GABAergic Signalling in the Peripheral Somatosensory System: Local and Multicellular Mechanisms Underlying Sensory Modulation

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Abstract

Primary sensory neurons of the dorsal root ganglia (DRGs) constitute the first relay in somatosensory signal transmission and play a central role in nociception and pain. These neurons exhibit a unique pseudo-unipolar morphology, with a bifurcating stem axon that imposes distinct biophysical constraints on action potential propagation. In particular, the axonal bifurcation, or T-junction, functions as a frequency-dependent filter regulating the transmission of sensory signals from the periphery to the spinal cord. While nociceptive processing has traditionally been considered neuron-centric, increasing evidence indicates that glial and immune cells critically modulate sensory signalling under both physiological and pathological conditions.

This thesis investigates mechanisms regulating nociceptive signal transmission within the peripheral nervous system (PNS), with a focus on GABAergic signalling and neuro-glial-immune interactions. The organization and functional properties of primary sensory neurons are examined, highlighting how axonal geometry, ion channel distribution, and excitability shape filtering at the DRG T-junction. Using compartmentalized *in vitro* models, the spatially distinct effects of GABA receptor activation across neuronal compartments are characterized.

Furthermore, Schwann cells are identified as active contributors to peripheral GABAergic signalling. They are shown to express a complete GABAergic molecular machinery and to release a bioactive ligand capable of activating $GABA_A$ receptors. Peripheral nerve injury induces a downregulation of Schwann cell GABAergic components, suggesting that loss of this inhibitory glial tone contributes to pathological sensory signalling. Finally, GABAergic signalling is shown to attenuate pro-inflammatory macrophage activation and reduce immune cell accumulation in models of neuropathic pain.

Together, these findings support a model in which nociceptive transmission is regulated by integrated interactions between neurons, glia, and immune cells in the PNS, identifying peripheral GABAergic signalling as a potential therapeutic target for chronic pain.

Contents

Publications

Li, X., Prudente, A. S., Prato, V., Guo, X., Hao, H., Jones, F., **Figoli, S**., Mullen, P., Wang, Y., Tonnello, R., Lee, S. H., Shah, S., Maffei, B., Berta, T., Du, X., & Gamper, N. (2024). *Peripheral gating of mechanosensation by glial diazepam binding inhibitor*. J. Clin. Invest., 134(16), e176227. DOI: 10.1172/JCI176227

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Posters

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Abbreviations

Aα: Alpha motor fibre

Aβ: Beta sensory fibre

Aδ: Thinly myelinated A-delta fibre

AMPA: α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid

ASIC: Acid-sensing ion channel

ATP: Adenosine triphosphate

BBB: Blood–brain barrier

BDNF: Brain-derived neurotrophic factor

BMDM: Bone marrow–derived macrophage

BSA: Bovine serum albumin

C-fibre: Unmyelinated nociceptive fibre

Ca^{2+}: Calcium ion

cAMP: Cyclic adenosine monophosphate

CGRP: Calcitonin gene-related peptide

CIP: Congenital insensitivity to pain

CNS: Central nervous system

CNTF: Ciliary neurotrophic factor

CRMP2: Collapsin response mediator protein 2

CSF-1: Colony-stimulating factor-1

DBI: Diazepam binding inhibitor

DH: Dorsal horn

DIV: Days in vitro

DR: Dorsal root

DRG: Dorsal root ganglion

EAAT: Excitatory amino acid transporter

GABA: γ-Aminobutyric acid

$GABA_A$ R: GABA type A receptor

$GABA_B$ R: GABA type B receptor

GAD: Glutamic acid decarboxylase

GAT: GABA transporter

GFAP: Glial fibrillary acidic protein

GIRK: G-protein-gated inwardly rectifying potassium channel

GILT: Glutamate transporter

GPCR: G-protein-coupled receptor

IASP: International Association for the Study of Pain

IL: Interleukin

KCC2: Potassium–chloride cotransporter 2

LPS: Lipopolysaccharide

LTP: Long-term potentiation

MAP2: Microtubule-associated protein 2

MCP-1: Monocyte chemoattractant protein-1

MHC-II: Major histocompatibility complex class II

Nav: Voltage-gated sodium channel

NGF: Nerve growth factor

NKCC1: Sodium–potassium–chloride cotransporter 1

NMDA: N-Methyl-D-aspartate

OCT: Optimal cutting temperature compound

ON: Overnight

PAD: Primary afferent depolarization

PBS: Phosphate-buffered saline

PAG: Periaqueductal gray

PBN: Parabrachial nucleus

PFA: Paraformaldehyde

PGE_2: Prostaglandin E_2

PMSF: Phenylmethylsulfonyl fluoride

PNS: Peripheral nervous system

PO: Posterior thalamic nucleus

PSNL: Partial sciatic nerve ligation

qPCR: Quantitative polymerase chain reaction

RIPA: Radioimmunoprecipitation assay buffer

ROS: Reactive oxygen species

RT-qPCR: Real-time quantitative polymerase chain reaction

RVM: Rostroventral medulla

S1 / S2: Primary / secondary somatosensory cortex

SDS: Sodium dodecyl sulfate

SGC: Satellite glial cell

SN: Sciatic nerve

SNP: Single nucleotide polymorphism

SNI: Spared nerve injury

TNF-α: Tumour necrosis factor alpha

TRP: Transient receptor potential

TRPA1: Transient receptor potential ankyrin 1

TRPM: Transient receptor potential melastatin

TRPV: Transient receptor potential vanilloid

VEGF: Vascular endothelial growth factor

VGAT: Vesicular GABA transporter

VPN: Ventral posterolateral thalamic nucleus

VPM: Ventral posteromedial thalamic nucleus

1 Chapter 1: Introduction

1.1 The somatosensory system: organization and function of primary sensory neurons

The somatosensory system is a major sensory system responsible for detecting and processing information about the mechanical, thermal, chemical, and nociceptive state of the body. It enables the perception of touch, pressure, vibration, temperature, pain, and body position, playing a crucial role in interaction with the external environment and in the maintenance of homeostasis. Somatosensory information is detected by specialized peripheral sensory neurons and conveyed to the central nervous system (CNS), where it is integrated and interpreted. This section focuses on the organization and properties of peripheral primary sensory neurons, which constitute the first level of sensory signal detection and transmission.

Primary sensory neurons are the first-order neurons of the somatosensory system and are responsible for detecting and transmitting information about the external and internal environment, from the periphery to the CNS. Primary sensory neurons are pseudo-unipolar neurons made up by a cell body which resides within the dorsal root, trigeminal and nodose ganglia, and a single process, the stem axon, which originates from the soma and bifurcates within the ganglion into peripheral and central branches (Herrick, 1909; Tandrup, 1995). Dorsal root ganglia (DRGs) contain sensory neurons innervating the trunk, limbs, and visceral organs and project to the spinal cord, whereas the trigeminal ganglion houses sensory neurons innervating the face and cranial structures and projects to the brainstem. In addition to DRGs neurons, visceral sensory information is conveyed by neurons whose cell bodies reside in cranial sensory ganglia, most notably the nodose ganglion. The nodose ganglion contains the somata of primary sensory neurons associated with the vagus nerve and are primarily involved in the detection visceral, cardiovascular, and respiratory information. The peripheral branch of DRG

neurons extends to innervate peripheral target tissues, including the skin, muscles, joints, and visceral organs, where it terminates in specialized sensory endings responsible for detecting mechanical, thermal, chemical, and noxious stimuli. Through these specialized peripheral terminals, DRG neurons transduce a wide range of somatosensory modalities - including touch, pressure, vibration, temperature, proprioception, and nociception - into electrical activity. The peripherally-generated signals travel along the peripheral axon, through the bifurcation, to the central axon which, *via* the dorsal root (DR), enters the spinal cord where it synapses with the second-order neuron in the dorsal horn (DH). Somatosensory information is then relayed to higher brain centres and it ultimately reaches the somatosensory cortex for processing and perception. Two major ascending pathways convey this information: the dorsal column-medial lemniscus pathway, which primarily transmits proprioceptive information, fine touch, and pressure, and the spinothalamic tract, which is mainly involved in the transmission of pain, temperature, and crude touch (Basbaum et al., 2009) (Figure 1.1).

Anatomically, DRGs are located in the intervertebral foramina alongside the spinal cord and form a swelling in the dorsal root of each spinal nerve, containing an accumulation of the cell bodies of thousands of sensory neurons. The number of neurons per DRG varies across spinal segments, correlating with ganglion size, and also differs across species. In humans, estimates suggest that a single DRG can contain up to ~70,000 neurons, particularly in young adults, whereas considerably lower numbers have been reported in rodents (e.g., ~15,000 neurons in rats) (Nagashima & Oota, 1974; Tandrup, 2004; West et al., 2012). Neuronal somata within the DRGs are surrounded by one or several layers of satellite glial cells (SGCs) that support, nourish and protect these neurons (Hanani, 2005; Jessen & Mirsky, 2005). Similarly, Schwann cells support and nourish the axons of primary sensory neurons. Depending on the axon type, Schwann cells either form myelin sheaths around large-diameter axons to facilitate rapid saltatory conduction or remain non-myelinating, enveloping multiple small-diameter axons within Remak bundles (Robertson, 1957; Taveggia & Feltri, 2022; Wilson et al., 2021).

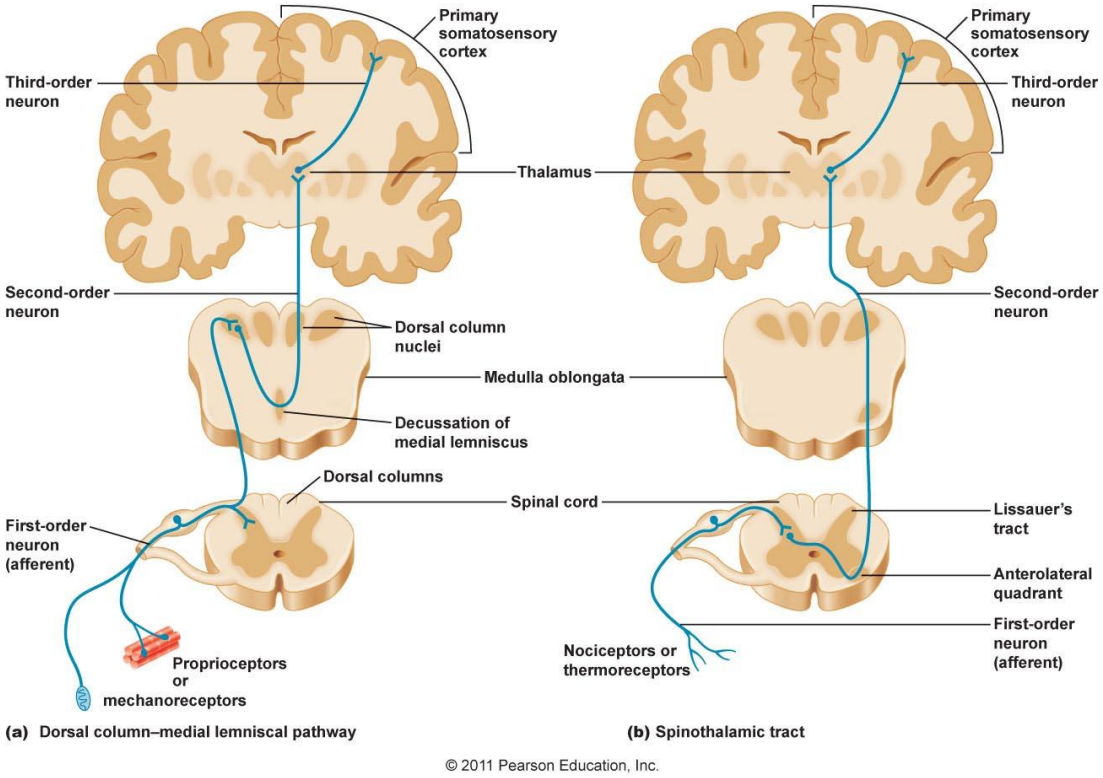


Figure 1.1 Ascending sensory pathways.
The dorsal column-medial lemniscal pathway transmits fine touch, vibration, pressure, and conscious proprioception from mechanoreceptors and proprioceptors. First-order afferent neurons enter the spinal cord and ascend ipsilaterally in the dorsal columns to synapse in the dorsal column nuclei of the medulla oblongata. Second-order neurons decussate in the medulla as the medial lemniscus and ascend to the thalamus, where third-order neurons project to the primary somatosensory cortex (A). The spinothalamic (anterolateral) pathway conveys pain, temperature, and crude touch from nociceptors and thermoreceptors. First-order afferent neurons enter the spinal cord, travel briefly in Lissauer's tract, and synapse in the dorsal horn. Second-order neurons decussate within the spinal cord and ascend contralaterally in the anterolateral quadrant to the thalamus. Third-order neurons then project to the primary somatosensory cortex (B) (Pearson Education, 2011).

Differential expression of a broad range of ion channels, receptors, and signalling molecules enables distinct sensory modalities to be selectively detected and transmitted by distinct populations of sensory neurons. In addition, other functional and anatomical characteristics, such as conduction velocity, activation threshold and peripheral target innervation, are often shared within the same neuronal subpopulation, further defining modality-specific classes of primary sensory neurons (Lawson, 2005; PERL, 1992). For instance, small diameter neurons are commonly identified as nociceptors, characterized by unmyelinated (C) or lightly myelinated (Aδ) slow conducting fibres and high activation threshold (Burgess & Perl, 1967; Dubin & Patapoutian, 2010; Harper & Lawson, 1985; Lawson, 2002). On the other hand, neurons with large diameters convey proprioception and mechanoreception, they are low threshold and fast conducting neurons equipped with myelinated large fibres (Aα and Aβ) (Table 1.1) (Abraira & Ginty, 2013; Brown & Iggo, 1967; Le Pichon & Chesler, 2014; Mountcastle et al., 1967). The development of new technologies as transcriptome and proteome investigations, enabled the subdivision into additional subgroups based on molecular markers.

Table 1.1 Sensory neuron classification in humans.

Fibre type	Primary function	Receptor type	Myelination	Axon diameter (μm)	Conduction velocity (m/s)
Aα (Ia, Ib)	Proprioception (muscle length, tension)	Muscle spindles (Ia), Golgi tendon organs (Ib)	Heavily myelinated	13–20	80–120
Aβ (II)	Fine touch, vibration, pressure	Cutaneous mechanoreceptors (Meissner, Merkel, Pacinian, Ruffini)	Myelinated	6–12	35–75
Aδ (III)	Fast (sharp) pain, cold temperature, crude touch	Free nerve endings (nociceptors, thermoreceptors)	Thinly myelinated	1–5	5–30
C (IV)	Slow (dull/burning) pain, warmth, itch	Free nerve endings (nociceptors, thermoreceptors, pruriceptors)	Unmyelinated	0.2–1.5	0.5–2

1.1.1 Pseudo-unipolarization and axonal specialization in DRG neurons

The pseudo-unipolar nature of DRG neurons was first demonstrated in 1904 by Ramón y Cajal, using the silver impregnation method (Figure 1.2). Successively, they summarized the sequence of events leading to the formation of the two branches in developing DRG neurons (Ramón y Cajal & Azoulay, 1955). Diverse hypothesis have been developed throughout the years and nowadays the debate is still open. It is commonly accepted that in early stages of development, most DRG neurons are spindle-shaped bipolar, with neurites emanating from opposite sides of the cell body. Although several reports indicate the pseudo-unipolarization derives from the fusion of the two opposing branches (Boubakar et al., 2017; Kiernan & Barr, 2009; Pomp et al., 2005), a different hypothesis indicates that cell body bulging gives rise to the stem axon. Following this view, the soma elongates and constricts between the initial segments of the two processes to form the stem axon from which the two axonal branches protrude (Figure 1.3) (Matsuda et al., 1996, 2000).

The onset of pseudo-unipolarization varies between species, but the process is likely concluded in late embryonic development. In rats, at E14, only 7% of neurons are pseudo-unipolar, while at E19 this percentage abruptly increases to 94% (Matsuda et al., 1996). Although the cellular and molecular basis of pseudo-unipolarization is unknown, some evidence suggests that other cell types, including Schwann cells and SGCs, may contribute to the process (Mudge, 1984; Takahashi & Ninomiya, 1987). Interestingly, in mice, undifferentiated bipolar DRG neurons extend their central axons within their central synaptic target fields in the spinal cord, suggesting that central signalling may contribute to pseudo-unipolarization (Barber & Vaughn, 1986).

Although not much is known about anatomical and molecular differences between distinct segments of the DRG neurons axon, electron microscopy experiments carried

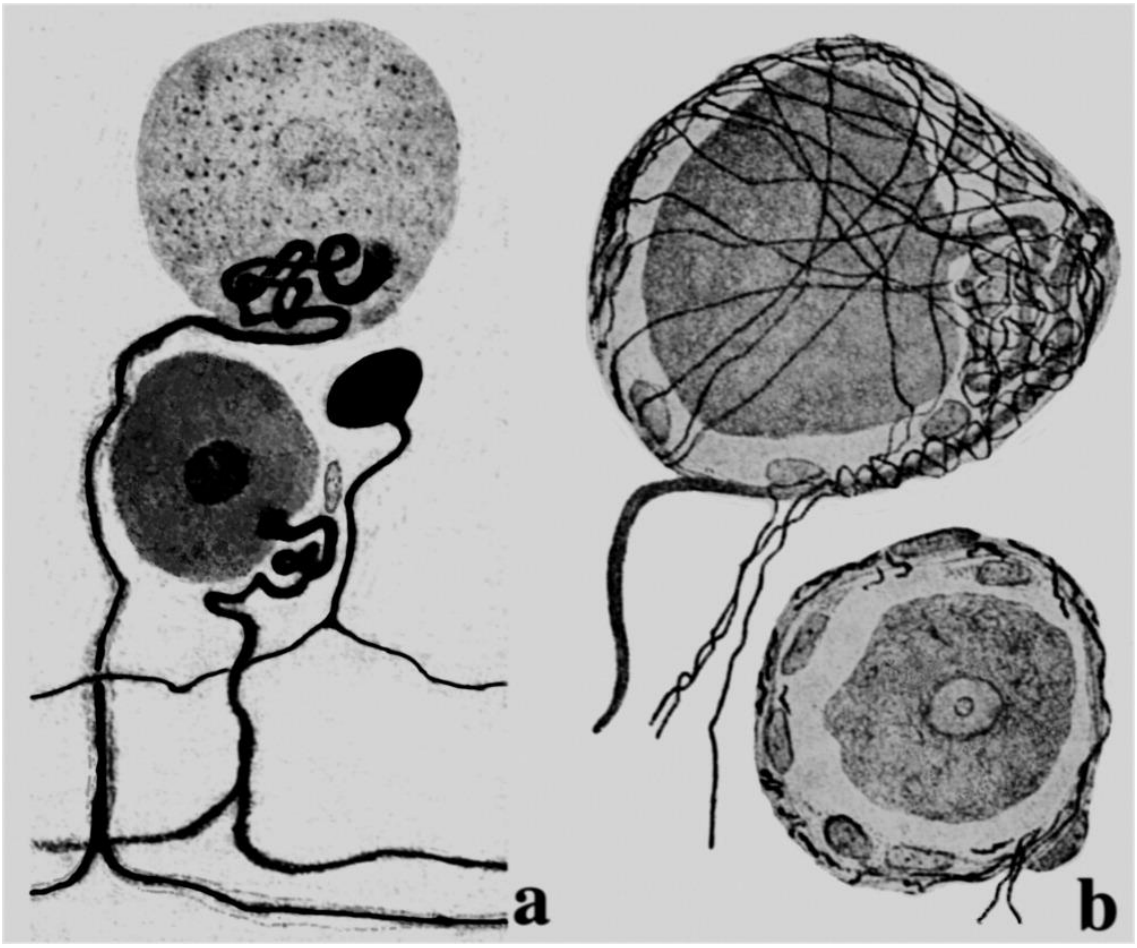


Figure 1.2 First drawings of DRG neuron pseudo-unipolar morphology by Ramón y Cajal. Drawings by Ramón y Cajal showing the initial glomerulus, a convolution of the stem axon near the parent cell body (A), and Dogiel's pericellular nests (PCNs) (B), which resembles balls of yarn made of thin unmyelinated fibres around DRG neuron. Adapted from from *(Ramón y Cajal & Azoulay, 1955)*.

out in myelinated neurons, showed that diverse axonal segments display different ultrastructural features. The proximal segment of the stem axon is always unmyelinated and enveloped by SGCs forming a multilayered coat continuous with that enwrapping the cell body (Zenker & Högl, 1976). This segment hosts a notable number of mitochondria and ribosomes and a higher density of microtubules and endoplasmic reticulum when compared to the distal region, which is ensheathed by Schwann cells and can be myelinated (Spencer et al., 1973; Zenker & Högl, 1976). The stem axon bifurcation has fasciculated microtubules and a triangular area occupied by clusters of mitochondria (Ha, 1970). Besides a notable difference in diameter, with the peripheral branch being the thickest, differences in microtubules and neurofilaments density have been observed between the two branches. In several species, microtubule density is significantly higher in the peripheral axon whereas the central one is enriched in neurofilaments (Fadić et al., 1985). Similarly, the degree of phosphorylation of the neurofilaments seems to be increased in the central branch, influencing the transport of neurofilaments, possibly contributing to central-peripheral differences in axon diameter (Watson et al., 1989).

A tight regulation of transport must be established along the stem axon of primary sensory neurons and successively, at the bifurcation, in order to control what is directed at the central branch and what instead, is required in the periphery. Due to technical difficulties, a small amount of data covers this topic. Early radiolabelling data suggested that similar proteins are transported to both axons and only a few studies demonstrated a differential distribution of some peptides. An asymmetrical distribution of microtubule-associated protein 2 (MAP2) and of an isoform of collapsin response mediator protein-2 was observed (CRMP2), with both proteins being highly expressed in the peripheral branch (Katano et al., 2006; Papasozomenos et al., 1985). MAP2 function has also been investigated at the level of the stem axon where it has been demonstrated to direct axonal cargo entry by coordinating the activities of molecular motors, especially in the proximal segment (Gumy et al., 2017).

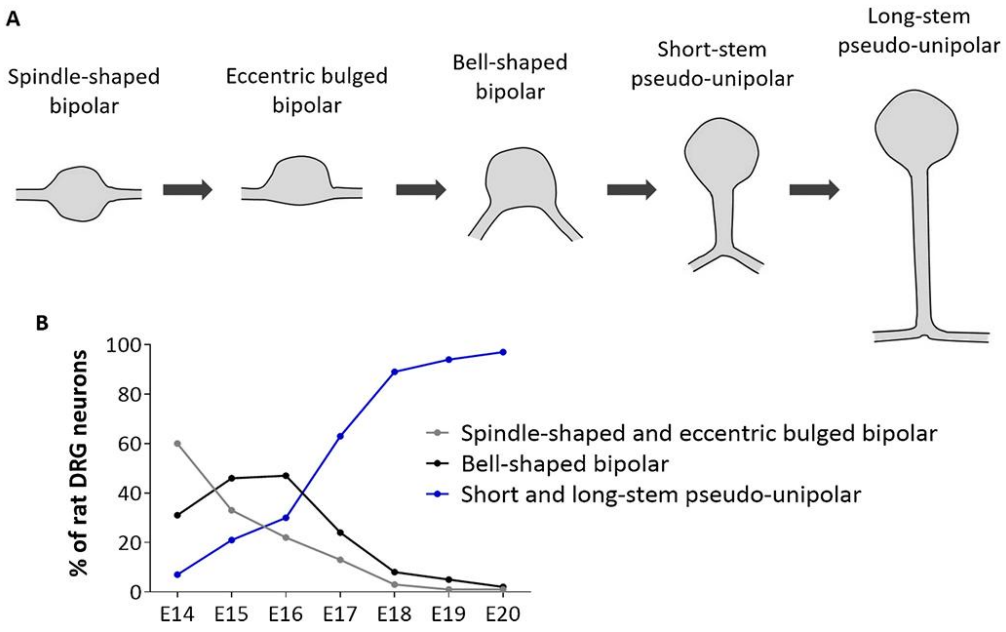


Figure 1.3 Development of DRG neuron pseudo-unipolarity.
Schematic drawings of DRG neuron pseudo-unipolarization (A). Percentage of spindle-shaped, bell-shaped bipolar and short and long-stem pseudo-unipolar neurons during embryonic development in thoracic rat DRGs (B) (Adapted from Nascimento et al., 2018).

MAP2 is commonly known to be enriched in the dendrites of neurons, raising the hypothesis that at the molecular level, the peripheral branch might have some dendritic-like feature. In addition to cytoskeletal and transport regulators, the selective delivery of sensory transduction machinery to the periphery is essential for neuronal function. Ion channels and receptors required for stimulus detection, such as transient receptor potential (TRP) channels, mechanosensitive Piezo channels, and other modality-specific receptors, must be efficiently transported and retained within the peripheral branch and its specialized terminals. The mechanisms ensuring the preferential targeting of these receptors to peripheral axons, while limiting their presence in the central branch, remain poorly understood and are likely to rely on branch-specific cargo sorting, motor regulation, and local trafficking cues. The limited sensitivity of early experimental approaches may have precluded the identification of additional differentially distributed proteins, and the molecular mechanisms underlying cargo filtering between the two axonal branches of DRG neurons remain an open question.

1.1.2 The T-junction: a filter for incoming inputs

DRG neurons pseudo-unipolar morphology is fundamental for their physiology. Early evidence showed that the sensory impulses are filtered at their passage through the DRG and the site of such blockage has been identified in the bifurcation point, also known as T-junction (Ducreux et al., 1993; Dun, 1955; C Luscher et al., 1994; Stoney Jr, 1990). Multiple factors contribute to these mechanisms and many are strongly related to the distinctive morphology of pseudo-unipolar neurons. The bifurcating geometry creates an impedance mismatch which lowers the safety factor for action potential propagation, thereby increasing the probability of spike failure at the T-junction (Debanne, 2004; C Luscher et al., 1994; Sundt et al., 2015). Axonal electrical impedance is determined by both geometric and biophysical parameters, including axon diameter, membrane resistance and capacitance, axial resistance, and myelination. In DRG neurons, the stem axon is typically unmyelinated, and the two daughter branches differ markedly in diameter and electrical load. Quantitative ultrastructural studies in rodents have shown

that the peripheral branch is substantially thicker than the central branch. In rat DRG neurons, unmyelinated peripheral axons typically measure approximately 0.8-1.0 µm in diameter, whereas the corresponding central branches are approximately 0.3-0.5 µm, representing an almost two-fold reduction in diameter (Ha, 1970; Hoheisel & Mense, 1986; Lee et al., 1986; Suh et al., 1984; Zhang et al., 1998). A similar, though less pronounced, reduction is observed in myelinated fibres, with central branches being approximately 30-50% thinner than their peripheral counterparts (Lee et al., 1986; Suh et al., 1984). These sudden transitions in axonal diameter and electrical properties create an impedance mismatch at the T-junction, such that an action potential arriving from the periphery must distribute its depolarizing current into two branches with different electrical loads (Debanne, 2004; Goldstein & Rall, 1974; Sundt et al., 2015; Zhou & Chiu, 2001). As a result, current may be insufficient to reliably depolarize the central branch to threshold, leading to attenuation, delayed propagation, or conduction failure, particularly for small-amplitude or slowly rising action potentials typical of unmyelinated nociceptive fibres (Gemes et al., 2013; Sundt et al., 2015). Functionally, the bifurcation can therefore act as a frequency-dependent filter, shaping the fidelity of sensory signal transmission from the periphery to the spinal cord and contributing to the modulation of sensory input at the level of the DRG (Figure 1.4). Experimentally, it has been observed that high frequency signals fail during their passage through the bifurcation both *in* vitro (C Luscher et al., 1994; Stoney Jr, 1990) and *ex vivo* in adult rat C-fibre neurons (Gemes et al., 2013). As mentioned above, several morphological parameters, including a longer stem axon, increase the probability of spike conduction through the bifurcation to the central axon, thus inhibiting low-pass filtering (Devor & Obermayer, 1984; Du et al., 2014; Sundt et al., 2015). Specifically, increasing the excitability of the DRG neuron cell body, for instance by enrichment in voltage-gated sodium channels (Na_v), could promote spike propagation and therefore inhibit low-pass filtering (Devor & Obermayer, 1984). For large

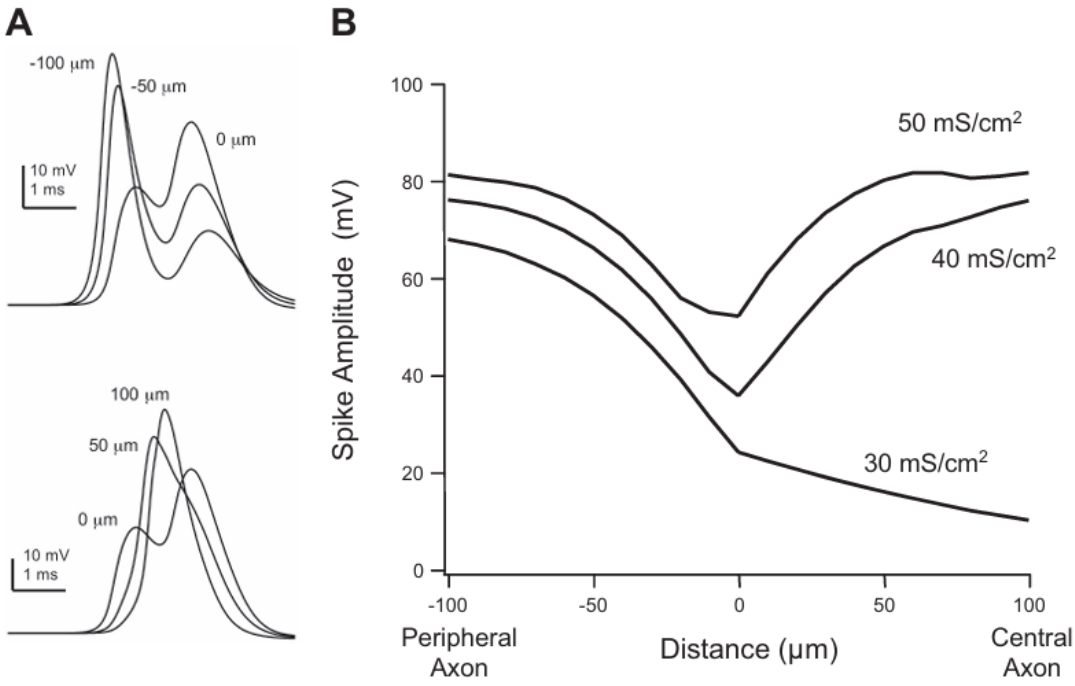


Figure 1.4 Simulated action potential filtering at the T-junction in an unmyelinated sensory neuron.

Simulated action potential waveforms at different locations along an unmyelinated sensory neuron, illustrating attenuation and waveform distortion as spikes propagate through the dorsal root ganglion T-junction. The change in waveform reflects the impedance mismatch at the bifurcation between the peripheral, central, and stem axons (A). Spatial profile of action potential peak amplitude along the neuron, highlighting a local reduction in amplitude in the region of the T-junction (B). This quantifies the filtering effect observed in (A) and demonstrates how neuronal geometry and ion channel distribution contribute to reduced spike propagation fidelity (Adapted from Sundt et al., 2015)

diameter DRG neurons, excitability of the soma appears to have little functional consequence on spike propagation, because the increased stem axon length electrically isolates the cell body (Amir & Devor, 2003; Du et al., 2014; Sundt et al., 2015). However, if the soma and proximal stem axon are electrotonically close enough to the bifurcation, which is the case in small diameter DRG neurons (Du et al., 2014; Sundt et al., 2015), they can have significant influence over the low-pass filtering. In fact, in small-diameter unmyelinated DRG neurons, increased membrane conductance and hyperpolarization of the somatic/perisomatic resting membrane potential have been suggested to contribute to filtering at the bifurcation (Du et al., 2014; Sundt et al., 2015; Hao et al., 2023). Because small-diameter, unmyelinated C-fibre neurons are particularly susceptible to frequency-dependent conduction failure, the bifurcation may act as an intrinsic gate that shapes pain signalling before it reaches the spinal cord. Importantly, this filtering is likely subject to modulation under pathological conditions such as inflammation or nerve injury, where changes in ion channel expression and membrane properties may alter impedance matching and contribute to aberrant sensory signalling (Wang et al., 2021; Waxman & Zamponi, 2014).

1.2 Physiological relevance of nociception

Nociception refers to the neural processes by which potentially harmful stimuli are detected, transduced, and transmitted to the CNS, ultimately leading to protective behavioural responses. It is important to distinguish nociception from pain, as the two terms are not synonymous. While nociception describes the sensory encoding and transmission of noxious stimuli by specialized peripheral and central neurons, pain is a subjective perceptual experience that arises from the integration of nociceptive signals with cognitive, emotional, and contextual factors at higher brain levels (IASP definition). As such, nociceptive activity can occur in the absence of pain, and conversely, pain may be experienced without ongoing nociceptor activation (Lee & Tracey, 2010, 2013).

Noxious stimuli are classically categorized into three main modalities: thermal, mechanical, and chemical. Thermal nociception is activated by extreme heat or cold that

threatens tissue integrity, mechanical nociception responds to high-threshold pressure or tissue deformation, and chemical nociception is triggered by irritant substances or endogenous mediators released during tissue damage and inflammation. These diverse stimuli are detected by specialized subsets of primary sensory neurons, named nociceptors, which are equipped with distinct molecular receptors and ion channels that confer modality specificity.

From a functional perspective, nociception plays a critical protective role by alerting the organism to potentially damaging conditions and promoting rapid withdrawal or avoidance behaviours. In addition to immediate reflexive responses, nociceptive signalling also contributes to longer-term adaptive processes, such as learning to avoid harmful environments (Wiech & Tracey, 2013). The fidelity and regulation of nociceptive transmission are therefore essential for maintaining tissue integrity and organismal survival (Karos et al., 2017; Leknes & Bastian, 2014).

The essential role of nociception in acute pain and tissue protection is strikingly illustrated by rare human conditions such as congenital insensitivity to pain (CIP). Individuals with CIP are unable to perceive pain despite otherwise intact sensory modalities, leading to recurrent injuries, burns and fractures (Nagasako et al., 2003; Schon et al., 2020). These clinical observations demonstrate that nociception, and the acute pain it elicits, is crucial for survival by enabling rapid detection of and response to harmful stimuli. Genetic studies of CIP have further revealed that loss-of-function mutations in key molecules involved in nociceptor excitability and signal propagation, such as Na_v channels and neurotrophins signalling components, selectively disrupt nociceptive pathways while sparing other sensory functions (Cox et al., 2006; Habib et al., 2018; Leipold et al., 2013; Rotthier et al., 2012).

Under normal physiological circumstances. nociceptive signals originate at peripheral sensory endings and are conveyed by C- and Aδ-fibres, high-threshold primary afferent neurons. Following peripheral transduction, action potentials propagate along the peripheral axon, pass through the DRG, and enter the spinal cord via the DR. Within the

DH, the central axonal branches of nociceptive sensory neurons arborize predominantly in laminae I and II, with some fibres extending deeper into lamina V of the DH (Figure1.5) (Todd, 2010). From there, nociceptive information is relayed to higher-order neurons and ascends through dedicated pathways, such as the spinothalamic tract, to reach supraspinal centres involved in sensory discrimination, affective processing, and motor responses.

Under physiological conditions, primary sensory neurons are typically considered to transmit action potentials uninterrupted from peripheral sensory terminals to the spinal cord, where synaptic communication with second-order neurons first occurs in the DH. Subsequent processing of somatosensory information takes place through distributed circuits within the spinal cord and across multiple supraspinal centres. Traditionally, peripheral sensory axons are thought to receive little to no synaptic input before entering the CNS, and the neuronal somata located within sensory ganglia have been viewed largely as passive conduits for signal transmission. However, increasing evidence suggests that sensory neuron cell bodies possess active signalling capabilities and can influence neuronal excitability, particularly under pathological conditions such as chronic pain. These observations position DRG neurons as an attractive and accessible target for the development of novel therapeutic strategies aimed at modulating pathological sensory signalling.

1.2.1 Nociceptors and nociceptive signal transmission

As mentioned in the previous sections, nociceptors are specialised primary sensory neurons responsible for the detection and transmission of noxious stimuli. They are distinguished from other sensory neuron subtypes by their higher activation threshold, slow conduction velocities and selective expression of receptors and ion channels that confer sensitivity to potentially damaging stimuli (Dubin & Patapoutian, 2010; PERL, 1992). Nociceptors represent a heterogeneous population that can be classified based on axon diameter, degree of myelination, conduction velocity, peripheral target innervation, and molecular identity.

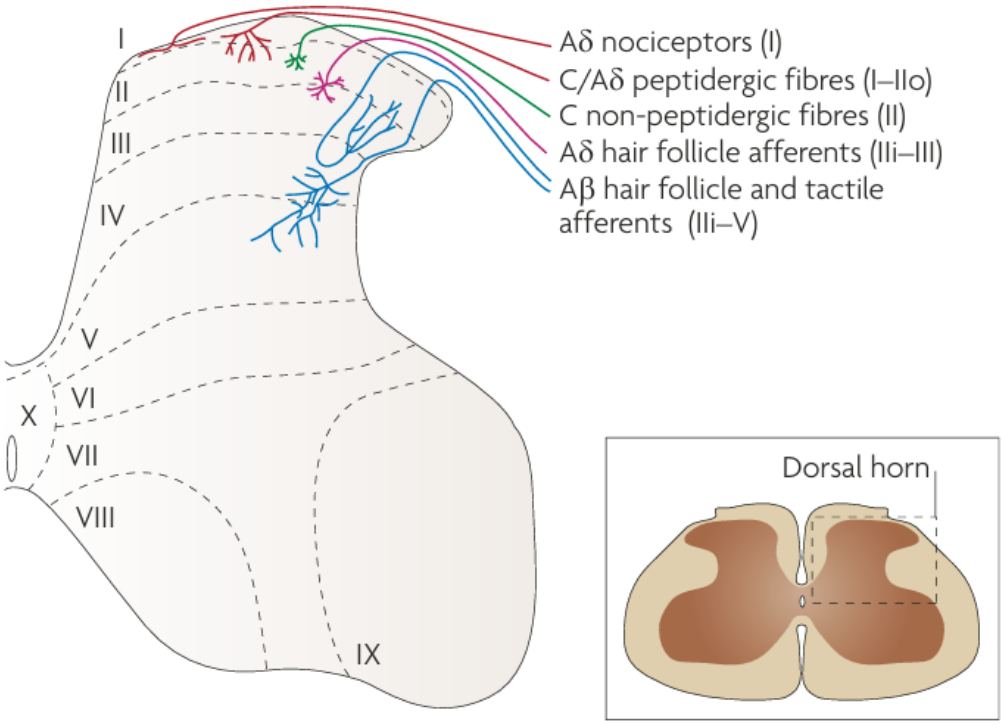


Figure 1.5 Laminar termination of primary somatosensory afferent subtypes in the spinal dorsal horn.
Schematic representation of the spinal DH illustrating the preferential termination of distinct primary afferent fibre classes within Rexed laminae I-V. Aδ nociceptors project mainly to lamina I, while peptidergic C- and Aδ-fibres terminate predominantly in laminae I–IIo. Non-peptidergic C-fibres project primarily to lamina IIi. Aβ hair follicle afferents terminate in laminae III–IV, whereas Aβ tactile afferents extend into deeper laminae III–V. The inset shows a transverse section of the spinal cord highlighting the location of the DH (Adapted from Todd, 2010).

From a functional perspective, nociceptors are commonly divided into two major classes: thinly myelinated Aδ fibres and unmyelinated C-fibres. Aδ nociceptors conduct action potentials at intermediate velocities and are often associated with the sharp and localized component of acute pain, whereas C-fibre nociceptors conduct slowly and are typically responsible for the delayed, diffuse, and persistent pain sensation (Burgess & Perl, 1967; Dubin & Patapoutian, 2010; Lawson, 2002; Samour et al., 2015; Treede et al., 1995; Willis & Westlund, 1997). Within these broad categories, nociceptors are further distinguished into polymodal nociceptors, which respond to multiple types of noxious stimuli, and modality-specific nociceptors that preferentially detect thermal, mechanical, or chemical stimuli. At molecular level, nociceptors can also be segregated into peptidergic and non-peptidergic populations, based on their expression of neuropeptides such as calcitonin gene-related peptide (CGRP) and substance P or their binding of isolectin B4, respectively (Hökfelt et al., 1975; Lawson, 2005; Snider & McMahon, 1998; Stucky & Lewin, 1999).

The ability of nociceptors to detect diverse noxious stimuli relies on the expression of specialised transduction channels and receptors at their peripheral terminals. Members of the TRP channel family play a prominent role in thermal and chemical nociception. Transient receptor potential vanilloid 1 (TRPV1) responds to noxious heat and capsaicin (Caterina et al., 1997), while transient receptor potential ankyrin 1 (TRPA1) is activated by a range of reactive chemical irritants and has also been implicated in certain forms of heat sensitivity (Bautista et al., 2006). In addition, transient receptor potential melastatin 2 (TRPM2) and TRPM3 have been shown to contribute to heat-evoked nociceptive responses (Tan & McNaughton, 2016; Vriens et al., 2011). In contrast, TRPM8 is primarily associated with the detection of innocuous cooling although more recent investigations suggest its involvement in warm perception (Dhaka et al., 2008; McKemy et al., 2002; Paricio-Montesinos et al., 2020). Although the molecular identity of sensors mediating noxious cold remains less well defined, several candidates, including glutamate receptor ionotropic kainate 2 (GluK2), have been proposed, but additional

cold-sensitive transducers are likely to exist (Cai et al., 2024). Mechanical nociception is supported by mechanosensitive ion channels, including Piezo1 and Piezo2 (Coste et al., 2010), although other nociceptive mechanotransducers are yet to be identified. Additional receptors, such as acid-sensing ion channels (ASICs), contribute to the detection of tissue acidosis often associated with injury and inflammation (Deval et al., 2010; Lingueglia et al., 1997) (Table 1.2).

Table 1.2 Major nociceptor subtypes and their associated transduction channels

Nociceptor subtype	Fibre type	Myelination	Conduction velocity	Primary modality	Key receptors / ion channels
Thermal nociceptors (heat)	Aδ- and C-fibres	Thinly myelinated / unmyelinated	Intermediate / slow	Noxious heat	TRPV1, TRPA1, TRPM2, TRPM3
Thermal nociceptors (cold)	Aδ- and C-fibres	Thinly myelinated / unmyelinated	Intermediate / slow	Noxious cold	GRIK2 (proposed), other unidentified cold-sensitive channels
Chemical nociceptors	Predominantly C-fibres	Unmyelinated	Slow	Chemical irritants	TRPA1, TRPV1
Mechanical nociceptors	Aδ- and C-fibres	Thinly myelinated / unmyelinated	Intermediate / slow	Noxious mechanical stimuli	Piezo2, additional unidentified mechanotransducers
Acid-sensitive nociceptors	Predominantly C-fibres	Unmyelinated	Slow	Tissue acidosis	ASICs (e.g. ASIC1, ASIC3)

Activation of these transduction mechanisms generates receptor potentials that, if sufficient in amplitude, trigger action potential firing at the peripheral terminal. Once initiated, nociceptive action potentials propagate along the peripheral axon toward the DRG and the CNS. This propagation is critically shaped by the intrinsic biophysical properties of nociceptor axons, including their small diameter, lack of myelination, and distinctive arrays of voltage-gated ion channels. A specific subset of Na_v channels, $Na_{v1.7}$, $Na_{v1.8}$, and $Na_{v1.9}$, are highly enriched in nociceptors and play essential roles in action potential initiation, propagation, and repetitive firing (Basbaum et al., 2009; Dib-Hajj et al., 2010; Nassar et al., 2004). The importance of these channels is highlighted by human

genetic studies, as loss-of-function mutations in $Na_{v1.7}$ cause CIP, whereas gain-of-function mutations result in painful neuropathies (Cox et al., 2006; Dib-Hajj et al., 2007).

Nociceptive signal transmission is further modulated as action potentials traverse the DRG. As discussed in the previous sections, the pseudo-unipolar morphology of DRG neurons imposes a unique biophysical constraint at the stem axon bifurcation, where impedance mismatch and other factors can lead to frequency-dependent filtering of incoming signals (Debanne, 2004; Goldstein & Rall, 1974). Small-diameter C-fibre nociceptors are particularly susceptible to propagation failure at the T-junction, such that only sufficiently strong or sustained peripheral activity successfully reaches the spinal cord (Gemes et al., 2013; Hao et al., 2023; Lall et al., 2025). In this way, axonal geometry and membrane excitability act together to regulate the flow of nociceptive information from the periphery to the CNS.

In addition to acute signal transmission, nociceptors exhibit marked plasticity in response to nerve damage or inflammation. Peripheral sensitisation can arise from rapid, reversible actions of inflammatory mediators, as well as from more persistent changes driven by transcriptional remodelling following nerve injury or axotomy (Costigan et al., 2009). Inflammatory mediators such as prostaglandins, bradykinin, and nerve growth factor (NGF) modulate the activity, expression, and trafficking of ion channels and receptors at peripheral terminals, while injury-induced transcriptional changes lead to sustained alterations in ion channel expression and intrinsic excitability (Basbaum et al., 2009; Dib-Hajj et al., 2010; Woolf & Ma, 2007). These changes lower activation thresholds and enhance firing frequency, increasing the probability that nociceptive signals overcome axonal filtering mechanisms and propagate centrally. Thus, alterations in nociceptor excitability at the periphery have direct consequences for signal transmission through the DRG and ultimately for pain perception.

1.2.2 Spinal processing

Following entry into the spinal cord via the DR, nociceptive primary afferents terminate within distinct laminae of the DH, where they synapse onto second-order neurons and local interneurons. Small-diameter C-fibres predominantly project to superficial laminae I and II, while Aδ fibres mainly terminate in laminae I and V, establishing the initial level of central processing for nociceptive information (Rexed, 1952; Todd, 2010). At this level, incoming sensory signals are subject to extensive modulation that critically shapes their transmission to higher brain centres.

Spinal DH circuits integrate nociceptive input with signals arising from low-threshold mechanoreceptors, propriospinal neurons, and descending pathways from supraspinal structures. Excitatory transmission is primarily mediated by glutamate acting on α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and N-methyl-D-aspartate (NMDA) receptors and by neuropeptides such as substance P and CGRP which enhance pain transmission (Basbaum et al., 2009; Iyengar et al., 2017; Malcangio et al., 2000; Todd, 2010). In parallel, inhibitory interneurons releasing GABA and glycine play a crucial role in limiting the spread and amplification of nociceptive signals within the DH.

In addition to relaying sensory information to supraspinal centres, spinal DH circuits play a crucial role in the generation of rapid protective reflexes. Nociceptive inputs activate polysynaptic networks of excitatory and inhibitory interneurons that couple sensory processing to motor output through connections with ventral horn motor neurons. The most extensively studied example is the nociceptive withdrawal (flexor) reflex, in which activation of peripheral nociceptors triggers the rapid withdrawal of an affected limb from a potentially damaging stimulus (Basbaum et al., 2009; Todd, 2010). This response occurs before conscious perception of pain and serves as an essential protective mechanism that minimizes tissue injury. In many cases, withdrawal is accompanied by activation of crossed-extensor reflex pathways, which facilitate extension of the contralateral limb to maintain posture and balance during movement. Although these reflexes can be generated entirely within spinal circuits, their excitability is subject to

modulation by both local inhibitory mechanisms and descending inputs from supraspinal regions (Heinricher et al., 2009). GABAergic and glycinergic interneurons play a particularly important role in regulating reflex gain and preventing excessie excitation. Following inflammation or peripheral nerve injury, alterations in inhibitory neurotransmission and enhanced excitatory signalling can lead to exaggerated reflex responses, reflecting broader processes of central sensitisation (Basbaum et al., 2009). Consequently, spinal reflex circuits represent an important functional interface between sensory processing, motor control, and pathological pain signalling, highlighting the integrative role of the spinal cord in both physiological and chronic pain states.

Beyond local spinal circuits, nociceptive processing is influenced by descending modulatory systems originating in brainstem regions such as the periaqueductal gray (PAG), rostroventral medulla (RVM), and locus coeruleus. These pathways can exert both inhibitory and facilitatory effects on spinal nociceptive transmission, depending on behavioural state, context, and prior experience (Fields, 2006; Heinricher et al., 2009). Descending control thus provides a mechanism through which cognitive and emotional factors can dynamically regulate nociceptive inputs at early stages of processing (Figure 1.6).

Importantly, nociceptive processing is not static but exhibits pronounced activity-dependent plasticity. Repetitive or intense nociceptor activation can enhance synaptic efficacy in DH neurons through mechanisms such as NMDA receptor-mediated long-term potentiation (LTP), increased receptor expression, and reduced inhibitory tone (Ji et al., 2003; Woolf, 2011). This form of plasticity can be triggered by natural noxious stimuli and tissue injury and may persist beyond the resolution of the initial insult, thereby increasing the gain of nociceptive circuits. As such, spinal LTP provides a cellular substrate for central sensitization and contributes to the transition from acute to chronic pain.

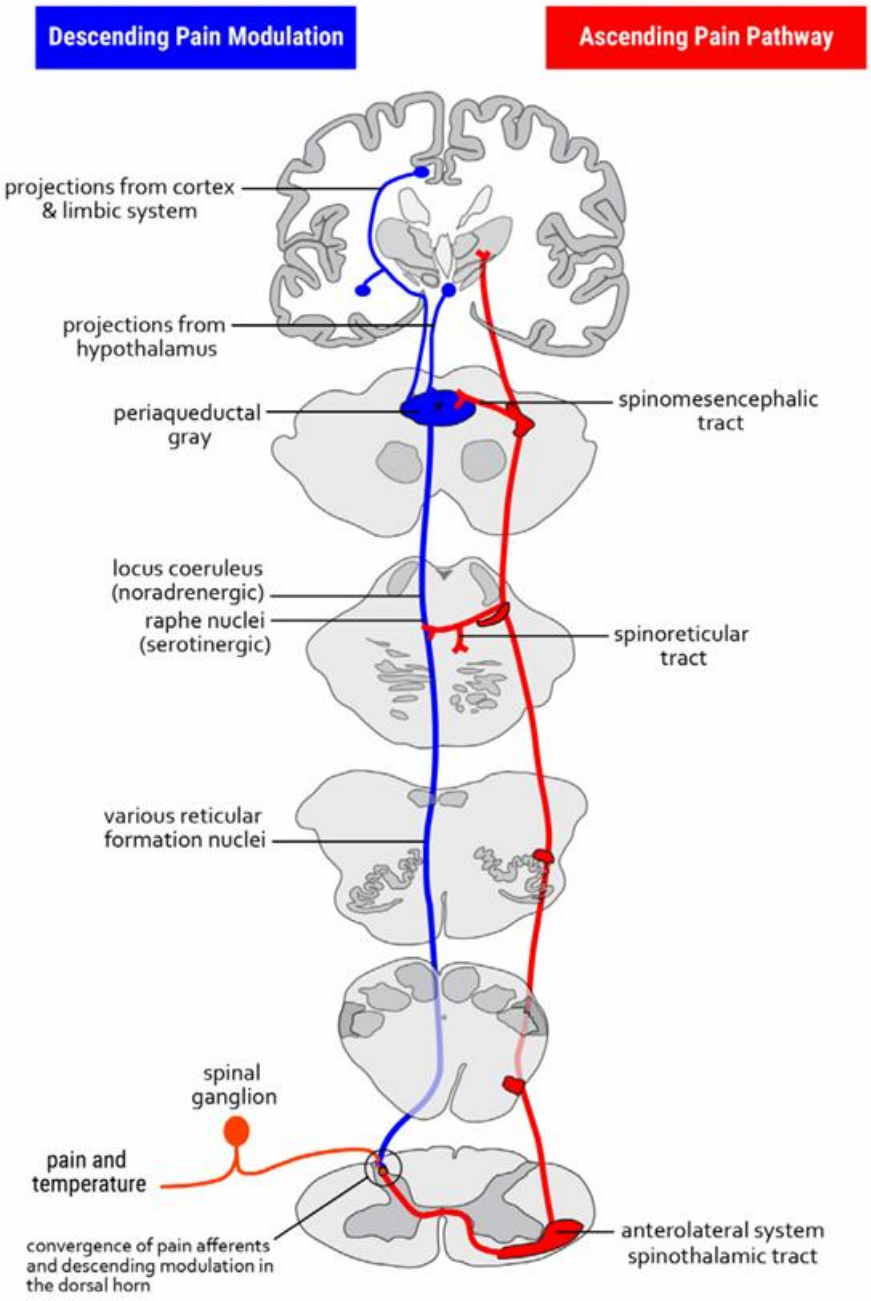


Figure 1.6 Ascending and descending pathways involved in pain transmission and modulation.
Schematic overview of nociceptive signal transmission from the periphery to supraspinal centres via the anterolateral system (spinothalamic, spinoreticular, and spinomesencephalic tracts; red), and descending pain modulatory pathways originating from cortical, limbic, and hypothalamic regions (blue). Descending projections engage the PAG, locus coeruleus (noradrenergic), and raphe nuclei (serotonergic), which in turn modulate nociceptive processing at the level of the spinal DH. The convergence of ascending nociceptive input and descending modulatory control within the DH is highlighted (Adapted from Krebs, 2024).

Together, these spinal and supraspinal mechanisms ensure that nociceptive information is not merely relayed but actively filtered, integrated, and modulated before reaching higher brain centres. This multilayered processing allows the nervous system to adapt nociceptive transmission to physiological demands, while also rendering it vulnerable to dysregulation in disease states.

1.2.3 Supraspinal processing of nociceptive signals

Following integration within the DH, nociceptive information is transmitted to supraspinal centres through multiple ascending pathways that segregate sensory signals to distinct brain regions involved in sensory discrimination, affective evaluation and motor responses. Supraspinal nociceptive processing involves parallel and partially segregated circuits that together generate the experience of pain.

The spinothalamic tract represents a major ascending pathway for nociceptive and thermosensory information. Projection neurons in lamina I and V of the DH extend axons that decussate and ascend to the thalamus, where they innervate nuclei such as the ventral posterolateral (VPL), ventral posteromedial (VPM), posterior (PO), and medial dorsal nuclei. These neurons relay discriminative aspects of nociception - such as stimulus location, intensity and modality - to primary and secondary somatosensory cortices (S1 and S2). Activity within these cortical areas underlies the spatial organization and perceptual features of pain (Apkarian et al., 2005; Willis & Westlund, 1997).

In parallel, nociceptive signals ascend via the spinoreticular and spinoparabranchial pathways, which are particularly important for mediating the affective, motivational, and autonomic components of pain. The spinoreticular pathway originates primarily from DH neurons located in deeper laminae VII and VIII, which integrate nociceptive input with convergent sensory, propriospinal and descending signals. Axons ascend bilaterally alongside the spinothalamic tract to reach the medullary and pontine reticular formation, thereby engaging the ascending reticular activating system (Willis, 1985). Rather than encoding precise sensory features, this pathway conveys slow, diffuse, and poorly

localised nociceptive signals, particularly those arising from deep tissues and viscera, and contributes to reflexive and autonomic responses (Al-Chaer et al., 1998; Basbaum et al., 2009; Willis & Westlund, 1997).

From the reticular formation, spinoreticular signals are relayed to intralaminar and medial thalamic nuclei, as well as to hypothalamic and limbic structures, which broadcast nociception-related activity to widespread cortical and subcortical regions. Through these connections, the spinoreticular pathway contributes to the affective-motivational and autonomic dimensions of pain, influencing emotional valence, arousal, and behavioural state rather than precise sensory discrimination (Fields, 2005). Subsequent projections engage cortical and limbic regions such as the anterior cingulate cortex, prefrontal cortex, amygdala, and hippocampus, where nociceptive input is integrated with cognitive appraisal, memory, and expectation (Apkarian et al., 2005; Bushnell et al., 2013).

In addition to its ascending role, the spinoreticular pathway is tightly coupled to descending modulatory systems. Reciprocal interactions with brainstem nuclei involved in pain control, including the nucleus raphe magnus and other bulbar reticular nuclei, allow spinoreticular signalling to engage inhibitory and facilitatory pathways that regulate DH excitability, such as diffuse noxious inhibitory control. This bidirectional organisation links nociceptive input to brainstem circuits governing arousal, autonomic function, and endogenous analgesia (Heinricher et al., 2009; Le Bars et al., 1979; Millan, 2002).

The spinoparabrachial pathway, originates predominantly from lamina I projection neurons in the DH whose axons project to the parabrachial nucleus (PBN) in the dorsolateral pons, a key integrative centre for nociceptive signals (Craig, 1995, 2003). From the PBN, information is relayed to limbic and hypothalamic structures, including the amygdala, hypothalamus, and bed nucleus of the stria terminalis, thereby coupling nociceptive input to emotional processing, autonomic responses, and homeostatic regulation (Chiang et al., 2019). The spinoparabrachial pathway is particularly important for encoding the aversive and affective components of pain and for linking noxious stimuli

to threat, stress, and learned avoidance behaviours (Basbaum et al., 2009; Palmiter, 2018).

Additional projections reach the midbrain via the spinomesencephalic pathway, which conveys nociceptive signals from DH projection neurons, primarily in lamina I and V, to midbrain structures such as the periaqueductal gray (PAG) (Keay & Bandler, 2002). The PAG integrates nociceptive input with contextual and emotional information and serves as a major control node for descending pain modulation. Through its connections with the rostroventral medulla and other brainstem nuclei, the PAG can exert powerful inhibitory or facilitatory influences on spinal nociceptive transmission, enabling flexible regulation of pain according to behavioural state, expectation, and prior experience (Fields, 2006; Heinricher et al., 2009).

As observed at subcortical levels, nociceptive processing at the cortical level is distributed across multiple, functionally specialised regions (Apkarian et al., 2005). While primary and secondary somatosensory cortices encode the sensory-discriminative features of nociception, including stimulus location and intensity, other cortical areas contribute to complementary functions. The anterior cingulate cortex and insular cortex are critically involved in the affective and interoceptive dimensions of pain, whereas prefrontal cortical regions support cognitive appraisal, expectation, and decision-making related to nociceptive input (Bushnell et al., 2013; Rainville et al., 1997; Wiech, 2016). Through their extensive reciprocal connections with limbic and subcortical structures, these cortical areas integrate nociceptive signals with emotional state, context, and behavioural goals, ensuring that pain perception reflects both sensory input and internal state.

1.2.4 The Gate Control Theory of Pain

A foundational framework for understanding the modulation of nociceptive transmission at spinal level is the Gate Control Theory, originally proposed by Melzack and Wall (Melzack & Wall, 1965). This theory postulates that nociceptive signals are not uniformly

transmitted from the periphery to the brain but are dynamically regulated - or “gated” - within the DH of the spinal cord. According to this model, whether pain signals reach higher brain centres depends on the interplay between activity in nociceptive and low-threshold mechanoreceptive afferents which eventually determines if the signal will or will not be transmitted from the spinal cord to the brain.

Specifically, activation of large-diameter Aβ fibres engages inhibitory interneurons within the DH, which suppress the transmission of nociceptive input from C and Aδ fibres to projection neurons. Conversely, strong activation of small-diameter nociceptors reduces inhibitory tone and facilitates nociceptive signal transmission. This mechanism provides a physiological explanation for everyday observations, such as the reduction of pain or itch through rubbing of the affected area, and it highlights the integrative nature of spinal sensory processing (Figure1.7).

Although the original formulation of gate control theory predates modern molecular and circuit-level insights, its core principles remain highly relevant. Subsequent experimental work has identified specific inhibitory interneuron populations, neurotransmitters, and synaptic mechanisms that underlie gating at the DH horn, granting strong empirical support to the model (Braz et al., 2014; Todd, 2010). Moreover, descending modulatory pathways from supraspinal centres can bias the gate toward inhibition or facilitation, further extending the original framework to incorporate cognitive and emotional influences on pain perception (Fields, 2006; Heinricher et al., 2009). Interestingly, recent work from our group suggested that in addition to the classical spinal gate, an earlier gating mechanism exist at the level of the peripheral ganglia (Du et al., 2017; Hao et al., 2023; Lall et al., 2025; Li et al., 2024; explained below).

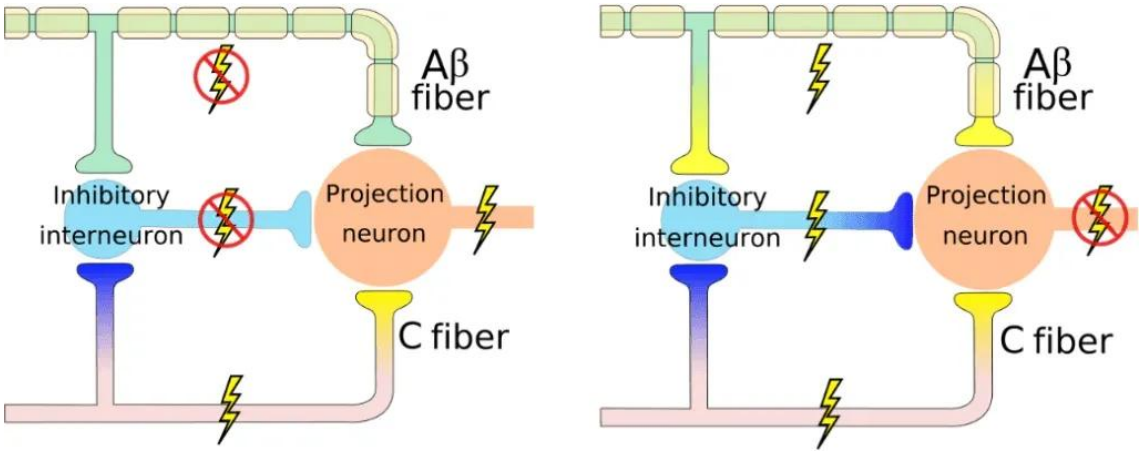


Figure 1.7 Schematic representation of The Gate Control Theory of Pain.
Schematic illustration of the modulation of nociceptive signaling by interactions between low-threshold Aβ-fibres, nociceptive C-fibres, inhibitory interneurons, and projection neurons in the spinal dorsal horn. Activation of Aβ-fibres enhances inhibitory interneuron activity, thereby suppressing excitation of projection neurons and reducing nociceptive signal transmission, whereas C-fibre input promotes projection neuron firing. This circuitry forms the basis of the gate control theory of pain

1.3 Chronic Pain

Chronic pain represents the leading cause of disability and disease globally. It's estimated to affect approximately 20% of adults worldwide, over 1.5 billion individuals, in various forms and severity (Goldberg & McGee, 2011; Lurie & Javaid, 2024). While acute pain is a crucial protective mechanism for the organism, when it persists beyond the resolution of the initial injury, it becomes chronic. Clinically, chronic pain is defined as pain which lasts longer than three months (Treede et al., 2019), even though it is better understood as a maladaptive disease state of the nervous system rather than a prolonged sensory response (IASP). The aetiology behind the disease can be various and the pathophysiology is sometimes not fully understood. Damage at the level of the nervous system can lead to neuropathic pain, whereas the inflammatory response to a tissue injury can be the cause behind the development of inflammatory pain, and these mechanisms are usually intertwined. Patients generally experience persistent pain in the absence of a nociceptive stimulus (allodynia) or exaggerated pain in response to a stimulus that already provokes pain (hyperalgesia).

1.3.1 Peripheral and central sensitization

While hyperalgesia occurring at the site of injury is known as primary hyperalgesia, increased pain sensitivity in the surrounding uninjured areas is identified as secondary hyperalgesia. Primary hyperalgesia is mainly due to peripheral sensitization whereas secondary hyperalgesia is caused by central sensitization. As mentioned in section 1.2.1, peripheral sensitization arises when tissue injury or disease triggers local biochemical and cellular changes that increase the excitability of primary sensory neurons.

Signals generated in the injured tissue trigger intracellular pathways within the nociceptors that modify the function and availability of transduction and voltage-gated ion channels. Inflammatory mediators released at the site of injury by both sensory nerve fibres and immune cells can either directly activate nociceptor terminals, as in the case of adenosine triphosphate (ATP), bradykinin, and protons, or exert modulatory effects

that enhance neuronal responsiveness. These processes lower activation thresholds, increase firing frequency, and may even induce spontaneous activity, thereby amplifying the afferent drive entering the CNS and providing a substrate for pain hypersensitivity. At molecular level, many inflammatory mediators act through G-protein-coupled receptors (GPCRs), initiating second-messenger signalling cascades that activate protein kinase A and protein kinase C. These kinases phosphorylate Na_v channels such as $Na_{v1.8}$ and $Na_{v1.9}$ as well as transducer proteins including TRPV1, resulting in prolonged channel opening and reduced activation thresholds (Laedermann et al., 2015). In parallel, intracellular signalling at peripheral terminals can be retrogradely conveyed to the cell bodies of DRG neurons, where it induces longer-lasting changes in gene expression. This transcriptional remodelling frequently involves upregulation of cytokines and chemokines receptors as well as ion channels and transducer proteins (e.g. NGF upregulates TRPV1) (Hensellek et al., 2007; Zhang et al., 2005). Interestingly, inflammation can induce a functional switch in primary sensory neurons, enabling normally non-nociceptive Aβ fibres to activate nociceptive circuits and drive pain signalling. For example, damage to the peripheral sensory fibres, up-regulates $Na_{v1.3}$ sodium channel, bradykinin B1 and TRPV1 receptors in myelinated neurons which greatly contribute to hyperalgesia (Ueda, 2006).

Central sensitization typically manifests in tactile allodynia and secondary hyperalgesia. The IASP defines central sensitization as the “increased responsiveness of nociceptive neurons in the CNS to their normal or subthreshold afferent input”. Functionally, this state is characterized by increased excitability of DH neurons, expansion of receptive fields and enhanced responses to both noxious and innocuous stimuli (Woolf, 2011). Similarly to peripheral sensitization, central sensitization is characterized by shorter lasting, activity-dependent mechanisms as well as long-lasting effects due to alteration in gene expression and membrane excitability. Repetitive C-fibres stimulation leads to increased glutamate and peptides release which results in increased postsynaptic depolarization. This process, known as homosynaptic plasticity, causes progressively increasing output

of the DH neurons leading to a temporary form of plasticity (Ikeda et al., 2003; Sandkühler, 2009; Woolf & Thompson, 1991). In contrast, heterosynaptic sensitization increases DH excitability for hours beyond the primary stimulus and it affects responses to multiple converging afferents (Keller et al., 2007; Woolf & King, 1990). As a result, previously subthreshold inputs from nociceptors as well as high-threshold Aβ fibres, can evoke suprathreshold responses (Torsney & MacDermott, 2006). Clinically, this leads to hyperalgesia, allodynia, and secondary hyperalgesia extending beyond the site of injury. Similarly to primary sensory neurons, DH neurons can undergo longer lasting changes including alteration in post-translational modifications and gene expression. An important hallmark of central sensitization involves the loss of the inhibitory tone normally sustained by GABAergic and glycinergic interneurons (Moore et al., 2002; Todd, 2010; Zeilhofer, 2008). As described by the Gate Control Theory, inhibitory DH interneurons limit the amount of nociceptive information transmitted to the brain and their impaired activity lifts this regulatory gate. The amplified pain information reaches the somatosensory cortex, contributing to allodynia and hyperalgesia. Aberrant activity of descending inhibitory pathways can lead to similar effects, affecting the physiology of central projecting neurons.

Beyond the spinal cord, persistent nociceptive drive can induce plastic changes within supraspinal pain-processing circuits. Functional and structural adaptations have been described in thalamic nuclei, brainstem centres, and cortical regions involved in sensory, affective, and cognitive dimensions of pain (Apkarian et al., 2009; Baliki & Apkarian, 2015). At the cortical level, prolonged nociceptive input is associated with altered activity and connectivity within the somatosensory cortex, anterior cingulate cortex, insula, and prefrontal cortex, reflecting changes in sensory representation, emotional salience, and cognitive appraisal of pain (Apkarian et al., 2011; Maihöfner et al., 2003). These supraspinal alterations contribute to the maintenance and amplification of pain perception, even in the absence of ongoing peripheral injury, supporting the view that

chronic pain arises from maladaptive plasticity across distributed CNS networks rather than from peripheral or spinal mechanisms.

1.3.2 Neuro-immune and Neuro-glia interactions in chronic pain

Chronic pain is not solely a neuronal disorder, but it rather reflects a complex, multi-cellular pathology whose development is significantly shaped by immune and glial cells. The last two decades have demonstrated that the cross-talk between neurons and surrounding glial and immune cells drives aberrant excitability and long-lasting sensitization (Grace et al., 2014; Ji et al., 2016; Scholz & Woolf, 2007).

As mentioned in the previous chapters, following tissue injury, nociceptors release neuropeptides (e.g. CGRP, substance P), ATP, chemokines (e.g. monocyte chemoattractant protein-1; MCP-1) and damage-associated molecular patterns (DAMPS) that activate resident and infiltrating immune and glial cells (Basbaum et al., 2009; Chiu et al., 2012). Concomitantly, immune cells rapidly accumulate at the site of injury and corresponding ganglia, where they produce pro-inflammatory mediators such as tumour necrosis factor-alpha (TNF-α), interleukin-6 (IL-6), interleukin 1-beta (IL1-β), NGF, histamines and prostaglandins. These compounds can act directly on nociceptors, lowering nociceptor's activation thresholds and promoting ion channels trafficking (e.g. NGF-TrkA signalling upregulates TRPV1 activity; prostaglandins increase Nav1.8 trafficking) (Liu et al., 2010; Zhang et al., 2005), or they can initiate intracellular cascades that modulate gene expression, thereby sustaining nociceptor plasticity and reinforcing peripheral sensitization (Zhang & An, 2007). Furthermore, these cytokines recruit additional immune cells from the circulation, amplifying the inflammatory response. Among immune cells, macrophages play a key role in the development and the maintenance of chronic pain. Following damage, resident macrophages rapidly expand in number and shift towards a pro-inflammatory phenotype while peripheral monocyte-derived macrophages infiltrate the DRGs from the blood. Neurons and macrophages communicate via a complex bidirectional cross-talk which involves multiple mediators: sensory neurons release colony stimulating factor-1 (CSF-1) and ATP that recruit and

activate macrophages, while macrophage-derived cytokines feed back onto neurons and satellite glia, amplifying both excitability and glial coupling (Figure1.8) (Chen et al., 2021; Peng et al., 2016). Macrophages are highly plastic cells that can shift from a pro-inflammatory (M1) to an anti-inflammatory, pro-resolving (M2) phenotype, in response to extracellular cues. This plasticity is a key determinant of whether inflammation results in transient, adaptive nociception or progresses toward persistent pathological pain. Importantly, M2-like macrophages play an essential role in terminating inflammatory responses, clearing cellular debris, releasing pro-resolving mediators, and promoting tissue repair (Chen et al., 2023; Xiaoye et al., 2024; Zhao et al., 2023). As such, successful resolution of releasing pro-resolving inflammation - and the subsequent return to normal nociceptive tone - depends critically on the presence and function of these pro-resolving macrophage populations.

At the level of the DRG, SGCs form a critical relay between immune signals and sensory neuron excitability. Inflammation and aberrant neuronal firing induce rapid and sustained activation of SGCs, marked by increased expression of glial fibrillary acidic protein (GFAP), proliferation, phenotypic switch and enhanced intracellular coupling (Avraham et al., 2020; Dublin & Hanani, 2007; Hanani & Spray, 2020; Ohara et al., 2009). The latter is caused by gap-junction coupling between adjacent SGCs, which dramatically increases electrical and chemical communication across neuronal units (Cherkas et al., 2004; Hanani et al., 2010). As a result, activity in one sensitized neuron can propagate indirectly to neighbouring neurons - even neurons that were not originally injured - thereby expanding pain sensitivity beyond the initial site of damage (Ji et al., 2013; Spray et al., 2019). One of the most critical drivers of SGCs activation is ATP-mediated signalling: depolarized nociceptors release ATP into the ganglion microenvironment, where it binds to purinergic receptors (P2X4, P2X7, P2Y12) on adjacent SGCs

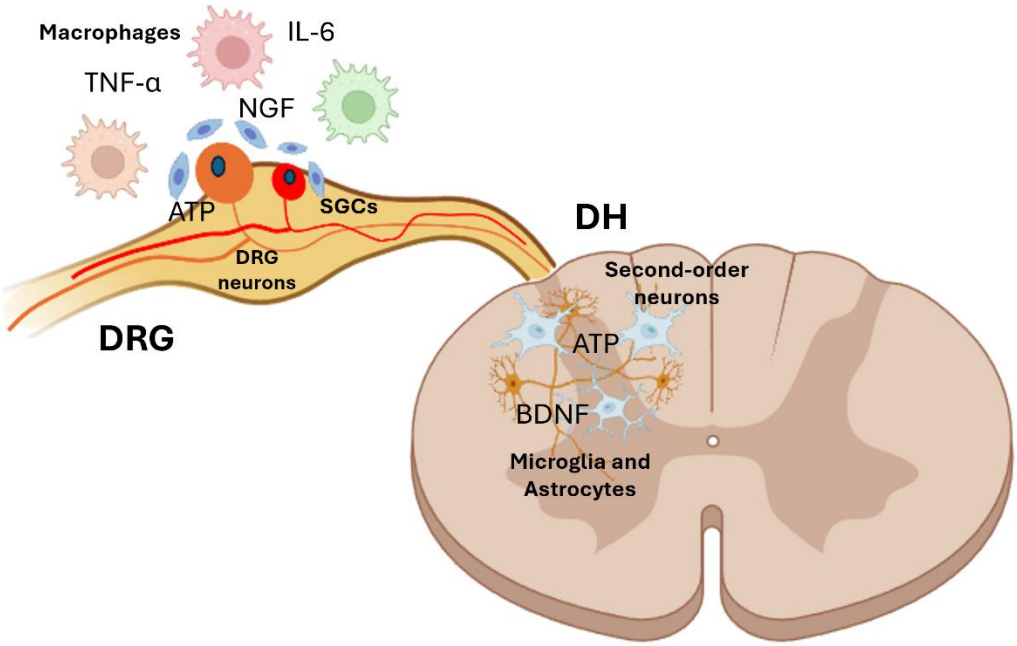


Figure 1.8 Neuro-immune and Neuro-glia interactions in chronic pain.
Schematic representation of inflammatory and glial modulation of nociceptive signalling at the DRG and DH. In the DRG, primary sensory neurons release signalling molecules that activate SGCs and macrophages which in turn secrete pro-inflammatory mediators, including TNF-α, IL-6, NGF, and ATP which enhance neuronal excitability and sustain peripheral sensitization. Action potentials propagate centrally to the DH, where second-order neurons release factors that activate microglia and astrocytes. Continued glial signalling, including the release of ATP and brain-derived neurotrophic factor BDNF, promotes synaptic plasticity, maintains central sensitization, and contributes to persistent pain

(Cherkas et al., 2004; Chessell et al., 2005; Hanani, 2012; Zhang et al., 2007). This triggers intracellular calcium (Ca^{2+}) signalling which downstream, it leads to cytokine production and further ATP release, creating a positive feedback loop that amplifies DRG excitability and support persistent pain states (Qiu et al., 2024). SGCs-released pro-inflammatory cytokines, which includes TNF-α, IL-1β and MCP-1, sensitise nearby neurons and amplify the immune response. In parallel, aberrant ion homeostasis in SGCs significantly promotes chronic pain. Specifically, downregulation of Kir4.1 channels impairs potassium (K^+) buffering which eventually leads to prolonged neuronal depolarization and ectopic firing by lowering firing thresholds (Vit et al., 2008).

Beyond the ganglion, similar mechanisms unfold within the spinal DH and supraspinal pain centres, where microglia and astrocytes both play a central role in shaping chronic pain. Following intense or repetitive nociceptive input, DH microglia become rapidly activated and transition into a pro-inflammatory phenotype, releasing cytokines, brain-derived neurotrophic factor (BDNF) and chemokines (Inoue & Tsuda, 2009; Milligan & Watkins, 2009; Tsuda et al., 2003). BDNF is known to enhance NMDA-dependent excitatory transmission in primary afferent terminals and to reduce GABAergic inhibition in the DH (Chen et al., 2014; Coull et al., 2005). Specifically, BDNF downregulates KCC2 in DH neurons, thereby disrupting chloride (Cl^-) homeostasis and reducing GABAergic inhibition - a mechanism that drives central sensitization (Chen et al., 2014; Coull et al., 2005). Astrocytes, in contrast, support slower but longer-lasting changes: gliosis, increased GFAP expression, and glutamate transporter down-regulation (EAAT2/GLT-1) which impairs neurotransmitter clearance, elevating extracellular glutamate and prolonging DH neuron depolarisation (Falnikar et al., 2016; Lu & Gao, 2023; Wang et al., 2008). Similarly to the periphery, glial cells in the spinal cord also communicate via ATP-mediated purinergic signalling. Activated microglia release ATP that, by acting on astrocytic P2Y receptors, triggers Ca^{2+} signalling reinforcing central sensitization (Ikeda et al., 2012; Trang et al., 2012).

At supraspinal sites - including the PAG, amygdala, and medial prefrontal cortex - chronic pain is increasingly recognized as a system-level disorder involving glial dysregulation, neuroinflammation, and maladaptive synaptic plasticity. Microglial and astrocytic activation within these regions has been shown to modify the activity of pain-modulatory circuits, leading to reduced descending inhibitory control and enhanced facilitation of nociceptive transmission (Chen et al., 2025; Ikeda et al., 2012; Yang et al., 2022; Zan et al., 2025).

Collectively, these neuro-immune and neuro-glial interactions demonstrate that chronic pain is sustained by communication between neurons, immune cells, and glia across multiple anatomical levels. However, a further layer of immuno-glial signalling exists within the peripheral nerve itself, where Schwann cells and macrophages form a dynamic axis that not only orchestrates axonal degeneration and repair, but also contributes to nociceptor sensitization. Given the central relevance of Schwann-macrophage interactions to the work presented in this thesis, the following section will focus on this peripheral cellular interface.

1.3.3 Schwann cells in neuropathic pain and nociceptive signalling

Schwann cells, traditionally viewed as passive myelinating cells, are now recognized as active modulators of nociception and drivers of neuropathic pain. Beyond enabling saltatory conduction, Schwann cells directly influence sensory neuron excitability through dynamic molecular communication and inflammatory signalling. Following peripheral nerve injury, Schwann cells undergo rapid phenotypic switch characterised by downregulation of myelin-associated genes and upregulation of a transcriptional repair programme (Arthur-Farraj et al., 2012).

The same processes that enable regeneration are intimately connected to the modulation of sensory signalling and the development of neuropathic pain. Schwann cells not only provide metabolic and structural support but also actively communicate with sensory neurons, macrophages, and other glial cells to shape the inflammatory and

nociceptive milieu after injury. Multiple receptors, channels and active molecules that contribute to neuropathic pain have been identified to be dysregulated in Schwann cells following nerve damage. Activated Schwann cells release cytokines and chemokines, including IL-6, TNF-α, and MCP-1, which support the immune response and sensitize nociceptors, contributing to the persistence of pain (Grothe et al., 2000; Tofaris et al., 2002; Wagner & Myers, 1996; Zhang et al., 2020). In addition, Schwann cell-derived ATP, neuropeptides, and other signalling molecules act in autocrine and paracrine fashion on neighbouring primary sensory neurons fibres, influencing their excitability (Fields & Stevens, 2000; Waltz et al., 2023). At the same time, molecules released by the damaged axons can affect Schwann cell behaviour. For instance, CGRP release from mouse cutaneous trigeminal fibres targets surrounding Schwann cells to evoke periorbital mechanical allodynia. CGRP signalling in Schwann cells triggers ROS production and release which in turn acts on the surrounding fibres in a pro-nociceptive feed-forward mechanism (De Logu et al., 2022).

Recent work further reframes a subpopulation of Schwann cells as sensory receptor cells that participate directly in touch, pressure and nociceptive transduction. In the skin this type of Schwann cells alongside the sensory fibres and other cellular subtypes, make up the sensory end-organ. In the Pacinian corpuscles, Meissner corpuscles, Ruffini corpuscle, and lanceolate endings of hair follicles terminal Schwann cells play an important structural function by anchoring LTMR nerves to the surrounding dermis and epidermis (Zelená, 1976, 1981; Zimmerman et al., 2014). In contrast to common knowledge, it was recently reported that in mouse, nociceptive nerve terminals are associated with specialized Schwann cells, which together form a mesh-like network in the subepidermal border with radial processes entering into the epidermis abutting to unmyelinated nociceptive nerves (Abdo et al., 2019). The nociceptive Schwann cells are inherently mechanosensitive, act as sensory receptor cells, and contribute to the initiation of pain. The ablation of nociceptive Schwann cells in mice results in nerve retraction and mechanical and thermal hyperalgesia (Rinwa et al., 2021). Nociceptive

nerves and nociceptive Schwann cells form a nociceptive glio-neural complex with two sensor-receptor cell types, the glia and the nerve, both influencing the sensation of pain (Abdo et al., 2019).

1.3.4 Bidirectional cross-talk between Schwann cells and macrophages

The timing and type of interaction between glial and immune cells are fundamental in orchestrating different steps following peripheral nerve injury. Immediately after damage, Schwann cells release pro-inflammatory chemokines and cytokines such as MCP-1, TNFα and IL-6, which activate local macrophages and recruit circulating monocytes to the injured site (Jessen & Mirsky, 2016; Stoll & Müller, 1999). Activated macrophages play a key role helping Schwann cells in the degradation and clearance of myelin debris, which is an important step following damage. A recent study showed that Schwann cell-released Interleukin-17B (IL-17B) is a key regulator of macrophage recruitment in early stages after injury. The lack of IL17-B reduces macrophage infiltration, myelin clearance, and axon regeneration. Interestingly, IL-17B pathway is defective in injured central nerves, possibly contributing to a more challenging regeneration (Huang et al., 2024).

In addition to canonical immune signalling, Schwann cells engage in cytokine-dependent pathways that propagate inflammation beyond the damaged nerve. A key example is the ciliary neurotrophic factor (CNTF)-STAT3-IL-6 axis. Following peripheral nerve injury, Schwann cells upregulate CNTF, which activates STAT3-dependent signalling in adjacent sensory axons. This injury-induced signalling is conveyed retrogradely to the neuronal soma, where it initiates transcriptional programmes that include the induction of IL-6 (Hu et al., 2020). Schwann cell-derived signals therefore promote IL-6 production within sensory neurons, which can act locally to enhance nociceptor excitability and, through subsequent neuron-glia interactions, contribute to microglial activation in the DH and the maintenance of central sensitisation. In this way, Schwann cells not only shape the immune microenvironment at the site of injury but also trigger a relay-based

neuroinflammatory cascade linking peripheral nerve damage to central pain-processing circuits.

If inflammation is not resolved over time, it can impair regeneration and contribute to the development of chronic pain. One mechanism sustaining pathological neuro-immune interactions involves TRPA1 activation on Schwann cells in response to oxidative stress. TRPA1 activation induces Ca^{2+} influx, which promotes nicotinamide adenine dinucleotide phosphate oxidase 1 (NOX1)-dependent production of reactive oxygen species, including hydrogen peroxide (H_2O_2). As a membrane-permeable signalling molecule, H_2O_2 acts in a paracrine manner to sustain macrophage recruitment and activation and to engage TRPA1 channels on adjacent nociceptor fibres, thereby enhancing neuronal excitability and promoting mechanical allodynia (De Logu et al., 2017). Schwann cell TRPA1 signalling is also implicated in prostaglandin E_2 (PGE_2)-induced pain. By activating the E-type prostanoid 2 (EP_2) receptor in Schwann cells, PGE_2 triggers protein kinase A (PKA)-mediated phosphorylation of TRPA1 which downstream, leads to ROS and H_2O_2 release. These molecules activate TRPA1 on adjacent nerve fibres eliciting a feed forward loop mechanism which promotes pain (Nassini et al., 2025). Interestingly, it has been recently shown that Schwann cells gain antigen-presenting function in a mouse model of chronic constriction injury (CCI). Major histocompatibility complex class II (MHC-II) expression by Schwann cells promotes post-traumatic inflammation in the peripheral nerve and subsequently exacerbates axonal loss and neuropathic pain in mice (Hartlehnert et al., 2017).

Many factors released by macrophages are fundamental for Schwann cell physiology after injury. Macrophages are known to shape Schwann cell and fibroblast migration trajectory during the formation of the nerve bridge at the site of injury through sLIT3-Robo1 signalling pathway (Blockus & Chédotal, 2016). The correct formation of the nerve bridge is crucial to guide axon regeneration. Macrophages can also indirectly regulate the function of Schwann cells through the vascular endothelial growth factor (VEGF) signalling pathway (Cattin et al., 2015). VEGF is an important neurotrophic factor that

promotes angiogenesis of nerve bridges. VEGF-A mediated signalling relieves hypoxia and favours Schwann to cross nerve bridges through the newly formed vessel tracks, ultimately guiding axonal regeneration (Figure1.9) (Cattin et al., 2015). In the later stages of regeneration, it is important that Schwann cells switch back to a myelination phenotype. Macrophages are key modulators of this process: through the release of factors such as Gas6, they promote the activation of a transcription profile in Schwann cells which powers up remyelination (Stratton et al., 2018). Together, these studies illustrate the dynamic and bidirectional interactions between Schwann cells and macrophages following peripheral nerve injury, highlighting their combined roles in regulating inflammation, regeneration, and the emergence of neuropathic pain.

In the next sections, several of the themes discussed so far are brought together to introduce the GABAergic system as a common signalling framework linking different cell types involved in nociception.

1.4 GABA as an inhibitory neurotransmitter

GABA was first identified as a by-product of plant metabolism in 1883. More recent studies showed that in plant, GABA production takes place in response to stress as drought, heat, cold and infection (Shelp et al., 2021). In 1950 GABA was detected for the first time in the human brain (Awapara et al.,1950). However, it was not until 1957 that GABA was identified as an inhibitory neurotransmitter by Florey and Bazemore (Bazemore, 1957; Florey & McLennan, 1959). A decade later Krnjevic and Schwarzt provided evidence that GABA functions as a major inhibitory neurotransmitter in the mammalian brain (Krnjević & Schwartz, 1967). Using intracellular recordings of neocortical neurons, they demonstrated that exogenous GABA application induced a pronounced membrane hyperpolarization accompanied by decreased input resistance and suppression of action potential firing. Importantly, these mechanisms were shown to be ion-dependent and reversible, suggesting a receptor-mediated mechanism rather

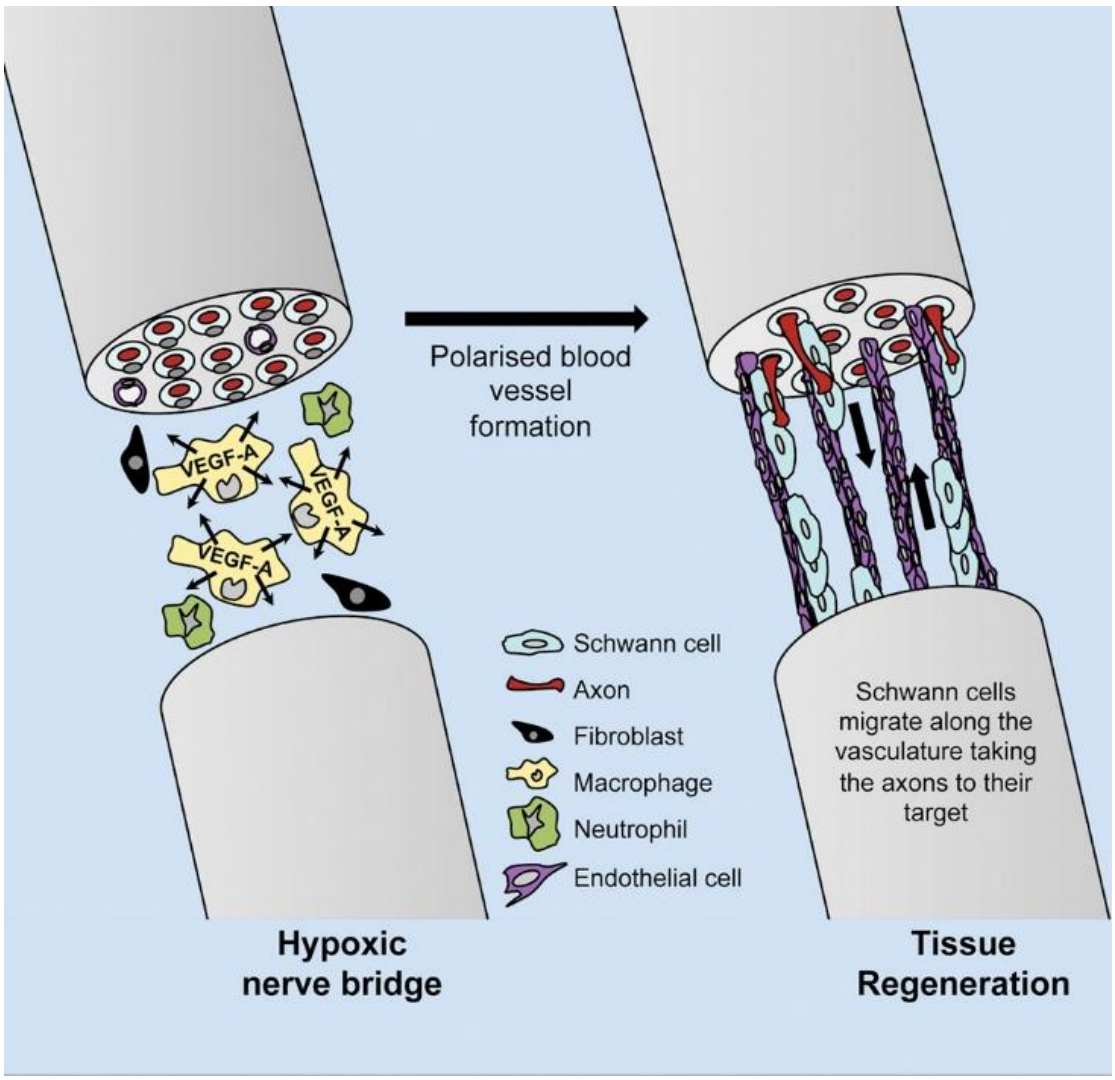


Figure 1.9 Macrophages-released VEGF-A polarizes blood vessel formation and guides Schwann cells migration.
Schematic representation showing that macrophages-released VEGF-A promotes polarized blood vessel formation. The newly formed vasculature provides guidance cues that direct Schwann cell migration, contributing to tissue remodelling and nerve regeneration (adapted from Cattin et al., 2015).

than non-specific chemical suppression of neuronal excitability (Krnjević & Schwartz, 1967).

1.4.1 GABA synthesis, release and metabolism

In neurons, GABA is synthetized primarily from the decarboxylation of glutamate via the enzymes glutamic acid decarboxylase (GAD). Two isoforms of GAD have been identified and named according to their different molecular weight: GAD65 and GAD67 (Soghomonian & Martin, 1998). GAD65 and GAD67 are produced by two different genes *Gad2* and *Gad1* respectively, and despite sharing sequence similarity, their biological regulation differs substantially. GAD67 is distributed throughout the neuronal cytoplasm, and it is responsible for constitutive GABA synthesis necessary for the overall inhibitory tone. In contrast, GAD65 mainly localizes in the nerve terminal, it is reversibly bound to the membrane of synaptic vesicles where rapid, activity-dependent GABA synthesis is needed for neurotransmission (Esclapez et al., 1997; Kaufman et al., 1991; Reetz et al., 1991; N. Tian et al., 1999). GAD65-/- mice displayed a decreased GABA release during sustained stimulation suggesting that the number of vesicles available for release is deficient in the absence of GAD65 (Tian et al., 1999).

In addition to the classical GAD-dependent pathway, an alternative biosynthetic route which involves the polyamine putrescine has been described. Putrescine, which is generated from ornithine via ornithine decarboxylase (ODC), undergoes oxidative deamination mediated by diamine oxidase (DAO) or monoamine oxidase (MAO), producing γ-aminobutyraldehyde, which is then converted into GABA by aldehyde dehydrogenase (ALDH) (Kim et al., 2015; Seiler & Sarhan, 1983). This pathway appears to be particularly relevant in non-neuronal cells, including astrocytes and other glial cells. In astrocytes, putrescine-derived GABA has been shown to contribute to tonic inhibition, acting through extrasynaptic $GABA_A$ receptors to modulate network activity (Yoon et al., 2014; Jo et al., 2014). Mutations to *ALDH1A1* have been linked to increased risk of alcoholism in humans (Liu et al., 2011; Sherva et al., 2009). Interestingly, ALDH1A1-mediates a non-canonical GABA synthesis pathway in mice midbrain dopaminergic

neurons which lack GAD enzymes, allowing these neurons to co-release GABA and inhibit dopaminergic signalling (Kim et al., 2015). Ethanol exposure attenuated GABA co-release at concentrations similar to those after binge drinking, potentially enhancing dopaminergic activity and contributing to addictive behaviours.

Following synthesis, GABA is packaged into synaptic vesicles by the vesicular GABA transporter (VGAT) and released through Ca^{2+}-dependent exocytosis in in the synaptic cleft (Chaudhry et al., 1998; Otsuka et al., 1966; Wu et al., 2007). GABA release can be phasic, generating rapid inhibitory post-synaptic currents (IPSCs), or tonic, producing persistent low-level inhibition that controls network excitability. Interestingly, GABA can also be released non-vesicularly via the reversal of GABA transporter-1 (GAT1) during strong depolarization or altered homeostasis (Wu et al., 2007).

Following release, GABA can engage $GABA_A$ and $GABA_B$ receptors, and it is subsequently cleared primarily through reuptake by GABA transporters (e.g. GAT1 in neurons, GAT3 in astrocytes), enabling termination of signalling and reuse in synaptic vesicles (Figure 1.10). A major recycling route involves the glutamate-GABA-glutamine pathway: following its uptake into astrocytes, GABA is converted into glutamate and then into glutamine, which is successively shuttled back to neurons and converted back to GABA.

1.4.2 GABA receptors

The effect of GABA is primarily mediated by two major types of receptors: the ionotropic $GABA_A$ receptors and the metabotropic $GABA_B$ receptors. These two receptor families differ in molecular structure, signalling mechanisms and temporal dynamics, allowing GABA to exert both fast and sustained inhibitory control over neuronal excitability. Although they are mostly known for their role in the inhibition of neurons, GABA receptors have also been identified in other cell types including glial cells (Schwann cells, Astrocytes) (Bernareggi et al., 2011; Fraser et al., 1994; Liu et al., 2022; Magnaghi et al., 2004; Procacci et al., 2013), immune cells (T cells, dendritic cells and macrophages)

(Barragan et al., 2015; Bhandage & Barragan, 2021; J. Tian et al., 1999) and pancreatic β-cells (Korol et al., 2018).

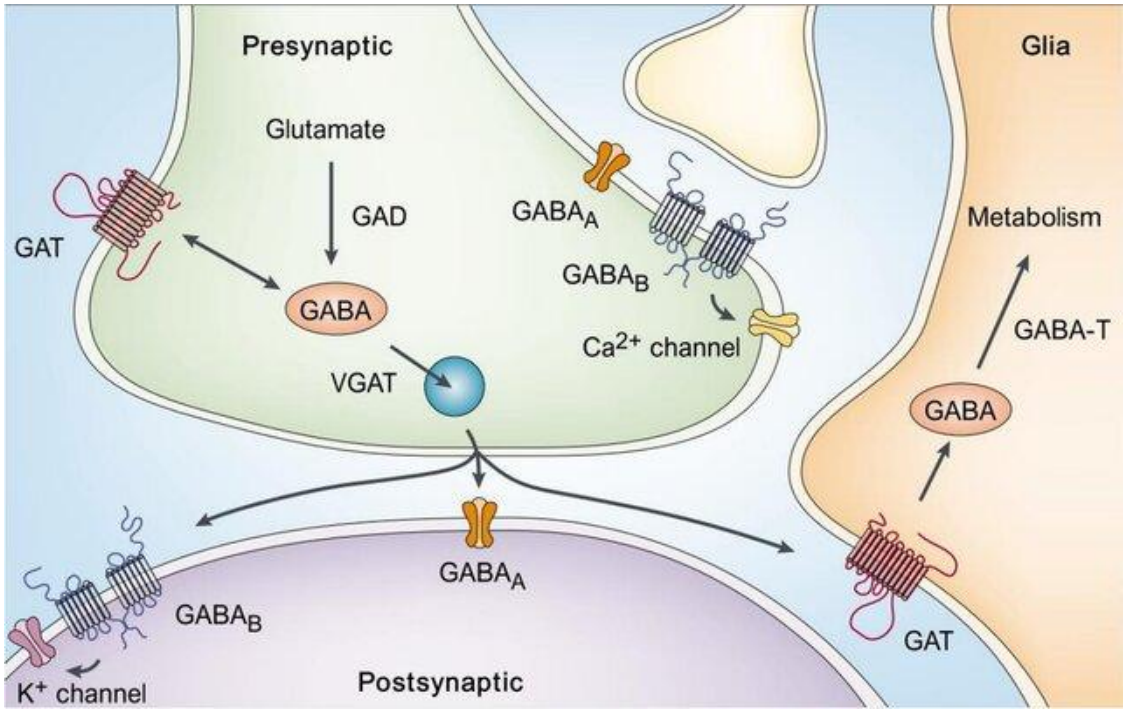


Figure 1.10 Overview of glutamate and GABA synthesis, packaging, release, transport, and metabolism in astrocytes, glutamatergic neurons, and GABAergic neurons.
In GABAergic neurons, glutamine is converted to glutamate by glutaminase and subsequently to GABA by GAD. GABA is then packaged into synaptic vesicles by VGAT and released into the synaptic cleft. Following release, GABA can act on $GABA_A$ and $GABA_B$ receptors expressed on the postsynaptic membrane. Successively, it is taken up by neurons and glial cells GATs, and it is then recycled or metabolized through enzymatic pathways (adapted from Knippenberg et al., 2023).GABA via GAD enzymes. Alternatively, GABA can be metabolized by GABA transaminase (GABA-T) to succinate and fed into the Krebs cycle.

1.4.2.1 $GABA_A$ receptors

$GABA_A$ receptors are ionotropic, ligand-gated Cl^- channels that belong to the Cys-loop superfamily. They are hetero-pentameric receptors assembled from a repertoire of 19 subunits (α_{1-6}, β_{1-3}, γ_{1-3}, δ, ε, θ, π). Each of the five subunits comprises a large extracellular N-terminal domain, four transmembrane α-helices (M1-M4), and an intracellular loop between M3 and M4. The extracellular domains of the five subunits assemble to form the ligand-binding region, while the M2 transmembrane segments from each subunit line the central pore and together constitute the Cl^--selective channel (Olsen & Sieghart, 2009). GABA binds at the interface between the α and β subunits, triggering conformational changes that open the channel (Figure 1.11) (Sigel & Steinmann, 2012).

The specific combination of subunits is tightly regulated, and it determines the receptor's biophysical properties as well as localization and pharmacology. In the CNS, the most common synaptic configuration comprises two α subunits, two β subunits, and one γ whereas δ-containing receptors are preferentially located extrasynaptically and mediate tonic inhibition (Sieghart & Sperk, 2002). Multiple subunits are expressed within DRG neurons, including α_{1-3}, β_{2-3} and γ_{1-2} (Du et al., 2017; Ma et al., 1993; Maddox et al., 2004; Obradovic et al., 2015). While synaptic $GABA_A$ receptors mediate phasic inhibition, extrasynaptic receptors display high affinity for ambient GABA and they provide a persistent control on neuronal excitability (Brickley & Mody, 2012).

Functionally, $GABA_A$ receptor activation increases Cl^- conductance, leading to membrane hyperpolarization or shunting inhibition. The inhibitory effect depends on the intracellular Cl^- concentration, which is tightly regulated by cation-Cl^- co-transporters as the Na^+-K^+-Cl^- co-transporter 1 (NKCC1) and the K^+-Cl^- co-transporter 2 (KCC2) which transport Cl^- in and out the neurons, respectively (Pressey et al., 2023; S. Zhang et al., 2021). In the majority of mature CNS neurons, intracellular Cl^- concentration is kept low (~5-10 mM) so that upon GABA binding, Cl^- flows inside the cells inducing hyperpolarization and inhibition of neuronal activity. Immature neurons have high

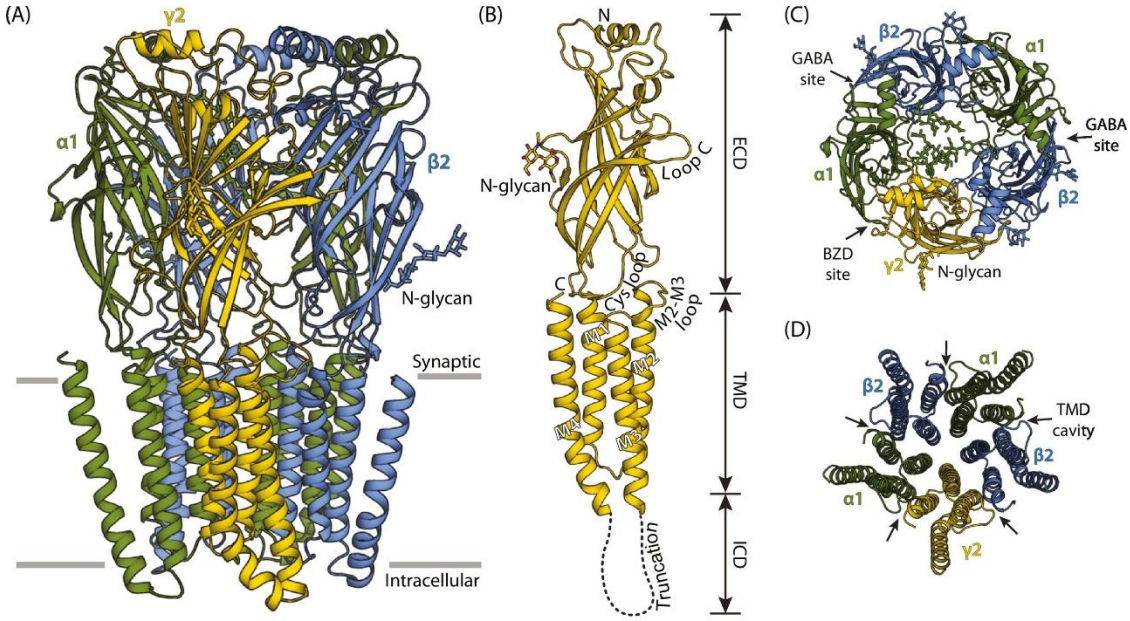


Figure 1.11 Structural overview of the $\alpha_1\beta_2\gamma_2$ $GABA_A$ receptor.
Side view of the $\alpha_1\beta_2\gamma_2$ subunit assembly in complex with GABA (A). Representative subunit architecture: a γ_2 subunit is shown (B). Views from the synaptic side of the extracellular domain (ECD) and transmembrane domain (TMD), respectively, highlighting binding sites for various small molecules (C-D) (adapted from Kim et al., 2021**).**

NKCC1 activity which causes intracellular Cl^- concentration to be high, leading to a depolarizing effect of GABA (Ben-Ari et al., 2007). Similarly, in DRG neurons from adult animals Cl^- concentration is ~30-40mM and although $GABA_A$ activation is depolarizing, when applied to the cell body, it leads to a net inhibitory effect on the throughput conductance due to a combination of shunting inhibition, Na_v channel inactivation and further impedance drop (Du et al., 2017; Kaneko et al., 2008).

$GABA_A$ receptors are also a major pharmacological target for multiple disorders as anxiety and epilepsy, and many compounds interact with sites within the receptor different from the GABA binding domain (Möhler, 2006). Benzodiazepine, barbiturates, neurosteroids and many anaesthetics are examples of exogenous positive allosteric modulators which enhance channel opening probability or prolong opening times (Olsen, 2018; Sieghart, 2006). Importantly, subunit composition determines drug sensitivity: for example, the γ_2 subunit is necessary for the high affinity benzodiazepine binding domain whereas the δ subunit confers sensitivity to neurosteroids (Benke et al., 1991; Luddens & Korpi, 1995; Smith et al., 2012). Orthosteric modulators interact directly with the receptor recognition site at the α-β subunit interface, and they are commonly used experimentally to probe receptor function. Orthosteric compounds include muscimol and bicuculline which function as $GABA_A$ receptor agonist and antagonist, respectively.

1.4.2.2 $GABA_B$ receptors

$GABA_B$ receptors were first described pharmacologically in 1981 as bicuculline-insensitive metabotropic receptors activated by the GABA analogue baclofen (Hill & Bowery, 1981). These are heterodimeric metabotropic GPCRs made up by a $GABA_{B1}$ and $GABA_{B2}$ subunits (Jones et al., 1998; Kaupmann et al., 1998). The $GABA_{B1}$ subunit contains the extracellular ligand-binding domain, while $GABA_{B2}$ is needed for G protein coupling and receptor dimer trafficking to the plasma membrane (Calver et al., 2000; Shen et al., 2021). Upon GABA binding, $GABA_B$ receptors activate $G_{i/o}$ proteins, predominantly engaging $G_{\alpha i}$ subunits that inhibit adenyl cyclase, leading to reduced intracellular cyclin AMP (cAMP) levels. In parallel, dissociation of the $G_{\beta\gamma}$ subunits directly

mediates inhibition of voltage-gated Ca^{2+} (Ca_V) channels and activation of G-protein-gated inwardly rectifying K^+ (GIRK) channels which cause membrane hyperpolarization and suppression of neuronal firing (Bettler et al., 2004; Bettler & Tiao, 2006; Bowery, 2002; Lüscher et al., 1997). Through these mechanisms, $GABA_B$ receptors provide a sustained, longer-lasting form of inhibition. Pharmacologically, the $GABA_B$ receptor agonist baclofen has been used both experimentally and clinically, particularly for the treatment of spasticity and certain pain conditions (Ertzgaard et al., 2017; Fromm et al., 1984; Hudgson & Weightman, 1971; Lind et al., 2008; Navarrete-Opazo et al., 2016; Penn et al., 1989).

1.4.3 Functions of GABAergic signalling in the brain

As mentioned above, GABA is the principal inhibitory neurotransmitter in the mammalian brain, and it plays a key role in shaping neuronal development and network function. By counterbalancing excitatory glutamatergic signalling, GABA maintains excitation-inhibition balance preventing hyperexcitability and stabilizing network activity (Farrant & Nusser, 2005; Isaacson & Scanziani, 2011; Krnjević & Schwartz, 1967b). This inhibitory control is highly dynamic, adapting to ongoing network activity and contributing to synaptic integration and plasticity.

Beyond its classical inhibitory role, GABA is a key player of brain development. During early development, immature neurons display high intracellular Cl^- concentration causing GABA to be excitatory. During these phases, GABA promotes neurogenesis, neuronal migration, synaptogenesis, axonal elongation as well as activity-dependent circuit refinement and critical periods development (Jelitai & Madarasz, 2005; Wu & Sun, 2015). Depletion of GABAergic excitatory signalling in the developing brain of rodents leads to aberrant proliferation of cortical neurons as well as reduced neurite length and dendritic processes (Antonopoulos et al., 1997; Brickley & Mody, 2012; Cancedda et al., 2007). GABAergic signalling is therefore a critical instructive signal which influences both spatial and functional organization of neuronal networks. The switch from excitatory to inhibitory mode is regulated by changes in the expression profile of Cl^-/cation transporters, as

KCC2, which cause Cl^- intracellular concentration to decrease. GABAergic inhibitory signalling also plays a pivotal role in the timing and regulation of critical periods, which are defined developmental windows during which neural circuits exhibit heightened plasticity. In a rodent model, pharmacological enhancement of GABAergic inhibition led to a precocious opening of the visual cortical critical period, whereas genetic deletion of the GABA-synthesizing enzyme GAD65 delayed its onset, demonstrating that appropriate levels of inhibition are required to initiate plastics refinement (Iwai et al., 2003).

In the mature brain, GABAergic signalling operates through both synaptic (phasic) and extrasynaptic (tonic) mechanisms mediated by $GABA_A$ and $GABA_B$ receptors. Phasic inhibition is mediated by synaptic GABA release and generates fast IPSCs, whereas tonic GABAergic signalling provides persistent inhibitory conductance - referred to as GABA tone - that modulates neuronal gain and network excitability (Brickley & Mody, 2012; Farrant & Nusser, 2005). This GABAergic tone is defined by the level of ambient extracellular GABA which induces sustained activation of extrasynaptic high affinity GABA receptors. Concentration of extracellular GABA depends on multiple factors including spillover from synaptic sites as well as the activity of neuronal and astrocytic GABA transporters, particularly GAT1 and GAT3 (Minelli et al., 1996; Schousboe et al., 2014).

GABAergic tone is a critical regulator of cognitive function, modulating attention, working memory, learning, and perceptual discrimination, and shaping critical periods of cortical plasticity by stabilizing synaptic circuits (Hensch, 2005; Muthukumaraswamy et al., 2009; Takesian & Hensch, 2013). In humans, magnetic resonance spectroscopy studies demonstrate that regional GABA levels correlate with motor learning, visual discrimination, and executive function, and that experimentally reducing GABA in the motor cortex using anodal transcranial direct current stimulation, enhances cortical excitability and short-term learning (Puts & Edden, 2012; Stagg, 2014).

Disruption of GABAergic signalling is a central feature of neurodevelopmental disorders, including autism spectrum disorder, schizophrenia, epilepsy, and intellectual disability, driven by altered GABA receptor expression, impaired interneuron development, and dysregulated chloride homeostasis (Lewis & Hashimoto, 2007; Nelson & Valakh, 2015; Rubenstein & Merzenich, 2003; Treiman, 2001). Post-mortem studies in schizophrenia reveal reduced cortical GABAergic interneurons and decreased expression of the interneuron-derived protein reelin (Eastwood & Harrison, 2006; Yang et al., 2014). Beyond development, injury and neuroinflammation induce maladaptive changes in GABA receptor function that disrupt excitatory-inhibitory balance and contribute to chronic neurological dysfunction (Clarkson et al., 2010; Redecker et al., 2000, 2002).

1.4.4 GABAergic inhibition in central pain processing

The function of the neurotransmitter GABA has been widely characterized in the spinal cord where it plays a crucial role in modulating the transmission of painful stimuli to higher brain centres. In the superficial laminae of the spinal cord - particularly in lamina II (substantia gelatinosa) - GABAergic interneurons form dense local networks that exert both presynaptic and postsynaptic inhibition, thereby regulating gating the painful input arriving from peripheral afferents (Todd, 2010; Zeilhofer et al., 2012). Through these mechanisms, GABA contributes to the fine tuning of pain perception and prevents excessive excitation to reach the brain.

Spinal GABAergic signalling is an important player of the previously described Gate Control Theory postulated by Melzack and Wall, which explains how nociceptive transmission integrates nociceptive and non-nociceptive afferent inputs and descending inhibition (Duan et al., 2014; Melzack & Wall, 1965; Zeilhofer et al., 2012).

Upon peripheral activation, Aβ fibres synapse onto GABAergic interneurons located in the lamina II of the spinal cord. The activated interneurons release GABA which exert its effect on: i) $GABA_A$ receptors located in primary afferent terminals of nociceptors which decreases the afferent input onto projection neurons (presynaptic inhibition) (Price et al.,

2009; Witschi et al., 2011) ii) $GABA_A$ and $GABA_B$ receptors in DH neurons by directly reducing their excitability through membrane hyperpolarization (postsynaptic inhibition) (Bardoni et al., 2013; Knabl et al., 2008; Zeilhofer et al., 2012).

As discussed above, postsynaptic inhibition is also regulated by descending pathways. Within this circuitries, GABAergic inhibition constitutes a major mechanism by which descending pathways suppress spinal nociceptive transmission. GABAergic neurons in the RVM project directly to the DH, where they synapse on the second order neurons to inhibit nociceptive signals transmission (Aicher et al., 2012; Kato et al., 2006) Kato and colleagues provided functional evidence for this mechanism using *in vivo* patch-clamp recordings from lamina II neurons during RVM stimulation. Their study demonstrated that RVM stimulation evoked inhibitory postsynaptic currents (IPSCs) and over 50% were mediated by GABAergic and glycinergic transmission. Importantly, noxious-evoked activity in the DH neurons were effectively suppressed by enhanced inhibitory input from the RVM (Kato et al., 2006). Complementing these physiological findings, anatomical and neurochemical tracing studies by Aicher and colleagues revealed that more than two-thirds of descending projections from the RVM to the lumbar spinal cord are GABAergic, as indicated by the expression of GAD67 (Aicher et al., 2012).

Importantly, loss of spinal GABAergic signalling is now recognized as a key mechanism underlying central sensitization and chronic pain maintenance. In multiple models of inflammatory and neuropathic pain, spinal GABAergic tone is reduced due to decreased GABA synthesis and release, dysfunction of inhibitory interneurons and impaired receptor signalling (Moore et al., 2002; Scholz et al., 2005; Zeilhofer et al., 2012). Behavioural and morphological studies showed that intrathecal application of either bicuculline or picrotoxin induced nocifensive behaviour and c-fos expression in DH neurons from the superficial laminae of the mouse DH (Cronin et al., 2004; Roberts et al., 1986). Electrophysiological recordings from primary afferent-evoked EPSCs from DH neurons in the lamina II of the spinal cord, demonstrated that bicuculline application led to significantly increased responses. Interestingly, the effect of bicuculline was less

pronounced in spinal cord slices from mice with spared nerve injury (SNI), possibly indicating that GABAergic signalling was already impaired in these mice (Baba et al., 1999). Microglia-released compounds, such as BDNF, impair GABAergic tone shifting the Cl^- equilibrium potential of DH neurons (Coull et al., 2003). By interacting with TrkB receptors on DH neurons, BDNF induces down-regulation of KCC2, ultimately increasing Cl^- intracellular concentration (Coull et al., 2005). Consequently, GABAergic input is rendered less inhibitory and may even acquire a net excitatory effect (Coull et al., 2003; Price et al., 2009). This disruption in Cl^- homeostasis promotes spontaneous neuronal activity and drives a functional shift in DH processing, whereby nociceptive-specific neurons lose high-threshold selectivity and acquire wide dynamic range-like properties, responding to both innocuous and noxious afferent input (Lavertu et al., 2014).

Consistent with a causal role for spinal disinhibition in neuropathic pain, transplantation of GABAergic neural progenitor cells into the adult DH restores inhibitory tone and significantly attenuates mechanical allodynia and thermal hyperalgesia in animal models of nerve injury, with graft-derived GABAergic neurons functionally integrating into host circuits and reducing exaggerated DH responses to stimulation (Etlin et al., 2016). Interestingly, in neuropathic mice as well as in animals injected with bicuculline, neuronal excitation resulting from electrical stimulation of Aβ fibres, spread from the deep DH to the superficial layers which normally only respond to noxious stimuli (Baba et al., 2003; Schoffnegger et al., 2008).

1.4.5 GABAergic signalling within primary sensory neurons

Although GABAergic inhibition is classically associated with central circuits, increasing evidence show that GABA plays a key modulatory role in the peripheral nociceptive processing. Component of the GABAergic system - including synthetizing enzymes, transporters and receptors - are expressed in primary sensory neurons, SGCs, Schwann cells and immune cells, indicating that GABAergic signalling is a key component of peripheral cells cross-talk (Du et al., 2017; Hao et al., 2023; Lall et al., 2025; Li et al., 2024; Magnaghi et al., 2004; Procacci et al., 2013) .

The presence of functional GABAergic mechanisms within the DRGs was first reported in studies from the 1970s-1980s which showed that GABA application to cat and rat DRG neurons induced depolarization (Bowery & Brown, 1974; De Groat, 1972; Feltz & Rasminsky, 1974; Hösli et al., 1978). Later work demonstrated that GABA triggered similar effects in human DRG neurons (Valeyev et al., 1996, 1999). Feltz and Rasminsky observed that although GABA depolarized DRG neurons, it was associated with an increase in Cl^- conductance that reduced action potential amplitude and neurotransmitter release at central terminals (Feltz & Rasminsky, 1974). These findings were pivotal in establishing the role of GABA in the regulation of PAD (primary afferent depolarization), a depolarizing yet inhibitory mechanism that underlies presynaptic inhibition at primary afferent terminals (Hochman et al., 2010; Peng & Frank, 1989; Rudomin & Schmidt, 1999; Takkala et al., 2016). Several mechanisms have been proposed to explain how GABA-mediated PAD suppresses synaptic transmission: i) depolarization-induced inactivation of Na_v channels in primary afferents impairs the transmission of action potential, ii) increased Cl^- conductance produces a shunting effect that attenuates the amplitude of action potentials reaching the presynaptic terminal, iii) inactivation of Ca_v channels reduces Ca^{2+} influx and therefore, neurotransmitter release (Price et al., 2009; Rudomin & Schmidt, 1999; Zeilhofer et al., 2012).

Interestingly, GABA can exert the opposite effect in a distinct anatomical context. Acting on $GABA_A$ receptors located at or near nodes of Ranvier and branch points along the primary sensory neuron central axon, interneuron-released GABA facilitates action potential propagation through the branch point. Activation of these receptors induces a mild depolarization at the nodes which brings the membrane potential closer to threshold, preventing spike failure and enhancing the reliability of sensory feedback to motoroneurons (Hari et al., 2022).

In addition to the well-established effect at the primary afferent terminals, recent studies from our group has demonstrated that GABAergic transmission also occurs within the cell bodies of primary sensory neurons in the DRGs (Du et al., 2017b; Hao et al., 2023;

Lall et al., 2025). DRG neurons are equipped with the molecular machinery necessary for the synthesis, release, and uptake of GABA and they express multiple $GABA_A$ receptor subunits. Functionally, nearly all small-diameter DRG neurons respond to GABA and many subtypes can release GABA via VGAT-mediated mechanisms, upon stimulation (Du et al., 2017; Hao et al., 2023). Local GABA application to the DRGs reduces thermal and mechanical allodynia in animal models of chronic pain, indicating that a local GABAergic tone within the DRG contributes to the modulation of nociceptive inputs before they reach the spinal cord. Interestingly, blockade of $GABA_A$ receptors with bicuculline in naïve animals induces nocifensive behaviour, further supporting the presence of an early peripheral gate at the level of the DRG (Figure 1.12) (Du et al., 2017).

As explained in section 1.1.2, the T-junction of DRG neurons acts as a low-pass filter for action potentials that travel from the periphery towards the spinal cord. Activation of GABAergic conductances at the cell body enhances T-junction filtering, increasing the probability of spike failure. Computational and experimental studies have shown that GABA-mediated currents at the primary sensory neurons somata, induce shunting inhibition at the T-junction by lowering membrane resistance, while also partially inactivating Na_V channels, thereby reducing action potential propagation toward the spinal cord (Du et al., 2017; Hao et al., 2023; Lall et al., 2025). Importantly, a significant downregulation of GABAergic signalling within the DRGs have been observed in animal models of chronic pain, suggesting that not only in the spinal cord, but also in the periphery dysregulation in GABAergic signalling occurs (Wang et al., 2021). Together, these findings indicate that GABAergic signalling exerts inhibitory control over nociceptive transmission at multiple anatomical checkpoints, including the central terminals, the somata, and the T-junction of primary sensory neurons, providing layered gating mechanisms along the primary afferent axis.

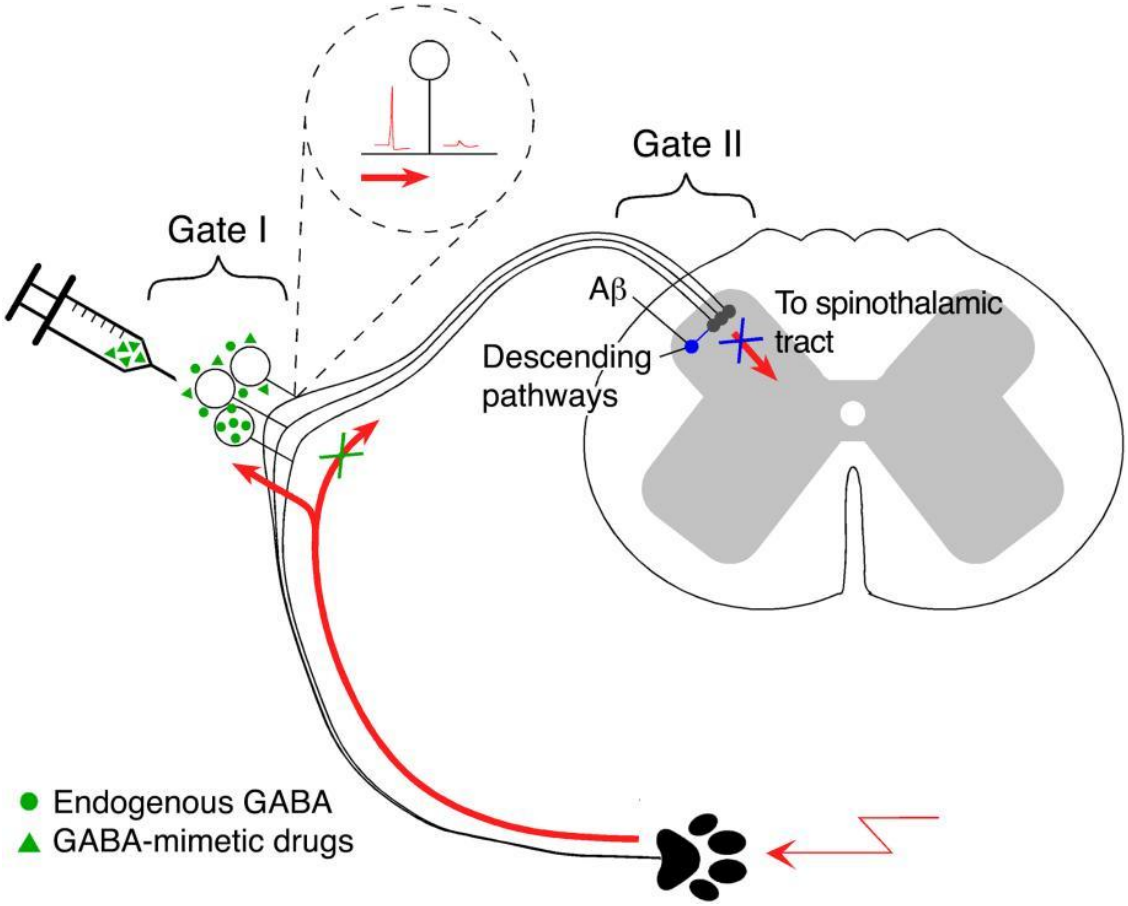


Figure 1.12 GABA-mediated filtering of nociceptive signals at the DRG.
Peripheral stimulation triggers activity-dependent release of GABA from somatic and perisomatic regions of primary sensory neurons in the DRG. GABA acts on $GABA_A$ receptors expressed on nocicepetive neurons and by shunting inhibition, it lowers the action potential safety factor at the T-junction, causing action potential failure and reduced excitatory input to the spinal cord. Similar effects can be achieved by local delivery of GABA or GABA mimetics, or by increasing GABA tone with reuptake inhibitors, highlighting the DRG as a functional “gate” in nociceptive transmission (adapted from Du et al., 2017)

Recent evidence highlights that GABAergic signalling in the DRG is not exclusively neuronal but it is also modulated by satellite glial cells (SGCs). A study from our group demonstrated that SGCs release diazepam binding inhibitor (DBI), a peptide that acts as a positive allosteric modulator and unconventional agonist of $GABA_A$ receptors on nearby sensory neurons (Li et al., 2024). DBI is selectively expressed in SGCs and is concentrated around mechanosensitive DRG neurons, suggesting a modality-specific inhibitory function. Functionally, DBI released from SGCs enhances GABAergic receptor activity, leading to increased inhibitory tone at the neuronal soma, which dampens sensory neuron excitability and reduces nociceptive signalling to the spinal cord. *In vivo*, knockdown of DBI in SGCs induced mechanical hypersensitivity, whereas SGC-targeted overexpression of DBI alleviated both inflammatory and neuropathic pain, highlighting its critical role in peripheral pain modulation. These findings establish a novel glia-neuron inhibitory pathway in the DRG, where SGCs fine-tune nociceptive input through GABAergic mechanisms before peripheral signals enter central pain circuits (Li et al., 2024).

In addition to DRG neuron somata and central terminal, GABA receptors and transporters have been detected along the peripheral sensory axons, where they may locally regulate axonal excitability. However, the functional outcome of peripheral axonal GABAergic signalling remains controversial. Some studies report antinociceptive effects, consistent with increased Cl^- conductance producing shunting inhibition and reducing action potential propagation or neurotransmitter release (Carlton et al., 1999; Hanack et al., 2015). In contrast, other reports suggest that GABA can exert pronociceptive actions by depolarizing peripheral axons due to their high intracellular Cl^- concentration, potentially facilitating ectopic firing or sensitization under pathological conditions (Bonalume et al., 2021; Jang et al., 2017). These divergent findings likely reflect differences in receptor subtype expression, local Cl^- homeostasis, fibre type, and inflammatory state of the tissue.

Finally, beyond neurons and SGCs, emerging evidence indicate that GABAergic signalling also operates within other peripheral cell types relevant to nociception, including Schwann cells and immune cells. Schwann cells express GABA receptors, transporters, and GABA-synthesizing enzymes, and GABAergic signalling in these cells has been implicated in the regulation of myelination, axonal support, and nerve regeneration following injury (Faroni et al., 2014, 2019; Magnaghi et al., 2004; Procacci et al., 2013). Although the direct contribution of Schwann cell-derived GABA to nociceptive processing remains incompletely understood, alterations in Schwann cell GABAergic signalling may indirectly influence sensory neuron excitability by modulating axonal function and the local inflammatory milieu.

In parallel, GABA receptors are widely expressed in peripheral immune cells, including macrophages, monocytes, T cells, and B cells, where GABAergic signalling exerts predominantly anti-inflammatory effects by limiting cytokine release, cellular activation, and immune-driven sensitization (Bhandage & Barragan, 2021; Yi et al., 2022; B. Zhang et al., 2021). In macrophages, GABA signalling has been shown to promote anti-inflammatory phenotypes and suppress pro-inflammatory cytokine production, while in lymphocytes it modulates proliferation, differentiation, and effector function. Although direct evidence for GABAergic mechanisms in DRG-resident macrophages remains limited, findings from other tissue-resident immune populations suggest that GABA acts as a broader immunomodulatory signal in peripheral tissues. Together, these observations support the view that peripheral GABAergic signalling extends beyond neurons to include glial and immune components, forming an integrated network that shapes nociceptive input before it reaches central pain circuits.

1.5 Aims and Objectives

The neurotransmitter GABA plays a pivotal role in the modulation of nociceptive signalling at multiple level of the nervous system, including the spinal cord, DRGs and peripheral sensory axons. Dysregulation of GABAergic signalling has been implicated in chronic pain development and maintenance, affecting both central and peripheral

inhibitory gates. Importantly, the effects of GABA are highly context-dependent: activation of $GABA_A$ receptors at different neuronal compartments - such as DRG neurons somata, T-junction, peripheral axon and central terminals - can lead to distinct outcomes, ranging from inhibition of action potential propagation to facilitation of spike transmission.

As described above, the T-junction is an important checkpoint controlling the transmission of painful stimuli travelling from the periphery to the spinal cord, functioning as a low-pass filter for action potential propagation. Despite its importance, the T-junction remains experimentally difficult to access due to its small size and deep anatomical location within the DRG. As a result, the mechanisms driving T-junction regulation - especially by local GABAergic signalling - remain poorly understood.

In addition, accumulating evidence indicates that non-neuronal cells, including SGCs, Schwann cells and immune cells, actively contribute to peripheral GABAergic signalling and nociceptive modulation. However, the cellular sources of peripheral GABA and the mechanisms by which neuron-glia-immune interactions shape inhibitory tone in pain conditions remain largely unexplored.

The overall aim of this study is to elucidate peripheral GABAergic cross-talk within neurons and non-neuronal cells of the peripheral somatosensory system and to define its contribution to nociceptive processing and chronic pain mechanisms. Specifically, this work aims to:

1. Investigate how compartment-specific GABAergic signalling differentially modulates signal transmission in primary sensory neurons. This will be achieved through the employment of a compartmentalized microfluidic DRG-DH co-culture system that recapitulates the morphological organization of primary sensory neurons and will enable investigation of spatially distinct $GABA_A$ receptor signalling.

2. Determine whether Schwann cells are a source of peripheral GABA and investigate how peripheral nerve injury alters the expression of GABA-related enzymes and transporters.
3. Assess how GABAergic signalling shapes macrophage polarization both *in vitro* and *in vivo* in animal models of chronic pain.

2 Chapter 2: Materials and Methods

2.1 Microfluidic experiments

2.1.1 Animals

All animal work carried out at the University of Leeds was approved by the University of Leeds Animal Welfare Ethical Review Body (AWERB) and performed under UK Home Office License P40AD29D7 and personal license I75542942 and in accordance with the regulations of the UK Animals (Scientific Procedures) Act 1986. All experiments were carried out on neonatal and adult Wistar rats (Charles River). Rats were housed in a regulated environment (23 ± 1 °C, 50 ± 5% humidity) with a 12-hour light-dark cycle with food and water ad libitum.

2.1.2 DRG neuron isolation

P0-P3 rat pups were sacrificed following Schedule 1 protocol using overdose of isoflurane and cervical dislocation. Spinal columns were then extracted and cut sagittally to expose DRGs down the left and right side in the intervertebral spaces. All DRGs were dissected and placed in ice-cold Hanks Balanced Salt Solution (HBSS, Gibco). DRGs were firstly incubated with collagenase (1mg/ml; Sigma) and dispase (10mg/ml; Invitrogen) dissolved in HBSS for 13 minutes at 37°. They were then mechanically dissociated and incubated for another 2-3 minutes, after which a second milder mechanical dissociation was carried out. DRGs were then washed with ice cold Neurobasal-A medium (Gibco) modified with B27 (Gibco), 2 mM Glutamax (Gibco), 100 U/ml penicillin (Sigma), 100 µg/ml streptomycin (Sigma) and pelleted by centrifugation at 800 rpm for 5 minutes at 4°C. After that, cells were washed in 15% BSA dissolved in modified Neurobasal-A medium, pelleted at 850 rpm for 10 minutes at 4°C and resuspended in 1 ml of medium. They were then counted and spinned down one last time for 5 minutes. They were then resuspended in a total volume of 25-50 µl, depending

on the number of chambers utilized (5 µl, which corresponded to ~50,000 neurons, were seeded in each chamber).

2.1.3 DH neuron isolation

P0-P3 mice pups were sacrificed following Schedule 1 protocol and spinal columns were then extracted. Laminectomy was then performed along the whole spinal column length and following the removal of the vertebras, the spinal cord was exposed. After the spinal cord was dissected from the spinal column, it was cut in a half from the dorsal side. From each half, the most external stripe, which corresponded to the DH stripe, was collected and placed in ice cold HBSS supplemented with 20 mM HEPES (Sigma). The tissue was then incubated with collagenase (1mg/ml) and dispase (10mg/ml) for 30 minutes. DH stripes were firstly washed with ice cold HBSS+HEPES twice and with modified Neurobasal-A cell medium once. After that, 5 rounds of mild mechanical dissociation were carried out with a fire polished glass pipette and the cells were gradually filtered in a 40 µm filter. A total volume of 3 ml was obtained, and it was then washed in 5% BSA dissolved in modified Neurobasal-A cell medium and pelleted at 1000 g for 5 minutes at 4°C. After resuspension in fresh cell medium, cells were counted and spinned down one last time for 5 minutes. The cells were resuspended in a total volume of 25-50 µl, depending on the number of chambers utilized (5 µl, which corresponded to ~100,000 neurons, were seeded in each chamber). After 1 hour, cell medium was added with a supplement of 50ng/ml of NGF to promote DRG neurons axons attraction, and 10µM AraC.

2.1.4 Cell culture in microfluidic devices

DRG and DH neurons were co-cultured in a tri-chambered microfluidic device (XONA microfluidics, TCND1000) assembled by non-plasma bonding on 40 mm x 20 mm glass coverslips which were then coated with 50 µg/ml poly-D-Lysin (Corning Life Sciences) and 20 µg/ml laminin (Sigma) dissolved in cell grade culture water. The coating was incubated overnight (ON), and it was washed twice with modified Neurobasal-A cell

medium before cell seeding. DRG and DH neurons were seeded in the central and in one of the later compartments of the device respectively, in modified Neurobasal-A medium and placed in a humidified incubator (5% CO_2, 37°C). Neurons were allowed to properly attach to the coverslips for 1 hour, and they were then flooded with prewarmed Neurobasal-A medium supplemented with AraC (10 µM; Sigma) to slow down glial proliferation. The medium placed in the empty lateral compartment which will successively host DRG neuron axons, was supplemented with 50 ng/ml NGF to favour axonal spreading, and it was replaced with normal medium at DIV3. A volume difference in volume of 80 µl was maintained between the compartments to enable fluidic isolation. Experiments were carried out between DIV 7 and DIV 14.

2.1.5 Dye tracing

To trace DRG neuron axons, DiO (green) or DiI (red) dyes (Thermo Fischer Scientific) were dissolved in culture medium at 1:200 dilution and added to the periphery or DH compartment. The dyes were allowed to diffuse for 24 hours before live imaging. Cell bodies extending axons into a single compartment were labelled either green or red, whereas neurons whose axons reached both compartments exhibited yellow labelling.

2.1.6 Immunocytochemistry

After carefully removing the chamber from the coverslips, the samples were washed twice with PBS and fixed with 2% paraformaldehyde (PFA; Sigma) for 20 minutes. They were then washed three times with a solution of PBS and Triton X-100 (Sigma) 0.001% and successively permeabilized with PBS and Triton X-100 0.1% for 10 minutes. Cells were then blocked with a solution of PBS and 5% of Goat Serum or Donkey Serum for 30 minutes. After this step, samples were incubated with the appropriate primary antibody (Table 2.1) ON at 4°C. The following day the cells were washed three times with PBS and they were then incubated with the appropriate secondary antibodies (diluted 1:500/1:1000) for 1 hour. The samples were then washed with PBS and mounted with Vectashield mounting media with DAPI on microscope coverglass supports. Images

were acquired at confocal microscope LSM800 (Zeiss) using a 20X and 63X magnification. Analysis was carried using the software ImageJ.

Table 2.1 Primary and secondary antibodies for immunocytochemistry

Antibodies	Production Company	Product code	Dilution	Species
α_1 subunit $GABA_A$ receptor	Invitrogen	PA5-114315	1:200	Rabbit
α_2 subunit $GABA_A$ receptor	Synaptic Systems	224103	1:500	Rabbit
β_3 subunit $GABA_A$ receptor	Thermo Scientific	PA5115261	1:500	Rabbit
γ_2 subunit $GABA_A$ receptor	Proteintech	14104-1-AP	1:200	Guinea Pig
MAP2	Abcam	Ab5392	1:10000	Chicken
Tuj-1	Sigma	T8578	1:500	Mouse
Secondary Antibodies				
Alexa Fluor 555	Thermo Fisher	A21429	1:500	Rabbit
Alexa Fluor 488	Thermo Fisher	A11039	1:500	Chicken
Alexa Fluor 647	Thermo Fisher	A21236	1:500	Mouse

2.1.6.1 Fluorescence quantification and statistics

To quantify immunofluorescence, we used the software ImageJ. Regions of interest (ROIs) were drawn along the two daughter branches of pseudo-unipolar neurons, beginning at the bifurcation point and extending approximately 20 µm along both the peripheral and central axons. Mean fluorescence and axonal diameter were measured within these ROIs. For pseudo-unipolarization analysis, the neurons were stained with MAP2, and the number of pseudo-unipolar and multi-polar cells was assessed. For statistical analysis, data were plotted with GraphPad Prism 10.0. Values are reported as the mean ± standard error of the mean (SEM). Sample distribution was assessed with

Kolmogorov-Smirnov test, D'Agostino & Pearson normality test and Shapiro-Wilk normality test. T test for unpaired data was used for samples with normal distribution, followed by Bonferroni correction. For data not normally distributed, statistical analysis was carried out with Mann-Whitney test. Significance was set at $p \leq 0.05$.

2.1.7 Ca^{2+} imaging

For ratiometric Ca^{2+} imaging, DRG or DH cultures were loaded with 2 μM Fura-2 AM (Life Technologies) in the presence of 0.01% pluronic F-127 (Sigma) dissolved in extracellular solution (EC) containing NaCl (160 mM), KCl (2.5 mM), $MgCl_2$ (1 mM), $CaCl_2$ (2 mM), Hepes (10 mM), and glucose (10 mM), at 37°C for 45 min. For depolarization experiments, a high-K^+ solution was prepared by increasing the KCl concentration to 50 mM. with equimolar substitution of NaCl. Imaging was carried out using a Nikon Eclipse TE-2000 microscope (inverted) with a 20X and 40X objectives. For Fura-2 imaging, cells were excited at 340 and 380 nm wavelengths using a LED (cool LED), while for GCaMP6s imaging, a FITC filter was used and cells were excited at 488 nm wavelength. In both cases, fluorescence images were acquired by DQC-FS camera at 500 ms using Nikon software. Compounds - GABA (100 μM), TTX (1 μM), Bicuculline (200 μM) - were applied selectively to either the DRG axonal or somata compartment for 60 seconds, owning to fluidic isolation, while Ca^{2+} responses were monitored in DRG or DH neuron cell bodies. Electrical stimuli were delivered using two DS3 isolated current stimulator (Digitimer) driven by DG2A signal generator (Digitimer). Coiled Pt wires were connected to the DS3 stimultors and submerged into each well of the periphery compartment. 5 Hz biphasic pulses (2 ms for each phase, 80 μA) robustly excited DRG neurons and a train of 5 pulses elicited Ca^{2+} responses in DH neurons.

To enable axonal imaging, DRG neurons were transduced with an AAV encoding the Ca^{2+} sensor GCaMP6s under the control of the CAG promoter (pAAV.CAG.GCaMP6s. WPRE.SV40; Addgene) diluted in medium to ~1.1×10^8 gene copies/μl. After 3 days it was washed out and replaced with standard medium and after a week, GCaMP was robustly expressed among DRG neurons. The day before GCaMP6s imaging, the red

dye Dio was delivered to the peripheral compartment. Dio-labelled neurons displaying pseudo-unipolar morphology and polarized branches, were selected and imaging was carried out with a 40X objective.

2.1.7.1 Image Analysis and statistics

Background fluorescence was estimated as the mean intensity of three ROIs placed in cell-free areas of each field and measured separately in the 340 nm and 380 nm excitation channels. Background values were subtracted frame-by-frame from the corresponding raw fluorescence signals in each channel prior to ratio calculation. Background-corrected Fura-2 ratios (340/380) were then calculated and normalized to baseline by expressing changes relative to the ratio at $t = 0$($\Delta F/F_0$). Cells were classified as responders when the increase in the normalized ratio exceeded three times the standard deviation of the baseline period. Peak responses were defined as the maximum $\Delta F/F_0$ reached during drug application.

GCaMP6s imaging was analysed using the same workflow as described for Fura-2 imaging. For signal acquisition, regions of interest were drawn around the peripheral-like thicker branch and the central-like thinner branch of pseudo-unipolar neurons, beginning at the bifurcation point and extending approximately 15 µm. Background fluorescence, measured from three cell-free ROIs per field, was subtracted frame-by-frame from raw fluorescence signals. Background-corrected traces were normalized to baseline and expressed as F/F_0. Cells were classified as responders when fluorescence increases exceeded three times the standard deviation of the baseline period, and peak responses were defined as the maximum F/F_0 during drug application.

For statistical analysis, data were plotted with GraphPad Prism 10.0. Values are reported as the mean ± SEM. Sample distribution was assessed with Kolmogorov-Smirnov test, D'Agostino & Pearson normality test and Shapiro-Wilk normality test. T test for paired data was used for samples with normal distribution, followed by Bonferroni correction. For data not normally distributed, statistical analysis was carried out with Wilcoxon-

Signed test. Comparisons involving multiple groups were analysed using one-way ANOVA followed by Tukey's post hoc multiple comparisons test for parametric data, whereas non-parametric datasets were analysed using the Kruskal-Wallis test followed by Dunn's multiple comparisons test. Significance was set at $p \leq 0.05$.

2.1.8 Iodide imaging

HEK cells co-transfected with EYFP-QL and $GABA_A$ receptor subunits α_1, β_2, γ_2 were co-cultured with DRG axons within the peripheral compartment to investigate GABA release. EYFP-QL is a mutated GFP which quenches its fluorescence when bond to Iodide (I^-). Extracellular solutions containing I^- instead of Cl^- was prepared by equimolar substitution of the NaCl with NaI (5 mM). The 50 mM K^+ solution was produced by subtracting the 47.5 mM increase in KCl (50 mM) from the NaCl content (160 mM to 112.5 mM) to maintain a balance in the ion content of the solution. Compounds were added to the NaI-containing solution while imaging: GABA (100 µM); Tocris); capsaicin (1 µM; Sigma); KCl (50 mM).

2.1.8.1 Image analysis and statistics

EYFP-QL fluorescence was analysed as changes in fluorescence intensity over time. ROIs were drawn around individual HEK cells expressing EYFP-QL. Fluorescence signals were normalized to baseline prior to stimulation and expressed as relative changes in fluorescence. Capsaicin (1 µM) or high-K^+ solution (50 mM KCl) were applied for 60 s, and I^--dependent fluorescence quenching was quantified during the stimulation period. GABA (100 µM) was applied at the end of each experiment as a positive control to confirm functional expression of $GABA_A$ receptors. Only cells that responded to GABA application were included in the analysis.

For statistical analysis, data were plotted with GraphPad Prism 10.0. Values are reported as the mean ± SEM. Sample distribution was assessed with Kolmogorov-Smirnov test, D'Agostino & Pearson normality test and Shapiro-Wilk normality test. Comparisons involving multiple groups were analysed using one-way ANOVA followed by Tukey's post

hoc multiple comparisons test for parametric data, whereas non-parametric datasets were analysed using the Kruskal-Wallis test followed by Dunn's multiple comparisons test. Significance was set at $p \leq 0.05$.

2.2 Schwann cell experiments

2.2.1 Primary Schwann cell culture

Primary Schwann cells were isolated from spinal nerves of neonatal P3-P6 Sprague-Dawley rats using an established dissociation and purification protocol adapted from previously published method (Wen et al., 2017). Neonatal rat pups were sacrificed following Schedule 1 protocol using overdose of isoflurane and cervical dislocation. Following sterilisation with 75% ethanol, animals were placed in a prone position on a dissecting board and secured. The dorsal skin was incised along the midline and reflected laterally to expose the musculature. Using fine dissection tools under a stereomicroscope, the shoulder girdle and vertebral laminae were carefully removed to expose the spinal cord. The spinal cord was gently removed to reveal the dorsal root ganglia and associated spinal nerves. Spinal nerves, including sciatic, brachial plexus, and intercostal nerves, were carefully isolated by grasping the ganglia and gently extracting the attached nerves through the intervertebral foramina. This approach allowed the collection of long nerve segments largely free of epineurial connective tissue. DRGs and any remaining connective tissue were removed, and the cleaned nerves were transferred into ice-cold HBSS. Collected nerves were cut into small fragments (approximately 0.5-1 mm in length) and transferred to microcentrifuge tubes containing pre-warmed 0.25% trypsin-EDTA (Sigma). Tissue was incubated at 37 °C for 30 min with gentle agitation at regular intervals. Enzymatic digestion was terminated by the addition of fetal bovine serum (FBS; Sigma), after which the tissue was gently triturated using a pipette to obtain a single-cell suspension. The resulting suspension was passed through a 100 µm cell strainer to remove undigested tissue and debris and then centrifuged at 106 g for 10 minutes. The cell pellet was resuspended in DMEM/F12 medium (Gibco)

supplemented with 10% FBS and 1% penicillin/streptomycin. Cells were plated onto poly-L-lysine-coated (Sigma) culture dishes at high density and allowed to attach for 2 h in a humidified incubator at 37 °C and 5% CO_2 before additional culture medium was added. To eliminate contaminating fibroblasts, cultures were treated 24 h after plating with DMEM/F12 containing 10% FBS and AraC for 48 hours. Following antimitotic treatment, the medium was replaced with Schwann cell growth medium consisting of DMEM/F12 supplemented with 3% FBS, heregulin (10 ng/mL), forskolin (3 μM), and penicillin/streptomycin (1%), to promote Schwann cell survival and proliferation. Cells were maintained in this medium and passaged when cultures reached approximately 90% confluence. Schwann cells were routinely used between passages 3 and 5 for all experiments, as cells within this range exhibited characteristic bipolar or tripolar morphology and robust proliferative capacity. The purity and identity of Schwann cell cultures were confirmed by immunocytochemical staining for established Schwann cell markers S100β.

2.2.2 Immunohistochemistry

1 month-old Wistar rats were sacrificed following Schedule 1 protocol and the sciatic nerves were collected in PBS. Briefly, the skin over the hind limb was incised along the femur, and the underlying muscles were bluntly dissected to expose the sciatic nerve. The sciatic nerve was carefully isolated from surrounding connective tissue using fine forceps. A segment of ~5cm of the nerve was excised and transferred to PBS. It was then washed twice in PBS and fixed in 4% PFA ON. The nerve was then washed twice with PBS and placed in PBS supplemented with 30% Sucrose at 4°ON. Once it had sunk, sciatic nerve was included in Optimal cutting temperature compound (OCT; VWR) and it was then placed at -80°. The embedded tissue was then cut into longitudinal 20 μm sections using a cryostat (Leica) and slices were stored at -20°. Before staining, slices were washed three times with PBS. They were then blocked with a solution consisting of 5% donkey/goat serum, 0.05% Tween20 and 0.25% Triton X-100 (all Sigma) dissolved in PBS for 2 hours. After this step, slices were incubated ON at 4° with

primary antibodies diluted in PBS and 50mg/ml BSA (Table 2.2). The following day, slices were washed 3 times with PBS and incubated with secondary antibodies in PBS and BSA 10mg/ml for 2 hours. They were then mounted and imaged with confocal microscope LSM800 using a 20X magnification. Analysis was carried out

using software ImageJ as previously described in 2.1.6.1.

2.2.3 Western blotting

Adult rat sciatic nerves were rapidly dissected as previously described and either processed immediately or frozen at -80°C. Tissue was homogenised on ice in radioimmunoprecipitation assay (RIPA) buffer containing 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, and 0.1% SDS (all SIGMA), supplemented immediately before use with a protease inhibitor cocktail at 1x final concentration (Merck) and 1 mM phenylmethylsulfonyl fluoride (PMSF; Sigma). Homogenates were incubated on ice with intermittent agitation and cleared by centrifugation at 12000 rpm at 4 °C for 10 minutes. Supernatants were collected for protein analysis. Total protein concentration was determined using the Bradford protein assay (Sigma) according to the manufacturer's instructions, and it ranged between 2 and 5 µg/µl. Equal amounts of protein (30-40 µg) were mixed with Laemmli sample buffer containing 62.5 mM Tris-HCl (pH 6.8), 2% SDS, 10% glycerol, 0.01% bromophenol blue, and the reducing agent β-mercaptoethanol (5% v/v), heated at 95 °C for 5 min, and resolved by SDS-polyacrylamide gel electrophoresis using pre-cast polyacrylamide gels (Bio-Rad). Electrophoresis was performed in running buffer containing 25 mM Tris, 192 mM glycine, and 0.1% SDS. Proteins were transferred onto polyvinylidene difluoride (PVDF) membranes (Bio-Rad) using a rapid semi-dry blotting system (fast blot; Bio-Rad) according to the manufacturer's instructions. Following transfer, membranes were briefly rinsed in Tris-buffered saline prior to blocking.

Membranes were blocked in Tris-buffered saline containing 0.1% Tween20 and 5% non-fat dry milk for 1 h at room temperature and incubated ON at 4 °C with primary antibodies

diluted in blocking solution. After washing, membranes were incubated with appropriate horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature. Immunoreactive bands were visualised using enhanced chemiluminescence and imaged using ChemiDoc imaging system (Bio-Rad) with initial automatic exposure followed by manual image acquisition at increasing exposure times (2 s, 5 s, 10 s, etc.) to ensure signals were within the linear detection range. Band intensities were quantified by densitometry and normalised to the corresponding loading control.

Table 2.2 Primary and secondary antibodies for immunohistochemistry and Western blot

Antibodies	Production Company	Product code	Dilution Immuno	Dilution WB	Species
VGAT	Proteintech	14471-1-AP	1:500	1:5000	Rabbit
GAD67	Proteintech	10408-1-AP	1:500	1:5000	Rabbit
GAT1	Invitrogen	PA585766	1:500	1:1000	Rabbit
DBI	Frontier Institute	MSFR101280	1:1000	1:1000	Rabbit
S100β	Synaptic System	287 006	1:500	-	Chicken
GAPDH	Proteintech	60004-1-1	-	1:2000	Mouse
Secondary Antibodies					
Alexa Fluor 555	Thermo Fisher	A21429	1:500		Rabbit
Alexa Fluor 488	Thermo Fisher	A11039	1:500		Chicken
Anti-mouse HRP	Cell Signalling	7076	1:1000		Mouse
Anti-rabbit HRP	Cell signalling	7074	1:1000		Rabbit

2.2.3.1 Western blot analysis and statistics

Western blot band intensities were quantified using ImageJ, following a standard densitometry workflow. High-resolution images of each blot were opened in ImageJ and converted to 8-bit grayscale to ensure consistent pixel intensity measurement. If necessary, images were inverted so that dark protein bands corresponded to high pixel values. The background signal was estimated from regions of the membrane without visible bands and subtracted from each measurement to isolate true band intensity. For each blot, rectangular ROIs of identical size were drawn around individual protein bands across all lanes. These ROIs were positioned to include the entire band and were used to measure the integrated density (total pixel intensity) of each band. Care was taken to use the same ROI size and placement for corresponding bands across samples to maintain consistency. Measured values were exported to a spreadsheet for processing. Background values were subtracted from each band measurement, and target protein intensities were normalised to their respective loading control band (e.g., GAPDH) from the same lane to account for variations in protein loading and transfer. Normalised band intensities were then expressed relative to control samples for statistical comparison.

For statistical analysis, data were plotted with GraphPad Prism 10.0. Values are reported as the mean ± SEM. Samples distribution was assessed with Kolmogorov-Smirnov test, D'Agostino & Pearson normality test and Shapiro-Wilk normality test. T test for unpaired data was used for samples with normal distribution, followed by Bonferroni correction. For data not normally distributed, statistical analysis was carried out with Mann-Whitney test. Significance was set at $p \leq 0.05$.

2.2.4 Partial sciatic nerve ligation (PSNL)

Partial sciatic nerve ligation was performed in adult rats as originally described by Seltzer and colleagues (Seltzer et al., 1990) to induce a model of peripheral neuropathic pain. Animals were anaesthetised by induction with 3-4% isoflurane delivered in 100% oxygen, followed by maintenance of anaesthesia with 1.5-2% isoflurane in oxygen for the duration

of the surgical procedure. The skin over the lateral aspect of the thigh was incised, and the underlying muscles were bluntly separated to expose the sciatic nerve at mid-thigh level. Using fine forceps under visual magnification, approximately one-third to one-half of the diameter of the sciatic nerve was tightly ligated with a fine non-absorbable suture (typically 8-0 or 9-0 nylon), taking care to avoid complete transection or excessive stretching of the nerve. The wound was then closed with sutures, and animals were allowed to recover with appropriate postoperative monitoring. Sham-operated animals underwent identical surgical exposure of the sciatic nerve without placement of the ligature. This procedure reliably induces long-lasting mechanical allodynia and thermal hypersensitivity in the ipsilateral hind limb while preserving partial nerve continuity.

2.3 Macrophage experiments

2.3.1 Animals

Studies at King's College London were conducted under under UK Home Office License PCD4DEDAC9 and PP4092047 and personal license I75542942 and in accordance with the regulations of the UK Animals (Scientific Procedures) Act 1986. All experiments were

33carried out on 8-12-week-old C57BL/6J male and female mice (in-house breeding). Mice were housed in a regulated environment (23 ± 1 °C, 50 ± 5% humidity) with a 12-hour light-dark cycle with food and water *ad libitum* housed in groups of maximum 5.

2.3.2 BMDMs primary culture

BMDMs were isolated and cultured as previously described (Marim et al., 2010). Briefly, bone marrows were flushed from femurs and tibias of adult mice. A single cell suspension was made from each mouse by filtering cells through a 70 µm cell strainer. Following centrifugation at 300 g, cells were resuspended and cultured in 10 cm petri dishes for 7 days in DMEM supplemented with 10% FBS; 10% L929 cell-conditioned medium as a source of macrophage colony stimulating factor, and 1% penicillin/streptomycin at 37° and 5% CO_2. On day 7, cells were detached and plated in

DMEM supplemented with 1% FBS. Subsequently, macrophages were incubated for classical polarisation protocols with either LPS (100ng/ml; Sigma) or PBS for 8 hours. GABA (Sigma), isoguvacine (Sigma) or PBS were added 5 hours after LPS. For RT-qPCR cells were plated at $1x10^6$ cells/well density.

2.3.3 Real-time quantitative PCR (RT-qPCR)

Total RNAs were isolated using Invitrogen Isolation kit according to manufacturer's instructions. Briefly, samples were lysed in a denaturing buffer to inactivate RNAses and disrupt cellular structures, followed by binding of RNA to a spin column. Contaminants were removed through a series of wash steps, and purified RNA was subsequently eluted in RNase-free water Concentration and purity were evaluated by a spectrophotometer (NanoDrop ND-100, Labtech). 500 ng of each RNA sample was reverse transcribed with a Quantitec Reverse Transcription Kit (Qiagen). RT-qPCR was carried out using SYBR Green I Master Mix (Qiagen) and specific primers for mouse genes (Table 2.3; Qiagen) in a LightCycler 480 (Roche).

2.3.3.1 RT-qPCR data analysis and statistics

Cycle threshold (Ct) values obtained from duplicate or triplicate reactions were averaged for each sample. Relative mRNA expression levels were calculated using the comparative Ct ($2^{\Delta\Delta Ct}$) method. Briefly, the Ct value of each target gene was first normalized to the Ct value of the housekeeping gene β-actin (βAct) to obtain the ΔCt value. Subsequently, ΔCt values from experimental samples were compared with those of the control group to calculate ΔΔCt values. Relative gene expression was then determined as $2^{\Delta\Delta Ct}$ and expressed as fold change relative to controls.

For statistical analysis, data were plotted with GraphPad Prism 10.0. Values are reported as the mean ± SEM. Samples distribution was assessed with Kolmogorov-Smirnov test, D'Agostino & Pearson normality test and Shapiro-Wilk normality test. T test for unpaired data was used for samples with normal distribution, followed by Bonferroni correction. For data not normally distributed, statistical analysis was carried out with Mann-Whitney

test. Comparisons involving multiple groups were analysed using one-way ANOVA followed by Tukey's post hoc multiple comparisons test for parametric data, whereas non-parametric datasets were analysed using the Kruskal-Wallis test followed by Dunn's multiple comparisons test. Significance was set at $p \leq 0.05$.

Table 2.3 Primers for qPCR

Gene symbol	Assay / Primer ID	Supplier	Catalogue number
Nos2	Mm_Nos2_1_SG	Qiagen	QT00100275
Il6	Mm_Il6_1_SG	Qiagen	QT00098875
Il1b	Mm_Il1b_2_SG	Qiagen	QT01048355
Tnfa	Tnfa PCR FWD and REV	Merck	SY190615463-095
Mrc1	Mrc1 PCR FWD and REV	Merck	SY190608845-031
Arg1	Arg1 PCR FWD and REV	Merck	SY190608845-017
βActin	Actin PCR FWD and REV	Merck	SY190320394-070-071
Gad1	Mm_Gad2_1_SG	Qiagen	QT00101416
Gad2	Mm_Gad1_1_SG	Qiagen	QT00163527
Slc6a12	Mm_Slc6a12_1_SG	Qiagen	QT00161287
Slc6a13	Mm_Slc6a13_1_SG	Qiagen	QT00153552
Gabrb3	Mm_Gabrb3_1_SG	Qiagen	QT00104349
Gabrd	Mm_Gabrd_1_SG	Qiagen	QT00154371

2.3.4 Animal model of spared nerve injury (SNI)

Spared nerve injury was performed in mice as previously described (Decosterd & Woolf, 2000a). Briefly, mice were anesthetized firstly with 5% and successively 2% of Isoflurane, and eye ointment was applied to prevent dryness. Buprenorphine (0.1 mg/kg) was administered for analgesia, and the surgical site on the left lateral thigh was shaved and disinfected with 70% ethanol. A small incision was made through the skin of the thigh and muscles fibres were separated with a blunt incision to access the sciatic nerve. Out of its three terminal branches, the peroneal and the tibial nerves were transected whereas the sural one was spared. The wound was then closed with surgical staples, and the animals were monitored until they recovered from the anaesthesia. Post-operative care included heat monitoring, administration of watery mashed food, and observation for two days following surgery. All procedures were performed using sterile techniques and in compliance with institutional animal care guidelines. At the end of the experiment, animals were culled with a schedule-1 method involving overdose by pentobarbital followed by exsanguination.

2.3.5 Isoguvacine administration and behavioural testing

Isoguvacine (Sigma) or PBS were delivered daily to the mice intraperitoneally (I.P) for a duration of 7 days. Prior the injection, Von Frey behavioural assessment was performed. Von Frey monofilaments (0.002-1.0 g) were applied to the plantar surface of the hind paw and mechanical thresholds were measured. Mice were placed in individual compartments where they were let to habituate for 30 minutes prior each behavioural experiment. The testing started with the 0.07 g filament which was then either gradually increased up to 1 g if the animal didn’t show any pain-like response or reduced to 0.002 g if there was a response from the stimulated paw. Filaments were applied to the right and left hind paw alternately using the described up and down method (Dixon & Chapman, 1980). Both hind paws were tested alternately, with the experimenter blinded to the treatment groups to prevent bias.

2.3.5.1 Behavioural data analysis and statistics

Mechanical withdrawal thresholds obtained from the Von Frey assay were calculated using the Dixon method, a statistical approach used to estimate the force required to elicit a withdrawal response in 50% of stimulations. In this method, the response to each filament application determines whether the next filament applied has a higher or lower force, and the final 50% withdrawal threshold is calculated from the pattern of responses (Dixon & Chapman, 1980).

Values are presented as mean ± SEM. Data distribution was assessed using the Kolmogorov-Smirnov, D'Agostino-Pearson, and Shapiro-Wilk normality tests. Since behavioural measurements were collected over time and across treatment groups, statistical analysis was performed using two-way ANOVA followed by Šídák's multiple comparisons test. Statistical significance was set at $p \leq 0.05$.

2.3.6 Flow cytometry

Mice were deeply anesthetized by intraperitoneal injection of pentobarbital (Pentoject) and perfused with ice cold PBS to remove circulating blood from vasculature. Lumbar DRGs L3, L4 and L5 and sciatic nerves were rapidly harvested and placed into ice-cold HBSS (Gibco). Single cell suspensions were obtained following enzymatic digestion using 3 mg/ml Dispase (Roche), 0.125% collagenase (Sigma), and 200 U/ml DNase I (Sigma) in F-12 medium (Gibco) for 30 minutes at 37°C, followed by centrifugation at 300g for 10 minutes and resuspension in FACS buffer r (0.5% BSA and 2 mM EDTA in PBS). To prevent antibodies nonspecific binding, cells were incubated with an anti-mouse CD16/CD32 blocker (1:100 in FACS buffer; Biolegend) for 10 minutes. Samples were stained with Live/Dead Fixable Near IR (Invitrogen) for 30 minutes to assess viability. Successively, conjugated antibodies were used to stain proteins of interests (Table 2.4) for 45 minutes and washed twice with FACS buffer. Finally, cells were fixed with BD-cell fix diluted in distilled wated (1:10). All staining steps were performed on ice and samples were kept at 4°C for maximum 48 hours before analysis.

2.3.6.1 Data analysis and statistics

Samples were analysed using an LSRII-Fortessa flow cytometer (BD Biosciences) and FlowJo software. Compensation was performed with single stain compensation beads (BD CompBeads, Cat#51-90-9001291/51-90-9000949) and events were gated using fluorescence minus one (FMO) controls. Monocytes were identified as CD11b+ cells and macrophages as CD11b+/F4/80+. M1-like macrophages were identified as CD11b+/ F4/80+/MHCII+/CD206-. M2-like macrophages were identified as CD11b+/F4/80+ MHCII-/CD206+.

For statistical analysis, data were plotted and analyzed using GraphPad Prism 10.0 software. Results are expressed as mean ± SEM. Normality of data distribution was assessed using the Kolmogorov-Smirnov, D'Agostino-Pearson, and Shapiro-Wilk tests. For normally distributed datasets, comparisons between two independent groups were performed using an unpaired Student's t-test, followed by Bonferroni correction for multiple comparisons when appropriate. Non-normally distributed data were analyzed using the Mann-Whitney test. Comparisons involving multiple groups or variables were analysed using one-way ANOVA followed by Tukey's post hoc multiple comparisons test for parametric data, whereas non-parametric datasets were analysed using the Kruskal–Wallis test followed by Dunn's multiple comparisons test. Comparisons of proportions between groups were performed using Fisher's exact test. Statistical significance was defined as $p \leq 0.05$.

Table 2.4 Antibodies for flow cytometry.

Target	Fluorophore	Clone	Supplier	Catalogue number	Dilution
CD11b	BV421	M1/70	BioLegend	101235	1:100
F4/80	APC	BM8	BioLegend	123116	1:100
MHC II	PerCP-Cy5.5	AF6-120.1	BioLegend	116416	1:100

Target	Fluorophore	Clone	Supplier	Catalogue number	Dilution
CD206	PE-Cy7	C068C2	BioLegend	141720	1:100

3 Chapter 3: Compartment-specific GABAergic modulation of primary sensory neuron signalling

3.1 Introduction

3.1.1 Physiologically relevant in vitro models

Primary sensory neurons are pseudo-unipolar cells, consisting of a soma located in the dorsal root, trigeminal and nodose ganglia and a single stem axon that bifurcates into peripheral and central branches. This architecture is fundamental for DRG neuron physiology: the T-junction formed at the bifurcation acts as a critical checkpoint for signal transmission and intracellular trafficking, while functional differences between the two branches - such as the larger diameter of the peripheral axon (Hoheisel & Mense, 1986; Lee et al., 1986; Suh et al., 1984) and enrichment of specific proteins like MAP2 (Papasozomenos et al., 1985) - influence spike propagation and suggest branch-specific molecular specialization. Consequently, proteins required for synaptic function must be selectively transported to the central branch, highlighting the need for models that faithfully preserve pseudo-unipolar morphology. Studying these compartment-specific mechanisms *in vivo* is challenging, as T-junctions are microscale structures hidden within the ganglia. In standard culture systems, DRG neurons typically sprout multiple neurites with no clear polarization, and the soma and axons are not physically separated, making it impossible to selectively manipulate or study different compartments. This underscores the importance of developing physiologically relevant *in vitro* models that maintain correct architecture and enable localized investigation of signal propagation and protein transport.

Several strategies have been used to promote pseudo-unipolar morphology *in vitro*. For instance, DRG neuron cultured with NGF displayed neurites that are positive for the somato-dendritic marker MAP2 (De Koninck et al., 1993), and co-culture with glial cells, including Schwann cells and SGCs, promoted pseudo-unipolarization (Costa et al., 2025; De Koninck et al., 1993; Mudge, 1984; Takahashi & Ninomiya, 1987). This suggests that DRG neuron morphology changes require interaction with glial cells whose role is crucial during development. Three-dimensional collagen gels also increase the proportion of pseudo-unipolar neurons, suggesting that dimensionality of the culture environment can influence morphology (Ribeiro et al., 2012). During development, pseudo-unipolarization occurs after primary sensory neurons extend projections towards the spinal cord, hinting that DH neurons may contribute to this process (Barber & Vaughn, 1986). DRG-DH neuron co-cultures have been exploited in the past to investigate synaptic transmission and axonal modulation (Ohshiro et al., 2007; Shipshina et al., 2011; Vikman et al., 2001).

In the more recent model developed by the Rouf group and adapted in this work, DRG and DH neurons were co-cultured in two interconnected chambers of a tri-compartment Xona microfluidic device (Vysokov et al., 2019). The DRG neurons, located in the central compartment, extend their axons through microgrooves to the lateral chambers, physically separating soma and axonal branches. This compartmentalization allows selective manipulation and analysis of different neuronal domains, enabling the study of local axonal phenomena, protein transport, and the first sensory synapse *in vitro*. Although the study did not focus on DRG neuron morphology - which we examined here - it represents a physiologically-relevant *in vitro* model to study the transmission of stimuli travelling from the periphery to the spinal cord. Maintaining the physiological compartmentalization *in vitro* not only allows the study of axonal transport and local signalling but is also crucial for investigating compartment-specific neurotransmitter modulation. In particular, GABA receptors are differentially expressed along somatic and

axonal compartments, and understanding their functional impact requires models where soma and axons can be selectively manipulated.

3.1.2 Compartment-Specific GABAergic Regulation in DRG Neurons

GABA is a major inhibitory neurotransmitter in the spinal cord, where it modulates pain transmission through pre and postsynaptic inhibition of primary afferents and spinal neurons, as described by the Gate Control Theory (Melzack & Wall, 1965; Witschi et al., 2011; Zeilhofer et al., 2012). Recent work from our group has extended this concept beyond the spinal cord, demonstrating that GABAergic signalling also operates locally within the DRG**.** Specifically, the T-junction of primary sensory neurons functions as a low-pass filter for action potentials and the activation of somatic GABAergic conductances regulates spike propagation, thereby establishing an early sensory gating mechanism at the level of the DRG (Du et al., 2017; Hao et al., 2023; Lall et al., 2025; Li et al., 2024).

GABA receptor expression in DRG neurons is not limited to the cell body but it extends to both axonal branches. The physiological role of these receptors has been mainly explored in the central terminals that innervate the spinal cord, where they are known to modulate signal transmission through PAD (Hochman et al., 2010; Peng & Frank, 1989; Rudomin & Schmidt, 1999; Takkala et al., 2016). In contrast, little is known about GABA receptors expressed in the peripheral axons and the evidence reported in literature leads to conflicting conclusions.

Both $GABA_A$ and $GABA_B$ receptor have been observed in the peripheral axons, where they play different roles (Bhisitkul et al., 1990; Brown & Marsh, 1978). Behavioural and functional studies indicate that the effects of peripheral $GABA_A$ receptor activation are conflicting and highly context dependent. In primary afferents, strong or sustained activation of peripheral $GABA_A$ receptors has been associated with pro-nociceptive effects. For example, intraplantar administration of the $GABA_A$ agonist muscimol attenuated formalin-induced nociceptive behaviour at low concentrations (2 μM),

whereas higher concentrations (1 mM) enhanced nociceptive responses in the cat, proposing that near-maximal activation of $GABA_A$ is required for conversion to a nociceptive role (Carlton et al., 1999). More recent work demonstrated that peripheral muscimol (1 mM) application evoked spontaneous nocifensive behaviour following inflammatory sensitization, implicating that $GABA_A$ receptor activation, and particularly α_5 subunit-containing receptors, facilitate nociceptive signalling under inflammatory conditions (Jang et al., 2017). In line with this, $GABA_A$-mediated depolarization of peripheral axons has been shown to enhance excitability and prolong firing in unmyelinated C-fibres via NKCC1-dependent Cl^- axonal loading (Bonalume et al., 2021). In contrast, other studies report inhibitory effects of peripheral $GABA_A$ receptor signalling, particularly in visceral afferents. Activation of $GABA_A$ receptors reduced colonic afferent excitability and attenuated visceromotor responses to noxious colorectal distension, suggesting an endogenous inhibitory role for peripheral GABAergic signalling in visceral pain (Gold & Loeza-Alcocer, 2024; Loeza-Alcocer & Gold, 2022). Together, these findings highlight the lack of consensus regarding the functional role of peripheral $GABA_A$ receptors and suggest that differences in afferent subtype, tissue environment, and Cl^- homeostasis critically shape the outcome of $GABA_A$ receptor activation.

In contrast, $GABA_B$ receptor-mediated signally consistently suppresses sensory neuron excitability through $G_{i/o}$-coupled mechanisms, including inhibition of Cav channels and activation of inwardly rectifying K^+ channels. Similarly to $GABA_A$ receptors, peripheral $GABA_B$ receptor activation reduces colonic afferent excitability and visceromotor responses to noxious colorectal distension (Gold & Loeza-Alcocer, 2024; Loeza-Alcocer & Gold, 2022). In line with this, α-Conotoxin Vc1.1, a small disulfide bonded peptide from the venom of the marine cone snail Conus victoriae, decreases the excitability of dissociated human DRG neurons and inhibits mouse colonic nociceptors via $GABA_B$ receptor engagement, with enhanced efficacy during chronic visceral hypersensitivity (Castro et al., 2017). Consistent with these findings, $GABA_B$ receptors expressed at peripheral nerve endings in the cornea and glabrous skin of the mouse attenuate TRPV1

sensitization induced by inflammatory mediators, suggesting an autocrine inhibitory feedback mechanism that limits peripheral nociceptor sensitization (Hanack et al., 2015).

Altogether these data indicate that both $GABA_A$ and $GABA_B$ receptors are expressed in different compartments of DRG neurons and that their activity differentially shapes nociceptive transmission. This suggests that GABAergic signalling can lead to different outcomes based on the type of receptor involved and on the precise subcellular localization (peripheral vs. central axon vs. soma; Figure 3.1).

3.2 Aim

Maintaining physiological compartmentalization *in vitro* not only enables the study of axonal transport and local signalling, but it is also essential for dissecting compartment-specific neurotransmitter modulation. In particular, $GABA_A$ receptors are differentially expressed along somatic and axonal domains of DRG neurons, and their functional impact cannot be resolved without selective access to these compartments. Accordingly, this study aims to: (i) determine whether DRG neurons cultured in this microfluidic system preserve the key morphological and functional features characteristic of their *in vivo* pseudo-unipolar organization; and (ii) use this platform to investigate how GABAergic signalling operates in distinct neuronal compartments, with a focus on axonal vs. somatic $GABA_A$ receptor function and its impact on action potential propagation and synaptic transmission.

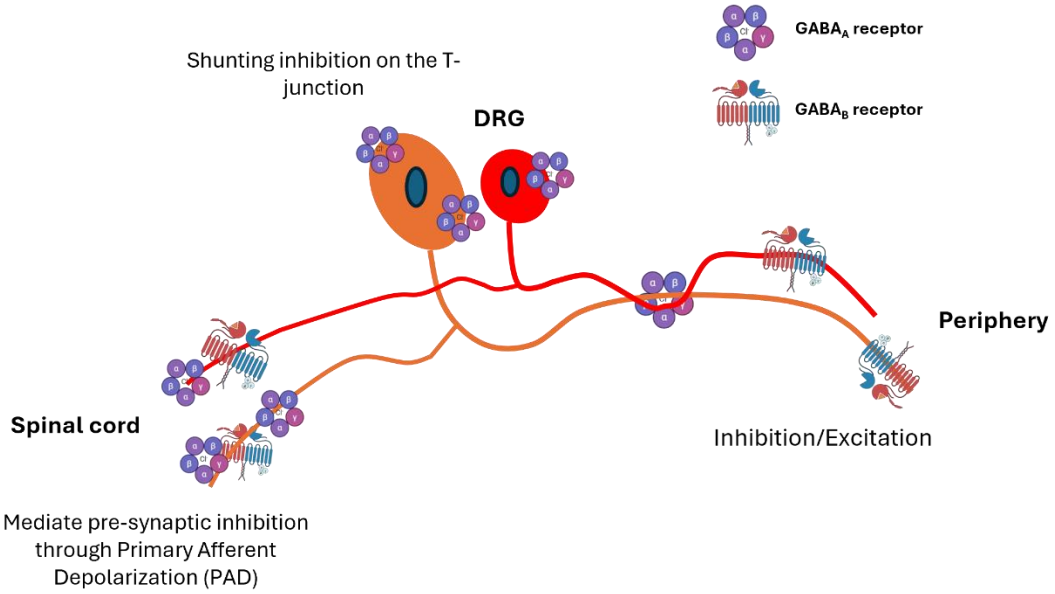


Figure 3.1 GABAergic signalling across DRG neuron compartments.
Schematic illustration of the distribution and functional roles of $GABA_A$ and $GABA_B$ receptors across distinct compartments of primary sensory neurons. $GABA_A$ receptors are expressed at central and peripheral axons as well as at the soma and T-junction, where their activation induces Cl^- efflux and depolarization due to high intracellular Cl^- levels. The functional outcome is compartment dependent: at central terminals, $GABA_A$ receptor activation mediates PAD and presynaptic inhibition, whereas at the soma and T-junction it promotes shunting inhibition and enhances spike filtering. In peripheral axons, $GABA_A$ receptor activation exerts context-dependent excitatory or inhibitory effects. In contrast, $GABA_B$ receptors consistently suppress neuronal excitability via Gi/o-coupled signalling mechanisms in both central and peripheral compartments.

3.3 Results

3.3.1 DRG neurons co-cultured with DH neurons in a compartmentalized microfluidic system mimic in vivo pseudo-unipolarity

To investigate GABAergic signalling across distinct DRG neuron compartments, we employed a tri-chambered microfluidic system that allowed sensory neurons to recapitulate their native axonal architecture (Vysokov et al., 2019). DRG neurons were seeded in the central compartment, whereas DH neurons were cultured in one of the two lateral compartments (method section 2.1). The chambers were connected by 1 mm long microgrooves with a restricted height of 3 µm, which allowed only axons to traverse. This configuration enabled DRG neuron axons to project into both lateral chambers, effectively mimicking the anatomical organization of the sensory pathway (Periphery - DRG -DH; Figure 3.2A).

To identify DRG neurons that extended axons bilaterally across the microfluidic device, we employed two lipophilic fluorescent tracers, DiI (red) and DiO (green), applied to the two lateral compartments. Axons projecting into each compartment incorporated the respective dye which subsequently diffused retrogradely to the cell bodies over 24 hours, enabling identification of DiO- and DiI-positive somata. Neurons that projected axons into both compartments were double-labelled, appearing yellow under fluorescence microscopy, thus confirming the presence of bilaterally projecting DRG neurons (Figure 3.2B).

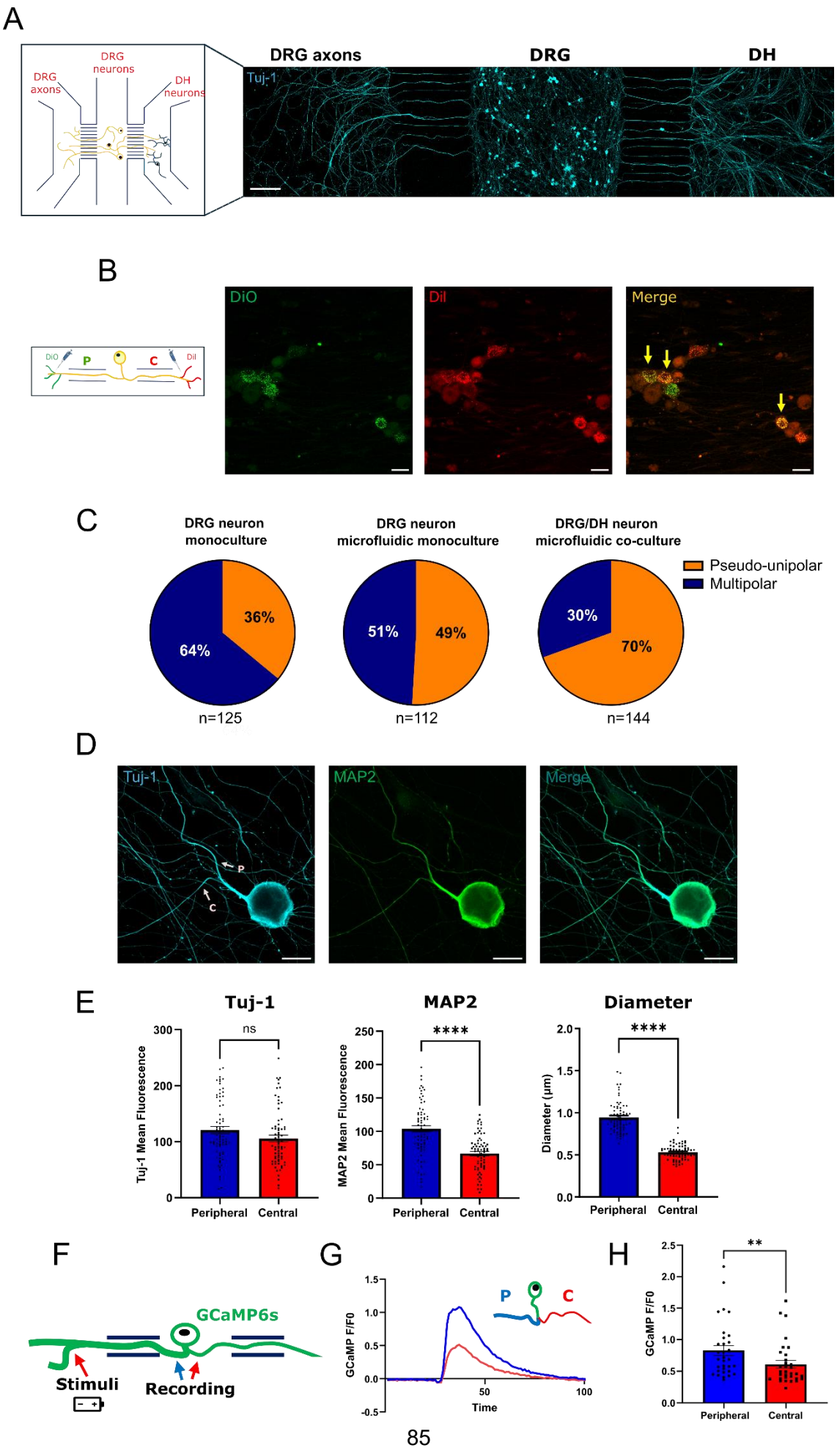
Under standard culture conditions, primary sensory neurons typically extend multiple neurites; in contrast, when cultured in microfluidic devices, a higher proportion exhibited pseudo-unipolar morphology (at DIV7 - 49% vs. 36% in standard culture; Figure 3.2C). Notably, co-culture with DH neurons further increased the proportion of pseudo-unipolar

DRG neurons (70%), indicating that DH-derived factors likely promote morphological maturation (Figure 3.2C).

In vivo, DRG neuron stem axon splits into a peripheral and central branch at the level of the T-junction. Diverse proteins, including MAP2, have been observed to be differentially transported between the two branches which also differ in diameter, with the peripheral being the thicker one (~1 µm peripheral vs. ~0.5 µm central) (Hoheisel & Mense, 1986; Lee et al., 1986; Suh et al., 1984). To assess branch polarization, we stained for MAP2 and a pan-neuronal marker, Tuj-1 (βIIITub; Figure 3.2D). MAP2 preferentially localized along the stem axon and proximal branches. Quantification was performed in ROIs extending approximately 20 µm from the bifurcation point along each daughter branch. MAP2 mean fluorescence was significantly higher in the thicker branch compared to the thinner branch (103.8 ± 4.76 vs. 66.7 ± 3.2; $p<0.0001$; N=4), whereas Tuj-1 was evenly distributed. Thus, we hypothesize that the higher-diameter, MAP2-enriched branch corresponds to the peripheral branch of the native nerve fibre, while smaller, MAP2-depleted branch corresponds to the central one. Consistently, the diameters measured in the two branches of cultured DRG neurons were comparable (peripheral branch 0.94 ± 0.02 vs central branch 0.53 ± 0.01; $p<0.0001$; N=4) to those described *in vivo* (Hoheisel & Mense, 1986; Lee et al., 1986; Suh et al., 1984), further supporting the physiological relevance of the microfluidic model (Figure 3.2E).

To determine whether pseudo-unipolarity had functional relevance, DRG neurons were infected with AAV9 expressing GCaMP6s which enabled axonal Ca^{2+} imaging at the T-junction (Figure 3.2F; method section 2.1.7). At DIV7, 1s 5Hz biphasic stimulation applied to the axonal compartment robustly depolarized DRG neuron somata and bifurcations. ROIs drawn around the peripheral and central branches near the junction, revealed that Ca^{2+} transients (F/F_0) were consistently and significantly lower in the central-like branch (peripheral 0.83 ± 0.08; central 0.61 ± 0.06; $p=0.005$; N=3), suggesting signal filtering at the bifurcation (Figure 3.2G-H), as it is reported to happen *in vivo* (Du et al., 2014; Gemes et al., 2013; Kent et al., 2018; Sundt et al., 2015).

Together, these data show that DRG neuron co-cultured with DH neurons in microfluidic devices acquire pseudo-unipolar morphology with functional compartmentalization reminiscent of their *in vivo* morphology.


A
DRG axons
DRG neurons
DH neurons
DRG axons
DRG
DH
Tuj-1
B
DiO
P
C
DiI
DiO
DiI
Merge
C
DRG neuron monoculture
DRG neuron microfluidic monoculture
DRG/DH neuron microfluidic co-culture
Pseudo-unipolar
Multipolar
36%
64%
n=125
51%
49%
n=112
30%
70%
n=144
D
Tuj-1
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Tuj-1 Mean Fluorescence
MAP2 Mean Fluorescence
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Central
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GCaMP6s
Stimuli
Recording
G
P
C
GCaMP F/F0
Time
H
**
GCaMP F/F0
Peripheral
Central

Figure 3.2 Microfluidic-cultured DRG neurons display physiological morphology and activity.
Schematic representation and Tuj-1 immunostaining of DRG and DH neurons co-cultured in the microfluidic device (A). DiI (red) and Dio (green) lipophilic dyes were added to the lateral compartments. Neurons in yellow picked up both dyes as they crossed to both compartments (B). Proportions of multi-polar vs. pseudo-unipolar DRG neurons in standard culture vs. microfluidic culture vs. microfluidic DRG-DH co-culture. Culture and even more co-culture in microfluidic devices increased the proportion of pseudo-unipolar DRG neurons compared to standard cultures (36% vs. 49% vs. 70%; n=125, n=112, n=144, N=3) (C). Immunostaining of microfluidic-cultured DRG neurons targeting Tuj-1 (green) and MAP2 (red) (Scale bar=20µm) (D). Mean fluorescence quantification showed that Tuj-1 does not change between the peripheral and central branch whereas MAP2 fluorescence is increased in the peripheral branch compared to the central one. Diameter measurements revealed that the peripheral branch is thicker than the central one (Mann-Whitney test, Tuj1 p=0.098, MAP2 ****p<0.0001; n=70, N=3; Unpaired t-test, Diameter ****p<0.0001; n=70, N=3) (E). Schematic representation of GCaMP6 imaging after peripheral electrical stimulation (1s, 5Hz). Imaging was performed in the peripheral and central branches, near the bifurcation (F). Representative traces of GCaMP6 F/F0 responses to peripheral stimulation in the peripheral (blue) and central (red) branches (G). GCaMP6 F/F0 quantification showed that the fluorescence peak was significantly higher in the peripheral branch when compared to the central one (Mann-Whitney test, **p=0.0046; n=32, N=3) (H).

3.3.2 GABA depolarizes DRG neuron axons through the engagement of $GABA_A$ receptors

Having established that DRG neurons in co-culture adopt functional pseudo-unipolar morphology, we next investigated the distribution and function of $GABA_A$ receptors along their processes. We focused on the $GABA_A$ receptor subunits known to be highly expressed in rat DRG neurons, which include the α_1, α_2, β_3 and γ_2 (Du et al., 2017b; Ma et al., 1993; Obradovic et al., 2015). After one week in culture, neurons were fixed and labelled with antibodies targeting these subunits along with MAP2, which enabled to distinguish between the two branches (method section 2.1.6). All four subunits were expressed in both the soma and along the axons of DRG neurons (Figure 3.3A). Quantification was performed in ROIs drawn from the bifurcation point and extending approximately 20 µm along each daughter branch. For all four $GABA_A$ subunits tested, the mean fluorescence intensity was higher in the peripheral-like branch when compared to the central-like one (Figure 3.3B), suggesting that in proximity to the bifurcation, $GABA_A$ receptors are preferentially transported to the peripheral axon.

Given that $GABA_A$ receptors were distributed throughout the axons in the periphery compartment we next examined the functional effect of GABA on peripheral axons (Figure 3.3C). The microfluidic system provides fluidic isolation between compartments, enabling selective compound application. We therefore locally delivered GABA (100 µM) to the periphery compartment, while Ca^{2+} dynamics were monitored in DRG neuron somata using Fura-2 imaging (method section 2.1.7). To identify which neurons extended axons to the periphery compartment, the day before the experiment we selectively applied the lipophilic fluorescent tracer DiO, which could then be detected in the corresponding cell bodies. GABA axonal stimulation depolarized a subset of DRG neurons, eliciting Ca^{2+} influx at the cell body (Figure 3.3D-E). The $GABA_A$ receptor antagonist bicuculline (200 µM), significantly attenuated the responses (40.4 ± 5.6% reduction; $p<0.0001$; N=3) suggesting that peripheral $GABA_A$ receptors mediate axonal responses to GABA (Figure 3.3D). Moreover, GABA-induced depolarization was

markedly reduced by TTX (1 μM) (33.8 ± 6.1% reduction; p=0.004; N=3), indicating that GABA stimulation at the axon likely initiates action potentials that propagate to the soma (Figure 3.3E)

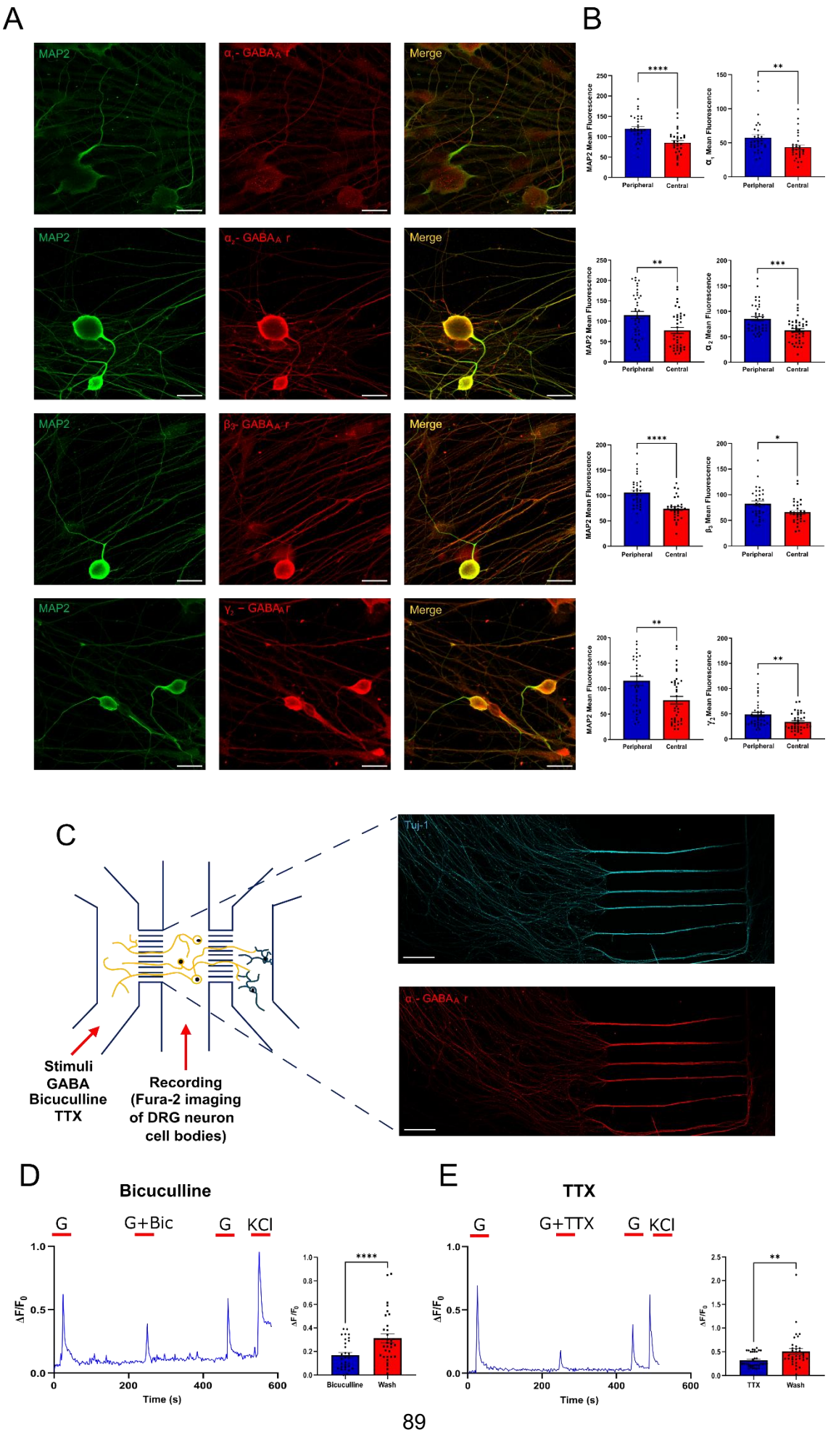

A
B
MAP2
α1- GABAA r
Merge
α2- GABAA r
β3- GABAA r
γ2 – GABAA r
MAP2 Mean Fluorescence
α1 Mean Fluorescence
α2 Mean Fluorescence
β3 Mean Fluorescence
γ2 Mean Fluorescence
Peripheral
Central
C
Tuj-1
Stimuli
GABA
Bicuculline
TTX
Recording
(Fura-2 imaging
of DRG neuron
cell bodies)
D
Bicuculline
G
G+Bic
KCl
ΔF/F0
Time (s)
Bicuculline
Wash
E
TTX
G+TTX
TTX
Wash

Figure 3.3 $GABA_A$ receptors are expressed and active along DRG neuron axons.
α_1, α_2, β_3 and γ_2 subunits of the $GABA_A$ receptor (red) were detected in the soma and axons of microfluidic-cultured DRG neurons, alongside MAP2 (green) (Scale bar=20μm) (A). ROIs were drawn at a distance of 15-20μm from the bifurcation in the two branches. MAP2 mean fluorescence was used to distinguish between the two branches. Mean fluorescence intensity quantification showed that all four subunits displayed increased fluorescence in the peripheral branch in comparison to the central one (Unpaired t-test, β_3 *p=0.013, γ_2 ***p=0.001; n=32, n=37, N=3; Mann-Whitney test, α_1 **p=0.005, α_2 **p=0.004; n=31, n=39 N=3) (B). Schematic of the imaging set-up. Stimuli (GABA, bicuculline and TTX) were delivered to the axonal compartment while Ca^{2+} activity was monitored in the cell bodies using Fura-2. Immunofluorescence of all four subunits (α_1 immunostaining is shown in red) was detected in the peripheral axons (Scale bar=100μm) (C). Representative Ca^{2+} responses (measured as normalized Fura-2 ΔF/F0) of DRG neurons somata following axonal stimulation with GABA (100μM) in the presence and absence of bicuculline (200μM) (D) or TTX (500nM) (E). Peak amplitude (ΔF/F0) by GABA stimulation is significantly reduced after the application of bicuculline and TTX (Wilcoxon test, Bic ****p<0.0001; n=31 N=3; Paired t-test, TTX **p=0.0097; n=35 N=3).

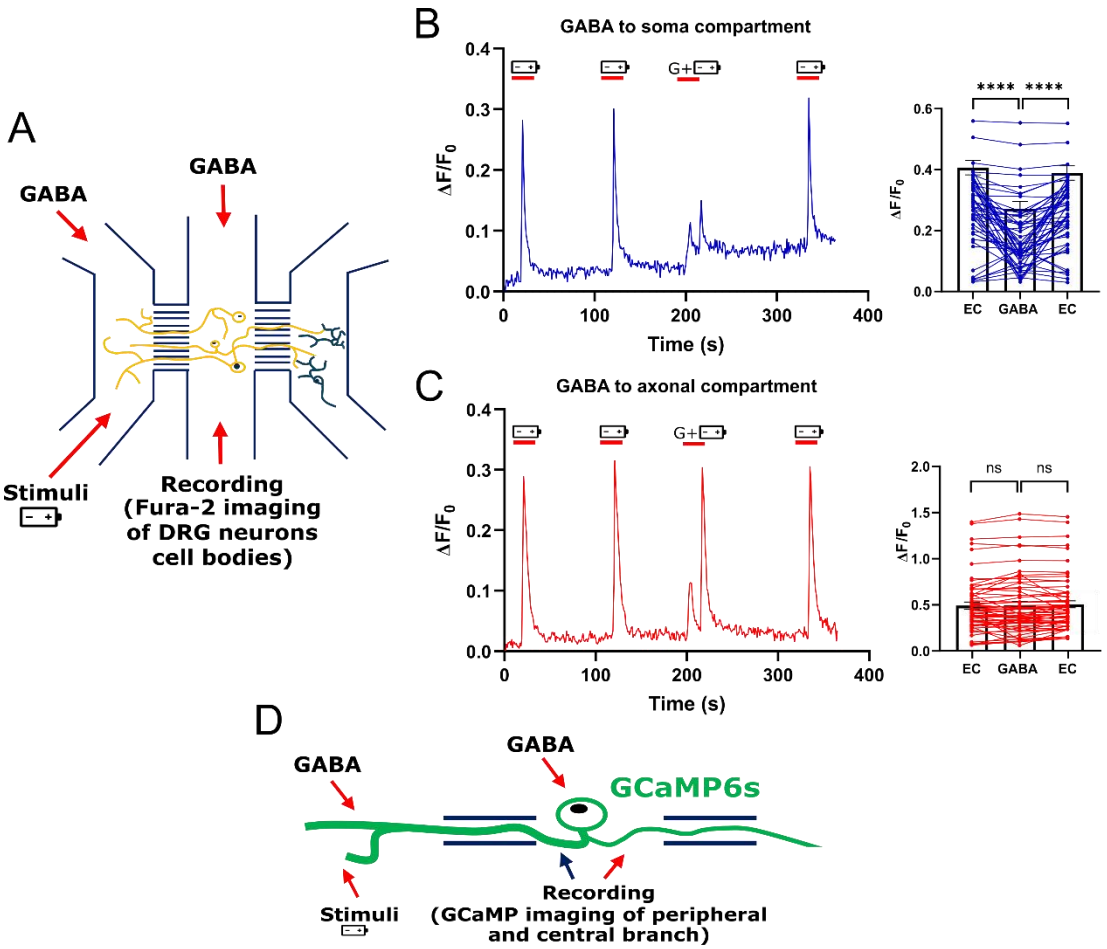
3.3.3 Soma but not axonal GABA application decreases DRG neuron responses to electrical stimulation

Previous work from our group demonstrated that GABA application to the DRGs reduces pain transmission through the ganglia (Du et al., 2017; Hao et al., 2023; Lall et al., 2025). Activation of somatic $GABA_A$ receptors increases Cl^- conductance, which lowers membrane resistance and produces shunting inhibition at the bifurcation. In addition, GABA-induced depolarization can inactivate Na_v channels at the T-junction (Du et al., 2017; Hao et al., 2023; Lall et al., 2025). Together, these mechanisms reduce the likelihood that action potentials generated in the periphery propagate to the spinal cord, by enhancing T-junction filtering. Based on these findings, we aimed to confirm whether this mechanism operates in our model system. In particular, we wanted to directly compare the effects of somatic vs. peripheral $GABA_A$ receptor activation, to determine whether compartment-specific GABA signalling differentially modulates action potential propagation.

Using our microfluidic setup, we applied GABA (100 µM) selectively to either the soma or axonal compartment of the Fura2-loaded microfluidic culture, while recording somatic Ca^{2+} responses to peripheral electrical stimulation (5Hz, 1s) (Figure 3.4A; method section 2.1.7). In both compartments GABA induced mild somatic depolarization which was detected as a small Ca^{2+} influx preceding the electrical stimulation. However, only somatic GABA application reduced the Ca^{2+} response evoked by peripheral electric field stimulation (19 ± 8% reduction; $p<0.0001$; N=4; Figure 3.4B). Axonal GABA application had no effect on the propagation of the peripheral signal ($p=0.62$; N=4), indicating that peripheral axonal $GABA_A$ receptors do not modulate somatic/T-junctional filtering (Figure 3.4C).

To further characterize activity at the T-junction, we expressed the Ca^{2+} sensor GCaMP6s in neurons via AAV9 transduction, as previously described. GCaMP6 enabled axonal Ca^{2+} imaging, with fluorescence monitored in ROIs drawn from the bifurcation

point and extending approximately 20 μm along each daughter branch. Branches were distinguished based on their diameter, with the thicker branch designated as peripheral-like and the thinner branch as central-like (Figure 3.4D). Pseudo-unipolar GCaMP6-positive neurons were selected and tested for responses to peripheral electrical stimuli (Figure 3.4E). We found that peripheral electrical stimulation evoked significantly smaller responses in both the peripheral (29.8 ± 6.6 % reduction, $p<0.0001$; N=3) and central branches (34.9 ± 8.7 % reduction; $p<0.0001$; N=3) when GABA was applied to the soma, but not to the axonal, compartment (Figure 3.4E-F-G). Notably, the reduction observed in the peripheral branch suggests that $GABA_A$ receptors located in the proximal axon, near the T-junction, contribute to this effect. Because the T-junction resides within the soma compartment, receptors located in this region are directly exposed to GABA delivered to the DRG compartment. Collectively, these results show that $GABA_A$ receptor activation at distinct neuronal compartments differentially modulates signal transmission.


A
GABA
GABA
Stimuli
Recording
(Fura-2 imaging
of DRG neurons
cell bodies)
B
GABA to soma compartment
G+
ΔF/F0
Time (s)
**** ****
EC GABA EC
C
GABA to axonal compartment
G+
ΔF/F0
Time (s)
ns ns
EC GABA EC
D
GABA
GABA
GCaMP6s
Stimuli
Recording
(GCaMP imaging of peripheral
and central branch)

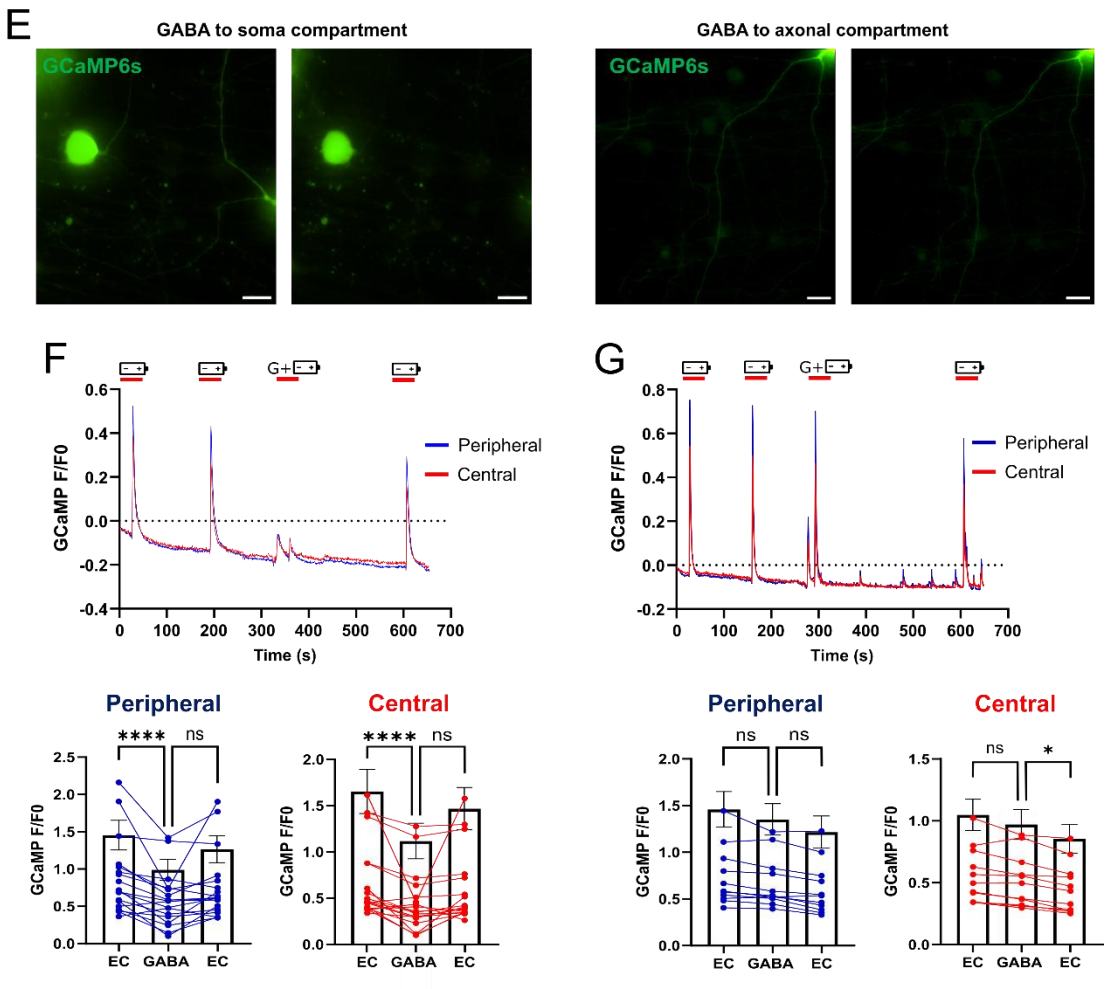

E
GABA to soma compartment
GABA to axonal compartment
GCaMP6s
GCaMP6s
F
G+
GCaMP F/F0
Peripheral
Central
Time (s)
G
G+
GCaMP F/F0
Peripheral
Central
Time (s)
Peripheral
Central
**** ns
**** ns
ns ns
ns *
GCaMP F/F0
EC GABA EC

Figure 3.4 Somatic but not axonal GABAergic signalling modulates DRG neuron activity. Schematic of the experiment. Electrical stimuli (5Hz, 1s) were delivered to the peripheral axonal compartment while Ca^{2+} imaging was performed in the neuronal somata (A). Representative trace from DRG neurons cell body showing Ca^{2+} responses to peripheral electrical stimulation before and after the application of GABA (100μM) to the soma compartment. Quantification and statistical analysis displaying a significant reduction to electrical stimulation upon GABA application (Friedman test with Dunn's multiple comparison test, ****p<0.0001; n=52, N=3) (B). Traces showing Ca^{2+} responses to electrical stimulation before and after GABA application to the axonal compartment. Quantification shows that axonal GABA application does not reduce the Ca^{2+} response to electrical stimulation (Friedman test with Dunn's multiple comparison test, p=0.626, p=0.06; n=62, N=3) (C). Schematic of GCaMP6 imaging after peripheral electrical stimulation (1s, 5Hz). Imaging performed in the peripheral and central branches, near the bifurcation (D). Representative images showing GCaMP6 expression in DRG neurons and responses to peripheral electrical stimulation before and after GABA application to the somatic (left) and axonal (right) compartment (Scale bar=20μm) (E). Representative traces and statistical quantification show that GABA delivery to the somatic compartment decreases GCaMP6 response in both the peripheral and central branch (Friedman test with Dunn's multiple comparison test, Peripheral ****p<0.0001, p=0.267; Central p<0.0001, p=0.06; n=18 N=3) (F). Representative traces and statistical quantification show that GABA delivery to the axonal compartment does not significantly decrease GCaMP6 response in the peripheral branch. The response is slightly decreased in the central branch, likely due to bleaching (Friedman test with Dunn's multiple comparison test, Peripheral p=0.1, p=0.1; Central p=0.1; p=0.05; n=12 N=3) (G).

3.3.4 Soma but not axonal GABA application to DRG neurons, decreases signal transmission to DH neurons

The microfluidic co-culture system enabled to reconstruct the first sensory synapse between the first- and the second-order neuron *in vitro*. By DIV10, DRG neuron axons had crossed multiple microgrooves and reached the DH compartment, where they established synaptic contacts with DH neurons. Consistent with previous reports (Vysokov et al., 2019), we observed that 5s, 5Hz electrical stimulation to the DRG neuron peripheral axons reliably elicited Ca^{2+} responses in a subset of DH neurons, indicating that functional synaptic contacts had been established between DRG and DH neurons (Figure 3.5A; method section 2.1.7).

We therefore set out to investigate how GABAergic signalling modulates DRG-to-DH transmission. Using the same experimental approach described before, we monitored Ca^{2+} responses to peripheral electrical stimulation (5 Hz, 5 s) in DH neurons using Fura-2 live imaging. Consistent with the reduction in DRG neuron activity observed following somatic GABA application, we found that GABA delivery to the DRG somatic compartment significantly attenuated stimulus-evoked Ca^{2+} responses in DH neurons (35.4 ± 4.9% reduction; $p<0.0001$; N=3; Figure 3.5B), indicating a corresponding decrease in DRG-to-DH synaptic transmission. In contrast, when GABA was delivered to the DRG axonal compartment, no significant changes were detected in DH neuron responses ($p=0.09$; N=3; Figure 3.5C). These findings support the hypothesis that only $GABA_A$ receptors located at the soma and T-junction of DRG neurons modulate junctional filtering which downstream, shapes central signal transmission.

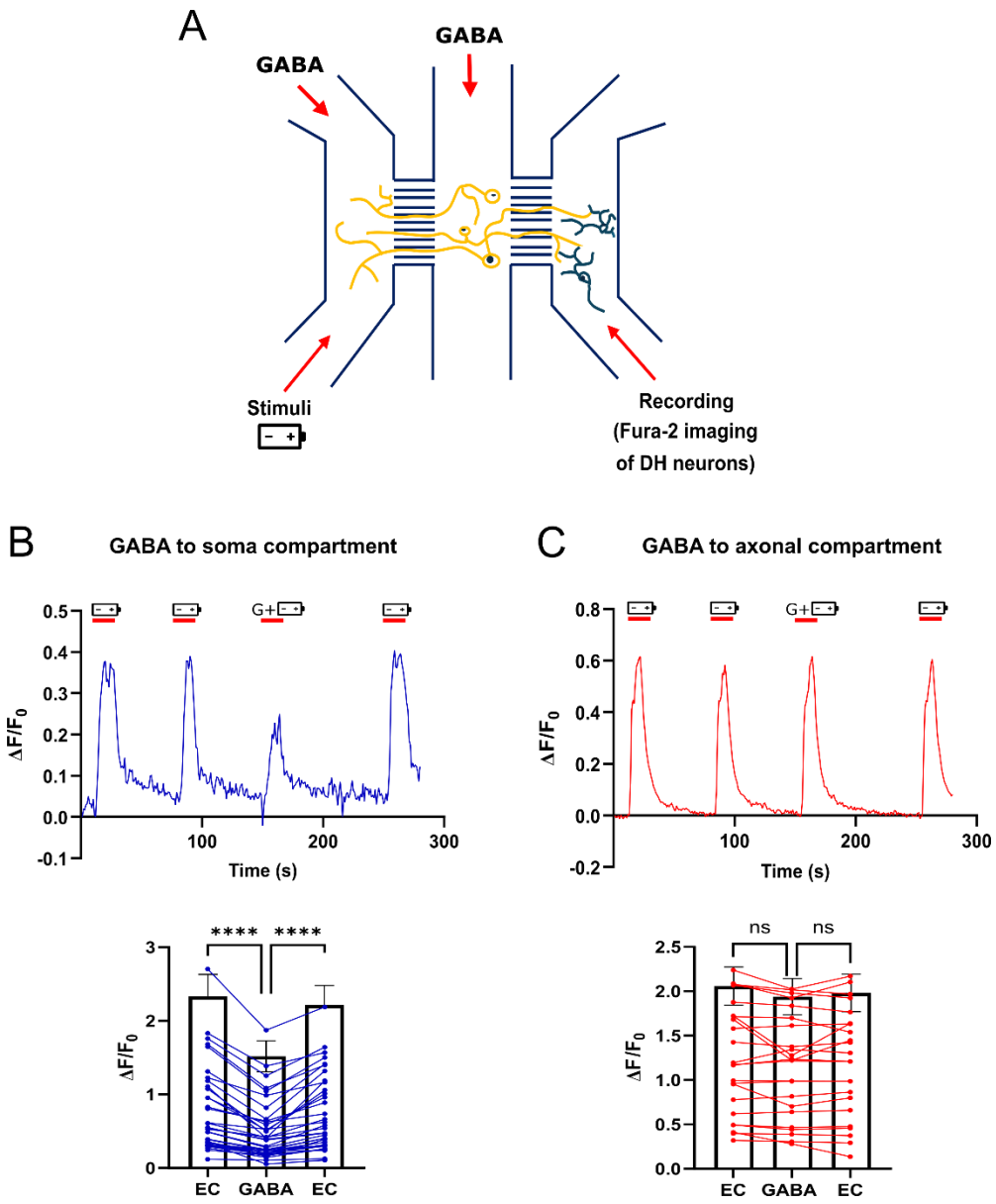


Figure 3.5 . Soma- but not axon-targeted GABA application modulates DRG-to-DH signal transmission in microfluidic co-cultures.
Schematic representation of the set-up (A). Representative Ca2+ traces and quantification showing that GABA application to the DRG somatic compartment significantly reduced the Ca2+ responses evoked in DH neurons (Friedman test with Dunn's multiple comparison test, ****p<0.0001; n=35, N=3) (B). In contrast, GABA application to the DRG axonal compartment did not alter DH calcium responses (Friedman test with Dunn's multiple comparison test, p=0.1, p=0.549; n=23, N=3) (C).

3.3.5 DRG neuron axons release GABA upon stimulation

DRG neuron somata both release and respond to GABA. Previous work from our group has shown that somatic GABA release occurs via a vesicular, VGAT-dependent mechanisms (Hao et al., 2023). In addition, Hanack and colleagues suggested that GABA can be released by peripheral nociceptive endings innervating the mouse skin, in an activity-dependent manner, although it was not functionally demonstrated (Hanack et al., 2015). Here we aimed to elucidate whether similar mechanisms occur at axonal level.

To monitor axonal GABA release, we used live imaging of HEK293 reporter cells co-transfected with the $GABA_A$ receptor subunits α_1, β_2 and γ_2 together with the I^- sensitive EYFP derivative H148Q/I152L (EYFP-QL), a method which has been already corroborated by our lab (Hao et al., 2023; Li et al., 2024; Shah et al., 2020) (method section 2.1.8). Following transfection, reporter HEK cells were co-cultured within the axonal compartment of the microfluidic device (Figure 3.6A). The following day EYFP-QL live imaging was performed. EYFP-QL fluorescence decreases upon I^- binding; thus, in the presence of 5 mM extracellular NaI, the activation of $GABA_A$ receptors expressed by HEK cells, opens Cl^--permeable channels, allowing I^- influx and resulting in the quenching of EYFP-QL fluorescence (Figure 3.6B).

EYFP-QL fluorescence was monitored in HEK reporter cells either co-cultured with DRG neuron axons or monocultured in a standard dish, which served as control. At the end of each experiment, GABA (100μM) was applied as a positive control and it was detected as a strong drop in EYFP-QL fluorescence. We observed that application of 50 mM extracellular KCl to the axonal compartment, predicted to strongly depolarize neurons, induced robust EYFP-QL quenching in HEK reporter cells co-cultured with DRG neuron axons, whereas minimal changes, likely due to leakage, were observed in monocultured HEK cells (F-F0: co-culture 0.04 ± 0.002 vs. monoculture 0.02 ± 0.002; p=0.02; N=4).

Bicuculline application to the periphery compartment significantly reduced KCl-evoked EYFP-QL quenching in co-cultured reporter cells (0.025 ± 0.001; p=0.01 vs. co-culture;

N=4), confirming depolarization induces GABA release from DRG neuron axons (Figure 3.6C). Similar results were obtained following capsaicin stimulation, further supporting activity-dependent axonal GABA release (F/F0: co-culture 0.03 ± 0.002 vs. monoculture 0.01 ± 0.002 vs. bicuculline 0.01 ± 0.0008; $p<0.0001$; N=4; Figure 3.6D).

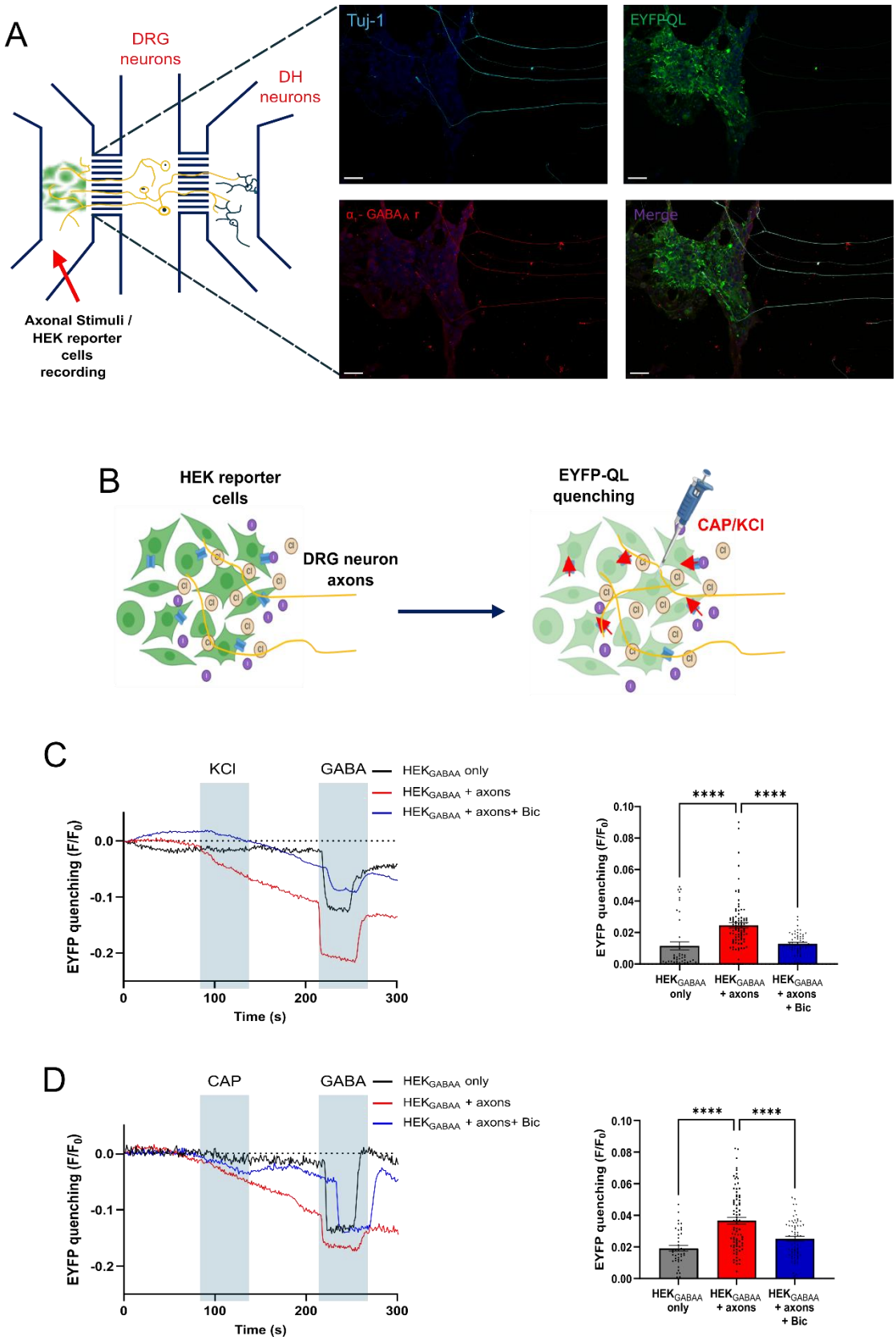


Figure 3.6 DRG neuron axons release GABA.
Schematic representation and immunofluorescence of HEK reporter cells co-transfected with α_1, β_2 and γ_2 subunits of the $GABA_A$ receptor together with the I^- sensitive EYFP derivative H148Q/I152L (EYFP-QL), co-cultured in the axonal compartment of microfluidic devices. Tuj-1-positive DRG neurons axons crossing to the periphery compartment are positive for V-GAT (Scale bar=20μm) (A). Schematic representation of EYFP-QL imaging. GABA release causes I^- influx in

the HEK reporter cells through $GABA_A$ channels. I^- binding to EYFP-QL quenches its fluorescence (B). Representative traces showing EYFP-QL fluorescence quenching in HEK reporter cells in monoculture (black), co-culture with DRG axons in absence (red) and presence (blue) of bicuculline. The trace and quantification show that upon KCl treatment, HEK cells EYFP-QL fluorescence is significantly quenched when the cells are co-cultured with DRG neurons axons, indicating GABA release. The effect is significantly attenuated in monoculture and in co-culture, when bicuculline is added (One-way Anova with Dunnett's multiple comparison test, ****p<0.0001; n=40, n=82, n=56; N=3) (C). A similar effect is obtained upon treatment with Capsaicin (One-way Anova with Dunnett's multiple comparison test, ****p<0.0001; n=41, n=86, n=60, N=3) (D*).*

3.3.6 Ex vivo analysis of $GABA_A$ receptor distribution in DRG and peripheral tissues

To confirm the expression of $GABA_A$ receptors across DRG neuron compartments *ex vivo*, we performed histochemical analyses of different tissue slices from P28 rats. To access different segments, whole DRGs with attached SN and DR, as well as sciatic nerves and glabrous skin from the paw were dissected. Following cryoprotection and OCT embedding, tissues were sectioned at 20 µm using a cryostat, stained with antibodies targeting the α_1, α_2, β_3 and γ_2 subunits, and imaged with confocal fluorescence microscopy.

Immunofluorescence for all subunits was detected in primary sensory neuron somata within the DRG and in both the SN and DR emerging from the ganglia (Figure 3.7A-B). However, due to the high density of neuronal somata and fibres, visualizing individual bifurcations was technically challenging. We were therefore unable to assess differences in distribution between peripheral and central branches near the T-junctions in these experiments.

All four subunits were also detected in the sciatic nerve, indicating peripheral localization of $GABA_A$ receptors (Figure 3.7C) Notably, Tuj-1 positive fibres were observed in the glabrous skin, but none of them showed $GABA_A$ receptor subunit staining, suggesting that receptor transport may not extend to the terminal nerve endings (Figure 3.8).

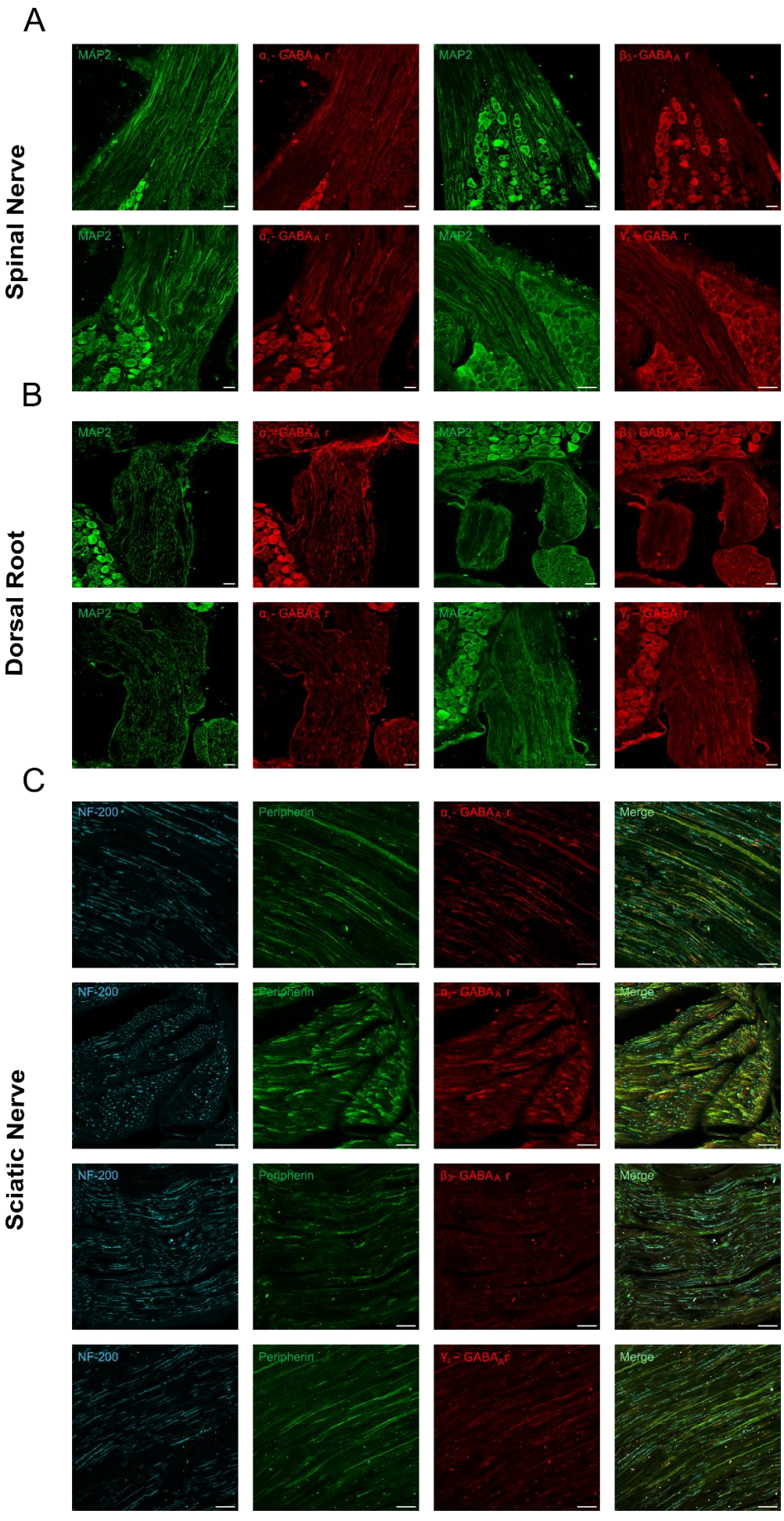

A
Spinal Nerve
MAP2
α1- GABAA r
MAP2
β3- GABAA r
MAP2
α2- GABAA r
MAP2
γ2- GABA r
B
Dorsal Root
MAP2
α1- GABAA r
MAP2
β3- GABAA r
MAP2
α2- GABAA r
MAP2
γ2- GABA r
C
Sciatic Nerve
NF-200
Peripherin
α1- GABAA r
Merge
NF-200
Peripherin
α2- GABAA r
Merge
NF-200
Peripherin
β3- GABAA r
Merge
NF-200
Peripherin
γ2- GABAA r
Merge

Figure 3.7 Expression of $GABA_A$ receptor subunits in the spinal nerve, dorsal root and sciatic nerve of the rat.
α_1, α_2, β_3 and γ_2 subunits of the $GABA_A$ receptor (red) were detected in the spinal nerve and dorsal root of rat DRG neurons alongside MAP2 (green). All subunits are expressed in the sciatic nerve where they co-localize with both NF200 (cyan) and Peripherin (green) (Scale bar=50μm).

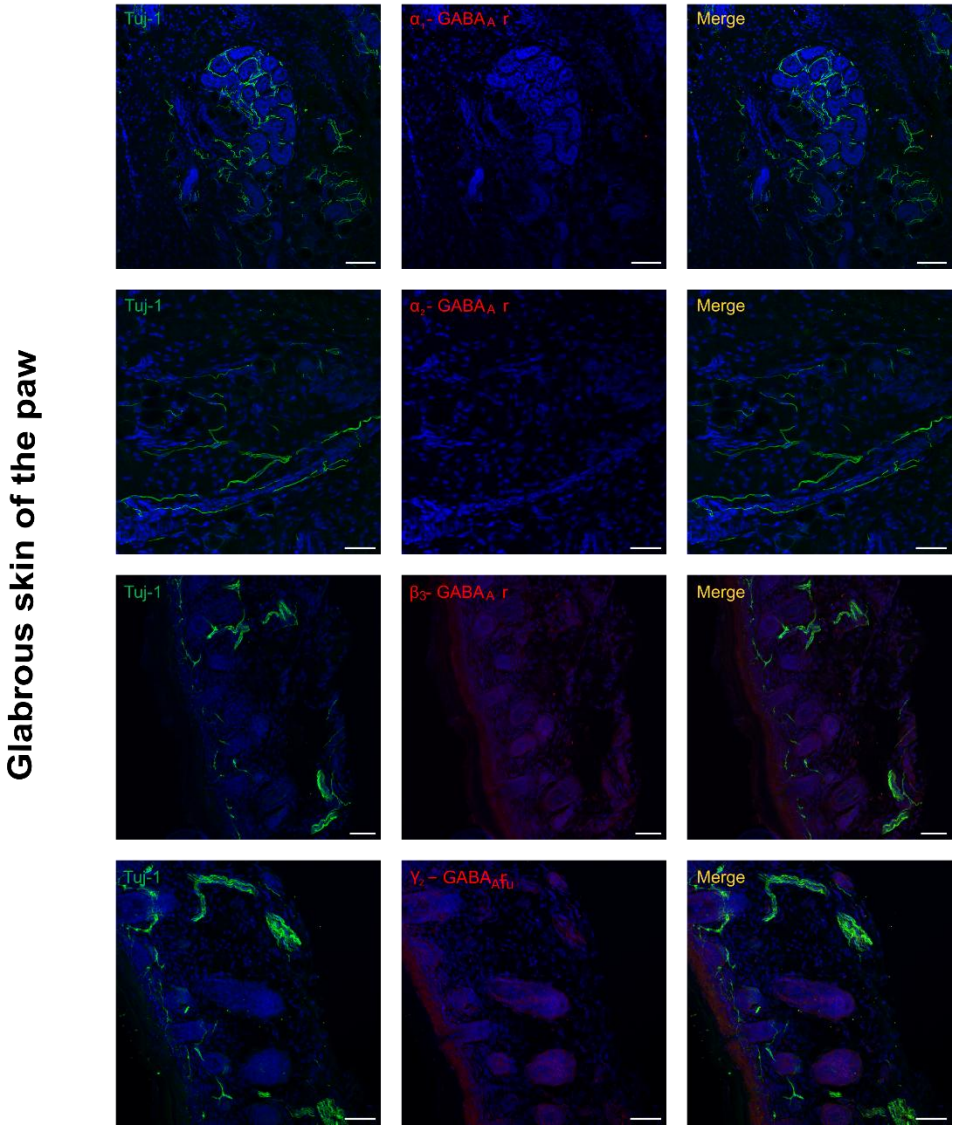


Figure 3.8 Expression of $GABA_A$ receptor subunits in the glabrous skin of the rat paw.
No fluorescence from α_1, α_2, β_3 and γ_2 subunits of the $GABA_A$ receptor was detected in the nerve endings innervating the glabrous skin of the rat. Axons are stained with Tuj-1 (green) (Scale bar=50μm) (A).

3.4 Discussion

The results obtained in this study further characterize the role of GABAergic signalling in primary sensory neurons. By employing an innovative co-culture *in vitro* model (Vysokov et al., 2019), we were able to investigate local events at distinct neuronal compartments, thereby deepening our understanding of local modulatory mechanisms within DRG neurons.

Our group had previously demonstrated that primary sensory neuron somata can both release and respond to GABA. GABAergic signalling within the ganglion establishes a local inhibitory tone that modulates the transmission of nociceptive information from the periphery to the spinal cord (Du et al., 2017; Hao et al., 2023; Lall et al., 2025). In this study, we focused on dissecting compartment-specific mechanisms, which are typically difficult to manipulate experimentally, to determine how local GABAergic activity shapes neuronal behaviour.

We showed that culturing DRG neurons in tri-compartment microfluidic devices promotes the acquisition of a pseudo-unipolar morphology, a feature that typically lacks in standard cultures. In addition, the compartmentalization of the chamber allowed for the separation of the somatic compartment from the axonal ones, enabling selective compound delivery and establishing a spatially organized configuration that recapitulates the peripheral axon, the ganglion region and the central axon synapsing onto DH neurons in the spinal cord. The slight positive pressure maintained in the soma compartment - by using a higher medium volume relative to the lateral compartments - likely facilitated bilateral axonal outgrowth and pseudo-unipolarization. Remarkably, co-culture with DH neurons, significantly increased the proportion of pseudo-unipolar DRG neurons. We hypothesize that DH neurons provide instructive cues that facilitate DRG neuron correct morphological maturation, consistent with developmental studies showing that in mice, DRG neurons pseudo-unipolarization follows spinal connections (Barber & Vaughn, 1986).

Immunocytochemical and morphological analysis validated the physiological relevance of this model, demonstrating that a subset of pseudo-unipolar neurons display polarized branches. The marker MAP2, previously reported to be expressed in the stem and peripheral axon, labelled predominantly the soma, the stem axon and showed enhanced intensity in the thicker branch emerging from the bifurcation, consistent with the literature (Gumy et al., 2017; Papasozomenos et al., 1985; Suh et al., 1984). Given recent evidence that MAP2 directs cargo entry at the stem axon by coordinating the activity of molecular motors (Gumy et al., 2017), it may also help control directional transport between the two branches, particularly in the peripheral branch, where it is predominantly expressed. Cultured DRG neurons exhibited two axonal branches with distinct diameters (~1 µm and ~0.5 µm), closely matching *in vivo* measurements (Hoheisel & Mense, 1986; Lee et al., 1986; Suh et al., 1984). Importantly, these neurons remained functionally competent, as peripheral axonal stimulation reliably evoked Ca^{2+} responses in DRG and DH neuron cell bodies, confirming preserved signal propagation across compartments. Although our microfluidic co-culture system reliably promotes the development of pseudo-unipolarization and polarized branches, we cannot definitely ascertain that in every neuron, the thicker branch consistently projects toward the peripheral compartment while the thinner one exclusively reaches the DH compartment. Nevertheless, the overall structural and functional features of the system closely resemble the *in vivo* organization of DRG neurons and first sensory synapse

Consistent with previous reports (Du et al., 2017; Ma et al., 1993; Maddox et al., 2004; Obradovic et al., 2015), immunocytochemical analysis revealed that $GABA_A$ receptor subunits, including α_1, α_2, β_3, γ_2, were expressed in both the soma and the axons of DRG neurons. Similar to the distribution of MAP2, these subunits were enriched in the stem axon and proximal regions near the bifurcation, with stronger expression in the peripheral-like branch, while remining detectable in distal axonal segments. Although we could not quantify fluorescence due to technical limitation, *ex vivo* immunohistochemistry confirmed the expression of the same $GABA_A$ receptor subunits in primary sensory

neuron somata and along the axons within the DRG and sciatic nerve. Due to the dense intermingling of fibres within the ganglion, we could not isolate individual T-junctions, precluding imaging and quantification at this site. Interestingly, we did not detect any $GABA_A$ receptor subunits at peripheral nerve endings in the glabrous skin of the rat paw, suggesting that these receptors are not transported to the most distal sensory terminals. In contrast, $GABA_A$ receptor fluorescence was observed at the axon terminals in the microfluidic co-culture system. This discrepancy likely reflects differences between *in vitro* and *in vivo* conditions, where altered trafficking due for instance by the absence of target-derived cues, may allow ectopic receptor expression in culture.

To investigate the functional role of GABAergic receptors at different subcellular localizations, we selectively applied GABA either to the axonal or somatic compartment of the microfluidic device, taking advantage of fluidic isolation to restrict the compound diffusion. Local application of GABA to the peripheral axonal compartment, elicited Ca^{2+} influx in DRG neuron somata which was significantly attenuated by both bicuculline and TTX, indicating that activation of axonal $GABA_A$ receptors induces depolarization sufficient to trigger action potentials that propagate toward the cell body. These findings demonstrate that peripheral GABAergic signalling is depolarizing and can promote excitability at the level of the axon, in accordance with early and more recent studies (Bhisitkul et al., 1990; Bonalume et al., 2021; Jang et al., 2017).

At the level of the soma, GABAergic signalling is also depolarizing due to the high intracellular Cl^- concentration characteristic of DRG neurons (Bowery & Brown, 1974; De Groat, 1972; Feltz & Rasminsky, 1974; Hösli et al., 1978; Wilke et al., 2020). However, although we observed that somatic GABA application produced a detectable Ca^{2+} signal, it also decreased neuronal excitation, in line with what has been reported by our group in previous investigations (Du et al., 2017; Hao et al., 2023; Lall et al., 2025). Specifically, we observed that somatic, but not axonal, GABA application significantly reduced somatic Ca^{2+} responses evoked by electrical stimulation in the peripheral compartment. This can be explained by the unique electrotonic properties of the T-junction: $GABA_A$

mediated conductances at the soma and proximal axon induced shunting inhibition at the T-junction by lowering membrane resistance, while also partially inactivating Na_v channels, thereby reducing action potential propagation through the bifurcation (Du et al., 2017; Hao et al., 2023).

Beyond somatic imaging, using the Ca^{2+} sensor GCaMP6s, we managed to investigate Ca^{2+} activity at the T-junction. We observed that Ca^{2+} responses evoked by peripheral electrical stimulation in both the peripheral and central branches adjacent to the bifurcation were attenuated following GABA application to the somatic compartment, but not to the peripheral compartment. In most neurons GABA itself induced depolarization at the T-junction, however, when applied to the DRG somatic compartment, it still produced a net inhibition of signal transmission. Interestingly, the reduced Ca^{2+} response was detected in both branches generating from the bifurcation, suggesting receptors located in the proximal peripheral branch contribute to the modulation of signal transmission. Given that *in vivo*, T-junctions are distributed through the ganglion, they could lie near neighbouring cell somata and $GABA_A$ receptors located at the bifurcation could therefore be affected by local GABA release (e.g. via volume transmission). This data supports the concept that activation of $GABA_A$ receptors at distinct locations along primary sensory neurons differentially affect signal transmission. While $GABA_A$ receptors expressed along the distal peripheral axon appear to be primarily depolarizing and, under this experimental condition, do not significantly regulate spike propagation, receptors located in the proximal peripheral axon near the bifurcation, shape signal transmission, likely via shunting inhibition. This spatially restricted organization provides a mechanistic framework to reconcile previous conflicting reports on peripheral GABAergic effects and underscores the importance of anatomical localization when interpreting the functional consequences of $GABA_A$ receptor activation in primary sensory neurons.

Having established that GABAergic signalling at the primary sensory neuron soma and T-junction exerts a strong inhibitory control over action potential propagation, we next asked whether these compartment-specific effects translate into altered signalling at the

first central synapse. Although cultured DH neurons can exhibit spontaneous activity, their evoked responses to peripheral stimulation depend on neurotransmitter release at the central terminals. We therefore examined how localized modulation at different DRG neuron compartments affects DH neuron responses. In line with previous observations, peripheral electrical stimulation of DRG neuron axons elicited Ca^{2+} responses in DH neurons, suggesting functional synaptic transmission (Vysokov et al., 2019). DH neuron responses were attenuated by GABA application to the DRG somatic compartment, whereas peripheral axonal GABA stimulation did not produce any detectable effects. This confirms that GABAergic modulation at the soma and T-junction directly affect central transmission, likely by reducing neurotransmitter release through decreased upstream excitability.

Given that $GABA_A$ receptors are expressed throughout the peripheral axon, we sought to explore peripheral sources of GABA. Our group had already demonstrated that DRG neuron somata can release GABA and we therefore wanted to investigate whether similar mechanisms took place at axonal level. Using our “sniffing” imaging approach in which indicator $HEK_{GABAA\text{-}EYFP}$ cells were co-cultured with DRG neuron axons in the periphery compartment, we detected GABA release from primary sensory neuron axons upon stimulation with capsaicin or high K^+. This is in line with what has been reported by Hanack and colleagues, who suggested that GABA was released by peripheral nerve endings in the cornea and glabrous skin of the mouse, following capsaicin stimulation (Hanack et al., 2015). Although our data did not show any modulatory effect of peripheral GABAergic stimulation on signal transmission, it is plausible that in different settings, axonal GABA release exerts autocrine or paracrine effects on peripheral fibres.

In summary, our findings delineate a compartment-specific organization of GABAergic signalling within DRG neurons and identify the soma and T-junction region as a pivotal site for modulation of sensory input. Using a physiologically relevant microfluidic co-culture system that preserves pseudo-unipolar morphology and functional connectivity with DH neurons, we demonstrate that local GABAergic activity within the ganglion exerts

inhibitory control over signal propagation and central transmission. These results expand the traditional view of GABA as a purely spinal inhibitory neurotransmitter, revealing its additional role as a local modulator of primary sensory neuron excitability, thus suggesting potential strategies to modulate pain transmission through selective targeting of soma/T-junction GABAergic mechanisms.

4 Chapter 4: Schwann Cells as a peripheral source of GABA

4.1 Introduction

4.1.1 Schwann cells are key regulators of peripheral nerve regeneration and pain modulation

Schwann cells are the principal glial cells of the PNS and play an essential role in axonal development, maintenance of regeneration. Their exceptional capacity to transition between differentiated and reparative states underlies the unique regenerative potential of the PNS. When a peripheral nerve is damaged, Schwann cells are among the first responders: they initiate a coordinated program of molecular and phenotypic changes that shape different stages of regeneration (Jessen et al., 2015, Wei et al., 2019). These same processes also contribute to the modulation of nociception and the development of neuropathic pain, placing Schwann cells at the intersection of tissue repair and pathological sensory processing.

This reprogramming is controlled by intrinsic transcriptional regulators and cues from the surrounding microenvironment which together drive the suppression of myelin-associated genes while activating a distinct repair-promoting gene expression program (Arthur-Farraj et al., 2012). Dedifferentiated Schwann cells acquire the ability to phagocytose myelin debris, a critical step in creating a permissive environment for regeneration (Nocera et al., 2020). Within the first week after injury, they can clear roughly half of the myelin remnants, a process later amplified by the recruitment of macrophages (Hirata et al., 2002). In parallel, Schwann cells adopt an elongated bipolar morphology and assemble into Büngner bands - longitudinal cellular columns that provide structural tracks for regenerating axons to navigate toward their targets (Jessen et al., 2019 Jessen et al., 2016; Gomez-Sanchez et al., 2017). Throughout these early phases, Schwann cells also secrete neurotrophic factors such as NGF, BDNF, and

GDNF, which support neuronal survival and stimulate axonal elongation (Brushart et al., 2013; Xu et al., 2009).

The reparative actions of Schwann cells are intimately linked to sensory signalling and can contribute to neuropathic pain. Activated Schwann cells release a broad range of cytokines and chemokines, including IL-6, TNF-α, and MCP-1, which not only recruit immune cells, but also sensitize nociceptors, thereby altering pain thresholds (Nazareth et al. 2022). They also secrete ATP, neuropeptides, and ROS that act on nearby sensory fibres in a paracrine manner to enhance neuronal excitability. Their communication with nerve-resident and invading macrophages is equally essential: Schwann cell-derived mediators initiate macrophage activation and monocyte recruitment, while macrophage-secreted factors direct Schwann cell migration, promote angiogenesis in the developing nerve bridge (Blockus & Chedotal 2016; Cattin et al. 2015; Koch et al. 2011), and later trigger their transition back to a myelinating state (Stratton et al. 2018). Together, these multifaceted roles position Schwann cells as central coordinators of peripheral nerve regeneration and key modulators of sensory physiology.

4.1.2 Schwann cells release and respond to GABA

Beyond their well-established roles in axon repair, immune regulation, and nociceptive signalling, Schwann cells also engage in a distinct form of neurotransmitter-like communication. Emerging evidence shows that Schwann cells can both produce and respond to the inhibitory neurotransmitter GABA, adding an additional regulatory axis that links Schwann cell differentiation, neuron-glia communication, and inflammatory modulation. Multiple studies - both *in vitro* and *in vivo* - have demonstrated expression of $GABA_B$ receptor subunits ($GABA_{B1a}$, $GABA_{B1b}$ and $GABA_{B2}$) and $GABA_A$ (α_2, α_3 and $\beta_{1\text{-}3}$) in Schwann cells, Schwann cells precursors and adult bone marrow stem cell-derived Schwann cells (Faroni et al., 2019; Procacci et al., 2013). The engagement of the two receptors activates diverse intracellular signalling pathways which lead to different functional outcomes.

$GABA_B$ receptors are metabotropic receptors coupled to $G_{i/o}$ type of G_α proteins; their activation suppresses adenylate cyclase activity, which, by lowering intracellular cAMP, suppresses PKA/CREB-mediated transcriptional program controlling proliferation and myelin-formation (Magnaghi et al., 2004). *In vivo*, conditional deletion of $GABA_B$ receptors in Schwann cells leads to an increase in small unmyelinated fibres and Remak bundles, altered peripheral myelin morphology and a behavioural phenotype of hyperalgesia. This highlights that $GABA_B$-mediated signalling in Schwann cells influences axon-glia interactions, myelination and nociceptive fibres architecture (Faroni et al., 2014; Magnaghi et al., 2004). Conversely, $GABA_A$ receptors are ionotropic receptors that form a Cl^- channel; these are also expressed by SC-like bone marrow-derived stem cells, and their activation has been shown to promote Schwann cell differentiation, driving the transition from immature, proliferative states to more mature, myelinating phenotypes (Faroni et al., 2012).

Notably, RNAseq data of the rodent sciatic nerve have shown expression of several $GABA_A$ related genes in Schwann cells, although expression appears to vary with developmental stage and Schwann cell subtype. Diverse subunits of the $GABA_A$ receptor have been detected in Schwann cell precursors and in differentiated non-myelinating Schwann cells (Gerber et al., 2021). The α_1 was enriched primarily in Schwann cell precursors, while the α_3 was detected in precursors and persisted into adulthood, particularly in non-myelinating Schwann cells. Additional subunits including β_2, β_3 were detected in early embryonic days whereas the ε and θ subunits were also present in differentiated non-myelinating Schwann cells. Interestingly, the α_3, ε and θ subunits were reported to be downregulated Schwann cells in a transcriptomic analysis carried out on injured mouse nerves (Clements et al., 2017).

Schwann cells also possess the enzymatic machinery necessary to synthetize, release and uptake GABA, expressing the glutamate decarboxylases GAD65, GAD67 and GABA transporters. Magnaghi and colleagues showed that Schwann cells can synthetize GABA *in vitro* and that treatment with neuroactive steroids such as

allopregnanolone further enhances GAD67 expression and GABA synthesis via a cAMP/PKA/CREB-dependent pathway (Magnaghi et al., 2010). Transcriptomic datasets support this biosynthetic capacity: Gad1 and Gad2, encoding GAD67 and GAD65 respectively, are most strongly expressed during early developmental stages, with Gad2 remaining detectable at lower levels in adulthood. Genes associated with GABA transport are also represented, including robust Slc6a1 (GAT1) expression, particularly in myelinating Schwann cells (Gerber et al., 2021). Immunohistochemical studies in developing peripheral nerve further localized GAD65/67, GABA and $GABA_B$ receptor subunits in pre-myelinating and non-myelinating Schwann cells predominately. $GABA_{B1}$ and $GABA_{B2}$ expression in the sciatic nerve decreases over time, but it's still detectable in the adult nerve (Corell et al., 2015; Gerber et al., 2021).

Notably, RNAseq data show that non-myelinating Schwann cells also express DBI, a peptide belonging to the endozepine family (Renthal et al., 2020). DBI acts as an endogenous $GABA_A$ receptor modulator, binding to the benzodiazepine binding site of the $GABA_A$ receptors to allosterically modulate its activity (Li et al., 2024; Tonon et al., 2020). A different transcriptomic dataset following peripheral nerve transection indicates downregulation of DBI expression after injury, suggesting that endogenous modulation of GABA receptor signalling may be dynamically regulated during nerve degeneration and repair (Clements et al., 2017). Together, these findings suggest that Schwann cells can not only synthesize and respond to GABA but also fine-tune receptor activity, adding an additional layer of regulatory complexity to peripheral GABAergic signalling.

Beyond neuron-glia communication, GABA has been shown to modulate immune cells, including macrophage polarization, promoting an M2 anti-inflammatory phenotype and reducing pro-inflammatory responses (Bhandage & Barragan, 2021; Jin et al., 2013). These findings raise the possibility that Schwann cell-derived GABA may also participate in glia-immune communication, shaping the local inflammatory environment following nerve injury and potentially contributing to both nerve regeneration and pain modulation. Collectively, these observations highlight a multifaceted role for Schwann cells as both

sources and modulators of peripheral GABAergic signalling, influencing neuronal, glial, and immune components within the injured nerve.

4.2 Aim

The aim of this chapter is to determine whether Schwann cells express key components of the GABAergic signalling machinery and to define their cellular and subcellular distribution within the peripheral nerve. Specifically, we seek to: (i) assess and localize the expression of proteins involved in GABA synthesis (GAD67), release (VGAT), uptake (GAT1) and modulation of the $GABA_A$ receptor function (DBI), in primary Schwann cell culture and sciatic nerve tissue using western blotting and immunofluorescence co-labelling; (ii) functionally assess GABA release from Schwann cells; (iii) investigate whether peripheral nerve injury affects the expression of GABAergic proteins in Schwann cells. Together, these approaches are designed to establish whether Schwann cells possess the molecular machinery required for GABA synthesis, transport, and modulation, thereby providing a foundation for understanding potential roles of GABAergic signalling in Schwann cell physiology and peripheral nerve function.

4.3 Results

4.3.1 Schwann cells express the enzymes necessary for the synthesis and release of GABA

Data from the literature indicate that Schwann cells express key proteins involved in GABAergic transmission, including multiple $GABA_A$ and $GABA_B$ receptor subunits, the GABA-synthesizing enzyme GAD67, and the plasma membrane GABA transporter GAT1 (Faroni et al., 2012, 2019; Gerber et al., 2021; Magnaghi et al., 2004, 2010; Procacci et al., 2013). However, the presence of the vesicular GABA transporter (VGAT), which mediates GABA loading into synaptic vesicles in neurons, has not been reported in Schwann cells. As we aimed to characterize potential peripheral sources of GABA, we

sought to determine whether rat Schwann cells express the molecular machinery necessary for GABA synthesis, transport, and release.

To this end, immunohistochemical and Western blot analyses were performed on primary Schwann cell cultures and whole sciatic nerve extracts (method section 2.2.2-2.2.3). We focused on GAD67, VGAT, and GAT1, which represent key elements of the GABA synthesis and transport pathways, and also examined DBI, a positive allosteric modulator of the $GABA_A$ receptor, that is highly expressed in SGCs in the DRG (Li et al., 2024). The Schwann cell marker S100β was used to reliably identify these cells in culture and tissue sections (Figure 4.1A-B).

Primary Schwann cell cultures were obtained from the dissociation of peripheral nerves of newborn rats and reached high purity after two passages, as indicated by strong and uniform S100β staining (method section 2.1.1). For *ex vivo* analysis, whole sciatic nerves (~5cm) were dissected from ~1 month old rats and either fixed for immunohistochemistry or dissociated for Western blotting. Immunofluorescence revealed strong labelling of all proteins of interest in both cultured Schwann cells and sciatic nerve tissue, where they co-localized with S100β (Figure 4.1A-B). In the sciatic nerve, VGAT and GAD67 were also detected in cells not labelled by S100β, suggesting that additional cell types, such as neurons, may also express GABA-related proteins in the peripheral nerve environment.

Western blot analysis confirmed the presence of all four proteins in both primary Schwann cell lysates and sciatic nerve extracts. Bands corresponding to GAD67 (~67 kDa), GAT1 (~67 kDa), and DBI (~10 kDa) were detected at their expected molecular weights. Interestingly, VGAT appeared as a single band at ~50 kDa in Schwann cell cultures but as a doublet (approximately 50 and 57 kDa) in sciatic nerve extracts, suggesting the presence of distinct VGAT isoforms or post-translationally modified forms in the intact nerve (Figure 4.1C). Protein extracts from the spinal cord, where GABAergic signalling is well characterized, were used as positive controls for all targets and consistently displayed the expected expression profiles, thereby confirming antibody

specificity and validating the analyses. These data support a form of non-synaptic, vesicle-mediated GABA release or storage in peripheral nerves, suggesting a previously unrecognized mechanism by which Schwann cells might modulate the local GABAergic microenvironment.

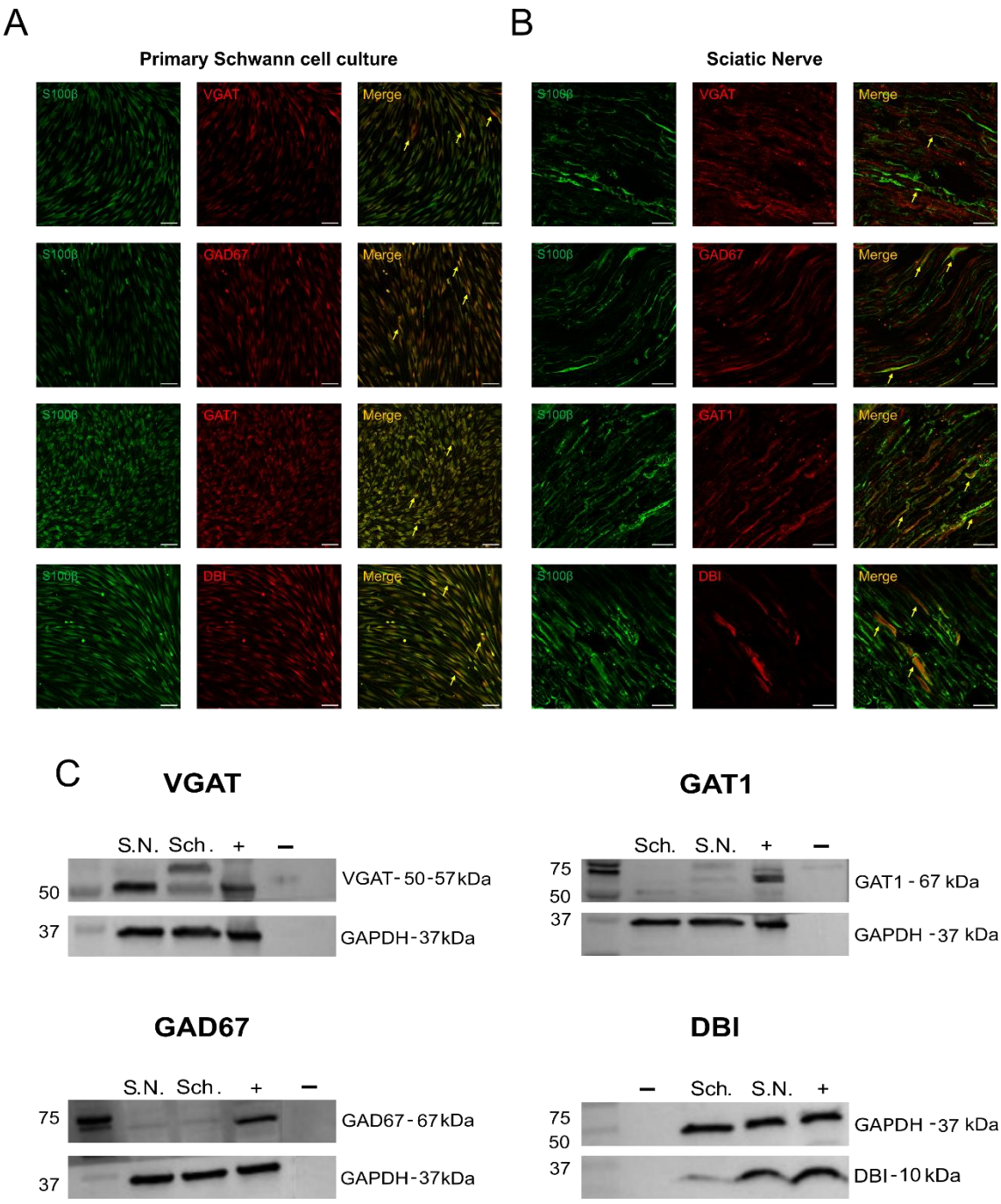


Figure 4.1 VGAT, GAD67, GAT1 and DBI are expressed in rat Schwann cells.
Representative immunofluorescence images of primary rat Schwann cell cultures showing VGAT, GAD67, GAT1, and DBI (red) co-stained with the Schwann cell marker S100β (green) (Scale bar=50µm) (A). In sciatic nerve sections, all four proteins co-localize with S100β$^+$ Schwann cells. VGAT and GAD67 display expression also outside Schwann cells suggesting expression in different cell types (B). Western blot analysis confirmed expression of VGAT (50-57kDa), GAD67 (67 kDa), GAT1 (67 kDa) and DBI (10 kDa) in lysates from primary Schwann cells cultures and sciatic nerves (N=3). Spinal cord tissue was used as positive control whereas lysis buffer served as negative control (C).

4.3.2 Schwann cells tonically release a bioactive ligand of the $GABA_A$ receptor in vitro

To functionally test whether Schwann cells release GABA *in vitro*, we employed the previously described I^- imaging assay (Hao et al., 2023; Shah et al., 2020) (method section 2.1.8). Briefly, HEK293T reporter cells co-transfected with the $GABA_A$ receptor subunits α_1, β_2 and γ_2 together with the I^- sensitive fluorescence protein EYFP-QL, were co-cultured with purified rat Schwann cells (Figure 4.2A). Activation of $GABA_A$ receptors enables channel opening, allowing extracellular I^- (supplied as 5 mM NaI) to enter the HEK298T reporting cells and quench EYFP-QL fluorescence. Therefore, if Schwann cells release a $GABA_A$ receptor ligand or modulator, it would be detected as a reduction in EYFP-QL fluorescence in reporter cells.

Live fluorescence recordings were obtained from Schwann/Reporter HEK co-cultures (Figure 4.2B) and from reporter HEK cells monocultures. Monocultures were used as baseline controls to assess I^- leakage through the $GABA_A$ receptors and/or spontaneous bleaching of EYFP-QL. After a 30s baseline recording under standard extracellular conditions, a solution containing 5mM of NaI was supplemented into the chamber and perfusion was then stopped for 5 min to allow release of signalling molecules from the cells. Perfusion was then resumed, and GABA (100 µM) was applied at the end of each recording as a positive control (Figure 4.2C).

Quantification of EYFP-QL fluorescence quenching during the 5-min stop-flow period revealed a robust and significant decrease in EYFP-QL fluorescence in HEK cells co-cultured with Schwann cells compared to monoculture controls (24 ± 5% quenching in co-culture vs. 13 ± 1% monoculture, p=0.04, N=3; Figure 4.2D). Control HEK reporter cells displayed minimal quenching during stop-flow, indicating a low level of baseline $GABA_A$ receptor activation in the absence of Schwann cell-derived signals. In contrast, the presence of Schwann cells consistently enhanced EYFP-QL quenching, demonstrating that Schwann cells release a factor capable of activating $GABA_A$

receptors on neighbouring HEK reporter cells. As we did not test specific $GABA_A$ receptor inhibitors and considering that both DBI and components of the GABAergic synthetic machinery are expressed by Schwann cells, we cannot conclusively identify the molecular nature of the released factor. It is therefore possible that the observed $GABA_A$ receptor activation is mediated by the release of GABA itself, DBI, or a combination of both.

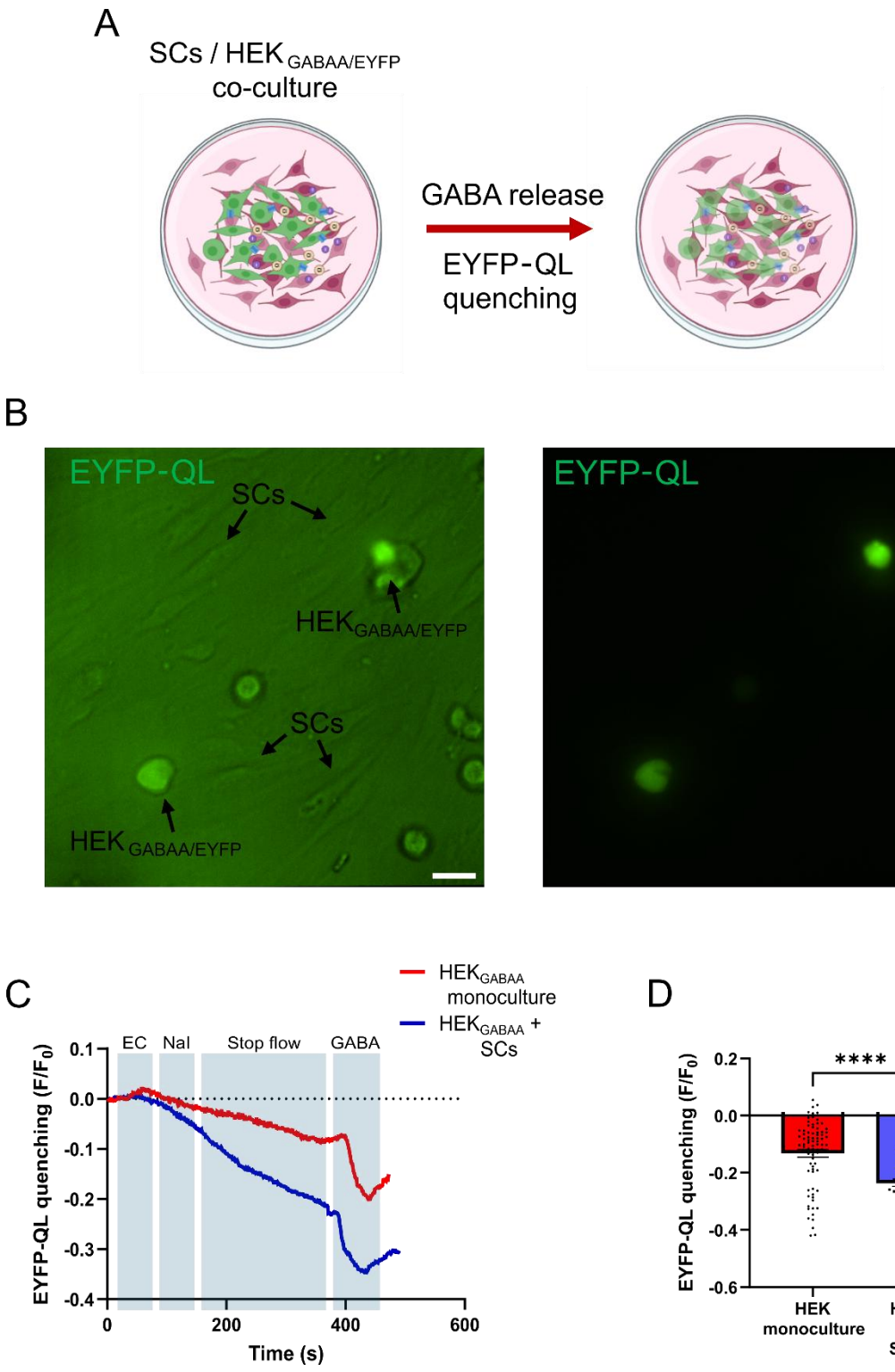


Figure 4.2 Schwann cells release GABA *in vitro*.
Schematic representation of I^- assay in HEK reporter cells co-cultured with Schwann cells. GABA release causes I^- influx in the HEK reporter cells through $GABA_A$ channels which in turn, quenches EYFP-QL fluorescence (A). Representative image of EYFP-QL imaging. HEK reporter cells co-transfected with α_1, β_2 and γ_2 subunits of the $GABA_A$ receptor together with the I^- sensitive EYFP derivative (green), co-cultured with rat primary Schwann cells (Scale bar=20µm) (SCs) culture (B). Representative traces showing EYFP-QL fluorescence quenching in HEK reporter cells in monoculture (red) and co-culture with Schwann cells (blue) (C). Quantification of EYFP-QL quenching during 5 minutes of no flow. EYFP-QL fluorescence is significantly decreased in HEK cells co-cultured with Schwann cells compared to monoculture (Data presented as mean ± SEM; Mann-Whitney test, ****p<0.0001; n=78, n=74, N=3) (C).

4.3.3 PSNL downregulates the expression of GABAergic proteins GAD67, GAT1, VGAT and DBI

Chronic pain has been associated with a downregulation of GABAergic tone both in the spinal cord and within the DRGs, a change that contributes to increased neuronal excitability and nociceptive transmission (Coull et al., 2005; Moore et al., 2002; Scholz et al., 2005; Wang et al., 2021). Given this reduction in inhibitory control, and the emerging evidence that Schwann cells express components of the GABAergic system, we sought to determine whether peripheral nerve injury alters the expression of GABA-related enzymes in Schwann cells.

To address this question, we performed PSNL on adult rats (method section 2.2.4). At 15 or 30 days post-surgery, ~ 5cm sciatic nerve segments were collected at the site of injury from PSNL animals and along the nerve of SHAM controls and they were processed for immunohistochemical and biochemical analyses. Immunohistochemistry was performed to evaluate the expression of GAD67, VGAT, GAT1, and DBI in Schwann cells along the injured sciatic nerve. In control nerves, all markers were clearly detectable in S100β+ Schwann cells and distributed throughout the endoneurial compartment. Following PSNL, fluorescence for all four markers appeared reduced at 15 days and further diminished at 30 days post-injury (Figure 4.3A-B-C-D; Figure 4.4A-B-C-D). Quantitative analysis of fluorescence intensity in S100β+ cells confirmed this observation, revealing a significant decrease in mean signal intensity relative to control samples for GAD67, GAT1 and DBI at 15 days (45 ± 1.7% reduction for GAD67, 13 ± 1.8% for GAT1 and 10 ± 3% for DBI, compared to SHAM controls, N=5; Figure 4.3F-G-H) , with the most pronounced reduction observed at 30 days (56 ± 0.7% reduction for GAD67, 53 ± 1.7 for GAT1, 53 ± 1.2% for DBI, compared to SHAM, N=4; Figure 4.4F-G-H). As fluorescence intensity was quantified specifically within S100β+ Schwann cells, the observed reductions indicate decreased marker expression within Schwann cells themselves. Interestingly, although VGAT fluorescence in the injured sciatic nerve appeared reduced, when we selected S100β+ cells, fluorescence quantification showed

minimal decrease between the two experimental group (no significant reduction at 15 days and 9 ± 2% reduction at 30 days, p=0.01, N=4), indicating that more robust VGAT downregulation may be taking place in other cell types (Figure 4.3E; Figure 4.4E).

To further substantiate these findings, VGAT expression was examined by Western blotting (Figure 4.5A-C). Densitometric analysis of VGAT protein bands (~50 kDa) showed a consistent downregulation over time, with a moderate decrease detected at 15 days and a marked reduction at 30 days post-injury (25 ± 11.7% reduction at 15 days, p=0.32, N=4; 69 ± 13.8% reduction at 30 days, p=0.01; compared to SHAM controls, N=5; Figure 4.5B-D). This indicates that PSNL decreases VGAT expression, possibly in multiple cell types. These results collectively indicate that peripheral nerve injury induces a time-dependent downregulation of GABAergic components in Schwann cells, suggesting a progressive impairment of GABA metabolism and signalling in the injured peripheral nerve microenvironment.

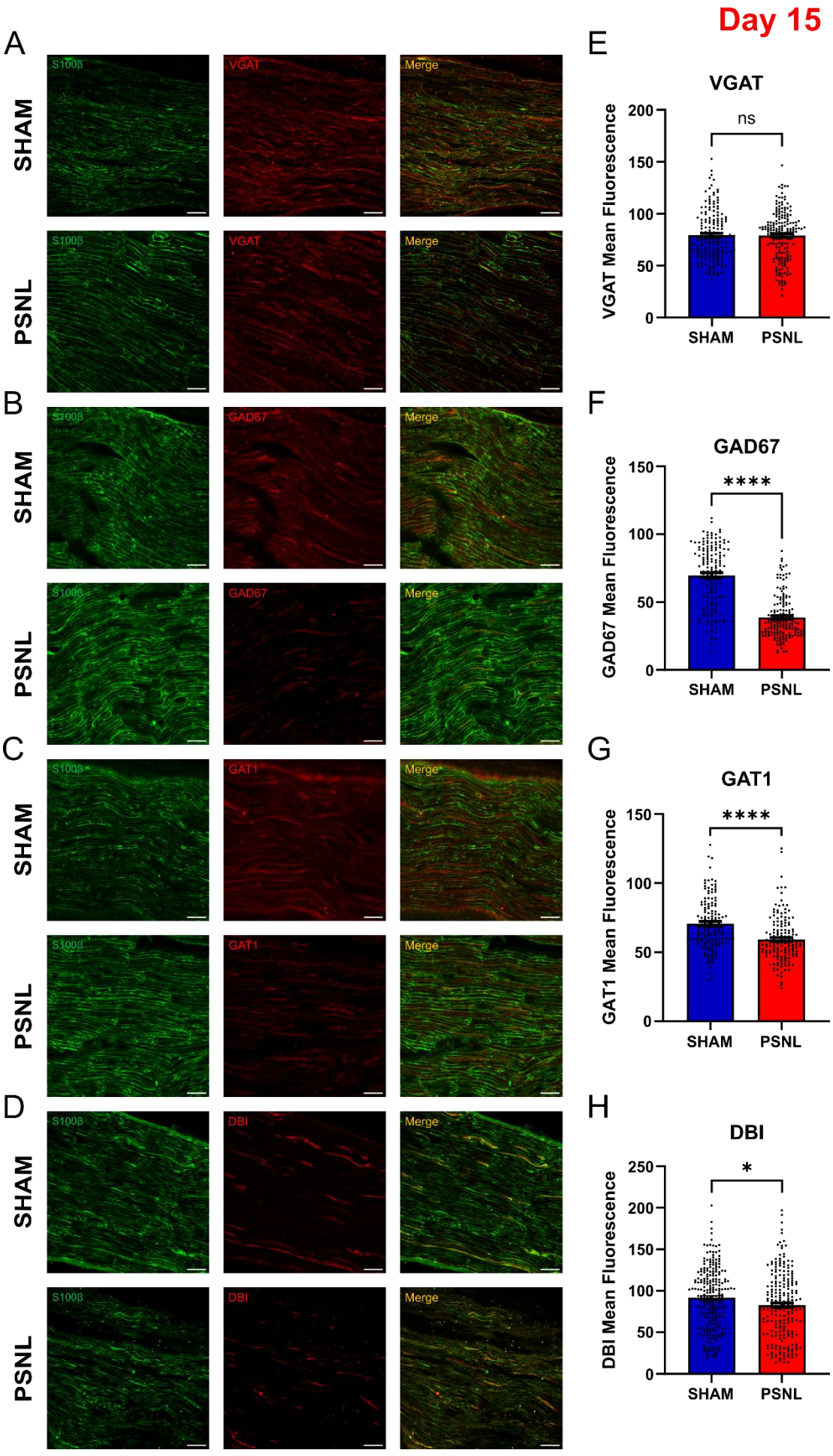

Day 15
A
SHAM
PSNL
S100β
VGAT
Merge
B
GAD67
C
GAT1
D
DBI
E
VGAT
ns
VGAT Mean Fluorescence
200
150
100
50
0
SHAM
PSNL
F
GAD67
****
GAD67 Mean Fluorescence
G
GAT1
GAT1 Mean Fluorescence
H
DBI
*
DBI Mean Fluorescence
250

Figure 4.3 Expression of GABAergic markers in the rat sciatic nerve 15 days after PSNL. Representative images of immunohistochemistry of sciatic nerves collected at 15 days post-injury shows VGAT, GAD67, GAT1 and DBI (red) co-stained with S100β (green) in PSNL vs. control SHAM nerves (Scale bar=50μm) (A-B-C-D). While at 15 days VGAT did not show any significant changes (no reduction compared to SHAM, p=0.6; n=171, n=191, N=4), fluorescence quantification revealed a decrease of GAD67, GAT1 and DBI fluorescence compared to SHAM controls (E-F-G-H) (Data presented as mean ± SEM; reduction of 45 ± 1.7% GAD67, n=150, n=167 ; 13 ± 1.8% GAT1, n=146, n=155 ;10 ± 3% DBI, n=235, n=220; Mann-Whitney test, ****p<0.0001, *p=0.01; N=4).

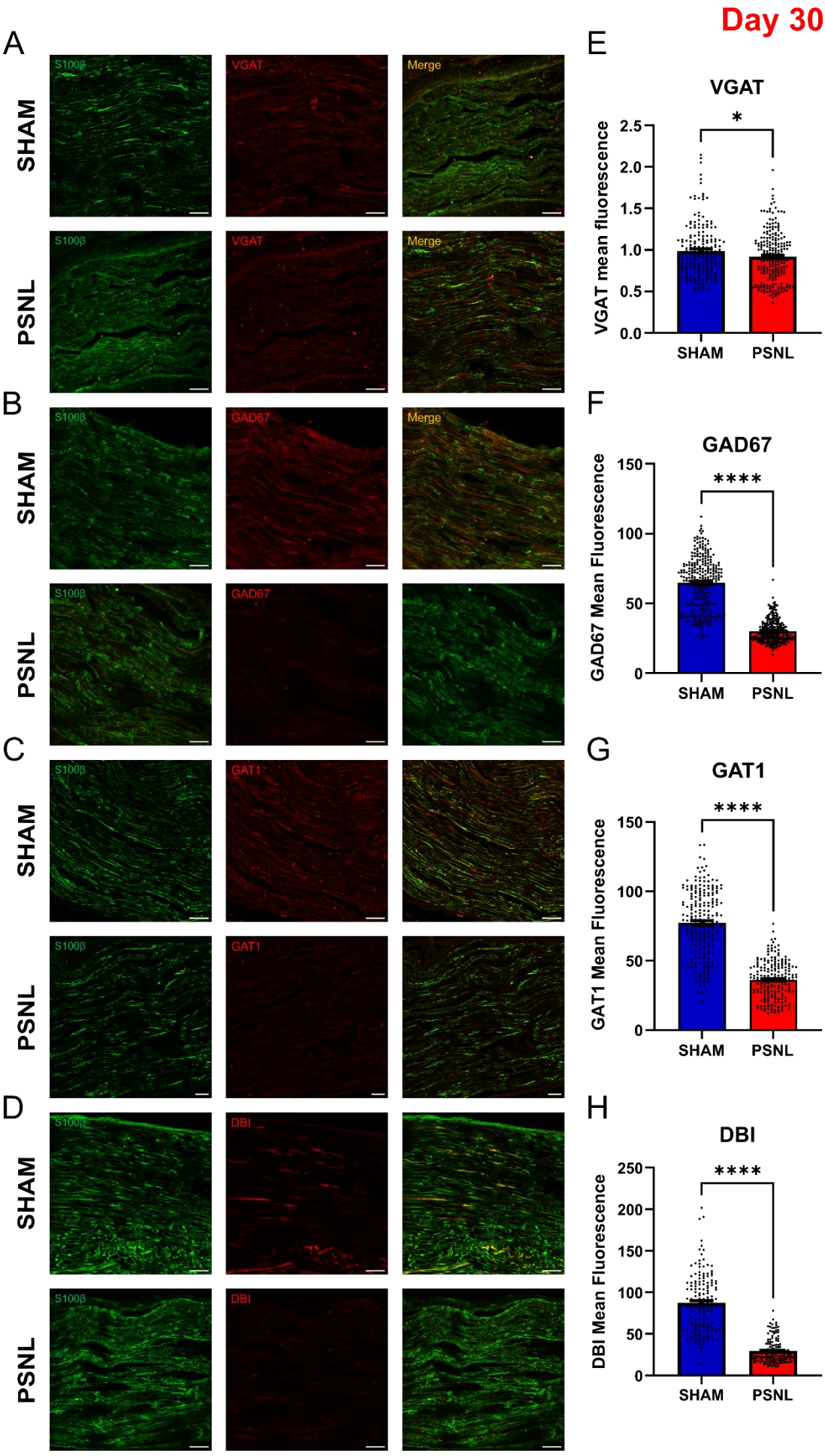

Day 30
A
SHAM
PSNL
S100β
VGAT
Merge
B
GAD67
C
GAT1
D
DBI
E
VGAT
VGAT mean fluorescence
*
F
GAD67
GAD67 Mean Fluorescence
****
G
GAT1
GAT1 Mean Fluorescence
H
DBI
DBI Mean Fluorescence
SHAM
PSNL

Figure 4.4 Expression of GABAergic markers in the rat sciatic nerve 30 days after PSNL. Representative images of immunohistochemistry shows VGAT, GAD67, GAT1 and DBI (red) co-stained with S100β (green) in 30-day PSNL vs. control SHAM nerves (Scale bar=50μm) (A-B-C-D). At 30 days, immunofluorescence level for all proteins was further decreased compared to controls (E-F-G-H) (VGAT 9 ± 2%, n=196, n=219; GAD67 56 ± 0.7%, n=277, n=276; GAT1 53 ± 1.7%, n=277, n=255; DBI 53 ± 1.2%, n=225, n=204; Mann-Whitney test ****p<0.0001, *p=0.01; N=4).

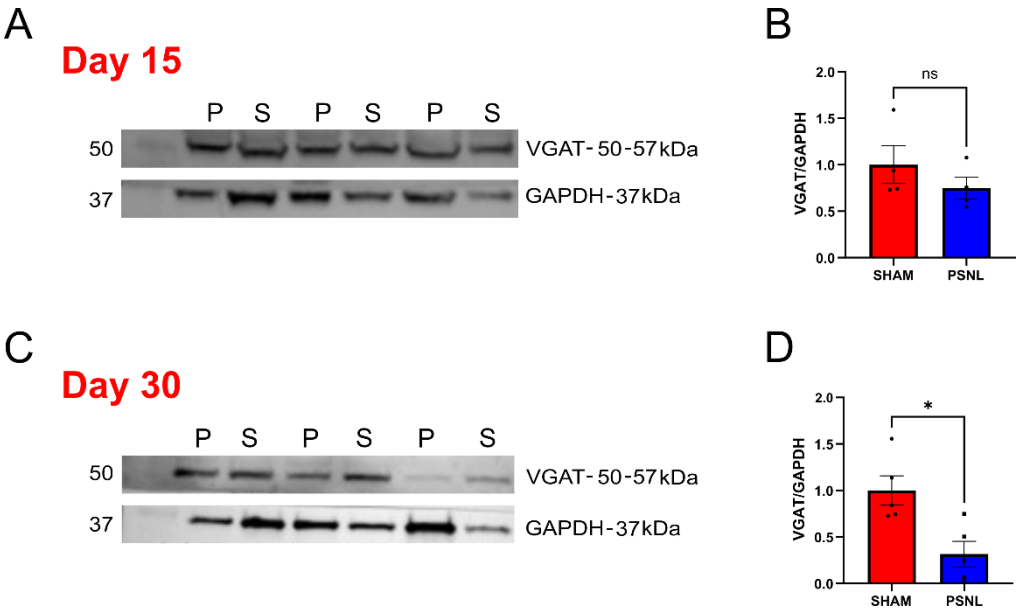


Figure 4.5 Western blot of VGAT at 15 and 30 days post PSNL. Representative bands of VGAT from PSNL (P) and SHAM (S) controls at 15 days post injury (A). Quantitative analysis of VGAT bands showed 25 ± 11.7% mean decrease in protein expression in injured nerves 15 days post PSNL compared to SHAM controls but it was not statistically significant (Data presented as mean ± SEM; Unpaired t-test, p=0.3; N=4) (B). Representative bands of VGAT from PSNL (P) and SHAM (S) controls at 30 days post injury (C). At 30 days the reduction increased to 69 ± 13.8% (D) (Data presented as mean ± SEM; Unpaired t-test, p=0.01; N=5).

4.4 Discussion

In this chapter, we demonstrate that Schwann cells possess a complete and functional GABAergic signalling machinery and that this system is dynamically regulated following peripheral nerve injury. Our findings show that Schwann cells express the essential molecular components required for GABA synthesis, transport and release, and that they tonically release GABA *in vitro*. Additionally, Schwann cells express DBI, an endogenous allosteric modulator of the $GABA_A$ receptor, indicating that they not only produce and respond to GABA but may also fine-tune their own receptor sensitivity. These findings identify Schwann cells as a previously underappreciated peripheral source of GABA and suggest that GABAergic signalling constitutes an additional layer of regulation within the peripheral neuro-glial and immune-glial network.

Previous investigations had established that Schwann cells express $GABA_A$ and $GABA_B$ receptors and that their activity is important during development for correct myelination and axon-glia interaction (Faroni et al., 2012, 2019; Magnaghi et al., 2004; Procacci et al., 2013). A few studies have reported GABA synthesis in Schwann cells, but it has never been demonstrated functionally (Magnaghi et al., 2010). Here we confirmed the expression of enzymes necessary for the synthesis (GAD67), release (VGAT) and uptake (GAT1) of GABA by Schwann cells, both *in vitro* and *ex vivo*.

Using histochemical and biochemical techniques, we detected GAD67, GAT1 and VGAT expression in both isolated Schwann cells and along the adult rat sciatic nerve, where they co-localized with the Schwann cells marker S100β. In addition, VGAT and GAD67 immunofluorescence was also observed outside S100β+ cells, suggesting that these proteins may be expressed by additional cell types within the nerve. As both GAD67 and VGAT are known to be expressed in primary sensory neurons, it is plausible that they are synthesized in the neuronal soma and subsequently transported along peripheral axons.

While GAD67 and GAT1 expression in Schwann cells had been previously reported (Gerber et al., 2021; Magnaghi et al., 2010; Petrov et al., 2025), VGAT expression was unexpected. Although GAT1 is classically associated with GABA uptake, it is known to operate bidirectionally depending on ionic gradients and membrane potential. In support of a potential role for transporter-mediated GABA release from Schwann cells, Petrov and colleagues recently demonstrated that at the neuromuscular junction, activation of α7 nicotinic acetylcholine receptors on terminal Schwann cells triggers GABA release, which then acts on presynaptic $GABA_B$ receptors to inhibit acetylcholine release (Petrov et al., 2025). The authors proposed that this GABA release occurs via reversal of GAT1, which could be triggered by increased intracellular Na^+ concentration and membrane depolarization. This is an interesting suggestion which implies that GAT1 could represent a more rapid route for GABA release under specific physiological or pathological conditions.

In contrast, VGAT is classically associated with the loading of GABA into synaptic vesicles at axon terminals of GABAergic neurons, and Schwann cells are not known to possess the canonical synaptic machinery required for fast neurotransmitter release. Nevertheless, VGAT function depends primarily on the presence of an appropriate proton electrochemical gradient rather than on synaptic vesicle identity per se. Schwann cells contain multiple acidified intracellular compartments, including endosomal and multivesicular bodies (MVBs), raising the possibility that VGAT loads GABA into non-synaptic vesicular compartments. Importantly, Schwann cells are established sources of exosomes and microvesicles that mediate bidirectional Schwann cell-axon communication and contribute to neuronal maintenance and regeneration (Lopez-Verrilli et al., 2013). These vesicles, derived from MVBs or plasma membrane budding, are actively exchanged between Schwann cells and axons and represent a well-characterised pathway of intercellular signalling in the peripheral nervous system.

Within this framework, VGAT could in principle interact with non-synaptic vesicular compartments involved in EV biogenesis, potentially contributing to intracellular GABA

handling outside canonical synaptic vesicles. However, whether VGAT localises to these compartments or participates in vesicle-associated trafficking in Schwann cells remains unresolved. Notably, the subcellular localization of VGAT in Schwann cells - whether at endosomal membranes, multivesicular bodies, intraluminal vesicles, or other compartments - will require direct experimental investigation.

Direct evidence supporting extracellular vesicle-mediated release of GABA is currently lacking, as GABA is primarily released through vesicular exocytosis in neurons or via transporter-mediated non-vesicular mechanisms (Kalra et al., 2012; Mathivanan et al., 2012; Petrov et al., 2025). In contrast DBI has been detected in extracellular environments and reported in proteomic analyses of EV fractions, suggesting a potential association with vesicle-based trafficking pathways, although the mechanisms governing its incorporation into EVs remain unresolved (Cherubini & Conti, 2001). Together, these observations support the possibility that DBI may be released in association with extracellular vesicles, whereas a similar mechanism for GABA remains unsubstantiated and likely indirect.

In this work we functionally demonstrated via I^- imaging, that Schwann cells release a bioactive ligand (GABA or DBI) capable of activating $GABA_A$ receptors. While the present study does not define the underlying release mechanism, these findings align with the broader capacity of Schwann cell-derived vesicular and non-vesicular signalling pathways to regulate axon-glia communication. Given the established role of GABA in promoting Schwann cell differentiation through $GABA_A$ receptors and inhibiting cAMP-dependent transcriptional programs via $GABA_B$ receptors (Faroni et al., 2014; Magnaghi et al., 2004), Schwann cell-derived GABA may act both in an autocrine and paracrine fashion to regulate phenotypic transitions during development, maintenance, and injury response. DBI, by modulating $GABA_A$ receptor sensitivity, may further refine this regulatory axis, enabling Schwann cells to dynamically adjust their responsiveness to endogenous GABA depending on physiological or pathological demands.

This novel glial signalling may also have implications for the communication with immune cells, including macrophages. Increasing evidence demonstrated that GABA shapes macrophage phenotype, promoting M2-like anti-inflammatory profiles and limiting pro-inflammatory cytokines production (Bhandage & Barragan, 2021; Jin et al., 2013). Our data, combined with these studies, support the possibility that Schwann cell-derived GABA may contribute to the resolution of inflammation following nerve injury. In this context, GABA signalling may complement established pathways such as IL-10, Gas6, and neuregulin, acting as another axis through which Schwann cells orchestrate the local immune environment (Stratton et al., 2018). Considering its role as positive allosteric modulator of the $GABA_A$ receptor, DBI could also contribute to these mechanisms, fine tuning the effect of GABA.

An important finding of our study revealed a robust, time-dependent downregulation of GABAergic proteins in Schwann cells following PSNL. Our group has previously demonstrated that peripheral nerve injury induces a downregulation of GABAergic signalling in DRG neurons (Wang et al., 2021), and data presented in this chapter suggest that similar mechanisms may take place in Schwann cells. Notably, transcriptomic analysis following peripheral nerve transection has previously reported reduced expression of both GAT1 and DBI in Schwann cells during the injury response (Clements et al., 2017), supporting the notion that suppression of GABAergic signalling represents a conserved feature of Schwann cell adaptation to nerve damage. Consistent with these observations, between 15 and 30 days post-injury, Schwann cells display marked reductions in GAD67, GAT1, DBI, and VGAT, both at the histological and biochemical level. This temporal profile is consistent with the transition from an active repair Schwann cell phenotype to a more dysfunctional state associated with prolonged inflammation and chronic injury. We speculate that loss of GABA synthesis may deprive the nerve microenvironment not only of an anti-inflammatory signal for immune cells but also of critical autocrine and paracrine cues for Schwann cells themselves. This disruption could impair Schwann cell phenotypic transitions and reparative functions,

while simultaneously exacerbating macrophage activation, oxidative stress and overall structural recovery of the injured nerve. Given DBI's role as an endogenous enhancer of $GABA_A$ receptor function, its downregulation may intensify these effects by further reducing the efficacy of residual GABAergic signalling. Further investigations are required to better understand the role of GABAergic signalling in Schwann cells and to characterize GABAergic cross-talk mechanisms with other cell types within the PNS, including sensory neurons, immune cells, and other glial populations. A more comprehensive understanding of GABAergic communication across distinct cellular compartments of the PNS will be essential to elucidate its contribution to nerve repair, inflammation, and the persistence of neuropathic pain.

Overall, our results indicate that Schwann cells possess the molecular machinery for peripheral GABAergic signalling and that the expression of key components, including GAD67, VGAT, GAT1, and DBI, is downregulated following nerve injury. These findings suggest that Schwann cells-derived GABA could potentially influence the local microenvironment, either through autocrine or paracrine effects on other Schwann cells or by modulating immune cells such as macrophages. However, the precise mechanisms of GABA release, the modes of Schwann cell communication, and whether GABA directly interacts with DRG axons *in vivo* remain unknown. Further studies will be needed to clarify how Schwann cell GABAergic signalling functions under physiological conditions and how its disruption contributes to nerve injury responses.

5 Chapter 5: $GABA_A$ receptor-mediated modulation of macrophage function in neuropathic pain

5.1 Introduction

5.1.1 Macrophages as central orchestrators of neuroinflammation in neuropathic pain

Neuropathic pain arises from damage to the somatosensory system, and its pathogenesis involves complex interactions between neurons, glial cells, and immune cells. Following peripheral nerve injury, DRG neurons, glial cells and resident immune cells, release a broad range of pro-inflammatory cytokines and chemokines which activate tissue-resident immune cells, but also recruit circulating monocytes to the DRGs and injured nerve, creating a sustained inflammatory environment that contributes to chronic pain (Gehrmann et al., 1992; White et al., 2005; Esper et al., 2004; Colloca et al., 2017).

Macrophages have emerged as one of the most important cell subpopulations involved in the neuro-immune interactions associated with neuropathic pain. Two major macrophage populations contribute to these responses: recruited monocyte-derived macrophages and resident sensory neuron-associated macrophages (sNAMs) (Old et al., 2014; Silva et al., 2021). Monocyte-derived macrophages originate from circulating monocytes produced in the bone marrow and they are drawn to the injured nerve and DRGs by chemokines such as MCP-1 and CX3CL1 (Kurihara et al., 1997; Silva et al., 2017; Tsou et al., 2007). Once they infiltrate the tissue, they differentiate into macrophages that adopt predominantly pro-inflammatory (M1) phenotype. In parallel, the DRGs and peripheral nerves contain long-lived population of resident macrophages that maintain close physical contact with sensory neurons and act as early responders to neuronal stress (Silva et al., 2021; Yu et al., 2020). Recent work has identified a specialized subset of $CD163^+$ resident macrophages positioned along the blood-DRG

barrier, where they actively monitor vascular permeability and respond to barrier disruption (Lund et al., 2023). These cells actively interact with endothelial cells and associated pericytes, and they are implicated in detecting and responding to injury-induced increases in blood-DRG barrier permeability, highlighting an additional mechanism through which macrophages may shape inflammatory signalling and sensory neuron excitability in neuropathic pain (Lund et al., 2023).

After injury, both macrophage populations expand and contribute to the inflammatory microenvironment producing pro-nociceptive mediators such as TNF-α, IL-6 and ROS, thereby promoting peripheral sensitization (Malcangio, 2020; Silva et al., 2021). Notably, both populations retain the capacity to convert to an anti-inflammatory, pro-resolving phenotype (M2), illustrating their remarkable plasticity and the potential for macrophage-targeted therapeutic strategies.

5.1.2 GABA polarizes diverse subpopulations of macrophages towards an anti-inflammatory phenotype

Emerging evidence indicates that GABA exerts significant modulatory effects on macrophage biology, linking neuronal inhibitory signalling to innate immune regulation. Diverse macrophage populations, including pulmonary, renal, as well as peritoneal and BMDMs, express key components of the GABAergic system, including GABA receptors, GABA transporters and GABA-synthesizing enzymes (Bhat et al., 2010; Januzi et al., 2018; Kim et al., 2018; Xia et al., 2021; B. Zhang et al., 2021).

Mechanistically, recent studies demonstrated that GABA suppresses macrophage inflammatory phenotype and function by reshaping intracellular metabolic and signalling pathways. For instance, GABA treatment decreases IL-1β expression in pro-inflammatory macrophages through inhibition of the NF-kB-driven pathway (Fu et al., 2022). Consistently, *in vivo* GABA administration alleviates inflammation in a mouse model of LPS-induced sepsis and high-fat-diet-induced obesity (Fu et al., 2022). Bhat and colleagues showed that peritoneal macrophages display $GABA_A$-mediated

electrophysiological responses to GABA and that GABA treatment reduces the expression of pro-inflammatory cytokines following LPS stimulation *in vitro* (Bhat et al., 2010). Interestingly, enhancement of GABAergic signalling delayed disease onset and ameliorated disease severity in an experimental autoimmune encephalomyelitis model, highlighting the anti-inflammatory potential of GABA *in vivo* (Bhat et al., 2010). In the tumour microenvironment, B cell-derived GABA promotes macrophage polarization toward an anti-inflammatory phenotype characterized by IL-10 secretion, thereby modulating anti-tumour immune responses (S. Zhang et al., 2021). Collectively these findings establish GABA as an important immunomodulatory molecule capable of shaping macrophage phenotype across multiple tissues and pathological contexts. However, whether GABAergic signalling similarly regulates macrophages involved in neuropathic pain, particularly those associated with sensory neurons and peripheral nerve injury, remains largely unexplored.

5.2 Aim

Given accumulating evidence that GABA polarizes diverse macrophage populations toward an anti-inflammatory phenotype, this chapter investigates whether this immunomodulatory effect also applies to macrophages involved in chronic pain. Specifically, we aim to: (i) determine how GABA and $GABA_A$ receptor activation modulates inflammatory phenotypes in LPS-treated BMDMs, by assessing changes in pro- and anti-inflammatory marker expression *in vitro*; (ii) assess whether inflammatory activation alters the expression of GABA-related genes in macrophages; and (iii) evaluate the impact of peripheral $GABA_A$ receptor agonism on pain behaviour and macrophage dynamics in an animal model of neuropathic pain.

5.3 Results

5.3.1 GABAergic signalling attenuates the pro-inflammatory response of LPS-challenged BMDMs

To investigate whether GABA modulates macrophage polarization, we examined the effect of GABA and the $GABA_A$-receptor agonist isoguvacine on LPS-challenged BMDMs obtained from adult mouse bone marrow (method section 2.3.2-2.3.3). LPS is a component of the outer membrane of Gram-negative bacteria and it therefore acts as a potent pro-inflammatory signal for macrophages (Meng & Lowell, 1997). Upon stimulation, LPS triggers a robust secretion of pro-inflammatory cytokines such as TNFα and IL-6 and it upregulates genes linked to inflammation as *Nos2* (Duque et al., 2025; Li et al., 2002; Meng & Lowell, 1997). Conversely, it downregulates the expression of anti-inflammatory genes characteristic of the M2 phenotype, including *Mrc1*, *Tgfβ* and *Arg1* (Allen et al., 2020; Orecchioni et al., 2019). Here we sought to determine whether the activation of GABAergic signalling could counteract the effect of the LPS-induced inflammatory program in BMDMs cultures.

As expected, 8-hour LPS (100ng/ml) stimulation resulted in a marked increase in mRNA levels of the pro-inflammatory markers *Tnfα* (~80 fold increase), *Il6* (~1500 fold increase) and *Nos2* (~2000 fold increase), accompanied by a significant downregulation in the expression of anti-inflammatory genes *Mrc1* (~3 fold decrease), *Tgfβ* (~2.5 fold decrease) and *Arg1* (~2 fold decrease) (Figure 5.1A-B-C). When GABA (900 μM) was added 3 hours after the start of LPS treatment, *Tnfα*, *Il6* and *Nos2* transcript levels displayed approximately 2-fold reduction compared to vehicle-treated controls (*Tnfα*: 0.5 ± 0.12-fold, p=0.04; *Il6*: 0.58 ± 0.11-fold, p=0.004; *Nos2*: 0.5 ± 0.07-fold, p<0.0001; N=6; Figure 5.1B). Among anti-inflammatory genes, only *Mrc1* expression was significantly increased following GABA treatment (*Mrc1* 2.38 ± 0.46-fold increase, p=0.002; N=6), whereas *Tgfβ* and *Arg1* showed notable mean increase that did not reach statistical

significance likely due to high variability (*Tgfβ* 1.51 ± 0.28-fold, p=0.1; *Arg1* 1.75 ± 0.6-fold, p=0.4; N=6; Figure 5.1C).

The $GABA_A$ agonist, isoguvacine (900 μM) confirmed these observations as it partially counteracted the LPS-induced upregulation of *Tnfα* and *Nos2* mRNAs compared to vehicle-treated controls (TNFα 0.53 ± 0.14-fold, p=0.04; Nos2 0.49 ± 0.13-fold, p=0.04; N=5; Figure 5.1D-E). Among anti-inflammatory genes, only *Mrc1* expression was significantly affected by isoguvacine treatment following LPS (2.95 ± 0.27-fold increase, p=0.04; N=5; Figure 5.1F).

To further determine whether the anti-inflammatory effect of isoguvacine was mediated specifically through $GABA_A$ receptors, we examined the effect of the selective $GABA_A$ receptor antagonist bicuculline in LPS-challenged BMDMs treated with isoguvacine. Cells were stimulated with LPS, followed by treatment with isoguvacine (900 μM) alone or in combination with bicuculline (200 μM) for an additional 5 hours (Figure 5.2A). qPCR analysis revealed that bicuculline partially reversed the anti-inflammatory effects induced by isoguvacine as shown by the increased expression of *Tnfα* and *Nos2* compared to isoguvacine-treated cells (*Tnfα*: 1.5 ± 0.08-fold, p=0.02; *Il6*: 1.22 ± 0.15-fold, p=0.5; *Nos2*: 1.8 ± 0.43-fold, p=0.04; N=4; Fig. 5.2B). In contrast, bicuculline had no marked effect on the expression of anti-inflammatory genes (Figure 5.2C). These results show that bicuculline partially reversed the effect of isoguvacine on the expression of pro-inflammatory markers in LPS-treated BMDMs.

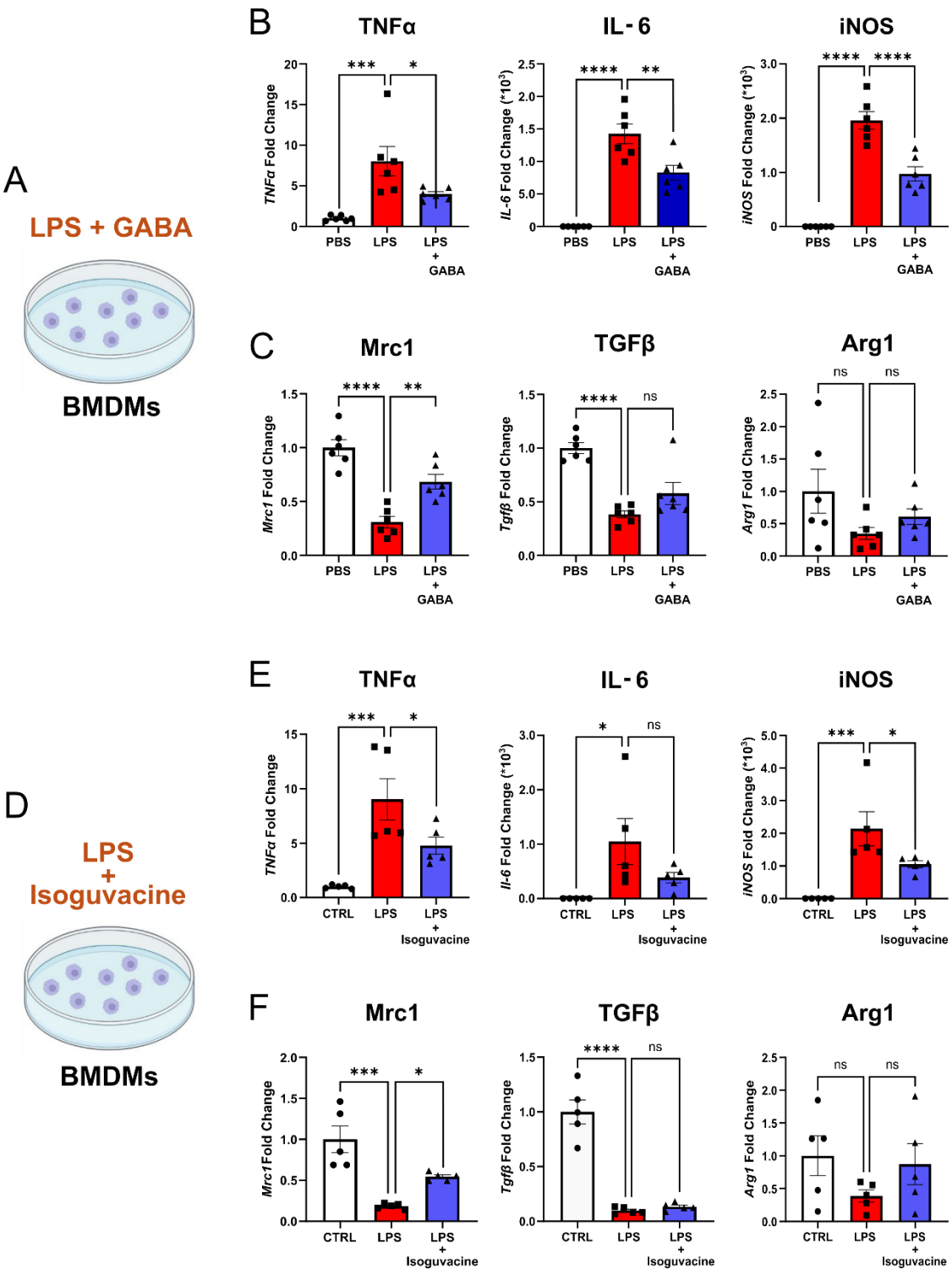


Figure 5.1 GABA and Isoguvacine treatment of LPS-challenged BMDMs partially counteract the pro-inflammatory effect of LPS.
Cultured BMDMs were treated with LPS (100 ng/ml) or PBS (control) for 3 hours, followed by GABA (900μM) or vehicle for 5 hours (A). Quantification of mRNA of pro-inflammatory (B) and anti-inflammatory (C) markers assessed by qPCR shows that GABA partially counteracts LPS effect. In a parallel experiment, isoguvacine was added for 5 hours following LPS treatment (D). mRNA quantification of pro-inflammatory (E) and anti-inflammatory (F) genes indicates that isoguvacine counterbalances LPS-induced inflammatory response (Data are mean ± SEM; One-way ANOVA, with Tukey's multiple-comparison test; *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 compared to controls; N=5 to 6).

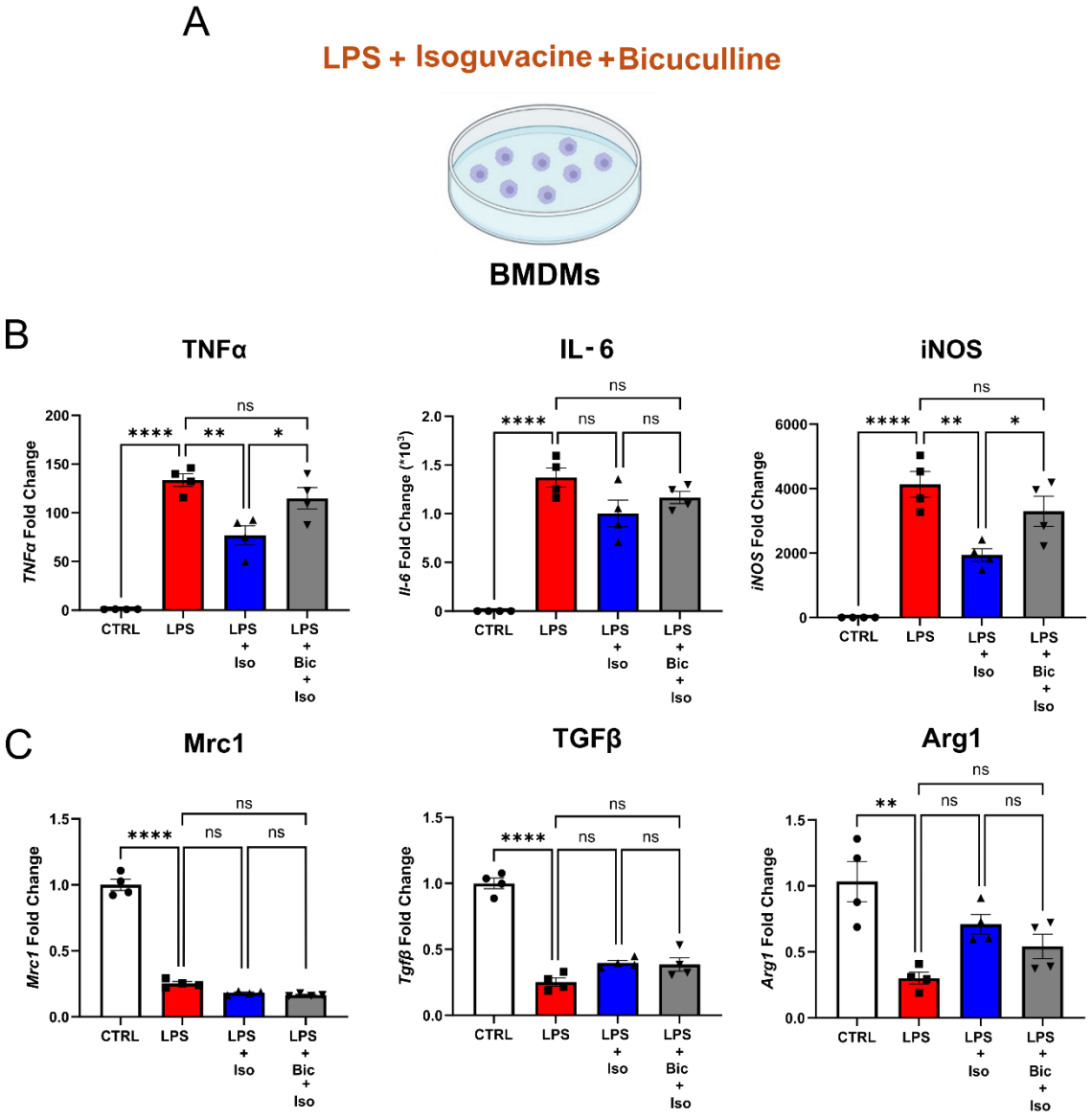


Figure 5.2 Bicuculline partially blocks the anti-inflammatory effect of Isoguvacine. Cultured BMDMs were treated with LPS (100 ng/ml) or PBS (control) for 3 hours, followed by Isoguvacine (900μM) or Isoguvacine + Bicuculline (200 μM) for 5 hours (A). Quantification of mRNA of pro-inflammatory (B) and anti-inflammatory (C) markers assessed by qPCR shows that Bicuculline partially counteract Isoguvacine effect. (Data are mean ± SEM; One-way ANOVA, with Tukey's multiple-comparison test; *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 compared to controls; N=4).

5.3.2 LPS alters expression of GABA related genes in cultured macrophages

As reported in literature, macrophages express several enzymes necessary for the synthesis and transport of GABA, including GAD65/67, GAT2, GAT3, as well as the β3 and δ subunits of $GABA_A$ receptor (Bhat et al., 2010; Fu et al., 2022; Januzi et al., 2018; Kim et al., 2018; B. Zhang et al., 2021). Based on these observations, we sought to investigate whether LPS treatment modulates the expression of these GABA-related genes in BMDMs. To determine whether the GABAergic system is altered in pro-inflammatory macrophages, cultured BMDMs were challenged with LPS for 8 hours, after which the expression of GABA-related transcripts was assessed by RT-qPCR (Figure 5.3A).

mRNA levels of *Gad1* (GAD67) and *Gad2* (GAD65), enzymes responsible for the decarboxylation of glutamate into GABA, were significantly upregulated in LPS-stimulated macrophages when compared to untreated controls (*Gad1* 2.48 ± 0.94-fold increase, p=0.01; *Gad2* 11.12 ± 2.5-fold increase, p=0.0003; N=5; Figure 5.3B). Similarly, transcripts coding for GABA transporters GAT2 (*Slc6a12*) and GAT3 (*Slc6a13*) were increased following LPS exposure (*Slc6a12* 4.15 ± 1.2-fold increase, p=0.02; *Slc6a13* 62.4 ± 16.7-fold increase, p=0.006; N=5 to 6; Figure 5.3C). Consistent with this pattern, the mRNA level of *Gabrb3* (β_3 subunit of the $GABA_A$ receptor) was also significantly upregulated in LPS-challenged macrophages (5.72 ± 2.45-fold increase, p=0.03; N=5) whereas the δ subunit did not show any significant changes (Figure 5.3D). Collectively, these results indicate that LPS stimulation markedly alters the expression of genes involved in GABA synthesis, uptake, $GABA_A$ receptor composition, suggesting that the GABAergic machinery is dynamically regulated during macrophage activation.

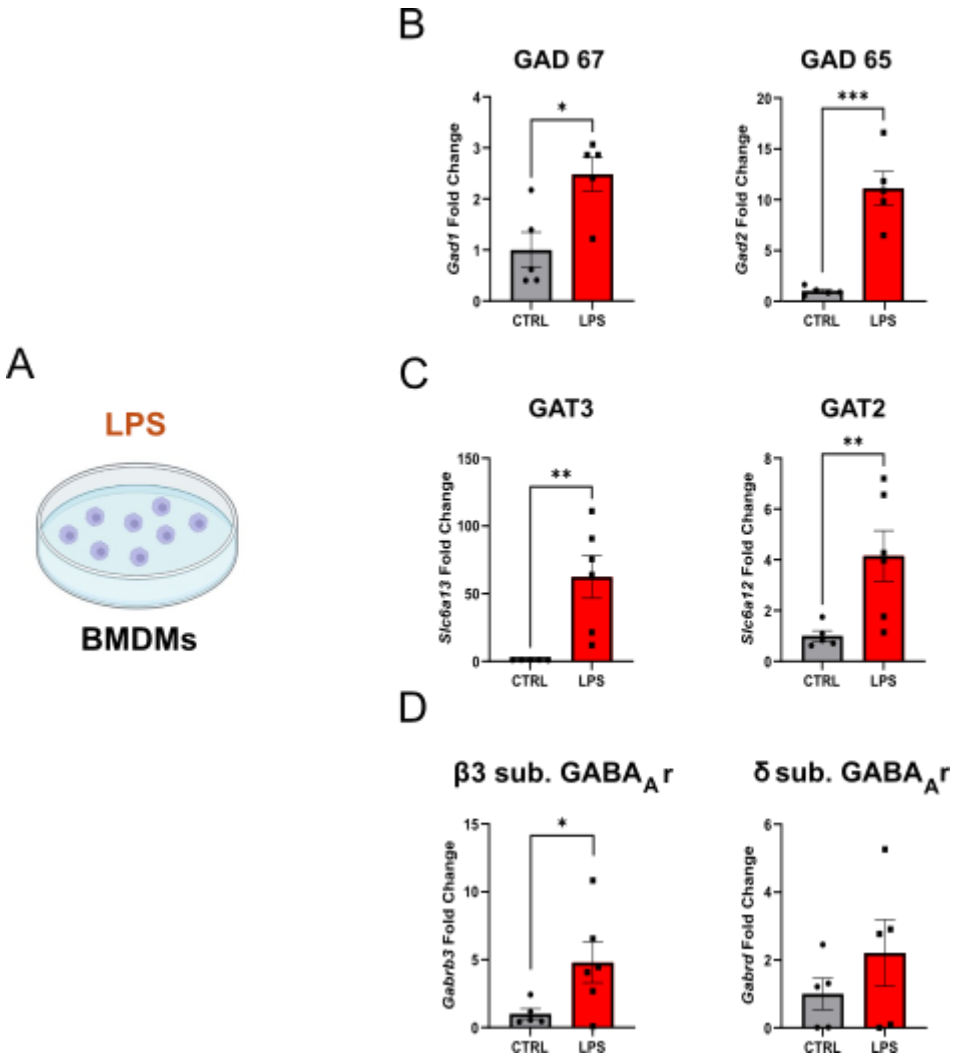


Figure 5.3 LPS stimulation alters the expression of GABA-related genes in cultured BMDMs.
Cultured BMDMs challenged with either LPS (100 ng/ml for 8 hours) or vehicle (A). mRNA quantification of glutamate decarboxylases GAD65 and GAD67 (B), GABA transporters GAT2 and GAT3 (C) and GABAA receptor subunits β3 and δ (C), assessed by qPCR shows that LPS treatment increases their expression. (Data are mean ± SEM; Unpaired t-test; *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 compared to controls; N=5 to 6).

5.3.3 GABA administration attenuates mechanical hypersensitivity in an animal model of neuropathic pain

To provide *in vivo* relevance to our *in vitro* findings, we sought to investigate the effect of the $GABA_A$ agonist isoguvacine on the development of mechanical hyperalgesia in a mouse model of chronic pain. Isoguvacine was selected instead of GABA because of its greater bioavailability and its inability to cross the BBB, thus confining its action to the periphery (Krogsgaard-Larsen & Falch, 1981; Li et al., 2024; Orefice et al., 2019).

Male and female mice underwent SNI on day 0 and were then injected intraperitoneally with isoguvacine (2 mg/kg) or PBS daily for 7 consecutive days (Figure 5.4A; method section 2.3.4-2.3.5). During SNI, the sciatic nerve is exposed at the level of its trifurcation into the tibial, common peroneal, and sural branches. The sural nerve is left intact, whereas the tibial and common peroneal nerves are transacted, leading to the development of chronic pain in the hind limb (Decosterd & Woolf, 2000). Sensitivity to mechanical stimuli was assessed using Von Frey assay each day, 2 hours prior the injection. 50% PWT, corresponding to the force that elicits a 50% probability of withdrawal, was subsequently calculated using the up-down Dixon method (Dixon & Chapman, 1980).

Baselines (BL) responses measured before surgery showed an expected 50% PWT of approximately 1g. After SNI, the withdrawal threshold of the injured paw dropped to 0.002g in both experimental groups, confirming a marked increase in mechanical sensitivity, while the values for the contralateral paw remained comparable to BL. To evaluate potential acute effects of isoguvacine, we performed additional Von Frey testing on the first postoperative day at 1, 2, and 6 hours after the first injection. No differences were observed between isoguvacine- and vehicle-treated mice, suggesting that Isoguvacine does not exert immediate neuronal effects in our model (Figure 5.4B).

Notably, from day 4 onward and until the end of the experiment (day 7), mice treated with isoguvacine displayed a moderate but consistent improvement in mechanical

hypersensitivity compared with vehicle controls, with the maximal effect observed at day 6 (0.028 ± 0.004g Vehicle vs. 0.144 ± 0.046g isoguvacine; p=0.001, N=4 to 5; Figure 5.4C). The timeframe for isoguvacine activity coincides with the time at which macrophages play a predominant role in sustaining the chronic pain state, suggesting that Isoguvacine is likely acting on this cell population (Chen et al., 2020; Guimarães et al., 2023; Msheik et al., 2022; Yu et al., 2020).

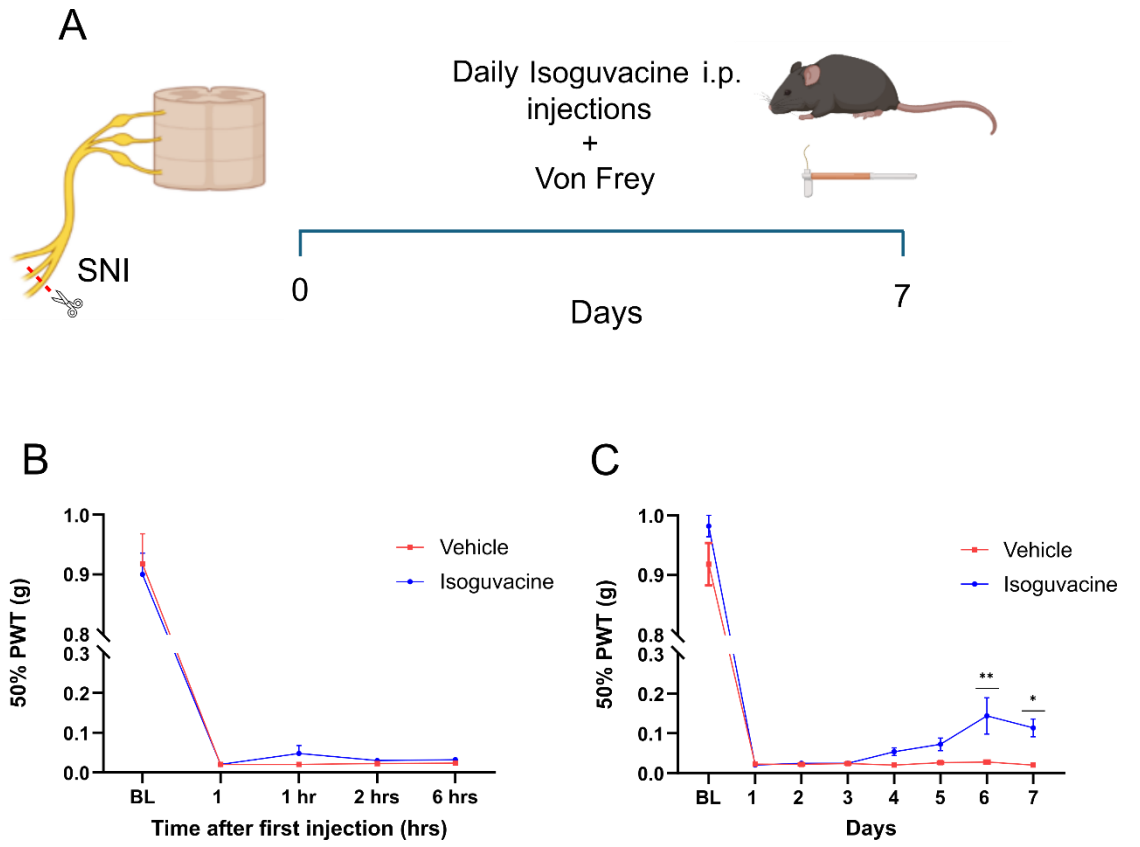


Figure 5.4 Repeated systemic application of Isoguvacine ameliorated mechanical hypersensitivity elicited by SNI.
SNI was performed on day 0 on male and female mice. Isoguvacine was injected i.p. daily followed by Von Frey assay (A). Mechanical allodynia developed following SNI. Isoguvacine did not elicit any acute effect at 1,2 and 6 hours post-injection (B). Isoguvacine treated group (blue) showed decreased mechanical hypersensitivity from day 4 to day 7 when compared to PBS-treated controls (red) (Data are mean ± SEM; Two-way ANOVA with Šídák's multiple comparisons test; *p<0.05, **p<0.01, compared to controls; N=4 to 5).

5.3.4 GABA treatment is associated with reduced macrophages infiltration in the DRGs and Spinal Nerve

Given the moderate improvement in mechanical hypersensitivity observed in isoguvacine-treated mice, particularly peaking at day 6 after SNI, we next aimed to determine whether this behavioural effect was associated with changes in immune cell dynamics at the site of nerve injury. Macrophages are key contributors to the maintenance of neuropathic pain, and our *in vitro* data demonstrated that GABAergic signalling modulates their inflammatory profile. Building on these observations, we investigated whether systemic isoguvacine administration alters macrophage infiltration and polarization *in vivo*.

At day 7 post-SNI, mice were euthanized, and DRGs from L3-L4 and L5, along with segments of the ipsilateral injured sciatic nerve, were dissected and processed for flow cytometry (method section 2.3.6). Mice treated with isoguvacine (2mg/kg i.p.) showed a trend toward reduced numbers of CD11b+ myeloid cells in the DRGs (69.7 ± 14.2% reduction, $p=0.06$, N=5) as well as of F4/80+ macrophages (63.5 ± 15.7% reduction, $p=0.09$, N=5; Figure 5.5A-B). As a consequence of this global reduction in macrophages, absolute numbers of both M1- (CD206-/MHCII+) to M2-like (CD208+/MHCII-) macrophages were decreased in isoguvacine-treated mice (Figure 5.5C). Interestingly, analysis of macrophage composition within the two groups, revealed a significant shift in phenotype distribution. Isoguvacine-treated mice exhibited a lower proportion of M1-like macrophages and a higher proportion of M2-like macrophages (M1 71% - M2 29%) relative to vehicle-treated controls (M1 56% - M2 44%), indicating that the macrophages within the remaining population were skewed towards a less inflammatory phenotype by isoguvacine ($p<0.0001$, N=5; Figure 5.5D).

Similarly, at the site of nerve injury in the sciatic nerve, the number of CD11b+ (71.02 ± 8.6% decrease, $p=0.001$, N=5) and F4/80+ cells (74 ± 8.7% decrease, $p=0.04$, N=5) was significantly reduced in the isoguvacine-treated group compared to vehicle-treated

controls (Figure 5.5E-F). The distribution of macrophage phenotypes at the injury site, however, did not differ significantly between the two groups ($p=0.22$, $N=5$; Figure 5.5G-H).

FACS data acquisition and analysis for this experiment were carried out by Dr. Lynda Zeboudj and Dr. Francesca Picco at King's College London.

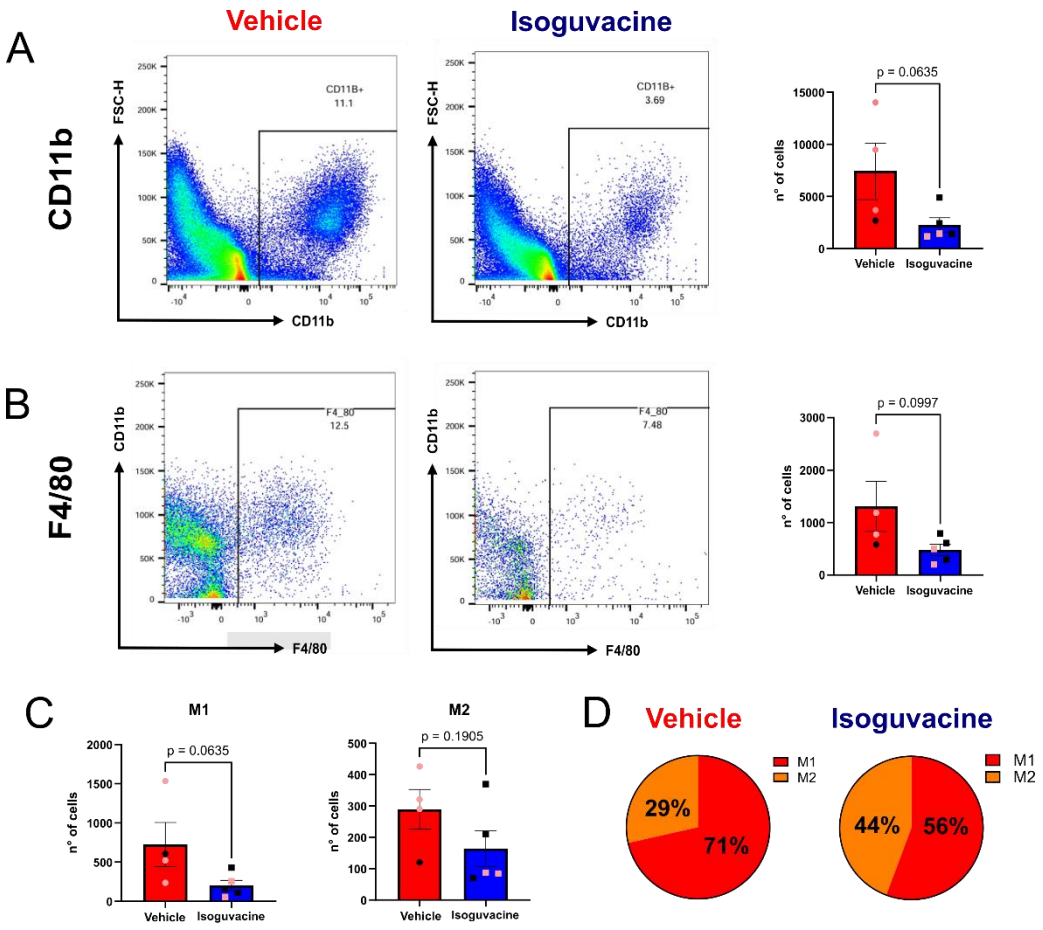

A
Vehicle
Isoguvacine
CD11b
FSC-H
CD11B+
11.1
CD11B+
3.69
CD11b
p = 0.0635
n° of cells
Vehicle
Isoguvacine
B
F4/80
CD11b
F4_80
12.5
F4_80
7.48
F4/80
p = 0.0997
C
M1
M2
p = 0.0635
p = 0.1905
D
Vehicle
Isoguvacine
M1
M2
29%
71%
44%
56%
E
Vehicle
Isoguvacine
CD11b
CD11B+
2.95
CD11B+
7.38
**
F
F4/80
F4_80
13.8
F4_80
7.89
*
G
M1
M2
*
*
H
Vehicle
Isoguvacine
M1
M2
45%
55%
44%
56%

Figure 5.5 Isoguvacine treatment is associated with decreased immune cells and M1 macrophages recruitment in DRGs and sciatic nerve after SNI.
Representative scatterplots of immune cells sorted from L3, L4 and L5 DRGs dissected at 7 days after SNI from isoguvacine - and vehicle-treated mice (A-B). Cells were gated on CD11b+ and F4/80+. Macrophages were defined as CD11b+/F4/80+ and further analyzed for M1 (MHCII+/CD206-) and M2(MHCII-/CD206+). Quantification of CD11b+ myeloid cells and F4/80+ macrophages shows decreased number in lumbar DRGs from isoguvacine-treated mice compared to controls (Data are mean ± SEM; Mann-Whitney test; p=0.06; p=0.09; N=5) (A-B). Absolute values of single positive cells for (MHCII+/CD206-) and (MHCII-/CD206+) display a non-significant decrease for both populations in the isoguvacine group (Data are mean ± SEM; Mann Whitney test; p=0.06; p=0.19; N=5) (C). M1/M2 ratio is decreased within the macrophage DRG population of Isoguvacine-treated mice as compared to controls (Fisher's exact test; ****p<0.00001; N=5) (D). Lower number of myeloid cells and macrophages was also detected in sciatic nerves of Isoguvacine-treated animals relative to controls (Data are mean ± SEM; Unpaired t-test; *p=0.01; **p=0.001; N=5) (E-F). Absolute values of M1 and M2 number of cells showed a significant decrease in the isoguvacine group compared to control (Unpaired t-test; *p=0.01; N=5) M1/M2 proportion did not show significant changes among the two groups (Fisher's exact test; p=0.22) (G-H).

5.4 Discussion

In this study we investigated whether GABAergic signalling modulates macrophage function in the context of neuropathic pain. Using *in vitro* and *in vivo* approaches, we showed that activation of $GABA_A$ receptors attenuates the pro-inflammatory response of macrophages. GABA and isoguvacine treatment partially counteracted LPS-induced polarization of BMDMs while LPS altered the expression of GABAergic enzymes and $GABA_A$ receptor subunits. Importantly, peripheral administration of the $GABA_A$ receptor agonist isoguvacine reduced mechanical allodynia in a mouse model of neuropathic pain and was associated with decreased macrophage accumulation at the site of nerve injury and within the DRGs.

Neuropathic pain is sustained by complex interactions between neurons, glial cells and immune cells, among which macrophages play a central role (Domoto et al., 2021; Yu et al., 2020). Following peripheral nerve injury, macrophages accumulate at the site of injury and within the DRGs, where they contribute to the establishment and maintenance of chronic pain by releasing pro-inflammatory cytokines, ROS, and other mediators that promote peripheral sensitization (Liu et al., 2010; Malcangio, 2020; Silva et al., 2021). At the same time, macrophages exhibit considerable plasticity which enables them to adopt either a pro- or anti-inflammatory phenotype, depending on the signals coming from the environment. Understanding how macrophage polarization can be shifted to an anti-inflammatory M2 state is therefore essential for the development of novel therapeutic approaches to chronic pain. Given accumulating evidence that GABA promotes M2 polarization across diverse macrophage populations (Bhat et al., 2010; Fu et al., 2022; Kim et al., 2018), we sought to determine whether this immunomodulatory effect also applies to macrophages involved in chronic pain.

Our *in vitro* data demonstrate that both GABA and the selective $GABA_A$ receptor agonist isoguvacine, significantly attenuate the pro-inflammatory transcriptional program induced by LPS stimulation in BMDMs. As expected, LPS elicited a robust upregulation of canonical pro-inflammatory genes, including *TNFα, IL6* and *Nos2*, accompanied by a

marked repression of anti-inflammatory genes such as *Mrc1*, *TGFβ*, and *Arg1.* The addition of GABA or isoguvacine partially reversed this effect, reducing the expression of pro-inflammatory genes and promoting, albeit modestly, the expression of anti-inflammatory markers, confirming its anti-inflammatory role. These findings are consistent with previous studies showing that GABA suppresses macrophage inflammatory responses, including the inhibition of pro-inflammatory cytokine production and NF-κB-dependent signalling pathways (Fu et al., 2022; Bhat et al., 2010). Furthermore, GABAergic signalling has been reported to promote anti-inflammatory macrophage polarization and to reduce inflammation in several *in vivo* disease models, highlighting its broader immunomodulatory potential (Fu et al., 2022; Bhat et al., 2010; Zhang et al., 2021; 2018; Reyes-García et al., 2007). The partial reversal of isoguvacine effects by the $GABA_A$ receptor antagonist bicuculline supports the involvement of $GABA_A$ receptors in mediating these anti-inflammatory responses in BMDMs.

Interestingly, our data suggest that inflammatory activation not only responds to GABAergic signalling but may also affect the expression of components of the GABAergic machinery itself. In the present study, LPS stimulation increased the expression of genes involved in GABA synthesis, (*Gad1* and *Gad2*), GABA transport (*Slc6a12* and *Slc6a13*) and $GABA_A$ receptor signalling (*Gabrb3*).

These findings raise the possibility that activation of the GABAergic system represents an endogenous feedback mechanism that acts to restrain excessive inflammatory responses in macrophages. The particularly strong induction of GAT3 expression points to a potential role for regulated GABA uptake in shaping extracellular GABA availability or intracellular metabolic pathways linked to macrophage function. Whether macrophages actively release GABA in inflammatory settings and how endogenous GABA contributes to autocrine or paracrine signalling remain important questions for future investigation.

An important unresolved question concerns the source of GABA acting on macrophages in inflammatory settings. One possibility is that macrophages themselves produce and

release GABA, enabling autocrine feedback signalling. However, macrophages *in vivo* are embedded within a complex tissue environment and are also likely exposed to GABA released by neighbouring cells. Sensory neurons within the DRGs and Schwann cells in peripheral nerves have both been shown to synthesize and release GABA, positioning them as plausible paracrine sources of GABA during nerve injury and inflammation (Du et al., 2017; Magnaghi et al., 2010). Thus, macrophage GABAergic signalling may reflect an integration of cell-intrinsic GABA production with extrinsic GABA derived from neural and glial sources.

To establish whether these findings had any *in vivo* relevance, we examined the effect of systemic administration of the peripherally restricted $GABA_A$ receptor agonist isoguvacine in the SNI model of neuropathic pain. Repeated isoguvacine treatment resulted in a moderate but consistent attenuation of mechanical hypersensitivity, which started at day 4 after injury. The absence of any acute analgesic effects shortly after injection suggests that isoguvacine does not directly alter sensory neuron excitability, but rather exerts its effect through slower, indirect mechanisms. This delayed time course coincides with the phase during which macrophages are known to play a dominant role in sustaining chronic pain (Chen et al., 2020; Guimarães et al., 2023; Msheik et al., 2022; Yu et al., 2020), supporting the idea that immune modulation underlies the observed behavioural improvement.

Notably, in a previous study from our group, isoguvacine alleviated mechanical allodynia induced by DBI knockdown in SGCs, within a few hours of injection, consistent with a more direct neuronal mechanism (Li et al., 2024). The discrepancy likely reflects fundamental differences between the models: DBI knockdown represents a more restricted perturbation of inhibitory signalling, whereas SNI induces a much more invasive injury associated with more drastic alterations. These considerations suggest that while isoguvacine can acutely modulate neuronal excitability under specific conditions, its predominant analgesic effect in the SNI model is mediated by slower, immune-dependent processes. The relatively modest effect observed in the SNI model

may also be related to the dose used, which was selected based on previous experiments and may not have been sufficient to achieve maximal efficacy in this more severe injury context.

Consistent with this interpretation, isoguvacine treatment was associated with reduced macrophage accumulation in both the DRGs and sciatic nerve of PSNL animals. At the site of injury, the number of infiltrating myeloid cells and macrophages was significantly decreased, indicating that peripheral $GABA_A$ receptor activation limits immune cell recruitment. Similarly, in the DRGs we observed a strong trend toward reduced myeloid cells and macrophages which also displayed a higher proportion of M2-like cells compared to the vehicle-treated controls. Although some of these changes did not reach statistical significance, likely due to limited sample size and inherent biological variability, their magnitude and consistency suggest a biologically meaningful effect. The discrepancy between the clear reduction in macrophage numbers and the more subtle changes in polarization may reflect tissue-specific differences in macrophage regulation or temporal dynamics that were not captured at the single time point analysed.

The mechanisms by which GABAergic signalling reduces macrophage infiltration remain to be elucidated. One possibility is that GABA dampens the production of chemokines and other inflammatory mediators that drive monocyte recruitment, thereby disrupting the feed-forward inflammatory loop that characterizes neuropathic pain. Alternatively, GABA may directly impair macrophage migratory capacity or survival within inflamed tissues. In this context, macrophages located in injured nerves and DRGs are likely exposed to extracellular GABA released by neighbouring cells, as both sensory neurons in the DRGs and Schwann cells have been shown to synthesize and release GABA (Du et al., 2017; Magnaghi et al., 2010). Such neuron- or glia-derived GABA could therefore act as a local paracrine signal modulating macrophage recruitment and behaviour. It is also conceivable that GABA acts on other peripheral cell types, such as Schwann cells or endothelial cells, indirectly influencing immune cell trafficking.

Although peripheral $GABA_A$ receptor activation produced a modest attenuation of neuropathic pain, this effect likely reflects the multifactorial nature of the condition, and it suggests that further optimization of dosing, treatment regime, or combination with complementary therapeutic strategies may be necessary to enhance efficacy. In addition, investigations at multiple post-injury time points will be essential to better define the temporal contribution of GABAergic signalling to macrophage recruitment, polarization and function during both the establishment and maintenance of chronic pain. Despite these considerations, our findings identify GABAergic signalling as an important modulator of macrophage function in neuropathic pain. By attenuating pro-inflammatory activation, reshaping the macrophage GABAergic machinery, and reducing immune cell accumulation in injured tissues, peripheral $GABA_A$ receptor activation emerges as a novel mechanism linking neurotransmitter signalling to immune regulation in chronic pain. These results broaden our understanding of neuro-immune communication and highlight GABAergic pathways as promising targets for therapeutic intervention in neuropathic pain and potentially other inflammatory disorders.

6 Chapter 6: General Discussion

Chronic pain arises from maladaptive interactions across multiple levels of the nervous system, involving not only neurons, but also glial and immune cells which together shape nociceptive signalling. While GABAergic inhibition in the spinal cord - mediated by both pre- and postsynaptic mechanisms - has long been recognized as a central mechanism modulating pain transmission (Bardoni et al., 2013; Price et al., 2009; Witschi et al., 2011; Zeilhofer et al., 2012), its role in the PNS had just started to be explored. Traditionally, GABAergic signalling in DRG neurons has been primarily associated with PAD at central terminals, where activation of presynaptic $GABA_A$ and $GABA_B$ receptors limits afferent excitability and neurotransmitter release within the spinal cord (Price et al., 2009; Witschi et al., 2011; Zeilhofer et al., 2012). However, recent findings from our group showed that enhancing GABAergic signalling at the DRG itself is sufficient to reduce both acute and chronic pain, identifying the DRG as a peripheral inhibitory gate (Du et al., 2017; Hao et al., 2023; Lall et al., 2025; Li et al., 2024). Growing evidence indicates that GABAergic signalling exerts different effects at diverse segments of primary sensory neurons: in addition to its action at the cell body and central terminals, GABA receptors are expressed along peripheral axons, although their function has not been fully elucidated yet. Moreover, GABA has been shown to facilitate, rather than inhibit, action potential propagation at branch points of myelinated proprioceptive sensory afferents within the spinal cord, particularly at axonal bifurcations that project to motor circuits (Hari et al., 2022). At these sites, mild GABA-mediated depolarization reduces spike failure and enhances transmission reliability through the branch point. These findings highlight that the functional outcome of GABAergic signalling in primary sensory neurons is strongly determined by its anatomical location, and that GABA can exert inhibitory or facilitatory effects based on the neuronal compartment engaged.

In parallel with neuronal mechanisms, increasing evidence indicates that GABAergic signalling also operates in peripheral non-neuronal cells, highlighting an additional layer of complexity to GABAergic modulation. In the PNS, glial cells, such as SGCs and

Schwann cells, express components of the GABAergic machinery and can both respond to and shape local GABAergic tone, thereby influencing neuronal excitability (Faroni et al., 2012, 2019; Li et al., 2024; Magnaghi et al., 2010; Procacci et al., 2013; Serrano-Regal et al., 2020). Beyond glial cells, immune cells, including macrophages, have been identified as important targets of GABA signalling. In several peripheral tissues, GABA has been shown to promote an anti-inflammatory, pro-resolving M2 macrophage phenotype, which contributes to tissue homeostasis and repair (Bhat et al., 2010; Fu et al., 2022; Januzi et al., 2018; Kim et al., 2018; B. Zhang et al., 2021). However, whether similar mechanisms operate within the DRG and in context of chronic pain remains unclear. Given the well-established role of macrophages in chronic pain development and maintenance (Domoto et al., 2021; Guimarães et al., 2023; Malcangio, 2020; Silva et al., 2021c; Yu et al., 2020), the possibility that local GABAergic communication influences macrophage polarization represents a novel and potentially relevant mechanism driving neuro-immune and glial-immune interactions in pain regulation.

The work presented in this thesis was designed to address these gaps by dissecting peripheral GABAergic mechanisms, with a specific focus on compartment-specific GABAergic neuronal signalling and neuron-glia-immune interactions. The key findings demonstrated that $GABA_A$ receptors are expressed throughout DRG neuron cell bodies and axons and that GABA stimulation at the peripheral axons is depolarizing. Using live-cell Ca^{2+} imaging we monitored neuronal activity in the microfluidic DRG-DH co-culture. We showed that localized GABA application to the somatic compartment of DRG neurons is depolarizing but it decreases neuronal firing induced by electrical stimulation of the peripheral axons, presumably by shunting inhibition. Similarly, GABA application to the peripheral axonal compartment produced a mild depolarization in the neuron somata but it did not affect the propagation of action potentials elicited by peripheral electrical stimulation. Moreover, using EYFP imaging we observed that peripheral axons release GABA upon stimulation. Together, these findings demonstrate that compartment-specific GABAergic signalling within pseudo-unipolar DRG neurons

differentially regulates axonal excitability and spike propagation, highlighting the importance of neuronal geometry in peripheral sensory gating.

In addition to neuronal mechanisms, this work identifies non-neuronal cells as active contributors to the peripheral GABAergic tone. We show that Schwann cells within the sciatic nerve express enzymes necessary for GABA synthesis and release, and they secrete a bioactive $GABA_A$ receptor ligand *in vitro*. Importantly, their expression was altered following peripheral nerve injury, suggesting that injury-induced changes in Schwann cell metabolism may reshape local inhibitory signalling. Moreover, our data demonstrate that GABA modulates macrophage polarization both *in vitro* and in animal models of chronic pain, providing evidence for a previously underappreciated role of GABA as in immunomodulatory signal in the PNS.

6.1 Overcoming experimental barriers to study pseudo-unipolar DRG neurons

Understanding how local signalling events regulate action potential propagation in primary sensory neurons remains challenging due to technical limitations. *In vivo*, the T-junction of DRG neurons is deeply embedded within a densely packed ganglionic environment, surrounded by neuronal somata, glial cells, and intersecting axons, with the whole structure hidden within vertebral foramen (Nascimento et al., 2018). This complex anatomy makes it difficult to selectively access, visualize, or manipulate defined neuronal compartments in a spatially restricted manner. As a result, despite longstanding theoretical predictions identifying the T-junction as a critical site for regulating spike propagation, direct experimental investigations of local modulatory mechanisms at this site have remained limited.

In parallel, classical *in vitro* approaches have hindered the study of compartment-specific mechanisms. Standard dissociated DRG neuron cultures typically lack spatial compartmentalization and only a small subset of neurons preserves the pseudo-unipolar morphology that characterizes primary sensory neurons *in vivo*. As a consequence, key

anatomical features such as the stem axon and T-junction are absent or poorly defined, precluding investigations of how localized signalling influences sensory transmission. Moreover, in standard cultures neurites broadly sprout and intertwine with cell bodies, precluding spatially restricted, selective manipulations. Together, these limitations have limited mechanistic analysis of compartment-specific signalling along the primary afferent axis, including GABAergic modulation. Some strategies have been proposed to overcome these limitations, including three-dimensional collagen gels and co-culture systems with glial cells, which promote aspects of pseudo-unipolarization (Costa et al., 2025; Mudge, 1984; Ribeiro et al., 2012), but remain limited in their ability to isolate defined neuronal compartments.

To overcome these challenges, in this study we employed a tri-compartment microfluidic DRG-DH co-culture system originally developed by the Raouf group (Vysokov et al., 2019). This configuration enables spatial segregation of neuronal compartments while preserving functional connectivity between primary sensory neurons and their central targets. In this setup DRG neurons reside in the central chamber and extend axons bilaterally through microgrooves, establishing a spatial organization that recapitulates key features of peripheral and central projections. Notably, a substantial proportion of neurons in this system develop polarized axonal branches emerging from a clearly defined bifurcation, resembling the pseudo-unipolar organization observed *in vivo*. The presence of DH neurons in co-culture further enhances this effect, increasing the proportion of DRG neurons that acquire polarized morphology, consistent with developmental evidence that interactions with central targets provide instructive cues for proper maturation (Barber & Vaughn, 1986). Beyond morphology, these neurons preserve functional integrity, as signal propagates from the periphery all the way to the DH compartment. It should be noted, however, that while the microfluidic system generates clearly bifurcated and polarized axonal branches, we cannot definitively ascertain that each peripheral and central branch originating from the T-junction, reliably reaches the right compartment. Nevertheless, the overall pattern of polarized axonal

branches closely resembles the pseudo-unipolar organization observed *in vivo*, providing a robust structural and functional framework for studying how localized signals, including GABAergic activity, influence neuronal excitability.

6.2 Compartment-specific GABAergic modulation of primary sensory neuron excitability

Using the compartmentalized microfluidic setup, this thesis examined how GABAergic signalling differentially regulates excitability along distinct regions of primary sensory neurons. Previous studies have established that GABAergic effects in sensory afferents are highly location-dependent, with divergent outcomes reported at somata (Du et al., 2017; Hao et al., 2023; Lall et al., 2025), peripheral axons (Bonalume et al., 2021; Hanack et al., 2015; Jang et al., 2017), branch points of central axons (Hari et al., 2022), and presynaptic terminals (Hochman et al., 2010; Price et al., 2009; Rudomin & Schmidt, 1999; Witschi et al., 2011; Zeilhofer et al., 2012). While GABA-mediated depolarization is a well-recognized feature of primary sensory neurons due to their elevated intracellular Cl^- concentration, depolarization does not necessarily equate to excitation at cellular level. Rather, the net effect of GABAergic signalling reflects a dynamic interplay between membrane depolarization, conductance changes, and local electrotonic properties, highlighting the importance of neuronal morphology in shaping functional outcomes.

The findings support a spatially stratified model of GABAergic modulation, in which distinct compartments of the neuron serve diverse functional roles. $GABA_A$ receptors are broadly distributed along microfluidic-cultured DRG neurons, including both somatic and axonal regions, with pronounced enrichment in proximal axonal segments near the bifurcation. Activation of $GABA_A$ receptors along distal axons in the periphery compartment, produces depolarization sufficient to trigger action potentials to travel to the cell body, consistent with prior reports of excitatory GABAergic effects in peripheral fibers (Bhisitkul et al., 1990; Bonalume et al., 2021; Brown & Marsh, 1978; Jang et al., 2017). However, although peripherally applied GABA can locally depolarize distal axons

and increase excitability, under baseline conditions it does not affect the propagation of action potentials evoked by peripheral electrical stimulation, indicating a limited role in regulating sensory transmission.

In contrast, GABAergic modulation at the soma and proximal axonal regions exerts a powerful inhibitory effect on signal propagation, as previously reported by our group (Du et al., 2017; Hao et al., 2023; Lall et al., 2025). Despite causing local depolarization, activation of somatic $GABA_A$ receptors reduces the reliability of action potential transmission through the T-junction. This effect likely arises from the unique electrotonic properties of the bifurcation, where increased membrane conductance and partial inactivation of Na_v channels, lower the safety factor for spike propagation (Du et al., 2017; Lall et al., 2025). Although GABAergic inhibition acts throughout the somatic area, the T-junction is likely to represent the most effective site for spike filtering, owing to the intrinsic impedance mismatch introduced by axonal bifurcation. Notably, this inhibitory effect is observed in both the proximal peripheral and central branches of the bifurcating axon, indicating that GABAergic modulation is likely to also occur upstream of the bifurcation, rather than being confined to the T-junction itself. Because *in vivo* T-junctions often lie near neighbouring neuronal somata within the ganglion, GABA released locally may act not only on other neurons' cell bodies, but also on $GABA_A$ receptors located at the T-junction (e.g. via volume transmission), directly contributing to the action potentials filtering.

The functional consequences of this compartment-specific regulation extend beyond the ganglion. By modulating spike reliability at the soma-T-junction region, intraganglionic GABAergic signalling effectively controls neurotransmitter release at the first sensory synapse, shaping DH neuron responses. In our experiments, we observed that local GABA application to the DRG somatic compartment reduced activity in DH neurons in response to electrical stimulation of peripheral DRG axon terminals, whereas activation of $GABA_A$ receptors along distal peripheral axons did not alter DH neuron responses. These findings directly support the view that the soma-T-junction region serves as a

critical inhibitory site, capable of regulating action potential propagation before spikes reach the central terminal. This mechanism positions the DRG as an active integrative hub, fine-tuning nociceptive information from the periphery to the spinal cord. Importantly, the observation that a single neurotransmitter can elicit distinct effects, helps explain previously conflicting reports on peripheral GABAergic signalling, demonstrating how excitatory and inhibitory outcomes can coexist within the same neuron depending on precise anatomical localization. While the role of the soma-T-junction region in spike filtering has been proposed in earlier work, the experimental approach developed here enables its direct functional interrogation and allows the underlying biophysical mechanisms of filtering to be examined with spatial specificity. Together, these findings provide a mechanistic framework for understanding how intraganglionic GABAergic signalling shapes nociceptive transmission and open new avenues for spatially targeted modulation of sensory processing.

6.3 Peripheral sources of GABA: Schwann cells and primary sensory neuron axons

Beyond the compartment-specific modulation of sensory neurons, GABAergic signalling has been found to shape other non-neuronal cell physiology, including Schwann cells and immune cells. Multiple studies have shown that Schwann cells can respond to GABA via $GABA_A$ and $GABA_B$ receptors, which regulate critical processes as development, myelination and nociceptive fibre architecture (Faroni et al., 2012, 2014; Magnaghi et al., 2004; Procacci et al., 2013; Serrano-Regal et al., 2020). In addition, the expression of the GABA-synthetizing enzymes GAD65 and GAD67 was reported in pre-myelinating and non-myelinating Schwann cells (Corell et al., 2015; Gerber et al., 2021) and the neuroactive steroid allopregnanolone was found to enhance GAD67 expression in these cells *in vitro* (Magnaghi et al., 2010).

Our findings extend this body of work demonstrating that Schwann cells express a complete GABAergic machinery, including GAD67, VGAT, GAT1 and the $GABA_A$

receptor allosteric modulator DBI, as detected by immunostaining both in primary cultures and *ex vivo* along the adult rat sciatic nerve. Importantly, we provide functional evidence that Schwann cells tonically release a bioactive ligand capable of activating $GABA_A$ receptors *in vitro*, although we did not pharmacologically discriminate between GABA and DBI. Following peripheral nerve injury, we observed a downregulation of these key GABAergic components in Schwann cells at the site of injury, including GAT1 and DBI, consistent with previous transcriptomic observations following nerve transection (Clements et al., 2017). These findings suggest that Schwann cell-mediated modulation of the local microenvironment is dynamically regulated under pathological conditions.

In parallel, we observed that primary sensory neurons can also release GABA from their peripheral axons, as previously suggested by Hanack and colleagues (Hanack et al., 2015). Using our microfluidic system, we observed that stimulation of DRG neurons axons with capsaicin or high K^+, triggered local GABA release, which was measured as EYFP quenching in HEK_{GABAA} reporter cells. Although this axon-mediated release did not measurably modulate action potential propagation under baseline conditions in our experimental setup, it nonetheless demonstrates that sensory axons are capable of locally supplying GABA to the periphery.

The functional implications of these peripheral sources extend to both neuronal and non-neuronal targets. Autocrine and paracrine signalling may regulate Schwann cell phenotypic transitions, supporting differentiation, maintenance, and repair programs via $GABA_A$ and $GABA_B$ receptor-mediated pathways. For instance, $GABA_A$ receptor signalling mediates Schwann cells differentiation from an immature, proliferative state to a myelinating phenotype (Faroni et al., 2012, 2014; Magnaghi et al., 2004), a process which may be particularly important in later stages of regeneration. In parallel, $GABA_B$ receptor activation has been implicated in axon-glia interactions, and the regulation of nociceptive fibers architecture (Magnaghi et al., 2004; Faroni 2014). Disruption of these pathways following nerve injury, as observed in our study, may therefore compromise

autocrine and paracrine control of Schwann cell plasticity, impairing appropriate phenotypic transitions required for effective repair and remyelination. This dysregulation may also exacerbate maladaptive interactions with immune cells, contributing to prolonged inflammation and the persistence of pathological states following injury.

In addition, peripherally-released GABA has the potential to directly act on nearby axons. Although distal axonal GABA application did not significantly alter the propagation of action potential elicited by peripheral electrical stimulation in our microfluidic model, the widespread expression of functional $GABA_A$ receptors along sensory axons indicates that axon-axon communication via GABA remains a plausible mechanism. *In vivo*, this form of signalling may become functionally relevant under specific conditions, such as high-frequency firing, injury, or inflammatory states. Consistent with this, Hanack and colleagues demonstrated that GABA released from peripheral sensory fibers exerts an autocrine and paracrine function engaging axonal $GABA_B$ receptors, which, by modulating TRPV1 activity, suppress nociceptive transmission. Importantly, this mechanism was shown to reduce nocifensive responses in models of inflammatory pain, highlighting the functional relevance of axon-derived GABA in pathological conditions (Hanack et al., 2015).

Together, Schwann cells and DRG neuron axons constitute complementary peripheral sources of GABA that likely act at a multicellular level, given the broad distribution of GABA receptors in the PNS. Although the precise functional role of this cross-talk remains unknown, this distributed signalling system may support peripheral homeostasis and represents a potential target in pathological conditions such as neuropathic pain and chronic inflammation. Future studies will be essential to clarify the precise mechanisms of Schwann cell and axonal GABA release *in vivo*, the extent of their interaction, and how this multi-source system integrates with central GABAergic circuits to regulate sensory processing and neuro-glial-immune communication. How this locally released GABA shapes immune cell behaviour, particularly macrophage polarization, is discussed in the following section.

6.4 GABAergic signalling shapes macrophage polarization

In addition to neuronal and glial mechanisms, the work presented in this thesis identifies macrophages as possible downstream targets of peripheral GABAergic signalling in neuropathic pain. Macrophages are now widely recognized as central drivers of chronic pain development and maintenance following nerve injury, accumulating both at the lesion site and within DRGs, where they release pro-inflammatory mediators that contribute to peripheral sensitization (Malcangio, 2020; Yu et al., 2020). At the same time, macrophages retain marked phenotypic plasticity, allowing their functional state to be shaped by local environmental cues (Chen et al., 2023; Xiaoye et al., 2024; Zhao et al., 2023). Our findings indicate that activation of $GABA_A$ receptors on macrophages attenuates their pro-inflammatory phenotype, reducing the expression of classical inflammatory genes *in vitro* and limiting macrophage accumulation in the injured nerve and DRGs *in vivo*, suggesting that peripheral GABAergic signals can restrain maladaptive immune responses during neuropathic pain.

These observations are consistent with a broader body of literature demonstrating that GABAergic signalling modulates diverse types of tissue-resident macrophages. For instance, GABA alleviated LPS-induced sepsis and high-fat-diet-induced obesity in mice mitigating macrophage-mediated acute and chronic inflammation (Fu et al., 2022). Similarly, GABA had strong anti-inflammatory effects in a mouse experimental autoimmune encephalomyelitis model (Li et al., 2024), and it has been found to modulate anti-tumor immune responses by polarizing tumor-associated macrophages towards a M2 phenotype (Zhang et al., 2021). These studies suggest that GABA functions as a conserved immunomodulatory signal across macrophage populations, capable of limiting pro-inflammatory activity and promoting resolution in multiple tissues.

Functionally, peripheral GABAergic modulation of macrophages in the PNS mirrors this established immunomodulatory role. In this study, using the SNI model, we demonstrated that repeated administration of the peripherally restricted $GABA_A$ receptor agonist isoguvacine moderately but consistently attenuated mechanical allodynia, with

the most significative effects emerging several days after injury. The delayed time course suggests that analgesia is mediated indirectly, likely through modulation of macrophage accumulation and polarization, rather than through acute changes in neuronal excitability. Consistent with this, isoguvacine treatment reduced macrophage numbers at the site of injury and in the DRG, where it promoted a higher proportion of M2-like macrophages. Notably, previous work from our group showed that isoguvacine also produced measurable effects on neuronal excitability and nocifensive behaviour when pain was elicited by downregulation of the endogenous $GABA_A$ receptor modulator DBI, in SGCs (Li et al., 2024). The difference between the neuronal effects observed in that model and the more modest neuronal effects in SNI likely reflects the more drastic nature of nerve injury in SNI, which involves widespread immune activation, structural changes, and complex maladaptive plasticity.

A recent study from Poslusney and colleagues demonstrated that a peripherally restricted $GABA_A$ receptor agonist attenuates visceral pain in a rat model of irritable bowel syndrome (Poslusney et al., 2026). Because the compound was chemically designed not to cross the BBB, its site of action must reside in the periphery. Accordingly, the authors proposed peripheral sensory neurons as likely targets and suggested that non-neuronal cells, including immune cells, may also contribute to the observed analgesic effects. Together, these findings reinforce the concept that peripheral GABAergic signalling can influence both macrophages and neurons, with context-dependent contributions to pain modulation.

Our data further indicate that macrophages actively remodel their GABAergic signalling machinery in response to inflammatory activation. Pro-inflammatory stimulation *in vitro* was associated with increased expression of enzymes involved in GABA synthesis, transport, and GABAergic signalling, suggesting that macrophages enhance their GABAergic machinery under inflammatory conditions. The prominent upregulation of GABA transport pathways is particularly notable, as it points to a potential role for macrophages in shaping local GABA availability within injured or inflamed tissues. By

regulating GABA uptake, macrophages may fine-tune inhibitory signalling in their microenvironment, thereby modulating not only their own activation state but also the behaviour of neighbouring cells. Such a mechanism is consistent with a feedback model in which inflammatory activation triggers compensatory inhibitory signalling aimed at limiting excessive or prolonged inflammation. Although we did not directly assess GABA release from macrophages, the coordinated expression of synthetic enzymes, transporters, and receptors supports the plausibility of both autocrine and paracrine GABAergic regulation within immune niches.

6.5 Concluding remarks and future perspectives

Together, the work presented in this thesis deepens the understanding of peripheral GABAergic cross-talk and it highlights its potential as a therapeutical target for chronic pain. We found that peripheral GABAergic signalling is a spatially organized, multicellular modulatory system that operates across neurons, glial cells and immune cells to shape nociceptive processing.

At neuronal level, our microfluidic data corroborate previous findings from our group which identified GABAergic signalling in the DRG as a crucial regulator of nociceptive transmission (Du et al., 2017; Hao et al., 2023; Lall et al., 2025; Li et al., 2024). Using compartmentalized cultures, we provide evidence that $GABA_A$ receptor activity at the soma and T-junction strongly influences DRG neuron excitability. While our approach enables spatially restricted application of GABA, further confirmation of junction-specific mechanisms would benefit from the use of caged GABA, which would allow focal release directly at the bifurcation, enabling a clearer dissociation between somatic and junctional signalling.

In contrast, GABAergic signalling along distal peripheral axons was found to be depolarizing per se, but it did not significantly alter DRG neuron excitability following electrical stimulation. The microfluidic platform proved to be a valid method to dissect GABAergic effects across distinct neuronal compartments, revealing a marked functional

divergence between proximal and distal axonal regions. Future investigations extending this approach to the central axonal compartment may further complete this spatial framework and clarify how peripheral and central GABAergic mechanisms are integrated to regulate sensory transmission.

While our microfluidic system reveals clear compartment-specific effects of peripheral GABAergic signalling, the functional relevance of axon- and glia-derived GABA is likely to be highly context-dependent and may differ substantially *in vivo*, where firing patterns, inflammatory cues, and cellular interactions dynamically shape peripheral signalling. Schwann cells, which we reported to express a full GABAergic machinery and release a bioactive $GABA_A$ receptor ligand *in vitro*, likely provide and additional source of inhibitory tone that can influence both neuronal excitability and the local microenvironment. Injury-induced downregulation of Schwann cell GABAergic components observed in our study suggests that disruption of this glial source may reduce the peripheral GABAergic tone as well as impair autocrine and paracrine regulatory mechanisms, compromising Schwann cell plasticity, remyelination, and repair processes. A similar downregulation of GABAergic signalling has been observed in DRG neurons and within the DH following nerve injury (Baba et al., 2003; Chen et al., 2014; Moore et al., 2002; Wang et al., 2021). The reduced GABAergic tone may further affect other cell-types, including tissue-resident macrophages and microglia.

Our data demonstrate that GABA polarizes macrophages toward an anti-inflammatory phenotype and that in a neuropathic pain model, peripheral administration of the $GABA_A$ agonist isoguvacine significantly reduces macrophage accumulation at the site of injury and in the corresponding DRGs. This raises the intriguing possibility that neuron- and glia-derived GABA may act *in vivo* to maintain tissue-resident macrophages in a non-activated state, restraining excessive inflammation under physiological conditions. Future experiments will be important to determine how macrophage GABAergic machinery is regulated *in vivo* physiologically and during injury, and whether cross-talk between neurons, Schwann cells, and immune cells contributes to shaping the peripheral

GABA-responsive niche. Clinically, these findings also suggest that peripherally targeted GABAergic therapies, such as isoguvacine - which does not readily cross the BBB - could represent a promising approach for pain management. Such strategies may provide analgesic and immunomodulatory benefits while avoiding the central side effects associated with currently available drugs.

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