosts the 3 cardinal glial cell types: astrocytes, oligodendrocytes, and microglia. During embryogenesis, macroglia which are composed of astrocytes and oligodendrocytes arise from neural progenitor cells in the embryonic neural tube while microglia are generated in the yolk sac (Li and Barres, 2018).

In the adult CNS, astrocytes are the most abundant cell type and are classified as protoplasmic (gray matter) or fibrous (white matter). Protoplasmic astrocytes of the gray matter are highly ramified, containing endfeet that contact blood vessels, as well as terminal projections that surrounds neuronal structures, particularly synapses. This connection allows the transportation of nutrients from the blood to neurons for metabolic support and regulates blood flow to brain regions in response to neuronal activity. Additionally, astrocytes have long been known to support synaptic transmission, by removing and recycling neurotransmitters from the synaptic cleft and buffering extracellular potassium (Chung et al., 2013). More recently, astrocytes were shown to be involved in key aspects of structural plasticity such as controlling the formation and removal of synaptic connections (Liddelow et al., 2017).

The other macroglia, oligodendrocytes, are the central counterparts of myelinating Schwann cells in the PNS. Oligodendrocytes select axons with diameters above 0.2 µm and wrap their plasma membrane around neuronal processes. Besides myelination, axon-oligodendrocyte interactions in white matter tracts and within the gray matter provide neurons with fuel, and neurotrophic supply, and participate in sodium channel clustering, as well as waste removal (Zuchero and Barres, 2015, Kaplan et al., 1997).

Microglia, the resident macrophages of the CNS, maintain dorsal horn homeostasis with their processes constantly surveilling local territories and interacting with all surrounding cell types. As immune cells, microglial phenotype is dynamic and influenced by changes in the microenvironment in different physiological and pathological states (Zuchero and Barres, 2015, Hammond et al., 2019). In the developing CNS, microglia are actively involved in synapse remodeling and maturation of the neural network (Wu et al., 2015). In the mature CNS, microglia continue these constant close interactions with neural components, including synaptic clefts, but do not physically manipulate these structures (Nimmerjahn et al., 2005). Moreover, microglia also contribute to many CNS diseases through aberrant activation of inflammatory and synapse removal pathways (Li and Barres, 2018). These versatile responses are mediated by transcriptionally distinct microglia which have the potential to change over the course of development or in response to injury and disease (Hammond et al., 2019). Recent studies revealed that microglia regulate neuronal activity, similarly to inhibitory neurons, and are essential for protecting the brain from excessive activation in health and disease (Badimon et al., 2020). It was only very recently that experimental evidence emerged to support such close interactions between microglial activity and functional plasticity.

1.3 Pathophysiological mechanisms of neuropathic pain

Neural damage triggers a cascade of changes within the somatosensory system that initiates and sustains neuropathic pain. Typically, maladaptive changes in the periphery occur early in response to the initial neural injury and participate in the induction phase of neuropathic pain, while central changes develop later and contribute largely to the maintenance of the pathology. Understanding the mechanisms responsible for these maladaptive changes is necessary for developing effective treatments. Here, we overview the key mechanisms that are proposed to cause neuropathic pain.

1.3.1 Peripheral sensitization

In physiological conditions, primary afferents are specialized to respond to a certain type and intensity of sensory stimuli (Dhaka et al., 2006). However, in the presence of tissue damage and inflammation, some primary afferents such as nociceptors are capable of reducing their

threshold of activation and membrane excitability by increasing the production, transport, and membrane insertion of transducer channels and voltage-gated ion channels. This physiological response is a transient adaptation necessary for protecting the damaged tissue and to mediate repair processes (Hucho and Levine, 2007, Woolf and Salter, 2000). In patients with peripheral nerve lesion, the sensory thresholds of primary afferents remain low long after the initial injury is healed, which results in a phenomenon known as peripheral sensitization (Fields et al., 1998). In some cases, the decrease in sensory threshold can be so marked that a response is generated in the absence of an identifiable stimuli, resulting in spontaneous activity. Typically, spontaneous activity of primary afferents is mediated by ectopic action potentials generated at the site of injury with aborted axon growth (so called, “neuroma”), but it can also be initiated in the cell body of injured DRG neurons, as well as in neighboring intact DRG cell bodies and afferents (Amir et al., 2005, Bostock et al., 2005, Wu et al., 2002). Ectopic activity of primary afferents explains spontaneous pain, a neuropathic pain-specific symptom which is defined as shooting electrical attacks that last for seconds.

Sensory threshold and excitability of primary afferents are largely determined by the expression of voltage-gated ion channels. Neural injury triggers massive transcriptional and translational changes including altered regulation and modulation of a large array of ion channels important for pain transduction (i.e., TRPV1, Nav 1.3, Nav 1.7, HCN, KCNQ, and Cav 2.2)(Xiao et al., 2002, Uttam et al., 2018). Besides injury, other factors, such as signals generated from denervated sensory organs and Schwann cells, immune neuromodulators, neuropeptides, and growth factors, also contribute to translational and posttranslational changes in both injured and uninjured primary afferents (Wu et al., 2002, Jin and Gereau, 2006, Zhu and Oxford, 2007). Since ion channels mediate the increase in membrane excitability and contribute to the generation of ectopic firing, most of the current pharmacological strategies have focused on targeting these dysregulated channels in primary afferents (Costigan et al., 2002). Recent advances in human genetics and protein structure-based drug design opened some new and exciting avenues for identifying key target channels and designing more selective analgesics (Yekkirala et al., 2017).

Maladaptive functional changes in primary afferents are accompanied by remodeling of the peripheral terminals of sensory neurons and sympathetic fibers. This remodeling can be in the form of collateral sprouting which results from migration of undamaged afferents from neighboring territories into the denervated areas. Alternatively, damaged nerves can regenerate and take over new sensory areas resulting in regenerative plasticity (Kuner and Flor, 2017). To what degree these structural changes contribute to peripheral sensitization is unclear as the functional capacity of the sprouted fibers is not known due to technical limitations.

1.3.2 Central sensitization

Peripheral nerve damage and sensitization promote increased excitability and synaptic efficacy within the central nociceptive pathway that results in distortion or amplification of pain in a manner that no longer represents the qualities of peripheral noxious stimuli, but rather the functional states of central circuits. This phenomenon is referred to as central sensitization which was first used to describe long-lasting but reversible synaptic facilitation that occurs in the dorsal horn (Woolf, 2011). Later research revealed that similar synaptic changes occur in higher CNS areas of the nociceptive pathway including the amygdala, and prefrontal cortex which likely contribute to the prolonged cognitive and mood changes associated with neuropathic pain (Pedersen et al., 2007, Governo et al., 2006).

Both presynaptic and postsynaptic changes contribute to the increased excitability of central nociceptive pathways. Repeated or high-intensity nociceptive impulses resulting from peripheral nerve injury increase synaptic strength through synthesis and release of transmitters and neuromodulators, changes in the presynaptic calcium channel expression, downregulation of metabotropic G protein-coupled receptor including μ-opioid receptors, $GABA_B$, and adenosine receptors, as well as increased expression, and phosphorylation of postsynaptic N-methyl-D-aspartate (NMDA) receptors (Ultenius et al., 2006, Hendrich et al., 2008, Sharif-Naeini and Basbaum, 2011, Latremoliere and Woolf, 2009). A number of mechanisms associated with central sensitization have been clinically targeted in neuropathic pain but have dose-limiting side effects.

1.3.3 Centralization

Centralization theory hypothesizes that, after peripheral nerve injury, maladaptive changes originating in the periphery migrate centrally and permanently modify the way sensory information is meant to be processed in the CNS. These changes include structural alterations in synaptic circuitry, remodelling of primary afferent central terminals, degeneration of inhibitory interneurons, and alterations in the brain stem and cortical regulation of nociceptive transmission (Devor, 2006, Kuner and Flor, 2017). Emerging research on the mechanisms underlying centralization presents novel targets with disease-modifying properties to prevent the development of neuropathic pain (Yekkirala et al., 2017).

1.3.4 Dorsal horn disinhibition

Melzack and Wall put forward the gate control theory of pain in 1965 (Melzack and Wall, 1965). In their original model, non-nociceptive input was proposed to block nociceptive transmission through activation of spinal inhibitory interneurons which serve as the "pain gate". Although some of the predicated neuronal connections are now considered to be inaccurate, the gate control theory was the first to recognize the pivotal role of spinal inhibition in regulating the interactions between different sensory modalities in the physiological and pathological processing of pain. Later pharmacological work by Yaksh and colleagues provided direct proof for the contribution of fast inhibitory neurotransmission to dorsal horn pain control. They showed that suppressing inhibition by intrathecal administration of selective antagonists of $GABA_A$ or glycine receptors produced signs of mechanical hypersensitivity, which resembled those seen after nerve injury (Yaksh, 1989). More recently, targeted ablation, silencing, and activation of different classes of inhibitory interneurons further revealed that inhibitory neurons have a key role in pain (and itch) transmission (Foster et al., 2015, Zeilhofer et al., 2012).

It is now well established that dorsal horn inhibitory interneurons control sensory transmission through at least four specific mechanisms: 1) attenuation of the intensity of incoming nociceptive inputs to dorsal horn neurons to achieve an appropriate level of activation in response to painful stimuli; 2) muting the spontaneous activity in dorsal horn neurons including projection cells; 3) separating different modalities by preventing crosstalk between touch and pain processing

circuits; and 4) limiting the spatial spread of sensory information, allowing accurate identification of the body regions that correspond to a particular sensation. Loss of inhibition results in disruption of these mechanisms and can lead to the various symptoms of neuropathic pain (Sandkuhler, 2009).

Several different structural, molecular, and neurochemical mechanisms were proposed for spinal disinhibition after peripheral nerve injury. Some of these mechanisms involve presynaptic deficits in inhibition and can explain the reduced frequency of miniature inhibitory postsynaptic currents (mIPSCs). These presynaptic mechanisms include the selective loss of inhibitory interneurons due to apoptosis, depletion of presynaptic inhibitory neurotransmitters, reduced excitation of inhibitory interneurons, and reduced excitability of inhibitory interneurons (Scholz et al., 2005, Baba et al., 2003, Moore et al., 2002, Leitner et al., 2013, Schoffnegger et al., 2006, Boyle et al., 2019, Petitjean et al., 2015, Inquimbert et al., 2018). Although the available evidence for large-scale loss of inhibitory interneurons in the dorsal horn circuitry is still debated (Polgár et al., 2005), it is possible that such changes happen at a smaller scale for a subset of interneurons.

Dorsal horn inhibition is also affected by postsynaptic changes that result in a decrease in the effectiveness of inhibitory transmission and the amplitude of miniature IPSCs. Chloride (Cl^-) homeostasis in the postsynaptic neuron is a critical determinant of neuronal excitability as well as the strength and robustness of fast inhibitory transmission mediated by $GABA_A$ and glycine receptors. In the adult CNS, potassium-chloride cotransporter 2 (KCC2), the main regulator of intracellular Cl^- within CNS neurons, extrude Cl^- against its chemical concentration gradient resulting in low intracellular Cl^- concentrations. This allows influx of Cl^- through the activation of $GABA_A$ and glycine receptors which mediate inhibition through generating hyperpolarization or preventing depolarization mediated by concurrent excitatory inputs (i.e. shunting) (Doyon et al., 2016). In a seminal study, De Koninck and colleagues reported that peripheral nerve injury results in down regulation of KCC2 in lamina I neurons, leading to a dramatic rise in intracellular Cl^- concentrations (Coull et al., 2003a). This finding indicated that in the neuropathic state, opening of ionotropic GABA and glycine receptors will not produce sufficient hyperpolarization for effective inhibition of dorsal horn neurons. Although some shunting effects may remain because

of conductance of anions through open $GABA_A$ or glycine channels, it cannot compensate for the loss of hyperpolarizing current. Therefore, downregulation of KCC2 reduces the ability of the inhibitory circuitry to control neuronal spiking and puts dorsal horn neuronal circuits in a hyperactive state. In males, the signaling pathway that results in downregulation of KCC2 is mediated through the activation of Tropomyosin receptor kinase B (TrkB) expressed by spinal cord interneurons. Different lines of evidence suggest that brain-derived neurotrophic factor (BDNF), a TrkB-specific ligand, released from activated microglia is responsible for the alteration in KCC2 (Coull et al., 2005, Ferrini et al., 2013). The synthesis and release of BDNF from microglia is through a purinergic receptor P2X4-evoked increase in calcium and activation of p38-Mitogen-Activated Protein Kinase (MAPK) (Ulmann et al., 2008, Tsuda et al., 2003, Trang et al., 2009). To target this mechanism, different p38-MAPK kinase inhibitors and KCC2 enhancers have been developed and tested for their clinical efficacy in treating neuropathic pain (Gagnon et al., 2013, Yekkirala et al., 2017). Although many of the compounds that target P38-MAPK and KCC2 showed promising results in preclinical studies, not much is known about their effectiveness in the human condition.

1.3.5 Descending facilitation of dorsal horn circuitry

Generally, the activity of the descending modulating system is enhanced by injury to compensate for increased nociceptive inputs. However, there are reports suggesting possible disruption in the descending modulatory circuits that results in facilitation of nociception and maintenance of neuropathic pain. For instance, after nerve injury, descending tonic noradrenergic or serotoninergic inputs lose effectiveness or switch from inhibition to facilitation (Bee and Dickenson, 2008, Matsuzawa-Yanagida et al., 2008, Finnerup et al., 2021, Huang et al., 2019).

As a main physiological apparatus for controlling pain, descending modulatory systems are logical sites for targeting neuropathic pain. One of the main current treatments for neuropathic pain are amine uptake inhibitors and serotonin norepinephrine reuptake inhibitors which boost descending inhibition by increasing the levels of norepinephrine and serotonin. Moreover, PAG-RVM descending pathways are sensitive to opioids, cannabinoids, acetylcholine, substance P and

many other pro- and anti-nociceptive compounds which are important targets for drug design (Yekkirala et al., 2017).

1.3.6 Neuropathic pain in the brain

Chronic pain is associated with global changes in the brain which include massive functional and structural alterations in pain processing centers as well as other areas like amygdala, hippocampus, hypothalamus, ventral striatum, thalamus, and some areas within the cerebellum (Kucyi and Davis, 2015, Kuner and Flor, 2017, Schweinhardt and Bushnell, 2010). Earlier studies in primates provided the first evidence for modification of the cortical somatosensory maps as the result of nerve damage from amputation or deafferentation (Pons et al., 1991). Later, imaging studies in humans also showed comparable changes in sensory maps in patients with neuropathic pain (Makin et al., 2015). These gross structural changes are accompanied with functional changes, including abnormal activation patterns consistent with sensitization within and outside of the primary sensory pathway (Becerra et al., 2006). Like the spinal cord, evidence suggests that brain circuits are also impacted by an imbalance between excitatory and inhibitory neurotransmission in the chronic pain state (Pomares et al., 2020). However, less is known about the specific mechanisms underlying this global imbalance. We are only just beginning to unravel the specifics of the neural coding of chronic pain state in the brain.

So far, no brain areas or networks have been specifically and exclusively linked to any form of chronic pain. Many changes that are associated with chronic pain are not limited to the sensory aspect of pain processing and can also occur in many other states. For example, many abnormalities observed in chronic pain conditions are also present in depression, anxiety, and other situations. These sensory unrelated changes could explain chronic pain comorbidities such as mood disorders (Schweinhardt and Bushnell, 2010).

1.3.7 Neuroinflammation

Accumulating evidence suggests that non-neuronal cells such as immune cells and glia, play active roles in the pathogenesis of neuropathic pain. Nerve injury induces drastic changes in non-neuronal cells throughout the entire sensory pathway, and specific pharmacological and genetic inhibition of these cells reduce neuropathic pain behavior in animal models (Ji et al., 2016, Sorge

et al., 2015, Peng et al., 2016). Following nerve injury, activated macrophages clear cellular debris and communicate with T-lymphocytes through cytokine production and their surface antigens (Vicuna et al., 2015, Amaya et al., 2000, Koizumi et al., 2007). Cytokines released from macrophages and T-cells sensitize neurons, while stimulating Schwann cells, and satellite cells (Ji et al., 2016). Peripheral macrophage activation promotes retrograde degeneration of the distal end of injured axons (Wallerian degeneration) and formation of neuroma which appears to contribute to pain hypersensitivity (Vargas and Barres, 2007). Meanwhile, DRG satellite cells signaling through IL-1β among many factors promote expression and release of colony stimulating factor 1 (CSF1) from damaged primary afferents which recruits spinal microglia to the dorsal horn region close to the terminals of injured afferents (Guan et al., 2016, Lim et al., 2017). Microglial CSF1 receptor (CSF1R) signaling through Src-family kinases (SFKs) can activate DAP12-dependent signaling and trigger myeloid cells 2 (TREM2) receptors which either directly or indirectly induces the expression of several key genes, including DAP12, IRF8, IRF5, P2RX4 and CX3CR1, TNFα, IL1 β, BDNF and CTSS. The expression of these genes generates a functionally active state of dorsal horn microglia and contributes to the induction and maintenance of neuropathic pain by altering neuronal function (Tsuda, 2016). In parallel with microglial activation, spinal astrocytes proliferate through activation of transcription 3 (STAT3)-mediated signaling (Tsuda et al., 2011). Although there is no evidence suggesting an irreversible phenotypic transformation of cytotoxic astrocytes, several lines of evidence support a role of astrocytes in the pathogenesis of pain through signaling with other CNS cells and alterations in their fundamental homeostatic functions (Ji et al., 2019).

Despite the diversity of immune and glial cells, they use many common neuromodulatory substances in response to nerve injury which either promote or diminish pain depending on the specific nature of the mediators involved. Most glial cells broadly express pro-nociceptive mediators which include pro-inflammatory cytokines (i.e., IL-1β, TNFα, IFNγ) and anti-nociceptive mediators such as anti-inflammatory cytokines (i.e., IL10, IL4). There are other pro-nociceptive mediators that are more cell specific. For instance, oligodendrocytes express IL33 in the periphery and microglia release BDNF in the dorsal horn of the spinal cord whereas astrocytes have the most diverse secretory profile among glial cells. In addition to anti-nociceptive agents

such as IFN-α, astrocytes release different pro-nociceptive molecules including matrix metalloproteinases, matrix proteins (TSP-4), pro-inflammatory chemokines (CCL2, CX3CL1), ATP, and glutamate (Ji et al., 2016, Zarpelon et al., 2016, Kim et al., 2016a, Tsuda, 2016). These mediators affect neuronal gene expression and function ultimately contributing to both peripheral and central sensitization. Based on the mounting basic science evidence, there is a growing interest in glial cells and their mediators as promising therapeutic targets for treating neuropathic pain.

1.3.7.1 Microglial signaling pathways in neuropathic pain

In 1975, Gilmore *et al.* noticed that peripheral nerve injury induced a significant increase in the number of non-neuronal cells within the dorsal horn. These cells were later identified as microglia and became a main focus in pain research over the following decades (Gilmore, 1975). There are over 40 microglia-specific molecules that contribute to the pathophysiology of neuropathic pain, and details of several microglial signaling pathways have been uncovered in rodent models of neuropathic pain (Tsuda, 2016).

In general, nerve injury induces upregulation of microglial surface receptors including different types of purinergic receptors, cytokine and chemokine receptors, as well as many others that are important for microglia interaction with the extracellular matrix and phagocytic activity. Downstream to these signaling receptors, there are key enzymes and protein kinases that are activated and result in gene expression changes that govern cellular alterations (i.e., cell cycle and morphological alterations) and production of potent diffusible factors that modify the activity of the surrounding cells. Here, we briefly overview key microglial pathways that are activated in neuropathic pain pathology.

1.3.7.1.1 Microglial key pro-inflammatory pathways

Key pro-inflammatory cytokines, IL-1β, and TNFα, are primarily produced by microglia through Toll-like receptor 2 (TLR2) activation via nuclear factor-κB (NF-κB) signaling pathway (Heneka et al., 2014, Masuda et al., 2012). Along with microglial P2X7 receptors, TLR2 also promotes post-translational processing of IL-1β which is a critical regulatory step. P2X7 also signals through p38 MAPKs to control the release of the lysosomal cysteine protease cathepsin S (CTSS) which can

cleave membrane-bound fractalkine (CX3CL1) on dorsal horn neurons (Clark and Malcangio, 2014, Clark et al., 2015). Soluble CX3CR1 activates CX_3C-chemokine receptor 1 (CX_3CR1) on microglial cells and leads to more IL-1β secretion and likely increases in other CX_3CR1 dependent activities such as phagocytosis (Limatola and Ransohoff, 2014, Gunner et al., 2019). Released IL-1β also acts on dorsal horn neurons to enhance glutamate-dependent excitatory synaptic transmission.

In addition to TLR2, nerve injury can promote microglial TNFα production through P2X7, P2Y12, CX_3CR1, and receptor tyrosine-protein kinase erbB2 (ERBB2) activation via p38 MAPKs signaling (Inoue et al., 2018, Jin et al., 2003, Tsuda et al., 2004). TNFα release results in dorsal horn hyperexcitability through acting on dorsal horn neurons (Kawasaki et al., 2008, Kronschläger et al., 2016), terminals of primary afferents (Park et al., 2011), microglia (Liu et al., 2017), astrocytes (Liddelow et al., 2017) and endothelial cells (Kanda et al., 2017).

Given the importance of the TLR2 receptor and p38 MAPK signaling in promoting pro-inflammatory pathways in microglia, the effect of TLR4 and p38 MAPKs inhibitors have been tested on neuropathic pain in preclinical settings (Bettoni et al., 2008, Taves et al., 2016). Interestingly, targeting these pathways is only effective in male mice, suggesting a sexual dimorphism associated with the contribution of pro-inflammatory signaling to the pathophysiology of neuropathic pain (Sorge et al., 2011, Taves et al., 2016).

1.3.7.1.2 Microglial signaling pathway regulating KCC2

Following peripheral nerve injury, activated microglia increase the expression of interferon regulatory factor 8 coding gene (*Irf8*) via CSF1R signaling (Guan et al., 2016). IRF8 directly regulates *Irf5* expression (Masuda et al., 2014). IRF5 is then translocated to the nucleus and binds to the promoter region of *P2rx4* gene to induce its expression (Masuda et al., 2014). CC-chemokine ligand 21 (CCL21) released from primary afferents is also reported to be involved in P2X4 upregulation through microglial-specific CCR gene (Biber et al., 2011). P2X4 is activated by extracellular ATP released by dorsal horn interneurons in response to nerve injury (Masuda et al., 2016) and promotes the production and release of BDNF which can bind to TrkB receptor on dorsal horn neurons and cause hyperexcitability via downregulation of KCC2. TrkB signaling also

potentiates glutamatergic excitation via receptors (NMDARs) (Coull et al., 2005, Hildebrand et al., 2016, Trang et al., 2009). The net result of the microglial P2X4–BDNF–TrkB signaling pathway is dorsal horn hyperexcitability which underlies mechanical hypersensitivity, a symptom of neuropathic pain.

Although KCC2 downregulation was reported in both male and female preclinical models of neuropathic pain (Mapplebeck et al., 2019), the P2X4–BDNF–TrkB pathway seems to be male specific (Sorge et al., 2015). Further studies are needed to unravel the signaling pathways upstream to KCC2 downregulation in females.

1.3.7.2 Beyond the known microglial functions

In recent decades, an accumulating body of literature has provided compelling evidence for the importance of microglia in the pathophysiology of neuropathic pain. However, there remains several core microglial functions that are much less studied in the pain field. Axonal remodeling and synapse pruning is an important microglial role that is essential for normal development of the nervous system. In recent years microglia-mediated synapse pruning pathways were discovered to be reactivated in various neurological disorders. This body of work is primarily focused on understanding the synapse pruning activity of microglia in neuropathic pain.

1.4 Synapse pruning

During development, there is an excess of synapses and neurons that are produced within the nervous system. To achieve an optimized neuronal architecture, excess synapses and neurons are selected for and later removed in an activity-dependent process known as synapse pruning (Neniskyte and Gross, 2017, Hong and Stevens, 2016). In the mammalian CNS, synaptic pruning occurs during two temporally distinct neurodevelopmental phases through the activity of glial cells, including microglia and astrocytes (Johnson and Stevens, 2018). The selectivity of this process depends on various signaling molecules, including those mediating glial chemotaxis, target recognition molecules and receptors, as well as the molecular machinery of phagocytosis. Although mechanisms underlying synapse pruning are still being uncovered, several signaling pathways were described in different neuronal systems during postnatal development in

rodents. The following is a brief review of the current state of knowledge regarding the molecular mechanisms involved in synapse pruning within the mammalian CNS.

1.4.1 Microglia mediated synapse pruning pathways

Dynamics of synaptic density throughout human lifespan was initially described by Peter Huttenlocher in the 1970s. He reported that synaptic density in the human cerebral cortex increases rapidly after birth, peaking at about 2 years of age, and then drops significantly during adolescence (Huttenlocher, 1979). About 4 decades later, Cornelius Gross noticed an upregulation of a neuro-immune signaling molecule called fractalkine during the peak of synaptic maturation in mice. Fractalkine (CX3CL1) is produced by neurons to communicate with microglia, the primary immune cell that express fractalkine receptor, CX3CR1 (Paolicelli et al., 2011a). This observation initiated a series of studies that investigated the role of microglia in synapse pruning. In fact, CX3CR1 signaling was shown to be critical for recruiting microglia to the hippocampal and cortical areas and microglia-mediated synaptic pruning (Gunner et al., 2019, Harrison et al., 1998). Another key mechanism through which microglia eliminate extra synapses was first reported in the developing visual system (Stevens et al., 2007). In this model, the key initiating signal was mediated by complement proteins, components of the innate immune system that bind to unwanted materials and microbes and flag them for removal by phagocytes. During different phases of postnatal development, complement components C1q, C4, C3 localize to weak synapses and promote engulfment of synaptic material through interaction with the

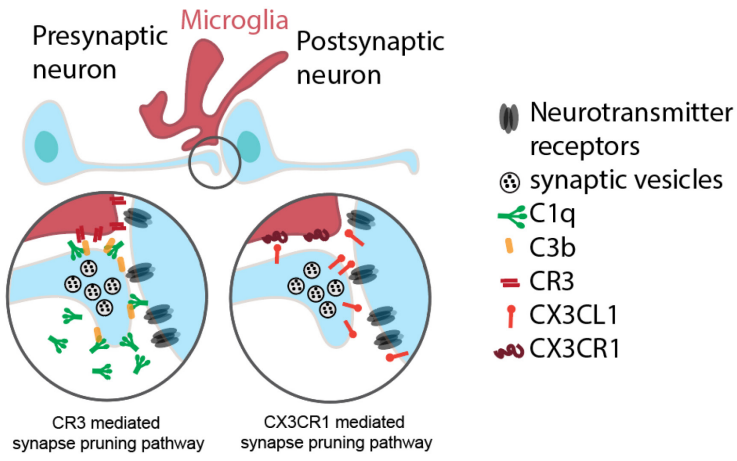

Figure 5 Major synapse pruning pathways in the CNS

The schematic illustrates microglia and neuronal molecules that participate in CR3 and CX3CR1 pruning mechanisms. In the CR3 synapse pruning pathway, complement component C1q induces the formation of C3b through the activation of the classical complement pathway. C3b induces engulfment of synapses by microglia expressing complement receptor 3 (CR3). Microglial CX3CR1 synaptic pruning is through signaling of CX3CL1, and chemokine receptor 1 (CX3CR1).

microglia-specific C3 receptor (CR3)(Schafer et al., 2012) (Figure 5). Other microglia-specific phagocytic receptors such as TREM2 and P2Y12 are reported to be important for synapse pruning (Neniskyte and Gross, 2017). However, details about their signaling pathways and their specificity to synapse pruning are less understood.

Until recently, the role of microglia in sculpting synaptic networks was thought to involve a full elimination of synapses through the engulfment and phagocytosis of synaptic inputs. Recently, a more sophisticated role for microglia was described which is called trogocytosis. In this process, microglia do not prune full synapses, but only "nibble" presynaptic structures to sculpt the presynaptic input (Weinhard et al., 2018). The signaling pathways that govern different microglial interactions with synapses and the functional consequence of synaptic trogocytosis remain to be understood.

1.4.2 Astrocyte mediated synapse pruning pathways

Following the discovery of the role of microglia in synapse pruning, research focused on investigating the contribution of other glial cells to synapse pruning. Astrocytes were found to have a crucial role in regulating synapse elimination in addition to providing trophic support for neuronal growth and synapse formation. This dual role of astrocytes in formation and removal of synapses during development is essential for normal organization of the neuronal network. For instance, the glycoprotein hevin (SPARCL1), which is secreted by astrocytes and localizes to excitatory synapses is required for the formation of excitatory inputs in the visual cortex, but in certain conditions it also regulates the removal of some of these connections (Singh et al., 2016, Kucukdereli et al., 2011).

One of the key phagocytic signaling pathways in astrocytes is mediated through astrocyte-specific phagocytic receptor Proto-oncogene tyrosine-protein kinase MER (MERTK). Mice lacking this receptor have comparable deficiencies in their visual synaptic network to complement-deficient mice (Chung et al., 2013). Interestingly, ablating MERTK promotes a transient increase in microglial engulfment, suggesting that microglia can compensate for reduced astrocyte phagocytosis. The collaborative role of microglia and astrocytes in synapse elimination was also reported in other signaling contexts and CNS regions (Vainchtein et al., 2018).

Astrocytes may also mediate synapse elimination through mechanisms other than phagocytosis. For instance, astrocytes release ATP in response to neuronal activity through inositol 1,4,5-trisphosphate receptor signaling which contributes to the normal process of synapse elimination during development (Yang et al., 2016).

Although our current molecular knowledge on how astrocyte–synapse interactions are mediated is still very limited, these studies show that alongside microglia, astrocytes have a powerful ability to construct and shape the synaptic network.

1.4.3 Neural activity and “eat me” signals regulate synapse pruning

Glia-mediated synapse pruning is regulated by neuronal activity. In the visual system, visual experience appears to modify the phagocytic capacity of microglia in the visual cortex. Both light deprivation and application of tetrodotoxin (TTX), an action potential blocking agent, during the refinement period disrupts microglial and astrocytic dependent mechanisms underlying normal synapse pruning (Hashimoto and Kano, 2003, Schafer et al., 2012, Chung et al., 2013, Favuzzi et al., 2021, Tremblay et al., 2010a).

Besides neuronal activity, expression of molecular cues known as “eat me” signals at the presynaptic sites enable recognition and removal of synapses by microglia. Multiple studies have identified a role for local caspases and initiation of the ubiquitin-proteasome system in synapse elimination. In addition, other molecular cues, such as ATP, UDP, TGFβ, and CX3CL1 have been implicated in the removal of transient synaptic structures. Recently, extracellularly exposed phosphatidylserine (PS) was characterized as an important neuronal "eat-me" signal involved in microglial-mediated pruning (Gyorffy et al., 2018, Scott-Hewitt et al., 2020, Elward and Gasque, 2003, Bialas and Stevens, 2013). However, is it does not clear how changes in neuronal electrical activity is converted to these “eat me” signals at the synaptic site.

1.4.4 The role of synapse pruning in neural diseases and injury

Recent influential studies have reported dysregulation or reactivation of synapse pruning pathways in multiple human neurobiological diseases (Hong et al., 2016b). It appears that synaptic dysfunction, dysregulation of immune gene expression, and abnormalities in glial function are common mechanisms shared between many neurodevelopmental and

neurodegenerative diseases. Recent genetic, neuroimaging, and animal model studies implicate synaptic pruning activities in neurodevelopmental diseases such as autism spectrum disorders (ASDs), and schizophrenia (Sekar et al., 2016, Schafer et al., 2016, Andreasen et al., 2011, Sellgren et al., 2019). Similarly, several lines of evidence collected from patients and animal models suggest that glia-mediated synaptic elimination may be operating in the neurodegenerative pathologies including multiple sclerosis, frontotemporal dementia, as well as Alzheimer's, and Parkinson's diseases (Hong et al., 2016a, Lui et al., 2016, Werneburg et al., 2020, Helton et al., 2008). Although it is not clear whether initiation of pruning pathways contributes to the disease progression or is just a collateral response to the existing neuronal pathology, targeting synapse pruning pathways were shown to have promising therapeutics effects in animal models of neural diseases giving new hope for discovering a novel class of disease biomarkers as well as potential therapeutic targets.

1.5 Thesis rationale and objectives

As treating neuropathic pain remains to be a clinical challenge, patients continue to suffer from the various debilitating, sometimes unbearable symptoms. Management of neuropathic pain requires more effective pharmacological approaches with disease-modifying capacity that are based on pathophysiological mechanisms underlying the sensory signs.

As described above, there is mounting evidence for spinal structural plasticity and reorganization in chronic pain. Given the key role of the spinal cord in normal transmission of sensory information, in this body of work we comprehensively characterize dynamics of dorsal horn synaptic density, the structural basis of neural coding, in different models of neuropathic pain. To find the mechanism underlying dorsal horn synaptic changes, we explore the role of microglia in dorsal horn circuitry remodeling and introduce a mechanism-based intervention for neuropathic pain. Finally, to build a foundation for future cell-specific studies of our proposed mechanism, we present an imaging-based approach to assess cell specific dynamics of pre- and post-synaptic structural changes in dorsal horn neurons.

The main hypothesis of this thesis is that: *Nerve injury-activated microglia mediate structural changes in dorsal horn synaptic circuitry. These changes result in loss of dorsal horn inhibition which contributes to the maintenance of neuropathic pain.*

To address this hypothesis, the following specific aims were followed in preclinical models of neuropathic pain:

1. Investigate the role of microglia in removal of dorsal horn synapses.
2. Demonstrate the molecular mechanisms for synapse pruning.
3. Investigate structural changes that drive inhibition in dorsal horn interneurons.

Chapter 2

Microglia Mediate Loss of Spinal Cord Inhibitory Synapses in Neuropathic Pain

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2.1 Abstract

Spinal dorsal horn disinhibition has been implicated in neuropathic pain, a debilitating condition resulting from damage to the nervous system. Loss of dorsal horn inhibitory synapses has been proposed to contribute to the reduction of inhibitory tone in neuropathic pain. However, how nerve injury affects inhibitory synapses in relation to other synaptic populations and mechanisms mediating synaptic loss remain unknown. Using a nerve injury rodent model of neuropathic pain, we show that microglia selectively engulf synapses intrinsic to the dorsal horn, but not terminals of primary afferents. Furthermore, we reveal that microglia specifically remove complement-tagged inhibitory synapses that are presynaptic to dorsal horn interneurons and terminals of primary afferents. Inhibiting or depleting microglia prevented inhibitory synapse loss without affecting excitatory synapses and attenuated pain behavior at chronic stages of neuropathic pain. These results define a new role for microglia in neuropathic pain pathogenesis: the selective pruning of spinal inhibitory synapses.

2.2 Introduction

Neuropathic pain is a type of severe chronic pain that is caused by a lesion or disease of the nervous system and is often resistant to treatment. Affected patients suffer from a range of debilitating sensory abnormalities which are driven by a variety of pathological changes in neural circuits responsible for processing and transmitting sensory information (Bouhassira et al., 2008). Important for normal integration of sensory information (e.g., the discrimination of pain, touch, and itch), complex circuitries of inhibitory and excitatory interneurons in the spinal dorsal horn (DH) receive and process a variety of sensory inputs (Abraira et al., 2017, Melzack, 1965). Based on insights gained from peripheral nerve injury (PNI) models of neuropathic pain and targeted modulation of inhibitory neurons, loss of inhibition (disinhibition) in the DH is thought to substantially contribute to the development of pain hypersensitivity (Foster et al., 2015, Prescott et al., 2014). Specifically, altered functional plasticity of DH inhibitory circuits has been demonstrated (Coull et al., 2003b, Sandkuhler, 2009) and evidence suggests a reduction in the number of DH inhibitory synapses (Lorenzo et al., 2014, Lorenzo et al., 2020, Batti et al., 2016, Petitjean et al., 2015, Bailey and Ribeiro-da-Silva, 2006). However, little is known about the effect

of nerve injury on different DH synaptic populations and, especially, the mechanisms underlying synaptic loss (Kuner and Flor, 2017) remain elusive.

In recent decades, studies have provided compelling evidence for the involvement of microglia, the tissue-resident macrophages of the central nervous system (CNS), in the development and maintenance of neuropathic pain (Inoue and Tsuda, 2018). Through the release of bioactive, diffusible modulators, activated DH microglia have been shown to contribute to altered sensory transmission in neuropathic pain (Coull et al., 2005, Clark et al., 2013, Echeverry et al., 2017, Yao et al., 2016). A classic microglial function, synapse pruning, is well studied in the context of CNS development, and was recently shown to be mediated by the complement system (Schafer et al., 2012). Microglial synapse pruning has also been implicated in CNS disorders that are associated with extensive synapse loss, such as Alzheimer's disease, schizophrenia, and autism (Hong et al., 2016a, Schafer et al., 2016, Sekar et al., 2016, Vasek et al., 2016, Lui et al., 2016). Notably, it remains unknown if microglia play a role in the process of DH synapse loss in neuropathic pain.

In this study, we tested the hypothesis that microglia participate in DH disinhibition and contribute to neuropathic hypersensitivity by removing complement-tagged inhibitory synapses. Using an experimental rat model of neuropathic pain, we performed a quantitative analysis to assess the effect of PNI on the density of five non-overlapping types of DH synapses. Our analysis included inhibitory and excitatory synapses primarily formed by spinal interneurons, and excitatory synapses formed by primary afferents. Then, we assessed microglial engulfment of presynaptic elements for each synaptic population and inhibited microglia to test for an effect on pain behavior and synapse density. Finally, we investigated the association between complement proteins and spinal cord synapses to find clues for the selectivity of microglia-mediated synaptic loss in neuropathic pain.

We showed that PNI induces a preferential elimination of a subset of excitatory synapses formed by primary afferents and inhibitory synapses of DH interneurons. Remarkably, we found that microglia are only responsible for the loss of inhibitory synapses as targeting microglia prevents both loss of inhibitory synapses and pain-related behavior, without affecting excitatory synapses. Consistent with the finding of selective pruning, we found that complement factors were

upregulated and selectively tagged inhibitory synapses after nerve injury, sparing excitatory synapses. This previously unknown role of microglia can cause long-lasting deficits in DH inhibition and contribute to the development of hypersensitivity following nerve injury.

2.3 Methods

2.3.1 Animals

Experiments were performed on male Sprague Dawley (Charles River) rats (weighing 200 g). Rats were kept on a 12-hour light/dark cycle with food and water available *ad libitum*. All animal experiments were performed in accordance with institutional and Canadian guidelines and approved by the McGill University Institutional Animal Care and Use Committee.

2.3.2 Pain-related behavior

Pain-related behavior was assessed at the baseline and different time points post-cuff surgery using von Frey filaments (Chaplan et al., 1994b, Dixon, 1980). Rats (n=8-10) were placed on a metal grid floor and were allowed to habituate to the testing environment for an hour prior to testing. Von Frey filaments were applied to the plantar surface of the hind paw and responses were recorded using the up-and-down method to measure the 50% withdrawal threshold (Dixon, 1980). Mechanical allodynia on the ipsilateral side was considered as a significant reduction in withdrawal threshold when compared to contralateral measurements.

2.3.3 Peripheral nerve injury

One day following baseline behavioral testing, an incision was made in the lateral left thigh under isoflurane anesthesia and the sciatic nerve was exposed to perform the cuff variation of chronic constriction nerve injury. The cuff, consisting of a 2 mm piece of split PE-60 polyethylene tubing with an inner diameter of 0.76 mm (Intramedic PE-60, Fisher Scientific, Canada), was placed around the sciatic nerve. The sham control group received a similar surgery without dissection of the nerve (Lorenzo et al., 2014, Pitcher et al., 1999).

2.3.4 Drugs

Minocycline hydrochloride (30 mg/kg, i.p.; Sigma-Aldrich) was dissolved in 0.1 M PBS, pH 7.4, and administered to rats at the day of nerve injury and every 12 h until the end of experiment. To

avoid an acute effect of minocycline on pain behavior, the drug was administered at least 8 h before behavior sessions. PLX3397 (0.2 mg, i.t.; Plexxikon Inc.) was dissolved in 10% DMSO + 45% PEG 300 + 5% tween+ddH2O at 20 mg/ml, i.t. injections (10 µl over 30 s under isoflurane anesthesia) started one day before the surgeries and were repeated every two days until the end of experiment.

2.3.5 Tissue preparation and immunohistochemistry for light microscopy

At 10 and 20 days post-surgery, animals were anesthetized (0.3 ml/100g of body weight of Equithesin containing 6.5 mg of chloral hydrate and 3 mg sodium pentobarbital), and perfused transcardially with perfusion buffer followed by 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (PB), pH 7.4, for 30 min. After fixation, spinal cords were extracted and post-fixed in the same fixative overnight at 4°C and then transferred to 30 % sucrose in PB for cryoprotection. For immunohistochemistry, transverse spinal cord sections from the lumbar enlargement (L4–L6) were cut on a cryostat (Leica, Germany) at −20 °C. Twenty-five-µm-thick sections were prepared for Airyscan confocal microscopy and 10-µm-thick sections were prepared for Structural Illumination Microscopy. Sections were then permeabilized with 0.2% Triton-X in 0.01M PBS (PBST) and blocked for 1 hour at room temperature in 10% normal donkey or goat serum. Sections were incubated in a cocktail of primary antibodies in 5% blocking solution diluted in PBST for 12 h at 4°C. Primary antibodies were rabbit anti-Iba1 (Wako, 1:1000), guinea pig anti-Iba1 (Synaptic Systems, 1:500), mouse anti-CD68 (Bio-Rad, 1:500), mouse anti-vesicular GABA transporter (VGAT, Synaptic Systems, 1:1000), rabbit anti-VGAT (Synaptic Systems, 1:2000), mouse anti-CD11b (Bio-Rad, 1:100), guinea pig anti-vesicular glutamate transporter 1 (VGLUT1, Millipore, 1:1000), guinea pig anti-VGLUT2 (Millipore, 1:2000), rabbit anti-VGLUT3 (Synaptic Systems, 1:1000), mouse anti-gephyrin (Synaptic Systems, 1:400), rabbit anti-Homer1 (Synaptic Systems, 1:200), mouse anti-Homer1 (Synaptic Systems, 1:200), mouse anti-GFAP (Cell Signaling, 1:1000), goat anti-rat C3 (MP Biomedicals, 1:100) and rabbit anti-C1q (Abcam, 1:400). Primary antibody labelling was detected using species-specific secondary antibodies conjugated to Alexa 488, Alexa 568, and Alexa 647 (Invitrogen, 1:800, incubated at room temperature for 2 hours). Sections were mounted on gelatin subbed slides and coverslipped using Prolong Gold Antifade mounting medium (Invitrogen) and Zeiss cover slips.

Microglia were also labelled with rabbit anti-Iba1 (Wako, 1:2000) antibody using a brightfield protocol for morphometric analysis. For this, endogenous peroxidase was quenched with 10% methanol and 3% H_2O_2 for 30 minutes. After 3 washes with PBST, sections were blocked with 10% donkey serum for 1 hour and then incubated in the primary antibody solution overnight at 4 °C. Sections were then washed and incubated for 2 hours with biotinylated goat anti-rabbit secondary antibody (1:200 in PBST, Vector Laboratories), which was detected using the Avidin-Biotin Complex (ABC) staining kit (VECTASTAIN) with horseradish peroxidase (HRP). The peroxidase activity was detected using 3,3'-diaminobenzidine (DAB) in the presence of H_2O_2.

2.3.6 Tissue preparation and immunolabeling for electron microscopy

Rats were perfused transcardially with a mixture of 4% PFA and 0.5% glutaraldehyde followed by 4% PFA only and 10% sucrose, all in PB, pH 7.4. Spinal cords were then placed in a 30% sucrose solution in 0.1 M PB for 24 hours. The lumbar spinal cord was dissected and underwent freeze thaw process by immersion in liquid nitrogen for 30 s followed by thawing in PB at room temperature. Transverse sections of the L4–L5 spinal cord segments were cut at 50 µm thickness on a Vibratome (TPI, St. Louis, MO, USA). Collected sections were treated with 1% sodium borohydride in PBS to reduce free aldehyde groups. Sections were incubated with 5% donkey serum in PBS for 1 hour prior to incubation with guinea pig anti-VGLUT1 (Millipore, 1:4000) or anti-Iba1 (Wako, 1:1000) overnight at 4°C. Sections were then incubated with donkey anti-guinea pig secondary antibody (1:400) and ABC staining kit (VECTASTAIN), each for 2 hours. Peroxidase activity was detected by DAB in the presence of H_2O_2. Sections were then osmicated (1% OsO4 in PB for 1 hr), dehydrated in ascending alcohols and propylene oxide, and flat-embedded in Epon. For each animal, serial ultrathin sections were cut from tissue blocks containing laminae I–IV (LI-IV) and collected on Formvar-coated mesh grids (Tremblay et al., 2010b).

For a double-labeling of VGLUT1 and GABA, ultrathin sections from blocks of material immunostained for VGLUT1 were cut and collected onto mesh nickel grids. Post-embedding immunostaining for GABA was performed as described previously (Ribeiro-da-Silva et al., 1993). After washes in Tris-buffered-saline (TBS), an anti-rabbit IgG antibody conjugated to 10 nm gold particles (British BioCell) was used. All grids were counterstained with uranyl acetate and lead

citrate and were viewed with a Philips CM120 transmission electron microscope equipped with a Gatan camera (Philips Electron Optics Canada, Toronto, Ontario, Canada) (Pawlowski et al., 2013).

2.3.7 Western blot

Saline-perfused rat spinal cords were extracted and homogenized on ice in lysis buffer (50 mM HEPES, 5 mM CaCl2, 1 mM MgCl2, 145 mM NaCl, pH 7.4) containing protease and phosphatase inhibitors (Sigma). Large debris were removed by centrifugation at 1000 × g for 5 minutes at 4 ˚C. Protein concentrations were determined by Bradford protein assay (Bio-Rad) and SDS samples were prepared with 1/3 standard Laemmli buffer, 1/10 ß-mercaptoethanol, sample and PBS, and 25 µg of protein was loaded onto 4–15% TGX precast gels (Bio-Rad) and separated by electrophoresis at 100 V. Proteins were subsequently transferred to PVDF membranes (Bio-Rad) at 22 mA for 2 hours. The membranes were rinsed, and then incubated with primary antibodies in Tris-buffered saline containing 0.1% Tween 20 (TBST) and 2.5% bovine serum albumin (BSA) for 1 hour at room temperature. Primary antibodies used: rabbit anti-CD11b (Novus Biologicals, 1:100) and mouse monoclonal [6C5] to GAPDH (Abcam, 1:500). After washing with TBST, the blots were incubated for 2 hours with peroxidase-conjugated secondary antibodies (Jackson ImmunoResearch, 1:10000), and washed again. Immunoreactive protein bands were detected using the ECL-plus kit (GE Healthcare). Signals were acquired using Bio-Rad Chemidoc MP imaging system and analyzed using ImageJ.

2.3.8 Analysis of synaptic elements

Images were collected from the central portion of inner LII (LIIi) and dorsal part of LIII at the single synapse level (see framed area in Figure 1A1 as the example of area of measurement). This area was selected for synapse analysis because of its importance in modulation of pain and touch information and the possibility to clearly differentiate three excitatory synapse subtypes based on the expression of vesicular glutamate transporters (Todd et al., 2003, Seal et al., 2009). Structured Illumination Microscopy (SIM) and a Zeiss LSM 880 confocal microscope with Airyscan were used to assess synapse densities. For SIM, cells were imaged with a 100x, NA = 1.42, oil immersion objective on a Vision OMX V4 Blaze system (GE) equipped with 488 nm and 592 nm

lasers and two Evolve EM-CCD cameras (Photometrics, Huntington Beach, CA, USA). Image stacks of 2 µm were reconstructed in Deltavision softWoRx 6.1.1 software with a Wiener filter of 0.01 using channel-specific optical transfer functions (Gustafsson et al., 2008). Captured synapse densities were analyzed using the spot function on IMARIS software (Bitplane) in combination with Matlab tool, colocalize spots XTention. A synapse was counted if the distance between the center point of a presynaptic punctum and a postsynaptic punctum was equal to or less than the radius of the presynaptic puncta + 0.1 µm, an empirically determined scaling factor (Hong et al., 2017). A similar approach was used to quantify the colocalization of the complement factor C1q with synaptic elements.

2.3.9 Electron microscopy quantification

For each grid, all immunoreactive VGLUT1 terminals or microglial processes in 4 square mesh in inner LII (LIIi) and LIII were imaged. For synapse analysis, both number and type of synapses were assessed using ImageJ by an experimenter blind to the condition. For each animal, two grids were quantified, and the number of inhibitory synapses was calculated per VGLUT1+ terminal. Images of terminals with ambiguous synaptic contacts were discarded from quantification. For quantitative analysis of phagocytosis, each captured Iba1+ microglial process was analyzed for phagocytic inclusion body density assessed by counting the vacuoles and endosomes containing cellular materials such as axon terminals with 40-nm synaptic vesicles per Iba1+ process (Tremblay et al., 2012).

2.3.10 Microglia density quantification

Low magnification images were captured using a Zeiss AxioImager M2 Imaging microscope with the Zeiss ZenPro software v.2.3 (Zeiss Canada). A Zeiss Axiocam 506 Colour camera was used for brightfield imaging, and an Axiocam 512 Monochrome camera was used for fluorescence imaging. For capturing images to analyze microglia morphology, z-stacks (spanning 30 µm with 1 µm step) were captured with a 40x oil immersion objective. To assess microglial morphology, the stacked images were converted into a single plane image using “the extended depth of focus tool” function on the Zeiss software. Morphology of microglia was assessed by the ratio of the length to area of each cell (Hanzel et al., 2014).

2.3.11 Engulfment assay

For assessing phagocytosis of synaptic elements, we used the method described by Schafer et al (Schafer et al., 2014), of high-resolution confocal microscopy followed by 3D reconstruction and surface rendering, with few modifications. Briefly, fixed sections immunolabelled with lysosomal marker CD68, microglial marker iba1 and a presynaptic marker were imaged. Two images were captured in LII for each section by 63x oil immersion objective (NA 1.4) using 2 µm z-steps using Zeiss LSM 800 confocal microscope. 3D volume surface renderings were created for each signal of z-stack using IMARIS software and for each microglial cell in the image, the volume of internalized synaptic elements colocalized with CD68 was calculated and normalized to the cell volume. Additionally, we assessed the proportion of the CD68 signal that was colocalized with internalized synaptic markers for each cell.

2.3.12 Complement quantification

The average intensity of complement C1q and C3 was quantified in ImageJ software (NIH) using images captured by 20x objective for time course assessments, this was the same approach used to quantify expression of CD11b. For assessing C1q signal inside (intracellular) and outside microglia (extracellular), confocal image stacks of 2 µm z-steps were used. In IMARIS, the channel corresponding to microglia labeling (Iba1+) was thresholded using a cut-off value determined empirically, and Iba1-immunoreactive areas were identified as ROI. These ROI were then applied as an overlay to the channel corresponding to the immunolabeled-complement, and total fluorescence intensity was separately quantified exclusively within (intracellular) or outside (extracellular) those regions.

2.3.13 Experimental design and statistical analyses

Sample sizes were determined based on standard practices in the field and pilot experiments. All statistical tests were completed using Windows GraphPad Prism version 8. Analyses used include only parametric tests: one-way ANOVA, two-way repeated measures ANOVA, and two-way ANOVA followed by Bonferroni's multiple comparison tests, and unpaired or paired two-tailed t tests. All p values and statistical tests employed are indicated in figure legends. All N values are reported and refer to the number of animals. All experiments were analyzed blind to groups.

2.4 Results

2.4.1 PNI Induces selective loss of DH synapses

After confirming the development of mechanical hypersensitivity at day 20 post-PNI, when hypersensitivity peaks in the cuff variation of the chronic constriction injury model of neuropathic pain (Nascimento et al., 2015), we quantified the density of different populations of DH synapses at this time point. First, we assessed inhibitory synapses, which are primarily formed by inhibitory interneurons. The major form of spinal inhibition involves the release of GABA and glycine at axodendritic or axosomatic synapses which can be identified by the presence of presynaptic vesicular GABA transporter (VGAT) and postsynaptic gephyrin and is known as post-synaptic inhibition. Another form of DH inhibition is implicated in controlling the flow of sensory information from primary afferents to DH neurons by presynaptic inhibition of central terminals of primary afferents through GABA release at axo-axonic synapses. Quantification of paired VGAT and gephyrin apposition density showed a significant reduction of the density of inhibitory synapses from 0.43±0.02 synapses per µm3 in sham to 0.23±0.02 in ipsilateral DH (Figure 1A1-4). The great majority of vesicular glutamate transporter (VGLUT1) expressing terminals in LIIi belong to touch coding primary afferents and represent the central element of complex glomerular synaptic structures. In these structures, axo-axonic GABAergic inhibitory inputs can be identified using electron microscopy (EM). In the contralateral DH, VGLUT1+ central terminals were surrounded by neuronal profiles, easily identifiable as glomerular structures (Figure 1B1-2). In the ipsilateral side, inhibitory synapse density at central terminals was significantly reduced by 51% compared to contralateral side (P=0.0098, Figure 1B3-5). Furthermore, signs of degeneration such as a more irregular shape and frequent presence of intracellular vacuoles suggest that glomerular structure integrity was compromised (Figures 1B3-4). In addition, an increased presence of glial processes surrounding the terminals was observed (Figures 1B3-4). Together, these results indicate that PNI induces a loss of synapses mediating both pre- and post-synaptic DH inhibition.

Subtypes of excitatory DH synapses were identified by the presence of different isoforms of VGLUTs, as markers of presynaptic elements (Todd et al., 2003, Seal et al., 2009), paired with excitatory postsynaptic marker Homer1 (Gutierrez-Mecinas et al., 2016). In LII-III, VGLUT1

primarily labels synapses formed by a subpopulation of primary afferents that transmit touch-related information, VGLUT2 labels synapses of all excitatory DH interneurons and synapses formed by a subpopulation of primary afferents that transmit pain-related information, and VGLUT3 is expressed in the terminals of a unique subpopulation of primary afferents that code for pleasant touch (Seal et al., 2009, Todd et al., 2003). Quantification of different subtypes of excitatory synapses revealed that VGLUT1+ synapse density (i.e. juxtaposed Homer1 and VGLUT1 puncta) was significantly reduced by 30% in the ipsilateral DH following PNI as compared to the contralateral side and to values in sham animals (P=0.0011, Figure 1C1-4), and no significant change in VGLUT2+ or VGLUT3+ synapse densities were detected (Figure 1D-E). In sham animals, and on the contralateral sides, VGLUT1+ and VGLUT3+ terminals were found at the centre of glomerular-like structures with multiple Homer1-positive appositions (Figure 1C3, 1E3 and insets). In general, the VGLUT3+ structures were notably smaller than the VGLUT1+, and were unaffected by PNI. On the ipsilateral side, where VGLUT1+ synapse density was reduced, the remaining terminals made multiple excitatory synaptic connections (Figure 1C3, inset). Together, these results show that PNI induces selective loss of VGLUT1+ primary afferents terminals and, unexpectedly, of DH inhibitory synapses per terminal onto both spinal interneurons and the remaining VGLUT1+ primary afferents boutons.

2.4.2 PNI activates phagocytic microglia in spinal DH

To determine if PNI-induced synapse loss is mediated by microglia, we first assessed microglia phenotypic changes including morphological changes associated with phagocytosis, upregulation of phagocytosis-related proteins, and microglial phagocytic inclusions. As expected, PNI induced significant microgliosis in the DH (Gilmore, 1975). At day 20 post-PNI, ipsilateral microglia displayed altered morphology and an increased density compared to the contralateral side (Figure 2A). Ipsilateral microglia presented a less ramified morphology, characterized by a larger cell body and short, thick processes, features classically associated with increased microglial phagocytotic ability. Interestingly, in the ipsilateral DH, a notable interlaminar difference in microgliosis was observed. Although microgliosis was present across all ipsilateral laminae, a larger increase in microglial density was found in deeper laminae (LIII-IV) compared to the superficial laminae (LI-II) (Figure 2A-C). Although there was a significant difference in microglia

morphology between the ipsi- vs contralateral sides in deeper laminae, no difference was found in the superficial DH. We assessed the expression of microglia-specific phagocytosis-related proteins CD68 and CD11b. CD68, a macrophage-specific lysosomal protein, was upregulated in ipsilateral microglia compared to both sham and contralateral DH, with a smaller increase between contralateral and sham DH (Figure 2D-E). CD11b, a microglial surface receptor that mediates phagocytosis, was upregulated in the ipsilateral DH compared to both sham and contralateral DH (Figure 2F-G).

To provide direct evidence of synaptic phagocytosis by microglia, we analysed the presence of inclusion bodies in microglia using EM (Figure 2H). We found that the density of inclusion bodies was increased in ipsilateral DH microglial processes compared to the contralateral side (Figure 2H-I). Ultrastructural examination of the phagocytic inclusions revealed the presence of engulfed neuronal elements including synaptic elements such as synaptic vesicles (Figure 2H). Taken together, these data show that PNI induced phagocytotic activity in DH microglia, which engulf neuronal elements including synaptic components.

2.4.3 Microglia selectively engulf synapses intrinsic to the spinal cord

To determine the type of synapses which are phagocytosed by microglia after PNI, we performed an engulfment assay to assess the presence of excitatory and inhibitory DH synaptic markers in microglial lysosomes. In both ipsilateral and contralateral DH samples, microglial processes were found to be in close association with synapses of all types. In ipsilateral DH, immunofluorescence for presynaptic markers was found inside microglial processes but colocalization with CD68 was rarely observed in contralateral images (Figure 3A1-2, B1-2, C1-4, D1-4). We found no evidence of VGLUT1 or VGLUT3 engulfment (Figure 3B, D) in contralateral or ipsilateral images. However, significant engulfment of VGAT and VGLUT2 was observed in the ipsilateral DH as compared to the contralateral, where little to no engulfed material was detected (Figure 3A, C). Engulfed VGAT and VGLUT2 colocalized with CD68 were most notable in the cell body or in bulbous process sections. To ensure that PNI-induced morphological changes and altered microglial volume were not affecting the engulfment results, we assessed the proportion of CD68 immunofluorescence that was occupied by internalized synaptic markers. Consistent with the engulfment assay

results, VGAT and VGLUT2 were the only synaptic markers that showed elevated colocalization with CD68 in ipsilateral as compared to contralateral side (Figure 3A6, B4, C6, D4). These data demonstrate that following PNI, microglia selectively engulf inhibitory and excitatory synapses that are intrinsic to the spinal cord but do not engulf synapses formed by primary afferents.

2.4.4 Chronic microglia inhibition attenuates hypersensitivity and phagocytotic marker upregulation

To confirm that synapse loss in the DH after PNI is mediated by microglia, we chronically inhibited microglia using minocycline, an established inhibitor of microglial activation. Minocycline treatment (30 mg/kg i.p., every 12 hours) reduced mechanical allodynia at both 10 days and 20 days following nerve injury with a greater effect at the later time point (Figure 4A). The drug effectively reduced PNI-induced microgliosis in the ipsilateral DH at day 20 post-neuropathic surgery (Figure 4B and C). To study whether minocycline supressed microglial phagocytotic activity, we first assessed the expression of microglial phagocytosis-related proteins CD68 and CD11b (Figure 4D-H). The PNI-induced CD68 increase was attenuated by minocycline treatment as demonstrated by a significant decrease in the proportion of CD68+ microglia (Figure 4D). Analysis of CD11b expression by western blot in the lumbar spinal cord showed that minocycline supressed PNI-induced CD11b upregulation (Figure 4E and F). In addition, and consistent with the western blot data, minocycline reduced ipsilateral CD11b detected by immunohistochemistry (IHC). Although there was a slight trend towards a remaining increased ipsilateral CD11b signal with minocycline treatment, this was not statistically significant (Figure 4G and H). Collectively, these results show that chronic administration of minocycline attenuated mechanical allodynia and effectively reduced markers of microglia phagocytotic activity.

2.4.5 Blocking microglial phagocytosis prevents inhibitory synapse loss

Based on our finding of DH synaptic phagocytosis by microglia and synaptic loss following PNI, we hypothesized that microglial inhibition should reduce synapse engulfment and, consequently, prevent synapse loss. To test this possibility, microglia-mediated engulfment of inhibitory and excitatory synaptic elements was assessed in the DH of vehicle- and minocycline-treated PNI animals. Since only VGAT+ (presynaptic inhibitory elements) and VGLUT2+ (a subtype of

presynaptic excitatory elements) synapses were engulfed by microglia, we assessed the effect of microglial inhibition on these synapse types. Microglial inhibition significantly reduced ipsilateral DH VGAT engulfment by 65% comparing vehicle and minocycline treated animals (Figure 5A1-4, B). Minocycline treatment also significantly reduced VGLUT2 engulfment (Figures 5C1-4, D) suggesting that both DH inhibitory and excitatory synapses are phagocytosed by microglia. We next examined the effect of microglia inhibition on PNI-induced synapse loss by analysing pairs of pre- and post-synaptic markers. Consistent with the VGAT engulfment results, chronic microglial inhibition prevented loss of inhibitory synapses in the ipsilateral DH, identified by VGAT and gephyrin appositions (Figure 5 E-F). In contrast, the density of VGLUT2+ excitatory synapses was not affected by PNI or microglial inhibition (Figure 5 G-H). Taken together, these data reveal that microglial phagocytosis of DH synapses leads to the selective reduction in DH inhibitory synapse density but not excitatory synapses after PNI.

2.4.6 Microglia depletion prevents hypersensitivity, synapse engulfment, and inhibitory synapse loss

To further confirm the role of microglia in remodelling of spinal cord synaptic connectivity in neuropathic pain, we depleted microglia using a potent colony stimulating factor 1 receptor (CSF1R) inhibitor, PLX3397. Although PLX3397 blocks CSF1R which is essential for survival of all macrophages, intrathecal injection preferentially targets spinal cord microglia while sparing blood macrophages. As expected, following PNI, vehicle-treated rats displayed lasting tactile hypersensitivity as shown by decreased paw withdrawal threshold to von Frey filaments. Development of mechanical hypersensitivity was alleviated by PLX3397 treatment, as shown by an increase in paw withdrawal thresholds compared to vehicle-treated rats (Figure 6A). Staining for microglia marker Iba1 showed that ipsilateral microglia density was reduced in drug-treated rats compared to vehicle-treated animals (Figure 6 B-C), confirming that the PLX3397 treatment effectively depleted spinal microglia. To assess the effect of microglial depletion on DH synaptic subpopulations, different synapse types were examined using high-resolution structured illumination microscopy (SIM).

Consistent with our previous findings, the number of DH inhibitory synaptic contacts was reduced following PNI (Figure 6D). Interestingly, a differential effect of microglia depletion on distinct synapse types was revealed. Similarly, to minocycline treatment, the loss of postsynaptic connections (i.e., juxtaposed VGAT and gephyrin puncta) was fully prevented by microglial depletion (Figure 6D). Unlike postsynaptic inhibitory inputs, loss of presynaptic inhibitory synapses on primary afferents, detected by the density of juxtaposed VGAT+ appositions on VGLUT1+ terminals, was only partially prevented by this treatment (Figure 6E).

Lastly, the effect of microglial depletion on excitatory synapses was evaluated. We confirmed the loss of VGLUT1+ terminals following PNI and that the remaining terminals were apposed by fewer Homer1 puncta. However, unlike the inhibitory terminals, the reduction in VGLUT1+ excitatory synapse density was not prevented by microglial depletion (Figure 6F). Consistent with our previous results, PNI did not induce loss of VGLUT2+ synapses and depletion of microglia had no effect on the density of these synapses (Figure 6G). These results show that a microglia-independent mechanism causes primary afferent terminal loss. Furthermore, microglia are directly implicated as selective mediators of DH anatomical rewiring following nerve injury that is specifically directed to inhibitory synaptic circuitry.

2.4.7 DH complement factors are upregulated and selectively tag inhibitory synapses

To study molecular mechanisms that attract microglia and mediate synapse elimination, we investigated plausible candidates complement proteins C1q and C3. C1q, the initiating protein in the classical complement cascade, and the downstream complement protein C3 are implicated in selective synaptic pruning by microglial phagocytosis (Stevens et al., 2007). In this process, complement signalling is initiated by C1q, which tags synapses for elimination. Therefore, we assessed DH expression of C1q at multiple time points post-PNI. Following PNI, ipsilateral DH C1q expression was significantly increased at days 7 and 14 post-PNI (Figure 7A-C). C3 is the ligand of microglial phagocytic receptor CD11b, which is increased at 14 days post-PNI (Figures 2 and 4). Consistent with C1q upregulation, ipsilateral DH C3 immunoreactivity was also significantly increased (Figure 7A, C). A closer look at the ipsilateral C1q staining pattern revealed a bright, punctate signal within microglia and a diffused increase in extracellular immunoreactivity outside

of microglia (Figure 7A and D). Both the microglial intracellular C1q signal and the extracellular immunoreactivity were significantly increased in the ipsilateral DH (Figure 7A, D and E). Using SIM, we observed a preferential colocalization of condensed extracellular C1q puncta at inhibitory (VGAT+) synapses as compared to excitatory synapses (VGLUT2+) in the ipsilateral DH (Figure 7F-H). These results provide evidence for a role of the complement system in the removal of inhibitory synapses by microglia in neuropathic pain.

2.5 Figures

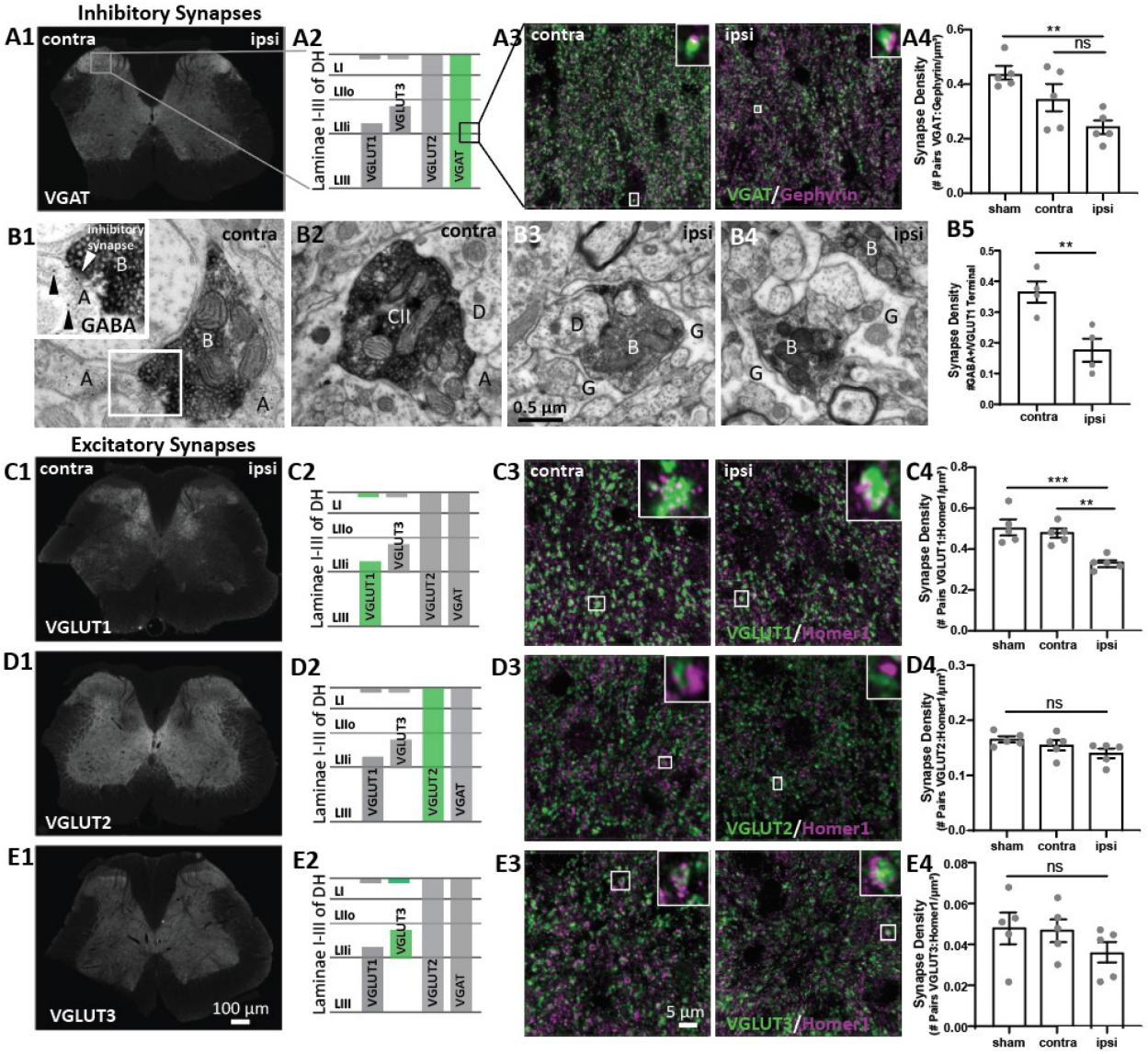

Figure 1. PNI induces selective loss of DH synapses. Representative images and quantifications show that distinct DH synaptic population densities are affected at 20 days post-PNI. Subpopulations of inhibitory (A-B) and excitatory synapses (C-E) were identified with VGAT paired with gephyrin (A), expression of GABA and synapse morphology (B), and homer1 paired with VGLUT1 (C), VGLUT2 (D), and VGLUT3 (E). Shown here, both types of inhibitory synapses but only VGLUT1+ excitatory synapses are lost. In A, C, D and E, the first panels show low magnification images, the second panels show schematic expression pattern representation of each pre-synaptic markers through DH LI to LIII. A1, inset indicates the DH region of analyses, where high

resolution airyscan images in the third panels of A, C, D and E were captured and show pre-synaptic (green) and post-synaptic (magenta) elements. Synapses mediating A, postsynaptic DH inhibition and B, presynaptic inhibition of VGLUT1+ primary afferents. B1-5, EM images of the ultrastructure of VGLUT1+ boutons in lamina IIi of rat spinal cord. B1-2, Contralateral VGLUT1+ boutons (B, and enlarged inset white arrowhead) are post-synaptic to GABA-IR inhibitory axons (A, and black arrowhead). B2, A central bouton of a type II glomerulus (CII) can be seen to have synaptic connections with multiple dendritic (D) profiles and an axonal profile (A). B3-4, VGLUT1+ boutons (B) sampled from ipsilateral spinal cord are surrounded by glial structures (G) and form fewer synaptic contacts. Insets in A3, C3-E3images show enlarged views of synaptic contacts, note that VGLUT1+ and VGLUT3+ primary afferent terminals form multiple excitatory connections. Data are mean ± SEM. *$p < 0.05$, **$p < 0.01$, and ***$P < 0.001$ using one-way ANOVA followed by Bonferroni post hoc test (A4, C4D4) and unpaired t-test (B4), n=4-5.

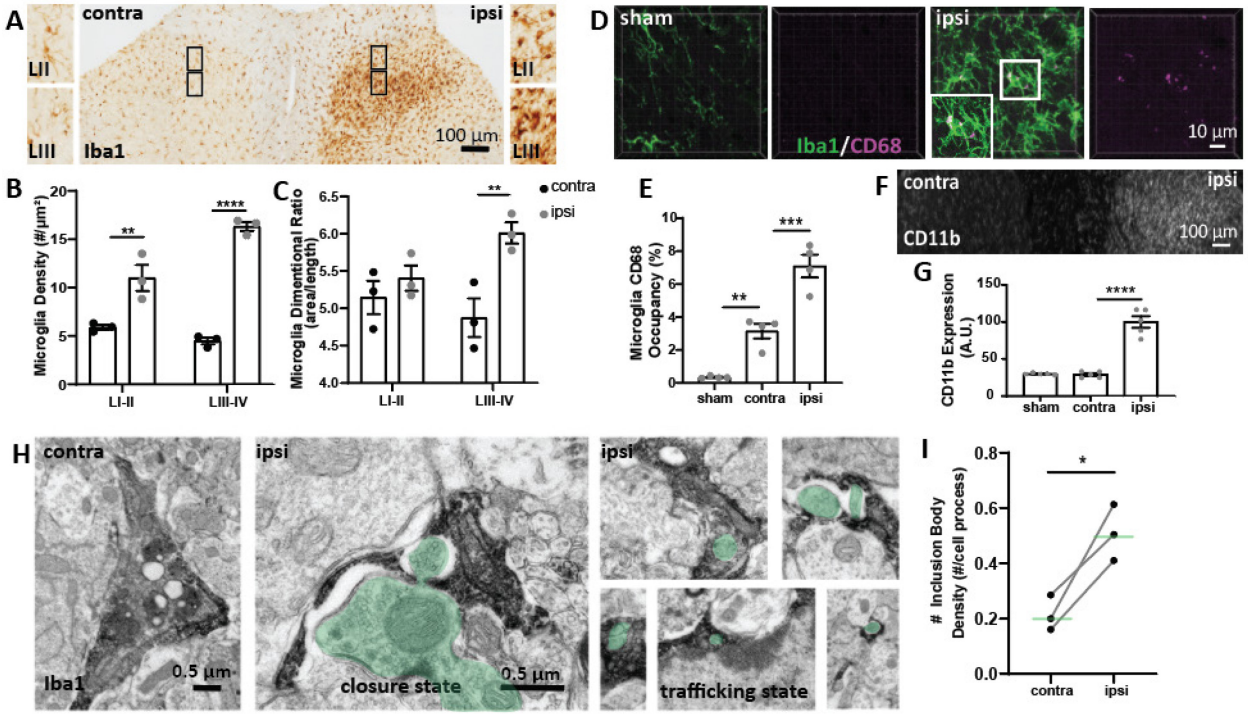

Figure 2. PNI activates phagocytic microglia in spinal DH. A, Representative images of DH Iba1 labeling at 20 days post-PNI, demonstrating that both microglia proliferation (B) and morphological changes (C) occur. D, Representative 3D images of Iba1 (green) and CD68 (magenta) in sham and ipsilateral DH show increased ipsilateral microglial CD68 expression (quantified in E). F, Representative image of DH CD11b-IR shows marked ipsilateral increase (quantified in G). H, Representative EM images show the presence of ipsilateral phagocytic inclusions (green) with ultrastructural features of neuronal elements, such pre-synaptic axon terminal material containing vesicles in Iba1-stained microglial processes (quantified in I). *p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.001 using two-way ANOVA (B and C), one-way ANOVA (E and G) and paired two-tailed t-test (I), followed by Bonferroni post hoc test, n=3-4.

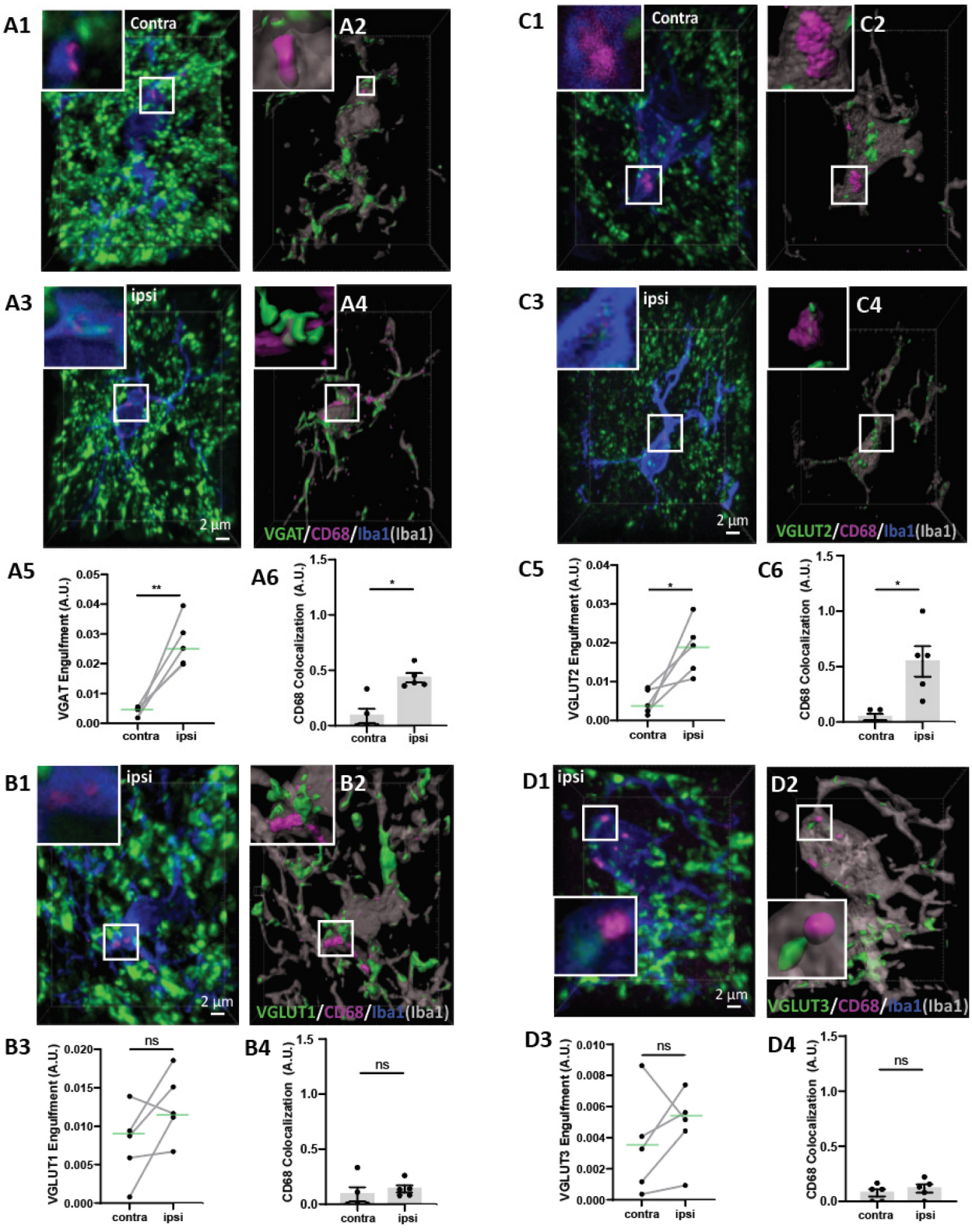
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Figure 3. Microglia selectively engulf synapses intrinsic to the spinal cord. Representative 3D reconstructions of high-resolution confocal images of microglia (blue) sampled from DH show lysosome (magenta) colocalization with pre-synaptic markers (green) in panels 1-4, quantified in subpanels 5-6. A, VGAT; B, VGLUT1; C, VGLUT2; D, VGLUT3; and D, VGAT. Only ipsilateral VGAT (A) and VGLUT2 (C) engulfment was detected at 20 days post-PNI. Insets are single plane images selected from stacks illustrating the presence (A and C) or absence (B and D) of CD68+ lysosomal colocalization with synapse markers. *$p < 0.05$, and **$p < 0.01$ using paired two-tailed t-test for all analysis, n= 5.

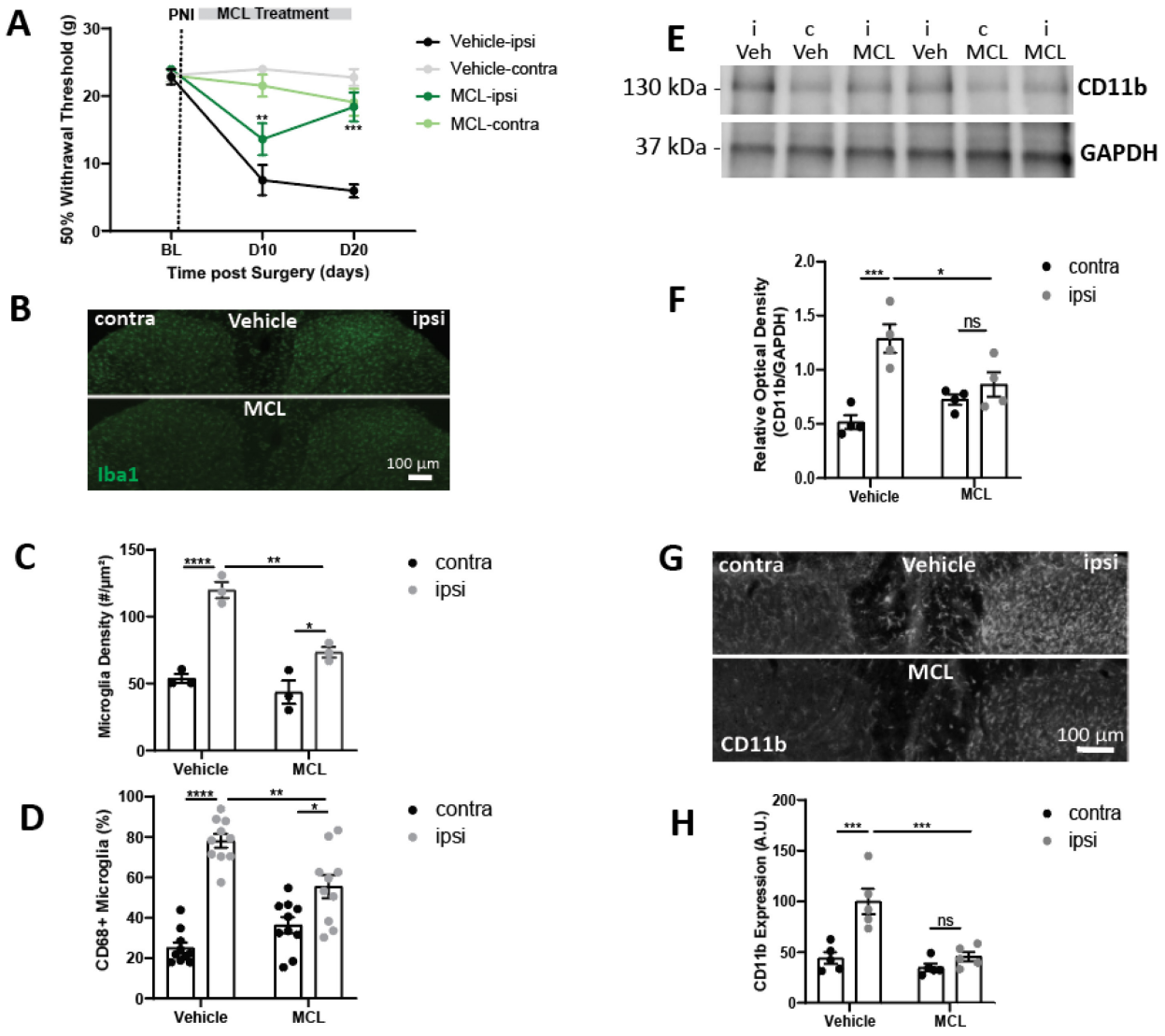


Figure 4. Chronic microglia inhibition attenuates hypersensitivity and upregulation of phagocytotic markers. A, Reversal of PNI-induced mechanical allodynia by chronic administration of minocycline. Symbols represent mean ± SEM 50% withdrawal threshold from von Frey filaments (n = 10 per group). B-H, show reduced DH microglia activation at day 20 post-PNI by twice-daily minocycline treatment. B, Immunohistochemistry for Iba1 (green) (quantified in C). D, Quantification of the proportion microglia that are CD68+ shows that minocycline attenuates the number of CD68+ microglia in the ipsilateral DH. E-F Western blot analysis of lumbar spinal cord tissue shows decreased CD11b expression by minocycline treatment; E, representative blot and F, quantification (normalized to GAPDH). G-H, CD11b immunohistochemistry shows ipsilateral upregulation is prevented by minocycline. Data are

shown as mean ± SEM. *p < 0.05, **p < 0.01, ***P < 0.001 analyzed with two-way ANOVA with Bonferroni post hoc test, n = 3-10.

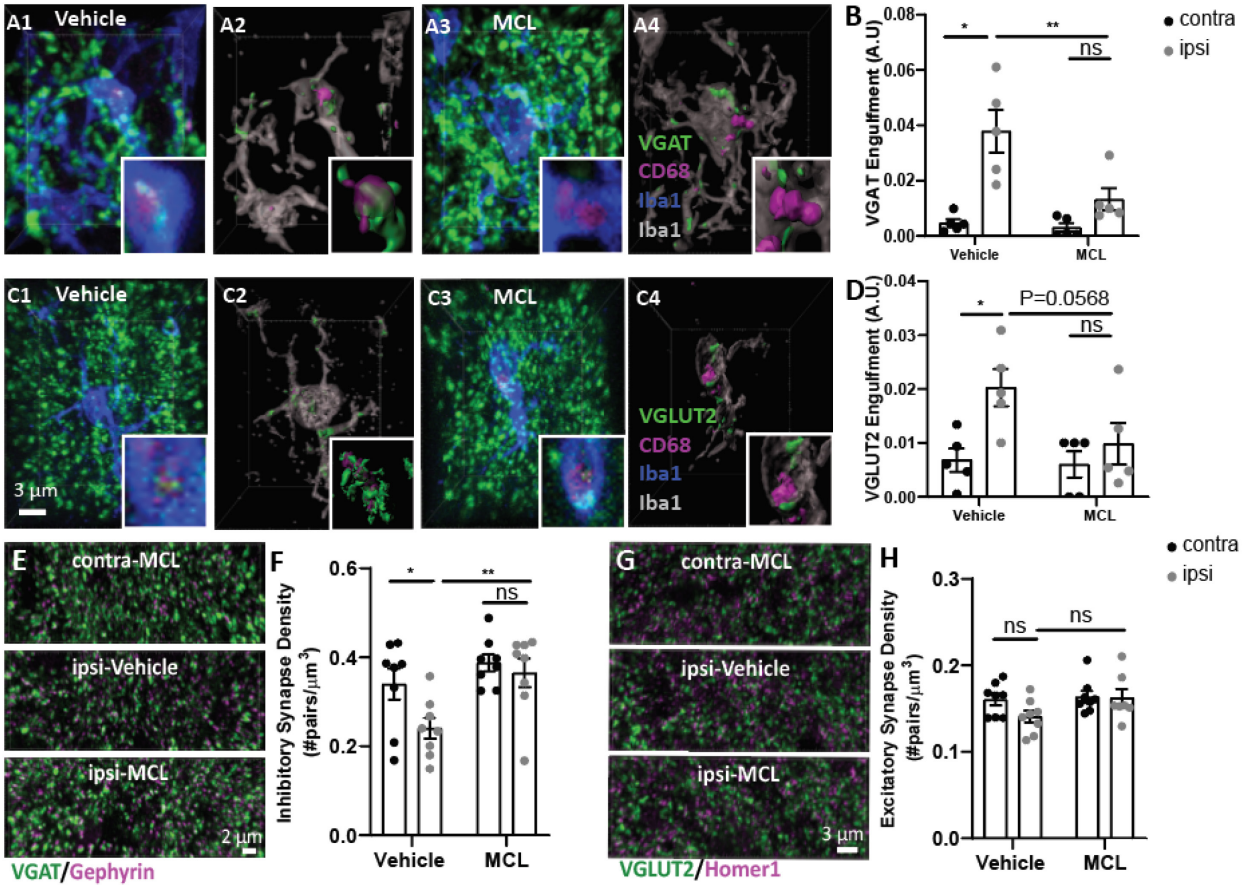
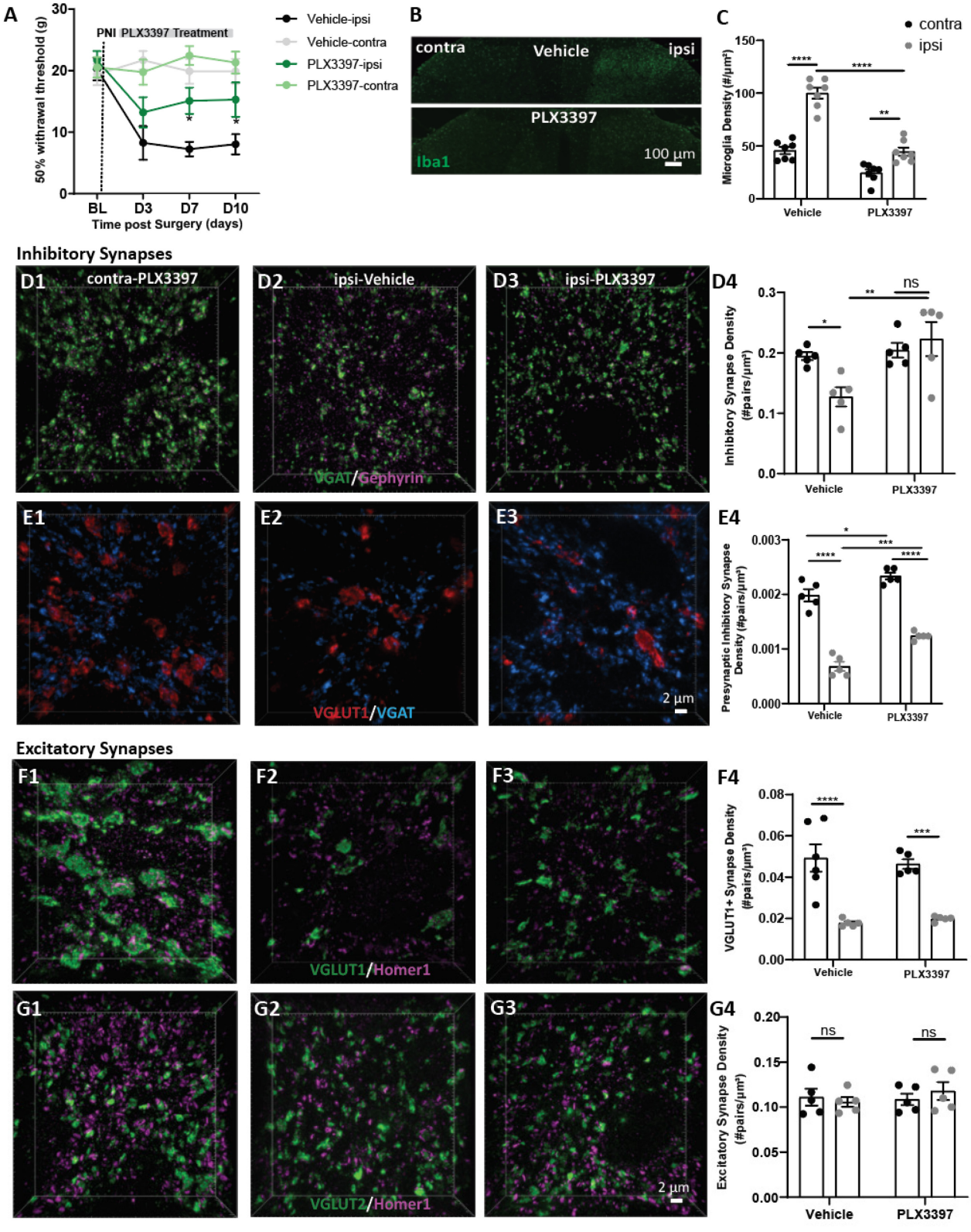


Figure 5. Blocking microglial phagocytosis prevents inhibitory synapse loss. A-D, Microglial engulfment of presynaptic markers at 20 days post-cuff is prevented by minocycline treatment. Representative reconstructed high resolution confocal images in the left-hand panels (A and C) of microglia (blue) show lysosomal (magenta) colocalization with pre-synaptic markers (green) beside surface renderings in the right-hand panels (microglia shown in grey) sampled from ipsilateral DH of rats treated with vehicle (A1 and C1) or minocycline (A3 and C3), shown quantified in B and D. A-B, VGAT; C-D, VGLUT2. E-F, Inhibitory synapse loss at 20 days post-PNI is prevented by minocycline treatment. G-H. Excitatory synapse loss is unaffected by PNI or minocycline treatment. Left hand panels show representative super-resolution confocal images of pairs of pre-synaptic (green) and post-synaptic elements (magenta), shown quantified in right hand panels. Data are shown as mean ± SEM. *p < 0.05, **p < 0.01 analyzed with two-way ANOVA with Bonferroni post hoc test, n= 5-8.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B
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Vehicle
ipsi
PLX3397
Iba1
100 µm
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ipsi
Microglia Density (#/µm²)
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D1 contra-PLX3397
D2 ipsi-Vehicle
D3 ipsi-PLX3397
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F3
VGLUT1/Homer1
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VGLUT1+ Synapse Density (#pairs/µm³)
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G3
VGLUT2/Homer1
G4
Excitatory Synapse Density (#pairs/µm³)

Figure 6. Microglia depletion prevents hypersensitivity, synapse engulfment and inhibitory synapse loss. A, Mechanical allodynia is reduced by chronic administration of intrathecal PLX3397. Values represent mean ± SEM 50% withdrawal threshold from von Frey filaments (n = 8 per group). B, Iba1 immunohistochemistry and quantification of DH microglia density (C) show reduced DH microgliosis by PLX3397 treatment. D-G Representative SIM images of pre-synaptic and post-synaptic elements and quantifications show that PLX3397 prevented inhibitory synapse loss (D-E) and had no effect on excitatory synapse density (F-G). D, Synapses identified by VGAT (green) and gephyrin (magenta) that mediate post-synaptic inhibition. E, Pre-synaptic (blue) inhibitory inputs on terminals of VGLUT1+ (red) primary afferents. F-G, Excitatory terminals identified with homer1 (Magenta) and VGLUT1 in F (green) or VGLUT2 in G (green). Data are shown as mean ± SEM at 10 days after PNI. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ and ****$p < 0.0001$, two-way ANOVA with Bonferroni post hoc test.

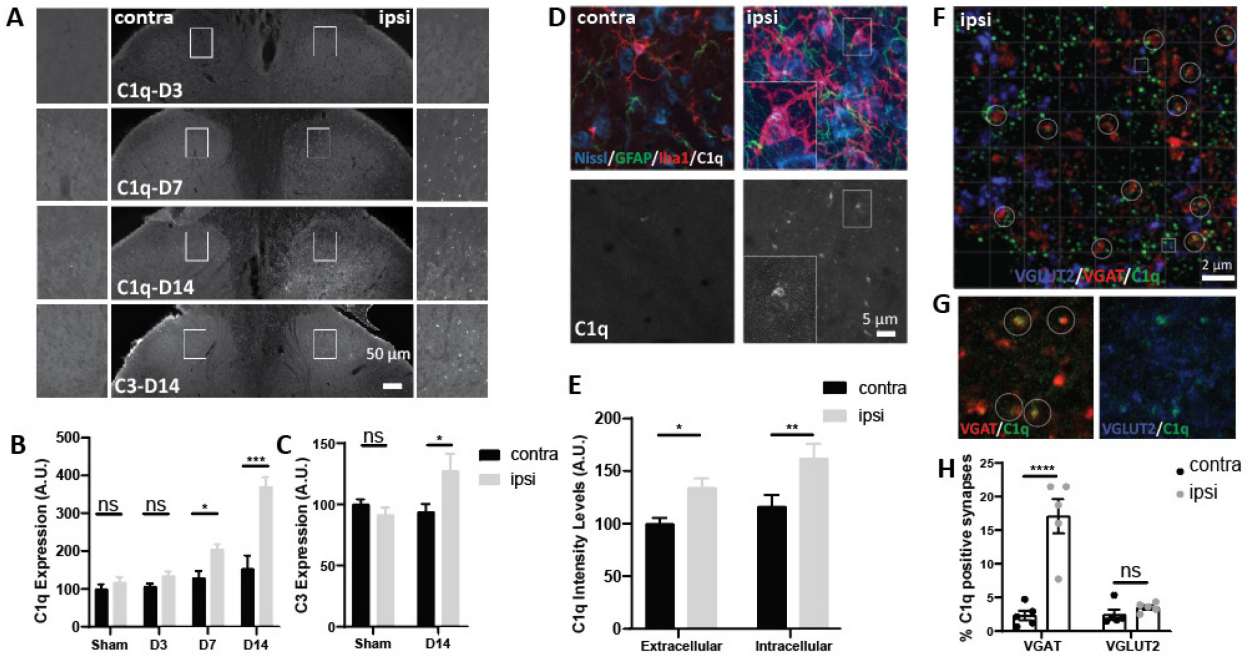

Figure 7. **DH complement factors are upregulated and selectively tag inhibitory synapses.** A, Representative images of C1q and C3 expression show a progressive ipsilateral DH upregulation of C1q, with peak C1q and C3 expression at 14 days post-PNI (quantified in B and C). Insets show enlarged fields from ipsilateral (left) and contralateral (right) DH. D, stacked confocal images with markers of neurons (blue), astrocytes (green), and microglia (red) show ipsilateral C1q (white) as a punctate intracellular, microglial signal and a diffuse extracellular signal. Both signals are increased after PNI (quantified in E). F-H, Ipsilateral C1q (green) preferentially colocalizes with inhibitory synapses (VGAT in red) indicated by overlaid circles, compared to excitatory (VGLUT2 in blue and overlaid squares), shown in representative SIM (F) and confocal images (G, quantified in H). Data are shown as mean ± SEM. *p < 0.5, **p < 0.01, ***p < 0.001 and ****p < 0.0001, two-way ANOVA with Bonferroni post hoc test.

2.6 Discussion

In this study, we demonstrate a significant and selective remodelling of DH inhibitory synaptic connections by microglia in a neuropathic pain model. Specifically, we show that: (1) The densities of both pre- and postsynaptic inhibitory synapses and VGLUT1 expressing excitatory synapses are selectively reduced following PNI. (2) Phagocytic PNI-activated microglia engulf DH neuronal elements that belong to DH intrinsic inhibitory and excitatory synapses but do not engulf synapses formed by primary afferents. (3) Microglial inhibition or depletion selectively prevents inhibitory synapse loss, without affecting other synapse types. (4) The classical complement system preferentially tags inhibitory synapses implicating it in the selective elimination of DH inhibitory synapses. Together, these findings provide evidence for a novel role of microglia in the pathophysiology of neuropathic pain. Overall, we propose that PNI induces a substantial structural modification in the DH circuitry resulting in an impairment of normal processing of sensory information —loss of inhibition— associated with long-lasting symptoms of neuropathic pain hypersensitivity. Most importantly, our results suggest that the structural basis of the deficit in DH inhibitory transmission can be prevented by targeting microglia.

2.6.1 Distinct synapse subpopulations are differentially affected by PNI

Our finding that PNI induces selective loss of DH synapses adds to the growing evidence that PNI induces remodeling of DH circuits which are essential for normal processing of sensory information (Petitjean et al., 2015, Moore et al., 2002, Lorenzo et al., 2020). In this study, we systematically analyzed DH synapses based on the type of coded sensory information (differential VGLUT expression in subtypes of primary afferents), origin (peripheral and central), function (inhibitory and excitatory), and post-synaptic targets (pre and postsynaptic inhibitory inputs).

In LIIi and dorsal LIII, the primary afferent markers that we focused on, VGLUT1 and VGLUT3, primarily label terminals of Aδ- and C- low-threshold mechanoreceptors (LTMRs), respectively, which share common peripheral sensory targets (Li et al., 2011). We found that PNI induces a significant loss of VGLUT1+ synapses without affecting VGLUT3+ synapse density. The simplest explanation for this observation lies with the properties of PNI model used. The cuff variation of

chronic constriction nerve injury involves partial damage to a peripheral nerve and is reminiscent of entrapment neuropathies(Bennett et al., 2003, Pitcher et al., 1999). In this model, the sciatic nerve appears normal proximal to the constriction, whereas marked degeneration of distal to injury primary afferents occurs. This peripheral degeneration was characterized by massive demyelination and nearly total loss of A fibers whereas C fibers are less affected (Munger et al., 1992). Therefore, it is plausible that VGLUT1+ but not VGLUT3+ synapse loss is the result of a preferential A fibers degeneration in response to PNI. Alternatively, it is conceivable that the expression of different subtype of VGLUTs in these terminals is related to differential functional dynamics after PNI. The primary functional difference between VGLUT1- and VGLUT3-expressing excitatory synapses is in quantal size and the rate of neurotransmitter release, both of which potentially affect the strength and duration of synaptic transmission (Weston et al., 2011, Wojcik et al., 2004). These differences are essential for understanding whether these synapses function distinctively in response to PNI and raise the possibility of the involvement of microglia independent activity-dependant synapse stripping mechanisms, which were previously described (Stevens et al., 2007, Gunner et al., 2019, Wu et al., 2015). Notably, we found no evidence of sprouting or excess synapse formation for Aδ- and C-LTMRs suggesting no regenerative plasticity of these terminals (Griffin et al., 2010).

Similar to many other neurological disorders (Saba et al., 2016, Di Liberto et al., 2018, Palop and Mucke, 2016), the dysregulated balance between excitatory and inhibitory control is associated with the abnormal sensations observed in the neuropathic state, including tactile allodynia. Although the organization of the DH circuitry that regulates inhibition-excitation balance to ensure normal sensory transmission and the nature of its alterations after PNI are not completely understood, in recent years several DH microcircuits have been identified (Koch et al., 2018, Peirs and Seal, 2016). So far, the effect of PNI on synaptic connectivity within these microcircuits has only been evaluated in a few studies (Petitjean et al., 2015, Boyle et al., 2019). Interestingly, there is evidence for and against structural plasticity at a circuit-specific level, suggesting that certain synaptic networks are more vulnerable to PNI-induced structural plasticity. To avoid possible microcircuit specific alterations induced by nerve injury in the DH circuitry, we decided to take a global approach to examine the global pool of interneuron synaptic connections by assessing

VGLUT2+ and VGAT+ synapses. We found no change in excitatory VGLUT2+ synapse density, but a significant loss of inhibitory inputs following PNI. Selective loss of inhibitory synapses can explain the reduced inhibitory postsynaptic currents (IPSCs) reported after nerve injury (Scholz et al., 2005, Yowtak et al., 2011, Lu et al., 2013, Moore et al., 2002). Other mechanisms for decreased IPSCs in neuropathic pain, including cellular stress (commonly present in dying neurons (Moore et al., 2002, Yowtak et al., 2011, Inquimbert et al., 2018)), and reduced neurotransmitter release could potentially increase the vulnerability of inhibitory synapses to be eliminated. Regardless of the mechanism, the selective loss of inhibitory synapses in the DH can lead to increased excitability of DH circuits, and abnormal, unrepressed functional connectivity in a neuropathic state (Torsney and MacDermott, 2006, Bardoni et al., 2013). We showed that inhibitory synapse types responsible for two different forms of DH inhibition, pre- and post-synaptic inhibition, are both lost after nerve injury. In addition to the general increase in DH excitability, caused by the loss of inhibitory synapses, our findings may have implications for the specific symptom of allodynia. Through the loss of presynaptic inhibitory input onto LTMRs, low threshold input may be escaping normal presynaptic inhibitory control. This in turn could lead to the activation of nociceptive pathways by touch (Torsney and MacDermott, 2006). Thus, although there is a loss of VGLUT1+ afferents, due to the decreased presynaptic inhibitory synapse density, the remaining VGLUT1+ terminals could contribute to enhanced sensory transmission. Microglia depletion resulted in only a partial recovery of presynaptic inhibitory synapse loss in the presence of almost a complete attenuation of mechanical hypersensitivity, suggesting that presynaptic inhibition of primary afferents may be important for more than the prevention of nociception from touch sensation. Importantly, neuropathic pain symptoms go beyond tactile sensitivity, with a wide range of altered touch-related sensations. Patient reports span across sensory loss and numbness, sensitivities, and chronic bothersome sensations, such as prickling, tingling, pins and needles, and stabbing. It is conceivable that the altered DH connections in different synaptic populations shown here could contribute to these phenotypes, but these are challenging to model experimentally.

2.6.2 Targeting microglia only protects DH inhibitory synapses

Our findings provide evidence for both microglia-dependent and -independent mechanisms of PNI-induced DH synapse elimination. Synapses originating from local DH circuitry were engulfed by microglia and although the DH density of VGLUT1+ primary afferent terminals was decreased, microglia do not seem to be required for this process. The phagocytic role of microglia following PNI was previously studied, and it was shown that at earlier time points after injury, microglia/macrophages remove myelinated axons via P2Y12 signaling (Maeda et al., 2010). We found that this phagocytic activity does not mediate loss of VGLUT1+ primary afferents synapses as microglial engulfment of VGLUT1 was not detected and VGLUT1+ terminal loss occurs when microglia are depleted. Possibly, microglia only contribute to early removal of myelinated debris following degeneration of A fibers and do not modify their synaptic inputs. Other immune cell types such as natural killer cells, which are involved in removing degenerating sensory axons at early timepoints, may contribute to loss of peripheral synapses at later time points (Davies et al., 2019). It remains unclear whether loss of primary afferent terminals following PNI is purely a degenerative process or is mediated by other cell types.

One of our most striking findings was the selectivity of inhibitory synapse removal by microglia. Although increased engulfment for both excitatory VGLUT2+ and inhibitory VGAT+ synapses was detected following PNI, there was no evident change in excitatory synapse density, and reduced inhibitory synapse density was apparent. One explanation for the mismatch between absence of VGLUT2+ synapse loss and increased VGLUT2+ engulfment is *de novo* excitatory synapse formation. There are different lines of evidence suggesting increased excitatory synaptogenesis following PNI (Lu et al., 2015, Simonetti et al., 2013). Moreover, it is possible for microglia to engulf synapses but not fully remove them. Recently, Gross et al. showed that microglia can participate in remodeling synapses by presynaptic trogocytosis without completely removing the synapse itself (Weinhard et al., 2018).

Unexpectedly, within inhibitory synapse subtypes, there was a differential attribution of synapse loss to microglia. Microglia were only necessary for complete recovery of the loss of inhibitory synapses that mediate post-synaptic inhibition. In addition to the microglia-independent loss of

primary afferents, the decreased density of presynaptic inhibitory terminals was also shown to be partially microglia independent. It is possible that at least a fraction of presynaptic inhibitory synapse loss is due to the decrease in afferent terminals. Together, these results show that although microglia are responsible for inhibitory and not excitatory synapse loss, not all inhibitory synapse loss is mediated by microglia. It remains unknown if the loss of presynaptic inhibitory synapses that is not driven by microglial phagocytosis is purely degenerative, active, autonomous, or mediated by another cell population.

While many studies have focused on microglial pruning of excitatory synapses (Schafer et al., 2012, Weinhard et al., 2018), less is known about microglial modulation of inhibitory synapses. Here we showed for the first time that microglia are necessary for loss of DH inhibitory synapses, and C1q, a known microglial phagocytosis effector, selectively accumulated at inhibitory synapses. Interestingly, there is evidence linking microglia with preferential elimination of inhibitory synapses in other CNS disorders (Di Liberto et al., 2018, Lui et al., 2016). However, to our knowledge, our study is the first to provide direct evidence for the engulfment of inhibitory synapses by microglia. Furthermore, we identified C1q as a molecular tag of inhibitory synapses that may mediate the selective removal of these synapses by microglia. The complement protein C1q recognizes specific proteome alterations in vulnerable synapses and initiates the complement cascade that eventually activates C3/CD11b synapse pruning pathway in microglia (Schafer et al., 2012). In many areas of the CNS, structural synaptic remodeling appears to coincide with functional changes, leading to sustained alteration in activity levels of the neuronal network(Inquimbert et al., 2018, Hofer et al., 2006, Hofer et al., 2009). There are reports suggesting that PNI lowers inhibitory drive through population-specific reduction in excitability of inhibitory neurons (Boyle et al., 2019, Leitner et al., 2013, Schoffnegger et al., 2006). Such chronic functional changes in neural activity can potentially drive synapse elimination through activity-dependent mechanisms. Therefore, it is plausible that a PNI-induced deficit in DH inhibitory transmission is the primary trigger for selective inhibitory synapse loss in neuropathic pain. Further, although specific molecular changes attracting C1q to synapses are not completely understood, general alterations in synaptic proteome compositions that are associated with

reduced synaptic activity and apoptotic-like mechanisms were shown to attract C1q to synaptic compartments (Gyorffy et al., 2018).

2.6.3 A new role for microglia in neuropathic pain

Our findings show that structural loss and reorganization of synapses in the DH circuitry take place in response to PNI. These structural modifications can contribute to the chronicity as well as abnormal sensory symptoms of neuropathic pain. Additionally, we show a key role for microglia in remodeling the DH circuitry through selective removal of synapses. Our discovery builds on the current knowledge that microglia play a major role in the pathology of neuropathic pain (Tsuda, 2016). Over 40 different microglial specific pathways have been identified that contribute to various aspects of neuropathic pain including efficacy of inhibition, microglia-astrocyte signaling, and inflammatory responses (Inoue and Tsuda, 2018). Specific targeting of each of these pathways lead to reversal of pain related behavior in animal models of neuropathic pain (Tsuda et al., 2003, Kobayashi et al., 2011, Batti et al., 2016, Griffin et al., 2007, Kobayashi et al., 2016, Maeda et al., 2010). Therefore, the analgesia that we observed as the result of global inhibition or depletion of microglia is likely due to targeting a constellation of changes, not only DH synaptic reorganization. Nevertheless, such a gross change in synaptic connectivity, specifically with respect to loss of inhibitory synapses, suggests a structural basis for reduced DH inhibitory drive and is conceptually relevant to the pathophysiology of neuropathic pain. Braz et al. (2012) achieved full recovery of pain related behavior by transplanting GABAergic precursor cells in the DH of neuropathic animals. This suggests that enhancing inhibition only by targeting structural plasticity of inhibitory neurons has therapeutic potentials (Braz et al., 2012).

In conclusion, our findings provide an overall assessment of structural dynamics of different populations of DH synapses, unravelling a new role of microglia as a mediator of selective inhibitory synapse loss in neuropathic pain. Altogether, this work contributes to the mechanistic understanding of neuropathic pain by demonstrating DH structural changes which maintain a maladaptive network function underlying symptom of neuropathic pain.

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2.8 Author contributions

NY designed and planned the entire study with the help of SL. NY carried out the majority of the experiments, prepared the figures and wrote the first manuscript version. SL edited the manuscript and performed minocycline injections. CW performed a portion of engulfment and synapse quantifications. HD proofread the manuscript and performed PLX3397 injections. LM quantified changes in microglial density. MSL and JO assisted with histology and EM. AK supported the super-resolution microscopy and manuscript reviewing. YDK contributed to the overall idea and manuscript reviewing. ARS oversaw the project as a whole and reviewed the manuscript.

Declaration of interests

The authors declare no competing interests.

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Connecting text: Chapter 2 to Chapter 3

In Chapter 2, in a rat model of chronic constriction nerve injury, we showed preferential elimination of a subset of excitatory synapses formed by primary afferents and inhibitory synapses of dorsal horn interneurons. We found that microglia were only responsible for the loss of inhibitory synapses since targeting microglia prevented both loss of inhibitory synapses and pain-related behavior, without affecting excitatory synapses. Consistent with this finding of selective pruning, we found that complement factors were upregulated and selectively tagged inhibitory synapses after nerve injury, sparing excitatory synapses.

Moving forward, in the next chapter, using a combination of complementary pharmacological and genetic approaches, we explored the molecular mechanisms underlying microglia-mediated synapse pruning in a mouse model of spared nerve injury (SNI). Specifically, we investigated the role of microglial CR3 and CX3CR1 signaling pathways as they are the major synapse pruning mechanisms described in the literature.

Chapter 3

Complement protein C1q is a therapeutic target for neuropathic pain

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3.1 Abstract

Activation of spinal microglia by peripheral nerve injury is a central component of neuropathic pain pathology. While microglia-mediated immune and neurotrophic signalling has been extensively studied in this disease, less is known about the phagocytic and synapse pruning capacities of microglia. Using a mouse model of neuropathic pain, we demonstrated that peripheral nerve injury induces engulfment of dorsal horn synapses by microglia, leading to a preferential loss of inhibitory synapses over excitatory synapses. This synapse removal was dependent on the microglial complement-mediated synapse pruning pathway, as mice deficient in complement C3 did not exhibit synapse elimination. Furthermore, pharmacological inhibition of complement protein C1q, prevented dorsal horn inhibitory synapse loss and interfered with the chronicity of neuropathic pain. Thus, we revealed a novel microglial mechanism in the pathology of neuropathic pain and identified C1q as a potential therapeutic target.

3.2 Introduction

Damage or disease of the nervous system can provoke a form of pathological pain condition known as neuropathic pain (Costigan et al., 2009). Symptoms of this debilitating neural disorder can include spontaneous, ongoing, or shooting pain, in addition to an amplified pain sensation in response to both noxious and innocuous stimuli (Baron et al., 2010). It is becoming increasingly evident that activation of spinal microglia, the resident macrophages of the central nervous system, plays a crucial role in the development of symptoms of neuropathic pain (Inoue and Tsuda, 2018). Following nerve injury, spinal microglia increase in number and undergo drastic morphological changes (Gilmore and Skinner, 1979). These changes are accompanied by an upregulation of genes associated with reactive states, the production and release of potent immune and neuro modulators, and an increase in phagocytotic activity (Coull et al., 2005, Echeverry et al., 2017, Jeong et al., 2016). Of these functions, far less is known about the role of microglial phagocytosis within the context of synapse pruning, and its implications in modulating spinal cord synaptic connectivity in neuropathic pain. However, this function is particularly important because maladaptive structural changes in the dorsal horn, such as pruning of inhibitory synapses, are likely key contributing factors to the chronic nature of neuropathic pain (Kuner and Flor, 2017).

In the context of development, various phagocytic signalling pathways are employed by microglia to regulate synapse pruning (Neniskyte and Gross, 2017). For example, in early postnatal life, microglia use the classical complement pathway to identify and remove weak synapses in the visual system (Schafer et al., 2012). In this model, complement proteins C3 and C1q bind to synapses, which can then be targeted and engulfed by microglia through their expression of complement receptor 3 (CR3). Similarly, microglia use the fractalkine receptor, CX3CR1, to regulate dendritic spine formation on hippocampal neurons (Paolicelli et al., 2011b). In this pathway, communication between CX3CR1, and its ligand, neuronal CX3CL1, results in the elimination of weak synapses. Interestingly, these pathways have been shown to be reactivated in a number of neurological disorders in adults (Gunner et al., 2019, Hong et al., 2016a, Lui et al., 2016). In animal models of neuropathic pain, microglial surface receptors CR3 and CX3CR1, are upregulated in the spinal cord, suggesting the possibility that these pruning pathways are active in this disease state (Clark and Malcangio, 2014, Griffin et al., 2007). However, whether they directly contribute to neuropathic pain remains unknown.

Here, we used a preclinical model of neuropathic pain to investigate engulfment of synaptic elements in combination with changes in synapse numbers in lamina II of the dorsal spinal cord. Lamina II was the region of interest, as it contains neuronal circuits that are critical for processing sensory information (Peirs and Seal, 2016). We demonstrated that nerve injury activated microglia engulf both spinal inhibitory and excitatory terminals, resulting in marked loss of inhibitory synapses with no effect on excitatory synapse count. Furthermore, we used a combination of complementary pharmacological and genetic approaches to identify the signaling pathway responsible for this synapse loss. We found that global depletion of microglia, genetic deletion of complement C3, and pharmacological inhibition of C1q protect against inhibitory synapse loss and result in an increase in excitatory synapse count. Therefore, we concluded that synapse elimination in spinal cord is mediated by the complement pathway. Most importantly, pharmacological inhibition of C1q reduced mechanical hypersensitivity at chronic time-points after nerve injury. Together, these results uncover a previously uncharacterized role of the CR3 synapse pruning pathway in neuropathic pain pathology. Moreover, our findings reveal that

targeting C1q appears to be a novel disease-modifying strategy; aimed at preventing maladaptive dorsal horn synaptic reorganization and circuit rewiring in neuropathic pain.

3.3 Methods

3.3.1 Animals

All mouse experiments were approved by the Animal Care Committee at McGill University and complied with Canadian Council on Animal Care guidelines. Following weaning, all mice were housed with their same-sex littermates and were kept in a temperature- and humidity-controlled room with a 12-h light/dark cycle and received food and water *ad libitum*. Wildtype C57BL/6 mice (6-8 weeks of age) of both sexes were purchased from The Jackson Laboratory (Bar Harbor, ME) for pharmacology experiments and used to breed mice of both sexes in-house for remaining experiments. Knock-in mouse lines used in this study include Gad2-tdTom and Tac1-tdTom mouse lines which were bred from the following commercially available parental lines: Rosa26LSL-tdTomato (Ai14) (JAX, stock #007908), Tac1-IRES2-Cre-D (JAX, stock #021877), and Gad2-IRES-Cre (JAX, stock #019022). Knock-out (KO) mouse lines include homozygous C3 KO (JAX, stock #003641), and CX3CR-1^{GFP} KO (JAX, stock #005582) which were bred in-house.

3.3.2 Pain related behavior

Baseline hind paw paw-withdrawal thresholds and the temporal development of mechanical hypersensitivity in mice were assessed using von Frey fibers and the up-down method of Dixon (Chaplan et al., 1994a). For this, mice were placed inside Plexiglas cubicles (5.3x8.5x3.6 cm) with a perforated metal floor and were permitted to habituate for at least 1 hour prior to testing. Using the up-down method of Dixon, mice were tested using an ascending series of von Frey filaments, starting with the lowest filament (0.008 g) until threshold was reached. In this approach, filaments were applied to the plantar surface of the hind paw for 3 seconds and responses were recorded. At least 2 consecutive measures were taken on each hind paw at each time point and averaged. The 50% response threshold was then calculated using the formula: 50% threshold (g) = $10^{(X+kd)}/10^4$, where X = the value (in log units) of the final von Frey filament, k = tabular value for the response pattern and d = the average increment (in log units) between von Frey filaments (Sorge et al., 2014). All experiments took place during the light cycle

and male and female animals were tested on separate days. All experimenters were blinded to the genotype of the mouse and/or the treatment group.

3.3.3 Peripheral nerve injury surgery

In all experiments, mice received a spared nerve injury (SNI) on the left side, which was performed under isoflurane/oxygen anesthesia as previously described (Decosterd and Woolf, 2000). For this, the sciatic nerve was exposed after making an incision on the skin on the lateral surface of the mouse thigh and sectioning through the biceps femoris muscle. Two of the three terminal branches of the sciatic nerve were tightly ligated with 7.0 silk (Covidien, S-1768K) and 2–4 mm of the nerve distal to the ligation were removed, avoiding any disturbance of the spared nerve. The muscle and skin were closed in separate layers using coated Vicryl (Ethicon, J489G). For experiments in which the sural nerve was left intact, von Frey testing was performed on the lateral part of the hind paw. In one experiment in which tibial nerve was intact, von Frey filaments were targeted to the mid-plantar hind paw (Shields et al., 2003). In all experiments mice were retested for mechanical hypersensitivity every 7 days for 3 weeks.

3.3.4 Drugs

PLX3397 (Selleckchem) (30 mg/kg, intraperitoneally (i.p), daily for a week) was dissolved in 10% DMSO+45% PEG 300+5% tween 80+ddH_2O at 20 mg/ml and administrated for 6 consecutive days. For the C1q neutralizing experiment, animals received i.p injections of either the ANX-M1 anti-C1q function blocking antibody (Annexon Biosciences) or IgG isotype control (Annexon Biosciences) at 100 mg/kg every 4 days starting 1 day prior to peripheral nerve injury until the experimental endpoint.

3.3.5 Tissue preparation and immunohistochemistry

At different timepoints post-surgery, for immunohistochemical analyses, mice were anesthetized (0.3 ml/100g of body weight of Equithesin containing 6.5 mg of chloral hydrate and 3mg sodium pentobarbital), and perfused transcardially with perfusion buffer (0.5 g/L $NaHCO_3$ in PBS) followed by 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (PB), pH 7.4, for 10 min. After fixation, spinal cords were extracted and post-fixed in the same fixative overnight at 4°C and then transferred to 30 % sucrose in PB for cryoprotection. For immunohistochemistry, transverse

spinal cord sections from the lumbar enlargement (L3–L5) were cut on a cryostat (Leica, Germany) at −20 °C. Twenty-five-µm-thick sections were prepared for Airyscan confocal microscopy (Carl Zeiss LSM 880) and 10-µm-thick sections were prepared for Structural Illumination Microscopy (SIM) (DeltaVision OMX SR). Sections were then permeabilized and blocked at room temperature for 1 hour in 10% normal donkey or goat serum and 0.2% Triton-X in PBS (PBST). This step was followed by incubation of sections in a cocktail of primary antibodies in 5% blocking solution at 4°C. Primary antibodies were rabbit anti-Iba1 (Wako, 1:1000), guinea pig anti-Iba1 (Synaptic Systems, 1:500), rat anti-CD68 (Bio-Rad, 1:500), mouse anti-vesicular GABA transporter (VGAT, Synaptic Systems, 1:1000), rabbit anti-VGAT (Synaptic Systems, 1:2000), rat anti-CD11b (Bio-Rad, 1:100), guinea pig anti-VGLUT2 (Millipore, 1:2000), mouse anti-gephyrin (Synaptic Systems, 1:400), rabbit anti-Homer1 (Synaptic Systems, 1:200), goat anti-mouse C3 (MP Biomedicals, 1:100), rabbit-anti NeuN (Millipore, 1:1000), rabbit anti-C1q (Abcam, 1:400), goat anti-CX3CL1 (R&D systems, 1:500), and rabbit anti-CX3CR1 (Thermofisher, 1:200). Twenty-four hours later, appropriate Alexa-fluorophore-conjugated secondary antibodies were applied to sections (Invitrogen, 1:800), which were incubated at room temperature for 2 hours. In some experiments, isolectin B4 (IB4) conjugated to Alexa 647 fluorophore was used in the mixture of secondary antibodies (Invitrogen, 1:200). Sections were then mounted and coverslipped using Prolong Gold Antifade mounting medium (Invitrogen) and Zeiss cover slips.

3.3.6 Microglia density quantification

Low magnification images were captured using a Zeiss AxioImager M2 Imaging microscope with the Zeiss ZenPro software v.2.3 (Zeiss Canada). For quantification of microglia density, 4 sections per animal were assessed. To capture the entire dorsal horn, a 10X field was imaged. To calculate the density of microglia, the number of microglia was divided by the area of the dorsal horn measured using ImageJ software (NIH).

3.3.7 Engulfment quantification

For assessing phagocytosis of synaptic elements, we used an engulfment quantification method inspired by Schafer *et al* (Schafer et al., 2014). Briefly, sections immunolabelled with CD68, Iba1, and a marker for the neuronal structure of interest were imaged by 63x oil immersion objective

(NA 1.4) using 0.2 µm z-steps using a Zeiss LSM 800 confocal microscope. Images were processed with IMARIS software (Bitplane) to produce 3D surface rendering of engulfed material within microglial lysosomes and the total volume of the associated surface was recorded for each microglia. For each animal, graphed values represent the mean of a total of 8-12 microglia sampled from 4 sections analyzed. Only microglia that had their full cell bodies imaged were selected for quantification. Same selection criteria and thresholding values were applied to all microglia. All experimenters were blinded to the genotype of the mouse, sex, and/or the treatment condition. Blinding to laterality in untreated mice was not possible because of the evident morphological changes in ipsilateral microglia.

3.3.8 Analysis of synaptic elements

SIM (Structured Illumination Microscopy) was used to assess synapse densities. Cells were imaged with a 100x, NA = 1.42, oil immersion objective on a Vision OMX V_4 Blaze system (GE) equipped with 488 nm, 592 nm, and 647 nm lasers and two Evolve EM-CCD cameras (Photometrics, Huntington Beach, CA, USA). Image stacks of 2 µm were reconstructed in Deltavision softWoRx 6.1.1 software with a Wiener filter of 0.01 using channel-specific optical transfer functions (Gustafsson et al., 2008). Captured synapses were analyzed with IMARIS software. A synapse was counted if the distance between the center point of a presynaptic and postsynaptic puncta was equal to or less than the radius of the presynapatic puncta + 0.1 µm, an empirically determined scaling factor (Hong et al., 2017). All experiments were analyzed blind to groups.

3.3.9 Western blot

Mice were perfused with cold saline and their spinal cords were extracted and homogenized on ice in lysis buffer (50 mM HEPES, 5 mM CaCl2, 1 mM MgCl2, 145 mM NaCl, pH 7.4) containing protease and phosphatase inhibitors (Sigma). After removal of large debris by centrifugation (1000 x g for 5 minutes at 4 °C) protein concentrations were determined by Bradford protein assay (Bio-Rad). Samples were prepared with 1/3 standard Laemmli buffer, 1/10 ß-mercaptoethanol, spinal cord homogenate and PBS, and 25 µg of protein was loaded onto 4–15% TGX precast gels (Bio-Rad) and separated by electrophoresis at 100 V. Proteins were

subsequently transferred to PVDF membranes (Bio-Rad) at 22 mA for 2 hours. The membranes were rinsed and subsequently treated with different primary antibodies made in Tris-buffered saline containing 0.1% Tween 20 (TBST) and 2.5% bovine serum albumin (BSA) for 1 hour at room temperature. Primary antibodies used: rabbit anti-TNF-α (1:500, Novus Biologicals), mouse anti-IL1α (1:200, R&D systems); and β-actin (1:3000, Cell Signaling Technology). After washing with TBST, the blots were incubated for 2 hours with peroxidase-conjugated secondary antibodies (Jackson ImmunoResearch, 1:10000), and washed again. Immunoreactive bands corresponding were visualized with ECL (GE Healthcare Life Sciences) using the Kodak Biomax XAR imaging film kit. The films were digitized, and the optical density of individual bands was measured with ImageJ software. Levels of TNF-α and IL1α immunoreactivity were normalized with respect to the β-actin levels in each sample.

3.3.10 RNA-seq analysis

Transcriptomics data of spinal cord from naïve mice and SNI-injured mice was taken from Gene Expression Omnibus accession GSE111216 (Parisien et al., 2019).

In short, RNA-Seq data have been trimmed with Trimmomatic v0.32 (Bolger et al., 2014), then mapped on UCSC's mouse genome version mm10 (grabbed from ftp://ussd-ftp.illumina.com/Mus_musculus/UCSC/mm10/) using tophat v2.0.11 (Kim et al., 2013) and bowtie v1.0.0 (Langmead et al., 2009). Differential gene expression detected at gene level from experimental triplicates using cuffdiff v2.2.1 (Trapnell et al., 2013).

3.3.11 RNAscope

The RNAscope Fluorescent Multiplex Assay (ACD Biosystems) was performed according to the ACD protocol for fresh-frozen tissue. Spinal cords from animals at 7 days after injury which had been perfusion fixed were sectioned at 10 µm on a cryostat and hybridized with C1qa mRNA probe (ACD cat#: 441221). The ACD 3-plex served as a negative control and applied on one section per slide to confirm signal specificity. The probes were amplified according to manufacturer's instructions and labeled with Alexa 488nm fluorophores.

3.3.12 Identification of mouse estrous cycle stages

The phase of the estrous cycle was determined by analysis of vaginal smears on three consecutive days. Smear slides of vaginal cells were collected and stained with Crystal violet stain (0.1%) solution. The smears were examined using light microscopy to determine cell types present for assessing stages of the estrus cycle. Each phase of the estrous cycle was defined as follows: proestrus (100% intact epithelial cells), estrus (100% cornified epithelial cells), metestrus (~50% cornified epithelial cells or exfoliated epithelial cells and 50% leukocytes), and diestrus (cell debris, some cornified epithelial cells or leukocytes).

3.3.13 Experimental design and statistical analyses

Sample sizes were determined based on pilot studies and in the absence of sex difference, male and female animals were pooled. All statistical tests were completed using Windows GraphPad Prism version 8. Analyses used include only parametric tests: one-way ANOVA, two-way repeated measures ANOVA, and two-way ANOVA followed by Bonferroni's multiple comparison tests, and unpaired or paired two-tailed t tests. All p values and statistical tests employed are indicated in results and figure legends.

3.4 Results

3.4.1 Nerve injury triggers engulfment of presynaptic terminals by microglia

We first assessed the phagocytic capacity of spinal microglia to engulf inhibitory and excitatory neuronal elements at different timepoints after peripheral nerve injury. Mice were subjected to a spared nerve injury (SNI) and engulfment of neuronal elements by dorsal horn microglia was quantified at day 3, 7, 14, and 21 post-SNI. To identify and differentiate inhibitory and excitatory elements, mice expressing TdTomato in inhibitory neurons ($GAD2^{Cre}$; tdTomato, referred to as GAD2-tdTom, Figure 1A) and substance P expressing excitatory neurons ($TAC1^{Cre}$; tdTomato, referred to as TAC1-tdTom, Figure 1B) were used. In GAD2-tdTom mice, all inhibitory neurons were labelled by Cre-dependent expression of tdTomato fluorescent protein (Figure 1A). Using the same Cre-lox recombination system in TAC1-tdTom mice, substance P expressing excitatory neurons were identified (Figure 1B). To quantify engulfment of genetically labelled inhibitory and excitatory neuronal elements, for each genotype, high-resolution Airy scan three-dimensional

stacks of dorsal horn (lamina II) microglia were analyzed for the presence of neural-derived tdTomato+ elements within microglial CD68+ lysosomes (Figures 1C-D and S1A-B). At day 7 post-SNI, but not at day 3, a significant increase in the total volume of inhibitory and excitatory neuronal elements within lysosomes of ipsilateral microglia was observed (GAD2-tdTom: t_{88} = 9.666, p<0.0001; Tac1-tdTom: t_{88} = 7.479, p<0.0001) (Figures 1E and 1F). Regardless of the nature of the engulfed material, the phagocytic activity of microglia peaked at 7 days after injury and gradually declined in the following two weeks. However, compared to contralateral levels, the ipsilateral increase in engulfment remained significant at the latest timepoint (day 21) for both neuronal types (GAD2-tdTom: t_{88} =37.59, P=0.0012; Tac1-tdTom: t_{88} = 3.382, p=0.0043). Although, temporal analysis showed no significant difference in the time-course of engulfment of inhibitory and excitatory elements, at day 7 post-SNI, a greater volume of inhibitory structures was engulfed compared to excitatory (t_{22} =1.316, P<0.0001) (Figure 1E and 1F).

To further distinguish what sub-cellular component of dorsal horn neurons had been engulfed by microglia we used high resolution Airyscan microscopy. We performed immuno-colocalization analyses of CD68, and markers of different neuronal compartments within Iba1-labelled microglia. For assessing the engulfment of synaptic terminals of inhibitory and excitatory interneurons, we used immunohistochemistry to detect synapse-specific proteins, VGAT and VGLUT2 respectively (Figures 1G and 1H). Quantifications of engulfment for specific terminal markers showed a similar trend as engulfment for neuronal elements (Figure 1I-J). At 7 days post-injury, there was a two-fold increase in the total volume of VGAT+ and VGLUT2+ terminals within microglial lysosomes sampled from the ipsilateral dorsal horn as compared to the contralateral side (VGAT: t_{88} =8.566, P<0.0001; VGLUT2: t_{88} = 4.771, P<0.0001) (Figures 1I and 1J). Consistent with the previous results, we detected an increased engulfment at day 14 and day 21 post-SNI, however, engulfment of inhibitory synapses was reduced at later timepoints as compared to day 7 (Figures 1I and 1J). To determine whether other neuronal structures are engulfed by microglia, we examined the engulfment of neuronal cell bodies (NeuN) as well as inhibitory (gephyrin) and excitatory (homer1) postsynaptic structures and found no evidence of their engulfment at 3,7,14, and 21 days post-SNI (Figures S2A-C). Additionally, to verify that the increase in engulfment of neuronal structures and presynaptic elements was not accompanied by neuronal loss, we

counted dorsal horn neurons and observed no significant decrease in overall number of inhibitory (GAD2+) and excitatory (TAC1+) cells at day 21 post-injury (Figure S2D). Therefore, we concluded that the observed tdTomato+ neuronal elements phagocytosed by microglia in our transgenic lines, are primarily enriched for presynaptic terminals. Thus, nerve injury activated microglia engulf both inhibitory and excitatory presynaptic terminals in the dorsal horn of neuropathic mice.

3.4.2 Transient depletion of microglia attenuates mechanical hypersensitivity and prevents dorsal horn synapse loss

It is well-established that microglia play an important role in neuropathic pain pathology (Tsuda, 2016) as both chronic or acute targeting of microglia have been shown to prevent and reverse pain behavior in models of neuropathic pain. To study the contribution of the synapse engulfment function of microglia to pain behavior, we transiently depleted microglia during the peak of their engulfment activity based on our temporal analysis of synapse engulfment (Figures 1I-J). To this end, we pharmacologically blocked microglial survival signaling, using PLX3397 (a specific colony stimulating factor 1, CSF1, inhibitor), for 6 consecutive days starting from day 5 to day 10 post-injury (Figure 2A). PLX3397 depleted 91% of dorsal horn microglia and eliminated the difference in microglial density between ipsilateral and contralateral dorsal horn by the end of the treatment period (vehicle: t_{40} =9.242, P<0.0001; PLX3397: t_{40} = 0.2486, P>0.9999) (Figures 2B-C). Within 10 days following the end of treatment, microglia repopulated the dorsal horn with a greater number of cells in the ipsilateral side compared to the contralateral (vehicle: t_{40} =8.934, P<0.0001; PLX3397: t_{40} = 3.698, P=0.0026) (Figures 2B and 2C). Next, we assessed the effect of transient depletion of microglia on mouse pain-related behavior. This analysis showed a reduction of mechanical hypersensitivity at all timepoints following SNI in PLX3397-treated mice as compared to vehicle-treated mice (treatment × repeated measures: $F_{3,120}$= 25.83, p<0.0001) (Figure 2D).

To determine whether nerve injury induces synaptic loss and if this loss is caused by microglia-mediated engulfment of presynaptic terminals, dorsal horn inhibitory and excitatory synapse densities were quantified in neuropathic mice using SIM. For inhibitory synapses, presynaptic terminals were labeled with an antibody against VGAT and postsynaptic sites were labeled with

anti-gephyrin. A synapse was counted when VGAT and gephyrin immunoreactive puncta were in close apposition (Figure 2E). Consistent with previous reports (Lorenzo et al., 2020), peripheral nerve injury resulted in significant reduction in the number of lamina II inhibitory synapses quantified as density of VGAT and gephyrin pairs (vehicle: t_{96} = 4.004, P=0.0002) (Figure 2F). In line with our hypothesis, depletion of microglia prevented loss of inhibitory synapses in the ipsilateral dorsal horn without affecting the contralateral synapse density (PLX3397: t_{96} = 0.3284. P> 0.9999) (Figure 2 F). For assessing excitatory synapse density, presynaptic terminals were labeled with anti-VGLUT2 antibody and postsynaptic excitatory sites were labeled with anti-homer1 (Figure G). In lamina II of the dorsal horn, VGLUT2 marks terminals of IB4 binding peripheral sensory neurons as well as those of spinal cord excitatory interneurons. To exclude VGLUT2+ terminals of sensory neurons from our analysis, we only quantified pairs between VGLUT2 positive terminals that were IB4 negative and homer1-positive postsynaptic puncta. Quantifications of VGLUT2+ excitatory synapses showed no difference between ipsilateral and contralateral dorsal horns in vehicle treated animals (vehicle: t_{96} = 1.499, P=0.2480) (Figure 2H). Surprisingly, microglial depletion resulted in 13% increase in ipsilateral excitatory synapse density (PLX3397: t_{96} = 1.499, P=0.2480) (Figure 2H). Although there was no sex difference in the effect of injury or treatment on densities of inhibitory and excitatory synapses, a greater variability was found among synapse density values in females. We attempted to determine whether the source of this variability is associated with female sex-hormones. For this, stages of the estrous cycle which correlates with levels of sex-hormones were determined for each female mouse at 21 days post-injury (Figure S3A-D). Considering the uneven distribution of animals within estrus cycle stages, we could not find any clear relationship between synapse density and different stages of the estrus cycle. Taken together, these data suggest that in both sexes, microglia contribute to the maintenance of mechanical hypersensitivity through removal of dorsal horn inhibitory and excitatory synapses in the SNI model of neuropathic pain.

3.4.3 Peripheral nerve injury induces microglia specific CR3 and CX3CR1 mediated synapse pruning pathways

We next investigated which microglial synapse pruning pathway mediates elimination of presynaptic terminals in in neuropathic pain. RNA-seq data of spinal cord at day 7 post-SNI when

microglial engulfment activity peaks, was analyzed using selected Gene Ontology (GO) annotations and revealed that synapse pruning pathways are significantly up-regulated in spinal cord of SNI mice as compared to naive animals (ES=+0.99, P=1.0x10^{-2}, FDR=11%)(Parisien et al., 2019). Specifically, genes related to CR3 and fractalkine receptor (CX3CR1) pathways, which are two main molecular pathways associated with synapse pruning, were significantly upregulated (Figure 3A). We next assessed protein levels of the main signaling molecules within these pathways in the spinal cord of neuropathic mice.

CR3 synapse pruning pathway consists of the initiating complement molecule C1q and downstream complement factor C3, which both localize to synapses, and CR3 which is expressed by microglia (Figure 3B) (Schafer et al., 2012). Quantification of C1q, and C3 protein levels in dorsal horn of neuropathic mice at day 7 post-injury revealed a significant increase in C1q and C3 expression (C1q: t_9 = 17.92, P<0.0001; C3: t_9= 5.614, P=0.0003) (Figures 3C1-2, and 3D1-2). Moreover, the number of CR3 expressing microglia was significantly increased (~3.5 fold) in ipsilateral dorsal horn at the same timepoint (CR3: t_7= 11.79, P<0.0001) (Figures 3E1-2). Time-course analysis of C1q, C3, and CR3 expression showed an earlier and greater increase in CR3 levels starting from day 3 following injury (Figure S4A). This was followed by a significant increase of C1q and C3 at day 7 post-injury. By the end of the third week after injury, C1q expression showed a modest decline while C3 and CR3 levels remained stable (Figure S4A).

The other major synapse pruning pathway, which was also identified in our RNA-seq dataset, operates through the communication between neuronal chemokine fractalkine (CX3CL1) and its microglial receptor CX3CR1 (Figure 3B) (Paolicelli et al., 2011b). Using CX3CL1 antibodies, we observed immunoreactivity of CX3CL1 in the dorsal the spinal cord, however, further analysis showed no overall (Figures 3F1-2) or cell specific changes (Figures S5A-C) in CX3CL1 expression 7 days following injury. Conversely, there was a significant increase in CX3CR1 expression at the same timepoint, quantified as overall fluorescence intensity within dorsal horn area (t_5= 3.663, P=0.0146) (Figures 3G1-2) as well as per individual microglia (t_{20}= 3.431, P=0.0026) (Figures 3H1-2). Time-course analysis of CX3CR1 expression after injury revealed a stable increase, starting on day 3, and persisting for three weeks after injury (Figure S4B). Taken together, these experiments

provide evidence for upregulation of CR3 and CX3CR1 synapse pruning pathways in our neuropathic pain model.

3.4.4 Nerve injury induced engulfment of spinal presynaptic terminals is mediated by CR3 pathway

Nerve injury-induced increase in components of CR3 and CX3CR1 pruning pathways in dorsal spinal cord supports the involvement of these pathways in synapse pruning. To fully assess the respective contribution of the CR3 and CX3CR1 pathways to spinal cord synapse removal in neuropathic pain, levels of engulfment of inhibitory and excitatory presynaptic terminals were measured at the peak of synapse engulfment (i.e., 7 days after injury) in mice lacking complement factor C3 (C3 KO) and CX3CR1 (CX3CR1 KO). In both KO mouse lines, peripheral nerve injury induced microgliosis (C3 KO: t_{30}= 8.762, P<0.0001; CX3CR1 KO: t_{30}= 6.088, P<0.0001) (Figures S6A-B) and a significant increase in the volume of CD68+ lysosomal compartments in the ipsilateral dorsal horn (C3 KO: t_{30}= 4.210, P=0.0006; CX3CR1 KO: t_{30}= 3.173, P=0.0104) (Figures S6A and C). Although the increase in the number of microglia and lysosomal volumes in CX3CR1 KO mice was smaller as compared to C3 KOs and wildtype littermates (Figures S6A-C), engulfed inhibitory and excitatory terminals were still detected within CX3CR1 KO lysosomes in the ipsilateral dorsal horn (Figures 4A and 4C).

Quantifications of the total volume of VGAT+ inhibitory terminals within microglial lysosomes showed a similar increase in microglia sampled from ipsilateral dorsal horn of CX3CR1 KOs and wild type mice as compared to contralateral side (Figures 4A-B). Significant engulfment of VGLUT2+ excitatory terminals was also detected in CX3CR1 KO mice but was less pronounced compared to wild type (t_{42}= 4.210, P=0.0024) (Figures 4C-D). Remarkably, C3 KO mice showed no significant engulfment of both inhibitory and excitatory presynaptic terminals as demonstrated by significant reductions of the total volume of VGAT and VGLUT2 immunoreactive puncta within microglial lysosomes (Figures 4B and 4D). Therefore, unlike the CX3CR1 pathway, the CR3 signaling pathway is necessary for the engulfment of terminals by microglia.

We next evaluated the role of CR3 and CX3CR1 pruning pathways in remodeling of dorsal horn synaptic connectivity in the SNI model of neuropathic pain. To this end, we quantified inhibitory and excitatory synapse densities in C3 KO mice and CX3CR1 KO mice 21 days after injury. Similar

to wild type mice, we found a 1.2-fold decrease in ipsilateral inhibitory synapse density (i.e., VGAT puncta juxtaposed gephyrin puncta) in CX3CR1 KO mice as compared to contralateral side (CX3CR1 KO: t_{42}= 3.375, P=0.0048) (Figures 4E-F). In contrast, ipsilateral inhibitory synapse density in C3 KO mice was completely preserved (Figures 4E-F). With respect to excitatory synapses, we observed no statistically significant change in VGLUT2+ excitatory synapse density in CX3CR1 KO mice as compared to wildtype animals (Figures 4G-H). However, ipsilateral VGLUT2+ excitatory synapse density in C3 KOs was increased by 9% compared to contralateral side (t_{42}= 4.210, P=0.0104) (Figure 4H). Interestingly, comparing contralateral synapse densities revealed that C3 KO mice had 20% increase in inhibitory synapse density and 24% in excitatory synapse density as compared to wildtype littermate controls indicating a potential developmental deficit in refinement of dorsal horn synaptic connections (Figures 4F and 4H). Taken together, these data implicate the CR3 synapse pruning pathway, but not the CX3CR1 pathway, as a mechanism modulating dorsal horn synaptic connectivity in the SNI model of neuropathic pain.

3.4.5 Complement protein C1q is expressed by nerve injury activated microglia and localized to dorsal horn synapses

Complement protein C1q is the initiating factor of the classical complement cascade and has been implicated in the selective elimination or "pruning" of synapses through the CR3 pathway (Schafer et al., 2012). C1q is normally low in the adult CNS; however, normal aging, injury or diseases can stimulate C1q expression (Presumey et al., 2017). Previous work in the CNS reported that both neurons and glia are capable of synthesizing C1q (Bialas and Stevens, 2013, Stevens et al., 2007). To determine whether neurons are the source of C1q in neuropathic pain, we performed fluorescent *in situ* hybridization for *C1qA* mRNA on spinal cord sections of GAD2-tdTOM and TAC1-tdTOM mice collected at 7 days post injury (Figure 5A). Consistent with the RNA-seq results, there was a significant upregulation (≈8-fold) of *C1qA* mRNA assessed as the number of C1q mRNA immunoreactive puncta per DAPI+ dorsal horn cell nuclei (t_8=11.24, P<0.0001) (Figures 5A-B). Interestingly, the *C1qA* mRNA signal was almost completely excluded from GAD2+ and TAC1+ cell bodies suggesting that these cells are not the source of C1qA (Figures 5A and C). We next closely examined the expression pattern of C1q protein in the dorsal horn of

neuropathic mice. We observed bright, distinct C1q immunoreactivity localized within microglia which was significantly elevated in the ipsilateral dorsal horn (t_{10}=6.382, P<0.0001) (Figures 5D-E). In addition to the marked increase of C1q protein within microglia, a relatively smaller, punctate signal was scattered outside of microglia which was only found in the ipsilateral dorsal horn (Figure S7). To test if this extracellular C1q signal was associated with synaptic structures, we performed SIM on spinal cord sections immunolabelled for C1q as well as presynaptic markers for inhibitory (VAGT+) and excitatory (VGLUT2+) synapses. Consistent with our prediction, most extracellular C1q signals were in close proximity of presynaptic markers (Figure 5F). Quantification of the number of synapses colocalized with C1q revealed a higher percentage of both inhibitory and excitatory synapses colocalized with C1q in ipsilateral dorsal horn as compared to contralateral (VGAT: t_{36}=12.48, P<0.0001; VGLUT2: t_{36}=3.510, P=0.0024) (Figure 5G). Interestingly, nerve injury induced C1q expression, showed a greater preference for inhibitory synapses as compared to excitatory (t_{18}=9.149, P<0.0001) (Figure 5G). To find whether C1q deposited on synapses is secreted by microglia, we chronically depleted microglia using PLX3397 and assessed C1q protein expression in the dorsal horn at day 7 post-injury. Depletion of microglia resulted in almost complete elimination of C1q immunoreactivity in the dorsal horn which included C1q deposition on synapses (Figures 5H-I and S8).

After establishing that C1q is released from spinal microglia after injury and deposited on synapses, we asked whether expression of apoptotic protein, cleaved caspase-3 (CC3), was present at vulnerable synapses attracting C1q. To answer this question, we analyzed inhibitory and excitatory presynaptic terminals for the presence of CC3 and C1q. Co-localization analysis showed that CC3 signal was rarely found at presynaptic terminals, and this was independent of C1q deposition at the synaptic site (Figures S9A-C), indicating that CC3 does not drive injury induced synapse pruning in the spinal cord.

Together, these results demonstrate that C1q protein is produced by microglia and preferentially marks dorsal horn inhibitory synapses for elimination in neuropathic pain.

3.4.6 Inhibiting C1q attenuates neuropathic pain and protects dorsal horn synaptic circuitry

To determine whether induction of C1q expression in response to peripheral nerve injury causes synaptic reorganization in dorsal horn, we pharmacologically blocked C1q using anti-C1q

antibodies (ANX M1.21 kindly provided by Annexon Biosciences). Anti-C1q antibodies were administered intraperitoneally every 4 days, starting 24 hours before SNI surgery. Mechanical hypersensitivity associated with neuropathic pain was assessed weekly (Figure 6A). Mice in both control (IgG isotope) and drug treated groups developed mechanical hypersensitivity as shown by decreases in withdrawal thresholds of the ipsilateral hind paw (Figure 6B). Remarkably, at day 21 after injury, unlike IgG-isotope control treated mice, both male and female ANX M1.21 treated animals showed a significant reduction in mechanical hypersensitivity (t_{152}=13.207, P=0.0065) (Figure 6B). At this timepoint, SIM revealed a marked decrease in the number of ipsilateral C1q-associated inhibitory synapses in ANX M1.21 treated groups as compared to IgG-isotope control treated (t_{20}=7.328, P<0.0001) (Figures 6C-D).

To study whether the effect of ANX M1.21 on neuropathic pain-related behavior was associated with disruption of the CR3 microglia mediated synapse pruning pathway, we quantified engulfment of inhibitory and excitatory terminals by microglia in control and treated animals. In line with our hypothesis, there was a significant reduction in inhibitory and excitatory presynaptic elements within ipsilateral microglial lysosomes of ANX M1.21 treated mice as compared to controls (inhibitory synapse engulfment: t_{36}=12.79, P<0.0001; excitatory synapse engulfment:t_{36}=2.872, P=0.0136) (Figures 6E-H). To test whether blocking C1q leads to protection against nerve injury induced synapse loss, we conducted synaptic quantification in the dorsal horn of ANX M1.21 and IgG control mice 21 days after injury. ANX M1.21 prevented inhibitory synapse loss in the spinal dorsal horn (Figure 6I-L) resulting in a similar protective effect as observed with PLX3397 treatment and C3 KO (ipsilateral inhibitory synapses: t_{76}=5.882, P<0.0001; ipsilateral excitatory synapses: t_{76}=5.249, P<0.0001).

In addition to synapse pruning, C1q is implicated in pathological mechanisms through stimulating the production of neurotoxic astrocytes (A1) (Liddelow et al., 2017). We asked whether A1 astrocytes are present in the spinal cord after peripheral nerve injury. At day 21 after injury, we quantified protein levels of Il-1α, and TNFα that along with C1q have been shown to induce A1 astrocytes. Although TNFα was significantly increased in ipsilateral dorsal horn, levels of Il-1α remained unchanged (Figures S10A-D). Moreover, C3, the main characteristic gene in A1 astrocytes, was not expressed within the ipsilateral spinal cord (Figure S10E). We did not find

evidence that A1-like reactive astrocytes play a significant role in the spinal cord of neuropathic mice and, therefore, ruled out the possibility that blocking C1q influences astrocytic transformation in this model of neuropathic pain.

Altogether, these results show that targeting C1q, the initiating signaling protein of the CR3 synapse pruning pathway, can protect the dorsal horn synaptic network and attenuate chronic neuropathic pain through reducing microglia mediated synapse engulfment.

3.5 Figures

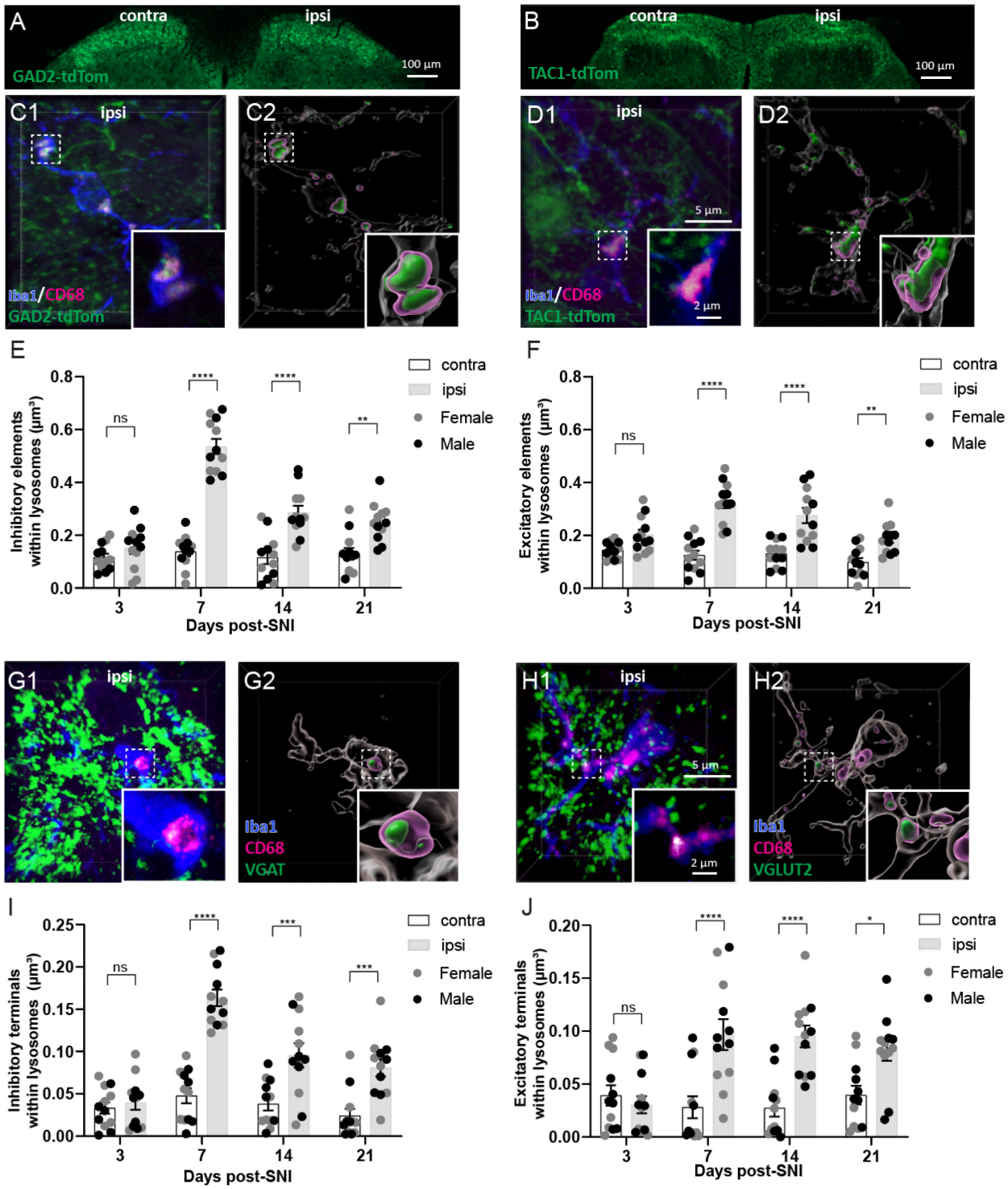

Figure 1. Nerve injury triggers engulfment of presynaptic terminals by microglia. A-B, the expression of tdTomato fluorescent protein reporter in dorsal horn of Gad2-tdTom (A) and Tac1-

tdTom (B) mice to demonstrate the distributions of inhibitory GAD65-expressing and excitatory substance P-expressing neurons, respectively. C-D, Representative 3D reconstructions (C1, D1) and surface rendering (C2, D2) of microglia (blue) sampled from ipsilateral dorsal horn (lamina II) to show internalized inhibitory and excitatory neuronal elements within lysosomal compartments (magenta) of ipsilateral microglia. Insets within raw images are single plane enlarged images selected from confocal stacks illustrating the presence of tdTomato fluorescent signals inside lysosomes. Insets within 3D reconstructions are enlarged views of the engulfed volumes from the same regions. E-F, quantification of temporal changes of engulfment of inhibitory (E) and excitatory (F) tdTomato+ neuronal structures by microglia. G-H, Three-dimensional fluorescent images of microglia (blue) from ipsilateral dorsal horn labeled for lysosomal marker CD68 (magenta), marker of presynaptic inhibitory terminals (VGAT; green) (G1) and marker of presynaptic excitatory terminals (Vglut2; green) (H1). Representative 3D surface-rendered images are illustrating the engulfment of presynaptic elements (G2 and H2). Insets within raw images are single plane enlarged images selected from confocal stacks to illustrate the co-localization between CD68 and markers of presynaptic terminals. Insets within 3D reconstructions are enlarged views of the engulfed volumes from the same regions. I-J, Quantification of temporal changes of engulfment of inhibitory (I) and excitatory (J) presynaptic terminals by microglia. *$p<0.05$,**$p<0.01$, ***<0.001, and ****$P<0.0001$ analyzed with two-way ANOVA followed by Bonferroni post hoc test. Means are plotted with individual data points ± SEM.

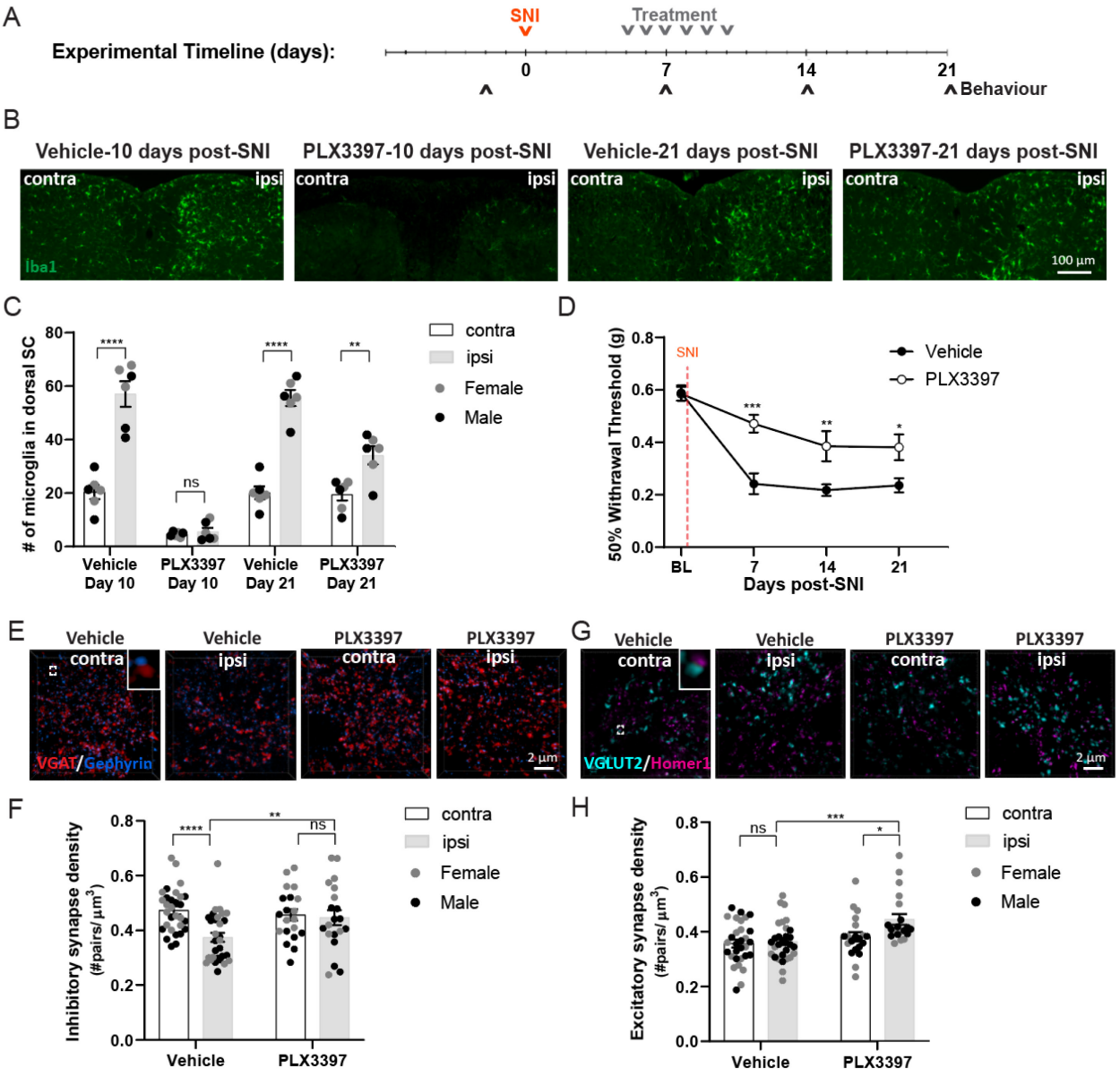

Figure 2. Transient microglia depletion during the peak of synapse engulfment prevents the normal development of mechanical hypersensitivity and protects dorsal horn synaptic connectivity. A, an experimental diagram showing the timeline of drug treatments, SNI surgery, and behavioral tests. B, dorsal horn images labeled for Iba1 (green) from vehicle and drug (PLX3397) treated groups imaged on the last day of treatment and after a 10-day wash-out period. C, quantification of the number of microglia in dorsal spinal cord. D, 50% withdrawal threshold responses to von Frey filaments in neuropathic mice treated either with PLX3397 or vehicle (n = 16 per group; 8 per sex). E, representative SIM images of inhibitory synapses labelled

with a pre-synaptic marker (VGAT; red) and a post-synaptic marker (gephyrin; blue) in different conditions. The inset within the first image is an enlarged view of an inhibitory synapse selected from the 3D confocal stack to illustrate the co-localization between the presynaptic and postsynaptic elements. F, Quantification of inhibitory synapse density in lamina II layer of the dorsal horn. G, representative SIM images of excitatory synapses labelled with a pre-synaptic marker (VGLUT2; teal blue) and a post-synaptic (homer1; magenta) in different conditions. The inset within the first image is an enlarged view of an excitatory synapse selected from the 3D confocal stack to illustrate the co-localization between the presynaptic and postsynaptic elements. H, Quantification of inhibitory synapse density in lamina II layer of the dorsal horn. *p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001 analyzed with repeated measures two-way ANOVA with Bonferroni post hoc test (D) and two-way ANOVA with Bonferroni post hoc test (C, F, and H). Means are plotted with individual data points ± SEM.

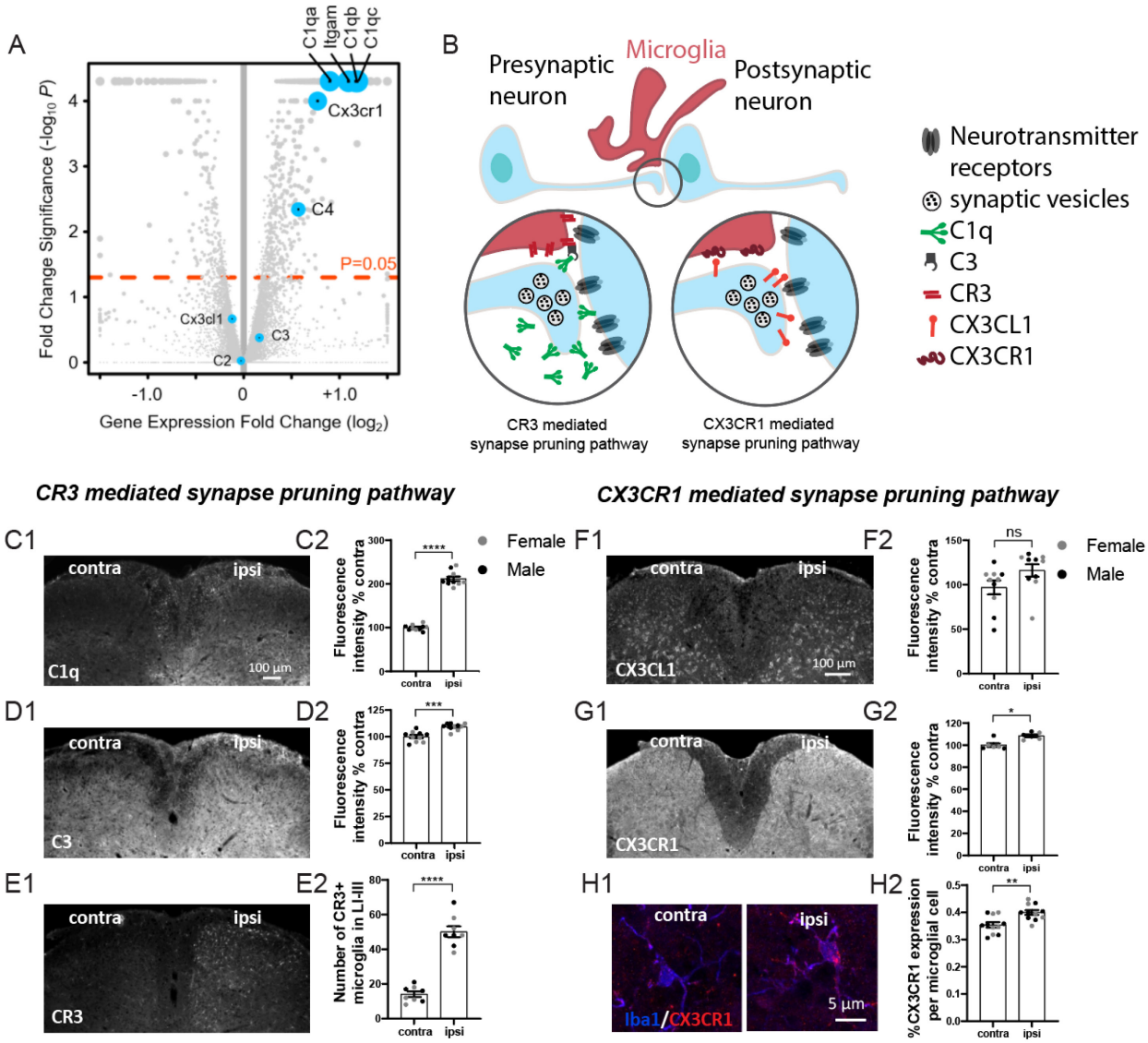

Figure 3. CR3 and CX3CR1 mediated synapse pruning pathways are upregulated in neuropathic pain. A, Volcano plot showing gene expression changes in neuropathic mice at day 7 post SNI. Each dot is a gene. Positive fold change indicates higher expression in the spinal cord of SNI-injured mice than in naïve. Genes highlighted in blue are related to CR3 and CX3CR1 pruning pathways. Dashed horizontal orange line indicates P-value of 0.05. B, the schematic illustrates microglia and neuronal molecules that participate in CR3 and CX3CR1 pruning mechanisms. C1-E1, representative images of C1q, C3, and CR3 labeling in the dorsal horn of SNI mice 7 days after injury. C2-E2, quantifications of fluorescence intensity of dorsal horn C1q, and C3, as well as number of CR3+ microglia in laminae I-III. F1, Representative dorsal horn image of CX3CL1

immunolabelling in SNI mice 7 days after injury (quantified in F2). G1, low-magnification representative images of CX3CR1 expression in the dorsal horn of SNI mice (quantified in G2). H1, High-resolution image of CX3CR1 (red) expression in microglia (blue) sampled from ipsilateral and contralateral dorsal horn (quantified F2). **p <0.01, ***p<0.001, and ****p<0.0001 analyzed with paired two-tailed t-test (C2-G2) and unpaired t-test (H2). Means are plotted with individual data points ± SEM.

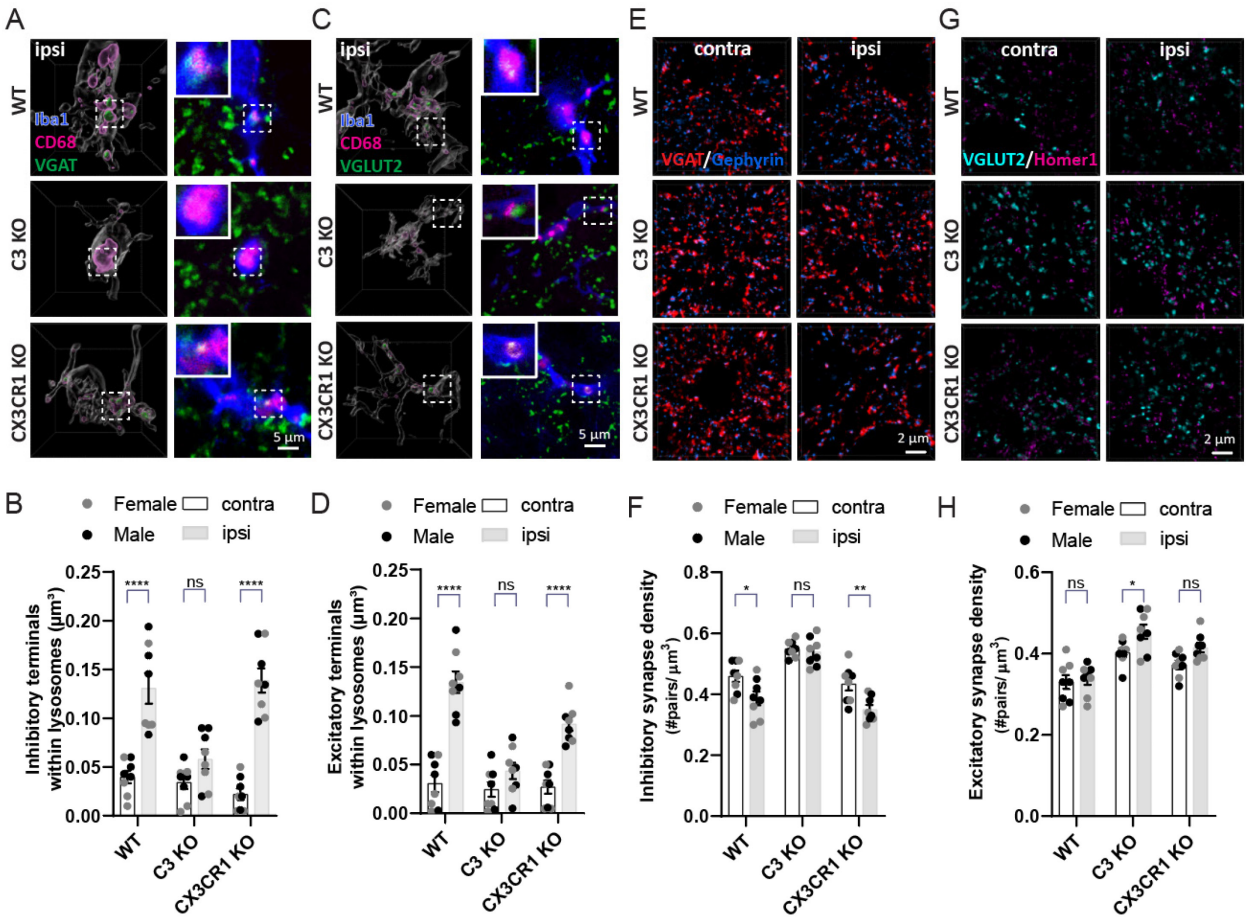

Figure 4. Nerve injury induced engulfment of spinal presynaptic terminals is mediated by CR3 Pathway. A and C, images in the first column are representative surface rendering 3D reconstructions of microglia (white) sampled from ipsilateral dorsal horns of neuropathic wildtype (WT), C3 KOs, and CX3CR1 KOs and demonstrate lysosome (magenta) co-localization with inhibitory (A) and excitatory (C) pre-synaptic markers (green). Single plane images selected from confocal stacks illustrating the presence or absence of CD68+ lysosomal co-localization with synapse markers are shown in the second column. B and C, quantification of engulfment of inhibitory (B) and excitatory (C) presynaptic terminals by microglia in different genotypes. E and G, three-dimensional representative images of inhibitory (A1: VGAT in red and gephyrin in blue) and excitatory (B1: VGLUT2 in teal blue and homer1 in magenta) terminals captured from ipsilateral and contralateral dorsal horns of WT, C3 KO, and CX3CR1 KO neuropathic mice. F and H, quantifications of dorsal horn inhibitory (G) and excitatory (I) synapse densities for

neuropathic mice of different genotypes. *p<0.05, **p<0.01, and ****p<0.0001 analyzed by two-way ANOVA with Bonferroni post hoc test. Means are plotted with individual data points ± SEM.

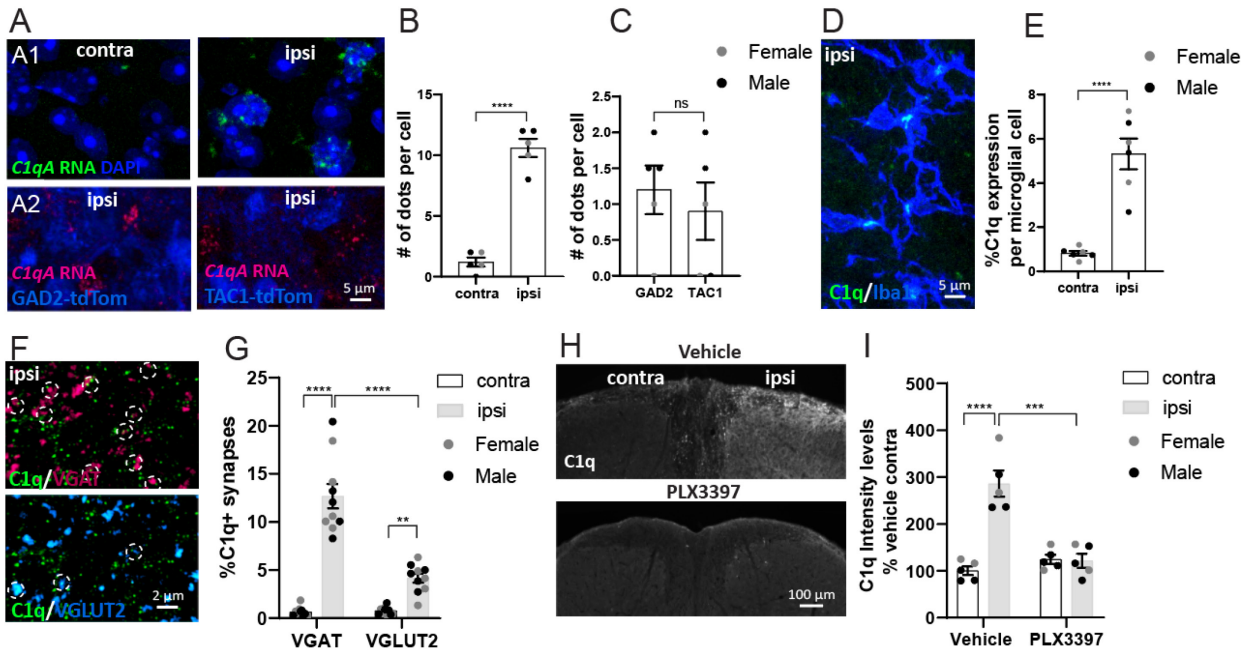

Figure 5. complement protein C1q is expressed by nerve injury activated microglia and localized to dorsal horn synapses. A, RNA scope for C1qA (A1: green) in ipsilateral and contralateral dorsal horn cells (quantified in B). Absence of C1qA (A2: red) expression in inhibitory and excitatory substance P neurons (blue) (quantified in C). D-E, representing high-magnification confocal image of C1q protein (green) in microglia (blue) (quantified in E). Representative SIM images (F) captured from ipsilateral dorsal horn at 7 days after injury show the preferential co-localization of C1q (green) with inhibitory synapses (VGAT in red), compared to excitatory (VGLUT2 in blue). Dotted circles show synapses that are colocalized with C1q. G, quantifications of C1q co-localization with inhibitory and excitatory synapses. H, C1q staining patterns in the dorsal horn of neuropathic mice chronically treated with vehicle and PLX3397 (quantified in I). ***p<0.001, and ****p<0.0001 analyzed by two-way ANOVA with Bonferroni post hoc test (G-H) and unpaired t-test (B-C, and E). Means are plotted with individual data points ± SEM.

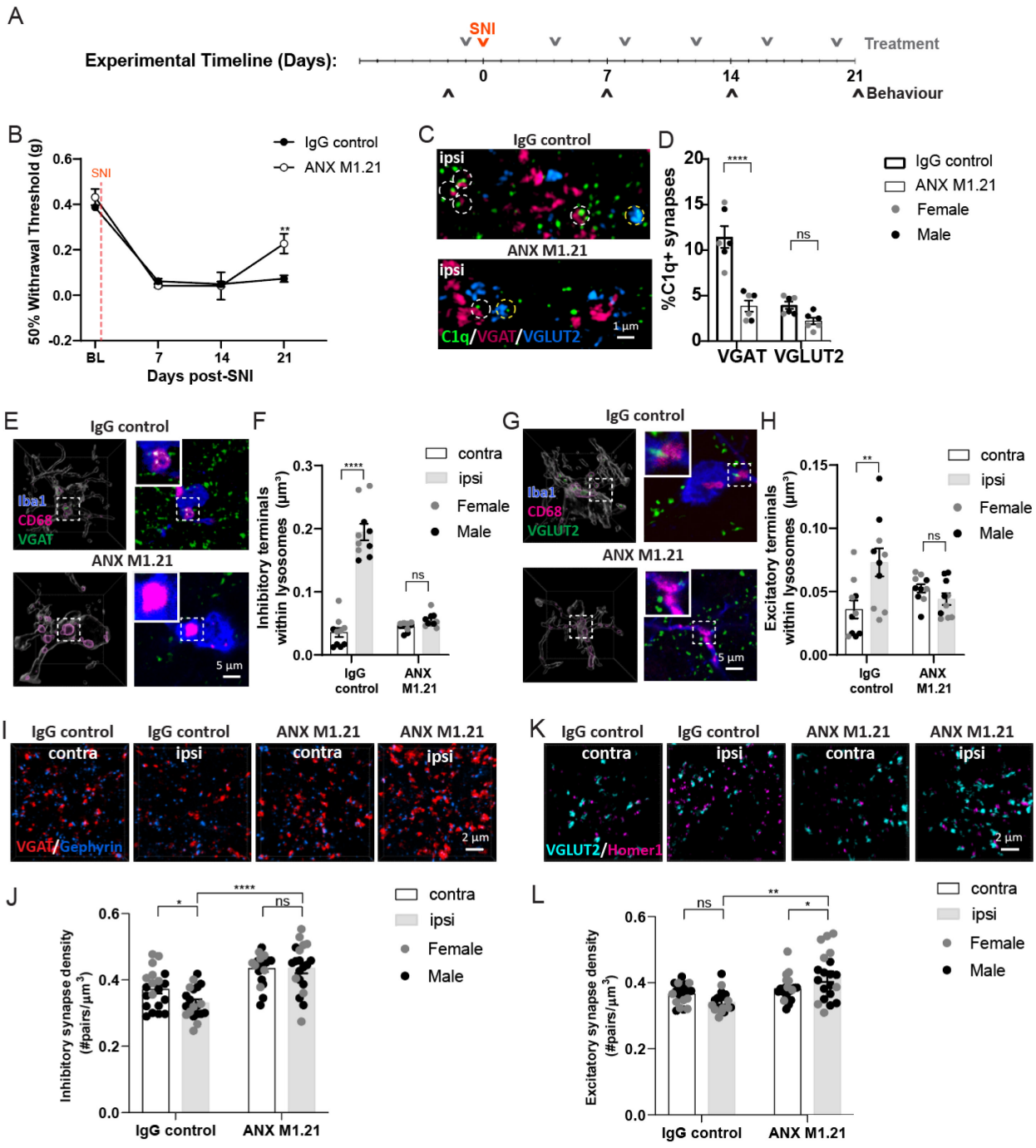

Figure 6. Functional blocking of C1q alleviates mechanical hypersensitivity, reduces synapse pruning, and prevents dorsal horn synapse loss. A, an experimental diagram showing the timeline of drug treatments, SNI surgery, and behavioral tests. B, 50% withdrawal threshold responses to von Frey filaments in neuropathic mice treated either with ANX-M1.21 or IgG control (n = 20 per group; 12 male and 8 female). C, Representative SIM images (F) captured from

ipsilateral dorsal horn show the co-localization of C1q (green) with inhibitory synapses (VGAT; red), compared to excitatory (VGLUT2; blue) in mice treated with ANX-M1.21 or IgG control. Dotted circles show synapses that are colocalized with C1q. D, Quantifications of C1q co-localization with inhibitory and excitatory synapses. E and G, representative 3D surface rendering of microglia (white) from ipsilateral dorsal horn of IgG control and ANX-M1.21 treated mice. A single plane enlarged image selected from the original confocal stacks illustrating the presence or absence of CD68 (magenta) co-localization with VGAT (E: green) or VGLUT2 (G: green). Insets within 3D reconstructions are enlarged views of VGAT (E) or VGLUT2 (G) co-localization with CD68 which represents engulfment (quantified in F). I, Representative SIM images of inhibitory presynaptic (VGAT; red) and postsynaptic (gephyrin; blue) elements captured from different conditions and quantification in J. K, representative SIM images of excitatory pre-synaptic (VGLUT2; teal blue) and post-synaptic (homer1; magenta) elements captured from different conditions and quantification in L. *$p<0.05$, **$p<0.01$, and ****$p<0.0001$ analyzed with repeated measures two-way ANOVA with Bonferroni post hoc test (B) and two-way ANOVA with Bonferroni post hoc test (D,F,H,J, and L). Means are plotted with individual data points ± SEM.

3.6 Discussion

We have identified a novel mechanism by which microglia contribute to the chronicity of neuropathic pain through reorganizing the synaptic network of the spinal dorsal horn. We show that following peripheral nerve injury, activated microglia engage the classical complement pathway, via production and release of complement protein C1q which is deposited on dorsal horn synapses, to prune these synapses in the spinal cord. Pharmacological inhibition of C1q protected spinal cord circuitry by disrupting synapse engulfment and ameliorated neuropathic pain at chronic stages of the pathology. These data implicate the CR3 synapse pruning pathway as a major driver of structural synaptic plasticity in neuropathic pain and identify a therapeutic strategy to prevent the chronification of the disease.

3.6.1 Activation of spinal CR3 synapse pruning pathway in neuropathic pain

It is well established that microglia-mediated synapse pruning plays a key role in sculpting a functional neural network within the developing central nervous system (Neniskyte and Gross, 2017). Emerging research has implicated different microglial signaling pathways in this process, many of which are reactivated later in life in different neurological disorders (Hong et al., 2016a, Yilmaz et al., 2021, Lui et al., 2016). For example, pathological involvement of CR3 synaptic pruning pathway has been implicated in Alzheimer's disease, frontotemporal dementia, and schizophrenia. CX3CR1 signaling was implicated in epilepsy and sensory-lesion-induced synaptic pruning (Gunner et al., 2019, Ali et al., 2015). Given the induction of both CR3 and CX3CR1 pathways in the spinal cord after nerve injury, we explored the possibility of their involvement in remodeling of dorsal horn circuits in neuropathic pain. The evidence for a role of the CR3 synapse pruning pathway in neuropathic pain was abundant as specific disruptions at multiple levels of this pathway protected both dorsal horn inhibitory and excitatory synapses and prevented synapse engulfment. Unlike the CR3 pathway, the CX3CR1 pathway did not appear to play a significant role in dorsal horn synapse pruning. The modest reduction in engulfment of excitatory synapses in neuropathic CX3CR1 KO mice can be explained by other defects in CX3CR1 deficient microglia (Sheridan and Murphy, 2013, Fong et al., 2002). These include problems with basic

homeostatic functions like cell adhesion, and motility which affect the phagocytic activity of microglia required for synapse pruning (Fong et al., 2002, Sheridan and Murphy, 2013).

Apart from synapse pruning pathways, phagocytosis receptors including P2Y12, P2Y6, and TMEM16F were reported to be upregulated in neuropathic pain and pharmacological blockade or genetic deletion of these receptors were shown to reduce neuropathic pain (Barragan-Iglesias et al., 2014, Maeda et al., 2010, Batti et al., 2016). Many of these receptors have been implicated in synapse removal in other contexts (Sipe et al., 2016, Koizumi et al., 2007). While we cannot rule out the possibility that these pathways might also contribute to dorsal horn synapse removal in neuropathic pain, our data suggest that the CR3 pathway has a central role in this process. The complete protection of the dorsal horn synaptic network in C3 KOs and in mice treated with a C1q inhibitor supports the necessity of the CR3 pathway for synaptic remodeling in the neuropathic pain. These findings were comparable to those observed in mice with complete microglial depletion. However, further studies are necessary to show whether deficits in other microglial phagocytic pathways can affect the efficiency of synapse removal through CR3 signaling cascade which would further our understanding of the complex interactions that are at play in this disease.

3.6.2 Microglia rewire dorsal horn circuitry in neuropathic pain

Our data demonstrate that microglia reorganize dorsal horn synaptic circuitry through the CR3 dependent signaling pathway. This function of microglia results in two main changes in dorsal horn synaptic network: 1) major deficit in inhibitory synapses; 2) reorganization of excitatory synapses without an apparent net loss in excitatory synapse density. In contrast to what was observed in inhibitory synapses, the elevated engulfment of excitatory synaptic terminals, although smaller than the engulfment of inhibitory synapses, does not lead to a net loss of excitatory synapses. This suggests that a compensatory mechanism, specific to excitatory system, is in place to replenish excitatory synapses. Interestingly, blocking engulfment results in an increase in excitatory synapse density which unmasks the dynamic process of excitatory synapse formation in the spinal cord. This is consistent with the emerging studies demonstrating activity-dependent excitatory synaptogenesis as a contributing mechanism to neuropathic pain (Simonetti et al., 2013, Yu et al., 2018). Interestingly, the dynamics of dorsal horn excitatory

synapses in neuropathic pain resembles the later phase of development when excitatory synaptogenesis accompanies synapse pruning (Tau and Peterson, 2010). Much like the developmental case, in the neuropathic pain state, spinal inhibition is highly compromised, and the spinal cord microenvironment is fueled with neurotrophic and neurogenetic factors to support excitatory plasticity (Coull et al., 2003b, Coull et al., 2005, Kaila et al., 2014).

One of the fundamental pathological features of neuropathic pain is the imbalance between excitation and inhibition of dorsal horn neuronal circuits (Inquimbert et al., 2018, Lee et al., 2019). In this study, we provided neuroanatomical basis for this imbalance which is mediated by synaptic pruning activity of microglia. At the molecular level, like the developmental model, our results show that C1q deposition on synapses is necessary for the recruitment of microglia to the synapse side. Interestingly, we found that within the spinal cord of neuropathic animals, C1q has a greater preference towards inhibitory synapses compared to excitatory. It is possible that differential changes in neuronal activity between inhibitory and excitatory dorsal horn neurons trigger cell type specific expression of C1q receptors or other recognition molecules at the synapse site (Scott-Hewitt et al., 2020, Gyorffy et al., 2018, Bialas and Stevens, 2013). This preferential removal of inhibitory synapses, in addition to the failure of the inhibitory system to compensate for the net synapse loss, contribute to the imbalance between inhibition and excitatory forces and increased excitability in neuropathic pain. This is particularly important for the generation of centralization, an enhancement in nociceptive pathways in response to sensory stimuli (Latremoliere and Woolf, 2009). Future studies are necessary to uncover the connection between neuronal activity and production of signaling molecules that attract C1q in neuropathic pain.

3.6.3 Targeting C1q interferes with the chronicity of neuropathic pain

We also showed that chronic inhibition of C1q is effective at reducing neuropathic pain only at chronic time-points. The delayed effect of anti-C1q antibodies treatment suggests that the structural remodeling of the dorsal horn synaptic circuitry is important for late but not early phases of neuropathic pain. Moreover, despite the robust effects of inhibiting C1q in reducing mechanical hypersensitivity in neuropathic mice, it is important to note that inhibition of C1q

does not completely eliminate neuropathic pain behavior. This suggests that other pathogenic factors, such as proinflammatory mediators (Echeverry et al., 2017), KCC2 downregulation (Coull et al., 2005), and peripheral sensitization (Costigan et al., 2009), likely also contribute to the pathology of the disease. Longer time-course studies are necessary to evaluate the relative contribution of these different mechanisms to neuropathic pain. Furthermore, the aberrant increase of excitatory synapses in anti-C1q treated group may contribute to the incomplete reversal of the pain behavior. To explore how much this increase contributes to the incomplete behavior effect of anti-C1q antibodies, both synaptogenesis and synapse pruning pathways should be inhibited at the same time. This would require new genetic tools to allow the selective and simultaneous manipulation of both pathways.

In addition to its relevance to remodeling of spinal circuits in neuropathic pain, our findings have important implications for understanding dorsal horn structural plasticity in other forms of chronic pain including different types of arthritis. Recent studies unraveled common pathological pathways between neuropathic pain and other forms of chronic pain (Locke et al., 2020, Locke et al., 2021b). Indeed, structural evidence for loss of inhibitory terminals and association between C1q and synaptic elements were observed in preclinical models of osteoarthritis and rheumatoid arthritis (Locke et al., 2021a). Therefore, targeting C1q has the potential as a therapeutic approach for preventing pathological synaptic rewiring in the spinal cord in other forms of chronic pain, interfering with the progression of pain pathology.

3.7 Conclusion

Overall, these results highlight the importance of microglia and the CR3 mediated synapse pruning pathway in pathological dorsal horn synaptic rewiring in neuropathic pain and presents a potentially clinically relevant novel therapeutic strategy that targets complement proteins to mitigate dorsal horn synapse loss and prevent the chronification of neuropathic pain.

3.8 Acknowledgments

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3.9 Author contributions

NY designed and planned the entire study with the help of ST and SL. NY carried out the majority of the experiments, prepared the figures and wrote the first manuscript version. ST designed and performed ANX M1.21 behavior experiments. BS and JSA performed neuropathic pain surgeries. JSA performed genetic data analysis. VB, VC, MSL and KL assisted with the histology experiments. HD proofread the manuscript. CW performed a portion of engulfment and synapse quantifications. AK supported the super-resolution microscopy, transgenic studies, and contributed to manuscript editing. VM, TY and YAZ contributed to the design of the ANX M1.21 component of the study. YDK and LD contributed to the overall idea and manuscript reviewing. PS and JM reviewed the manuscript. ARS oversaw the project as a whole and contributed to the writing and editing of the manuscript.

Declaration of interests

The authors declare no competing interests.

3.10 Supplementary figures

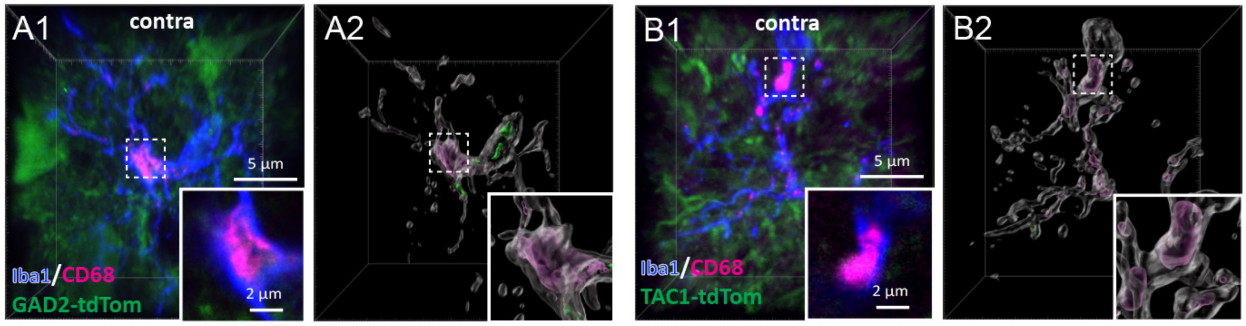


Figure S1. Microglia mediated engulfment of neuronal elements is absent in contralateral spinal dorsal horn. Representative 3D reconstructions (A1, B1) and surface rendering (A2, B2) of microglia (blue) sampled from contralateral dorsal horn to show the absence of internalized inhibitory and excitatory substance P neuronal elements within CD68+ lysosomal compartments (magenta) of contralateral microglia.

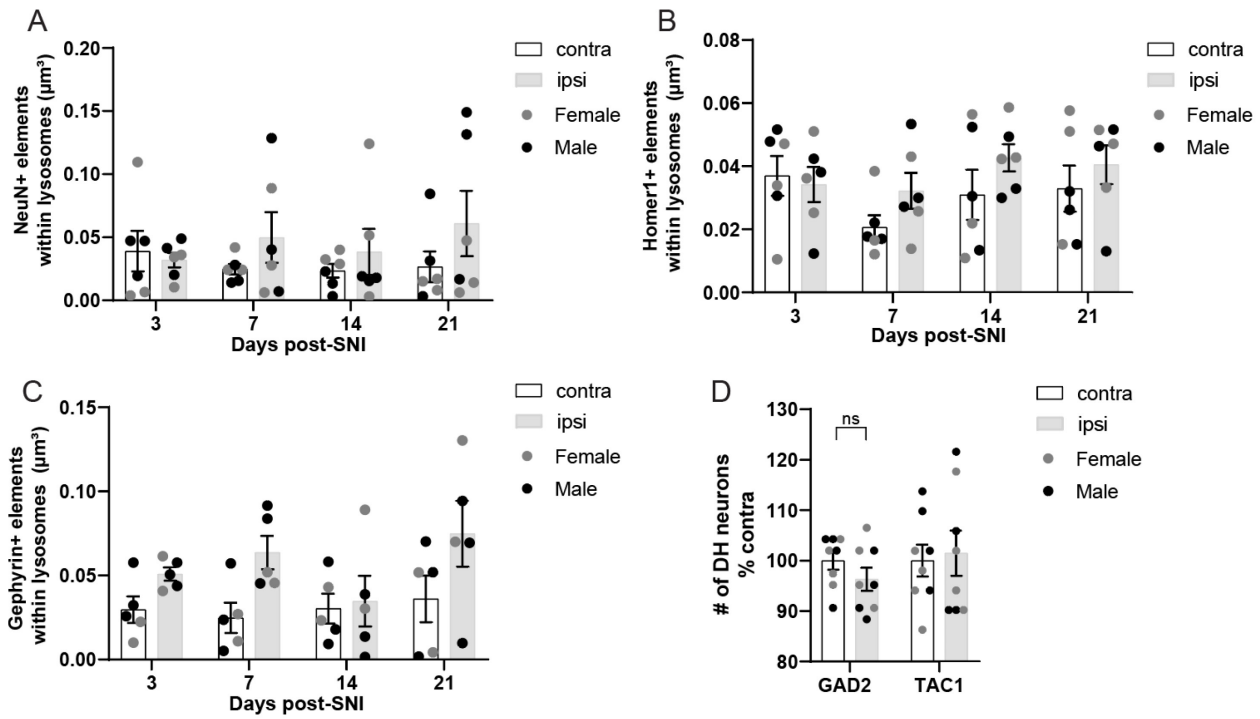


Figure S2. Postsynaptic elements and neuronal cell bodies are not engulfed by spinal microglia in the neuropathic pain. A-B, Quantification of temporal changes of engulfment of postsynaptic inhibitory (A) and excitatory (B) elements by microglia. C, Quantification of temporal changes of engulfment of neuronal cell bodies by microglia. D, Quantification of tdTomato expressing inhibitory and excitatory substance P dorsal horn neurons at 21 days after injury. Means are plotted with individual data points ± SEM.

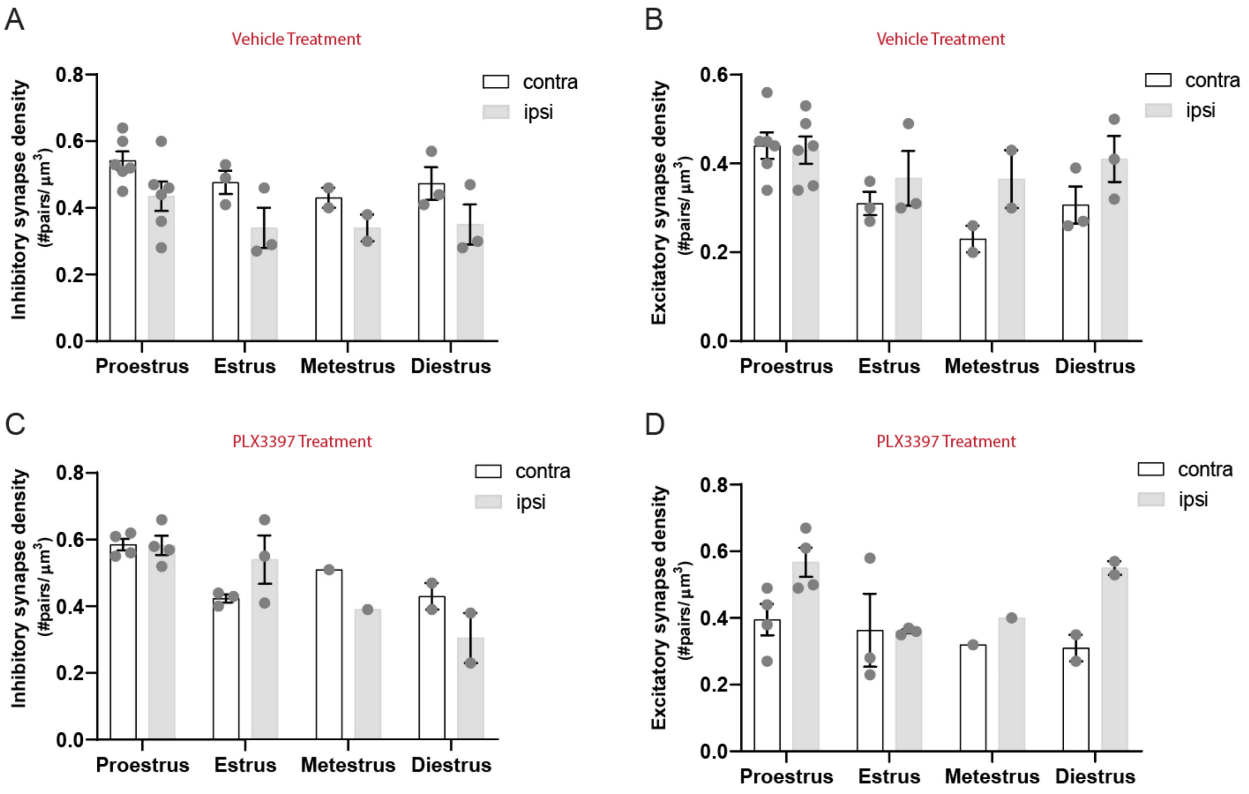


Figure S3. Spinal synapse density in different stages of the female mouse estrus cycle A-D, Quantification of inhibitory (A and C) and excitatory synapse density (B and D) in proestrus-estrus-, metestrus-, and diestrus- neuropathic female mice which received vehicle (A and B) or PLX3397 (C and D) treatments.

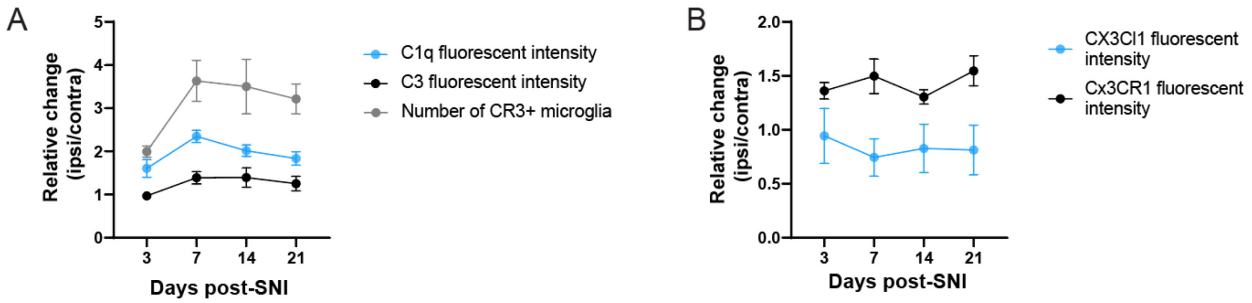
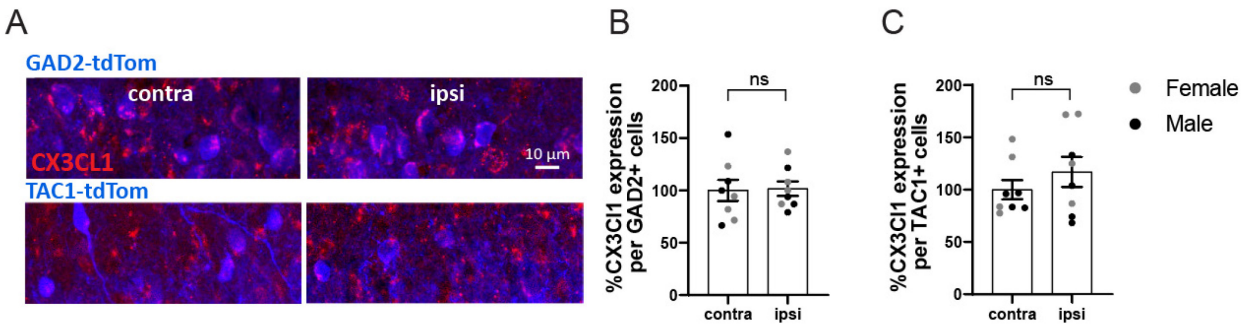
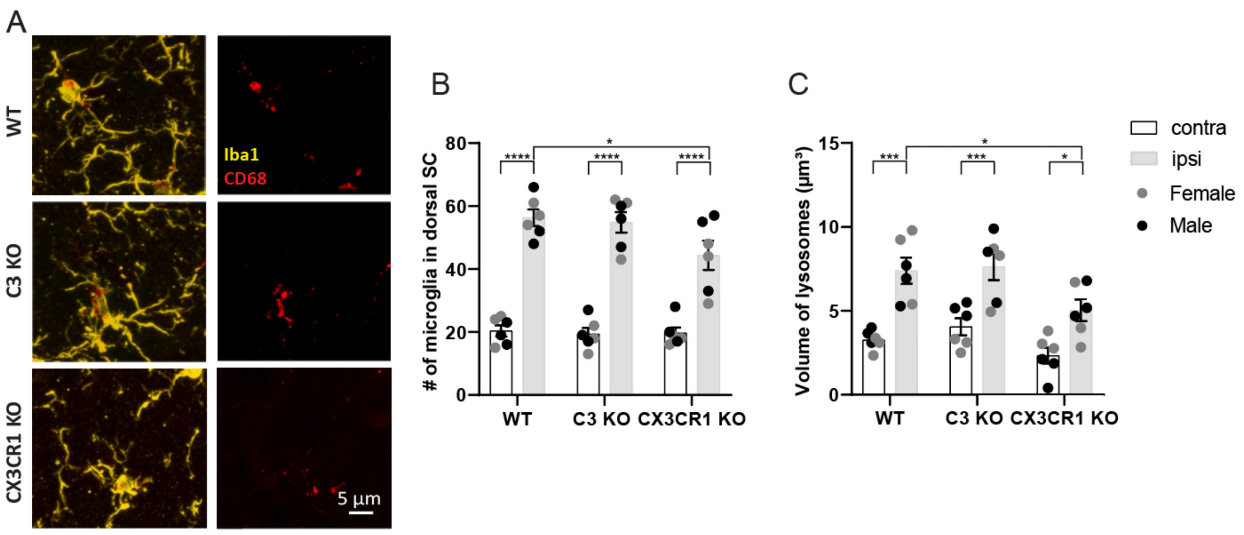


Figure S4. **Temporal expression pattern of components of the CR3 and CX3CR1 synapse pruning pathways.** A, temporal fold-change expression of C1q, C3, and CR3 relative to contra. B, temporal fold-change expression of CX3CR1, and CX3CL1 relative to contra.


A
GAD2-tdTom
contra
ipsi
CX3CL1
10 µm
TAC1-tdTom
B
%CX3Cl1 expression per GAD2+ cells
ns
200
150
100
50
0
contra
ipsi
C
%CX3Cl1 expression per TAC1+ cells
ns
200
150
100
50
0
contra
ipsi
Female
Male


A
WT
C3 KO
CX3CR1 KO
Iba1
CD68
5 µm
B
# of microglia in dorsal SC
0
20
40
60
80
****
*
****
****
WT
C3 KO
CX3CR1 KO
C
Volume of lysosomes (µm³)
0
5
10
15
***
*
***
*
WT
C3 KO
CX3CR1 KO
contra
ipsi
Female
Male

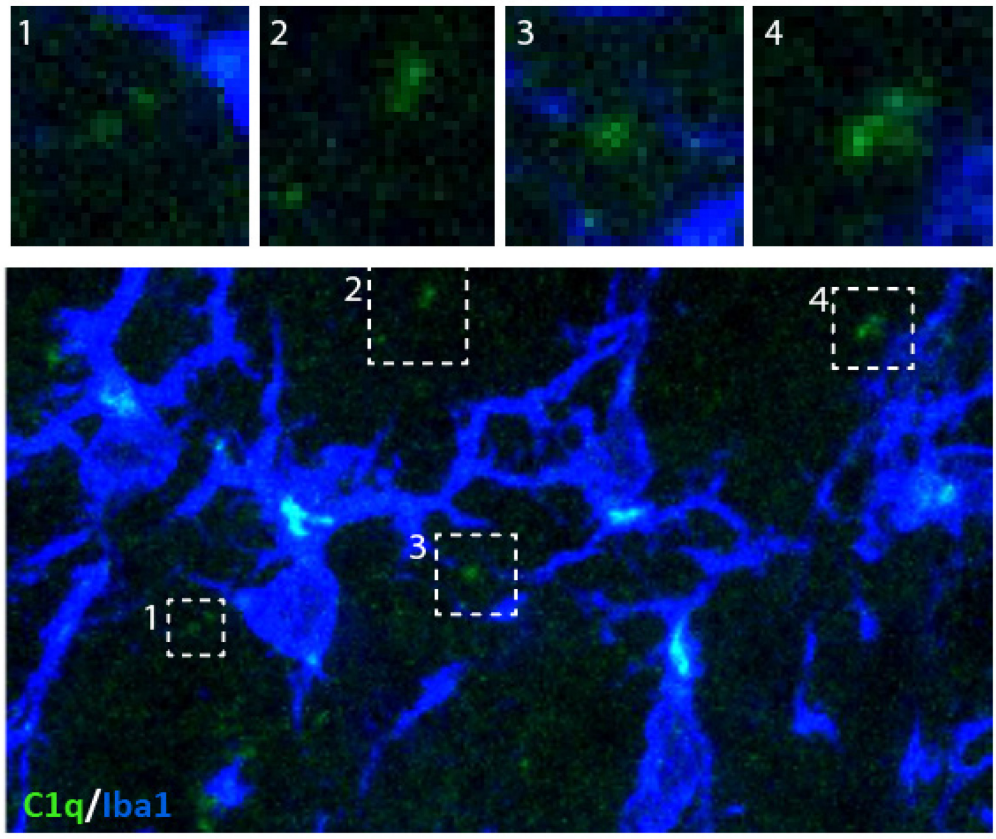


Figure S7. Extracellular C1q signal in the ipsilateral dorsal horn. An enlarged view of Figure5D confocal image to better show the extracellular C1q protein (green) outside microglia (blue). Examples of extracellular C1q signal are selected (box 1-4) and the corresponding magnified version are shown in the top row (images 1-4).

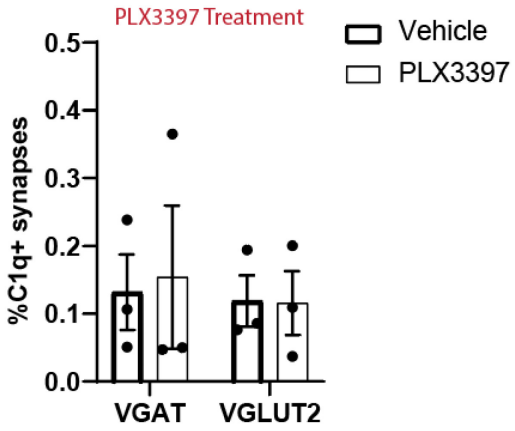
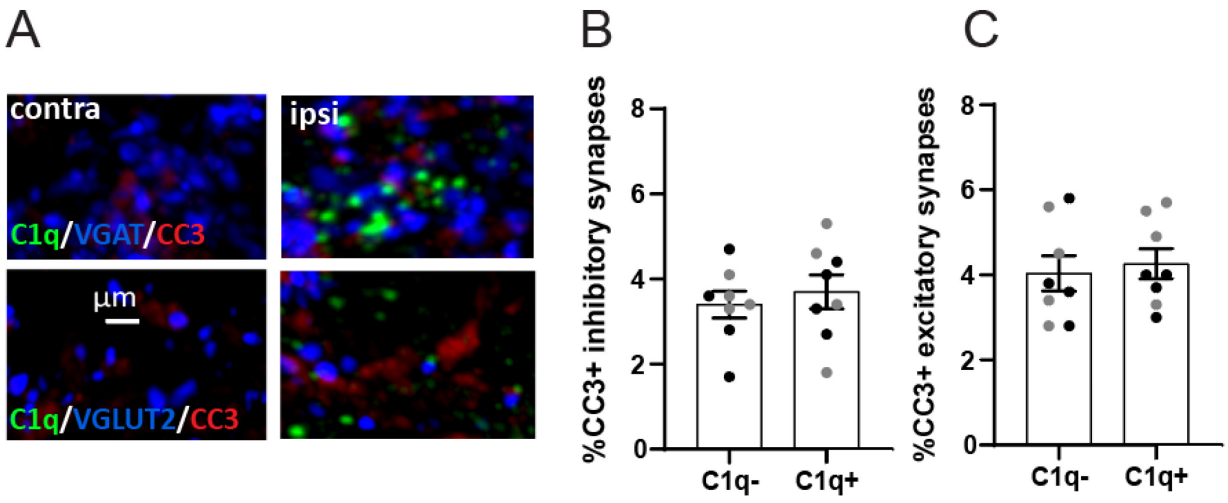
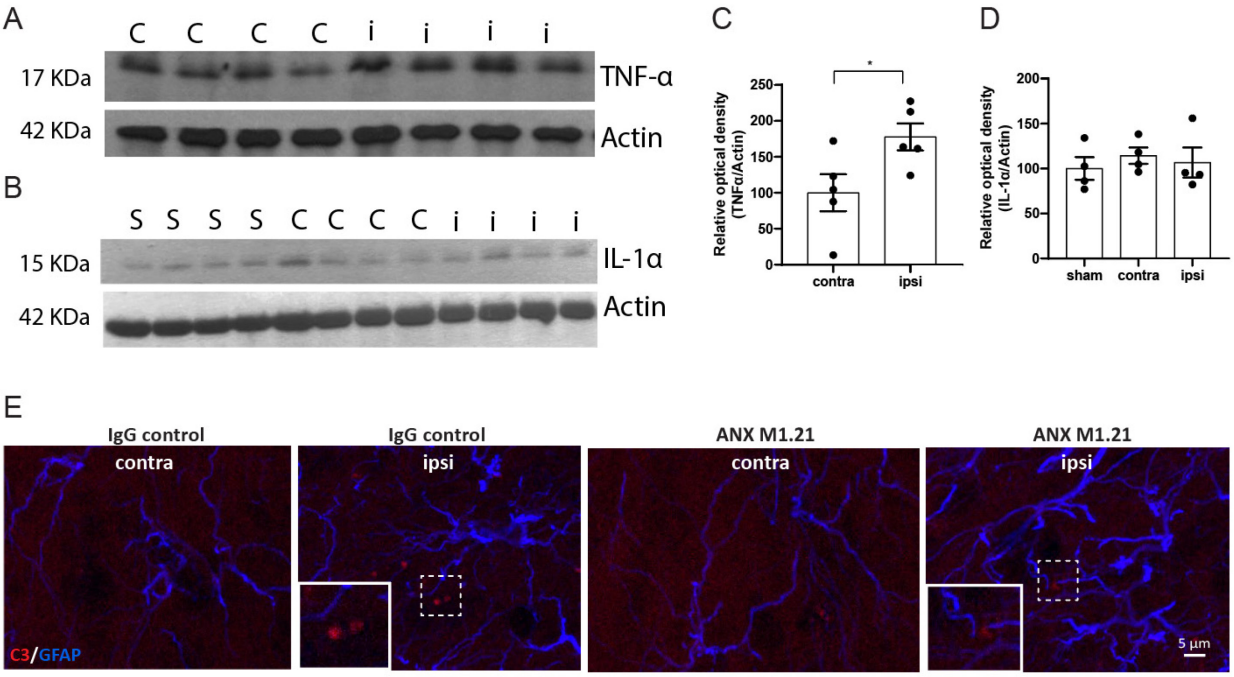


Figure S8. Chronic depletion of microglia eliminates C1q depositions on dorsal horn synapses. quantifications of C1q co-localization with inhibitory and excitatory synapses in vehicle and PLX3397 treated mice.


A
contra
ipsi
C1q/VGAT/CC3
µm
C1q/VGLUT2/CC3
B
%CC3+ inhibitory synapses
0
2
4
6
8
C1q-
C1q+
C
%CC3+ excitatory synapses
0
2
4
6
8
C1q-
C1q+


A
C C C C i i i i
17 KDa
TNF-α
42 KDa
Actin
B
S S S S C C C C i i i i
15 KDa
IL-1α
42 KDa
Actin
C
Relative optical density (TNFα/Actin)
250
200
150
100
50
0
*
contra
ipsi
D
Relative optical density (IL-1α/Actin)
200
150
100
50
0
sham
contra
ipsi
E
IgG control
contra
IgG control
ipsi
ANX M1.21
contra
ANX M1.21
ipsi
C3/GFAP
5 μm

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Connecting text: Chapter 3 to Chapter 4

In Chapter 2 we characterized dynamics of dorsal horn synaptic density, and later in Chapter 3 we demonstrated that such structural changes contribute to the sensory symptoms of neuropathic pain.

The approach we used in previous chapters to assess global changes in dorsal horn synapse density did not provide information about how different sub-populations of neurons and circuits were affected by synapse loss. This is an important question that should be investigated in future studies. Therefore, in Chapter 4 we present an imaging-based approach to assess cell specific dynamics of pre- and post-synaptic structural changes in dorsal horn neurons to build a technical foundation for future studies of this nature in this line of research.

Chapter 4

Inhibition of dorsal horn neurons is affected differentially by synapse removal and KCC2 downregulation

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4.1 Abstract

Spinal cord inhibition has a main role in normal processing of primary sensory information such as pain and touch. In neuropathic pain, a debilitating condition caused by nerve damage, spinal inhibition is severely compromised (disinhibition), resulting in symptoms like hypersensitivity to gentle touch. Disinhibition is caused by different mechanisms including downregulation of the potassium-chloride co-transporter 2 (KCC2), and inhibitory synapse loss. Here we used a novel image analysis approach to characterize the temporal and cell type specific pattern of membrane KCC2 changes and inhibitory synapse loss in the dorsal horn of a mouse model of neuropathic pain. We found that KCC2 downregulation occurred before inhibitory synapse loss and both inhibitory and excitatory neurons were affected by these pathological components of disinhibition. We also observed that at baseline, excitatory neurons expressed more KCC2 and received a higher density of inhibitory inputs compared to inhibitory neurons. Moreover, excitatory neurons lost a higher proportion of KCC2 protein compared to their inhibitory counterparts making them more vulnerable to chloride dysregulation and disinhibition. These results suggest that spinal cord neurons are not affected uniformly by different mechanisms of disinhibition and the relative contribution of these mechanisms are time and cell type dependent.

4.2 Introduction

Neuropathic pain is caused by damage or impairment afflicting the somatosensory nervous system and is notoriously difficult to treat (Woolf, 2010a). One key symptom of neuropathic pain is mechanical allodynia which is defined as pain following tactile stimulation such as gentle touch (Jensen and Finnerup, 2014). Mechanical allodynia can be reproduced in animal models without nerve damage through blocking synaptic inhibition at the spinal level, indicating that disinhibition may be an underlying mechanism of mechanical allodynia (Yaksh, 1989, Miraucourt et al., 2009, Foster et al., 2015). In fact, studies have shown that nerve damage can induce a deficit in normal synaptic inhibition in the spinal dorsal horn of neuropathic animal models (Coull et al., 2003b, Prescott, 2015, Moore et al., 2002). Several possible causes have been reported for spinal disinhibition such as decreased negative current flow through activated $GABA_A$ or glycine receptors, and loss of inhibitory synapses (Coull et al., 2003b, Lorenzo et al., 2014, Lorenzo et al., 2020). The former is caused by dysregulation of intracellular chloride due to downregulation and

hypofunction of potassium chloride cotransporter 2 (KCC2) (Coull et al., 2003b), and the latter is mediated by synapse pruning (Yousefpour et al., 2021). Both loss of KCC2 and inhibitory synapses contribute significantly to nerve injury-induced disinhibition as enhancing KCC2 function or interfering with synapse pruning pathway reduce injury-induced allodynia (Lorenzo et al., 2020, Yousefpour et al., 2021).

Although the global effect of KCC2 downregulation and inhibitory synapse loss is well documented within the spinal cord, less is known about their temporal and cell type specific dynamics—including amount of membrane localization—independently or in relation to each other (Lorenzo et al., 2020). Devising an effective therapeutic approach for neuropathic pain requires a clear understanding of how contrasting and parallel mechanisms of disinhibition contribute to the disruption of sensory information processing (Woolf, 2010a). For instance, the net functional impact of a deficit in chloride homeostasis resulting from membrane KCC2 downregulation can vary depending on whether inhibitory synapses are intact or compromised. Therefore, in the absence of a comprehensive map of the dorsal horn circuitry, the first step towards understanding how spinal disinhibition occurs at the network level is to characterize the effect of membrane KCC2 downregulation and loss of inhibitory inputs on inhibitory and excitatory dorsal horn neurons.

Since assessing functional parameters associated with KCC2 downregulation and synapse loss for specific cells is technically challenging, especially at a large scale, we developed an immunohistochemistry-based method using high-resolution imaging and a detailed three-dimensional image analysis algorithm. With this tool, we quantified temporal and cell specific changes in KCC2 expression and inhibitory inputs for individual cells within the lamina II (LII) of the dorsal horn. This area was selected as it contains most of the microcircuits implicated in mechanical allodynia (Peirs and Seal, 2016). Using a mouse model of neuropathic pain, we demonstrated that peripheral nerve injury first triggers a loss of membrane KCC2 and later results in a reduction of axosomatic inhibitory inputs on LII dorsal horn neurons. At the baseline level, LII excitatory cell bodies express more membrane KCC2 and receive a higher density of inhibitory inputs compared to inhibitory cell bodies. In the neuropathic pain state, excitatory neurons lose

more membrane KCC2 compared to inhibitory neurons while both cell types are affected similarly by loss of inhibitory inputs. These findings suggest that LII excitatory neurons are more vulnerable to disinhibition mediated by membrane KCC2 downregulation.

4.3 Methods

4.3.1 Animals

All mouse experiments were approved by the Animal Care Committee at McGill University and complied with Canadian Council on Animal Care guidelines. Wildtype C57BL/6 mice (6-8 weeks of age) of both sexes were bred in-house at the McGill University animal facility. Depending on the experiment, neonatal (3 and 7 days old) and adult (6-8 weeks old) mice of both sexes were used. All experimental adult mice were housed with their same-sex littermates and kept in a temperature- and humidity-controlled room with a 12-h light/dark cycle and received food and water *ad libitum*.

4.3.2 Peripheral nerve injury

To model neuropathic pain, spared nerve injury (SNI) was performed on the left leg, under isoflurane/oxygen anesthesia as previously described (Decosterd and Woolf, 2000) For this, the sciatic nerve was exposed after making an incision on the skin on the lateral surface of the thigh and sectioning through the biceps femoris muscle. Two of the three terminal branches of the sciatic nerve were tightly ligated with 7.0 silk (Covidien, S-1768K) and 2–4 mm of the nerve distal to the ligation were removed, avoiding any disturbance of the spared nerve (sural). The muscle and skin were closed in separate layers using coated Vicryl (Ethicon, J489G).

4.3.3 Tissue preparation and immunohistochemistry

At different time points post-neuropathic surgery (days 3, 7, and 14), animals were anaesthetised (0.3 ml/100g of body weight of Equithesin containing 6.5 mg of chloral hydrate and 3 mg sodium pentobarbital), and perfused transcardially with perfusion buffer followed by 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (PB), pH 7.4, for 30 min. Spinal cords of neonatal mice (P3 and P7) were removed quickly after decapitation following anesthesia and fixed by immersion for 48 hr in 4% PFA at 4°C. After fixation, perfused spinal cords were extracted

and post-fixed in the same fixative overnight at 4°C. All samples were then transferred to 30% sucrose in 0.1 M PB for cryoprotection.

For immunohistochemistry, transverse spinal cord sections from the lumbar enlargement (L3–L5) were cut on a cryostat (Leica, Germany) at −20 °C. To achieve a complete and uniform penetration of all antibodies, relatively thin 15-µm-thick sections were prepared for Airyscan confocal microscopy. For every experiment, sections from P3, and P7 mice were included along with the samples from male and female neuropathic animals. Sections were washed with PBS and blocked for 1 hour at room temperature in 10% normal donkey serum. Sections were then incubated in a cocktail of primary antibodies in 5% blocking solution diluted in PBS for 12 h at 4°C. Primary antibodies were rabbit anti-KCC2 (Millipore, 1:1000), mouse anti-VGAT (Synaptic Systems, 1:500), goat anti-Pax2 (Bio-Rad, 1:500), and guinea pig anti-NeuN (Millipore, 1:1000). Primary antibody labelling was detected using species-specific secondary antibodies conjugated to Alexa 488, Alexa 568, and Alexa 647 (Invitrogen), diluted 1:800, incubated at room temperature for 2 hours. Sections were mounted on gelatin subbed slides and coverslipped using Prolong Gold Antifade mounting medium (Invitrogen) and Zeiss #1.5 cover slips.

4.3.4 KCC2 and VGAT imaging and quantification

For each animal, five spinal cord sections were selected for imaging. Images were acquired on an Airyscan LSM 880 confocal microscope at 63X using 0.2 µm z-steps. 16-bit images (1024 x 1024 µm) were acquired with a pixel dwell of 0.76 µs and an averaging of four by line (1 pixel = 0.31 µm). For each dorsal horn, two fields were selected and imaged from the central portion of LII in the ipsilateral side and two fields were imaged from the same area in the contralateral side (minimum of four fields per section, 20 fields per animal). Sections from P3 and P7 animals were imaged using the same acquisition parameters (two fields per section). Subsequent images were processed with Imaris software (Bitplane). For this, 3D volume surface rendering of each z-stack was performed for the NeuN channel to determine the volume of individual neuronal cell bodies. Pax2 signal was only used to identify inhibitory cells. Both KCC2 and VGAT channels were masked on to the NeuN channel to isolate signal associated with the neuronal cell bodies (Figure 1A). To measure the volume of membrane KCC2 and inhibitory inputs, 3D volume surface renderings for

both isolated channels were generated and the volumes of membrane KCC2 and inhibitory inputs associated with each individual cell were calculated. The same parameters were used for surface rendering of all channels which were previously determined in a pilot study. To calculate the densities of membrane KCC2 and inhibitory inputs, the following calculation was used: Volume of membrane KCC2 or inhibitory input (μm^3)/Volume of the neuronal cell body (μm^3). For this quantification, only cells bodies that were completely captured were included. All analyses were performed blind to the sex, and the experimental condition.

4.3.5 Experimental design and statistical analyses

Sample size was determined based on the effect size and the variance of the quantification method. This information was obtained from a pilot study previously conducted in male animals (data not shown). To assess sex differences, the same number of male and female animals were used and when no sex difference was found animals of both sexes were combined. All statistical tests were conducted in Windows GraphPad Prism version 9. Statistical analyses included two-way ANOVA followed by Bonferroni's multiple comparison test, unpaired two-tailed t tests, and Pearson's correlation coefficient test. All tests employed are indicated in figure legends. All N values are reported and refer to the number of animals.

4.4 Results

4.4.1 Membrane KCC2 downregulation precedes loss of inhibitory inputs in LII neurons of neuropathic mice

To assess the temporal pattern of KCC2 downregulation and inhibitory synapse loss in the spinal cord of neuropathic mice, we developed an immunohistochemistry-based quantification approach to reliably measure changes in membrane-bound KCC2 ($KCC2_M$) expression and number of axosomatic inhibitory inputs per individual LII neuronal cell body. For this, we used Airyscan high resolution confocal imaging to capture 3D stacks of ipsilateral and contralateral LII neurons. Neuronal cell bodies were labeled with antibodies directed against the neuronal nuclear protein (NeuN), KCC2 transporter, and the marker of presynaptic terminals, vesicular GABA transporter (VGAT). Within the spinal cord, KCC2 immunoreactivity is detectable in neuronal

membranes and in intracellular neuronal structures. Since intracellular KCC2 does not have a functional significance, we eliminated intracellular KCC2 labelling using triton-free experimental conditions which allowed successful isolation of KCC2 staining signal at the membrane ($KCC2_M$) without compromising signals associated with other markers (Figure 1A). More importantly, using this approach, we were still able to detect the loss of inhibitory inputs and $KCC2_M$ expression for individual cells sampled from the ipsilateral dorsal horn as compared to contralateral (Figure 1A).

To find $KCC2_M$ density levels that corresponded to hyperpolarizing $GABA_AR$ responses, we measured $KCC2_M$ density of individual LII neurons at postnatal days 3 and 7 (P3 and P7) when low levels of KCC2 result in hyperpolarizing $GABA_AR$ signaling (Figure 1B) (Cordero-Erausquin et al., 2005, Baccei and Fitzgerald, 2004). Consistent with previous functional studies, we found a 1.5-fold increase in $KCC2_M$ density at P7 compared to P3 which is in line with the temporal pattern of postnatal KCC2 gene expression ($KCC2_M$ density: $t_6 = 5.011$, $p = 0.0024$) (Figure 1B) (Stein et al., 2004). In all subsequent experiments, we used spinal cord sections from P7 mice along with those from neuropathic adults to compare the mean values of $KCC2_M$ density between these groups. The mean value of $KCC2_M$ density at P7 for each experiment was used as a threshold that represents critically low levels of $KCC2_M$. We speculate that inhibition on cells below this threshold is likely ineffective.

In adult neuropathic mice, at days 3, 7, and 14 after peripheral nerve injury (post-SNI), we investigated the temporal pattern of $KCC2_M$ downregulation and inhibitory synapse loss in LII neurons (Figure 1C). At day 3 post-SNI, no significant change in $KCC2_M$ and inhibitory inputs was detected ($KCC2_M$ density (day 3): $t_{42} = 1.342$, $p = 0.56$). However, a significant loss of $KCC2_M$ was found one-week post-SNI ($KCC2_M$ density (day 7): $t_{42} = 3.426$, $p = 0.0041$) (Figure 1C3). Moreover, at this timepoint, there was a 5% increase in the proportion of cells expressing lower levels of $KCC2_M$ than the mean $KCC2_M$ density at postnatal day 7 (=0.27) (Figure 1C4). At the same timepoint, no loss of inhibitory inputs on neuronal cell bodies was identified suggesting that $KCC2_M$ downregulation precedes inhibitory synapse loss (VGAT density (day 7): $t_{42} = 1.361$, $p = 0.54$) (Figure 1D). At two weeks post-SNI, reduction of $KCC2_M$ remained significant and was

accompanied by loss of inhibitory inputs on LII dorsal horn neurons ($KCC2_M$ density (day 14): t_{42} = 6.296, p<0.0001; VGAT density (day 7): t_{42} = 5.37, p<0.0001) (Figures 1C2, 1D). Taken together, these data show that nerve injury initially triggers $KCC2_M$ downregulation and later results in a reduction of inhibitory inputs on LII dorsal horn neurons.

4.4.2 LII excitatory and inhibitory neurons are impacted differentially by $KCC2_M$ loss in neuropathic mice

Nerve injury results in a global downregulation of $KCC2_M$ and loss of inhibitory synaptic inputs. To determine the magnitude of these changes at the single cell level, changes in $KCC2_M$ expression and inhibitory synapse density were assessed in cell bodies of inhibitory and excitatory interneurons sampled from LII. For this, dorsal horn cell bodies were labelled with NeuN, and inhibitory neurons were labelled using antibodies against paired box protein, Pax2. Excitatory cells were identified as cells that were positive for NeuN and negative for Pax2 (Figure 2A1 and 2B1). At two weeks post-SNI, contralateral excitatory neurons expressed more $KCC2_M$ on their cell bodies as compared to inhibitory neurons. This indicated an intrinsic physiological difference in $KCC2_M$ expression between functionally distinct LII cells at baseline ($KCC2_M$ density (day 14, contra): t_{40} = 2.636, p=0.0238) (Figure 2A2). Consistent with previous studies, peripheral nerve injury resulted in a significant loss of $KCC2_M$ detected in both inhibitory and excitatory cell bodies ($KCC2_M$ density (day 14, excitatory): t_{40} = 7.047, p<0.0001; $KCC2_M$ density (day 14, inhibitory): t_{40} = 3.666, p=0.0014) (Figure 2A2). However, a greater proportion of excitatory cells (22%) as compared to inhibitory cells (7%) expressed lower levels of $KCC2_M$ than the mean $KCC2_M$ density at postnatal day 7 (=0.18) (Figure 2A3). Interestingly, cell bodies of excitatory LII neurons had a greater change (ipsi subtracted from contra) of $KCC2_M$ expression compared to their inhibitory counterparts ($KCC2_M$ density (day 14, contra-ipsi): t_{20} = 2.353, p=0.0290) (Figure 2A4).

At two weeks post-SNI, in parallel with $KCC2_M$ density measurements, density of axosomatic inhibitory inputs was assessed on LII neurons. On the contralateral side, excitatory cell bodies received more inhibitory inputs compared to inhibitory ones ($KCC2_M$ density (day 14, contra): t_{40} = 2.462, p=0.0364) (Figure 2B2). However, both cell types lost a significant fraction (≈40%) of their inhibitory inputs (ipsi subtracted from contra) at two weeks post-SNI ($KCC2_M$ density (day

14, excitatory): t_{40} = 4.958, p<0.0001; $KCC2_M$ density (day 14, inhibitory): t_{40} = 3.594, p=0.0018) (Figure 2B2). The degree of this loss was comparable between inhibitory and excitatory cells ($KCC2_M$ density: t_{20} = 0.9977, p = 0.33) (Figure 2B3).

We further assessed whether there was a relationship between levels of $KCC2_M$ expression and axosomatic inhibitory inputs per LII neurons. For this, we correlated the mean density of $KCC2_M$ and inhibitory inputs for each animal. Results of the Pearson correlation indicated that on the contralateral side, there was a significant positive association between levels of $KCC2_M$ expression and inhibitory synaptic inputs (R^2 = 0.19, p = 0.04). However, this association was lost in the ipsilateral side (R^2 = 0.039, p=0.37) (Figure 2D).

Taken together, these results demonstrate that, at baseline, LII excitatory cells bodies express more $KCC2_M$ and receive a higher density of inhibitory inputs as compared to their inhibitory counterparts. Furthermore, although LII inhibitory and excitatory cells are affected differentially by loss of $KCC2_M$, they are similarly affected by loss of inhibitory inputs. The differential changes in these two variables possibly explains the loss of association between $KCC2_M$ expression and density of inhibitory inputs after injury.

4.5 Figures

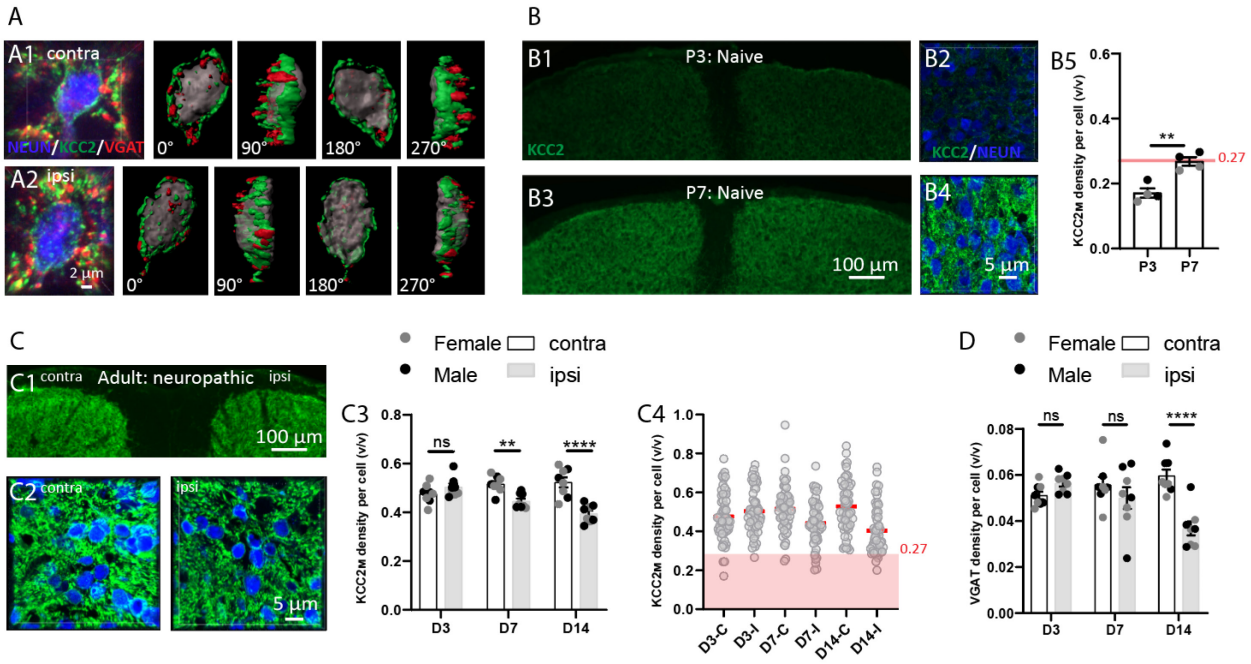

Figure 1. Single-cell quantification of $KCC2_M$ expression and inhibitory appositions in LII neuronal cell bodies reveals a different temporal pattern for downregulation of $KCC2_M$ and inhibitory appositions in neuropathic mice. A1-2, representative 3D reconstructions of LII neuronal cell bodies (labelled with NeuN in blue) sampled from contralateral (A1) and ipsilateral (A2) dorsal horns (DH) illustrating the expression of $KCC2_M$ (green), and presence of inhibitory appositions (labelled with VGAT in red). Surface rendering of 3D confocal images at different angles show reduction in $KCC2_M$ and VGAT immunoreactivities on the perimeter of the cell body. Low magnification full view of baseline levels of DH KCC2 expression (green) at P3 (B1), and P7 (B3) time-points along with high magnification images (B2, and B4) showing KCC2 expression on neuronal cell bodies (quantified in B5). C1, overall downregulation of KCC2 in the ipsilateral DH of neuropathic mice 14 days post-SNI. C2, high magnification views used for cell specific quantifications. C3, quantification show a temporal pattern of KCC2 downregulation per individual LII cell in neuropathic mice. C4, scattered plot illustrates individual cells analyzed for $KCC2_M$ expression; inhibition on cells below the red threshold is likely ineffective. D, quantification of temporal changes of inhibitory inputs per individual LII cell in neuropathic mice. **p<0.01, and ***p<0.0001 analyzed with unpaired t-test (B5) and two-way ANOVA followed by

Bonferroni post hoc test (C2 and D), ns = non-significant. Measurements for individual cells are plotted in C3 and means with individual data points ± SEM are plotted in B5, C2, and D.

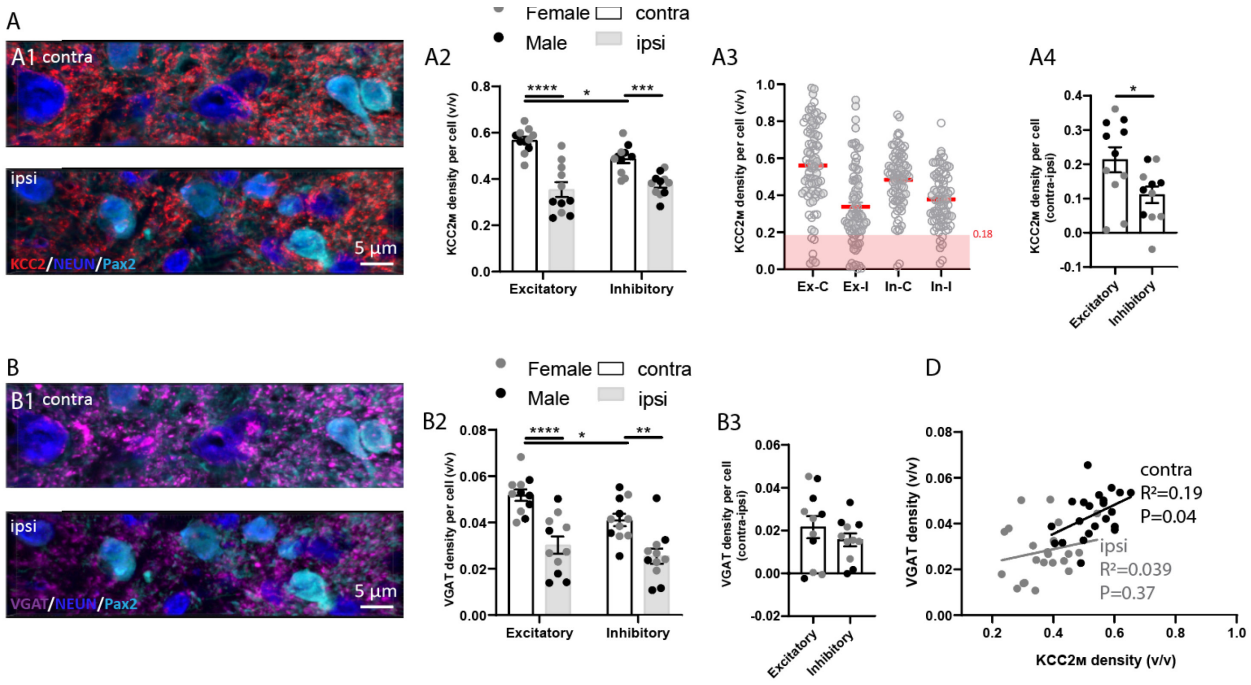

Figure 2. LII excitatory and inhibitory neurons are impacted differentially by $KCC2_M$ expression and inhibitory inputs at the baseline and neuropathic states. A1, representative 3D reconstructions of LII neuronal cell bodies (blue) sampled from contralateral and ipsilateral dorsal horns illustrating the expression of $KCC2_M$ (red). Excitatory cell bodies are differentiated from inhibitory ones by expression of Pax2 (cyan). A2, Quantification of $KCC2_M$ for excitatory and inhibitory neuronal cell bodies in LII. A3, Quantification data for each individual cell. Inhibition on cells below the red threshold is likely ineffective. A4, Graph illustrating the relative difference between contralateral and ipsilateral $KCC2_M$ expression for excitatory and inhibitory cells. B1, representative 3D reconstructions of LII neuronal cell bodies (blue) sampled from contralateral and ipsilateral dorsal horns illustrating density of inhibitory terminals (purple) per excitatory (blue) and inhibitory (cyan) neurons (quantified in B2). B3, Graph illustrating the relative difference between contralateral and ipsilateral inhibitory inputs on excitatory and inhibitory cells. D, Scatter plot showing the correlation between levels of $KCC2_M$ expression and inhibitory inputs. *p<0.05, **p<0.01, ***p<0.001, and ****p<0.0001 analyzed with two-way ANOVA followed by Bonferroni post hoc test (A2, and B2), unpaired t-test (A4 and B3), and Pearson correlation (D). Measurements for individual cells are plotted in A3 and means with individual data points ± SEM are plotted in A2, A4, B2-3.

4.6 Discussion

Our results add to the growing body of evidence that suggest downregulation of $KCC2_M$ and loss of inhibitory inputs as mechanisms underlying spinal disinhibition in neuropathic pain. Specifically, we demonstrate that dorsal horn $KCC2_M$ downregulation and loss of inhibitory inputs have a distinct temporal pattern in the SNI model of neuropathic pain. Although loss of axosomatic inhibitory inputs seems to affect both LII inhibitory and excitatory neurons equally, loss of $KCC2_M$ is more pronounced in excitatory neurons. Out data suggest that the relative contribution of disinhibition mechanisms depends on the time course of the pathology and can be cell type specific.

4.6.1 Single-cell assessment of the dynamics of $KCC2_M$ expression and inhibitory inputs in LII neurons

Direct measurements of neural inhibition are primarily done using whole-cell patch clamp recording which is performed in *ex vivo* slices obtained from animal models (Coull et al., 2003b, Moore et al., 2002). These direct measurements have been the most valuable in providing insights into the dynamics and mechanisms of inhibition at a single cell level (Zeilhofer et al., 2012). Assessment of functional parameters associated with KCC2 (i.e., chloride reversal potential) and inhibitory synapse density (i.e., frequency and kinetics of mIPSCs) are usually complemented with histological approaches (Heubl et al., 2017, Ferrini et al., 2020, Mapplebeck et al., 2019). However, many of these histological methods assess global- or lamina-specific changes of KCC2 or synapses (Mapplebeck et al., 2019), and those few studies that perform single cell analysis do not assess the whole cell with great detail and accuracy (Ferrini et al., 2020). Here, we introduce a 3D single cell imaging and quantification approach for assessing $KCC2_M$ expression with higher resolution to better complement functional studies. Using $KCC2_M$ levels of neonatal spinal neurons as an indicator for low $KCC2_M$ levels allows interesting speculations about the cell specific pattern of $KCC2_M$ changes and the extent different cells can be impacted. These structural studies can be the foundation for future functional studies that assess the relative impact of $KCC2_M$ downregulation on inhibition of different classes of dorsal horn cells.

4.6.2 Temporal dynamics of LII $KCC2_M$ expression and axosomatic inhibitory inputs in the state of neuropathic pain

Previous work has shown downregulation of spinal $KCC2_M$ and inhibitory synapse loss in different models of neuropathic pain (Ferrini et al., 2020, Coull et al., 2003b, Lorenzo et al., 2020, Lorenzo et al., 2014). Our data provide a timeline for these changes and show that $KCC2_M$ downregulation precedes inhibitory synapse loss. Contrary to the physiological condition in which $KCC2_M$ expression is tuned locally by synaptic activity (Wang et al., 2006, Fiumelli et al., 2005, Lee et al., 2011, Heubl et al., 2017), in neuropathic pain nerve injury-induced signaling pathways (i.e., BDNF-TrkB) drive a global KCC2 downregulation at the transcriptional level (Coull et al., 2005, Rivera et al., 2004). Whether this significant postsynaptic deficit in inhibition can contribute to the loss of inhibitory inputs later in the neuropathic pain state is not clear. Interestingly, in other neurological conditions with compromised inhibition, both KCC2 hypofunction and loss of inhibitory synapses have been reported, suggesting that there could be a fundamental connection between the two changes (Chen et al., 2017, Garcia-Marin et al., 2009, Spreafico et al., 2000, Pathak et al., 2007). Further experiments are required to study the mechanistic link between $KCC2_M$ downregulation and inhibitory synapse loss.

At the longest time point of this study, we show both $KCC2_M$ downregulation and loss of inhibitory inputs. This raises the interesting question of how a combined deficit in $KCC2_M$ expression and inhibitory inputs affects cellular inhibition. The answer depends on many factors including the extent to which the chloride homeostasis is compromised at the cellular level, and the balance between excitatory and inhibitory forces in individual cells (Prescott, 2015). Our data show that dorsal horn cells express different levels of $KCC2_M$ at baseline and in the neuropathic state, while certain cells sustain a significant downregulation in $KCC2_M$ expression, and others do not. Ultimately, the extent to which these cell-specific changes in $KCC2_M$ expression and consequently chloride dysregulation disrupt inhibition depends on other pathologic and physiological forces that regulate the balance between excitation and inhibition such as synapse pruning and synapse scaling (Lorenzo et al., 2020).

4.6.3 Cell type specific dynamics of LII $KCC2_M$ expression and axosomatic inhibitory inputs in a neuropathic pain state

Our study revealed an intrinsic difference in $KCC2_M$ expression between LII excitatory and inhibitory neurons. Other studies in the brain have reported differences in the reversal potential of $GABA_A$ receptor mediated currents (E_{GABA}) among neuronal populations and brain structures (Chavas and Marty, 2003). These differences are thought to partially reflect changes in $KCC2_M$ expression and function (Watanabe and Fukuda, 2015). In addition to cell type specific variance in $KCC2_M$ expression at baseline, we found that excitatory cells lose a greater fraction of $KCC2_M$ compared to their inhibitory counterparts. The greater vulnerability of LII neurons to $KCC2_M$ downregulation can theoretically contribute to hyperexcitability of the spinal circuits in neuropathic pain. However, without knowing the map of dorsal horn circuitry, it is difficult to predict the consequence of differences in $KCC2_M$ downregulation in dorsal horn cells. In recent years, a number of microcircuits composed of subclasses of excitatory and inhibitory interneurons that are implicated in allodynia have been identified (reviewed in (Peirs and Seal, 2016)). Within these circuits, loss of inhibition on different classes of excitatory interneurons was shown to be necessary for mechanical allodynia. Whether there is a difference between these subsets of excitatory interneurons in terms of how they are affected by $KCC2_M$ downregulation in response to nerve damage remains to be investigated.

We and others have previously shown a global reduction of dorsal horn inhibitory synapses in neuropathic pain state (Lorenzo et al., 2020, Lorenzo et al., 2014). Here, we show that both inhibitory and excitatory neurons lose their axosomatic inhibitory inputs to a comparable degree. Our results support the notion that synapse loss is primarily driven by presynaptic mechanisms, because the effect of synapse loss is not specific to the type of postsynaptic cell affected (Bialas and Stevens, 2013). Interestingly, at baseline, LII excitatory neurons receive a higher density of inhibitory inputs compared to inhibitory neurons suggesting that these cells might be controlled with stronger inhibition and/or more neuronal partners as compared to inhibitory neurons. To properly validate this conclusion, further functional and structural studies are required to examine the relative contribution of both inhibitory and excitatory forces towards inhibition of different dorsal horn cell types.

In conclusion, using a new method for analyzing cell specific structural changes of $KCC2_M$ expression and axosomatic inhibitory inputs, we discovered that nerve injury results in temporal and cell specific changes of $KCC2_M$ expression and inhibitory inputs affecting the cell bodies of LII neurons. These findings can be a foundation for future studies to better understand how dorsal horn sensory processing is disrupted in neuropathic pain and results in debilitating symptoms like mechanical allodynia.

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4.8 Author contributions

NY designed and planned the entire study with the help of SL. NY carried out all the experiments, prepared the figures and wrote the first manuscript version. MKF assisted with troubleshooting experiments and edited the manuscript. ACC was involved in helpful discussions and helped in the editing of manuscript. ARS oversaw the project as a whole and reviewed the manuscript.

Declaration of interests

The authors declare no competing interests.

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Chapter 5

General Discussion

5.1 Overview

The work presented in this thesis is the first to demonstrate that microglia contribute to the pathophysiology of neuropathic pain through active remodeling of dorsal horn synaptic circuitry.

In Chapter 2, we described changes in dorsal horn synapse density resulting from peripheral nerve injury. These changes included the preferential elimination of a subset of synapses formed by primary afferents and dorsal horn interneurons. The key finding of this chapter was that microglia selectively remove dorsal horn synaptic inputs and specifically promote inhibitory synapse loss in a chronic constriction nerve injury model of neuropathic pain.

In Chapter 3, we further explored the mechanism underlying microglia mediated synapse removal in a spared nerve injury model of neuropathic pain. We showed that synapse removal was dependent on the microglial complement-mediated synapse pruning pathway, and proposed complement protein C1q as a new target for disease-modifying therapeutic strategies aimed at preventing the chronification of neuropathic pain.

Finally, in Chapter 4, we presented an imaging-based approach to assess the cell-specific dynamics of inhibitory synaptic inputs and expression of KCC2 in dorsal horn neurons. We showed that nerve injury resulted in temporal and cell-specific changes in the expression of KCC2 and inhibitory inputs on the cell bodies of dorsal neurons. These findings serve as a foundation for future studies to better understand how dorsal horn sensory processing is disrupted in neuropathic pain and results in debilitating sensory symptoms.

In this chapter, the significance of the presented findings will be discussed collectively within the context of the wider field of knowledge, where potential caveats and technical limitations will be highlighted. Finally, this chapter will present some of key questions raised by our findings and will suggest future directions of this line of research.

5.2 The curious case of microglia-mediated synapse removal in neuropathic pain

Distinct microglia-dependent mechanisms play a crucial role in shaping neural circuits in health and disease (Hong et al., 2016b). The main finding presented in this thesis is that microglia mediate spinal dorsal horn synapse removal in preclinical models of neuropathic pain.

Surprisingly, microglia modify centrally originated synaptic connections that are not physically compromised by nerve injury while sparing synapses established by potentially damaged primary afferents. This observation suggests that, unlike what happens in demyelinating diseases like multiple sclerosis and central traumatic injuries (Jafari et al., 2021, Werneburg et al., 2020, Jamjoom et al., 2020, Chen et al., 2014), injury-associated signaling at the synapse is not driving the microglial-mediated synapse removal pathway in neuropathic pain.

In recent years, influential studies explored synapse removal pathways in major neurological pathologies such as Parkinson's and Alzheimer's diseases. The main trigger of microglial removal pathways in these pathologies seemed to be disease-specific pathological proteins such as parkin and soluble Aβ oligomers (Helton et al., 2008, Hong et al., 2016a). Although no specific protein pathology is reported to be associated with neuropathic pain, it is well documented that nerve injury drastically modifies spinal cord microenvironment and promotes the production of pro-nociceptive (i.e., substance P, CGRP), neurotrophic (i.e., BDNF), and excitotoxic compounds (i.e., glutamate, ATP)(Woolf, 2004). However, even though microglia express receptors for all these molecules (Burmeister et al., 2017, An et al., 2021, Coull et al., 2005, Pocock and Kettenmann, 2007, Calovi et al., 2019), there is no evidence that these molecules are implicated in complement system recruitment and synapse pruning.

In general, activity-dependent synaptic competition drives microglia-mediated synapse pruning (Wang et al., 2020, Schafer et al., 2012, Hong et al., 2016b, Cheadle et al., 2020). In neurodegenrative diseases, neuronal activity is comprimized, whereas in the neuropathic pain condition — similar to the developing CNS — the spinal cord is in a hyperactive state (Bonin and De Koninck, 2014).

During postnatal development, maturation of the spinal nociceptive circuits relies heavily on spontaneous tactile input (Waldenström et al., 2003). External stimuli evoke and shape activity-dependent strengthening and pruning of synaptic connections, ultimatly setting up gating mechanisms that regulate dorsal horn information output (Zouikr and Karshikoff, 2017). Sensory deprivation during development can disrupt normal formation of neuronal circuitries through enhancing microglia-mediated synaptic engulfment (Gunner et al., 2019). Along with synapse

pruning, activation of other key mechanisms that regulate developmental neuroplasticity including KCC2 downregulation (Coull et al., 2003a), BDNF upregulation (Coull et al., 2005), and activation of matrix remodeling pathways (Parisien et al., 2019) suggest a great convincing resemblance between the states of neuropathic pain and postnatal development.

In the neuropathic pain state, peripheral nerve injury not only produces partial sensory deprivation (resulting from damaged afferents), but also triggers abnormal activation in recovered or healthy afferents. This significant change in the quality, intensity, and distribution of sensory input likely drives microglia-mediated synapse pruning to re-adjust the spinal circuitry for the new levels of incoming information. Further functional studies are necessary to explore the role of altered neuronal activity in nerve injury-induced spinal synapse pruning and rewiring.

5.3 Microglia-mediated structural plasticity of the inhibitory circuits: a new concept

While many studies have focused on pruning or sculpting of excitatory synapses, less is known about structural modulation of inhibitory synapses (Weinhard et al., 2018, Schafer et al., 2016, Hong and Stevens, 2016). In general, the arrangement and strength of inhibitory synapses are regulated by long-term changes in circuit activity to maintain homeostasis, keep excitation/inhibition balanced, and prevent abnormal activity (Wierenga et al., 2008, Lushnikova et al., 2011, Chen et al., 2012). Emerging studies show that, like in the excitatory network, alterations in the activity of inhibitory neurons can regulate synaptic remodeling although the underlying mechanism for theses presynaptic changes are not known (Chen et al., 2018a).

The contribution of microglia to structural plasticity of inhibitory networks was recently described in the developing nervous system. In an elegant study, Favuzzi et al. (2021) showed, for the first time, that GABA triggers a transcriptional synapse remodeling program within microglia expressing $GABA_B$ receptors. These specialized microglia regulate inhibitory synapse pruning and sculpting during a critical window of mouse postnatal development. Moreover, they showed that C1q expression impacted the rate of inhibitory synapse pruning mediated by these specialized microglia during development (Favuzzi et al., 2021). In Chapter 3 we show that microglia remove dorsal horn inhibitory and excitatory synapses through the CR3 synapse

pruning pathway. It is plausible that as a result of peripheral nerve injury, these specialized GABA receptive microglia are reactivated in the spinal cord and drive inhibitory synapse pruning.

Detailed transcriptomic studies on nerve injury induced spinal microglia is a logical approach to identify $GABA_B$ receptor expressing microglia subtypes and determine their transcription profile. This would open new avenues for identification of specific therapeutic targets to selectively manipulate this subpopulation of microglia with the goal of slowing down the progression of maladaptive structural changes only in the dorsal horn inhibitory circuits.

5.4 Time-course of spinal changes in the neuropathic pain pathology

As the neuropathic pain pathophysiology progresses over time, the relative contribution of different underlying mechanisms to the sensory phenotype changes. Although, a temporal analysis of the relative contributions of known mechanisms to neuropathic pain is still missing in the clinical setting, there are few pre-clinical studies that have explored these dynamic alterations over longer periods of time. For instance, Echeverry et al. (2017) showed that microglia rely mainly on TrkB-BDNF signaling for long-term maintenance of neuropathic pain, while at earlier stages of the pathology, microglial inflammatory responses play a more important role (Echeverry et al., 2017). In Chapters 3 and 4, we show that structural changes including reorganization of synapses and dynamics of membrane KCC2 expression have a specific temporal pattern. These structural changes must be accompanied with functional changes that explain different aspects of pain behavior in neuropathic animal models. In my view, successful management of neuropathic pain requires properly classifying the stage of the pathology and specifically targeting the most influential mechanism of the disease at that specific stage. For instance, targeting the microglial-mediated synapse pruning signaling pathway should be effective at earlier stages of the disease to prevent centralization and the transition of acute pain into a chronic disorder, while targeting central signaling pathways underlying the maintenance phase of the pathology (i.e., KCC2 hypoactivitiy) can be more effective when the chronic phase of the disorder is established.

In clinical settings, assessment of symptoms and signs can help determine which pathophysiological mechanisms are involved in specific neuropathic pain disorders as the time-

course of disease progression varies between patients (Haanpää et al., 2009, Cruccu et al., 2010, Rasmussen et al., 2004, Baron, 2006). Our clinical tools to identify the underlying mechanism contributing to an individual's neuropathic pain pathology is restricted to sensory tests, electrophysiological techniques, nerve biopsy samples, and the relative efficacy of different pharmacological interventions (Herrmann et al., 2006, Amir et al., 2005, Dworkin et al., 2010). Most of these tests only provide partial information about the peripheral progression of the neuropathy in cases like channelopathies and severe neuropathies, while they are less informative about the degree of central changes and stages of disease progression (Lauria and Lombardi, 2007, Truini et al., 2013, Themistocleous et al., 2018).

Identifying biomarker profiles associated with the key central changes in patients is a promising future avenue for devising proper treatment strategies. This is because detection and quantification of key neuropathic pain signaling factors such as complement proteins, pro-inflammatory and excitotoxic molecules, in blood serum and cerebrospinal fluid, are now technically feasible and reliable (Sandholm et al., 2019, Alexander et al., 2007). Unfortunately, this level of precision medicine remains a future hope as it requires a strong and persevering multidisciplinary collaboration between basic science and clinical pain research.

5.5 Dynamics of excitatory synapses differ between neuropathic pain models

We observed the phenomenon of microglia-mediated synapse removal in a rat model of chronic constriction nerve injury (cuff) (Chapter 2), and a mouse model of spared nerve injury (SNI) (Chapter 3). Although in both models, spinal microglia engulf inhibitory and excitatory presynaptic inputs, the impact of targeting microglia on inhibitory and excitatory synapse density was different between cuff and SNI animals. Microglial inhibition or depletion prevented loss of inhibitory synapses in both neuropathic models, but it had variable effects on excitatory synapses. In the cuff model, targeting microglia did not affect excitatory synapse density contrasting with the SNI model in which the same microglial manipulation resulted in an increase in the number of excitatory synapses. This striking inconsistency suggest that remodeling of dorsal horn connectivity might also be a function of the nature, intensity, and the extent of the peripheral nerve injury.

Differences observed in the dynamics of excitatory synapses between cuff and SNI neuropathic pain models can be explained by two factors: the nature of microglia-synapse interactions (phagocytosis versus trogocytosis), and the extent of synapse formation.

Unlike the case with inhibitory synapses, microglia-mediated engulfment of excitatory presynaptic terminals observed in both neuropathic models was not accompanied by a significant loss of spinal excitatory synapses. One complicating possibility, based on emerging studies on spinal synaptogenesis in neuropathic pain is formation of new excitatory synapses to replace the pruned ones (Yu et al., 2018, Brennan et al., 2021, Simonetti et al., 2013). This can explain our observations in the SNI model, as depletion of microglia or targeting the complement system resulted in an excess number of synapses. However, this mechanism alone cannot explain the lack of changes in excitatory synapse count in the cuff model when microglia are depleted. It is likely that in the cuff model, microglia are modifying presynaptic structures without completely removing them through trogocytosis which was recently shown to be dependent on the complement pathway (Weinhard et al., 2018, Lim and Ruthazer, 2021).

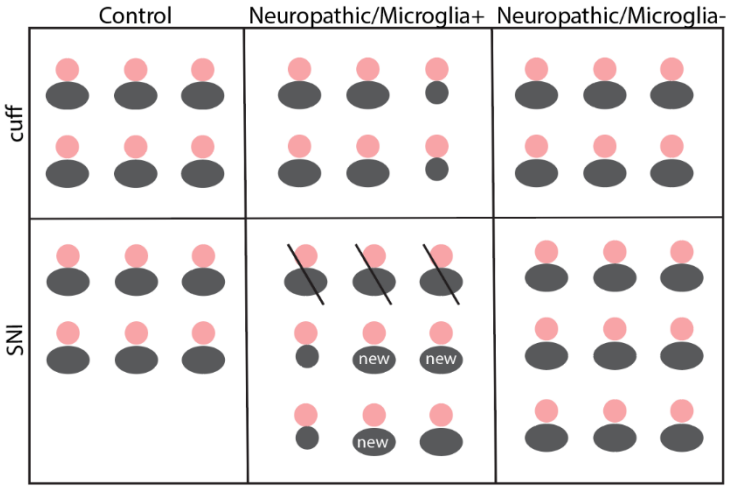

Figure 1 A hypothetical model of the structural dynamics of excitatory synapses in different models of neuropathic pain

Diagram showing a hypothetical model of the dynamics of spinal excitatory synapses in different experimental conditions. (Presynaptic and post synaptic elements are depicted in pink and black respectively)

The diagram in Figure 1 summarizes our hypothetical model of the dynamics of excitatory synapses in cuff and SNI animals under different experimental conditions. This hypothetical model can be tested by quantifying the changes in size of presynaptic terminals as a result of nerve injury and microglial depletion to evaluate the possibility of microglia-mediated trogocytosis. To assess whether excitatory synapse formation is contributing to synapse count changes, synaptogenesis and synapse pruning pathways should be inhibited at the same time.

Given the advances in genetic tools for selective and simultaneous manipulation of these pathways, finding the relative contribution of synapse removal and formation pathways to the net synapse density in the neuropathic pain models would be possible in the near future.

Taken together, the results presented in Chapters 2 and 3 suggest that chronic constriction (cuff model) and partial transection (SNI model) neuropathies can lead to different structural modifications within the spinal circuitry which likely contribute to the differences in pain-related behavior profiles in these models (Decosterd and Woolf, 2000, Bennett et al., 2003). It is plausible that similar variabilities in central changes exist in human painful peripheral neuropathies as they are mechanistically diverse (Fields et al., 1998).

5.6 Species choice (is rat=mouse=human?)

To address the main hypothesis of this thesis we used both mice and rats, the most commonly used species in basic science research of pain. Several core findings such as evidence for microglia-mediated synapse pruning, complement upregulation, and co-localization of C1q with synaptic elements were demonstrated in both models, suggesting that the synapse pruning pathway in response to peripheral nerve injury is evolutionarily conserved at least in rodents.

Although there are possible differences between rats, mice, and humans in the basic wirng of spinal neural circuitry that modulate, and transmit sensory information (Ribeiro-da-Silva, 2015, Todd, 2010), the underlying pathophysiological mechanisms responsible for reorganization of these networks seems to be similar across species. In fact, there are human genetic and postmortem histological evidence in support of the involvement of the complement-mediated synapse pruning pathway in psychiatric and demyelinating disorders in humans (Stevens et al., 2007, Werneburg et al., 2020). Whether this pathway is also activated in neuropathic pain patients and contributes to sensory symptoms remains to be explored in future studies.

5.7 No obvious sexual dimorphism in microglia-mediated structural plasticity in the spinal cord of neuropathic mice

There is growing evidence to support the existence of sexual dimorphism in key microglial pathways implicated in neuropathic pain such as TLR4 signaling, and P2X4–BDNF–TRKB (Sorge et

al., 2011, Sorge et al., 2015, Taves et al., 2016). However, no obvious sex differences have been reported with respect to microglial phagocytic signaling pathways including CX3CR1 (Staniland et al., 2010), TMEM16F (Batti et al., 2016), and P2Y12 (Gu et al., 2016) in neuropathic models. In Chapter 3, we showed that the CR3 signaling pathway mediates synapse pruning and contributes to neuropathic pain in both males and females. Moreover, we showed that transient depletion of microglia during the peak of synapse pruning reduced mechanical allodynia at later time-points in both sexes. Interestingly, using different drug administration paradigms, others have shown ineffectiveness of microglial depletion in female neuropathic mice (Sorge et al., 2015), indicating that the sexually dimorphic role of spinal microglia in pain is likely affected by the temporal changes of microglial function and the extent to which a particular or a set of microglial signaling pathways are contributing to sensory symptoms at a given time.

Classically, the use of female rodents was avoided and even discouraged in key reference papers that described neuropathic pain models (Bennett et al., 2003). The logic for excluding females was to avoid the potentially confounding effects of the estrus cycle on the behavioral assays (Mogil and Chanda, 2005). Given the overwhelming evidence for sex differences in pain mechanisms (Mogil, 2012), in Chapters 3 and 4, we investigated the mechanism of microglia-mediated synapse pruning and dynamics of membrane KCC2 expression in dorsal horn neurons in both sexes. Although we did not find any significant sex differences, we noticed larger variability in synapse counts, and membrane KCC2 levels in females which did not seem to be related to the estrus cycle phases. The underlying cause of the observed variability warrants further studies.

A note of caution that sex-specific differences in data variability can lead to false positive sex difference findings and, therefore, such differences should be carefully considered in experimental designs. This is to avoid planning studies that are sufficiently powered for males and are underpowered for females.

5.8 Restoring spinal neural circuits to treat neuropathic pain

5.8.1 How does synapse pruning modify the neuronal map of the dorsal horn?

Structural remodeling and rewiring of synapses, and neural circuits, within the central somatosensory network potentially contribute to the long-term and likely irreversible nature of neuropathic pain. The main goal of this thesis was to identify the mechanism underlying the structural remodeling of the spinal synaptic network to prevent such changes and assess their contribution to the chronicity of neuropathic pain. A key question raised by our study is how spinal circuits are modified by microglia mediated synapse pruning.

Recent studies have described several spinal cord microcircuits and within them, neurochemically distinct subpopulations of inhibitory interneurons were identified as gate keepers between touch and pain processing layers of the dorsal horn (see Figure 4 of Chapter 1). A popular working hypothesis in the field is that distinct subsets of inhibitory interneurons are affected differentially by both structral and functional presynaptic mechanisms of disinhibition in the neuropathic pain state (Petitjean et al., 2015, Petitjean et al., 2019, Boyle et al., 2019, Peirs and Seal, 2016, Lu et al., 2013, Miraucourt et al., 2007, Foster et al., 2015). Although, to a lesser extent, structural alterations in excitatory networks were also reported as a contributing factor to dorsal horn hyperexcitability in neuropathic pain (Simonetti et al., 2013, Leitner et al., 2013, Brennan et al., 2021). Determining which connections or neuronal subpopulations are more vulnerable to synapse remodeling, within the broader map of dorsal horn neural network, will pave new avenues for drug discovery. It is likely that the molecular profile of pre-synaptic sites (i.e., complement regulatory proteins, neurotransmitters, eat me and do not eat me signals, and molecules controlling microglia chemotaxis) are specific to neuron populations and circuits (Gyorffy et al., 2018). As reported in Chapters 2 and 3, we found more C1q signal on inhibitory synapses compared to VGLUT2+ excitatory synapses. Moreover, C1q was virtually absent from VGLUT1+ and VGLUT3+ synapses, supporting the hypothesis that there is a synapse specific molecular change attracting the C1q tag and the synapse pruning machinery. Future studies are needed to study neuron type and circuit specific structural changes resulting from synapse pruning, and to charactarize proteome changes associated with specific synapse types in neuropathic pain models.

5.8.2 Structral dynamics of spinal presynaptic inhibition in neuropathic pain

In Chapter 2, as part of an utrastructural study, we deliberately characterized the anatomical dynamics of presynaptic inhibition on terminals of touch-coding primary afferents and showed that microglia contribute to loss of these synapses in neuropathic pain. To my knowledge, this is the first EM study to report structural alterations of axo-axonic inhibition on these afferents in a model of neuropathic pain. Although, due to technical difficulties, the functional contribution of loss of presynaptic inhibition to neuropathic pain remains to be speculative, there are emerging genetic tools and more sensitive calcium imaging technologies that will allow accurate assessment of the functional dynamics of presynaptic inhibition at the physiological and neuropathic state (Zimmerman et al., 2019, Katona et al., 2012). The significance of our anatomical findings can be better evaluated when more information about the functional consequence of these changes becomes available.

5.9 The complement system: a new target for neuropathic pain

The role of the complement system in neuropathic pain is relatively underexplored and the few studies that have investigated the complement system in chronic pain primarily focused on the pro-inflammatory roles of complement proteins (Griffin et al., 2007, Levin et al., 2008). These studies have introduced potential drug targets within the C5a/C5aR1 axis in the complement system and promoted the generation of C5 component targeted therapies to ameliorate neuropathic pain (Giorgio et al., 2021). Activation of the C5a/C5aR1 axis recruits immune cells and triggers pro-inflammatory cytokine production, which likely contributes to the inflammatory response and sensory symptoms in neuropathic pain (Moriconi et al., 2014). Importantly, targeting the C5a/C5aR1 axis does not affect synapse pruning as the key complement factors involved in synapse pruning (i.e., C1q, C2, C3 and C4) are upstream to C5 production (Trouw et al., 2017).

Although targeting C1q affects C5 production through blocking the progression of the classical complement pathway, it is unlikely that this deficit contributes to the analgesic effect we observed with C1q neutralizing antibodies. This is because C5 can be formed independently from C1q through other complement pathways such as the alternative pathway (Trouw et al., 2017).

Moreover, C1q neutralizing antibodies result in a delayed analgesic effect on pain behavior which differs from the acute effects observed with complete depletion of the complement system (e.g., via Cobra Venom Factor) or with C5a/C5aR1 inhibitors (Levin et al., 2008, Griffin et al., 2007, Moriconi et al., 2014).

To target spinal synapse pruning in neuropathic pain, our strategy to interfere with complement activation (e.g., with Annexon's C1q blocking antibody) does not directly affect the monocyte-macrophage lineage, therefore most of the adverse effects associated with approaches that inhibit or deplete microglia (e.g., though blockade of CSF1 signaling) are avoided (Fu et al., 2020). Still, few concerns remain regarding targeting C1q that should be addressed in future studies. First, at the physiological state, C1q is highly expressed in different brain areas including the hippocampus, and a growing body of evidence suggests that it is implicated in aspects of normal synaptic plasticity such as memory formation (Wang et al., 2020, Stephan et al., 2013). Therefore, further studies are required to characterize the effect of C1q inhibition on these physiological processes. Second, other glia and immune cells within the CNS and peripheral systems express C1q receptors, but the role of C1q signaling in the physiology of immune and glial signaling is not fully understood (Kouser et al., 2015). Therefore, more research is necessary to determine the consequence of chronic inhibition of C1q on the immune response and glial function in health and disease.

Regardless of the remaining questions regarding the general impact of targeting C1q beyond synapse pruning, given the high specificity and efficiency of C1q inhibition in preventing maladaptive spinal synaptic reorganization, I believe C1q is a logical and relevant target for developing new therapeutic strategies to treat neuropathic pain in humans.

5.10 Limitations and future directions

There are number of technical limitations that may affect the conclusions drawn in this thesis. Some of the main issues will be addressed here and future experiments will be proposed for addressing these caveats.

5.10.1 Functional correlates

Although there is functional evidence (i.e., changes in the frequency of miniature inhibitory and excitatory postsynaptic currents – mIPSCs and mEPSCs) from neuropathic models that supports the loss of spinal inhibitory synapses without affecting excitatory synapses (Moore et al., 2002, Scholz et al., 2005, Yowtak et al., 2011, Lu et al., 2013, Inquimbert et al., 2018), it is not known how chronic depletion of microglia or blocking the synapse pruning pathway affects these functional parameters. Our results in Chapters 2 and 3 show that depletion or inhibition of microglia, as well as blocking the CR3 synapse pruning pathway, can prevent inhibitory synapse loss and have model-specific effects on excitatory synapses. Based on these results, we would expect that blocking synapse pruning activity would restore the frequency of mIPSCs and, depending on the neuropathic pain model, the frequency of mEPSCs would be decreased, not affected, or increased.

The importance of having functional correlates in our studies goes beyond the confirmation of the structural findings and is essential for fully understanding the role of synapse pruning in spinal structural and functional synapse plasticity. Indeed, using functional approaches, other valuable information such as the nature of synapses that are removed (i.e., GABAergic versus glycinergic), and the physiological effect of C1q inhibition on functional plasticity can be determined. Therefore, I propose future whole-cell patch clamp experiments to assess the frequency, amplitude, and kinetics of mIPSCs and mEPSCs of dorsal horn neurons in different experimental conditions to complement the work presented in this thesis.

5.10.2 Engulfment assay

The key observation in this thesis was the engulfment of spinal synapses by microglia in models of neuropathic pain. For this, we used the engulfment assay method (Schafer et al., 2014) in which the volume of synaptic material within microglial lysosomes was measured. In the original engulfment assay protocol, authors suggested the use of retrograde fluorescent dyes to label engulfed terminals since these dyes are resistant to lysosomal hydrolases. However, this approach was not feasible for identification of different synapse types in our rat experimental model, thus we used antibodies to label presynaptic terminals (the engulfed material) instead.

Although we still managed to detect and quantify synapse engulfment using an antibody-based approach, the amount of engulfed material was found to be relatively small. This is likely because most endogenous proteins are rapidly broken down once in lysosomes and not enough antigen remains for antibody detection as the engulfed material.

To better assess engulfment, in Chapter 3, transgenic mouse lines expressing fluorescent proteins in spinal neurons were used and engulfment of endogenous fluorescent protein was quantified. Although this approach was more robust, it was difficult to distinguish which part of the neuronal cell had been engulfed and complementary experiments were required to prove that the fluorescent engulfed material were in fact synaptic structures.

For future experiments, to simultaneously achieve robust and accurate engulfment assay, transgenic animals that encode fluorophores tagged to synapse-localized proteins are necessary.

5.10.3 Behavior

A caveat to the behavior data presented in this thesis is that we only utilized one behavioral assay. In all our behavioral studies, using the von Frey method, we assessed stimulus-evoked mechanical allodynia and demonstrated that microglia-mediated synapse pruning is implicated in "static" mechanical allodynia, a form of tactile hypersensitivity which results from gentle touch. Since all the key changes in synaptic densities were observed in laminae II and III of the dorsal horn where there are circuitries relevant for mechanical allodynia (Koch et al., 2018), the von Frey method seemed to be a logical choice for behavior. We considered doing other behavior assays to quantify "dynamic" allodynia, which is another form of tactile hypersensitivity that results from an object moving across the skin. However, we did not find the available methods sensitive and reliable enough to detect such behavioral changes in our experimental conditions (Field et al., 1999). Given the fact that dynamic allodynia is a pressing clinical problem (Jensen and Finnerup, 2014), development of reliable behavior assays to measure this sensory phenotype would be a significant progress for preclinical pain research.

Because of major concerns over the clinical translatability of stimulus-evoked pain behavior assays including the von Frey method, the pain field is gradually moving towards non-stimulus evoked behavior methods, such as grimace scales (Matsumiya et al., 2012). A next logical step to

evaluate the effectiveness of C1q neutralizing antibodies on pain-related behavior is through non-stimulus evoked behavior assays.

5.10.4 Difficulty studying synapse pruning mechanisms in knockout mouse models

Transgenic mice lacking essential proteins involved in synapse pruning (i.e., CX3CR1, C1q, CR3, C3, and C4) have documented abnormal synapse pruning during postnatal development in different brain regions (Gunner et al., 2019, Paolicelli et al., 2011a, Yilmaz et al., 2021, Stevens et al., 2007, Schafer et al., 2012). In Chapter 3, we report abnormal synapse density in the contralateral dorsal horn of C3 and CX3CR1 knockout mice suggesting that the spinal wiring in these animals is likely abnormal due to developmental deficits in synapse pruning.

Given the potential abnormalities in the dorsal horn circuitry, and possible compensatory mechanisms in the modulation and transmission of sensory information in the aforementioned global knockout mice, they are not the best tools for assessing the role of synapse pruning defects on spinal circuitry and pain-related behavior in neuropathic pain models. Generation of inducible conditional knockout mouse models for mediators of synapse pruning would be an important next step for future studies in this line of research.

5.10.5 Testing different experimental paradigms to determine the therapeutic potential of C1q neutralizing antibodies

In Chapter 3, we used C1q neutralizing antibodies in an experimental paradigm that was designed to assess the contribution of the CR3 synapse pruning pathway to pain-related behavior. For this reason, animals were treated chronically with C1q neutralizing antibodies for the entire duration of the study. However, the full therapeutic potential of C1q neutralizing antibodies in neuropathic pain remains to be explored.

Our main claim is that C1q neutralizing antibodies have disease-modifying properties since they can prevent maladaptive synaptic remodeling of the dorsal horn circuitry which is likely contributing to the chronification of neuropathic pain. To test this in a preclinical setting, I propose to treat neuropathic mice with C1q neutralizing antibodies for a month after SNI surgery to fully block C1q dependent synapse pruning and then stop the treatment and continue with assessing pain-related behavior for the following weeks. Based on the findings presented in this

thesis, I would expect mice that received the drug to have less pain-related behavior compared to controls even in the absence of further treatments.

Lastly, because our approach to block C1q was systemic, the effect on behavior can be also influenced by peripheral mechanisms. Therefore, it will be important for future efforts to isolate central and peripheral effects of inhibiting C1q using more restricted drug administration approaches (i.e., intrathecal injections).

5.11 Overall summary and conclusions

To conclude, the magnitude of the challenge to fully understand the mechanisms underlying neuropathic pain is great. Here, I have presented work that brings us closer to understanding how microglia and the complement system contribute to the long-term nature of chronic pain through structural reorganization of the dorsal horn circuitry in preclinical models of neuropathic pain.

Structural plasticity in spinal neural circuits of neuropathic models has long been known to be associated with the sensory symptoms (Lorenzo et al., 2014, Kuner and Flor, 2017). However, it was unclear whether these structural changes have a direct influence on the sensory symptoms of neuropathic pain. Finding a mechanism responsible for loss of spinal synapses revealed the key role of structural reorganization of the dorsal horn circuitry in the pathophysiology of neuropathic pain.

Identification of the CR3 synapse pruning pathway as the primary signaling mechanism driving dorsal horn synapse loss in neuropathic pain led to identification of C1q as a potential new target for treating neuropathic pain. Beyond C1q, another way forward is to identify other regulatory molecular mechanisms involved in attracting C1q to the vulnerable synapses to discover even more specific targets.

Another major focus of this thesis was to develop, and optimize, image capturing and analysis protocols to reliably detect small structural changes using basic immunohistochemistry and imaging techniques. Moving forward, these protocols can be easily adapted for future cell type and circuit cell-specific studies.

Overall, the data presented here is the foundation of a broader line of research concerning the role of spinal glia in mediating structural plasticity within the somatosensory nervous system and it is my hope that it will open new avenues for identifying novel targets for the treatment of neuropathic pain.

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