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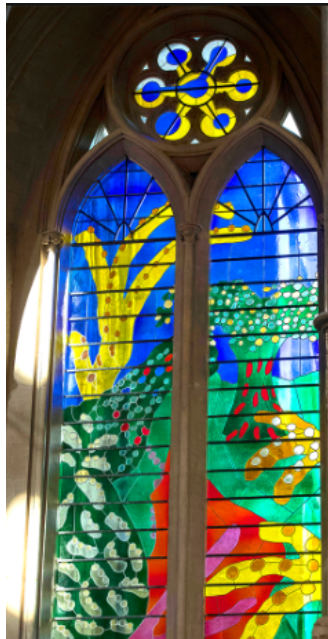
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Cellular, molecular and synaptic properties of pain: A microfluidic approach

Georgios P. Kimourtzis

Thesis submitted to King's College London for the degree of Doctor of Philosophy



A David Hockney design at Westminster Abbey
resembling a 3-compartment microfluidic device

Wolfson Centre for Age-Related Diseases
Institute of Psychiatry, Psychology & Neuroscience
King's College London, 2023

Declaration

I declare that the research presented in this thesis is my own work, unless stated otherwise in the text.

George Kimourtzis

September 2023

Abstract

The detection of stimuli that produce painful behaviors occurs within a subset of sensory neurons, namely nociceptors. The signals elicited by nociceptors are transmitted to the spinal cord through synaptic relay points in the dorsal horn. Nociceptors undergo significant plastic changes upon nerve injury and inflammation, where they augment painful signaling and can further modulate the excitability of central neurons, resulting in persistent pain. Elucidating the cellular and molecular properties of the somatosensory apparatus provides a deeper understanding of the physiology of painful signaling and opens therapeutic contingencies for the treatment of painful conditions. However, the plasticity of nociceptors and their contribution to painful pathologies has not been fully captured by existing methodologies. The current *in vivo* behavioral and electrophysiological techniques lack the spatial resolution to detect molecular contributions to nociceptor excitability, while *in vitro* studies have focused on the cell bodies of nociceptors and not the axons, that often represent the actual sites of injury.

To address this problem, we have employed a novel cell culture model which exploits advances in microfluidics. Microfluidic cultures allow the investigation of the functional properties of nociceptor axons separately from their respective cell bodies. Three problems are addressed using variations of this technique in my thesis. First, the properties that the inflammatory mediator prostaglandin E2 (PGE2) exerts on sensory axons are elucidated. Second, the periphery-to-center circuit between DRG and DH neurons is recapitulated and the formation of functional synapses between them is reported. Third, the pre-synaptic properties of transient receptor potential (TRP) and voltage-gated sodium (Nav) channels are examined and subsequently their contribution to DH excitability between axotomized and non-axotomized cultures is compared.

The results from the first investigation reveal a direct and persistent depolarization of sensory axons by PGE2. It is shown that the PGE2-evoked activity is dependent on the EP4/cAMP/PKA pathway. Evidence is provided for the mediation of this activity by the TTX-resistant sodium channel Nav1.8 and by the calcium activated chloride channel ANO1. Hence, it is proposed that PGE2 possesses a dual role as a sensitizer, but also as direct activator of sensory axons. The molecular mechanisms that underpin the latter phenomenon are delineated.

In the second investigation, mouse postnatal DRG and DH neurons are co-cultured in microfluidic devices. The co-culture model recapitulates salient features of synaptic transmission between DRG and DH, allowing the recording of DH neuron activity following DRG neuron stimulation. The model is important because it allows a spatially resolved interrogation of the molecular properties of nociceptors and the extent to which they contribute to synaptic transmission.

Last, the co-culture model is employed to investigate the contribution of pre-synaptic TRP and Nav channels to synaptic transmission. As a surrogate model of nerve transection, an axotomy is introduced and compared with the contribution of these channels under intact conditions. A role for Nav1.7 channels in synaptic transmission under intact conditions is reported, however it is lost following axotomy. Surprisingly, Nav1.6 channels emerge as principal contributors to synaptic transmission only following axotomy. A novel finding of this thesis regards the pre-synaptic role of TRPV1 and TRPA1 channels. Traditionally considered as transducer channels of thermal and chemical noxious stimuli, these channels show significant pre-synaptic activity where they dynamically contribute to synaptic transmission, a property which is lost following axotomy.

Overall, this thesis demonstrates the versatility of the microfluidic platform and unravels novel properties of a neuroimmune interaction relevant to inflammatory pain, synaptic transmission between DRG and DH neurons and finally, the pre-synaptic contribution of TRP and Nav channels in normal and axotomized conditions. The delineation of the molecular properties of nociceptors using pharmacological or genetic manipulations at distinct anatomical parts may further reveal unknown aspects about the physiology and pathophysiology of these neurons. The platform possesses an industrial utility too, as it can be used for the screening of novel analgesic molecules and the determination of how they affect the excitability of nociceptors or DH neurons.

Dedication

My thesis is dedicated equally to the most extraordinary people I have hoped to ever meet, my grandparents. The values, intellect and warmth over our common lifetimes have transformed into sociocultural and biological atavisms which I will ensure they endure beyond my generation. Words are unspeakably lame, but a written dedication is imperative; I love you and I miss you every day.

It is dedicated to my grandmother, Marika Kimourtzi, who would have celebrated this doctorate more than anyone. From your deepest blue eyes and playful smiles to your commitment to perfection, your unprecedented culinary skills and your literary knowledge, this is only the surface of your understated elegance and grace. You already knew about how everything would culminate, and I consider your departure as part of our telepathic understanding.

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To my grandmother Evangelia Mavri, the only one who will read this, I hope to be excused by English readers and address directly in Greek:

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Publications

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Abbreviations

5-HT	5-hydroxytryptamine/serotonin
ACC	Anterior cingulate cortex
Ach	Acetylcholine
AH-TTX	4,9-anhydrotetrodotoxin
ALT	Anterolateral tract neurons
AMPA	Amino-3-hydroxy-5-methyl-4-isoxazol propionate
ANO1	Anoctamin-1 channel
ANOVA	Analysis of variance
APs	Action potentials
AraC	Cytosine arabinoside
ATP	Adenosine triphosphate
ASIC	Acid sensing ion channel
BDNF	Brain-derived neurotrophic factor
BEST1	Bestorpin 1
BH4	Tetrahydrobiopterin
BK	Bradykinin
BoTox	Botulinum toxin
BSA	Bovine serum albumin
CAIA	Collagen-antibody induced arthritis
cAMP	Cyclic adenosine monophosphate

CC	Chemokine
CCI	Chronic constriction injury
CCK	Cholecystokinin
CeA	Central amygdala
CFA	Complete Freud's adjuvant
CGRP	Calcitonin gene-related peptide
CIP	Congenital insensitivity to pain
CIPN	Chemotherapy-induced peripheral neuropathy
CNS	Central nervous system
CR	Calretinin
Cryo-EM	Cryo-electron microscopy
COX	Cyclooxygenase enzyme
DAG	Diacylglycerol
DEG/ENaC	Degenerin/epithelial sodium channels
DH	Dorsal horn
DRG	Dorsal root ganglia
DY	Dynorphin
EP	Prostaglandin receptor
EPP	End-plate potentials or minis
FKN	Fractalkine
FOV	Field of view
GABA	Gamma aminobutyric acid

GAL	Galanin
GDNF	Glial cell line-derived neurotrophic factor
GFAP	Glial fibrillary acidic protein
GPCR	G-protein coupled receptor
HBSS	Hank's balanced salt solution
HCN	Hyperpolarization-activated cyclin nucleotide-gated channels
hiPSCs	Human induced pluripotent stem cells
IB4	Isolectin B4
IBS	Irritable bowel syndrome
IL	Interleukin
IM	Inflammatory mediators
IP3	Inositol 1,4,5-triphosphate
K2P	Two-pore domain potassium channel
Kca	Ca ²⁺ activated K ⁺ channels
Kir	Inward rectifier potassium channels
Kv	Voltage-gated potassium channels
KA	Kainate receptor
KCC2	K-Cl cotransporter 2
KCl	Potassium Chloride
KO	Knockout
KOR	Kappa opioid receptor
LTMR	Low-threshold mechanoreceptor

LTP	Long-term potentiation
MAPK	Mitogen-activated protein kinase
MFC	Microfluidic cultures
MRGPRD	Mas-related G-protein coupled receptor member D
mTOR	Mammalian target of rapamycin
Nav	Voltage-gated sodium channel subtype
NGF	Nerve Growth Factor
NK	Neurokinin receptor
NKCC1	Na-K-2Cl co-transporter
NMDA	N-methyl-D-aspartate receptor
NO	Nitric oxide
NOS	Nitric oxide synthase
NPFF	Neuropeptide FF
NPY	Neuropeptide Y
NSAID	Non-steroidal anti-inflammatory drug
NT-3	Neurotrophin-3
NTS	Neurotensin
OA	Osteoarthritis
P2X	Purinergic 2X receptor
p75	p75 neurotrophin receptor
PAG	Periaqueductal grey
PBS	Phosphate buffer saline

PEPD	Paroxysmal extreme pain disorder
PDMS	Polydimethylsiloxane
PFA	Paraformaldehyde
PGE2	Prostaglandin E2
PKA	Protein kinase A
PKC	Protein Kinase C
PLC	Phospholipase C
PLL	Poly-l-lysine
PNS	Peripheral nervous system
PV	Parvalbumin neurons
RAG	Regeneration-associated gene
ROI	Region of interest
S1	Primary somatosensory cortex
S2	Secondary somatosensory cortex
SEM	Standard error of means
SGCs	Satellite glial cells
SNARE	Snap receptor protein
SNI	Spared nerve injury
SP	Substance P
TENS	Transcutaneous electrical nerve stimulation
TG	Trigeminal ganglia
TNF	Tumor necrosis factor

TRAAK	TWIK-related arachidonic acid-stimulated K ⁺ channel
TREK-1	TWIK-1 K ⁺ channel
Trk	Tropomyosin hydroxylase
TLR	Toll-like receptor
TRPA	Transient receptor potential ankyrin channel
TRPC	Transient receptor potential canonical channel
TRPM	Transient receptor potential mechanical channel
TRPV	Transient receptor potential vanilloid channel
TTX	Tetrodotoxin
TTX-R	Tetrodotoxin-resistant
TTX-S	Tetrodotoxin-sensitive
V _m	Membrane potential
VGCC	Voltage-gated calcium channels
VGLUT	Vesicular glutamate transporter
VGSC	Voltage-gated sodium channels
WDR	Wide dynamic range neuron
WT	Wild type

1. Introduction

1.1 Pain: A multifaceted problem

1.1.1 The ontogeny of pain

Pain is a manifold sensory phenomenon upon which biological idiosyncrasy and empirical stochasticity converge to elicit a protective response and an unpleasant sensation. In an abstract context, pain describes “an awareness of an action to be rid of it” (Wall,1999), similar to homeostatic imbalances that require immediate relief and satiation such as the states of hunger or thirst. A “private and personal experience” (Melzack 1961), a formidable challenge is presented to express our own, let alone predict accurately another person’s magnitude of suffering. Virginia Woolf, fellow alumna of King’s College London, aphorizes in advocacy of that notion in her 1925 essay *On Being Ill*, stating that “the merest schoolgirl, when she falls in love, has Shakespeare, Donne, Keats to speak her mind for her; but let a sufferer describe a pain and language at once runs dry”. It is for subjective, conscious experiences such as the feeling of pain that philosophers of the mind introduced the ontological term *qualia* (Lewis,1929).

Yet, the ancient ancestors who inhabited my country of origin, Greece, had a remarkable repertoire of expressing variations of pain as found in Homer’s epic poems *Odyssey* and *Iliad*, in Sophocles’ tragedies or in Socrates’s and Aristotle’s philosophical works (Rey, 1995) (Cheng et al,2019). The word *algos* (άλγος), with its derivative terms analgesia and hyperalgesia extensively used in the pain field literature, possesses a predominantly sensory hue. In contrast, *odynē* (ωδίνη), the etymological root of allodynia, has a dual meaning: a localized description of labor pain in women and a wider description of internal sorrow applicable for both sexes. The word *achos* (άχος) was instead used as a descriptor of the emotional states of pain-induced anxiety but the connotation has changed since and every ache in contemporary English, from headache to heartache, is a derivative. It is however the term *pain* that has prevailed in modern times as the encompassing name of all things hurtful. Pain derives from the verb *penomai* (πένομαι) which means to be penalized or found in a condition of hardship and it has vernacularly transformed over the centuries into the Latin *poena* (penalty/punishment).

And for all the richness of the ancient greek vocabulary or Ronald Melzack’s ingenuity to catalogue all sensory, affective and evaluative word descriptors of pain in a questionnaire (Melzack,1975), an objective quantification measure of pain is lacking, yet (nearly) everyone has

experienced it. What is pain and what is the purpose of it? Jacques Monod, in his influential book *Chance and Necessity* (Monod,1974) introduces *teleonomy*, a tenet that biological adaptations may exert a self-purpose without an ulterior motive. Therefore, circumstances are the springboard of evolutionary adaptations, but the objects are not themselves endowed with intent. In compliance with the teleonomic principle introduced by Monod, pain as we know it probably has evolved from simple avoidance responses of prokaryotes to this ornate sensory and emotional experience that the most complex object known in the universe, the human nervous system has constructed (Walters & Williams,2019). If indeed true, then what are the evolutionary adaptations over the millennia that culminated in the feeling our species now recognizes as pain? The answers can be conjectured but a complete dissection of the phenomenon still eludes us.

The life kingdom is replete with examples of avoidance mechanisms, protective reflexes and pain-like behaviors. Despite its unicellular constitution, the ancient bacterium *Escherichia coli* (*E. coli*) responds to forces and changes in osmolarity by expressing the mechanosensitive channels MscL and MscS (Sukharev et al,1994). Bilaterally symmetric phyla with inchoate nervous systems such as annelids, arthropods mollusks and nematodes, among them being the commonly used animals for research *Aplysia*, *Caenorhabditis elegans* (*C.elegans*) and *Drosophila melanogaster* (*D.melanogaster*), possess nervous tissue that encodes mechanical, chemical and thermal stimuli (Kavaliers et al,1988) (Smith & Lewin, 2009). Octopi and squids, two cephalopod species, use chemotactile receptors that structurally resemble nicotinic acetylcholine receptors and which are expressed in their arms to evaluate chemosensory cues in their vicinity (Allard et al,2023) (Kang et al,2023).

Most vertebrate species, and in particular all mammals, detect noxious stimuli through peripheral nervous tissue. A few species have even evolved to resist extreme physiological conditions that are normally painful to most mammals. The naked mole rat survives anoxic conditions by anaerobically glycolyzing glucose (Park et al,2017). This remarkable species expresses a variant of the Nav1.7 channel that is blocked by protons to prevent action potential (AP) conduction upon acid-sensing (Smith et al,2011) while mutations in tropomyosin kinase A (TrkA) receptors impair potential painful sensitization by the nerve growth factor (NGF) (Omerbasic et al,2016). Neanderthals, on the other hand, possessed a few key amino acid substitutions in their Nav1.7 protein that caused a reduced inactivation of the channel and possibly resulted in enhanced painful

responses (**Zeberg et al,2020**). An enhanced painful sensation may have been a much-needed protective mechanism for the foraging lifestyle of our ancestors, and its loss may reflect an adaptation to a settled lifestyle as humanity entered the neolithic revolution of primitive societal structures and agricultural advances.

In humans, pain acts as the somatosensory correlate of alertness from actual or potential injury. It normally acts as a protective mechanism; it is suggestive of rapid mobilization to avoid danger or regional immobility while healing occurs (**Scholz & Woolf,2002**). Although naked mole rats have evolutionarily benefited from attenuated noxious sensitization, humans who are congenitally insensitive to pain have found themselves to ignore extensive burns, bruises and fractures (**Melzack & Wall, 1973**). Others, due to their inability to experience abdominal pain have been subjected to life-threatening appendicitis or untreated widespread infections. These anecdotal descriptions of congenital analgesia in humans have eventually been attributed to genetic loss-of-function mutation for key molecules that mediate and regulate peripheral nerve excitability such as the TrkA receptor (**Indo et al,1996**), the Nav1.7 channel (**Cox et al,2006**) and more recently of the transcriptional regulator ZFHX2 in sensory neurons (**Habib et al,2018**).

Less empirically verifiable but plausible conjectures on the evolutionary purpose of somatosensation in mammals may arise from systems and theoretical neuroscience. The somatosensory nervous system, detects and processes an exorbitant number of salient stimuli. The detection of palpable external stimuli allows the brain to create internal representations of the world. Based on prior beliefs created by the processing of sensory input, the brain allows for predictions to be made about the future and devises relevant behaviors in a process named active inference (**Sajid et al,2021**). Mismatches between actual sensory input and nervous system processing may cause inaccurate representations of reality, quantified as prediction error (**Schultz & Dickinson,2000**). An accurate representation of the world via associative distinctions of what is painful and what is not may minimize prediction error and prevent harm (**Ploghaus et al,2000**) (**Seymour et al,2005**). By possessing a finely tuned and operational somatosensory apparatus, the brain samples exteroceptive input optimally, reduces uncertainty and enables it to make more accurate predictions about the future (**Friston,2010**) (**Seymour,2019**). Subsequently, the avoidance of harm allows the maintenance of a functional homeostasis, hence optimizing the survival probability of a living system (**Friston et al,2006**). Thus, the threat of a painful event may

endanger the integrity of an organism. On the other hand, chronic pain manifestations might be an adaptive mechanism in the form of a persistent alert to avoid any injury that may jeopardize survival even further than before (**Seymour et al,2023**). An individual with congenital analgesia, by being oblivious to external threat, is an aberrant sampler of environmental cues. So is a person with gain-of-function painful mutations who is rendered immobile although the same environmental conditions are not posing a threat for their human peers.

Just as Ludwig Wittgenstein argued in *Tractatus Logico-philosophicus* that the breadth of our vocabulary shapes the limits of our world (**Wittgensetein,1929**), our magnitude of pain sets the limits of our actions. Mice and other experimental animals do not catastrophize about their future, their dialects are less sophisticated and their breadth of sampling the world is less analytical. On the contrary, human survival is dependent on both a sensory and an emotional integrity. Persistent pain diminishes the quality of life; it might deprive someone of their job, their social interactions, their sleep and sanity. The unprecedented complexity of human pain is perhaps best echoed in Dante's *Divine Comedy* (Canto IV 103-111, *Inferno*): "Remember now your science, which says that when a thing has more perfection, so much the greater is its pain or pleasure".

1.1.2 The socioeconomic impact of chronic pain

In addition to its deep biological and philosophical complexity, pain poses an equally formidable societal problem. Somewhat arbitrary in its clinical definition, any pain lasting for more than 3-6 months is also considered chronic (**Treede et al,2015**). Careful epidemiological studies in Europe (**Breivik et al,2006**) and the US (**Dahlhamer et al,2018**) have concluded that one fifth of the population lives with chronic pain. If the percentage is assumed analogous across all continents, it is translated into an absolute number of nearly 1.5 billion people worldwide living with some form of disability related to chronic pain. Lower back pain, migraine headache, neck pain and musculoskeletal pain are the disorders that appear with highest prevalence (**Vos et al,2016**). Pathologies where chronic pain is also very commonly reported include arthritis, cancer, diabetes, fibromyalgia and irritable bowel syndrome (IBS).

There are several factors that are associated with chronic pain that have demographic, lifestyle, or clinical origins. Age is a paramount contributing factor: a patient age stratification study in the UK

showed that chronic pain prevalence rose from 14.3% in ages 18-25 to a quadrupled 62% in ages over 75 (**Elliott et al,1999**). Gender differences are another very important factor as women report lower pain thresholds and tolerance (**Wiesenfeld-Hallin,2005**) and are more likely to experience chronic pain, pinpointing towards distinct biological mechanisms (**Bartley & Fillingim, 2013**). There are considerable ethnic disparities in pain perception which highlight the need to study diversely the genetic contributions to pain while also considering more carefully cultural contexts in healthcare and pain management (**Cambell & Edwards,2012**). For instance, racist stereotypes in the US about the enhanced tolerance of African Americans to pain have resulted in their undertreatment in pain management. These stereotypes have been debunked by clinical studies (**Losin et al,2020**) and have highlighted a systemic racial bias in the treatment of African Americans to painful conditions (**Hoffman et al,2016**). People with less privileged socioeconomic background have a higher prevalence of experiencing chronic pain (**Duenas et al,2016**). Lifestyle choices such as alcohol consumption, nutrition, smoking, physical activity and exposure to sunlight have all been correlated with chronic pain (**Mills et al,2019**). Deteriorating mental health, weight imbalance, sleep disorders, and genetic discrepancies add a clinical dimension that may further complicate management. Depression and anxiety impose potent co-morbidities where 52% of patients in pain clinics also report such negative symptoms (**Bair et al,2003**).

The individual detriment of being in chronic pain can be more cynically reflected in the socioeconomic costs both to the individual and to society. In the US, the financial consequence of having 100 million citizens with unrelieved chronic pain, costs the country's economy $\approx$ 600 billion dollars per year. Of the subsequent financial issues, the lion's share goes to direct healthcare costs. The problems further extend to the days of work missed and the lower salaries paid (**Smith & Hillner,2019**). In the US, the incremental costs of medical expenditures include payments to private insurances, the Medicare package but also direct payment out of pocket in uninsured cases (**Gaskin & Richard,2012**). Fortunately, access to healthcare in Europe and the UK is a public benefit of the welfare state, yet chronic pain still poses a financial challenge. In Austria, each patient pays $\approx$ 10k euros per year for pain treatment costs, which represents nearly one third of the yearly average salary (**Mayer et al,2019**). In total, the costs of managing pain in Europe approach 200 billion euros (**Barham,2012**) while in the US the estimate is more than triple at 635 billion dollars (**Gaskin & Richard,2012**).

Despite the meteoric financial burden of chronic pain in society, the landscape of available treatments for chronic pain has been unsatisfactory. The recommended line of pharmacological treatment for acute pain and inflammation (see **Types of Pain** section for definitions) are the non-steroidal anti-inflammatory drugs (NSAIDs), also efficacious for headaches and dysmenorrhea (period pain). However, NSAIDs cannot be chronically prescribed due to their adverse effects that include gastrointestinal bleeding, myocardial infarction and stroke prevalence (**Davis & Robson, 2016**). Gabapentinoids, tricyclic antidepressants and serotonin-noradrenaline reuptake inhibitors have been suggested as a first line of treatment for neuropathic pain (see **Types of Pain** section for definition), although they are ineffective to a large percentage of patients (**Finnerup et al, 2015**). On the opposite end of the spectrum, severe pain conditions are treated with opioid analgesics but their administration may underlie debilitating side effects of addiction, respiratory failure, constipation, nausea and vomiting (**Benyamin et al, 2008**). In the absence of more efficacious analgesic solutions, the abuse of opioid prescriptions in the US culminated in an epidemic where in its heyday, 238 million of narcotic analgesic prescriptions were made within one year and tens of thousands of lives were lost to complications of addiction (**Manchikanti et al, 2012**). Non-pharmacological options such as transcutaneous electrical nerve stimulation (TENS), have been used with controversial efficiency (**DeSantana et al, 2008**). More recently the neuromodulation of peripheral nerves via ultralow frequency stimulation has shown promise (**Jones et al, 2021**) but is still in nascent stages of development. It is topical and urgent to identify novel targets for pain relief and the only methodological approach to achieve this is through the mechanistic dissection of the physiological and pathophysiological correlates of pain at all molecular, cellular, circuits, behavioral and systems levels. If promising, therapeutic strategies can then be devised in drug discovery, genome editing, stem cell therapy, applied physics and bioengineering.

1.1.3 A brief anatomy of the pain pathway

The somatosensory nervous system is comprised of the sensory (or primary afferent) neurons of the peripheral nervous system, the spinal cord and several brain areas (**Basbaum et al,2008**). Sensory neurons are organized in clusters named dorsal root ganglia (DRG). The DRG cell bodies are found within the dorsal root of spinal nerve cavities and are enclosed by the spinal column. These neurons have a pseudo-unipolar structure, where a single axon, interconnected with the DRG cell body via a T-shaped junction protrudes bidirectionally. The axons innervate the skin and peripheral target organs at the distal site and central terminals at the proximal site. The DRG neurons can be broadly subdivided into three subtypes according to their diameter size, myelination density and conduction velocity. These subtypes are the A β -fibers, the A δ -fibers and the C-fibers (**Figure 1**). Of the three subtypes, A δ -fibers and C-fibers, via their distal axonal sites known as free nerve endings detect and process noxious stimuli such as heat, cold, pressure and noxious chemical stimuli and are called nociceptors.

Nociceptive fibers convey the detected noxious stimuli to distinct superficial laminae of the spinal cord's dorsal horn (**Figure 1.1**), forming a relay point which is henceforth referred to in this thesis as the first sensory synapse. There are complex circuitual pathways within the spinal cord that involve the conveyance of sensory information from the dorsal horn to excitatory or inhibitory interneurons which in turn transmit the information to projections neurons in the spinal cord. The ascending course of nociceptive signals culminates in the transmission of sensory information from the spinal cord neurons to the brainstem and then to the neocortex.

There are two well characterized ascending brain pathways for pain. The first pathway originates from the spinothalamic tract and regards mainly the discriminative and sensory processing of pain signaling (**Figure 1.1**). The second pathway is mediated through the spino-parabrachial tract and conveys sensory information to the limbic system, which processes the emotional and aversive components of pain (**Figure 1.1**). Also present but not shown, there are significant descending modulatory pathways which can suppress or enhance pain further.

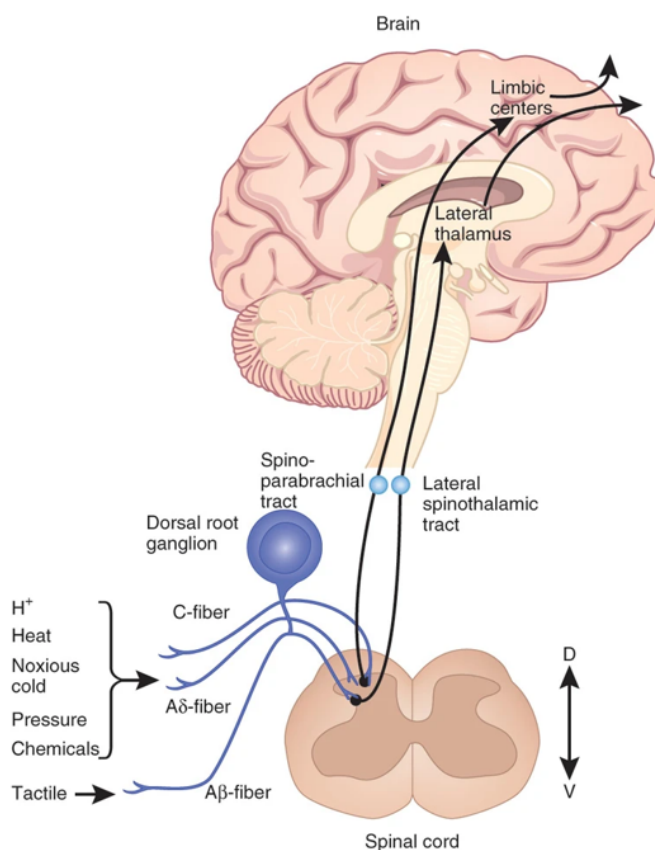


Figure 1.1 Basic anatomy of the pain pathway. Specialized sensory neurons called nociceptors are comprised of A δ - and the C-fibers and detect noxious stimuli in the periphery. The noxious signaling is transmitted to the dorsal horn of the spinal cord which is then further conveyed via ascending supraspinal pathways to be processed by the brain. **(Modified from Kuner, 2010)**

1.1.4 Theories of pain

Historical accounts of the study of pain can be traced since antiquity. Celsus of Tiberia and Areataeus of Cappadocia attempted to describe painful conditions and attribute probable anatomical causes, but it is Galen of Pergamum who suggested that pain is a sensation and impressively reported loss of sensory and motor function following spinal cord transection of pigs **(Rey,1995)**. Ibn Sina (Avicenna), a Muslim philosopher and physician in the 10th century AD, recognized that pain is a distinct sensation from touch and temperature **(Perl, 2007)**. The religious obscurantism of the Middle Ages stalled scientific enquiry but Renaissance and the re-emergence

of anatomy as a field of scientific study paved the way for Rene Descartes, who in the *Treatise of Man* (written in the 1630s) described the archetypical notion behind what came to be known as specificity theory. The cartesian view was that of a labeled line as demonstrated by La Forge's famous illustration where a boy's foot is exposed to a fire and a linear circuit originating from the foot and terminating to the brain gives rise to protective reflexes **(Moayedi & Davis,2013)**.

A formal articulation of the Specificity theory of pain came from the Scottish physician Charles Bell, when he suggested that distinct nerve bundles hold specialized sensory functions for different stimulation types while other nerve bundles exert motor functions **(Bell & Shaw,1968)**. The French physician Francois Magendie corroborated Bell's reports, spatially differentiating motor from sensory nerves' paths to and from the spinal cord, a neuroanatomical observation formulated as the Bell-Magendie law **(Moayedi & Davis,2013)**. More discoveries in the 19th century of end organ touch receptors such as the Pacinian and Meisner corpuscles, Merkel's discs and Ruffini's end organs further pointed towards a specialized nerve fiber function. Initially, Johannes Muller and then Max von Frey argued that the brain does not have access to objects but only to nerves, hence modalities such as heat, cold, pain and touch are nerve-detected **(Perl & Kruger,1996)**. The culmination of these ideas was expressed by Charles Sherrington by characterizing the functional integrative properties of the nervous system through robust experimental evidence. Sherrington suggested that specialized end-organ, cutaneous nerve fibers detect and process noxious stimuli, and named those fibers nociceptors **(Sherrington,1906)**. More than half a century later, Iggo in Europe **(Iggo,1960)** **(Brown & Iggo,1967)** and Perl in the US **(Burgess & Perl,1967)** demonstrated the existence of nociceptors by characterizing myelinated nerve fibers that respond to noxious mechanical stimulation and unmyelinated fibers that were high-threshold mechanosensitive as well as polymodal nociceptors **(Bessou & Perl,1969)**.

As a counterproposal to Specificity Theory, summative stimulation was suggested to be the source of pain magnitude, a theory that came to be known as Intensity theory. The theory argued that nerve fibers are not discriminated into low- and high-threshold, rather innocuous stimuli produce smaller discharges while noxious stimuli are signaled through more intense discharges **(Perl, 2007)**. A Patterning Theory was proposed by John Nafe **(Nafe, 1929)**, stating that each afferent responds with different intensity and that the composite pattern of the cumulation of afferent responses is responsible for the spatiotemporal encoding of sensory input to the central nervous

system. Elements of these two theories have held grains of truth, yet not at the peripheral nerve level. The discovery of wide dynamic range neurons in the posterior grey column of the spinal cord by Lorne Mendell (**Mendell & Wall,1965**) (**Mendell,1966**) supported that both painful and non-painful stimuli are encoded by these neurons, and in fact, repetitive stimulation of nerve fibers may increase the intensity of painful responses, leading to a phenomenon known as ‘wind-up’.

A decades long opposition between the Specificity and Patterning theories led Melzack and Wall to propose a new theory of pain, one that contextualizes and encompasses key observations of the previous ones but also introduces a role of the spinal cord in modulating peripheral sensory input. The Gate Control Theory (**Melzack & Wall,1965**) recognized from Specificity theory that indeed A δ - and C-fibers detect noxious stimuli and transmit signals to the spinal cord. However, in contrast to Specificity Theory, Melzack and Wall opposed the simplistic labelled line model of sensory input transmission to the higher orders of the central nervous system and instead placed the substantia gelatinosa neurons of the dorsal horn (DH) as the spinal gates that modulate ascending excitability. They considered that “gating” 2nd order spinal neurons in the substantia gelatinosa control the input and modulate the output of “transmitting” 3rd order spinal neurons which then inform the brain of a painful stimulus (**Figure 1.2**). Their theory thus meant that input from nociceptor A δ - and C-fibers can be antagonized and ameliorated by activation of A β - fibers which are touch receptors. Hence, upon injury one rubs themselves to modify the spinal gate and alleviate their pain. The model also predicted a descending modulatory pathway from the brain that gates sensory input too (**Melzack,1996**). It is now accepted that the intricacy of the spinal cord circuitry is not entirely captured by the gate control theory (**Todd,2010**) (**Mendell,2014**), but given the lack of technical advancements at the time, the leap of faith to articulate it was extraordinary. In the words of Wall himself, who felt that several aspects of the theory were misunderstood: “Most write of the spinal gate as an elaborate gain control system affecting a one-way, input-output, pain-producing mechanism. I think of it as the tip of a distributed and integrated feedback mechanism, one of whose functions is to produce pain as an output state” (**Fitzgerald,2022**).

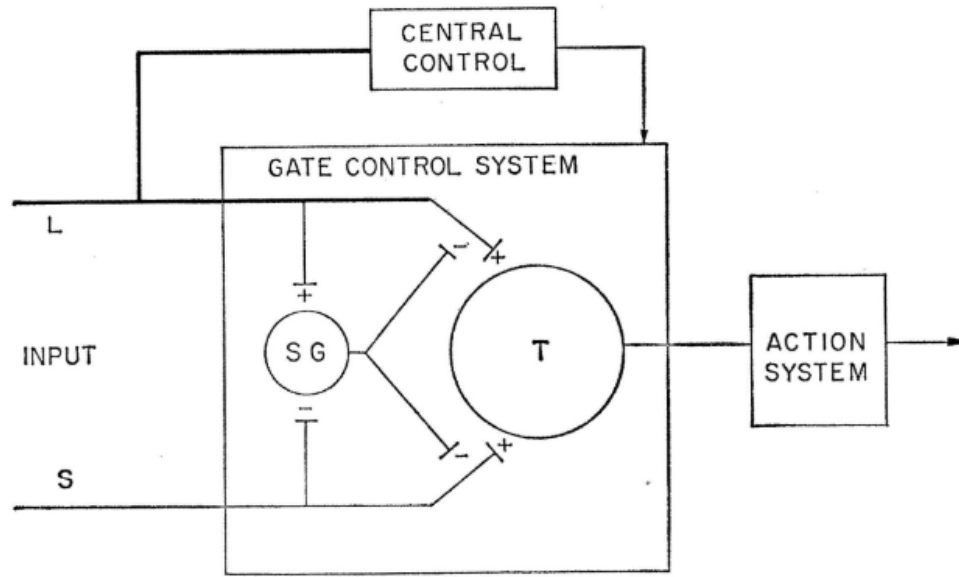


Figure 1.2 The gate control theory of pain. The dorsal horn of the spinal cord forms a circuit that receives input from touch receptors and nociceptors. Excitatory (+) or inhibitory (-) connections modulate gating (SG) dorsal horn neurons which in turn further modulate the output of the transmitting (T) spinal neurons. It is thus not linear that nociceptor input can produce pain rather it depends on the balance between nociceptor and touch receptor inputs. (Adapted from Wall & Melzack,1965).

Regardless of the extent of its validity, the Gate Control Theory established the study of pain as an experimental neuroscientific field in the 20th century. Building upon the theory, Wall and Sweet (1967) introduced transcutaneous electrical nerve stimulation (TENS), a therapeutic method where voltage stimulation of previously sensitive areas transiently minimized pain in certain patients. Melzack complimented the theory by further involving the brain, where he considered that a wired system of interconnected brain areas, called “the neuromatrix”, gives rise to the perceptual experience of pain (Melzack,1990). In that vein, the fMRI observation of a pain matrix brain signature was reported (Tracey & Mantyh,2007), a controversial subject amongst systems pain neuroscientists (Legrain et al,2011) (Salomons et al,2016). More recently, Gate Control has been challenged at the spinal circuitual level and evidence has been provided for distinct subpopulations of the DH that mediate allodynia caused by neuropathic or inflammatory pain (Peirs et al,2021), implying specificity at least at the DH level. A notion that has been recently brought forward is

that the gating occurs at the peripheral level of dorsal root ganglia (DRG), where proprioceptive sensory neuron deletion results in an enhanced nociceptor activity and the balanced wiring of sensory neurons is more critical than the spinal processing (**Fuller et al,2023**).

The *status quo*, especially after the influential studies of mouse DRG neuron identity using mouse single-cell RNA sequencing (**Usoskin et al,2015**) (**Sharma et al,2020**) and more recently human DRG RNA sequencing (**Nguyen et al,2021**) and spatial transcriptomics (**Tavares-Ferreira et al,2022**), is a consensus that peripheral sensory neurons encode noxious stimuli in a labelled line manner. The molecular taxonomy of primary afferent subtypes may however differ between species, while it is not unchangeable. There are several different forms of sensory input and abrupt physical interventions that can induce plastic changes and affect the molecular and functional identity of sensory neurons. Yet, it is the spinal cord's complexity that has not yielded a verifiable theory on whether painful stimuli are encoded as patterns or also follow labelled lines. Finally, the brain forms the last frontier in somatosensory processing, a black box where sensory input fuses with the emotional valence of pain perception in an unknown as-yet spatiotemporal manner.

1.1.5 Types of Pain

Every painful event is a unique occurrence in nervous system space-time. The classification of pain conditions can therefore be described in varied terms. The International Association for the Study of Pain (IASP) currently defines pain as “an unpleasant sensory and emotional experience associated with, or resembling that associated with, actual or potential tissue damage” (**Raja et al,2020**). Of course, any generalized definition of pain is antithetical to the individualistic perception of pain and it's a futile philosophical or clinical exercise. Despite the diversity in genetics, prior experiences and emotional processing mechanisms of a patient, it is certain universal characteristics regarding the duration and etiological nature of injury that end up defining pain subtypes. This subsection examines both temporal and qualitative dimensions that define pain states. A first division is made between transient and chronic forms of pain, highlighting certain benefits that each respective definition encapsulates but also stating important caveats to this dichotomy. Second, the suggested transitional states between acute and chronic pain are discussed. Last, the pain state classifications reflecting the broad etiologies of pain are described in

pathological settings. These latter subtypes have been widely accepted by both clinicians and basic researchers within the neurobiological field of pain and have been incorporated into its jargon.

1.1.5.1 Chronic and non-chronic pain

An initial subdivision can be made between chronic and non-chronic pain, a solely temporal dimension. This subdivision argues that if there is not a resolution of a defined pain within 3-6 months, then a pain is chronic (**Treede et al,2015**). There is some merit to this definition as the healing phase from most injuries rarely has a more long-lasting duration. We cut our skin during shaving or scratch our extremities on a rough surface, and it all subsides within a brief period. Certain forms of non-chronic pain can be incredibly transient, such as acute pain which immediately ceases after detaching oneself from the source of discomfort. Other forms of non-chronic pain can be moderate illnesses, bacterial infections, minor burns, a strained muscle or a minor sprain, a post-surgical pain or labor pain. Perhaps the most neatly regulated form of transitory pain in mammalian biology is dysmenorrhea. All these pain states, regardless of their temporarily perceived magnitude, are tentatively reversible but on some occasions pain that arises from them may persist chronically.

On the other hand, chronic pain involves a form of discomfort and unpleasantness that has resisted resolution. Common types of chronic pain are forms of arthritis, diabetic neuropathy, cancer, compressed nerves or lesions, fibromyalgia, stroke, angina, endometriosis and possibly all conditions ending in -itis (**Yaksh,2020**). An initial disturbance with or without defined injury that originated like all other pains has henceforth morphed into a persistent bane. In chronic pain, the conveyance of sensory information to the dorsal horn initiates several yet distinct cascades of physiological events where the sensitization of the peripheral nervous leads to a central nervous system sensitization (**Woolf,1983**). These cascades are finely orchestrated by molecular, neuronal and intercellular modulations in a stochastic fashion according to the modal nature, intensity and location of the damage. The modulatory phenomena following neuronal injury are encompassed under the term *maladaptive plasticity* and at the cellular level they involve a triad of cellular culprits: neurons, glia and immune cells (**Scholz and Woolf, 2007**). The contributing physiological mechanisms form an ornate mosaic, the decipherment of which requires microscopic investigations of the molecular interactions eventuating after injury, and more macroscopic

investigations of circuit plasticity that generate anatomical changes in periphery, spinal cord and the brain **(Reichling & Levine,2009) (Kuner & Flor,2017)**.

The stochasticity of chronic pain development and the multiple factors that weigh in the behaviors of individuals can be reflected in the most well-controlled clinical setting of inducing incisions during medical operations **(Kehlet et al,2006)**, where persistent postsurgical pain occurs to 10-50% of operations. In sports, where surgery is commonly required, there are several occasions where athletes cannot return to previous performance levels. There is much difficulty to discern whether this has happened because the damage has had an irreversible effect on their physiological integrity that has eluded the rectification of surgery or whether the performance is hampered by psychological reservations and enhanced caution **(Arden et al,2013)**. Conversely, it is documented by the American anesthesiologist Henry Beecher, that a percentage of men that were seriously wounded or even amputated in battle, would report lack of pain. The relief for their survival, their anticipation to see their beloved ones and the affirmation that their efforts will be recognized as heroic suppressed any sense of pain **(Beecher,1946)**. Hence, pain chronification, along with the peripheral mechanisms where pain originates, has a profound psychological component. The brain can prolong pain by catastrophizing or oppositely can be tricked to suppress it with various manifestations of the placebo effect **(Kaptchuk & Miller, 2015)**.

1.1.5.2 Acute to chronic pain transition

Painful sensation can be dissected into two broad physiological constituents. The first component regards the process of detecting, propagating and transmitting noxious stimuli, called nociception. The second component is an affective one, that characterizes an emotional and idiosyncratic interpretation of noxious stimulation that may result in aversion, unpleasantness and persevering discomfort. The emotional component of pain is epiphenomenal to a confirmed or probable affliction, and it is attributed under universal consensus to brain mechanisms that are recently becoming more tractable at a detailed circuitry level **(Hua et al,2020) (Yang et al,2021)**. Nociception on the other hand, involves presynaptic sensory neurons, postsynaptic dorsal horn neurons and higher order spinal and supraspinal circuitry. Whether there is an ascending anatomical site in the brain where nociception hands over the baton to the emotional component of pain or whether pain is an emergent property of the nervous system with a stochastic, immediate

imprint that holds sensory encoding entangled with emotive encoding is not clear. Yet, it is undeniable that adaptive responses to injury in the periphery and spinal cord greatly enhance the probability of manifesting persistent pain and it is postulated that there is a transitional process that accounts for this.

It has been proposed that the peripheral sensitization of nociceptors initiates molecular cascades that trigger the gradual sensitization of the central nervous system. The Levine lab at UCSF has developed a “hyperalgesic priming” model, where after carrageenan injection and the resolution of a first hyperalgesia, a second stimulation with inflammatory mediators triggers a long-lasting second hyperalgesic response (Aley et al,2000). Thus, a collection of plastic molecular changes that involve the regulated activation of Phospholipase C (PLC), Protein Kinase A (PKA), MAP kinases, C-jun and mTOR pathways along with post-translational modifications in the periphery can confer a prolonged hyperexcitability and a persistent sensitization. In turn, the prolonged amplification of input by sensory neurons can give rise to a central sensitization of the dorsal horn (Woolf,1983). The aberrant release of glutamate by the ‘primed’ sensory neurons may subsequently induce an engagement with post-synaptic N-methyl-D-Aspartate (NMDA) and amino-3-hydroxy-5-methyl-4-isoxazol prpionate (AMPA) receptors which in turn produce a sensitization of the spinal cord, representing a form of long-term potentiation (LTP) within the first sensory synapse (Woolf & Salter,2000) (Sandkuhler, 2007) (D’Mello & Dickenson,2008).

1.1.5.3 Acute, Inflammatory & Pathological Pain

In experimental neurobiology, the most convenient classification of pain subtypes regards the mode of actual or potential painful injury. Thus, pain is subdivided into acute, inflammatory and pathological pain (Woolf,2010). Acute or nociceptive pain regards the detection of thermal, chemical and pressure noxious stimuli which in turn elicits very transient pain behaviors and autonomic reflexes. It is an adaptive, high-threshold response which has a clear, immediate protective purpose (Figure 1.3). Certain responses are simple withdrawal reflexes to noxious stimulation, called nocifensive responses. Within this category, certain acute pains subside within seconds or minutes and leave no remnants of the initial cause of pain.

Inflammatory pain occurs after tissue injury or peripheral inflammation where immune cells secrete mediators that sensitize nociceptors, and it is associated with spontaneous pain and pain hypersensitivity, mostly exerted as hyperalgesia. Inflammatory pain is adaptive and low-threshold and its purpose is mid-lasting protection while healing occurs (**Figure 1.3**). There are temporary forms of inflammatory pain such as mild burns and bruises or the healing phase of a medical operation. As mentioned earlier, successive inflammatory applications can trigger more long-lasting effects (**Hucho & Levine,2008**). There are several pathological states of strong immune component and inflammatory pain profiles, especially osteoarthritis and rheumatoid arthritis.

Last, pathological pain may be subdivided into two further classifications: neuropathic pain and dysfunctional pain (**Figure 1.3**). Neuropathic pain occurs due to a lesion or damage to nerve fibers. It is also associated with spontaneous pain and hypersensitivity, but also with allodynia, the property where previously innocuous stimuli produce noxious responses. Causes of neuropathic pain are peripheral nerve injury, diabetic neuropathy, cancer pain, chemotherapy-induced peripheral neuropathy (CIPN), HIV infections, amputation, postherpetic neuralgia, trigeminal neuralgia, or radiculopathy. The cellular components that lead to neuropathic pain are not only neurons but also immune and glial cells (**Colloca et al,2018**). Because neuropathic pain regards a persistent maladaptation of the nervous system, it ends up defining a disease state by itself. Dysfunctional pain also results in the peripheral and central sensitization of the pain pathway. Conditions such as fibromyalgia or irritable bowel syndrome, where there is no obvious pathological cause or nerve lesion, represent dysfunctional pain. However, it has been recently shown that fibromyalgia may have neuroimmune origins (**Goebel et al, 2021**), and that distinct subpopulations of DRG afferents convey mechanosensitive signals during colon inflammation (**Wolfson et al,2023**) while the PIEZO2 channel regulates gut motility and colon distension (**Servin-Vences et al,2023**) pinpointing towards cellular and molecular therapeutic avenues in IBS.

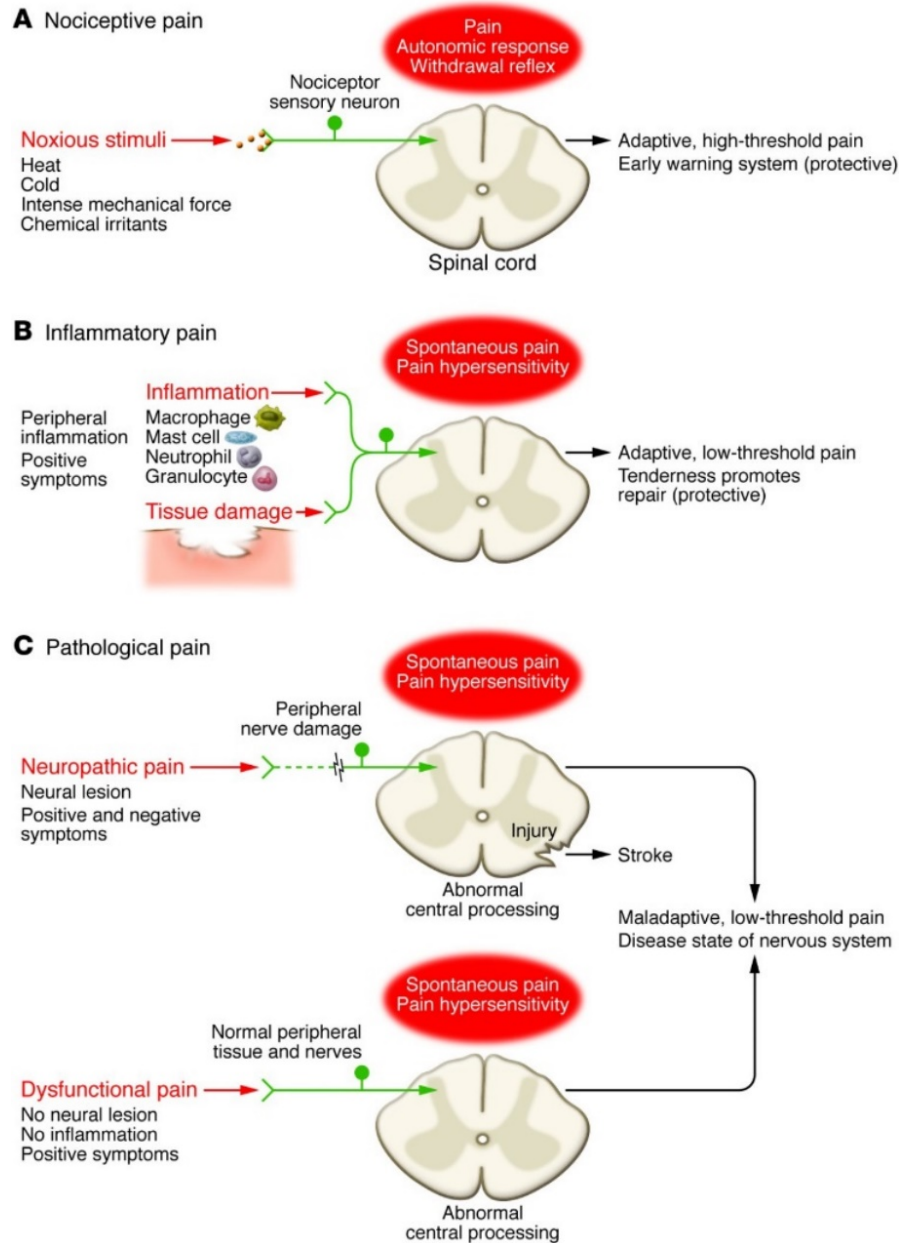


Figure 1.3 Pain type classification according to the mode of actual or potential injury. In experimental neurobiology, pain can be subdivided into acute/nociceptive pain (A), inflammatory pain (B) and pathological pain (C). Pathological pain encompasses both neuropathic pain phenomena of defined nerve injury and dysfunctional pain, which occurs under no obvious symptoms but also reveals a maladaptation of the nervous system. (Adapted from Woolf,2010)

1.2 Sensory Neuron Physiology

1.2.1 Sensory Neuron Classification

Sensory neurons have been famously assessed by Wall to “taste their environment” (**Koltzenburg,1999**) and rightfully so, as they detect distinct mechanical, thermal and chemical modalities. Depending on the anatomical innervation of sensory neurons, detected stimuli can be of exteroceptive or interoceptive origin. Exteroceptive stimuli can be hot/warm external temperatures, soft touches and hard pressures, environmental irritants or venomous poisons. In turn, interoceptive stimuli or endogenous ligands can range from inflammatory mediators to endocrinal secretions and changes in the pressure of internal organs. On either occasion, the encoding of this sensory input by peripheral sensory neurons allows for behavioral output of emotional and motor nature to be devised by the central nervous system.

Primary afferent neurons are organized in clusters named trigeminal ganglia (TG) and dorsal root ganglia (DRG). The TG are the clustered cell bodies of the trigeminal nerve located in a cavity of the dura mater called the Meckel’s cave. Despite their structural and functional similarity to the DRG and their unequivocal importance in the development of migraine (**Goadbsy & Edvinsson, 1993**), the study of TG is out of the scope of this thesis and hence further information about them is withheld. The DRG cell bodies are located in the dorsal root of spinal nerve cavities, enclosed by the spinal column. In descending caudal order, the DRG are separated into cervical, thoracic, lumbar and sacral. There are 60-62 DRG in rodents and humans, where each ganglion encapsulates approximately 10-15 thousand sensory neuron cell bodies of high molecular and functional heterogeneity. Sensory DRG neurons possess a pseudo-unipolar morphology where a singular axonal protrusion innervates both the peripheral (distal axonal side) and central terminals (proximal axonal side). The distal axonal side of sensory neurons targets the skin, viscera, muscle and bones while the proximal axonal side projects to the spinal cord and brainstem (**Meltzer et al,2021**). Most DRG sensory neurons are cutaneous (skin-innervating) while much scarcer is their visceral innervation. Although several painful conditions and physiological processes arise from the function or dysfunction of internal organs, sensory neurobiologists have predominantly studied pain in the context of cutaneous sensory neurons.

The axons of sensory neurons, called primary afferent fibers, are commonly classified into four broad categories depending on their myelination density: the thickly and moderately myelinated

A α - and A β -fibers respectively, the thinly myelinated A δ -fibers and the unmyelinated C-fibers (**Emery & Ernfors, 2018**). Myelination degree influences the diameter of sensory axons, where the largest diameter fibers are the A β -, while of smallest diameter are the C-fibers (**Table 1**). The degree of myelination for each primary afferent subtype affects the conduction velocity of APs across the axonal membrane. Increased myelination insulates the fibers and prevents current leaks while promoting faster current flow. A comparison by orders of magnitude is observed, as the conduction velocity of A α - and A β -fibers ranges between 70-120 m/s and 30-70 m/s respectively, for A δ -fibers between 5-30 m/s and for C-fibers between 0.5-1.5 m/s (**Li & Bak,1976**).

	A β -fibers	A δ -fibers	C-fibers
Myelination	Thickly myelinated	Thinly Myelinated	Unmyelinated
Cell diameter	6-12 μ m	1-5 μ m	0.2-1.5 μ m
Conduction velocity	30-70 m/s	5-30 m/s	0.5-2.0 m/s
Common Neurochemical markers	Parvalbumin Neurofilament	Neurofilament	Peripherin (universal) CGRP, Substance P (Peptidergic population) IB4 (Non-peptidergic population)
Modality or Function	Proprioception Low-Threshold Mechanoreception	Thermal Chemical Low-Threshold Mechanoreception High-Threshold Mechanoreception	Thermal Chemical Low-Threshold Mechanoreception High-Threshold Mechanoreception Pruriception

Table 1.1 Fundamental comparisons of primary afferent DRG neurons by morphological and electrophysiological properties, functional roles and immunohistology.

Earlier, the Sherringtonian concept of nociceptors was introduced; sensory neurons that specifically detect and respond to noxious stimuli. Not all sensory neurons are nociceptors, but the largest subset of them is. The C-fibers represent the major class among them, with the thinly myelinated A δ -fibers exerting important nociceptive roles and the moderately myelinated A β -fibers reported to infrequently convey noxious stimuli (**Djouhri & Lawson, 2004**) (**Lawson et al,2019**). Due to their differences in conduction velocity, the A δ -fibers mediate the acute, first response to noxious stimulation while C-fibers are mediators of the slow, “burning” secondary pain response.

An alternative classification of nociceptors regards the modality conveyed and the intensity of each stimulus as the determinant criterion of discrimination. Hence, animal and human studies categorize as noxious the encoded extremities of modalities like temperature (below $\sim 15^\circ\text{C}$ and above $\sim 42^\circ\text{C}$), intense pressure and chemical irritation (**Foulkes & Wood,2007**). The encoding of a single modality by a nociceptor subtype confers *unimodality*, while the encoding of multiple modalities confers *polymodality*. The percentage distribution of unimodal and polymodal nociceptors emerges as contentious between electrophysiological and *in vivo* calcium imaging studies. In electrophysiological studies (skin nerve preparation and microneurographic studies) an uninformative range of 35-100% of nociceptors is thought to be polymodal, a 10-15% to be mechanosensitive, a 10-25% to be heat sensitive and a 10-25% to be unresponsive during canonical conditions but unmasked upon damage (the so-called *silent nociceptors*) (**Ernfors & Emery,2021**). In *in vivo* calcium imaging studies, $\sim 25\%$ of nociceptors have been described as polymodal, with mechanical nociceptors representing $\sim 55\%$ of responses (**Emery et al,2016**) (**Wang et al,2018**), although polymodality has been attributed to 50-80% in another study (**Chisholm et al, 2018**). A layer of complexity is added to the functional characterization of nociceptors, as the transcriptional modifications of sensory neurons following injury or inflammation can result in a plastic redistribution of nociceptor properties (**Yarmolinsky et al,2016**) (**Nickolls et al,2020**).

A practical subdivision of nociceptors has been devised through molecular tracing of these subpopulations and their projections to the DH and periphery. These neurochemical marker genes are represented in the form of neuropeptides, neuronal membrane-expressed channels and receptors as well as calcium-binding molecules. Peptidergic nociceptors innervate the deeper skin

and other tissues; they express the calcitonin gene-related peptide (CGRP) and Substance P or somatostatin and are C-fibers or A δ -fibers. The non-peptidergic neurons mainly innervate the epidermis of the skin and bind to the isolectin B4 (IB4) (**Stucky & Lewin,1999**). Further, peptidergic nociceptors abundantly express TrkA, the high-affinity receptor for nerve growth factor (NGF) and less-abundantly the neuropeptide Y2 receptor (Npy2r). Non-peptidergic nociceptors express Ret which is the receptor of glial- derived neurotrophic factor (GDNF) and the GDNF-binding receptors GFR α 1 and GFR α 2. Three distinct subpopulations of non-peptidergic IB4- and RET-expressing nociceptors express different Mas-related G-protein coupled receptors (Mrgpr): Mrgprd, MrgprA1/C1 and MrgprB4 respectively. Other molecular classifications of nociceptors have been made according to the molecular expression of heat and cold transducers (TRPV1, TRPV2 and TRPM2 for heat, TRPM8 for cold) (**Hjerling-Leffler et al,2007**), chemical irritation transducers (TRPA1, Mrgprd), mechanical force transducers (PIEZO2) and acidic sensitivity transducers such as the acid-sensing ion channels (ASICs).

The molecular profiling of nociceptors through marker gene expression has acted as a useful blueprint for modern nociceptor classification methods, albeit has lacked the potential to elucidate their respective functional roles. The heterogeneity of mouse nociceptors has been more recently categorized (**Usoskin et al,2015**) (**Zeisel et al,2018**) through single cell RNA-sequencing experiments where 13 types of nociceptors subtypes have been reported in mice (**Figures 1.4A&1B**): 3 populations of noxious cold sensors (Trpm8.1, Trpm8.2 and Trpm8.3), 4 populations of peptidergic C-fibers (PEP1.1, PEP1.2, PEP1.3 and PEP1.4) that convey mechanical and heat noxious stimulation and inflammatory heat hyperalgesia, an A δ peptidergic population (PEP2) that conveys sharp pain stimuli, 2 non-peptidergic C-fiber populations that convey noxious touch and itch (NP1.1 and NP1.2), 2 non-peptidergic/Mrpra3-expressing C-fiber populations that convey chemical irritants (NP2.1 and NP2.2), and a non-peptidergic/somatostatin-expressing C-fiber population (NP3) that mediates acute and allergic itch sensation (**Gatto et al,2019**). A spatial transcriptomic study of human DRG neurons (**Tavares-Ferreira et al,2022**) has found several similarities but also differences with the murine single-cell RNA sequence analysis, where 5 classes of C-fibers, 1 C-low threshold mechanoreceptor, 2 A δ , 2 A β , 1 nociceptor A β and a proprioceptor DRG neuron subtype have been reported (**Figure 3C**). Similarities between murine and human DRG raise important considerations at the nociceptor functional level but also may inform or hinder translational efforts in analgesic drug development. For instance, human and

A

Late neurogenesis *Early neurogenesis*

FIBROTYPE	TRANSDUCERS	PROPORTION/INNervation	MARKERS	FUNCTION
C-Cold	Trpm8	6-8% Skin, Deep	Trpm8, Foxp2	Noxious innocuous cold
C-Nociceptor C-MH	Trpv1, Trpa1, Trpm3, Piezo2	25% Skin, Deep	Tac1, Adcyap1, Gal	Slow pain Heat Chemical Mechanical
C-LTMR	Piezo2	15-20% Hairy skin	Th, Wfdc2	Pleasant touch
C-Mechano & Pruriceptor	Piezo2, Mrgprd	25-32% Hairy/Glabr. skin	Mrgprd, Lpar3	Mechanical threshold Itch
C-Pruriceptor	Mrgpra3, Hth1	4-7% Hairy/Glabr. skin	Mrgpra3, Gfra1	Itch
C-Pruriceptor & Warmth?	Hth1, Piezo1, Trpm2	5-9% Skin	Sst, Nppb, Il13ra, Osmr, Htr1f	Inflammatory & chronic itch Warmth?
Aδ-H-Nociceptor	Trpv1	2.5% Skin, Deep?	Smr2, Asc1, Tmem233	Deep inflammatory pain? Sharp heat pain?
Aδ-HTMR Nociceptor	Piezo2, Trpm8 ^{low}	2.5% Skin, Deep?	Fam19a1, Kit	Sharp & fast mechanical pain Hair pull pain Noxious cold?
Aδ- & Aβ-LTMRs Proprioceptors	Piezo2	5% Skin, Muscles, Joints	Nefh, Ntrk2 (Aδ), Pvalb (Pr)	Touch/ Proprioception

B

P5 DRG (*Ayfl^{CreERT2}; Rosa26^{LSL-tdTomato}*)

● Aβ field/SA1
● Aβ RA-LTMR
● Aδ-LTMR
● C-LTMR
● CGRP+
● CGRP+
● CGRP+
● CGRP+
● MRGPRD+
● Proprioceptors
● SST
● Cold thermoreceptors
● Non-neuronal

tdTomato 7% (C-LTMR) 0% (Non-neuronal)

Per cent of cells with >1 tdTomato tag (Subtype)

17% (MRGPRD) 15% (SST) 19% (Aβ RA-LTMR) 6% (Cold thermo.) 5% (Aβ field/SA1) 4% (C-LTMR) 10% (Aβ RA-LTMR) 11% (Aβ field/SA1) 10% (Aβ-LTMR) 0% (Non-neuronal) 10% (Aβ-LTMR) 0% (Non-neuronal)

C

UMAP_1

Male Female

DEG - Surrounding

DEG - Cold nociceptors

DEG - Aδ-HTMRs

DEG - Aδ-LTMRs

DEG - Aβ RA-LTMRs

DEG - Aβ SA-LTMRs

DEG - Proprioceptors

DEG - Prurito-gen receptor enriched

DEG - TRPA1+ nociceptors

DEG - PenK+ nociceptors

DEG - Putative silent nociceptors

DEG - Putative C-LTMRs

45

The unbiased molecular characterization of nociceptors has elucidated important physiological aspects of these neurons for the summation of modalities and intensities encoded by each subtype (**Figure 1.4**). A hallmark of the approach is the functional prediction of a painful signal's trajectory and the premise of targeted, nociceptor subtype-specific strategies for different painful conditions induced in the periphery. However, as mentioned earlier, the plastic nature of sensory neurons is also reflected in alterations of their molecular identity, either through abruptly induced events such as nerve injury or more periodically through aging. Of future translational significance will be the determination of the extent to which human nociceptor classification studies align with rodent studies. Further, the use of human induced pluripotent stem cells (hiPSCs) should reach an optimization level where key protein and ion channel expression patterns may coincide with the human DRG data (**Cao et al,2016**) (**Alsaloum & Waxman,2021**) (**Deng et al,2023**). Much frustration has been caused by the lack of analgesic efficacy of pre-clinically validated analgesic drug targets, and it has been suggested that discrepancies in the molecular identities and mechanistic differences of sensory neurons may account for it. Detailed molecular cartographic maps of existing transcriptomic and novel spatiotemporally resolved translatomic techniques may shed sufficient light to address this (**Ren et al,2023**) (**Zeng et al,2023**).

Further, it will be useful to quantify the molecular re-organization of each nociceptor subtype following defined injuries and pathological models. Last, but perhaps most importantly, the molecular and functional identity of each sensory neuron subtype should be dissected based on each nerve's anatomy. Thus far, the structural and molecular identities of each specific axonal projection elude us, although a recent effort attempts to address this (**Qi et al,2023**).

It is imperative to determine how the nociceptor projections to the spinal cord and subsequent connectivity patterns shape the molecular labelled lines proposed. Beforehand, however, the modal nature and the functional properties of each nociceptor subtype in relation to their respective anatomical location must be validated beyond doubt. To achieve this, each peripheral nerve population must be extracted, dissected and characterized in both molecular and functional terms through a combination of transcriptomic and electrophysiological studies.

1.2.2 Sensory Neuron Excitability

Sensory neurons convert environmental and endogenous cues into electrical signals. The specialized detection of these signals generates APs and causes a depolarization of the sensory neuron membrane, in a process henceforth referred to as transduction. The generated electrical signals in the form of APs at the free nerve endings traverse long distances across their axonal membrane, a process henceforth named propagation. Last, the electrical signals terminate their sensory neuron course at central terminals as the generated APs and the local changes in membrane potential and ion flux trigger the chemical release of neurotransmitters that are diffused to distinct laminae of the DH, a process henceforth referred to as synaptic transmission. Responsible for the transduction, propagation and transmission of electric signals in sensory neurons are specialized ion channels whose activation allows the selective conduction flow of ions and results in the excitability of the neuronal membrane (**Figure 1.5**).

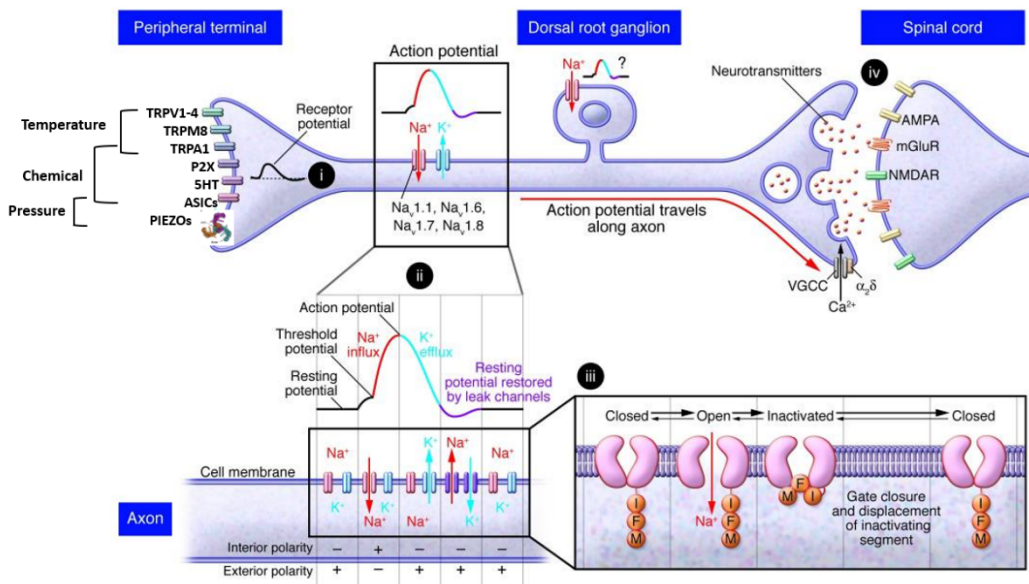


Figure 1.5 Schematic of ion channel topography and function across nociceptors. Free nerve endings convert temperature, chemical and pressure stimuli into generator potentials that depolarize the neuronal membrane (**transduction**). Upon reaching a depolarization threshold, voltage-gated sodium channels (VGSCs) become activated, generating APs while potassium channels repolarize the membrane thereafter (**propagation**). The generated APs reach the presynaptic terminal where the Ca^{2+} increase is detected by calcium channels and induces the release of neurotransmitters to the DH of the spinal cord (**transmission**). (Modified from Raouf et al,2010).

1.2.2.1 Transduction mechanisms

The molecular machinery responsible for the conversion of thermal, chemical and mechanical stimuli into APs are transducer ion channels, which have evolved to selectively distinguish between different modalities. The binding of a chemical ligand, exposure to a particular temperature or to a defined pressure intensity induces conformational changes to these channels. The opening of a transducer channel signifies the emergence of a generator potential which in turn depolarizes the sensory neuron's membrane.

The cloning of the first temperature sensor and transducer ion channel in mammalian sensory neurons from the Julius lab was that of the vanilloid receptor 1, known as TRPV1 (**Caterina et al,1997**). The channel displayed sensitivity to capsaicin as well as noxious heat (~42 °C) (**Tominaga et al,1998**). TRPV1 acts as the molecular thermostat of sensory neurons and possesses an important role in the pain pathway, especially in inflammatory pain (**Caterina,2000**). More TRPV channels were reported following the discovery of TRPV1, namely TRPV2, TRPV3 and TRPV4. The TRPV2 channels are activated by higher temperatures (~52 °C) and are expressed not only in nociceptors but also in medium and large diameter neurons as well as in non-neuronal cells (**Caterina et al, 1999**). In contrast TRPV3 channels are activated by moderately warm temperatures (~34 °C), they are involved in skin and taste sensing. TRPV3 channels are responsive to carvacrol, eugenol and thymol, all plant components of oregano, savory and clove (**Smith et al,2002**) (**Xu et al,2006**). The TRPV4 channel is also widely expressed beyond sensory neurons with polymodal properties of osmolarity and acidity detection, shear stress but also with a distinct temperature threshold (>27 °C) (**Liedtke et al,2000**) (**Alessandri-Haber et al,2003**). Belonging to the melastatin family of TRP channels, TRPM8 is a menthol-activated channel that is recognized as the *bona fide* cold transducer (**Peier et al,2002**) (**McKemy et al,2002**). The TRPM2 channel is a moderate heat sensor (**Tan & McNaughton,2016**) while TRPM3 channel responds to extreme heat and together with TRPV1 and TRPA1 are required to abolish noxious heat sensation in mice (**Vanderwauw et al,2018**). Last, TRP ankyrin1 (TRPA1) is perhaps the most promiscuous of the sensory neuron expressed TRP channels and responds to pungent compounds such as mustard oil and garlic oil and plays an important role in inflammatory pain (**Bautista et al, 2006**). A more detailed description of TRP channels, their properties and roles in the pain pathway is provided in the fifth chapter but have also been reviewed extensively elsewhere (**Levine & Alessandri-Haber,2007**) (**Julius,2013**).

The detection of chemical and environmental irritation is not limited to TRPA1. A component of endogenous inflammatory molecules secreted upon tissue injury is adenosine triphosphate (ATP) **(Hamilton et al,2000)**. The detection of ATP occurs via a purinergic receptor (P2X) channel expressed in sensory neurons, namely P2X3, that modulates thermal responses and inflammatory pain in mice **(Chen et al,1995)** **(Souslova et al,2000)**. The acid-sensing ion channels (ASICs), members of the degenerin/epithelial sodium (DEG/ENaC) channels are gated by protons and are activated by tissue acidification and low extracellular pH levels **(Wemmie et al,2013)**. The ASIC3 channels are expressed in sensory neurons and are activated by protons causing pain-related behaviors **(Yu et al,2010)** and external snake toxins potentiate the gating of protons in ASIC2a channels **(Bohlen et al,2011)**. Arachidonic acid, a precursor molecule of prostaglandins, activates ASIC1-3 channels providing an endogenous activator and thus implicating them in inflammatory pain **(Smith et al,2007)**.

The involvement of several DEG/ENaC channels has been reported for mammalian mechanosensation. The ASIC3/DRASIC channel has been shown to contribute to cutaneous touch sensation together with acidity changes **(Price et al, 2001)** and so have ASICs 1 and 2 **(Delmas et al,2018)**. The major breakthrough in mechanotransduction is the discovery of the PIEZO1 and PIEZO2 channels by the Patapoutian lab **(Coste et al,2010)**. The PIEZOs are two non-selective cationic mechanotransducers that exert a variety of physiological of functions, mediating touch sensation **(Ranade et al,2014)**, proprioception **(Woo et al,2015)** and tactile allodynia in sensory neurons **(Murthy et al,2018)** **(Szczot et al,2018)**. Very enigmatic is the mode of activation of these channels, as in contrast to molecular ligands, the detection of pressure occurs in waves and is intangible. Recent biophysical models influenced by the Helfrich theory of membrane bending have identified key elements of pressure gating by the propeller-like structured PIEZOs **(Haselwandter et al,2022a)** **(Haselwandter et al,2022b)**. Other mechanically gated ion channels are three two-pore potassium channels, namely TREK1, TREK2 and TRAAK **(Brohawn et al,2014)** which mediate both mechanical and temperature allodynia **(Noel et al,2009)**. More elusive is the identification of a noxious mechanotransducer channel, with the recent discovery of TACAN **(Beaulieu-Laroche et al,2020)** being disputed for both its mechanosensitivity and ion channel identity **(Niu et al, 2021)**. Recently emerging candidate mechanosensitive channels are members of the OSCA/TMEM63 family, the ELKIN1/TMEM87a and the Tentonin 3/TMEM150c proteins **(Kefauver et al,2020)** **(Delmas et al,2022)**. Patch clamp recordings of cultured DRG

neurons and ex vivo skin-nerve preparations show that two more mechanosensitive currents exist in sensory neurons in addition to PIEZO2, one possibly accounting for the noxious mechanotransducer channel whose identity remains unknown **(Murthy,2023)**.

Molecular transducers have long been sought after in the somatosensory field and the puzzle seems to be nearing completion. For a long time, it was speculated from proponents of the Specificity Theory that the identification of these transducer molecules would be the ideal therapeutic candidates to silence nociceptor activity in a modality-specific manner. However, the physiological roles of these channels transcend the sole attribution of transduction properties as mediators of the pain pathway. Hence, the development of analgesic molecules for TRPV1, TRPA1 and TRPM8 has been accompanied by body temperature dysregulation and adverse effects. However, a Trojan horse-imitating approach utilized the calcium gating properties by the large pore subunit domain of TRPV1 channels for the intrusion of charged VGSC blockers into activated nociceptors **(Binshtok et al,2007)**. Significant difficulty may be predicted in the development of selective PIEZO2 antagonists due to the intricate structure and gating properties of the channel. It is challenging in terms of structural biology to perturb a propeller-shaped channel gated by pressure. Despite the translational caveats of designing transducer-targeted analgesics for chronic pain conditions, there is optimism for cellular and molecular targeting and maladaptive re-wiring of nociceptors using gene editing tools that can be applied in a variety of targets **(Moreno et al, 2021)**.

1.2.2.2 Action Potential Propagation mechanisms

Sensory neurons are the longest cells in the mammalian nervous system, that can reach a meter in humans **(Haberberger et al,2019)**. The generator potentials elicited at the peripheral nerve terminals of nociceptors by transducer ion channels are insufficient to traverse alone the long distances across axonal membranes. It is, however, this initial depolarization by the transducer molecules that causes an ionic imbalance which in turn activates and deactivates voltage-gated channels that initiate and propagate the APs within and across the sensory axons.

The pioneering studies by Hodgkin and Huxley on the squid giant axon using the voltage clamp technique led them to identify that the generation of electrical signals in nerves is dependent on

the voltage-gated dependent initiation of Na^+ inward currents (**Hodgkin & Huxley,1952a**) (**Hodgkin & Huxley,1952b**). The inactivation of the sodium current after 1-2 ms is succeeded by a K^+ outward current that then restores the balance of charges in the axonal membrane. Their studies did not conclude a mechanism for this ionic permeability, however Hodgkin and Keynes, after observing abnormalities in the flux of K^+ ions stated that “a different way of explaining the anomalous flux ratios is to imagine that K^+ ions cross the membrane through narrow tubes or channels” (**Hodgkin & Keynes,1955**).

The inception of selective cationic channels was taken up and extended by the trio of Clay Armstrong, Bertil Hille and William Catterall who set out to characterize the saturation and blockade of Na^+ and K^+ ion permeation in lipid bilayers, efforts that have been extensively documented in the words of the protagonists (**Armstrong & Hille,1998**) (**Hille et al,1999**) (**Catterall,2012**). The development of the patch clamp technique by Neher and Sakmann made the trio's lives easier as they could record the opening and closing of channels by measuring changes in single-channel currents (**Neher & Sakmann,1976**). The cloning and sequencing of the α subunits of VGSCs led to the identification of an entire family of such channels (**Noda et al,1984**) (**Noda et al,1986**). The X-ray crystallographic studies by Roderick Mackinnon's group characterized in structural terms the K^+ channel ionic conductivity (**Doyle et al,1998**) and earned the youngest of the 3+1 voltage-gated ion channel musketeers a Nobel Prize.

Voltage Gated Sodium Channels

The family of VGSCs is comprised of 9 functional subtypes (Nav1.1-Nav1.9) which are encoded in the mammalian genome by *SCNA1-SCNA5* and *SCNA8-SCNA11* (**Figure 1.6B**). The Nav1.1-Nav1.7 channels display sensitivity to tetrodotoxin (TTX) blockade while Nav1.8 and Nav1.9 are resistant (**Hartshorne & Catterall,1981**). These channels are consisted of a pore forming α subunit and auxiliary β subunits. They are arranged in 4 domains where each domain is subdivided in six transmembrane segments (S1-S6) (**Bennett et al,2019**). The voltage-sensing properties of these channels are attributed to the S1-S4 segments and especially S4 (**Figure 1.6A**). The S5 and S6 segments form the pore region which is important for ionic selectivity. The conformational states that VGSCs can be found are the open, closed and inactive. When depolarized, the voltage sensing subunits rotate and move outwards, which within less than 1 ms subsequently opens the channel pore and allows the passage of sodium ions (**Figure 1.5**). In contrast, on a hyperpolarized membrane potential, the channels are closed. There are two states of channel inactivation that depends on an IFM motif found in domains III and IV of the cytoplasmic region: a fast one and a slower one which occurs after prolonged depolarizations (**Armstrong & Hollingworth 2018**). The inactivation gate facilitates the repolarization for the resting membrane potential and the return to a closed state. The intermittent period between closing of the inactivation gate and the opening of the inactivation one is called refractory.

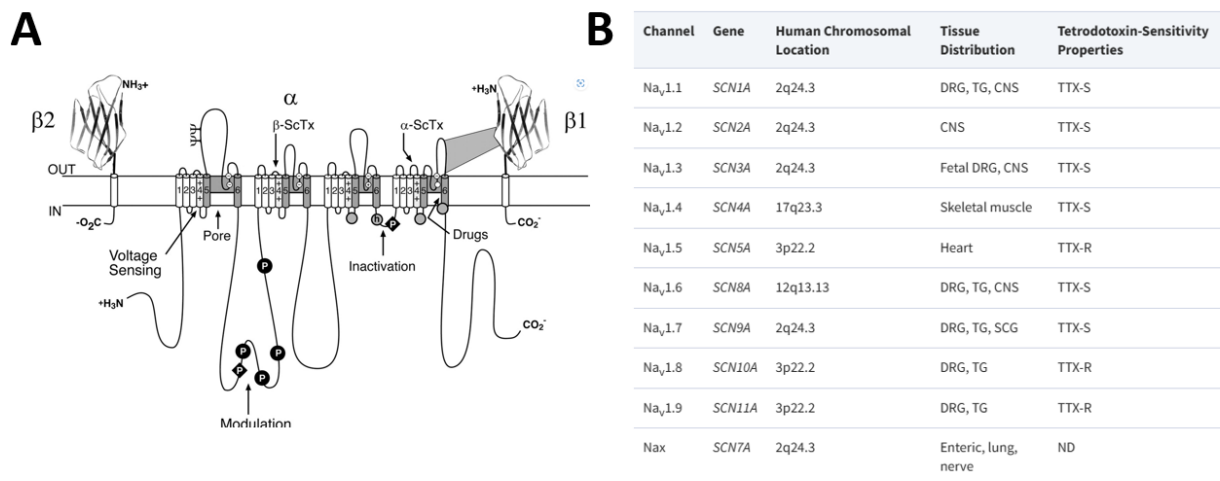


Figure 1.6 Structure and nomenclature of VGSCs A) Structural features of sodium channel subunits. Each sodium channel is consisted by a pore forming α subunit and associated with two β subunits (**Adapted from Catterall et al,2005**). B) Nomenclature of VGSC α subunits (**Adapted from Cummins et al,2020**).

The distribution of VGSC subtypes is widespread in the mammalian nervous system, although certain subtypes are preferentially expressed in different neuronal populations (**Wang et al,2017**). Mature sensory neurons express Nav1.1, Nav1.6, Nav1.7, Nav1.8 and Nav1.9 channels while Nav1.2, Nav1.3 and Nav1.5 are expressed during development but are downregulated thereafter (**Fukuoka et al,2008**). Both myelinated and unmyelinated fibers express Nav1.1, Nav1.6 and Nav1.7 channels. The Nav1.8 channels are almost exclusively nociceptor specific (**Zimmermann et al, 2007**) while Nav1.9 channels are preferentially expressed in the non-peptidergic C-fiber population (**Dibb-Hajj et al,2002**). In contrast, Nav1.6 channels are localized at the nodes of Ranvier of myelinated fibers (**Caldwell et al, 2000**) but are also expressed in unmyelinated neurons and are upregulated following injury (**Chen et al,2018**) (**Vysokov et al,2019**).

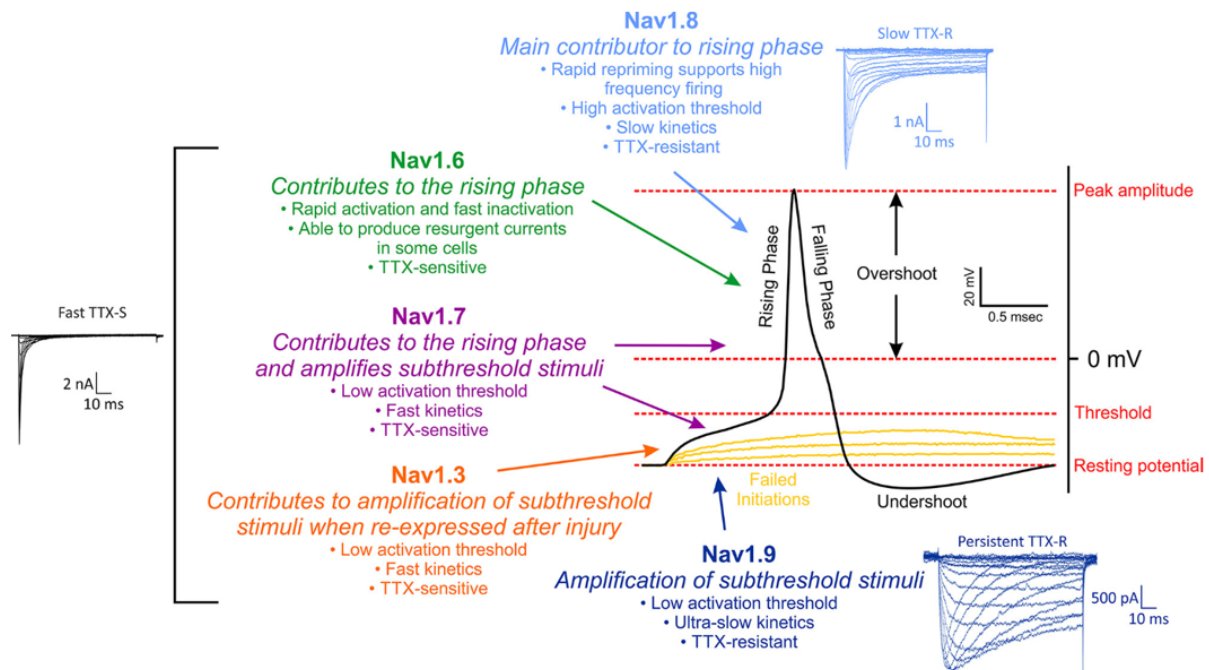


Figure 1.7 The contribution of VGSC subtypes to the AP waveform in rodent sensory neurons. The small diameter sensory neurons express Nav1.6, Nav1.7, Nav1.8 and Nav1.9 channels which are both TTX-sensitive and TTX-resistant. The large diameter, myelinated neurons, express mostly Nav1.1, Nav1.6 and Nav1.7 channels which are all TTX-sensitive.

The expression patterns of VGSCs on different sensory neurons also define the conduction speed and AP waveforms of those neurons. For instance, the myelinated DRG neurons predominantly express TTX-sensitive VGSCs such as Nav1.1, Nav1.6 and Nav1.7. In comparison, the unmyelinated C-fibers express the TTX-resistant Nav1.7, Nav1.8 and Nav1.9 along with Nav1.6. The nociceptor-expressed and TTX-R VGSCs are large contributors to the rising phase of the AP. The Nav1.7 channels have fast kinetics and a low activation threshold, acting as the molecular “amplifier” of APs (**Figure 1.7**). The Nav1.8 channels have a high activation threshold and display slow kinetics, while they contribute towards an increased firing frequency (**Figure 1.7**). In contrast, Nav1.6 channels display rapid activation and fast inactivation kinetics and Nav1.9 channels have slow kinetics and contribute towards the amplification of subthreshold stimuli (**Bennett et al,2019**). Thus, small diameter neurons display a longer decay time in APs firing (**Roy & Narahashi,1992**).

Potassium Channels

Potassium channels are remarkably heterogeneous in structural and functional properties. These channels are widely distributed across excitable tissues and are encoded by 78 genes in humans (**Tsantoulas & McMahon,2014**). They can be subdivided in several major classes: the voltage-gated (Kv) which are six transmembrane, the Ca²⁺-activated K⁺ channels (K_{ca}) which are also six transmembrane, the two-pore domain (K2P) which are four transmembrane found in dimeric arrangements and the inward rectifiers (Kir) which are two transmembrane. The activation of potassium channels in sensory neurons induces a rapid K⁺ efflux that causes the repolarization and in certain occasions the hyperpolarization of the neuronal membrane, thus affecting the firing frequency and generation of APs. In the anecdotal words of Tony Dickenson during his Pharmacology of Pain lecture (UCL,2017), “if sodium channels are the accelerator pedals of sensory neuron excitability, potassium channels are the brakes”. Potassium channel blockers produce aberrant firing activity (**Kirchoff et al,1992**) while openers suppress it (**Roza & Garcia-Lopez, 2008**). The agonism of potassium channels emerges as an important therapeutic target after nerve injury, as spontaneous firing occurs at the lesion site (**Everill & Kocsis,1999**) (**Kawano et al,2009**). As this thesis does not examine the role of potassium channels in sensory neuron excitability, only an epidermal account of their diversity, expression patterns and contribution to

APs will be given. The hyperpolarization-activated cyclin nucleotide-gated (HCN) channels which are both Na^+ and K^+ conducting are also mentioned by courtesy in this subsection.

The Kv channels are classified further according to their α subunits where Kv.1-4, Kv.7 and Kv.10-12 are pore-forming while Kv.5, Kv.6, Kv.8 and Kv.9 are not conditionally conducting upon association with pore-forming channels. Most of these channels exhibit slow activation kinetics and are called delayed rectifiers where certain subtypes such as the low threshold Kv.1 and Kv.4 channels filter the triggering of APs. Based on their biophysical properties and the respective currents contributions they are further separated into the delayed rectifiers (I_K), the transient slow inactivating (I_D), the transient fast-inactivating (I_A) and the non-inactivating (I_M) (**Tsantoulas & McMahon,2014**). The downregulation of Kv1.2 channels has been shown to contribute to mechanical and cold allodynia in neuropathic pain (**Zhao et al,2013**) and Kv1.1 ablation produces hyperalgesia in mice (**Clark & Tempel,1998**). The Kv.2 channels are delayed rectifiers (I_K) expressed in both unmyelinated and myelinated fibers. In contrast, Kv.3 and Kv.4 channels are fast inactivating and contribute towards repolarization and AP firing frequency respectively. Last, Kv.7 are non-inactivating (I_M) channels that facilitate the AP threshold. Retigabine potently acts on Kv7.2 and Kv7.3 subtypes enhancing the I_M currents to attenuate inflammatory and neuropathic pain (**Blackburn-Munro & Jensen,2003**).

The K_{Ca} channels modulate calcium influx in pre-synaptic terminals of nociceptors and contribute to membrane hyperpolarization. They are subdivided into the small conductance (SK_{Ca}), intermediate conductance (IK_{Ca}) and big conductance (BK_{Ca}). Antagonism of the K_{Ca} channels increases the firing rate and reduces the AP activation threshold of nociceptors (**Smith,2020**). Several $\text{K}_{2\text{P}}$ channels are expressed in sensory neurons, such as the previously mentioned mechanotransductive TREK1 and TRAAK. They contribute towards the hyperpolarization of sensory neurons and place them before firing threshold (**Alloui et al,2006**). Last, the HCN channels are important regulators of nociceptor firing frequency. There are 4 HCN subtypes activated after hyperpolarization that generate an inward I_h current (**Emery et al,2012**). The nociceptor-specific deletion of HCN2 has been shown to attenuate inflammatory and neuropathic pain in mice (**Emery et al,2011**). Despite the collective important role of potassium channels in nociceptor excitability, especially after nerve injury, their heterogeneity, distribution and expression across the PNS, CNS and cardiac cells poses a challenge for optimal drug discovery.

Chloride channels

A third cationic contribution in sensory neurons is that of chloride. There has been a well-established role for the ionic contribution of Cl^- in spinal excitation and inhibition (**Kaila et al,2014**). In the periphery, the role of chloride and chloride channels is controversial, as these channels have both been argued to hyperpolarize and depolarize nociceptors depending on the reversal potential and amount of chloride efflux upon channel or transporter activation (**Wilke et al,2020**). There are two neuronal chloride transporters that regulate the $[\text{Cl}^-]$ gradient with antithetical effects, the Na-K-2Cl cotransporter 1 (NKCC1) and the K-Cl cotransporter 2 (KCC2). The former is responsible for the influx of Cl^- ions into the cell and the latter for the efflux of Cl^- ions out of the cell. In DRG neurons, there is high expression of NKCC1 (**Price et al,2006**), while the expression of KCC2 is scarcely present in matured neurons (**Mao et al,2012**). The fluctuation of intracellular $[\text{Cl}^-]$, which is relatively high in nociceptors, reveals a dynamic modulation of chloride that depends on the activation of the transporters and ion channels expressed. Given that NKCC1 is preferentially expressed in nociceptors over KCC2, one would expect that the increased Cl^- influx would confer mostly depolarizing properties in nociceptors.

Several different chloride ion channels are expressed in sensory neurons such as the calcium-activated chloride channels Anoctamin1 (ANO1) and bestrophin 1 (Best 1), as well as certain ligand-gated GABA receptor subtypes. The ANO1 channel was identified in 2008 by 3 different groups (**Caputo et al,2008**) (**Schroeder et al,2008**) (**Yang et al,2008**) and when studied in DRG neurons it was found to be heat-sensitive and expressed mostly in small diameter neurons, particularly co-expressed with TRPV1 (**Cho et al,2012**). It has been indeed shown that TRPV1 and ANO1 interact in nociceptors to amplify nociceptive signaling (**Takayama et al,2015**) (**Shah et al,2020**). The loci of ANO1 expression across nociceptors have been proposed to be at the distal axons of nociceptors as well as their pre-synaptic terminals (**Oh & Jung,2018**). A role for ANO1 in thermal hyperalgesia and inflammatory pain has been postulated, as the secretion of inflammatory mediators has been shown to increase the intracellular $[\text{Ca}^{2+}]$ which in turn activates the channels (**Funk et al,2008**). Indeed, this thesis later presents evidence for the contribution of ANO1 in mediating PGE2-evoked depolarization of DRG axons (Chapter 3) (**Kimourtzis et al,2022**) and supports that at the level of nociceptor nerve endings, ANO1 channels possess depolarizing properties.

A contrasting role for gamma-aminobutyric acid (GABA), the principal inhibitory neurotransmitter, has been proposed in nociceptor excitability. The pre-synaptic expression of GABA_A receptors which are Cl⁻ conducting channels has been reported in nociceptors (**Labrakakis et al,2003**). It has been proposed that GABA_A receptor-induced increases in intracellular [Cl⁻] regulated by NKCC1 results in primary afferent depolarization. Counterintuitively, pre-synaptic GABA_A receptor treatment with diazepam, a positive allosteric modulator, possesses anti-hyperalgesic effects, presumably due to the inactivation of pre-synaptic VGSCs or shunting inhibition (**Witschi et al,2011**). More recent work has shown that GABA or GABA agonist application reduces nocifensive responses and alleviates neuropathic and inflammatory pain (**Du et al,2017**). The authors support that mechanistically, the GABA-mediated inhibition occurs at the cell bodies and T-junctions of nociceptors. The contradictory GABAergic effects on cell bodies and pre-synaptic terminals of nociceptors along with the dual promiscuity of chloride channels to induce depolarization or hyperpolarization under different intracellular [Cl⁻] conditions reveal a complex and dynamic landscape. The source of GABA production in sensory neurons remains elusive as well as the expression and local distribution of GABA receptors in DRG afferents.

1.2.2.3 Synaptic Transmission Mechanisms

It was described in the previous two subsections how transducer molecules generate graded potentials which in turn activate VGSCs that depolarize the axonal membrane and propagate APs across nociceptors. The course of nociceptor excitability ends at a central site consisting of the pre-synaptic terminals of these neurons and the distinct laminae of the DH that they innervate (**Figure 1.5**). Nociceptors release neurotransmitters stored at synaptic vesicles and peptides at that first relay point that depolarize their corresponding second order DH neurons. The pioneering experiments of Katz and Fatt studying the frog neuromuscular junction observed small and spontaneous depolarizations of the postsynaptic membrane potentials and called them miniature end-plate potentials (mini EPPs or minis) which occurred due to spontaneous release of Acetylcholine (ACh) (**Fatt & Katz,1952**). More importantly, del Castillo and Katz found that EPPs are evoked due to multiple, mini-resembling quanta of ACh, demonstrating the concept of quantal release in neurotransmitters (**del Castillo & Katz, 1954**). Later, Katz and Miledi showed

that synaptic transmission occurs due to a transient increase in intracellular $[Ca^{2+}]$ (**Katz & Miledi,1967**).

The molecular machinery and the mechanisms of neurotransmitter release have been thoroughly studied. The APs invade the presynaptic terminals and activate calcium channels that increase the local $[Ca^{2+}]$ in the presynaptic active zone. Within less than 100 microseconds (**Sabatini & Regehr,1996**), the increased $[Ca^{2+}]$ binds and activates synaptotagmins and complexins, a family of membrane trafficking proteins that initiate the exocytosis of neurotransmitter-containing synaptic vesicles (**Perin et al,1990**) (**Fernandez-Chacon et al,2001**). The synaptotagmins interact with SNARE-protein complexes to exocytose vesicles that diffuse the neurotransmitters within the synaptic clefts and are then taken up by post-synaptic receptors (**Sudhof,2012**). Nociceptors exclusively release glutamate which binds to post-synaptic NMDA and AMPA receptors in the dorsal horn (**Jahr & Jessell,1985**) (**Todd,2010**). The molecular architecture of pre-synaptic DRG axonal terminals and the post-synaptic DH neurons is more analytically discussed in Chapters 4& 5. The expression of ion channels in the pre-synaptic terminals and the transcriptional plasticity following nerve injury may influence the properties of the first sensory synapse and subsequently the excitability of DH neurons.

Besides targeting transducer molecules by “tackling pain at the source” (**Snider & McMahon,1998**) (**Patapoutian et al,2009**) or by silencing/amplifying the molecular accelerators (Nav channels) and breaks (Kv channels) of sensory neurons, the most clinically successful strategy thus far has been the blockade of the machinery that triggers neurotransmitter release, a concept known as pre-synaptic inhibition. Different strategies have been devised for the disruption of glutamate release in nociceptors. A first strategic line is the blockade of voltage-gated calcium channels (VGCCs) expressed in nociceptors and in particular those localized at the pre-synaptic terminals. There are subtypes of VGCCs distinguished by their functional properties and currents they generate. The L-type (Long-lasting) channels consist of 4 members, Cav1.1-Cav1.4. The P-type (Purkinje) the N-type (Neural) and the R-type (Residual) currents are found the three members of the Cav2 family (Cav2.1-Cav2.3). Last, T-type (Transient) currents are found in all members of the Cav3 family (Cav3.1-Cav3.3) (**Ertel et al,2000**). Structurally, the channels are heteromultimers comprised of $Cav_{\alpha 1}$ Cav_{β} and Cav_{δ} subunits that form functional complexes. The pre-synaptically expressed VGCCs in nociceptors belong to the Cav2 family. The Cav2.2 N-type

calcium channels are mainly responsible for neurotransmitter release in primary afferents (**Diaz & Dickenson,1997**) (**Saegusa et al,2001**).

Gabapentin, a commonly used analgesic for neuropathic pain, binds to the $\alpha 2\delta$ subunits of VGCCs and inhibits their trafficking resulting in the reduction of Cav2.1 and Ca2.2 (**Hendrich et al,2008**). Moreover, pregabalin downregulates the $\alpha 2\delta$ -1 subunit of Cav2 channels at the pre-synaptic terminals (**Bauer et al,2009**). There has been evidence for the involvement of the T-type Cav3 channels in synaptic release and especially of Cav3.2 (**Bourinet et al,2005**). For brevity, there are extensive reviews of the role of VGCC subtypes and their several splice variants in nociception and chronic pain conditions (**Bourinet et al,2014**) (**Zamponi et al,2015**). Other strategies targeted the SNARE protein complexes that prevent the subsequent exocytosis and neurotransmitter release. The botulinum toxin (BoTox) has been found to cleave synaptobrevin, a SNARE protein that enables membrane fusion and has been used for the silencing of nociceptors (**Maiaru et al,2018**). The synaptobrevin cleavage by BoTox is effective but its lack of selectivity amongst sensory neurons produces adverse effects and rigidity. An exciting frontier in sensory neuron research is the characterization of the pre-synaptic components of nociceptors and whether neurotransmitter release can be mitigated selectively in defined nociceptor subtypes.

The neuropeptides Substance P and CGRP, released by peptidergic C-fibers, are the two other important contributors to synaptic excitability. The expression of Substance P in sensory neurons (**Hokfelt et al,1975**) is co-localized with glutamate (**Battaglia & Rustioni,1988**) and its release elicits slow excitatory post-synaptic potentials in dorsal horn after noxious stimulation (**De Koninck & Henry,1991**). This peptide, which is secreted by sensory neurons and is found in abundance in dorsal horn neurons, binds to neurokinin (NK) receptors. There have been strategies to both sequester the ligand itself or its receptors to produce analgesia. In turn, CGRP is a potent vasodilator and neuronal sensitizer found in nociceptors and released in the central terminals to dorsal horn neurons (**Brain et al,1995**). The CGRP and its receptors play a role in both to inflammatory and neuropathic pain conditions where its secretion increases sensory neuron firing, hyperalgesic responses and ion channel trafficking (**Russell et al,2014**) (**Iyengar et al,2017**).

1.3 Peripheral pain mechanisms

Sensory neurons do not operate in isolation to convey painful signaling to the dorsal horn and the higher order neurons of the CNS. There are many molecular and cellular components that, depending on the nature of a pathology, contribute to sensory neuron hyperexcitability and consequently pain. There are neurotrophic factors that foster the survival of sensory neurons, but which acquire pathological roles in chronic pain conditions, especially in those of inflammatory nature. There are several inflammatory mediators which confer sensitization properties of sensory neurons through distinct downstream signaling cascades. The priming of sensory neurons to respond with enhanced excitability to defined stimuli further characterizes the phenomenon of hyperalgesia. Peripheral glia namely Schwann cells and satellite glial cells may contribute to pain hypersensitivity too. Skin cells (keratinocytes) are the first barriers to infectious signals and may interact with sensory neurons to modulate painful signaling. Last, acting both centrally and peripherally, an endogenous opiodergic and cannabinoid system modulates painful responses and can have long-lasting effects in pain perception [(Fields,2004) and (Woodhams et al,2015) for extensive reviews respectively].

1.3.1 Neurotrophic Factors

There are 3 principal neurotrophins involved in pain processing and these are the nerve growth factor (NGF), the brain-derived neurotrophic factor (BDNF) and neurotrophin 3 (NT-3). Each neurotrophin binds to respective tropomyosin—related kinase (Trk) receptors: NGF to TrkA receptors, BDNF to TrkB receptors and NT-3 to TrkC, while all neurotrophins also bind to a low affinity receptor, p75 (Pezet & McMahon,2006). Sensory neurons are NGF-dependent to survive during early development but upon maturation half of them lose this dependence (Ritter et al,1991) (Mantyh et al,2011). Under physiological conditions, NGF is absent despite the wide expression of TrkA and p75 in nociceptors, but in inflammatory states, there is an upregulation of NGF expression that is controlled by immune cells and especially mast cells that produce it (Leon et al,1994) (McMahon,1996). Application of NGF produces hyperalgesia in both neonates and adult rodents (Lewin et al, 1993) (Andreev et al,1995). The NGF-mediated sensitization of sensory neurons has been demonstrated in osteoarthritis, cancer pain and low back pain. Hence, its sequestration has been found to be a desirable strategy to alleviate pain (McMahon et al,1995).

A monoclonal antibody, Tanezumab produced remarkable analgesia in osteoarthritis, but the clinical trials were discontinued due to adverse effects such as the destruction of joint cartilage (Lane et al, 2010). The BDNF is important for the survival of both CNS and several subtypes of sensory neurons, where its genetic ablation causes important sensory deficits in proprioception (Ernfors et al,1994). The BDNF is found in small and medium diameter sensory neurons that are peptidergic but its high-affinity receptor, TrkB, is predominantly expressed in medium and large myelinated neurons (McMahon et al,1994). It has been shown that BDNF leads to the development and maintenance of chronic pain, inducing synaptic plasticity and contributing to central sensitization (Sikandar et al,2018). Last, NT-3 has contrasting pronociceptive and antinociceptive effects and has been shown to regulate ion channel expression (Wilson-Gerwing et al,2008).

1.3.2 Inflammatory Mediators

When there is tissue injury, there is a secreted collection of mediators by inflammatory and non-inflammatory cells which sensitizes nociceptors and produces hyperalgesia. The inflammatory mediator soup consists of prostaglandins, bradykinin, histamine, 5HT, protons and NGF (Figure 1.8). A relevant concept is that of *hyperalgesic priming*, where acute exposure to inflammatory mediators such as prostaglandin E2 (PGE2) causes a long-lasting hyperalgesia, thus rendering nociceptors in a prolonged hyperexcitable state (Parada et al,2003). These mediators both independently and collectively act through distinct molecular cascades to sensitize nociceptors (Petho & Reeh,2012 for extensive review). Of them, PGE2 is a principal contributor to inflammatory pain where it binds to 4 distinct receptors EP1-EP4, while exhibits high affinity for EP4 in nociceptors (Jang et al,2020). Binding to EP4 upregulates the intracellular cAMP concentration and increases PKA levels which in turn phosphorylate and activate TTX-resistant VGSCs and in particular Nav1.8 (England et al,1996) (Gold et al,1998). The role of PGE2 in inflammatory pain and pathological conditions such as osteoarthritis and rheumatoid arthritis has been well characterized. The development of COX inhibitors that disrupt the metabolic synthesis of PGE2 are a very effective line of treatment for acute inflammation but also for inflammatory pain, although they show short-term efficacy and adverse gastrointestinal and cardiac effects. Bradykinin is another important component of inflammation that induces potent hyperalgesia

(Dray and Perkins,1993). Sensory neurons express two receptors, BK1 and BK2, and the binding of bradykinin triggers the activation of phospholipase C (PLC) and protein kinase A (PKA) which activate ion channels such as TRPV1 and TTX-resistant VGSCs **(Wang et al,2008)**. Histamine acts through H1 receptors, which are G_s- protein coupled and lead to the downstream activation of PLC which in turn produces DAG and IP₃ **(Obara et al,2020)**. Via a PKC-mediated phosphorylation, histamine suppresses potassium channel activation and activates VGSCs, contributing to itch and inflammation **(Mobarakeh et al,2000) (Shim & Oh, 2008)**. In turn, serotonin (5-HT) acts on the 5HT-3 receptor subtype which is preferentially expressed in primary sensory afferents. The serotonergic signaling is particularly important in the context of visceral primary afferents where enterochromaffin cells secrete serotonin in nearby sensory neurons that in turn transmit nociceptive signals to the spinal cord to drive visceral pain **(Bellono et al,2017) (Bayrer et al,2023)**. Tissue acidification upon nerve injury allows protons and ATP to be released and which as previously mentioned, directly act on ASIC channels and P2X receptors respectively.

1.3.3 Neuroimmune Interactions

The release of inflammatory mediators upon tissue injury may also be encompassed under neuroimmune interactions. Some of them, such as PGE2 and the NGF play important roles in chronic pathological conditions. This subsection emphasizes the cellular and molecular components of neuroimmune interactions that are relevant to pain exacerbation or resolution. These cellular components include mast cells, macrophages, T-cells, neutrophils, B-cells, natural killer cells and microRNA-containing vesicles of diverse origins that secrete pro-inflammatory/pro-nociceptive or anti-inflammatory/anti-nociceptive agents.

Mast cells are first line responders to tissue injury that secrete NGF, Substance P, TNF- α and PGE2 to nociceptors **(Chatterjea & Martinov,2015 -for comprehensive review)**. In macrophages, the activation of Toll-like receptors (TLRs), TLR4 and TLR9 in particular, elevates the synthesis of inflammatory cytokines, namely Tumor Necrosis Factor (TNF), Interleukin 1 β (IL-1 β) and chemokines such as CCL2 and CXCL2 **(Chen et al,2020)**. The secreted chemokines and cytokines bind to specialized receptors in sensory neurons which in turn sensitize nociceptors. Emerging roles for macrophages support that they modulate acute pain sensitivity by tonically releasing NGF **(Tanaka et al,2023)** but also that the M2-type macrophages which are pro-inflammatory resolving

can ameliorate inflammatory pain conditions by transferring mitochondria to sensory neurons (**van der Vlist, et al,2022**). In a crosstalk between sensory neurons and macrophages, it has been suggested that miR21 is transferred to macrophages which in turn downregulates TGF- β and converts macrophages to a pronociceptive phenotype, thus causing allodynic symptoms (**Zeboudj et al,2023**).

The T-cells, components of the adaptive immune response, support B cells and promote pain resolution together with macrophages by secreting a number of cytokines, with interleukin 10 (IL10) being most prominent among them (**Kavelaars & Heijnen,2021**). Neutrophils have been shown to contribute to hyperalgesia (**Levine et al,1985**) and that they invade sensory neurons to confer mechanical hypersensitivity in mice, which is resolved upon their ablation (**Caxaria et al,2023**). The natural killer (NK) cells may emerge as contributors to the resolution of neuropathic pain (**Kim et al,2023**) while B cells have a remarkable capability of producing several cytokines that may contribute to pain (**Shen & Fillatreau,2015**). Monocytes and endothelial cells together with macrophages produce and secrete IL-6, a pro-nociceptive mediator (**Marchand et al,2005** for holistic review).

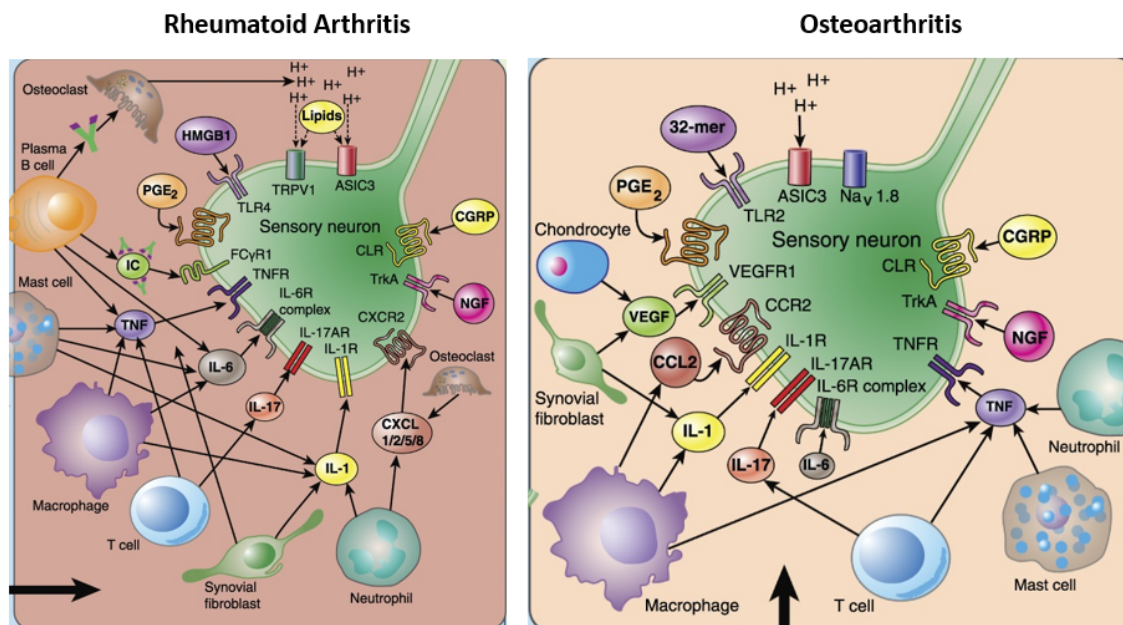


Figure 1.8 Intercellular interactions between immune cells and sensory neurons. Summary of inflammatory mediator actions secreted by resident and recruited immune cells or non-inflammatory cells and the mechanisms of nociceptor activation in two prevalent chronic inflammatory pain conditions: rheumatoid arthritis and osteoarthritis. (**Modified from Pattison et al, 2021**).

1.3.4 Peripheral Glial Cells

Novel lines of evidence involve peripheral glial cells such as Schwann cells and satellite glial cells in the initiation and maintenance of peripheral pain pathways. Since this is an emerging field in pain research and is not directly related to the content of this thesis, only a few topical ideas are mentioned here. A recent study challenged the notion of the free nerve endings in the periphery and counter proposed that a network of Schwann cells found in the subepidermal border may convey noxious thermal and mechanical stimuli (**Abdo et al,2019**). The same group has reported that damage to these specialized Schwann cells leads to neuropathic pain and hyperalgesia (**Rinwa et al, 2021**). It is also currently being pursued to determine how satellite glia cells (SGCs) contribute to pain hypersensitivity. There has been evidence that SGCs are activated by sensory neuron-expressed fractalkine (**Souza et al,2013**). In turn, the production of prostanoids, TNF- α and IL1- β by the SGCs is a contributor to the maintenance of inflammatory of pain. It has been more recently shown that there are transcriptional changes in these cells following peripheral nerve injury and has been further suggested that an increased activity of SGCs is observed in painful states (**Jager et al,2020**).

1.4 Spinal cord and supraspinal physiology

1.4.1 Dorsal Horn Organization

The spinal cord is a structure of remarkable and complex organization that constitutes the second order relay site for nociceptive information processing. Cutaneous and non-cutaneous primary afferent nociceptors convey sensory signals to the spinal cord which in turn are ascendingly propagated towards the brain. Nociceptors relay noxious information to distinct laminae of the dorsal horn through fast glutamatergic transmission, which is canonically excitatory. The recipients of dorsal horn input are then inhibitory GABAergic and excitatory glutamatergic interneurons for further processing within the spinal cord and directed to the brainstem. Simultaneously, descending brainstem input modulates these circuits.

Rexed divided the dorsal horn into six parallel laminae (I-VI) based on the packing density and neuronal size of the terminating primary afferents (**Rexed, 1952**). Dorsal horn laminae exhibit a somatotopic stratification where each lamina is the synaptic recipient of distinct primary afferent subtypes. The dorsoventral axis of the dorsal horn reveals a modality-specific organization of afferent input. However, the transcriptomic classification of nociceptors (**Usoskin et al,2015**) has not allocated yet the precise topographic sites of their central terminals. Using the traditional nociceptor categorization, most nociceptors terminate to the superficial laminae I and II (**Figure 1.9**). More specifically, nonpeptidergic A δ -fibers which are high-threshold, mechanosensitive afferents terminate on lamina I and outer lamina II. The peptidergic C-fibers and A δ -fibers terminate on laminae I and II, while the pruritoreceptive C-fiber/ MrgD⁺ fiber population terminates on middle lamina II. Virtually all DH neurons are interneurons, meaning that they project to other spinal cord regions. A small number (<1%) of DH neurons in lamina I which receive input from nociceptors, axonally project to supraspinal regions (**Todd,2017**). In lamina V, there is a population of projection neurons that respond to both innocuous and noxious stimulation, called wide dynamic range neurons (WDRs).

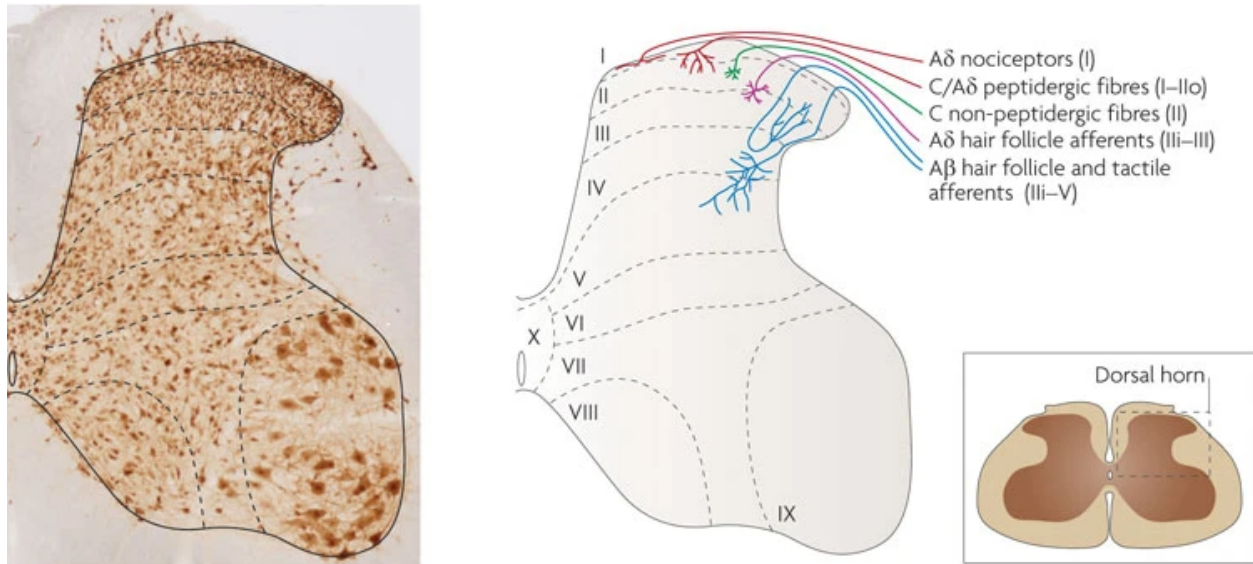


Figure 1.9 Dorsal organization and primary afferent innervation. The termination pattern of sensory neurons to the dorsal horn of the spinal cord reveals a somatotopic alignment of sensory neurons with distinct laminae. (Adapted from Todd,2010)

The DH interneurons are a highly diverse population that each possesses unique morphological, electrophysiological and neurochemical profiles. These interneurons can be excitatory and release glutamate or inhibitory where they can either release GABA or glycine. All excitatory interneurons express the vesicular glutamate transporter 2 (VGLUT2) (Todd et al,2003). Among the inhibitory interneuron population, the GABAergic interneurons reside in laminae I-III, while the glycinergic interneurons in laminae I and III-V. A transcriptomic study of DH populations has revealed distinct neurochemical markers that define each subtype (Haring et al,2018). Such markers for excitatory DH interneurons are neurokinin B (NKB), neurotensin (NTS), cholecystokinin (CCK), substance P (SP), neuropeptide FF (NPFF) and GRP-GFP cells form distinct populations, although there is some overlap between the NKB/NTS and the CCK/SP populations (Gutierrez-Mecinas et al,2019). In inhibitory interneurons subdivisions are made between parvalbumin (PV), neuronal nitric oxide synthase (NNOS), galanin (GAL), dynorphin (DYN), neuropeptide Y (NPY) and Calretinin (CR) neurons (Boyle et al,2017). The definite functions of these DH interneuron subpopulations have not yet been completely delineated. However, it has been proposed that distinct dorsal horn subtypes are responsible for the induction of mechanical allodynia according to the mode of injury (Peirs et al,2021). For instance, the CR neurons found in lamina II mediate

mechanical allodynia induced by inflammatory pain while PKC γ -expressing neurons in laminae II & III mediate allodynia of neuropathic origin, while CCK-positive neurons in laminae III & IV are crucial for both inflammatory and neuropathic pain-induced allodynia (**Peirs et al,2021**). The circuitry of the dorsal horn neurons and the synaptic inputs of DH interneurons to other interneurons and projection neurons is highly intricate and the known associations are summarized elsewhere (**Todd & Wang,2018**).

1.4.2 Central Sensitization

The concept of Central Sensitization was introduced to explain how changes that occur in the spinal cord following nerve injury confer an augmented AP output (**Woolf,1983**). Like the Gate Control Theory, the concept of Central Sensitization took an impressive leap to underscore that the intensity or the duration of peripheral sensory input is no longer driving pain sensitivity, rather dorsal horn excitability predominates the magnitude of excitability directed towards higher order neurons. The molecular correlates of central sensitization are the NMDA, AMPA and kainate (KA) receptors, abundantly expressed in the superficial laminae of the DH but also at the pre-synaptic terminals of DRG neurons. In addition, Substance P is co-released with glutamate and binds to the NK1 receptors found in the spinothalamic and spino-parabrachial neurons. Several molecules including CGRP, BDNF and inflammatory mediators trigger a range of modulatory phenomena mediated by downstream effectors that amplify the excitability of DH neurons. A manifestation of central sensitization is the induction of mechanical allodynia. Although central sensitization and ‘windup’, a phenomenon that characterizes a summative response to persistent small fiber stimulation (**Dickenson & Sullivan, 1987**), share similarities, the mechanisms which underlie them are distinct (**Mendell,2022**). For brevity, the collection of cellular and molecular changes that underlie central sensitization are summarized elsewhere (**Woolf & Salter, 2000**) (**Latremoliere & Woolf,2009**).

1.4.3 CNS glial cells

Three types of glial cells are found in the CNS, namely astrocytes, microglia and oligodendrocytes, of which the former two have well-characterized roles in pain processing. Microglia originate from monocytes and functionally resemble immune cells such as macrophages. The quiescence or lack thereof of microglia under non-pathological states is a contentious matter **(Ji et al,2013)**. There is strong evidence for the activation of spinal cord microglia following nerve injury that mediates tactile allodynia through a P2X4 receptor mechanism that enables BDNF release **(Tsuda et al,2003)**. It was subsequently reported that ATP stimulation evokes the microglial BDNF release to lamina 1 neurons contributing towards a neuropathic pain state **(Coull et al,2005)**. The microglia-originated lysosomal cysteine protease cathepsin S was subsequently identified as a mediator of the maintenance of neuropathic pain as it triggers the secretion of neuronal fractalkine (FKN) in a feedback mechanism **(Clark et al,2007)**. Cathepsin S release was subsequently found to be also dependent on ATP-induction that activates microglia via P2X7 receptors **(Clark et al,2010)**. In contrast to the traditional view of activated microglia as pronociceptive effectors in neuropathic pain, a study has identified a subpopulation of CD11c⁺ microglia which appears after the development of pain hypersensitivity and which promote recovery via interference with IGF1 signaling **(Kohno et al,2022)**. The antinociceptive effects of distinct microglial populations suggest a morphological, molecular and functional diversity which remains to be further characterized **(Paolicelli et al,2022)**. Astrocytes on the other hand, are the most abundant glial cells in the CNS and under physiological conditions they exert a metabolic role and regulate the ionic environment during synaptic development and transmission **(Ji et al,2019)**. The branches of astrocytes contain the glial fibrillary acidic protein (GFAP) **(Eng,1985)**, where astrogliosis affects GFAP expression and modulates neuropathic pain phenotypes. Several confirmed and putative molecular targets of microglia and astrocytes include the MAP kinase p38 which facilitates cytokine synthesis, the Toll receptors and especially TLR4 which is selectively expressed in microglia and last, downstream of p38, the NF-κB regulation in microglia and JNK activation in astrocytes **(McMahon & Malcangio,2009)**.

1.4.4 Supraspinal & Brain Pathways

The intricacy of ascending and brain pain pathways leaves massive space for enquiry. Several brain regions are innervated by subclasses of spinal interneurons and anterolateral tract (ALT) projection neurons. These projection neurons originate predominantly from lamina I of the spinal cord and more sparsely through deep laminae III-VI where they innervate the thalamus, the periaqueductal gray (PAG) the pons and the medulla (**McMahon & Wall,1985**). The landscape is much more complicated for the cellular and molecular identity of the interneurons projecting to ascending pain areas and summaries may be found elsewhere (**Todd & Wang,2018**) (**Tan & Kuner,2021**) (**Mercer Lindsay et al,2021**). The existence of distinct, parallel ascending pathways advocates that the feeling of pain is an emergent property of the brain. For instance, a well-characterized pathway originates from the spinothalamic tract and projects to the primary (S1) and secondary (S2) somatosensory cortices as well as the insular and cingulate cortices. Each of these areas affects different aspects of pain; S1 localizes and processes the discriminative aspects of pain (**Bushnell et al,1999**), S2 regards nociceptive learning and memory (**Ploner et al,1999**), the insular cortex mediates motivational responses and tunes cortical regions to cognitively process pain (**Starr et al,2009**) and the anterior cingulate cortex (ACC) fires following noxious thermal and mechanical stimulation (**Hutchison et al,1999**) while it is thought to process the negative valence of pain (**Shakman et al,2011**). The spino-parabrachial pathway innervates the limbic system to modulate dopamine responses following painful stimulation (**Yang et al,2021**). A central amygdala (CeA) GABAergic neuron circuit has been shown to encode the affective component of pain and whose activation abrogates pain (**Corder et al,2019**) (**Hua et al,2020**). Other studies implicate the prefrontal cortex in the encoding of long-term fear memories which are sustained throughout lifetime (**Stegemann et al,2023**). The silencing of these areas reverses chronic allodynic and hyperalgesic symptoms. In chronic pain, the brain gradually undergoes structural and functional changes that may cause shifts in cortical representations of nociceptive processing, glial activity, grey matter volume, white matter connectivity, resting-state connectivity and descending pathway reorganization (**Kuner & Flor,2017**). Optogenetic and circuit tracing advances, together with multi-omic brain mapping will enable a better understanding of pain processing in the brain. However, promising brain circuits for analgesic targeting may exhibit severe side effects, as said areas exert multiple sensory and cognitive functions. Re-wiring and manipulation of these circuits will require delicate and sophisticated strategies.

1.5 *In vitro* investigations of cellular and molecular pain mechanisms

Since this introductory chapter has given a holistic description of the bread and depth of phenomena underlying painful signaling and pain chronicity, why engage in the reductionism of molecular pain mechanisms and in particular use an *in vitro* model? Cori Bargmann and her husband Richard Axel, accomplished molecular neuroscientists themselves, aptly argue that “genes do not control behavior, nervous systems do” (Axel, 2018). However, the robust evidence that silencing nociceptor activity can provide effective analgesic relief, has anatomically narrowed the desired nervous system location sites that can potentially relieve painful perception. This idea has equally benefited and plagued the field and has propelled towards the optimization of target contingencies for analgesic development.

An *in vitro* model of studying the properties of the peripheral and dorsal horn apparatus provides three major advantages: 1) It allows a precise mechanistic delineation of the molecular pathways that contribute to pain 2) It accelerates the screening of multiple molecular hypotheses which would in contrast require significantly more animals and time to test and 3) It provides a platform for topographic precision for an observed phenomenon, which is masked *in vivo* due to the physical barrier obstruction of studying an intact organism and the intercellular interactions of neurons with non-neuronal tissue.

At the same time, *in vitro* models of sensory neuron physiology may fail to reveal the understanding of the complex cellular and molecular interactions between sensory neurons and other glial and non-neuronal cells. Further, the extraction of DRG neurons in primary cultures may result in the “priming” of nociceptors and subsequent transcriptional changes. Moreover, the sprouting of neurites from DRG cell bodies does not maintain the axonal pseudo-unipolarity observed *in vivo*. Last, the DRG-DH recapitulation of the first sensory synapse may not maintain the somatotopic alignment observed *in vivo*.

The tenet of this thesis ultimately considers that if a detailed cellular and molecular description of the periphery to spinal cord signaling does not suffice for the development of novel therapeutics for treatment of persistent pain conditions, it should only be then that the field should re-direct its focus towards translational studies of the higher central nervous system. The utilization of an *in vitro* platform for the delineation of the molecular mechanisms and screening of analgesic compounds is a substantial step towards accelerating enquiry around this objective. It can also

further elucidate intercellular interactions between nociceptors and glial or non-glial cells, resident and recruited immune cells as well as extracellular vesicular entities released across nociceptors.

1.5.1 Brief retrospective of *in vitro* neuronal models

To study the peripheral components of afferent physiology, several experimental models have been developed over the years. The existing *in vivo* techniques such as the skin nerve preparation and excised DRG preparation require demanding dexterity and they are insusceptible to genetic and occasionally precise pharmacological or electrophysiological manipulation **(Reeh,1986) (Banik & Brennan,2008)**. Hence, novel *in vitro* models act as essential experimental blueprints for discovery prior to or concomitantly with *in vivo* validation. The conventional neuronal culture models exhibit spatial limitations as they allow measurements of somatic but not axonal responses and often lead to ambiguous conclusions about the molecular mechanisms of APs initiation and propagation. Examination of protein trafficking in axons has been prohibited under the same rationale.

Efforts to accurately recapitulate *in vivo* microenvironments in neuronal cultures have circulated for decades in neurobiological research **(Millet & Gillette,2012)**. The first such attempt by Campenot, whose compartmentalized chambers are named after, spatially segregated neuronal bodies from the growing axons of sympathetic neurons and demonstrated the dependency of neurite outgrowth by NGF **(Campenot, 1977) (Campenot,1982)** and decades later it was repeated for other neurotrophin requirements **(Kimpinski et al,1997) (Salehi et al,2003)**. The Campenot chambers, assembled as a Teflon divider attached to a Petri dish with a mediating grease layer, do not guarantee fluidic isolation with soluble factors traversing from one compartment to another while neurites are mechanically or chemically perturbed due to the instability or leaking of the fabricated chamber.

Solutions to these practical concerns have been proposed as an amalgamation of Campenot's original idea along with advances in physicochemical sciences and chemical engineering. In compliance with Sydney Brenner's epistemological aphorism that "progress (in biology) depends on the interplay of techniques, discoveries and new ideas, probably in that order of decreasing importance" **(Brenner, 2002)**, well-developed microfluidic culture techniques have allowed a

precise, integrated and controlled *in vitro* recapitulation of *in vivo* axonal functions (**Gross et al,2012**) (**Terenzio et al,2017**) (**Neto et al,2016**). Microfluidics regard the biochemical engineering branch that exploits nanotechnological advances for precise spatiotemporal manipulation of fluids at the micrometer scale (**Sackman et al,2014**). With hindsight, the proposition for the use of microfluidic technologies in biomedical research was gradually embraced in several fields (**Whitesides, 2006**). In neuroscience, a model was adopted as the archetype of microfabrication, microfluidic and surface micropatterning techniques and was exploited for the construction of a compartmentalized cell culture (**Taylor et al,2003**). The same researchers later published proof-of-concept studies exploiting microfluidic devices for models of CNS axonal injury and synaptic transmission (**Taylor et al,2005**) (**Taylor et al,2010**), followed by another published protocol for reproducible mold fabrication using soft lithography (**Park et al,2006**).

1.5.2 The mechanics and benefits of microfluidics

The fabrication of microfluidic devices employs soft lithography to form molds made of polymer materials, usually the elastomer poly-dimethylsiloxane (PDMS). The material is susceptible to convenient 3D designs of channels, corridors and valves, while its transparency enables optical and fluorescent microscopy (**Gross et al,2012**). In addition, the PDMS-fabricated devices can convert hydrophobic surface properties towards hydrophilicity while can bond to a range of materials, most often experimentally to glass and plastic (**Chaudhury & Whitesides,1991**). The microenvironment constructed within the devices is controlled by governing physical effects such as the laminar flow, diffusion rate, fluidic resistance, surface tension and surface area to volume ratio (**Beebe, Mensing & Walker,2002**). Uncommon but potential hazards of PDMS-fabricated devices is the leaking of oligomeric structures into the culture medium or the absorption of small molecules that may critically affect cellular signaling dynamics (**Toepke & Beebe,2006**). Future platforms may utilize thermoplastic polymers such as polystyrene and polycarbonates to facilitate these issues (**Sackmann et al,2014**).

The microfluidic chambers arrangement commonly used in neuroscience research simply consists of the devices attached to glass coverslips (analytically described in Methods chapter). The PDMS devices possess embedded microgrooves which physically separate neuronal axons from cell

bodies into two identical compartments and facilitate the passage of axonal processes. There are variations of 4-well and 6-well devices where each well contains a reservoir. A common variation of the technique is to apply the neuronal bodies (between 1000-5000 neurons) diluted in media inside the somal reservoir and create an artificial volume difference (between 40-50 μ L) between the axonal and somal compartments. Initially, capillary action settles cultured cell bodies into one side of the channel **(Taylor et al, 2005)**. The imbalance inside the microgrooves provides a physical filter to allow the passage of neurites towards the axonal side without moving their respective cell bodies. A 72–96-hour incubation after neuronal plating and reservoir filling enables the growing axons to expand from the somal compartment and sprout their extensions towards the axonal compartment of the device.

The lab-on-a-chip approach is suitable for neuronal cells and especially sensory neurons as they extend very long processes. The successful exposure of their distinctive neuronal parts eases the unravelling of their respective molecular physiology. A convenient aspect of microfluidic DRG neuronal cultures is the survival of the small-sized C-fibers and the medium sized- A δ - fibers which constitute the nociceptors, while the A β -fibers, which predominantly acts as mechanoreceptors and mediate touch, do not survive **(Tsantoulas et al,2013)**.

The advantages of MFC cultures allow several and diverse technical enquiries that extend but are not limited to imaging, pharmacological and biochemical manipulations. It is possible to elucidate the functions of DRG axons and their respective peripheral hyperexcitability by directly stimulating the axons and using the somal compartment as readouts **(Tsantoulas et al,2013)**. Hence, the technique can be used in combination with imaging technologies such as calcium imaging and voltage imaging or classical electrophysiology through patch clamp recordings **(Tsantoulas et al,2013) (Clark et al,2018) (Vysokov et al,2019)**. The platforms enable the examination of transcriptomic modulations resulting in enhanced axonal excitability as well as ion channel trafficking in sensory neurons **(Akin et al,2021) (Higerd-Rusli et al,2023)**. The localization of pharmacological interventions allows a more realistic evaluation of how algogenic and sensitizing compounds exert their effects and clarify topographical features of the nociceptor functional architecture. Further, the interactive crosstalk between nociceptors and non-neuronal cells such as glia and inflammatory mediators can be studied in co-cultures as it has been established that these cells contribute to the modulation of peripheral hyperexcitability.

Microfluidic cultures can be also used to examine phenomena such as Wallerian degeneration following axotomy (**Gaudet et al,2011**) and elucidate the role of novel regeneration associated genes (RAGs) (**Xie et al,2023**). Last, it can reveal the intercellular interactions between sensory axons and particularly dorsal horn neurons which play a significant role in the integration of amplified painful signals. In its most industrial form, whether studying DRG axons in isolation or synaptic transmission between DRG-DH, the platform can be simplistically act as a drug screening platform in pre-clinical studies.

1.6 General aims and objectives of the thesis

The overarching aim of my thesis was to develop a microfluidic platform that possesses the versatility to study the cellular and molecular properties of nociceptors at distinct anatomical locations, such as the axonal, cell body and synaptic sites. A second aim was to model inflammatory and neuropathic pain conditions and dissect the molecular mechanisms involved within the *in vitro* environment.

To pursue these aims, a technical foundation was necessary to be laid. This foundation regarded first, the establishment of an adult DRG mouse model of distinctly studying axonal properties from cell body properties of nociceptors and second, the establishment of a postnatal mouse DRG-DH co-culture model that can recapitulate salient features of the first sensory synapse. Three representative aspects of peripheral pain signaling were chosen to be studied on each chapter:

- 1) The first chapter examines a principal neuroimmune interaction between the inflammatory mediator PGE2 and sensory axons and is followed by the delineation of the molecular mechanism underlying this phenomenon.
- 2) The second chapter regards the establishment of a microfluidic co-culture model of DRG and DH neurons where the examination of synaptic transmission properties is accurately quantifiable.
- 3) The third chapter examines plasticity in synaptic Nav and TRP channel function under intact and axotomized conditions.

2. Materials and Methods

2.1 Animals

For the work on the effects of PGE2 on sensory axons (Chapter 3), adult male C57BL/6 mice (8-16 weeks) were purchased from Charles-River UK. For the work on the establishment of the microfluidic co-cultures and on the role of TRP and Nav channels in synaptic transmission, female mice with litter, no older than P2, were purchased from Charles-River UK (Chapters 4&5). All animals were maintained in a designated facility according to the UK Home Office Code of Practice for the Housing and Care of Animals Used in Scientific Procedures. Adult male and female C57BL/6 mice were sacrificed through CO₂-anaesthetization followed by cervical dislocation in full compliance with UK Home Office Regulations and procedures under Schedule 1 of Animals (Scientific Procedures) Act 1986. Due to resistance to hypoxia, postnatal mice (P2-P4) were sacrificed only by cervical decapitation. All procedures were approved by the Animal Welfare and Ethical Review Body at King's College London (PPL U136).

2.2 Preparation of Microfluidic devices

The protocols used in this thesis are modified and upgraded versions of the protocols established and published by the lab (Tsantoulas et al 2013) (Vysokov et al,2019). Microfluidic devices with 2 compartments of 150 µm microgroove length (Xona Microfluidics, SND150 design) were used for experiments of adult mouse DRG cultures (**Figure 2.1A**). Microfluidic devices with 3 compartments consisting of 500 µm microgroove length (Xona Microfluidics, TCND500 design) were used in postnatal (P2-P4) mouse DRG-DH co-cultures (**Figure 2.1B**). Glass bottom dishes of 40mm diameter (WillCo Wells) were cleaned by sonication and sterilized with 70% ethanol. The dishes were previously coated with 0.5 mg/ml poly-L-lysine (Sigma). Microfluidic devices were non-plasma bonded onto the glass. The assembled devices were coated after assembly with 40 µg/ml laminin (Sigma) dissolved in Neurobasal-A medium for adult mice (Thermo Fisher) and in Neurobasal medium for postnatal mice. Before each use, the devices were re-sterilized in a consecutive series of sonication, methanol sterilization and 70% ethanol sterilization rounds. The

glass bottom dishes were discarded at the end of each experiment while the devices were kept in falcon tubes containing 70% ethanol.

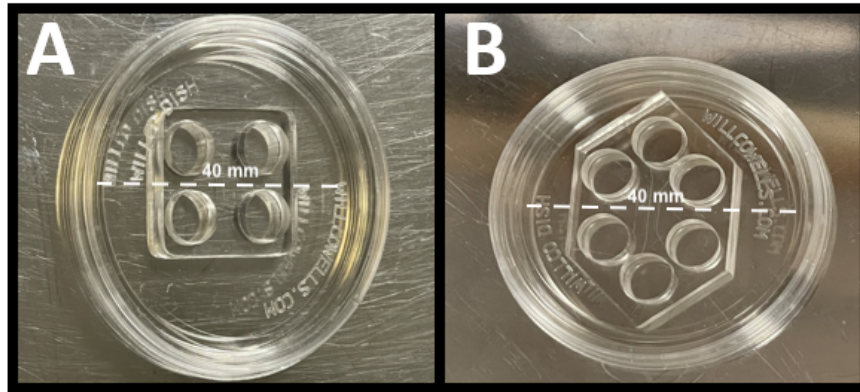


Figure 2.1 **A)** Image of a 2-compartment microfluidic device (150 μm microgroove length) attached to a 40 mm glass bottom dish. **B)** Image of a 3-compartment microfluidic device (500 μm microgroove length) attached to a 40 mm glass bottom dish.

2.3 DRG cultures of adult mouse sensory neurons

Adult male C57BL/6 mice (8-16 weeks) were sacrificed by CO_2 -anaesthetization and immediately followed by cervical dislocation. Preparation of dissociated primary neuron cultures was performed through aseptic excision of the spinal column. Cervical, thoracic, lumbar and sacral DRG-containing cavities were exposed. The DRG were collected and placed in 30 mm dish filled with HBSS +2% BSA. The ganglia were digested with an enzyme mixture containing 0.125 mg/ml collagenase (Sigma) and 10 mg/ml dispase (Thermo Fisher Scientific) for 40 minutes in a 37 $^{\circ}\text{C}$ and 5% CO_2 humidified incubator. Following mechanical trituration, the dissociated neurons were centrifuged on a 10% bovine serum albumin cushion in HBSS. The cell pellet was washed twice and resuspended in complete medium containing Neurobasal-A (Thermo Fisher), 1% B27 supplement (Invitrogen) and 1% Glutamax (Gibco), 100 units/ml penicillin and 100 $\mu\text{g}/\text{ml}$ streptomycin. The cells were loaded into prepared microfluidic devices and left to adhere for 60 minutes before being flooded with 200 μl containing 150ng/ml NGF (Gibco) on the somal side and 130 μl containing 200ng/ml NGF on the axonal side (**Figure 2.2**). The loaded devices are incubated for 5 days to allow neurite sprouting and crossing of sensory axons to the axonal compartment via

the microgrooves. Cell culture media was renewed every 2 days before use for imaging experiments.

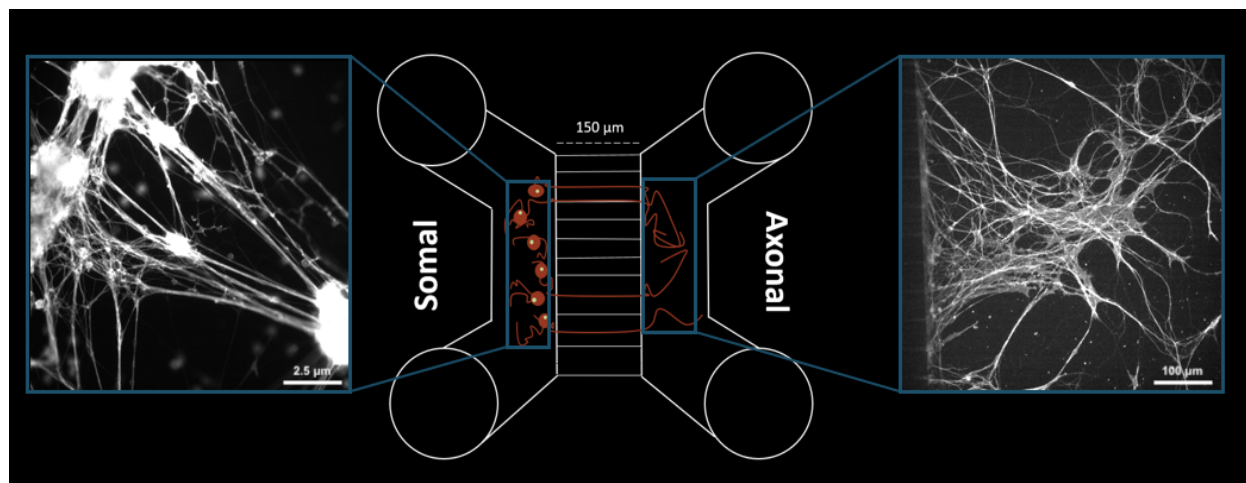


Figure 2.2 Schematic representation of a cultured 2-compartment microfluidic device where sensory cell bodies are loaded on the somal side (left) and the sprouting neurites gradually cross through the 150 μm microgrooves to reach the axonal side (right) where they are fluidically isolated from the cell bodies.

2.4 DRG-DH cultures of postnatal mouse neurons

P2-P4 C57BL/6 mice were sacrificed instantly by decapitation. Preparation of dissociated DRG and DH neuron cultures was performed through initial aseptic excision of the spinal column. The spinal cord is removed carefully while cutting through ventral roots that may still be attached, then the meninges are pulled apart. Approximately 1/3 of the dorsal side was cut off to isolate the DH which is placed in HBSS +2% BSA. For DRG neurons, the protocol used for adult mice was also applied to the postnatal (P2-P4) mice. Cervical, thoracic, lumbar and sacral DRG-containing cavities were exposed, the DRG were collected and placed in HBSS +2% BSA. The DRGs were digested with enzyme mixture containing 0.125 mg/ml collagenase (Sigma) and 10 mg/ml dispase (Thermo Fisher Scientific) and the DH were digested with 2 mg/ml papain (Sigma) enzyme mixture for 40 minutes in a 37 °C and 5% CO₂ humidified incubator.

Following mechanical trituration of DRG and DH neurons in their respective 30 mm petri dishes, the dissociated neurons were centrifuged respectively on a 10% and 15% bovine serum albumin cushion in HBSS. The cell pellet was washed twice and resuspended in complete medium

containing Neurobasal (Thermo Fisher), 1% B27 supplement (Invitrogen) and 1% Glutamax (Gibco), 100 units/ml penicillin and 100 µg/ml streptomycin.

Due to the gelatinous and fragile nature of postnatal mouse DRG and DH neurons, each co-culture contained the extracted and dissociated DRG and DH neurons of 2-3 mice. The DH neurons were loaded into one of the two corner compartments of the prepared microfluidic devices and were left to settle for 5 minutes (**Figure 2.3**). Then the DRG were loaded into the middle compartment of the same devices and were left to settle for 5 minutes. The co-cultured DRG and DH neurons were left to adhere for 60 minutes in the incubator before flooding with NGF-containing media. To ensure neurite sprouting, an NGF-gradient between somal and the two corner compartments was applied upon flooding, where 200µl containing 150ng/ml NGF (Gibco) on the somal side and 130µl containing 200ng/ml NGF on the two axonal sides were added. The devices were then left for 9-12 days in the incubator, with renewal of media every 2-3 days.

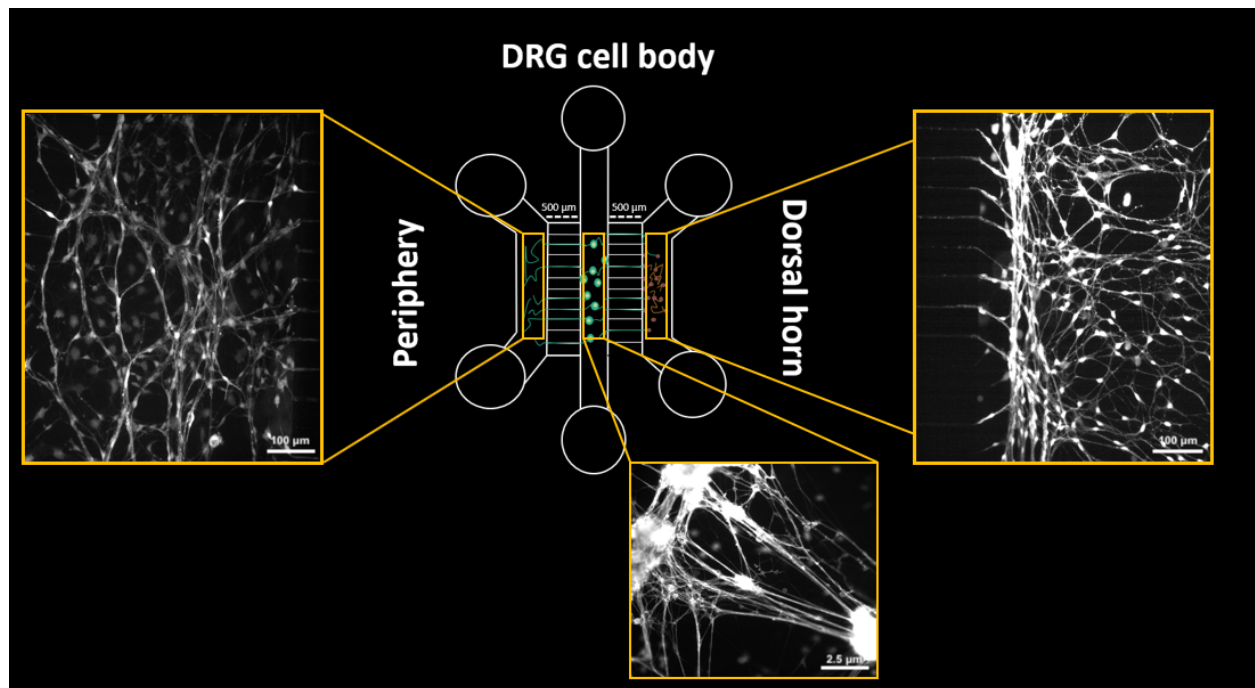


Figure 2.3 Schematic representation of a cultured 3-compartment microfluidic device recapitulating the physiology of the first sensory synapse. The DRG cell bodies are loaded on central compartment, and the sprouting neurites gradually cross through the 500 µm microgrooves to reach the two corner compartments. One corner compartment is co-cultured with DH neurons (right) and another corner compartment is left intact to mimic the free nerve endings of nociceptors.

2.5 Axotomy

In the DRG-DH co-cultures, at DIV5, the peripheral axons of sensory neurons are axotomized using manual application of force to cut them inside the MFC device corridor. A 1000 μL tip is filled with complete medium and was applied and suctioned forcefully and continuously until all axons were cut and removed from the peripheral axonal compartment.

2.6 DiI/DiO Tracing

The lipophilic trace dyes DiI and DiO were used to mark cell bodies with axons crossing the microgrooves to the axonal side of the microfluidic devices. 24 – 48 hours prior to the recording, the axonal compartments were incubated with the DiI and DiO solutions (1:200 in complete medium) for 1 hour at 37 °C and 5% CO_2 . Care was taken to ensure the microfluidic isolation remained intact. After washing with Neurobasal-A medium, and replacement with complete medium, the devices were placed in the incubator until use. The stained cell bodies were visualized immediately prior to live imaging on a Nikon TE200 microscope using a TRITC fluorescence filter set.

2.7 Calcium Imaging

For the DRG-DH co-cultures, between DIV9-12, all 3 compartments were incubated for 1 hour with 2 μM of the calcium indicator dye Fluo4 (Thermo Fisher) in imaging buffer consisting of HBSS [Ca^{2+} and Mg^{2+} - free] supplemented with 10 mM HEPES (Thermo Fisher), 2 mM CaCl_2 (Sigma), 1 mM MgCl_2 , 2 mM probenecid (Sigma), pH 7.4 with NaOH. Axonal and somal MFC compartments were washed twice with imaging buffer prior to recording. Prior to recording fluorescence activity of DH neurons, the central DRG and peripheral axonal compartment were filled with imaging buffer. The devices were mounted on an inverted fluorescent microscope (Nikon Eclipse TE200) equipped with 20X Plan Fluor 0.5 NA objective and an EasyRatioPro imaging unit (Horiba Scientific) (**Figure 4A**). Images were acquired using ORCA 4.2 sCMOS camera (Hamamatsu). Bright field and DiI staining were visualized first, and only neurons with staining in the soma (indicative of axonal crossing) were used for the analysis. The regions of interest (ROIs) were drawn around each corresponding soma and fluorescent images were acquired

at 510nm at 6.3 Hz. For the work on transmission across the first sensory synapse and the synaptic role of TRP and Nav channels, pharmacological blockade was applied at the DH compartment of and Fluo4 recordings were acquired from the DH compartment (**Figure 3**). Care was taken to keep the media volume level higher in the somal compartment and maintain fluidic isolation intact while performing the experiments.

2.8 Voltage Imaging

To record changes in axonal membrane potential, a voltage-sensitive probe FluoVOLT (Life Technologies) was employed. FluoVOLT (**Miller et al,2012**) belongs to a pursuit of large-scale quantification of changes in membrane potential among neurons (**Peterka et al,2011**) (**Knopfel & Song,2019**). FluoVOLT and relevant voltage-sensitive dyes optically detect changes in membrane by modulating a process called photo-induced electron transfer (PeT). The PeT occurs when an electron-rich donor is connected to a fluorescent reporter, through a molecular wire in the neuronal membrane (**Miller et al,2012**). In similar fashion to calcium-sensitive dyes, FluoVOLT allowed the measurement of percentage changes in fluorescence intensity. However, instead of detecting changes in calcium concentration, voltage-sensitive dyes determine the magnitude change in membrane potential which is a direct measure of neuronal depolarization. Protocols have been devised to perform voltage-imaging in brain slices (**Carlson & Coulter,2008**) and several voltage sensors (either genetically encoded or manufactured as dyes) have been tested in neurons (**Milosevic et al,2020**). To our knowledge, the data in this thesis is the first demonstration of imaging changes in membrane potential of DRG axons.

Axons were loaded with FluoVOLT according to the manufacturer's protocols (Life Technologies). Briefly, the axonal compartment was treated with 1:1000 FluoVOLT dissolved in imaging buffer for 20 minutes at 37°C. After loading, the axons were washed, and the devices were mounted on the imaging microscope connected to EasyRatioPro (Horiba) (**Figure 4A**). The excitation wavelength produced by the monochromator for fluorescent images is at 388 nm with an emission wavelength from the axonal compartment recorded at 535nm at 6.3 Hz. The application of drugs was performed at the axonal compartment using a perfusion system while recording from the somal compartment was performed using FluoVOLT (**Figure 2**). Care was taken to preserve the fluidic

isolation between somal and axonal compartments by maintaining the fluid level in the compartments.

2.9 Drug perfusion

A modified perfusion system (Digitimer) has been built for controlled pharmacological administration and avoidance of movement artefacts. The system is comprised of an 8-channel pen with a 360 μm removable tip (Automate Scientific) and regulated by a VC-8 Valve Controller (Warner Instruments) to precisely allow 50 μl of solution every 5 seconds upon turning on (**Figure 2.4B**).

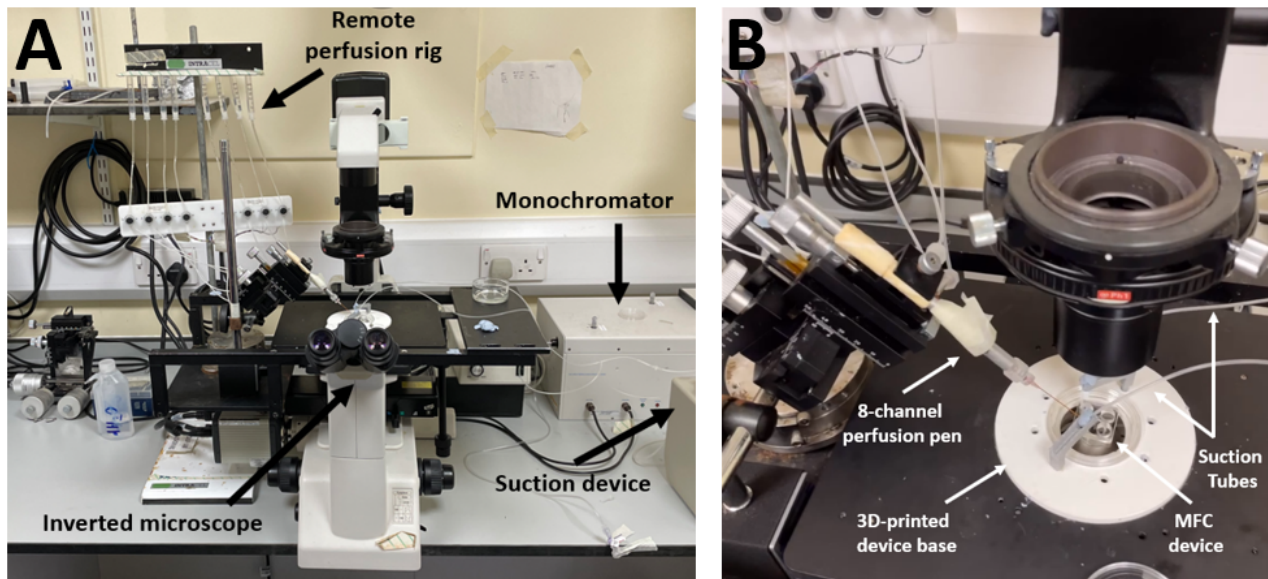


Figure 2.4 A) Calcium/Voltage imaging setup for microfluidic cultures consisted of an inverted microscope, a remote-control perfusion rig, a monochromator and a suction device. B) Surface of the inverted microscope consisted of a 3-D printed holder of the MFC devices, suction tubes and an 8-channel perfusion pen that allows the temporal and volume precision of drug application.

2.10 Electrical Stimulation

In the first sensory synapse recapitulation experiments (**Figure 2.3**), electrical stimulation is applied to the compartments to quantify the amplitude ($\Delta F/F$ ratios, see **Image Analysis** section) of DH or DRG neuron responses. A biphasic stimulator board was built comprised of three width/delay modules (NL405, NeuroLog/Digimer), a pulse generator module (NL301, NeuroLog/Digimer), a pulse buffer module (NL510A, NeuroLog/Digimer) and an analogue switch (NL506, NeuroLog/Digimer) (**Figure 2.5A**). The board was connected to two current stimulus isolators (NL800A, NeuroLog/Digimer) which provided input to handmade soldered electrodes made up of coiled AgCl pellets (**Figure 2.5B**). The electrodes were set to produce 8 biphasic pulses at 4Hz frequency x 2 ms @4 μ A (**Figure 2.5C**).

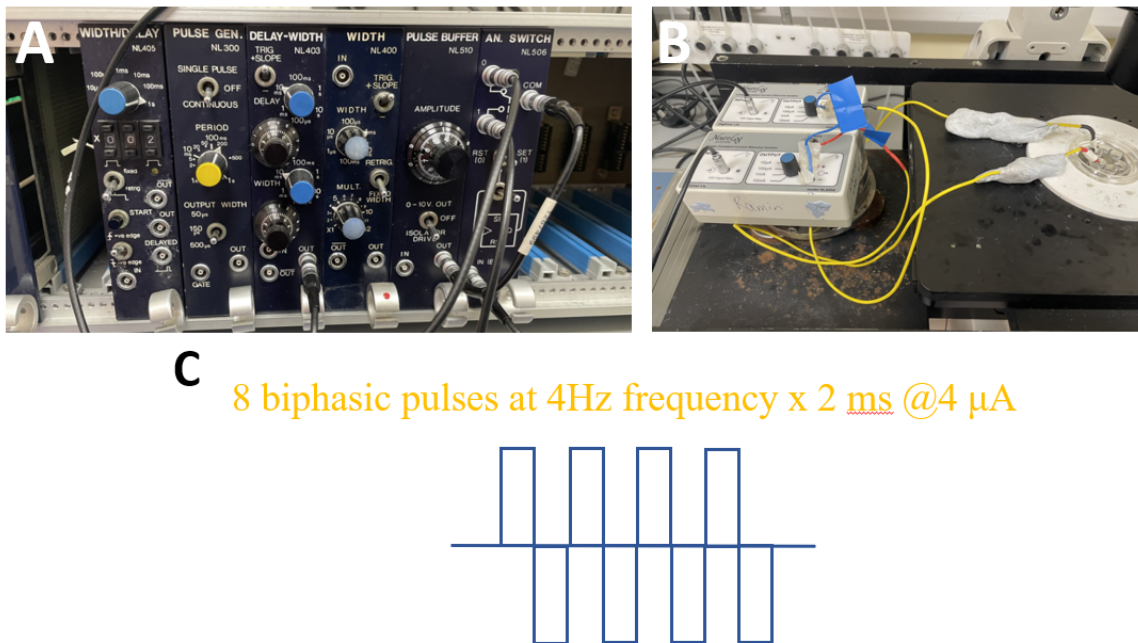


Figure 2.5 A) Customized biphasic stimulator board comprised of width/delay and pulse generator modules for train pulse timing and two additional width delay modules, a pulse buffer and an analogue switch module for modifying the width of biphasic pulses. **B)** Two current stimulus isolators receiving input from the board and connected to two soldered electrodes which are placed into the microfluidic device to stimulate the neurons. **C)** Optimized pulsed symmetric biphasic stimulation parameters used throughout the thesis.

2.11 Drugs

PGE2 stock (Sigma) was dissolved in DMSO at 10 mM. Tetrodotoxin (TTX) stock solution (Alomone Labs) was prepared in deionized H₂O at 1 mM; Nav1.8 blocker PF-01247324 (Tocris), Nav1.7 blocker PF-05089771 (Tocris) were prepared in DMSO at 1mM; the non-selective VGSCs blocker lidocaine, the cAMP antagonist cAMPS-Rp, Nav1.6 blocker 4-9-Anhydrotetrodotoxin (AHTTX), Nav1.8 blocker A-803467, EP4 inhibitor L-161,982, ANO1 blocker T16Ainh-A01, non-selective HCN channel blocker zatebradine hydrochloride, the NKCC1 inhibitor Bumetanide and the KCC2 inhibitor VU01240551 (all purchased from Tocris) were dissolved in DMSO. The TRPA1 blocker A-967079, the TRPV1 blocker AMG517 and the TRPM8 blocker M8-B hydrochloride (all purchased from Tocris) were dissolved in DMSO. All stock solutions were kept at -20 C until use.

2.12 Image analysis for calcium imaging

For calcium imaging experiments, the $\Delta F/F$ ratios were determined for designated regions of interest (ROIs) using EasyRatioPro software. The EasyRatioPro sequences saved from the live calcium imaging recordings were converted into frame-by-frame TIFF files. The TIFF files were added to FIJI and $\Delta F/F$ traces were acquired using that software. which were saved as comma delimited (.csv) files. The comma delimited files were uploaded on the specialized for electrophysiological analysis software Clampfit 9.0 (Molecular Devices). The amplitudes of the traces were determined using the software (**Figure 2.6**).

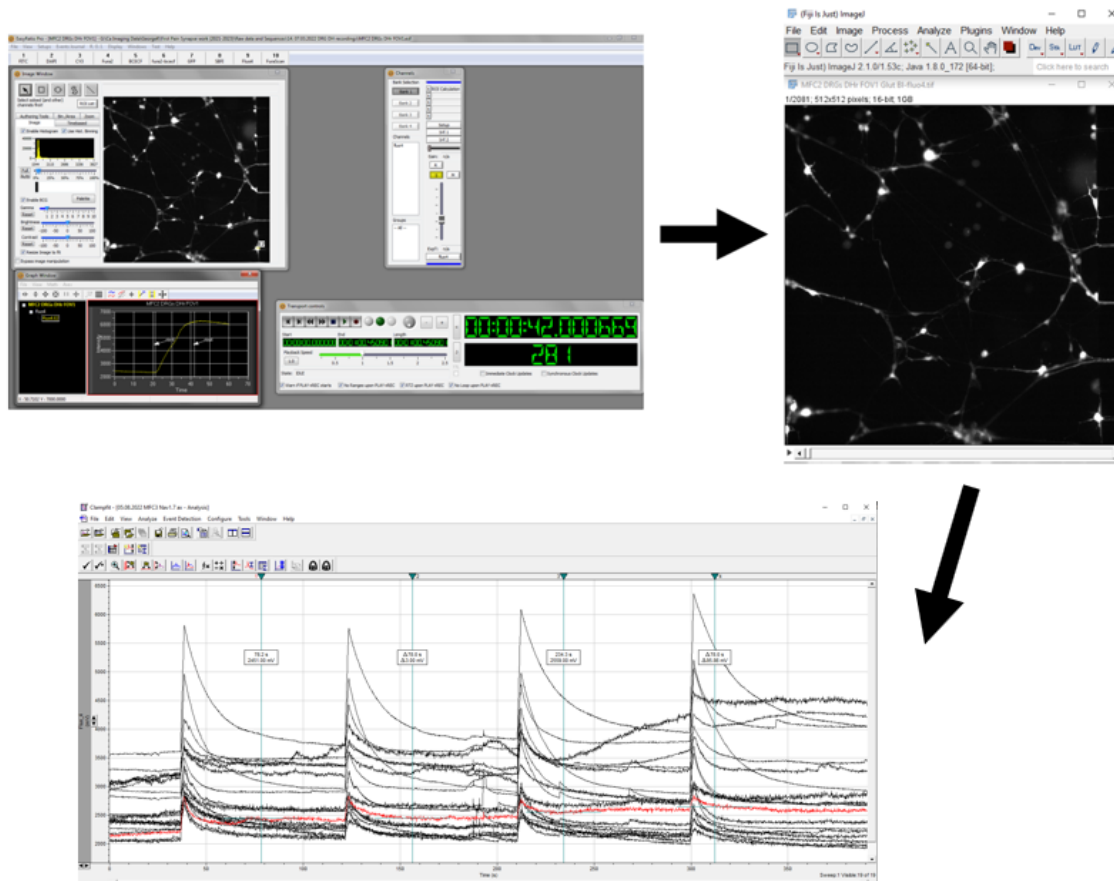


Figure 2.6 The workflow of image analysis for calcium and voltage imaging experiments. The live calcium imaging experiments captured by EasyRatio Pro were converted into frame-by-frame TIFF files. The TIFF files were processed with FIJI, where the baseline responses were subtracted from the peak responses to obtain $\Delta F/F$ traces. The $\Delta F/F$ traces were transferred to Clampfit 9.0 to calculate changes in $\Delta F/F$ amplitude.

The responses to stimulation were visually verified and confirmed if the maximum increase in F_{ratio} was at least three times greater than the standard deviations of the baseline calculated from at least 50s prior to drug application. The peak response (ΔF_{ratio}) was calculated as the maximum increase in fluorescence intensity above the baseline. The detection of Fluo4 fluorescence intensity changes was defined as the ($\Delta F/F$) between baseline responses taken at least 10 seconds prior to application of stimulus where no fluorescence activity was reported and ΔF maximum peaks within the timespan of application.

In certain calcium imaging experiments (**Figures 4.10-12 & 4.14**), together with changes in amplitude of fluorometric responses represented as $\Delta F/F$ values, the decay time (τ) of responder traces until they return to baseline was calculated. The τ values were represented as the following standard exponential curve function:

$$f(t) = \sum_{i=1}^n A_i e^{-t/\tau_i} + C$$

2.13 Image analysis for voltage imaging

For voltage imaging experiments, frame by frame TIFF files were transferred from EasyRatio Pro to FIJI, where axonal ROIs were selected according to brightness intensity changes (**Figure 6**). We quantified the changes in axonal membrane potential in microfluidic cultures by determining the average peak change in fluorescent intensity across all axonal regions in the field of view (FoV). The average peak change in fluorescence intensity was calculated by first determining a mean baseline image from 50 frames prior to drug application and subtracting the mean baseline image from all image stacks to create a ΔF image stack. The ΔF images were thresholded to demarcate the activated regions of in the axons. Particle Analysis in FIJI was used to assign the ROIs. The average peak in FluoVolt fluorescence intensity for each axonal ROI was calculated using the formula:

$$\Delta F/F = 100 * [(F_{\max} - F_{\text{baseline}}) / F_{\text{baseline}}]$$

We also determined the proportion of the total axonal area that showed a change in FluoVolt fluorescence signal intensity in the FoV. A limitation of FluoVolt is that it does not allow long recording times which are needed to determine the responses in the same FoV to the application of the inhibitors. Thus, we used the proportion of axonal area that showed an increase in FluoVolt signal to avoid any bias caused by the heterogeneity of the axonal responses between the experiments. The quantification of axonal areas that exhibited a change in membrane potential was represented as a percentage of the total axonal area that showed a change in FluoVolt fluorescence following drug application (**Figure 2.7**). Total axonal areas in each image, expressed as total

number of pixels, were determined prior to drug application by thresholding the images in Fiji and selecting the entire axonal regions over the background. The activated axonal areas were determined from background subtracted images that were thresholded to highlight the areas that showed a change in intensity following drug application. The percentage ratio of the activated area to the total axonal area was taken as a measure of the activated area expressed as:

$$\text{Activated area \%} = \text{Axonal Activated Area} / \text{Total Axonal Area}$$

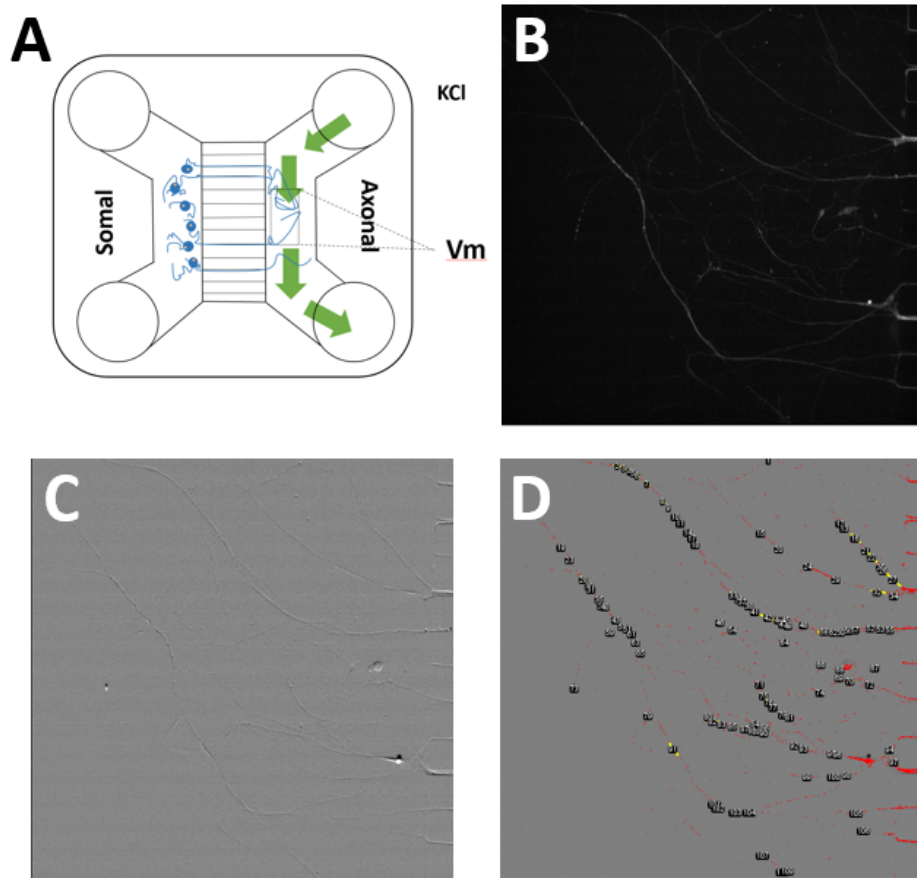


Figure 2.7 Image analysis workflow for voltage imaging **A)** Schematic setup of KCl (30 μ M) application to the axonal compartment and voltage imaging recording using FluoVolt. **B)** Fluorescence image of a chosen FoV designating the total axonal area of activation. **C)** Baseline subtracted image of the FOV in a 32-bit version of the TIFF file before the application of KCl. **D)** Baseline subtracted image of the FOV after the application of KCl with the responding axonal areas annotated for the calculation of the activated area %.

2.14 Voltage Imaging Trial Experiments

As a demonstration of the voltage imaging experimental protocol, a mini proof-of-concept set of experiments was run to determine whether FluoVolt can appreciably detect changes in membrane potential represented as $\Delta F/F$ FluoVolt fluorescence intensity. In a 2-compartment microfluidic device using adult DRG mice (**Figure 2.8A**), the FluoVolt responses of sensory axons to direct axonal KCl are recorded. A step depolarization experiment shows that successive applications of escalating KCl concentrations increase the detected FluoVolt fluorescence, thus confirming that the voltage-sensitive dye is responding to the increased depolarization of the axonal membrane by KCl (**Figure 2.8D**). A further trial experiment was the stimulation of sensory axons with KCl (30 μM) in the absence (**Figure 2.8B**) or presence (**Figure 2.8C**) of a selective Nav1.8 blocker (A-803467, 10 nM). Utilizing the methodology described in the **Image analysis for voltage imaging** section, comparisons of the average amplitude response of KCl in the presence or absence of the blocker are quantified using GraphPad Prism 9, showing that Nav1.8 blockade attenuates KCl-evoked amplitude responses (**Figure 2.8E**). However, for the second metric introduced regarding the activated axonal area of KCl-evoked responses, the concomitant addition of the blocker with KCl (30 μM) does not alter the total responding activated area (**Figure 2.8F**).

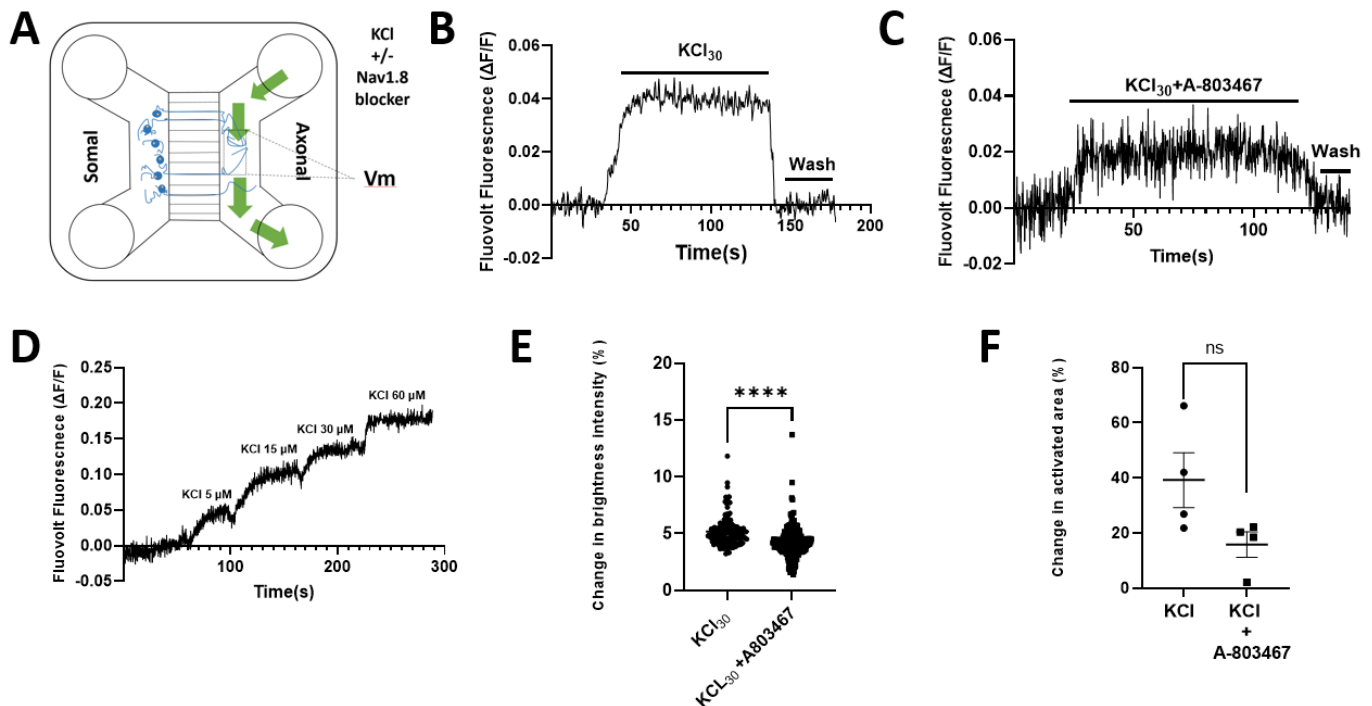


Figure 2.8 KCl induced depolarization of the axolemma of adult DRG mice is detectable using Fluovolt dye. **A)** Schematic of experimental set up used for data: 2-channel MFC compartment, where the somal side is separated from the axonal side. **B)** Representative trace indicating changes in brightness intensity following a 1-minute stimulation with 30 μ M of KCl **C)** Representative trace indicating changes in brightness intensity following a 1-minute stimulation with 30 μ M of KCl in the presence of 10 nM of a selective Nav1.8 blocker (A-803467) at an axonal ROI and expressed as Fluovolt Fluorescence ($\Delta F/F$) **D)** Representative trace of a step depolarization experiment where increasing concentrations of 5mM, 15mM, 30mM and 60mM applied. Changes in brightness intensity are expressed as Fluovolt Fluorescence ($\Delta F/F$), of one section of an axon in response to KCl application. n=1 device from 1 culture (1 mouse) **F)** Scatter plot describing changes in brightness intensity between KCl-treated and KCl+Nav1.8 blocker-treated axons. Application of 30mM KCl in the presence of the Nav1.8 blockers attenuates the average amplitudes of responses ($5.16 \pm 0.11\%$ for KCl alone vs $4.16 \pm 0.08\%$ for KCl+ A-803467. (**** $p < 0.0001$ Student's t-test, $n_{\text{ROIs}} = 135$ on 4 devices from 4 cultures (4 mice) for KCl and, $n_{\text{ROIs}} = 271$ on 4 devices from 3 cultures (3 mice) for KCl+A-803467). **G)** Scatter plot describing changes in the number of total activated axonal area in the presence of 30mM KCl in the presence of the Nav1.8 blocker. The Nav1.8 blocker exhibits an attenuating trend towards KCl-mediated activity of the total area covered by sensory axons. ns $p < 0.05$, Student's t-test, $n = 4$, devices from 4 cultures (4 mice) for KCl and $n = 4$ devices from 3 cultures (3 mice) for KCl+A-803467).

2.15 Immunocytochemistry

After calcium imaging, cells were washed with D-PBS and fixed in 4% paraformaldehyde (PFA) in phosphate buffer for 10-15 minutes, then washed and stored at 4 °C. After 3 consecutive D-PBS washes, cells were incubated with blocking solution (10% donkey serum + 0.1% Triton-X) for 1 hour at 25 °C. The primary antibodies for chicken anti-MAP2 (Novus Biologicals 1:4000 dilution), anti-Synapsin 1 (Invitrogen, 1:4000 dilution) or anti-VGlu2 polyclonal (Invitrogen, 1:10000 dilution) were added and incubated for 24 hours at 4 °C. Upon removal of the primary antibodies, the devices were washed 3 consecutive times with D-PBS. Respective secondary antibodies donkey anti chicken 649 (Invitrogen, 1:1000 dilution), donkey anti rabbit 488 (Invitrogen, 1:1000 dilution) and donkey anti rabbit 594 (Invitrogen, 1:1000 dilution) were incubated for 1 hours at 25 °C, which were followed by removal and subsequent D-PBS washes. The acquisition of confocal images was performed in an inverted Zeiss Imager.Z1 microscope with 20x objective and equipped with DAPI, TRITC and FITC filters within 24 hours after removal of secondary antibodies. The

experiments were performed once and following image acquisition, no further analysis and quantification was conducted.

2.16 Statistics

The data are reported as mean $\pm$ SEM unless otherwise stated. All experiments were repeated at least 3 times to determine statistical significance. In Chapter 3, the ROIs (axonal points), the number of devices, the number of cultures and the number of mice used are designated on each figure legend. In Chapters 4 & 5, the number of DH neurons, number of devices and number of cultures are designated in each figure legend. For quantifying changes in brightness intensity with Fluovolt or Fluo4, the corresponding values of ROIs were used in the statistical analysis. For quantifying changes in activated area, the axonal areas of each device were used for quantification.

All figures designed and statistical analysis have been performed using GraphPad Prism 9. Statistical significance was set at $p < 0.05$. Student t-test (unpaired, equal variance) was used to compare groups in calcium imaging analysis. One Way ANOVA with Tukey's multiple comparison test was used for comparisons between three or more independent groups in calcium imaging analysis. Brown-Forsythe and Welch ANOVA tests were used to determine significance between groups of equal and different variance respectively on voltage imaging analysis.

3. PGE2-mediated depolarization of sensory axons

Aims and objectives

PGE2 is an important contributor to inflammatory pain hyperalgesia and the aim of the chapter has been to investigate the extent to which it modulates the activity of nociceptive axons and delineate the mechanism responsible.

- 1) By isolating these nociceptor axons within microfluidic devices, a first objective was to determine whether PGE2 may sensitize responses to depolarizing stimulation but also whether it may elicit a direct and persistent axonal depolarization that propagates towards the cell body.
- 2) A second objective regarded the development of a novel method of recording changes in membrane potential directly at the axonal site of several axons at once, using a voltage-sensitive dye, FluoVolt.
- 3) Third, via pharmacological manipulations, it was sought to examine whether the PGE2-evoked depolarization is mediated by the EP4/cAMP/PKA pathway.
- 4) A fourth objective was to determine whether an observed PGE2-mediated activity on sensory axons is mediated by the TTX-sensitive Nav1.7 or the TTX-resistant Nav1.8 channels.
- 5) The determination of whether other ionic contributions such as changes in chloride concentration and more specifically the activation of the calcium-activated chloride channel ANO1 may contribute to the PGE2-evoked activity.

A general hypothesis regards the plausibility of PGE2 exerting a dual role in inflammatory pain by sensitizing the evoked responses in nociceptors but also by directly initiating painful signals through the activation of specific ion channels.

3.1 Introduction

Inflammatory pain occurs upon tissue injury or infection where released mediators by immune cells promote pain hypersensitivity while healing occurs. The manifestation of inflammatory pain can have a temporary duration or a persistent one. Several resident or infiltrated immune cells secrete inflammatory mediators and sensitizers that render the injured tissue in a hyperalgesic state and may define hallmarks of pathological pain conditions **(Shu and Mendell,1999) (Oggero et al,2022)**. In states of hyperalgesic pain, stimuli that were previously evoking a painful response of defined magnitude, are then perceived in an even more amplified manner; a hallmark of inflammatory pain conditions.

In acute injuries, following repair of damaged tissue, there is a resolution of inflammation and the hyperalgesic pain profile subsides. However, in chronic pain conditions such as those of autoimmune origin or those with an age-related component, the hyperalgesic profile persists, revealing a mode of prolonged, peripherally initiated sensitization **(Bhave and Gereau,2004)**. The persistent pain in cases of chronic inflammation may display an enormous complexity in terms of the cellular and molecular components involved. Inflamed tissue may progressively induce molecular, cellular and anatomic changes of neuronal and non-neuronal cells with both the peripheral and central nervous system becoming affected **(Hucho and Levine,2007) (McMahon and Malcangio,2009)**.

There are several molecular mediators of diverse cell origins which have been shown to contribute to hyperalgesic states and in inflammatory pain. The identified molecular mediators of inflammation include the prostaglandins, bradykinin, nerve growth factor (NGF), histamine, protons, ATP, 5-hydroxytryptamine (5-HT) and nitric oxide (NO). Discovered in 1960 **(Montalcini et al,1960)**, the NGF has since been shown to play an important role in the sensitivity of nociceptors, by binding to its high-affinity receptor trkA and low affinity receptor p75 **(Chao,2003) (Pezet and McMahon,2006)**. The NGF has been extensively implicated in inflammatory hyperalgesia and pain **(Andreev et al,1995)**, where major efforts have been conducted to develop analgesics targeting NGF or the complex between trkA and p75 **(Lewin et al,1993) (McMahon,1996)**. The sequestration of NGF using a trkA-IgG fusion molecule/neutralized recombinant antibody was demonstrated as a promising treatment of inflammatory pain and neuropathic pain **(McMahon et al,1995)**. Later, a monoclonal antibody,

tanezumab, underwent advanced clinical trial stages (**Lane et al,2010**) (**Katz et al,2011**) and although efficaciously pain-relieving, it produced adverse effects in the cartilage and bone integrity of the patient cohort. Bradykinin, a dilating agent, is another key mediator which acts through two receptors BK1 and BK2 in sensory neurons. It has also an established role in mediating inflammatory pain, although bradykinin antagonists are not as commonly used analgesics as COX inhibitors (**Steranka et al,1988**) (**Dray and Perkins,1993**). More recently, the tetrahydrobiopterin (BH4) pathway has been implicated in inflammatory and neuropathic pain conditions (**Latremoliere et al,2015**) (**Tegeder et al,2006**). In these models, inhibition of sepiapterin reductase, a crucial enzyme in the metabolic pathway of BH4, is explored as a promising target. It is believed that BH4 inhibition may ameliorate the serotonergic and nitric oxide synthase (NOS) production in sensory neurons, yet the mechanisms downstream of the metabolic pathway of BH4 remain to be elucidated (**Costigan et al,2012**).

Prostaglandin E2 is a major component of inflammatory pain and hyperalgesia as well as neuropathic pain (**Petho and Reeh, 2012 for review**). Since its discovery in 1962 by Bergstrom and colleagues, PGE2 has been implicated in several physiological processes (**Bergström, 1967**). In the absence of inflammation, PGE2 possesses homeostatic and regulatory roles within the nervous system. It is established that PGE2 not only interacts with sensory neurons (**Pitchford and Levine,1991**) but also mediates dorsal horn and spinal cord responses to inflammation and injury (**Baba et al,2001**) (**Samad et al, 2001**). More recently, it has been shown that it also mediates hypothalamic responses of the central nervous system to peripherally induced infection and inflammation (**Osterhout et al, 2022**).

PGE2 belongs to a class of abundantly expressed eicosanoids and is a product of the enzymatic catalysis of arachidonic acid. The production of PGE2 depends on cyclooxygenases, COX-1 and COX-2, which convert arachidonic acid into the precursor molecules PGG₂ and PGH₂. The precursor molecules are further enzymatically converted by specialized prostaglandin synthases into the prostaglandins E2, D2 and F2A, prostacyclin (PGI₂) and thromboxane (**Simmons et al,2004**). The disruption of the enzymatic pathway leading to the production of PGE2 and other prostaglandins has been the underlying rationale behind the development of non-steroidal anti-inflammatory drugs (NSAIDs) and more specifically COX inhibitors, such as aspirin and ibuprofen, which are extensively used as mild analgesics (**Vane & Botting, 1998**). Despite the

efficacy of COX inhibitors in treating acute inflammatory pain, the drugs are not as effective in chronic pain conditions. Further, adverse effects associated with the use of COX inhibitors, such as peptic ulcer development and elevated cardiovascular event risks (**Suleyman et al,2007 for review**), in combination with the contemporary dietary lifestyle and the increased lifespan of people in the 21st century, have rendered the development of new anti-inflammatory analgesics a matter that requires immediate resolution.

Downstream of PGE₂ binding, there are four receptor isoforms, called EP1-4. Cloned in the 90s, the EP receptors belong to the superfamily of G-protein coupled receptors and are expressed in several tissues including the sensory neurons (**Sugimoto et al, 1992**) (**Watabe et al, 1993**), (**Bastien et al, 1994**), (**Coleman et al,1994**). The EP2 and EP4 receptors form a G_s complex, while EP1 forms a G_q complex and EP3 forms a G_i complex. All four isoforms are expressed in sensory neurons (**Southall & Vasko,2001**), however, the high affinity receptor for PGE₂ in sensory neurons is EP4, which forms a G_s complex that activates adenylate cyclase and increases cyclic AMP (cAMP) production, (**Fujino et al,2005**), (**Lin et al,2006**). Recently, a cryo-EM structure of the PGE₂/EP4 complex was published revealing the detailed binding pockets and interaction sites between the ligand and its receptor (**Nojima et al,2021**).

The pronociceptive effects of PGE₂ and its role in peripheral sensitization through activation of EP receptors have been further established. In human synovial fluid samples from osteoarthritic patients, evidence has been provided for the involvement of the PGE₂/EP4 pathway in the pathology (**Attur,2008**). Similarly, in a collagen antibody-induced arthritis (CAIA) experimental model of rheumatoid arthritis, EP4 knockout mice showed reduced inflammation and modest severity of arthritis in comparison to the other 3 isoform EP receptors (**McCoy et al,2002**). The increased levels of cAMP due to the PGE₂/EP4, in turn leading to an increase in PKA activity, which is responsible for the phosphorylation and subsequent activation of ion channels such as TRPV1 and Nav1.8 that mediate neuronal excitability (**England et al,1996**) (**Cesare & McNaughton, 1997**) (**Moriyama et al,2005**). Several studies have shown the sensitization of nociceptive nerve endings in the skin and membrane targets by the PGE₂/EP4 and their contribution to inflammatory pain (Lin et al,2006) (**Jang et al,2020**). It has been also shown that PGE₂ can directly activate dorsal horn neurons of the spinal cord (**Baba et al,2001**).

Despite our understanding of the sensitization of nociceptive neurons by PGE2 leading to hyperalgesia, it is not clear if PGE2 can directly evoke pain. Early studies support the hypothesis of the excitation of nociceptors by prostaglandins, and in more recent work calcium responses evoked by PGE2 have been detected in cell bodies of cultures sensory neurons **(Coleridge et al,1976) (Materazzi et al,2008)**. In contrast, other studies have not detected PGE2-evoked spike discharges in visceral afferents and in skin nerve preparation **(Mizumura, 1993), (Brunsden & Grundy, 1999)**. Even less is known about the molecular mechanisms underpinning axonal responses to inflammatory mediators and how they affect their excitability. Approaches preceding microfluid cultures possessed technical limitations that did not enable the study of functional properties of nociceptor axons. Recent work has suggested that inflammatory mediator stimulation moderates the trafficking of ion channels across nociceptors distinct anatomical compartments, hence modulating their excitable properties **(Higerd-Rusli et al,2023)**. However, it is the axons and not the cell bodies of nociceptors that are often the actual sites of injury and the recipients of inflammatory mediator stimulation following tissue damage from the secreted immune cell milieu.

Utilizing the microfluidic platform developed in our lab, we have been able to investigate the function of sensory axons in isolation from nociceptor cell bodies **(Tsantoulas et al,2013)**. Microfluidic cultures, while they recapitulate salient features of the anatomy and physiology of nociceptors, they are also amenable to pharmacological manipulations of their respective compartments in isolation. Hence, we have sought to address the effect that local treatment with PGE2 exerts on sensory axons.

Previous studies in the lab have established and validated a microfluidic culture model to study the molecular mechanisms of PGE2-mediated sensitization of the sensory axons. The results described in this chapter were published together with experiments initiated by a previous doctoral student in the lab, Dr Natasha Rangwani in a pre-print manuscript **(Kimourtzis et al,2022)** The initial hypothesis of studying peripheral sensitization on nociceptive axons, was that application of an inflammatory mediator (IM) cocktail consisted of PGE2, histamine, bradykinin and serotonin, would potentiate the responses evoked by a depolarizing chemical stimulus such as potassium chloride. Indeed, the calcium imaging recordings from the DRG cell bodies before and after treatment with IMs showed profound changes in the amplitude of KCl-mediated responses. The number of respondent neurons was also increased in IM-treated axons, closely mimicking the

concept of silent nociceptor unmasking. These results confirmed the role of inflammatory mediators as predominant contributors of sensory neuron sensitization. The recapitulation of peripheral sensitization by IMs on sensory axons was examined for each mediator on an individual basis. Treatment with PGE2 potentiated the Ca^{2+} responses recorded from the cell body (**Figure 1B**). The HCN channels display pacemaking properties of DRG neuron firing frequency (**Emery et al,2012**) (**Lainez et al,2019**). Further, these channels are involved in neuropathic and inflammatory pain states (**Emery et al,2011**) (**Young et al,2014**). We found that zatebradine, a non-selective blocker of HCN blockers led to an amelioration of PGE2-mediated sensitization of axonal responses to KCl-evoked depolarization. This result is consistent with the lack of PGE2-induced thermal hyperalgesia in HCN2 gene deleted from in Nav1.8 expressing DRG neurons (**Emery et al,2011**). The application of an HCN blocker ameliorated the sensitizing effects of PGE2 in KCl-evoked responses (**Figure 3.1C**). Considered together, the microfluidic culture model of inflammatory mediator sensitization recapitulates accurately salient features of the *in vivo* physiology of nociceptors and confirmed the role of HCN channels as enhancers and pacemakers of sensory neuron excitability. Thus, it provides a platform for studying axonal mechanisms of pain sensitization.

A compelling observation from the PGE2 sensitization experiments, was that in several traces obtained, axonal application of PGE2 independently induced short, repetitive bursts of intracellular $[\text{Ca}^{2+}]$ transients detected in the somal compartment using calcium imaging. This phenomenon propelled to the direct application of PGE2 in sensory axons and the recording of the firing activity from the cell body (**Figure 3.1D**). The direct application of PGE2 to the distal compartment produced a persistent wave of calcium transients detected at the somal compartment, even following washout (**Figure 3.1E**). Thus, to localize and confirm that the site of activation of the observed persistent activity is the axonal one, the non-selective VGSC blocker lidocaine was applied at an isolated compartment. Lidocaine blocked completely the evoked persistent response detected at the soma, suggesting that the persistent responses elicited by PGE2 originated in the axon terminals (**Figure 3.1F**).

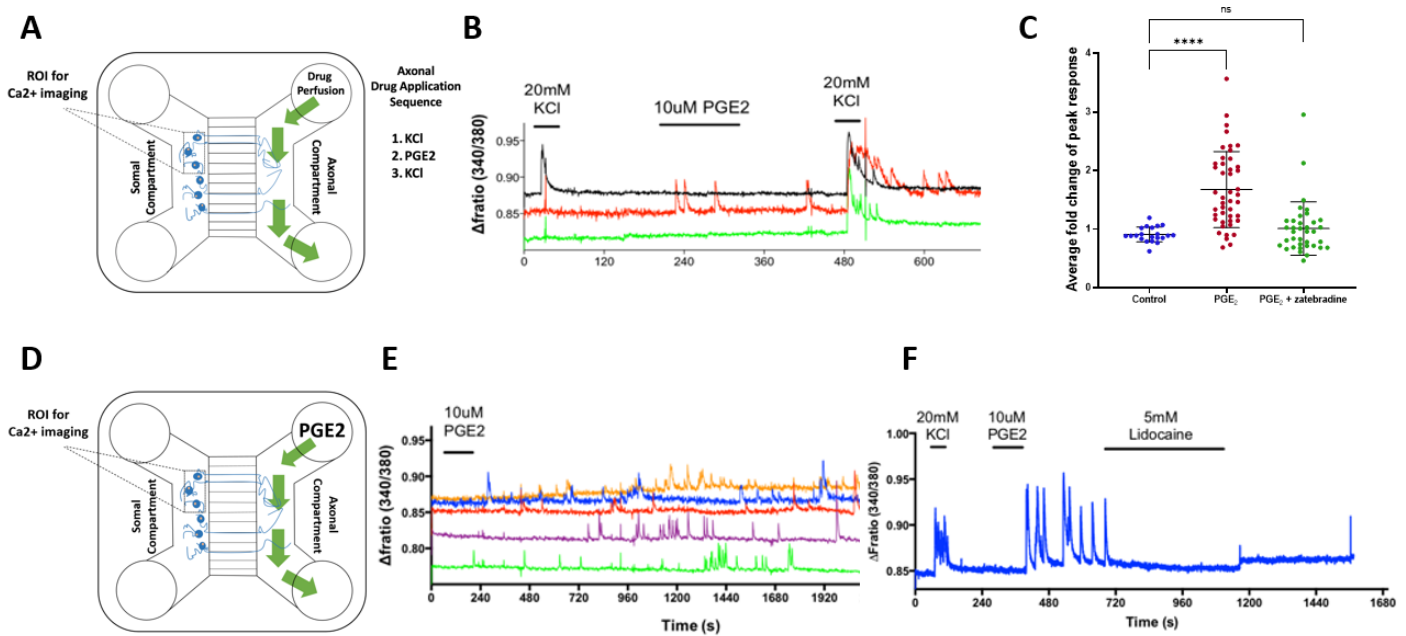


Figure 3.1 Dual effects of PGE2: sensitization to depolarization evoked by KCl stimulation and direct activation of sensory axons. A-C) The axonal application of PGE2 potentiates KCl-evoked responses detected at the cell bodies of nociceptors. The concomitant application of PGE2 with a non-selective HCN blocker, zatebradine abolished the PGE2-amplified responses evoked by KCl. **D-F)** The direct axonal application of PGE2 elicited an increase in calcium transients detected at the cell body. The addition of lidocaine abolished the PGE2-elicited calcium transients suggesting that the activity originates in the axons (Adapted from Kimourtzis et al,2022).

The persistent activity evoked by PGE2 raised the probability of the involvement of a downstream second messenger signaling mechanism. Immunocytochemical experiments showed that the high-affinity prostaglandin receptor, EP4, co-localizes with Nav1.8 channels on those DRG axons (**Figure 3.2A**). The co-expression pattern of EP4 and Nav1.8 confirmed that these EP4 receptors are expressed in a subset of Nav1.8 positive neurons, which are putatively nociceptors and underscored a potential interaction between the two proteins. Further evidence for the involvement of a G-protein coupled downstream messenger pathway was made through the application of a membrane permeant inhibitor of cAMP-dependent protein kinases called Rp-cAMPs (**Figure 3.2B**). The addition of the cAMP inhibitor ameliorated the PGE2-evoked activity, which was reversibly restored following washout, thus confirming that the persistent activity requires the generation of cAMP and PKA (**Figure 3.2C**).

Last, the contribution of VGSCs to the PGE2-evoked activity was examined. Application of TTX did not attenuate the PGE2-evoked persistent activity in the axonal compartment (**Figure 3.2E&F**), suggesting a lack of involvement for TTX-sensitive VGSCs in the generation or propagation of this persistent activity. Conversely, we examined the role of the TTX-resistant sodium channels Nav1.8, which have been previously shown to be modulated by PGE2 (**Gold et al., 1998**). Application of a Nav1.8 blocker PF-01247324, significantly attenuated the PGE2-evoked activity in the sensory axons, suggesting an important role for the TTX-resistant VGSC (Figure 3.2H&I).

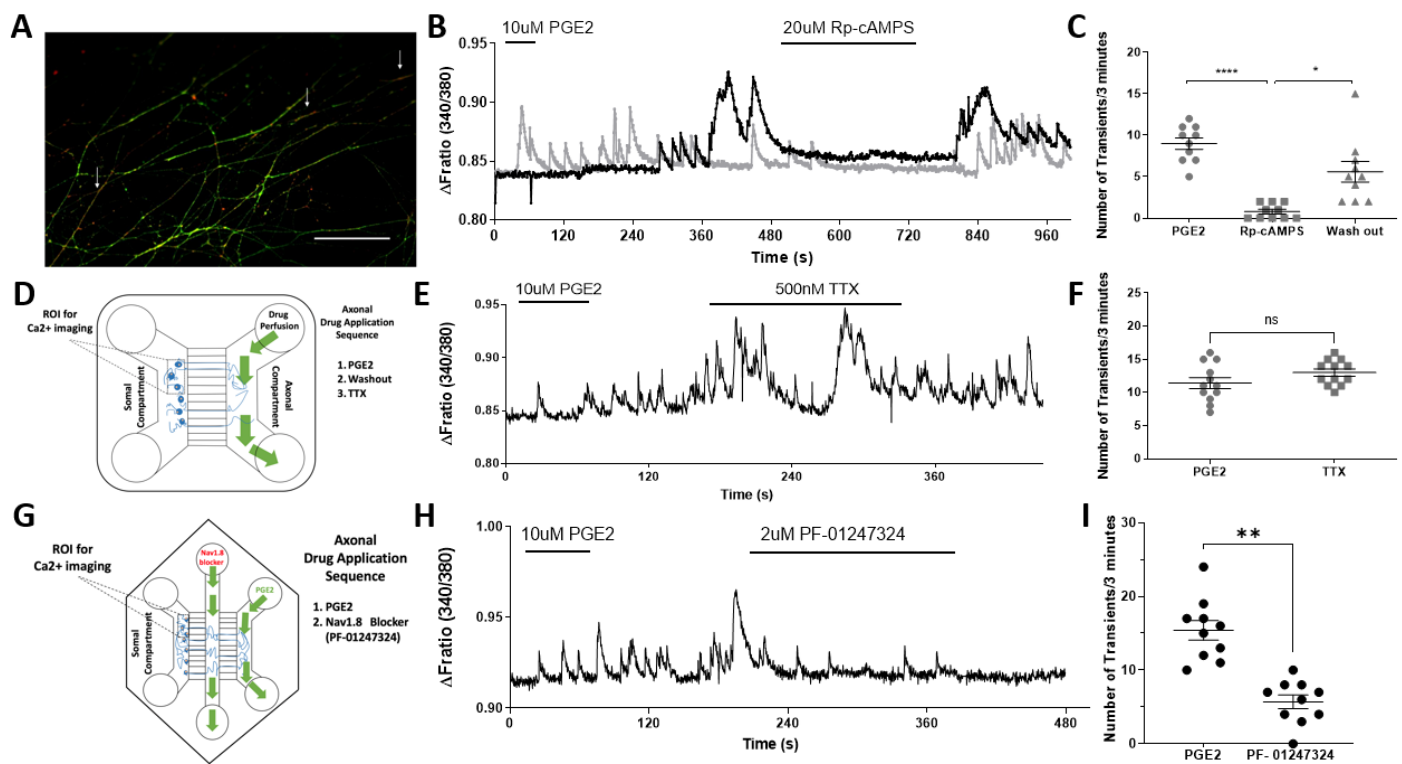


Figure 3.2 Evidence for the downstream mechanistic activity of direct and persistent PGE2-evoked calcium transients. A) Axonal staining of Nav1.8 channels (green) with EP4 receptors (red) reveal co-expression (white arrows). **B-C)** The increased calcium transients evoked by axonal application of PGE2 are abolished by cAMP/PKA inhibition. **D-F)** TTX-sensitive VGSC do not mediate the axonal PGE2-evoked calcium transients detected at the nociceptor cell bodies. **G-H)** Blockade of the TTX-resistant Nav1.8 channel attenuates the axonal PGE2-evoked calcium transients recorded at the cell bodies (**Adapted from Kimourtzis et al,2022**)

The previous experiments suggested that there was activation of axons by application of PGE₂, however, the mechanism and site of initiation of this activity remained unclear. In this chapter, we use a spatially resolved quantification methodology (**see Methods**) to validate the activation of axons by PGE₂, determine the site of activation and investigate the mechanism underlying the PGE₂-evoked activity in sensory axons. The voltage-sensitive dye FluoVolt was employed to record large-scale, direct changes in the membrane voltage of sensory axons.

It is thus postulated whether PGE₂-evoked activity originates from the nociceptor axons by locally modulating the axonal membrane potential, in the absence of other stimulation. The mechanistic basis of this depolarization was delineated, and evidence was provided for the involvement of the EP4/cAMP/PKA cascade mediating the PGE₂ evoked responses. Last, the ionic contribution of specific ion channels was examined and specifically the VGSC Nav1.8 and the calcium-activated chloride channel Anoctamin 1 (ANO1) are essential for the depolarization and persistent activity of PGE₂ in sensory axons.

3.2 Results

3.2.1 PGE2 directly depolarizes nociceptive axons

Once we observed that the persistent activity induced by PGE2 is initiated locally in the sensory axons (Kimourtzis et al,2022), we hypothesized that PGE2 depolarizes the axonal membrane, and the evoked APs are then propagated towards the neuronal body. We utilized a voltage-sensitive dye, FluoVolt (Miller et al,2012), where we measured ensued changes in axonal membrane potential following PGE2 stimulation. Similar to calcium-sensitive dyes, FluoVolt allowed us to measure percentage changes in fluorescence intensity to determine the magnitude change in membrane potential (Figures 3.3B -D). In each microfluidic device a separate experiment was conducted. Hence, there were two separate, matching groups where sensory axons were either treated with KCl or PGE2.

In the control experiments, application of KCl (30mM) for 90 seconds produced a change in fluorescence intensity of $5.18\% \pm 0.11\%$ which returned to baseline immediately after washing with buffer (Figure 3.3E). An application of PGE2 (10 μ M) produced a $5.89\% \pm 0.09\%$ increase in FluoVolt fluorescence intensity (Figure 3.3G), which is comparable in amplitude to the KCl-evoked response. In contrast to the KCl response, the PGE2-evoked depolarization exhibited slow activation kinetics and a sustained plateau following wash (Figure 3.3F). While responses of sensory axons to KCl returned to baseline following wash, (Figure 3.3E) the response of axons to PGE2 were sustained following its removal (Figure 3.3F). This kinetics supported the possibility that activation of a second messenger pathway induces persistent depolarization, an observation which may reflect the occurrence of tonic pain in inflammatory pain conditions. In conclusion, these data demonstrate a direct and local depolarization of the axonal membrane in response to PGE2.

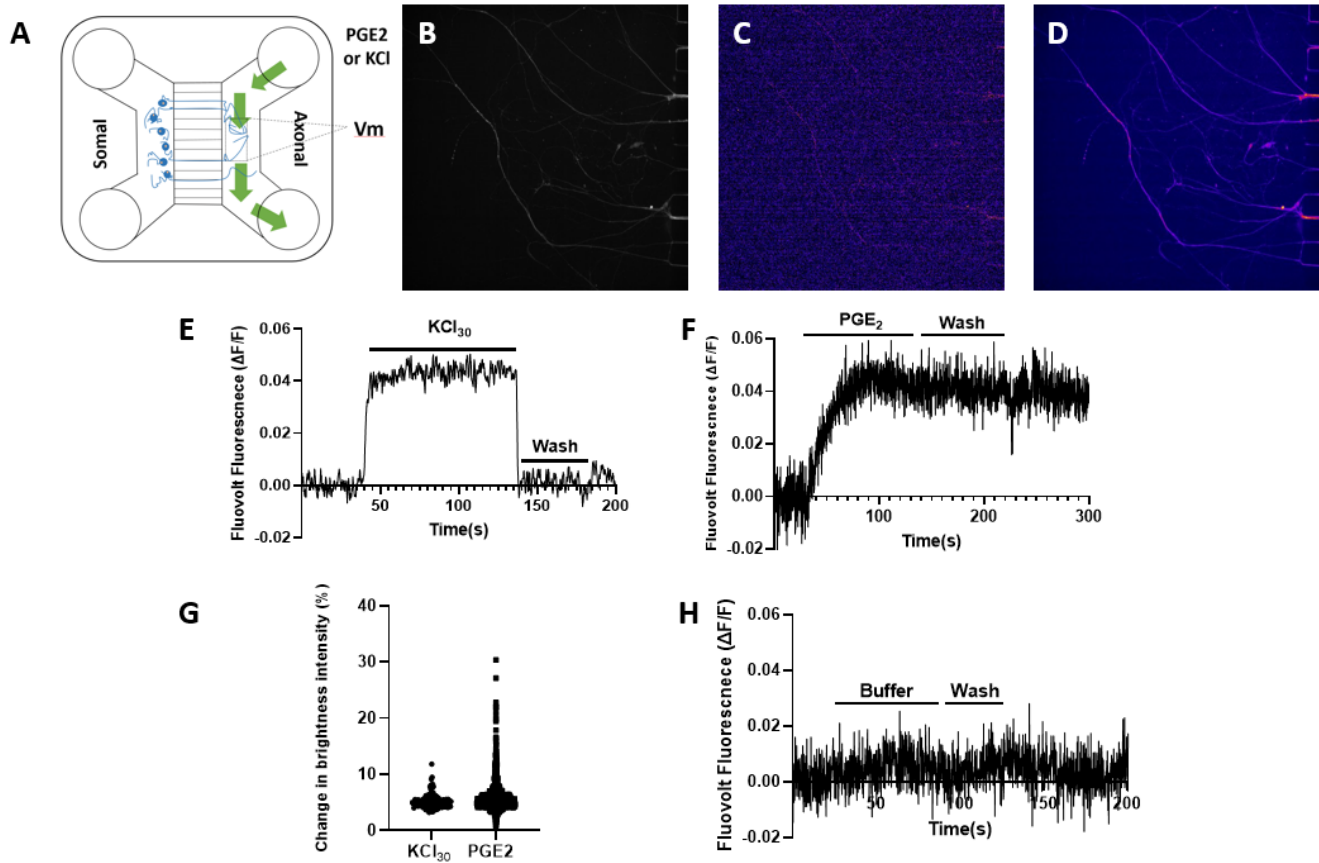


Figure 3.3 Application of PGE2 increases the membrane potential in the axons. **A)** Schematic of experimental set up for data shown in panels **B-G**. A two-compartment microfluidic configuration was used, where PGE2 or KCl were applied in the axonal side. FluoVolt fluorescence intensity was measured in the axonal compartment as a measure of membrane potential (V_m). **B)** Image showing a representative field of view (FoV) from the axonal compartment of a microfluidic culture stained with FluoVolt. **C)** Background subtracted image ($\Delta F/F$) of the FoV shown in **B**, prior to PGE2 or KCl application. No change in brightness intensity occurs prior to PGE2 application. **D)** Background subtracted image ($\Delta F/F$) of the FoV shown in **B**, after PGE2 application. Changes in FluoVolt brightness intensity upon PGE2 stimulation are highlight with arrows. **E and F)** Representative traces of FluoVolt brightness intensity from the axonal compartment showing increased intensity with 30mM of KCl (**E**) or 10 μ M PGE2 (**F**). The KCl trace returns to baseline after washout whereas the PGE2 trace remains elevated. **G)** Summary of the data describing peak KCl and PGE2 evoked changes in FluoVolt fluorescence. (Student's t-test, $p < 0.354$, $n_{ROI} = 925$ on 20 devices from 18 cultures (18 mice) for PGE2 and $n_{ROI} = 135$ on 4 devices from 3 cultures (3 mice) for KCl) **H)** Representative control trace of FluoVolt brightness intensity from the axonal compartment following stimulation with imaging buffer.

3.2.2 PGE2-induced depolarization is mediated by the EP4/cAMP/PKA signaling pathway

A previous immunocytochemical experiment showed that EP4, the high-affinity receptor of PGE2, is expressed in sensory axons (**Figure 3.2A**). To examine the potential functional role of EP4 receptors in mediating the PGE2-evoked activity, we performed voltage imaging experiments on axons following a 20-minute incubation with the EP4 inhibitor L-161,982 (10 μ M). The EP4 inhibitor L-161,982 has been previously used at 10 μ M to *in vitro* inhibit rat renal sensory nerve EP4 receptors (**Kopp et al,2004**). In our experiments, we decided to test the 10 nM concentration for the mouse sensory neurons. We assessed the effect of the EP4 inhibitor on membrane potential in two ways. We quantified the proportion of axonal area where the FluoVolt signal was increased by PGE2 stimulation (see Methods) either alone or in the presence of the EP4 inhibitor.

L-161,982 incubation abolished the PGE2-evoked axonal membrane depolarization (**Figure 3.4B**). As a comparison, the total axonal area activated by PGE2 in the absence of the EP4 inhibitor was 47.94% $\pm$ 4.39%, while in devices treated with the EP4 inhibitor, 5.92 $\pm$ 1.92% of the total axonal area was activated, a reduction of 88.7% of corresponding areas (**Figure 3.4C**). Further differences were found in the amplitude of FluoVolt fluorescence between the naïve and EP4-inhibited axons, where the fluorescence was significantly attenuated in the presence of the inhibitor (**Figure 3.4D**). Therefore, the data suggest that PGE2 depends mainly on the high-affinity EP4 receptor for the generation of the persistent activity observed in the DRG axons.

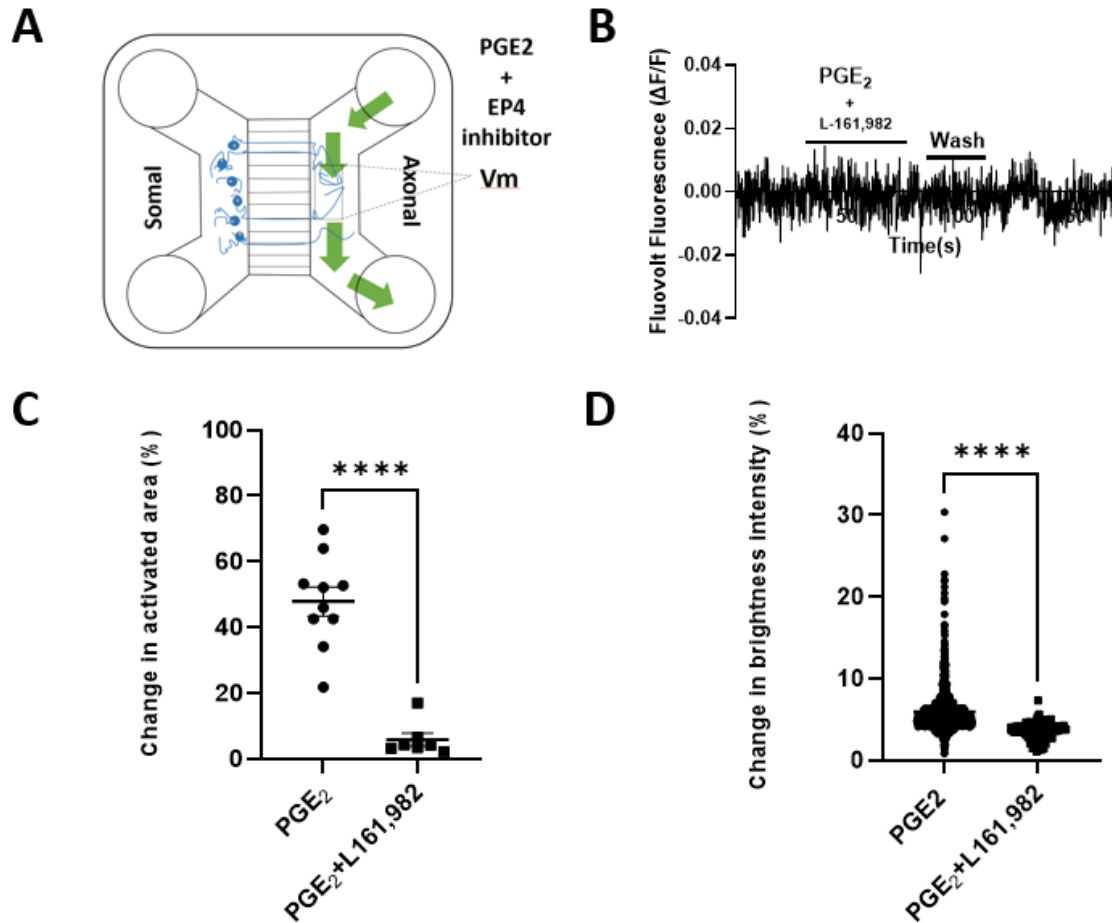


Figure 3.4 Inhibition of EP4 receptors abolishes PGE2-evoked axonal depolarization. **A)** Schematic of the experimental set up for data shown in B. Stimulation of the axonal side with PGE2 in the presence of a selective EP4-inhibitor, L-161,982 (10 μ M). **B)** Representative FluoVolt intensity trace from an axonal area following a one-minute stimulation with 10 μ M PGE2 in the presence of 10 nM L-161,982. PGE2 failed to evoke a change in membrane potential in the presence of the EP4 inhibitor **C)** Summary of the changes in total PGE2 activated axonal area in the presence of L-161,982. L-161,982 significantly reduced mean total PGE2 activated axonal area (**** $p < 0.0001$, Brown-Forsythe and Welch ANOVA tests, $n = 10$ devices from 10 cultures (10 mice) for PGE2 and $n = 7$ devices from 4 cultures (4 mice) for PGE2+L-161,982). **D)** Summary data describing the changes in the peak brightness intensity in the activated areas for PGE2-treated and PGE2+L-161,982 treated axons. (**** $p < 0.0001$, Student's t-test, $n_{\text{ROIs}} = 925$ on 20 devices from 18 cultures (18 mice) for PGE2 and $n_{\text{ROIs}} = 141$ on 7 devices from 4 cultures (4 mice) for PGE2+L-161,982).

3.2.3 PGE2-evoked persistent activity in sensory axons requires Nav1.8 but not TTX-S channels

To understand how PGE2 induces a persistent depolarization on sensory axons we sought to investigate further the involvement of ion channels to PGE2-evoked activity. Three lines of evidence compelled us to start with VGSCs. First, we showed the silencing of PGE2-evoked axonal activity by the application of lidocaine to the middle chamber, which was suggestive of a blockade to the propagation of generated actions potentials to the somal compartment (**Figure 3.1F**). Second, the application of TTX and the selective Nav1.8 blocker, pinpointed towards a TTX-resistant mechanism of PGE2-evoked activity (**Figures 3.2F&I**). Third, the evidence for the involvement of the EP4/cAMP/PKA cascade were indicative of a potential activation of ion channels downstream of the pathway, subject to PKA phosphorylation (**Figure 3.2B**).

The contribution of the abundantly expressed in nociceptors and TTX-sensitive Nav1.7 channel was first examined. Voltage imaging experiments were conducted where a selective Nav1.7 blocker, PF-05089771 (8nM), was applied to the axonal compartment in concomitance with PGE2 for 90 seconds. The selective Nav1.7 blocker PF-05089771 has been previously shown to attenuate C-fibre currents in both voltage-clamp and skin-nerve preparation experiments at the 10 nM concentration (**Middleton et al,2022**). We observed no significant change in the proportion of axonal membrane that exhibited a change in membrane potential, in response to PGE2 in the presence or absence of the Nav1.7 blocker (**Figure 3.5E**). There was, however, a slight decrease in the average axonal depolarization in the presence of the Nav1.7 blocker (**Figure 3.5D**), suggesting a slight yet significant contribution from Nav1.7 channels to the observed PGE2 evoked depolarization.

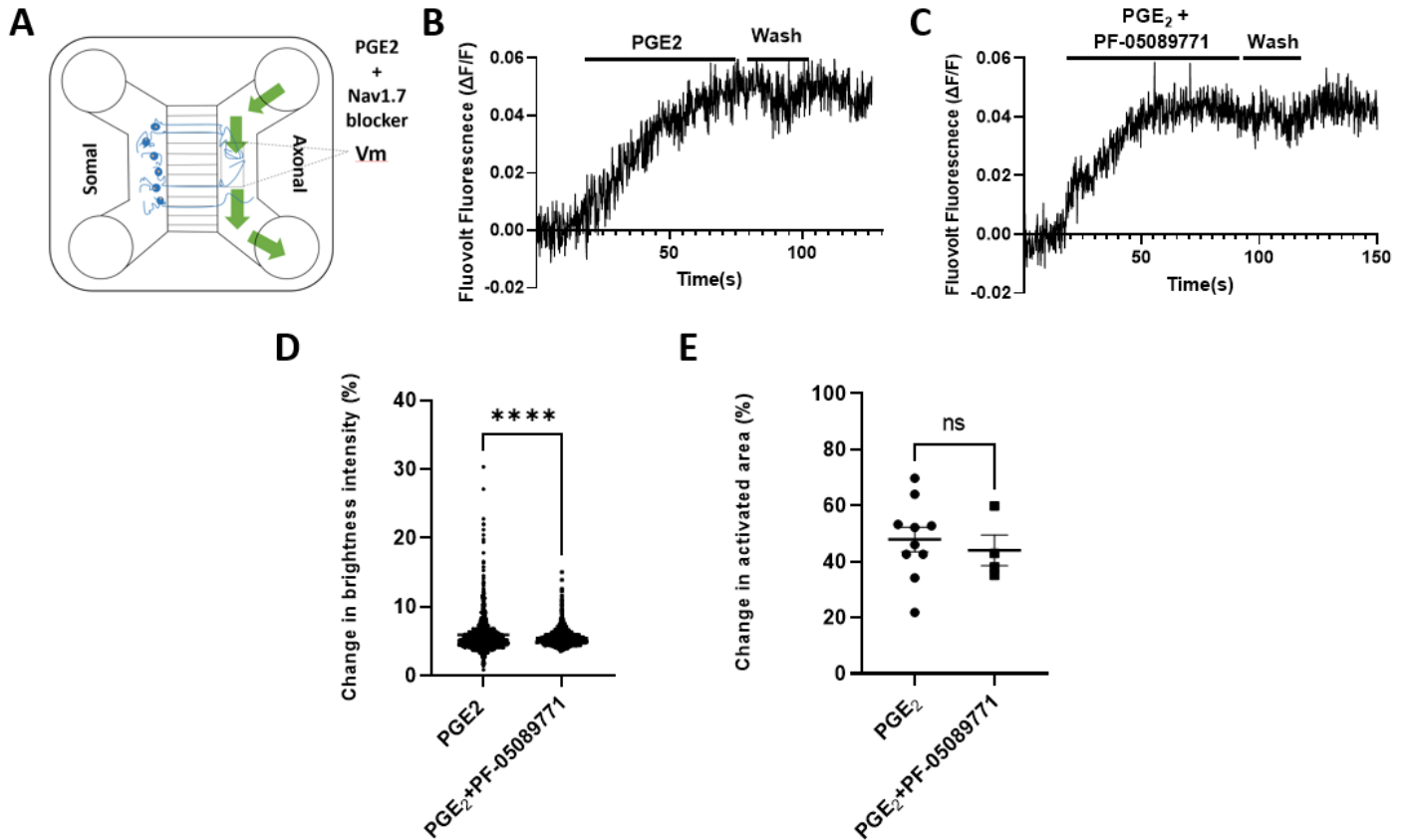


Figure 3.5 The PGE2-evoked persistent activity in the axons is not dependent on Nav1.7 channels.

A) Schematic of the experimental protocol for data shown in panels B and C. cultures). **B and C)** Representative traces of FluoVolt brightness intensity in the axons showing the increase following stimulation with 10 μ M PGE2 (D) and 10 μ M of PGE2 followed by application of 8nM the Nav1.7 blocker PF-05089771. **D)** Summary of the data in D and E. Application of PGE2+Nav1.7 blocker decreased the average peak brightness intensity to $5.47 \pm 0.03\%$ (**** $p < 0.0001$, Student's t-test, $n_{\text{ROIs}}=925$ on 20 devices from 18 cultures (18 mice) for PGE2, and $n_{\text{ROIs}}=1202$ on 6 devices from 4 cultures (4 mice) for PGE2+ PF-05089771). **E)** PGE2 application elicits activation of $47.94 \pm 4.39\%$ of the total axonal area, and addition of PF-05089771 with PGE2 did not significantly change the total activated area ($44.03 \pm 5.53\%$, $p < 0.5975$ Student's t-test, $n=10$ devices from 10 cultures (10 mice) for PGE2, and $n=6$ devices from 4 cultures (4 mice) for PGE2+ PF-05089771).

After examining the contribution of Nav1.7 channels as a candidate to the PGE₂-evoked desensitization, we shifted our focus to the TTX-resistant Nav1.8 channel. The effects of two Nav1.8-selective channel blockers were examined, PF-01247324 at (2 μ M) (Payne et al,2015) and A-803467 (10nM) (Jarvis et al,2007) (Figures 3.6C&D). While PGE₂ evoked a $5.89 \pm 0.09\%$ increase in the average FluoVolt fluorescence intensity in the axons, co-application of either PF-01247324 or A-803467 led to a statistically significant reduction in the observed fluorescence to $4.40 \pm 0.035\%$ and $3.46 \pm 0.31\%$, respectively (Figure 3.6E). Interestingly, in the presence of the Nav1.8 blockers there was a stark reduction in the axonal area activated by PGE₂ stimulation. We observed an almost complete abolishment (85% reduction) of PGE₂-evoked axonal depolarization by the Nav1.8 blockers (Figure 3.6F). Overall, these data suggest an important contribution of Nav1.8 channels to PGE₂-evoked depolarization of sensory axons.

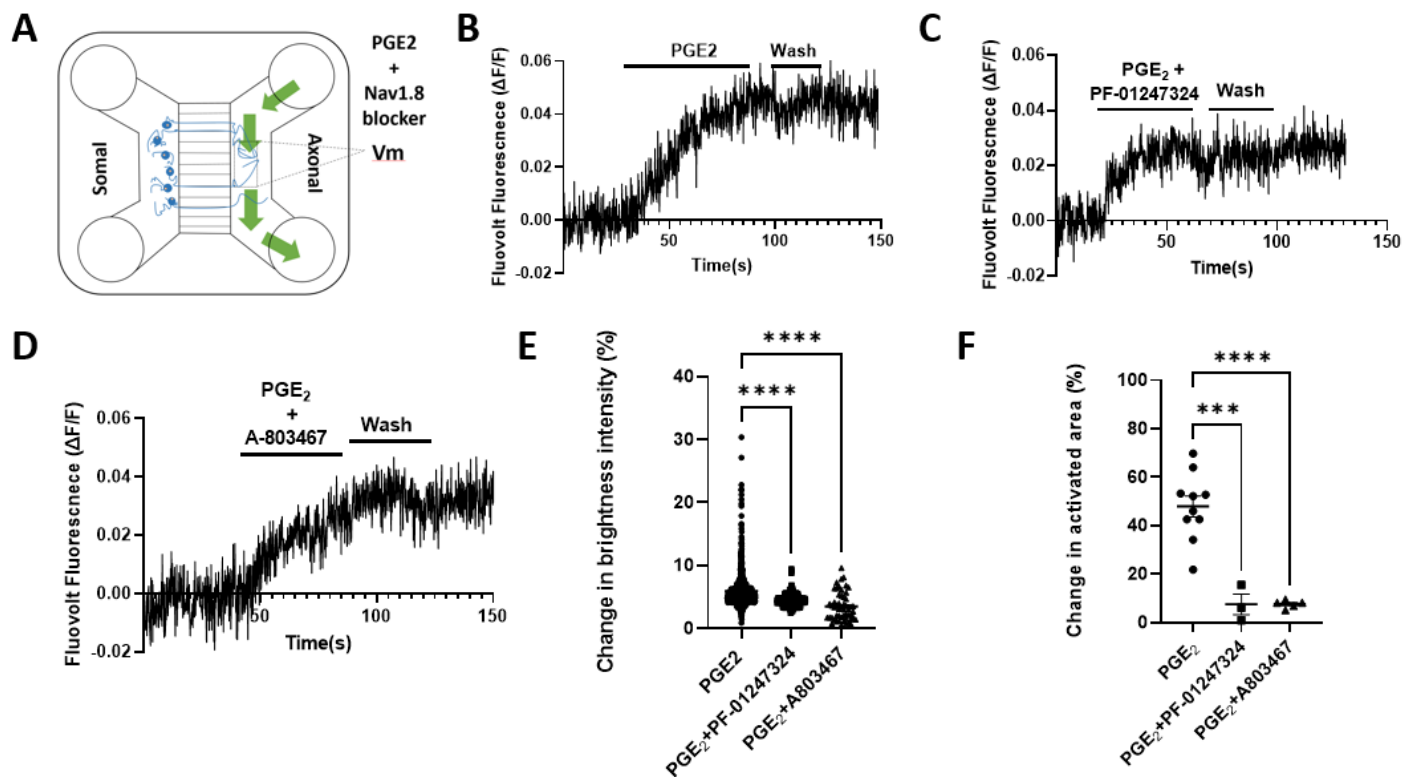


Figure 3.6 PGE₂-evoked persistent axonal firing is predominantly mediated by Nav1.8 channels A) Schematic of the experimental set up for data shown in B-D Application of 2 μ M PF-01247324 largely attenuated the response. B-D) Representative traces of FluoVolt intensity showing changes following treatment of the axonal compartment with PGE₂, PGE₂ with 2 μ M PF-01247324, and PGE₂ in the presence of 10 nM A-803467. Nav1.8 blockers attenuate the amplitude of the membrane depolarization evoked by

PGE2. **E)** Summary of the changes in the brightness intensity evoked by PGE2. Application of PGE2 in the presence of the Nav1.8 blockers significantly reduces the average amplitudes of responses to nearly half ($4.40 \pm 0.035\%$ for PF-01257324 and $3.46 \pm 0.31\%$ SEM for A-803467, **** $p < 0.0001$, Brown-Forsythe and Welch ANOVA tests, $n_{\text{ROIs}} = 518$ on 3 devices from 3 cultures (3 mice) for PGE2+PF-01247324, and $n_{\text{ROIs}} = 53$ on 5 devices from 5 cultures (5 mice) for PGE2+A-803467). **F)** Summary of the changes in total PGE2-evoked activated axonal area in the presence of Nav1.8 blockers. PGE2 application depolarizes $47.94 \pm 4.39\%$ of the total axonal area while Nav1.8 blockers significantly reduce the activated area to $7.53 \pm 4.25\%$ for PF-01257324 and $7.60 \pm 0.73\%$ for A-803467, *** $p < 0.0006$ and **** $p < 0.0001$ respectively, Brown-Forsythe and Welch ANOVA tests, $n = 3$ devices from 3 cultures (3 mice) for PGE2+PF-01247324 and $n = 5$ devices from 5 cultures (5 mice) for PGE2+A-803467 blocker).

3.2.4 PGE2-evoked axonal depolarization is dependent on the chloride gradient and is mediated by ANO1 channels

Given the established role of Nav1.8 channels in the rising phase of APs in TTX-resistant (TTX-R) DRG neurons (**Renganathan et al., 2001**) (**Bennett et al., 2019**) we hypothesized that a localized activation of another channel or channel complex leads the depolarization of axonal membrane and potentially the subsequent activation of Nav1.8 channels. We therefore further investigated other candidate ion channels that might initiate the PGE2-evoked depolarisation of the axons. We examined ANO1, a calcium activated chloride channel expressed in Nav1.8 expressing DRG neurons and known to be involved in inflammatory pain (**Cho et al., 2012**). We found that application of the selective ANO1 blocker T16inh-A01 at concentrations of $20\mu\text{M}$ and $100\mu\text{M}$ significantly inhibited the PGE2-evoked axonal depolarisation by $4.7 \pm 0.12\%$ and $53 \pm 0.73\%$ respectively (**Figures 3.7C-E**). The ANO1 blockade did not reduce however, the total area of PGE2-activated axons (**Figure 3.7F**). The result suggests that ANO1 is activated in response to PGE2 application and initiates the depolarization of the axonal membrane, yet it does not possess the amplificatory role that Nav1.8 exerts on PGE2-evoked responses. These data indicate that the localized depolarization of DRG axons evoked by PGE2 is dependent on the axonal Cl^- concentration gradient and is due to the opening of ANO1 channels.

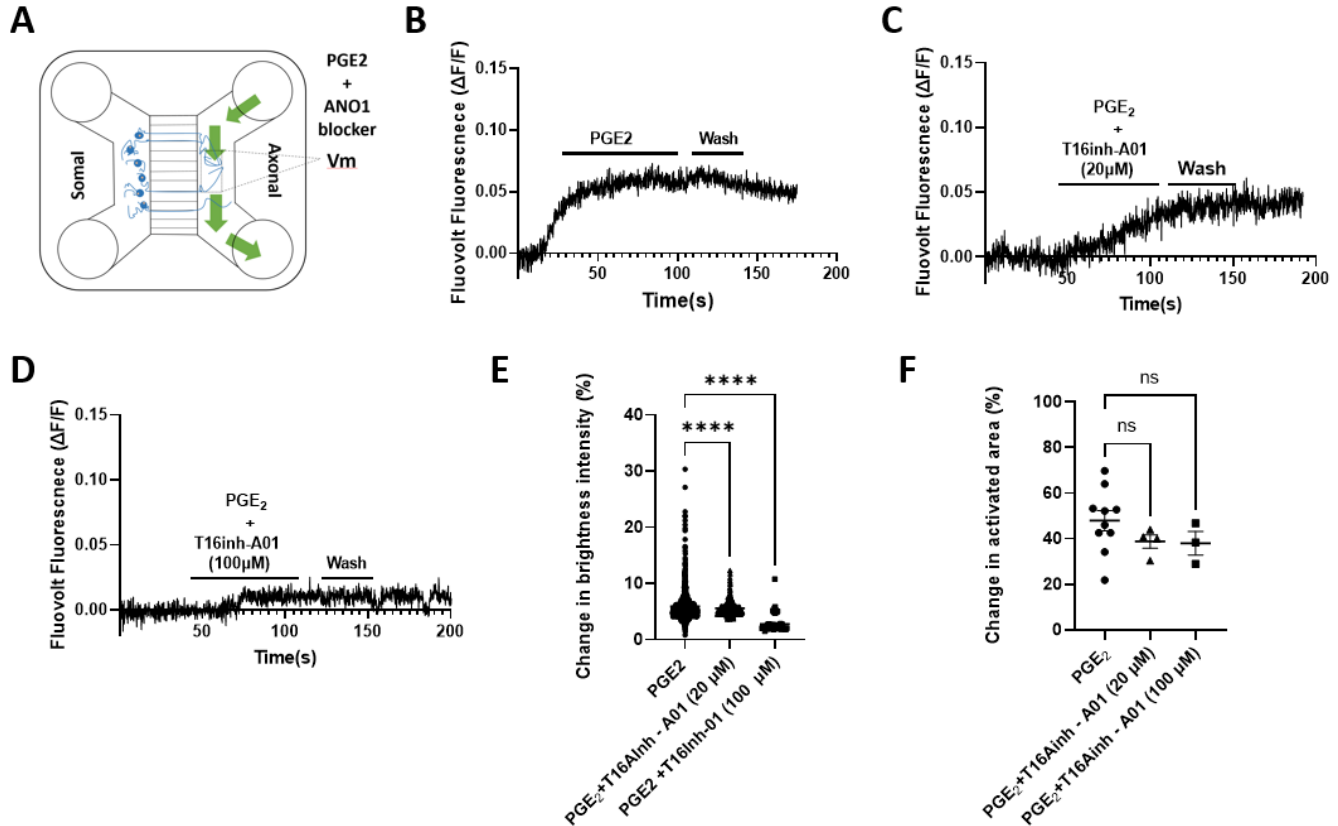


Figure 3.7 PGE2-induced axonal depolarization amplitude is attenuated by ANO1 blockade **A)** Schematic of the experimental set up for data shown in B-D. **B)** Representative trace showing repetitive persistent activity recorded from the soma in response to axonal stimulation with PGE2. **C&D)** Representative trace indicating changes in fluorescence expressed as Fluovolt Fluorescence ($\Delta F/F$), of an axonal ROI in response to application of PGE2 in the presence of a selective ANO1 blocker (T16inh-A01) at 20 μM and 100 μM respectively **E)** Summary of the changes in the brightness intensity evoked by PGE2. Application of PGE2 in the presence of the calcium-activated chloride channel ANO1 blocker significantly attenuates the average amplitudes of responses (from $5.92 \pm 0.098\%$ for PGE2 (10 μM) to $5.64 \pm 0.11\%$ for PGE2+T16inh-A01 (20 μM) and $2.78 \pm 0.17\%$ SEM for PGE2+T16inh-A01 (100 μM), **** $p < 0.0001$ and **** $p < 0.0001$ respectively, Brown-Forsythe and Welch ANOVA tests, $n_{ROIs} = 943$ on 23 devices from 21 cultures (21 mice) for PGE2, $n_{ROIs} = 317$ on 4 devices from 4 cultures (4 mice) for PGE2+ T16inh-A01 (20 μM) and $n_{ROIs} = 68$ on 3 devices from 3 cultures for PGE2+ T16inh-A01 (100 μM). **F)** Summary of the changes in total PGE2-evoked activated axonal area in the presence of ANO1 blockade. The ANO1 blockade at both 20 μM and 100 μM did not produce significant reduction of total axonal area activated. ns $p < 0.05$, Brown-Forsythe and Welch ANOVA tests, 13 devices from 13 cultures (13 mice) for PGE2 (10 μM), 4 devices from 4 cultures (4 mice) for PGE2+ T16inh-A01 (20 μM) and 3 devices from 3 cultures (3 mice) for PGE2+ T16inh-A01 (100 μM).

To further confirm that PGE2-evoked axonal depolarization is dependent on the Cl^- gradient, we used bumetanide (10 μM) (**Pieraut et al,2007**), a selective inhibitor of the chloride transporter NKCC1. NKCC1 has been shown to be highly expressed in mature DRG neurons and functions to increase the intracellular Cl^- concentration which could make chloride channel activation excitatory in these neurons (**Rivera et al., 1999**) (**Sung et al., 2000**). Bumetanide, an inhibitor of NKCC1, significantly reduced the amplitude of PGE2-evoked responses, in a similar manner to T16inh-A01 (**Figure 3.8C**). We found that bumetanide, in a similar fashion to T16inh-A01, significantly attenuated the amplitude of PGE2-evoked responses (**Figure 3.8E**) and did not alter the total activated axonal area (**Figure 3.8F**). We also employed VU0240551 (2 μM) (**Deisz et al,2014**), a chloride transporter inhibitor of KCC2 which functions to decrease Cl^- concentration in the neurons leading to hyperpolarizing responses in the neurons (**Price et al., 2009**). KCC2 and NKCC1 maintain the Cl^- -homeostasis in the central neurons, however, the expression of KCC2 in mature DRG is very low (**Lu et al., 1999**) (**Price et al, 2006**). The KCC2 inhibitor did not potentiate the PGE2-evoked axonal amplitude or the area of axonal depolarization (**Figures 3.8E-F**), concordant with its minimal expression in mature DRG neurons.

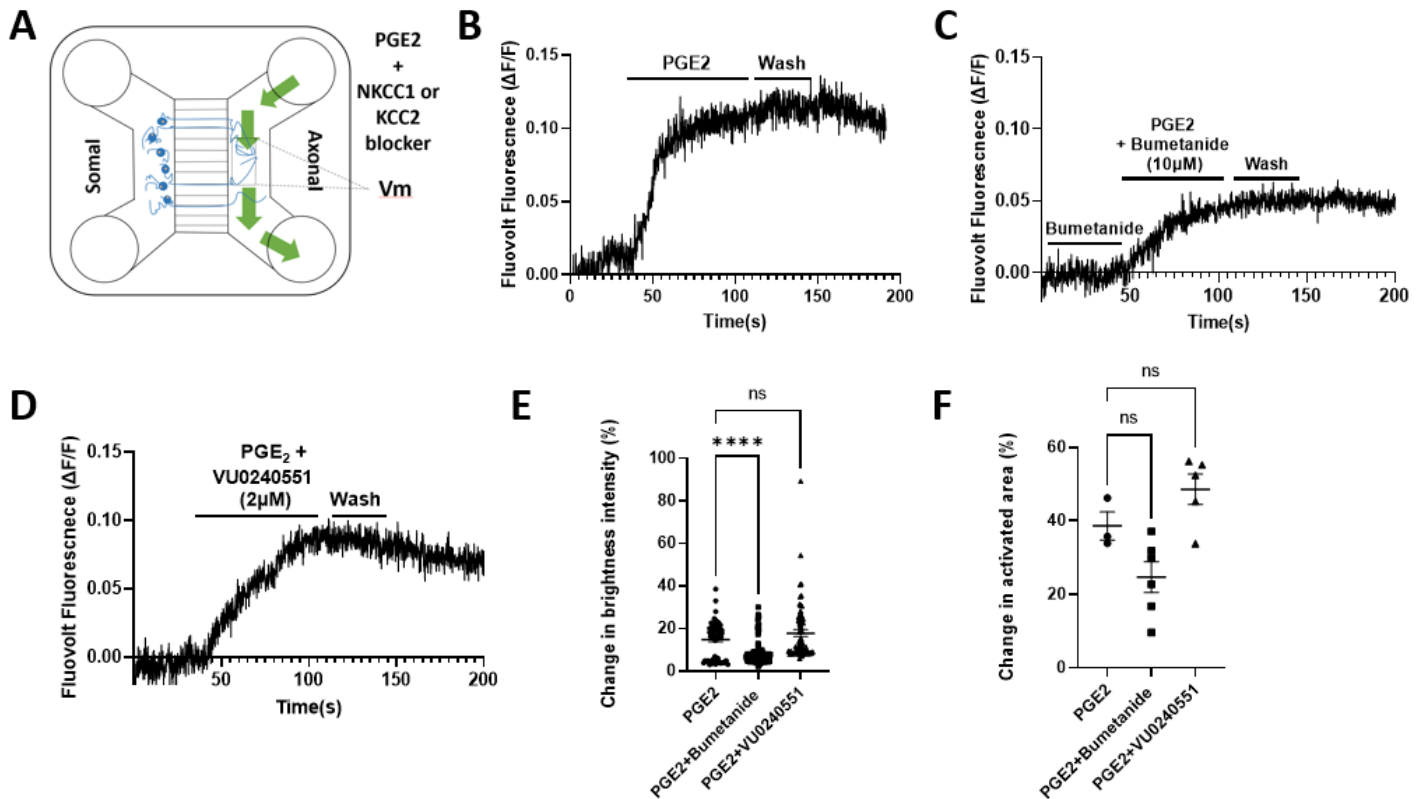


Figure 3.8 PGE2-induced axonal depolarization is dependent on the chloride gradient and its amplitude is attenuated by ANO1 blockade **A)** Schematic of the experimental set up for data shown in B-D. **B)** Representative trace showing repetitive persistent activity recorded from the soma in response to axonal stimulation with PGE2. **C)** Representative trace indicating changes in fluorescence expressed as Fluovolt Fluorescence ($\Delta F/F$), of an axonal ROI in response to application of PGE2 (10 μM) in concomitance with the NKCC1 inhibitor bumetanide (10 μM). **D)** Representative trace indicating changes in fluorescence expressed as Fluovolt Fluorescence ($\Delta F/F$), of an axonal ROI in response to application of PGE2 (10 μM) in concomitance with the NKCC1 inhibitor VU0240551 (2 μM). **E)** Summary of the changes in the brightness intensity evoked by PGE2. Application of PGE2 in the presence of the NKCC1 blocker ameliorates the average amplitudes of responses (from $14.81 \pm 1.069\%$ for PGE2 (10 μM) to $7.90 \pm 0.40\%$ for PGE2+Bumetanide (10 μM). In the presence of KCC2 inhibitor VU0240551 (2 μM), we observed no significant change ($17.83 \pm 1.63\%$ **** $p < 0.0001$ and $p > 0.223$ respectively, Brown-Forsythe and Welch ANOVA tests, $n_{\text{ROIs}} = 62$ on 3 devices from 4 cultures (4 mice) for PGE2, $n_{\text{ROIs}} = 151$ on 5 devices from 4 cultures (4 mice) for PGE2+ Bumetanide (10 μM) and $n_{\text{ROIs}} = 68$ on 4 devices from 4 cultures (4 mice) for PGE2+ VU0240551 (2 μM). **F)** Summary of the changes in total PGE2-evoked activated axonal area in the presence of chloride transporter inhibition. Both the NKCC1 and KCC2 inhibitors failed

to appreciably alter the activation of axonal areas in comparison to PGE₂. ns p<0.05 for both, Brown-Forsythe and Welch ANOVA tests (4 devices from 3 cultures (3 mice) for PGE₂, 5 devices from 4 cultures (4 mice) for PGE₂+ Bumetanide (10 μ M) and 4 devices from 4 cultures (4 mice) for PGE₂+ VU0240551 (2 μ M).

3.2.5 PGE₂-evoked persistent activity in sensory axons is not dependent on HCN channels

Last, after showing through the calcium imaging experiments an involvement of HCN channels in PGE₂-mediated sensitization of sensory axons (**Figure 1C**), we considered that it may also affect the PGE₂-evoked axonal depolarization. We applied zatebradine, a non-selective HCN blocker, in concomitance with PGE₂ (10 μ M) at 10 μ M and 50 μ M. We neither observed a reduction of the amplitude of PGE₂-evoked depolarization of axons (**Figure 3.9B-D**), nor a change in the total activated axonal area (**Figure 3.9E**). We therefore concluded that HCN channel activation is not a requirement for the PGE₂-evoked depolarization of axons.

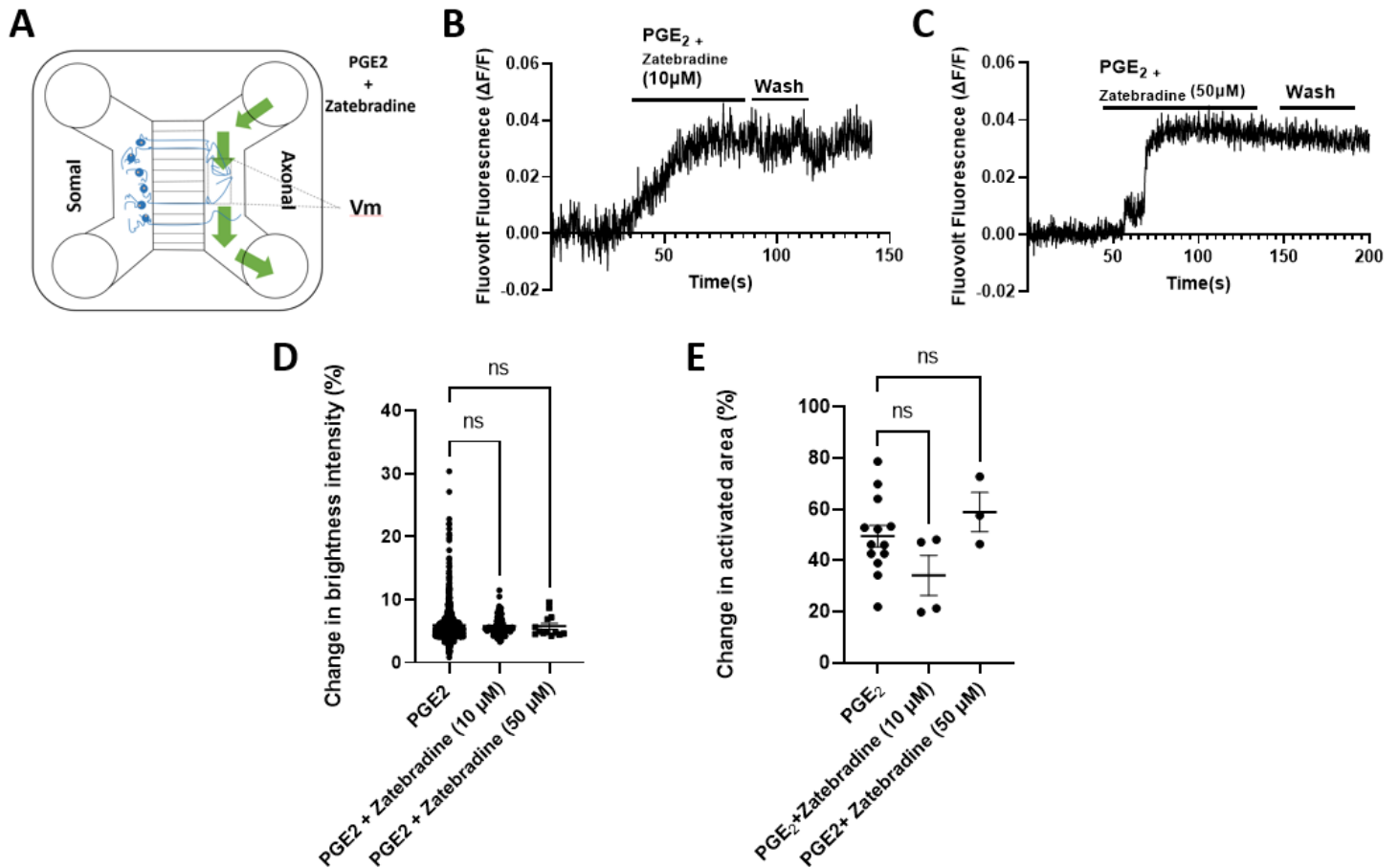


Figure 3.9 HCN channels do not mediate PGE2-evoked axonal depolarization. **A)** Schematic of the experimental set up for data shown in B-C. **B-C)** Representative traces indicating changes in fluorescence expressed as Fluovolt Fluorescence ($\Delta F/F$), of an axonal ROI in response to application of PGE2 in the presence of a non-selective HCN channel blocker (Zatebradine) at 10 μ M and 50 μ M respectively. **D)** Zatebradine does not alter the amplitude or the kinetics of PGE2 elicited responses. Application of PGE2 in the presence of the HCN channel blocker at 10 μ M and 50 μ M fails to attenuate the average amplitudes of responses (ns $p < 0.05$ for both, Brown-Forsythe and Welch ANOVA tests, $n_{\text{ROIs}} = 116$ on 4 devices from 3 cultures (3 mice) for PGE2+ Zatebradine (10 μ M) and $n_{\text{ROIs}} = 13$ on 3 devices from 3 cultures (3 mice) for PGE2+ Zatebradine (50 μ M)). **E)** Scatter plot of average changes in Fluovolt Fluorescence between PGE2 and Zatebradine at 10 μ M and 50 μ M respectively. PGE2 in the presence of HCN blockade does not produce any significant effect in total axonal area generated by PGE2. ns $p < 0.05$ for both (Brown-Forsythe and Welch ANOVA tests on 4 devices from 3 cultures (3 mice) for PGE2+ Zatebradine (10 μ M) and 3 devices from 3 cultures (3 mice) for PGE2+ Zatebradine (50 μ M)).

3.3 Discussion

The premise of utilizing microfluidic cultures for studying the cell and molecular physiology of sensory neurons derives from the need to understand the physiological properties not only of cell bodies but also of axons. Most existing *in vitro* studies are conducted using conventional cell cultures and the conclusions are derived from events that have occurred at the cell bodies of the DRG neurons. However, it is the nerve endings of nociceptors at the site of inflammation that are the targets of inflammatory mediators. Hence, the fluidic isolation of axons in the microfluidic cultures allows a more suitable model of recapitulating the stimulation of sensory neuron responses to the secreted mediators upon tissue damage. We subsequently exploited the technology to investigate the modulation of nociceptive axons by the inflammatory mediator PGE2.

Initially, we used the microfluidic platform to model in general the peripheral sensitization induced by inflammatory mediators in response to depolarizing stimuli such as KCl. It was demonstrated that acute treatment of fluidically isolated axons with an inflammatory mediator cocktail led to a robust enhancement of several local responses to KCl-induced depolarization. The observation that the application of the inflammatory cocktail produced an increase in calcium transients in the absence of any stimulation, compelled us to find out by process of elimination which mediator or combination thereof was responsible for this effect. When repeating the experiment with PGE2 alone, we observed an analogous enhancement to KCl. While it has been shown that PGE2 can mediate the sensitization of nociceptor cell bodies' *in vitro* (**Pitchford and Levine,1991**), it is the first time that the sensitization is demonstrated in axons *in vitro*. The physiological relevance of this finding supports the argument that microfluidic cultures can reliably recapitulate aspects of the *in vivo* conditions.

In the peripheral sensitization experiments with PGE2, we made the compelling observation that a set of sensory axons may be directly activated by application of PGE2 alone. The PGE2-evoked calcium signals generated at the axons were recorded from the somal compartment. We further demonstrated that axonal activation of the PGE2/EP4/cAMP pathway in adult mice DRG neurons induces a direct and persistent depolarization that elicits AP which are in turn propagated towards the soma.

Whether PGE2 can induce the excitation of nociceptive terminals has remained an issue of contention. In rat skin-saphenous nerve *in vitro* preparation, PGE2 failed to potentiate responses

to bradykinin (**Lang et al,1990**). In primary visceral afferents of rat jejunum, application of PGE2 failed to alter the basal firing, but potentiated the responses of the neurons to bradykinin (**Brunsdén and Grundy,1999**). In a canine preparation of testis-spermatic nerve preparations, PGE2 did not induce significant responses to discharge rates. However, extracellular recordings in the cat's knee joint did detect the induction of spike discharges elicited by PGE2 (**Schaible and Schmidt,1988**). In rat DRG neurons, application of PGE2 elicited an increase in intracellular $[Ca^{2+}]$ levels in DRG cell bodies (**Linhart et al,2003**), while in another study, the exogenous application of the same PGE2 concentration, sensitized the neurons but did not evoke an elevation in intracellular calcium (**Nicolson et al,2007**).

There are several reasons for potential inconsistencies in these observations. Initially, species differences may account for the observed discrepancies. Second, technical limitations in *in vivo* electrophysiological recordings such as conduction failure may confound the results. Another plausible explanation is the distribution of EP receptors and the subsequent activation of the downstream second messenger cascade across different types of nociceptors. For instance, visceral afferents may be less responsive to PGE2 than joint afferents or skin-innervating afferents, where acute tissue injury is more common. The topography and distribution of the PGE2 downstream signaling apparatus is unknown in these previous studies, while it is impossible to be determined by primary afferent cultures including DRG cell bodies from all cervical, thoracic, lumbar and sacral segments enclosed within the spinal column. Further, these studies on the excitatory action of PGE2 on sensory neurons in culture have primarily focused on the cell bodies rather than the axons.

In our study, using microfluidic cultures, we demonstrated a localized and sustained activation of sensory axons to PGE2. The voltage imaging experiments demonstrated a sustained depolarization of the sensory axons by PGE2 which persisted following wash, implying the activation of a second messenger downstream cascade. We hence showed that the PGE2-evoked axonal responses are downstream of the EP4/cAMP/PKA activation cascade in DRG axons. The spatiotemporal dynamics of cAMP has been studied extensively in peripheral neurons. It has been observed that the highest cAMP concentrations are generated in the axons while the lowest ones are generated in the cell body during signaling (**Nikolaev et al,2004**) (**Gervasi et al,2007**) (**Shelly et al,2010**). Hence, the discrepancies in cAMP concentration between axons and cell bodies may explain why

previous recordings of DRG cell bodies failed to detect response evoked by PGE2. A confounding variable of our observation would be that the NGF treatment at 200ng/ml used to promote neurite sprouting and crossing into the axonal compartment, but it may alter the intracellular signaling that mediates the PGE2-evoked effects, “priming” the cAMP levels (**Vasko et al,2014**). There is, however, important evidence that in inflammation and pathological states such as rheumatoid arthritis and fibromyalgia, there are elevated concentrations of NGF (**Seidel et al,2010**). The addition of NGF in our model may therefore reflect such changes which are relevant to inflammatory pain conditions, where NGF sequestration has been shown to effectively ameliorate pain (**McMahon et al,1995**). However, the concentrations of NGF that have been obtained in tissues fluctuate within the pg/ml range rather than the ng/ml (**Seidel et al,2010**), an observation which can be optimized in subsequent studies. We therefore have demonstrated a localized and sustained activation of sensory axons in response to PGE2, which is dependent on the EP4/cAMP cascade.

It has been shown in previous studies that prostaglandins can target many ion channels in the cell membrane and can potentiate their responses (**Moriyama et al,2005**). For instance, there is evidence for PGE2-mediated sensitization of TRPV1 channels through the PKA activity (**Schnitzler et al,2008**), while cyclopentenone prostaglandin metabolites have been shown to mediate TRPA1 activity in DRG cell bodies (**Materazzi et al,2008**). Since any noxious modality between heat, mechanical pressure or chemical irritation can depolarize nociceptor membranes of the nerve endings and induce a subsequent AP generation, the sensitization presented here may represent an inflammatory modulation independent of enhancement induced by activated local transducer channels such as TRPV1.

The voltage imaging experiments conducted showed that the PGE2-evoked axonal depolarization is dependent on its high-affinity receptor, EP4. Upon EP4 blockade the depolarization is silenced, corroborating that the EP4/cAMP/PKA cascade leads to a depolarization of DRG axons. The observation provides an explanation for the anti-hyperalgesic properties of EP4 antagonism in nociceptors (**Lin et al,2006**) (**Nakao et al,2007**) (**Zhu et al,2020**). We examined the role of VGSCs as downstream mediators of the PGE2-evoked activity, due to the prominent role of these channels in sensory neuron excitability and pain sensation. It has been shown that PGE2 modulates TTX-resistant but not TTX-sensitive currents via the cAMP/PKA cascade in DRG cell bodies

(England et al,1996) (Gold et al,1998) (Baker et al,2005). No contributions of TTX-sensitive channels to the PGE2-evoked axonal activation had been detected. Selective blockade of the Nav1.7 channel only produced a slight effect to the ensuing depolarization, in agreement with *in vivo* lack of evidence of PGE2-induced peripheral sensitization attenuation in Nav1.7 knockout mice (Emery et al,2016) (MacDonald et al,2021). To the contrary, selective blockade of the Nav1.8 channel almost abolished the PGE2-evoked axonal depolarization. The direct administration of PGE2 has potentiating effects in Nav1.8 current amplitudes and induces a hyperpolarizing shift in the current-voltage relationship (Gold et al,1998). The immunostaining experiments further bolstered our line of evidence (Figure 3.2A), as a co-localization of EP4 receptor with the Nav1.8 channels was demonstrated, underscoring the role of Nav1.8 in PGE2-evoked depolarization of these axons.

Despite the predominant involvement of Nav1.8 in mediating the PGE2-evoked axonal depolarization, it is not immediately clear how Nav1.8 channels could directly elicit the generation of APs, given that they contribute towards the rising phase of the AP (Bennett et al,2019). The changes in the magnitude of the depolarization of the evoked currents would not justify the depolarization observed in the axons. We therefore postulated that other ion channels are involved, that may mediate the threshold depolarization induced by PGE2, which would then be amplified by Nav1.8 activation. It has been suggested for instance that potentiation of the heat sensor TRPV1 by PGE2 might result in spontaneous, ongoing heat pain (Moriyama et al,2005) (Petho and Reeh,2012).

To delineate the clew of the molecular mechanisms underlying PGE2-evoked depolarization, we focused on other ion channels expressed in the DRG. Potential ionic mechanisms downstream of cAMP/PKA would include the opening of calcium activated chloride channels (Vaughn and Gold,2010) or the closing of potassium channels (Young et al,2014). The blockade of HCN channels did not produce a significant attenuation of PGE2-evoked responses recorded in the axons. We hypothesize that the channel plays an important role in mediating the firing frequency but not initiating the depolarization axonal responses evoked by PGE2. On the other hand, the ANO1 channels, expressed primarily in small diameter sensory neurons, is activated by thermal stimulation and calcium to induce the depolarization of DRG neurons (Cho et al,2012). The selective blockade of ANO1 largely inhibited the PGE2-evoked depolarization of the sensory

axons. This novel observation indicates a link between ANO1 activation and the PGE2/EP4 pathway.

The molecular mechanism underlying the PGE2-evoked axonal depolarization is largely addressed. The evidence for the involvement of the EP4/cAMP/PKA cascade indicated towards the contribution of ion channels and specifically Nav1.8 and ANO1 channels. It is not known, however, the exact molecular cascade sequence each channel requires for its activation. An interesting remainder question is whether ANO1 acts synergistically or independently with Nav1.8 to elicit the PGE2-evoked responses. A plausible contingency is that the two channels are activated independently. It has been previously shown that Nav1.8 channels are potentiated and activated by cAMP-dependent phosphorylation (**Fitzgerald et al,1999**) while the ANO1 channel is similarly activated by a cAMP dependent $[Ca^{2+}]$ increase evoked by PGE2 to initiate the depolarization (**Figure 3.10A**). A second, more intricate contingency is that ANO1 activation occurs via calcium permeable TRPV1 channel activation (**Figure 3.10B**). It has been shown that TRPV1 is sensitized and phosphorylated by PKA (**Schnizler et al,2008**). It is thus plausible that downstream of PKA, TRPV1 might become activated. Moreover, co-expression of ANO1 and TRPV1 has been recently shown in sensory neurons and an interaction between the two channels should not be excluded (**Takayama et al,2015**). The intracellular calcium levels could activate the calcium permeable TRPV1 channel could in turn activate directly or indirectly the ANO1 channels. In a more recent study (**Shah et al,2020**), it was shown that an IP_3 receptor isoform indispensable for the activation of ANO1 by TRPV1. Last, it is unclear whether ANO1 activation and the generated APs lead directly to the Nav1.8 channel activation, or whether Nav1.8 channels are activated by a distinct mechanism (**Figure 3.10C**). Nevertheless, the evidence provided for the depolarization of axons by PGE2 via the ANO1 and Nav1.8 channels are advancing our understanding of the mechanisms of inflammatory spontaneous pain. They also provide translational targets for analgesic discovery in acute and potentially chronic inflammatory pain conditions, extending beyond the classic paradigm of cyclooxygenase inhibition (**Zeilhofer and Brune,2006**).

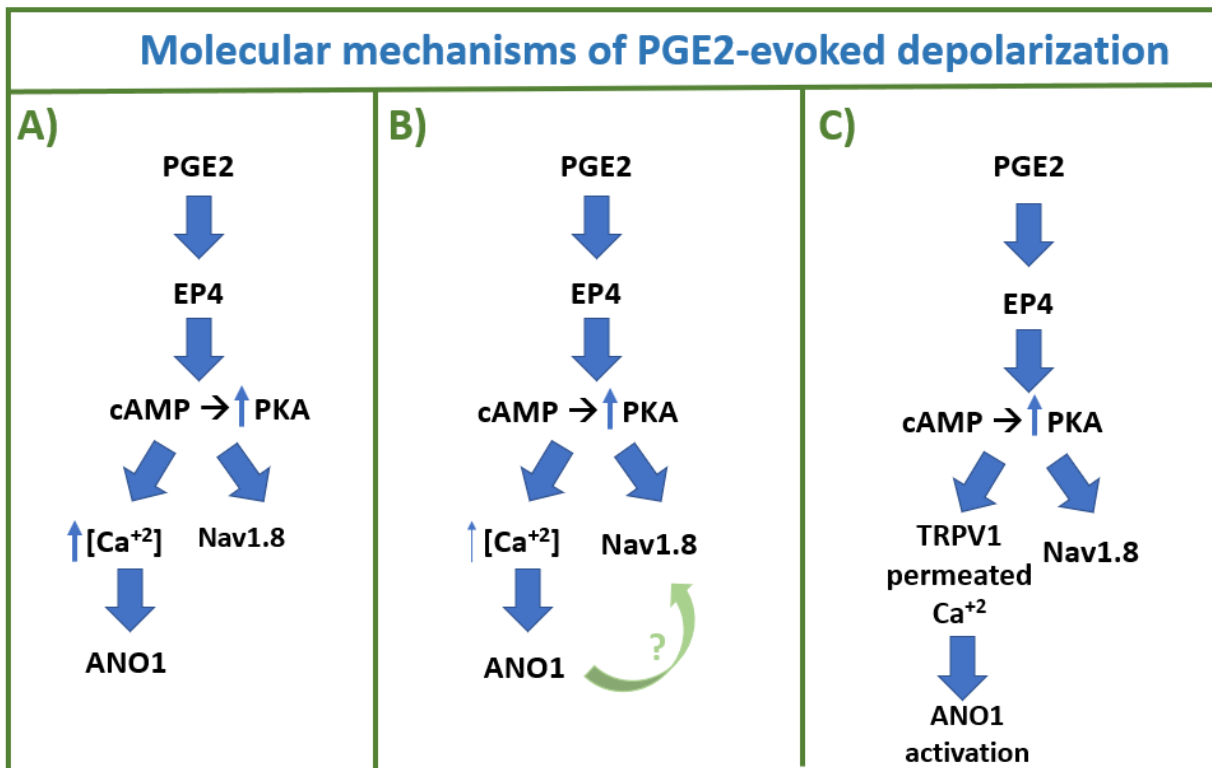


Figure 3.10 Schematic of the proposed molecular mechanisms of PGE2-evoked depolarization. **A)** The PGE2/EP4/cAMP cascade may directly activate ANO1 due to a cAMP dependent $[Ca^{2+}]$ increase and the Nav1.8 channels by PKA-mediated phosphorylation. **B)** In contrast to panel A, activation of Nav1.8 channels may be dependent on ANO1 activation instead of displaying distinct activation mechanisms. **C)** The activation of ANO1 channels may not be directly dependent on cAMP-mediated $[Ca^{2+}]$ elevation and instead it might be TRPV1-dependent. TRPV1 activation might be due to the presence of an endogenous agonist or via another molecular interaction.

The intracellular concentration of Cl^- is a major determinant of the membrane depolarization or hyperpolarization properties of Cl^- channels (Kaila et al,2014). Mature sensory neurons possess a high intracellular Cl^- concentration which is maintained by the high levels of expression of the sodium potassium chloride co-transporter NKCC1 (Price et al,2009). We used bumetanide, an inhibitor of NKCC1, and confirmed the dependence of the PGE2-evoked depolarization on the intracellular Cl^- concentration. Bumetanide greatly reduced the amplitude of the responder axons to the PGE2 evoked depolarization. It has been previously shown that bumetanide can relieve

thermal and mechanical hyperalgesia in a model of peripheral nerve injury (**Modol et al,2014**). The chloride potassium symporter 5 or KCC2 has opposing effect to NKCC1 as it extrudes intracellular Cl^- , thereby reducing the chloride concentration. It would be then expected that KCC2 inhibition would increase the intracellular Cl^- concentration and potentiate the excitatory effects of opening Cl^- channels as the ANO1. We found that inhibition of KCC2 did not alter the depolarizing properties of sensory axons by PGE2. Unlike its expression in central neurons, the expression of KCC2 in mature sensory neurons has not been detected (**Lu et al,1999**) (**Rivera et al,1999**). Hence, the most prevalent reason for not observing any potentiated responses by PGE2 in the presence of bumetanide is a lack of KCC2 expression in mature sensory neurons (**Lucas et al.2012**). Considering both experiments, the data demonstrate a requirement of a high intracellular Cl^- concentration which is maintained by NKCC1 in sensory neurons. Together with our ANO1 findings, we support the hypothesis that ANO1 activation by PGE2-evoked responses in sensory axons is enabled by the high intracellular Cl^- concentration, which is in turn maintained by NKCC1.

In conclusion, this chapter provides evidence for the dual role of the PGE2/EP4 pathway, acting both as an initiator and a sensitizer of painful stimulation in DRG sensory axons and neurons respectively. The latter phenomenon could explain tonic pain activity in inflammatory pain conditions, upon PGE2 secretion. The ANO1 and Nav1.8-mediated activation of nociceptive axons and the concomitant enhancement of axonal responses to depolarizing stimuli by PGE2, provides a plausible explanation for both acute and chronic manifestations of ongoing pain following tissue injury and inflammation, in an independent manner of the sensitization of specific receptor channels. The silencing of PGE2-evoked activity observed by blocking ANO1 and Nav1.8 channels, render them as desirable targets for analgesic drug development and nociceptor specific gene therapy approaches.

4. Establishment of an *in vitro* model of the first sensory synapse

Aims and objectives

It is not fully understood how synaptic plasticity may modulate the excitability of DH neurons. The aim of this chapter was to establish a microfluidic co-culture model of the first sensory synapse. Within this platform, the spatial properties of all involved neuronal compartments can be distinctly examined in the absence of non-neuronal cells.

- 1) The extent to which mouse postnatal DRG-DH co-cultures recapitulate salient features of the *in vivo* physiology is examined.
- 2) The percentage of axons sprouting bi-directionally to the two corner compartments is quantified. One corner side represents the free nerve endings while on the opposite side they form functional synapses with the co-cultured DH neurons.
- 3) Using immunocytochemistry, the expression of MAP2 co-localization with VGLUT2 is used to investigate the identity of the cultured spinal cord neurons. Further, the expression of Synapsin1 with MAP2 is used to determine the formation of functional synapses in the DH compartment.
- 4) A methodical electrophysiological characterization is used to validate whether the stimulation of sensory DRG axons results in calcium responses in DH neurons.
- 5) The glutamatergic nature of these synapses is examined using selective NMDA and AMPA receptors blockers.
- 6) As a surrogate model of peripheral nerve injury, axotomy is introduced to sensory axons where DH neuron responses are recorded and compared following stimulation of intact and axotomized DRG neurons.

The establishment of this versatile model may enable the characterization of DRG-DH synapses using pharmacological tools as well as study the molecular mechanisms underlying the modulation of these synapses.

4.1 Introduction

Fundamental understanding of the systemic workflow in pain processing has established that specialized primary afferent fibers called nociceptors detect and process a wide range of noxious stimuli **(Bessou and Perl, 1969) (Wall and Devor,1983)**. They are comprised of small-sized, unmyelinated C- fibers and medium-sized, thinly myelinated A δ -fibers **(Basbaum et al,2009)**. As mentioned in the introduction, the cell bodies of sensory neurons, including nociceptors, are found in clusters called dorsal root ganglia (DRG) located within the spinal column or trigeminal ganglia found close to the brainstem. Sensory neurons are pseudo-unipolar with long axonal processes. which bifurcates to form a T-shaped junction. The distal axonal projections of nociceptors innervate tissues such as the skin, visceral organs, muscles, and joints terminating in free nerve endings. On the opposite side, the axonal compartments of nociceptors located proximally to the spinal column project to distinct laminae of the spinal cord's dorsal horn **(Cook et al,1987) (Todd,2010)**.

Further in the introductory chapter, we described how nociceptors act as the cellular machinery that transmits information to the central nervous system **(Woolf and Ma,2007)**. Specialized transducer ion channels expressed at the nerve terminals of nociceptors detect noxious stimuli and generate electrical impulses. The evoked APs follow a propagation trajectory across nociceptors' long axons and cell bodies. The propagation of APs is predominantly dependent on the activity of voltage-gated sodium and potassium channels expressed selectively in the membranes of nociceptors **(Cummins et al, 2007) (Tsantoulas & McMahon,2014)**. Ultimately, the excitability of nociceptors terminates its peripheral course via synaptic transmission to specific dorsal horn laminae of the spinal cord.

It is an ostensible challenge in pain neurophysiology to infer how stimuli under normal or pathological states are converted into electrical signals and how these signals travel immense distances through diverse routes along the sensory machinery to elicit the unpleasant sensation of pain. In the periphery, where much is known about the somatic components of nociceptors, there has been a dearth of knowledge on the properties of their axons at the distal site but especially at their central, pre-synaptic site. The axonal compartments of sensory neurons are responsible for the initiation and propagation of APs but also, they contain a dynamic contingency of axonal mRNA for cytoskeletal and immune related molecules as well as ion channels **(Gumy et al,2011)**.

The molecular plasticity of sensory neurons can alter their excitability through expression of ion channels and second messenger molecules. Such newly adapted properties may modulate the DH neurons which receive synaptic input from them. It has been suggested from the Dibb-Hajj and Waxman group that the phenomenon may occur through the trafficking of ion channels across the axonal membrane (**Akin et al,2021**) (**Hagard-Rusli,2022**). Demonstrated at the axonal level of sensory neurons by the Fawcett group (**Gummy et al,2011**) and a possible contingency which has emerged from studies of long-term potentiation (LTP) in the CNS, is that neuronal and synaptic plasticity may be induced due to localized mRNA synthesis at distinct spatial sites (**Perez et al,2021**). Transcriptomic studies reveal a reprogramming of sensory neurons following nerve injury (**Renthal et al,2020**) while *in vitro* studies of synaptic transmission between DRG and DH have revealed pre-synaptic changes in VGSC contribution to DH excitability (**Vysokov et al,2019**). Thus far, the consensus remains inconclusive and the possibility of a co-existence of both trafficking and localized mRNA transcription mechanisms at the synaptic site should not be excluded.

The first sensory synapse is the initial relay point between the peripheral and central nervous system. It is formed by a pre-synaptic site found in the proximal axonal compartment of sensory DRG neurons and post-synaptic site on second order neurons found in the spinal cord's dorsal horn (**Clark et al, 2013**). Studies on synaptic transmission across the first sensory synapse highlight the role of nociceptors in modulating the excitability of spinal cord neurons. Once the generated APs reach the pre-synaptic terminals of nociceptors, the calcium influx activates a subset of voltage-gated calcium channels (VGCCs), called the N-type, which then triggers the exocytosis of synaptic vesicles (**Zamponi et al,2015**). In turn, the synaptic vesicles release excitatory neurotransmitters; primarily glutamate, as well as neuropeptides, namely Substance P and the calcitonin gene-related peptide (CGRP) (**Dickenson and Sullivan,1987**) (**Jessel et al,1978**) (**Benemei et al,2009**). Glutamate is stored in small vesicles while Substance P and CGRP are stored in large, dense-core vesicle in the same pre-synaptic terminals. The released neurotransmitters bind to their respective receptors, which are ligand-gated ion channels for glutamate and GPCRs for Substance P and CGRP and they are expressed in the post-synaptic sites of dorsal horn neurons. For glutamate, three families of ionotropic receptors are expressed in the pre- and post-synaptic sites within the first sensory synapse: the NMDA, AMPA and kainate receptors. Upon binding and subsequent activation of the post-synaptic receptors, APs are

generated and along with the activation of downstream second messenger mechanisms, they modulate the excitability of the spinal cord (**Woolf and Salter,2000**).

Several efforts have been directed towards analgesic relief by exploiting targets found within the first sensory synapse. The concept of pre-synaptic inhibition is relevant to analgesic drug discovery, as it regards the prevention of neurotransmitter release from the pre-synaptic nerve terminals. Gabapentinoid analgesics prescribed commonly for neuropathic pain such as pregabalin and gabapentin are shown to be acting on the $\alpha 2\text{-}\delta\text{-}1$ subunit of calcium channels at the pre-synaptic terminals of C-fiber nociceptors (**Field et al, 2006**) (**Bauer et al,2009**) (**Patel and Dickenson,2016**). These drugs are prescribed for neuropathic pain disorders including various neuropathies and fibromyalgia. It has been shown that gabapentinoids can inhibit the release of both glutamate (**Kumar et al,2010**) and the neuropeptides Substance P and CGRP (**Fehrenbacher et al,2003**). Selective NMDA and AMPA receptor antagonists block the binding of glutamate at the postsynaptic site of dorsal horn neurons. It is now established that the expression of NMDA and AMPA receptors also occurs at the pre-synaptic site on dorsal root ganglion neurons (**Bardoni et al,2004**) (**Xie et al,2022**). Commonly used anesthetics such as ketamine are pore blockers of NMDA receptors (**Himmelseher et al,2005**). The rationale for blocking the binding of glutamate to the post-synaptic terminals is to prevent a potential sensitization of the central nervous system that has been initiated by the peripherally injured tissue (**Latremoliere and Woolf, 2009**). The concept has been applied to post-operative pain; termed pre-emptive analgesia, it regards the administration of glutamate receptor blockers prior and throughout recovery of an operation to prevent the development of a chronic, central pain response (**Woolf and Chong,1993**).

The enormous complexity of the pre-synaptic active zone (**Sudhof,2012**) has provided several contingencies for disrupting synaptic transmission, including that between sensory and DH neurons. Thus, certain non-ion channel targets have been considered for the inhibition of neurotransmitter release in treatment of pathological pain. An early finding was that botulinum toxin proteolytically cleaves synaptobrevin, a key SNARE protein that drives membrane fusion during exocytosis (**Schiavo et al,1992**). The finding has been recently exploited to block transmission of pain signals from nociceptors (**Maiaru et al,2018**), however the lack of specificity in blocking transmission of signals from sensory neurons may act as an adverse effect causing rigidity and a loss of peripheral sensation (Matak et al,2019). A more recent, surprising finding is

the use of anthrax, a toxin of bacterial origin, silences nociceptor activity (Yang et al,2022). The edema toxin used in the study blocked partially blocked neurotransmission in a cAMP/PKA-dependent manner by binding to its cognate receptor ANTXR2. Still unknown is whether the mechanism of action is the pre-synaptic blockade of neurotransmitter release by cleaving SNARE proteins or a cAMP-dependent enhancement of GABA_A-receptor chloride channels (Yang et al,2022).

There are limited techniques that can readily examine both the pre- and post-synaptic properties of DRG and DH neurons respectively. An inventive recent *in vivo* methodology to measure post-synaptic excitability following peripheral stimulation was the use of two-photon imaging of a virally expressed glutamate sensor (MacDonald et al.2021). However, the technique was comparatively low throughput as few ROIs could be recorded from each anaesthetized animal. There is also limited knowledge on the topography of the molecules modulating the first sensory synapse and it is highly challenging to be examined *in vivo*. The development of spatiotemporally resolved transcriptomic techniques at molecular resolution have the potential to provide a blueprint for the involved molecules, which can then be confirmed using electrophysiological and fluorometric functional readouts (Shi et al,2022) (Ren et al,2023).

The *in vitro* recapitulation of the first sensory synapse has posed a compelling model for studying interactions between sensory and dorsal horn neurons. A strength of the model is the isolation and study of neurons in the absence or presence of non-neuronal cells. This feature can be readily controlled in contrast to the *in vivo* environment where non-neuronal cells are resident (Vysokov et al,2019). In the past, embryonic rat explants were used to study DRG and DH synaptic properties (Jahr & Jessel,1985) (Jessel, Yoshioka & Jahr,1986) (Tsuzuki et al,2004). The DRG-DH explant studies are amenable to intracellular recording techniques but are unable to monitor the excitability of several DH neurons at once. More recent studies have been conducted using conventional primary culture techniques (Ramgoolam and Dolphin,2023). Conventional DRG cultures have been traditionally applied to study the cellular properties of sensory neurons and have been widely used in combination with patch-clamping and fluorometric imaging to elucidate cellular and molecular mechanisms of nociception. The conventional cultures, however, are not amenable to isolated pharmacological treatments, therefore are not as illuminating about the spatial properties of the individual neurons present. Further limitations include the monitoring

of the timing of synapse formation and the potential multi-axonic input that each DH neuron receives in the non-isolated presence of several sensory neurons. A micro-island assay was developed to study synapse formation in a precise 1:1 manner between DRG and DH neurons (**Gu & McDermott,1997**) (**Joseph et al,2010**). It, however, fell short of allowing large scale, calcium-imaging recordings of several dorsal horn neurons receiving synaptic input from their corresponding sensory axons.

Technical progress in the field of microfluidics has paved new avenues into studying axonal pre-synaptic properties of nociceptors and transmission mechanisms across the first sensory synapse in an isolated manner. This chapter regards the establishment of a microfluidic model between sensory neurons and DH neurons. Whether rats or mice are a more accurate rodent model relevant to human nociceptor physiology is not settled (**Rostock et al,2019**). The use of mice, however, opens the contingency for the utilization of genetic tools such as knockout models which can be studied as a confirmation to observations from pharmacological interventions. Primarily due to the molecular revolution in neuroscience and the use of genetic and stem cell advancements, the mouse has become the prominent experimental model in pain research, overtaking rats as of 2019 (**Sadler et al,2022**).

In a previous effort of the lab, the microfluidic co-culture model was performed in embryonic rats (**Vysokov et al,2019**). The benefits of further optimizing the model and conducting these experiments in postnatal mice are multiple. Initially, the postnatal rat DRG cultures are very young and their development may not recapitulate the diversity of these neurons, since the cell integrity deteriorates approximately 10-12 days after culture. Moreover, the embryonic spinal neurons may also not follow the *in vivo* developmental path and may hinder the recapitulation of the plasticity observed *in vivo*. In contrast, postnatal mouse neurons are already older and more mature and can provide a more physiologically relevant model. Even more importantly, the mouse co-cultures open the contingency for the use of transgenic animals and the cross-verification of observations from pharmacological studies.

The objective of these experiments was to develop a modified culture model to recapitulate the first sensory synapse using postnatal mice. We show that our model recapitulates the first sensory synapse in culture. The microfluidic co-cultures are divided into 3 isolated compartments: a distal axonal compartment, a compartment for DRG cell bodies and a proximal axonal compartment

upon where synapses are formed with the DH neurons, and which mimics the central terminals of nociceptors. We show that using calcium imaging, we can monitor the excitability of multiple DH cell bodies following stimulation of the distal axonal DRG terminals or the DRG cell bodies. As a surrogate model of neuropathic injury, we incorporate in our model the axotomy of peripheral terminals, which we use in this chapter and in later ones to study the neuronal excitability of DH neurons.

4.2 Results

4.2.1 Microfluidic co-cultures of mouse sensory and DH neurons

The desired outcome of establishing the microfluidic co-cultures was to study synaptic transmission between DRG and DH neurons. We replicated the experimental setup (Vysokov et al,2019) but switched species to postnatal mice (P2-P4). The culture of postnatal spinal cord neurons has been notoriously difficult, yet a protocol was optimized for the co-culture. To limit our studies in the neuronal interactions between DRG and DH neurons in the absence of non-neuronal cells contributing to the excitability of either compartment, we applied in our cultures the mitotic inhibitor cytosine arabinoside (AraC). The microfluidic devices were comprised of 3 compartments (**Figure 4.1A**): DRG and DH neurons were plated to the middle and side compartments respectively. The sensory neurons sprouted neurites bidirectionally to both side compartments which are henceforth referred to as the DRG cell body compartment and DH compartments. The sprouted neurites from the DRG compartment were observed to cross to the third compartment (**Figure 4.1B**) mimicking the free nerve endings of sensory neurons. That compartment is henceforth called the periphery.

We also observed that sprouted neurites in DRG compartment (**Figure 4.1C**) crossed to the DH compartment (**Figure 4.1D**). Using this co-culture configuration, we were able to stimulate the DRG cell bodies or their axons in the peripheral compartments using electrical stimulation and measure the ensued activation of central neurons using calcium imaging at the DH neuronal soma at DIV8-10 as a functional readout (**Figure 4.1A**).

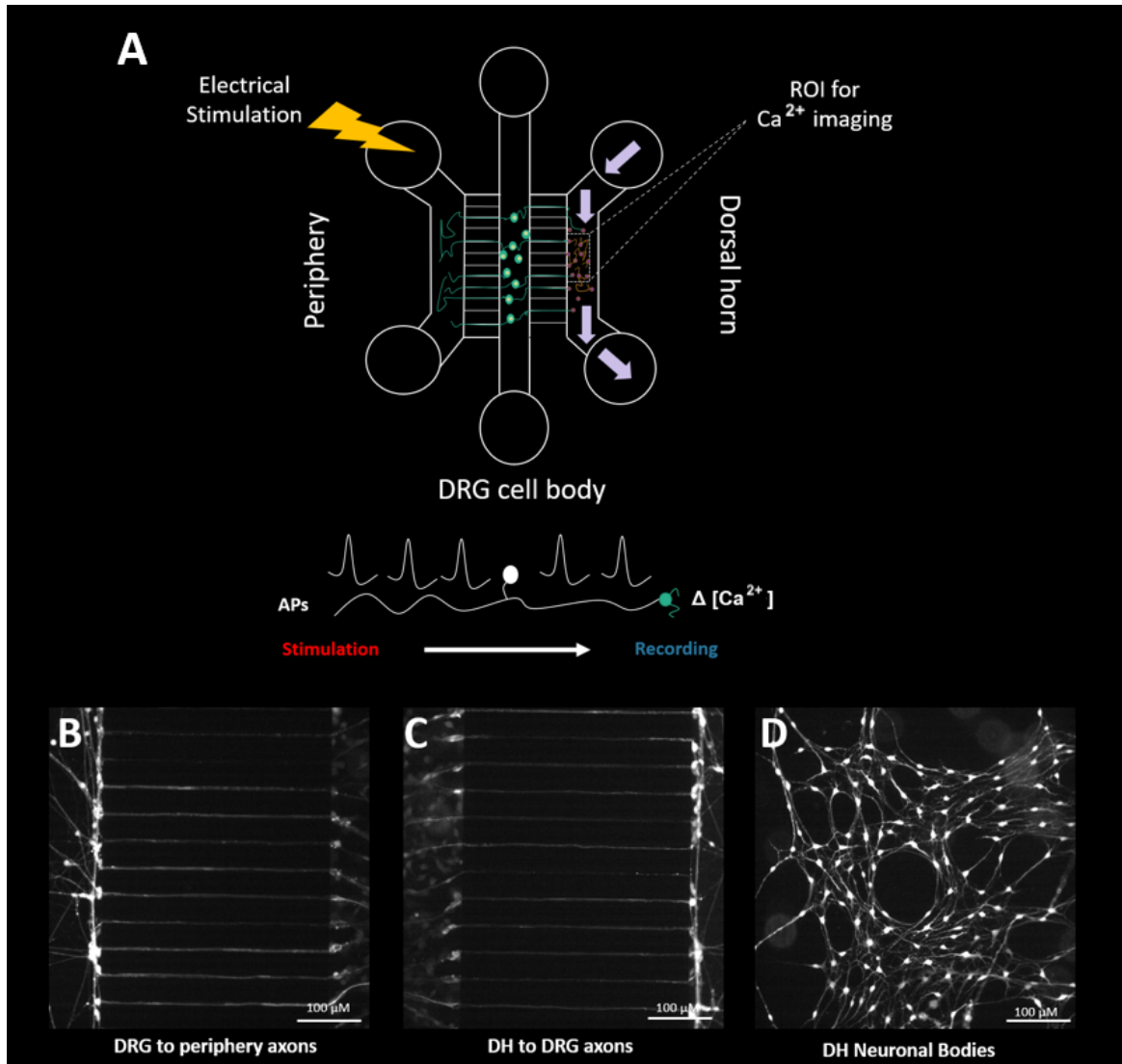


Figure 4.1 Microfluidic co-culture of DRG and DH neurons. **A)** Schematic representation of the co-culture setup. Mouse (P2-P4) DRG and spinal cord dorsal horn neurons (DH) were loaded in different compartments of the microfluidic devices and allowed to mature for 8-12 DIV. In our model, peripherally induced electrical stimuli were used to stimulate the axons which in turn triggered activation of neurons in the DH compartment. **B)** Brightfield image of the microfluidic corridors with neurites sprouting from the DRG cell bodies (left) to the periphery (right). **C)** Brightfield image of the microfluidic corridors with neurites sprouting from the DRG cell bodies (right) to the DH (left). **D)** Brightfield image of a network between DH neuronal cell bodies of postnatal mice.

4.2.2 Characterization of the microfluidic co-culture model: Axonal Crossings

The cultures were maintained for 8-12 days which has been shown to be sufficient time for markers of mature DRG neurons to appear, such as the nociceptor-subset specific Nav1.8 and TRPV1 channels (Vysokov et al,2019). Once we established the protocol for co-culturing the DRG and DH neurons, we sought to determine the percentage of DRG neurons with axons that traversed bidirectionally to the peripheral and the DH compartments. The percentage of bidirectionally crossing axons from the DRG cell bodies in the microfluidic cultures is an important determinant of the number of synaptic connections that can be formed between the incoming DRG axons and the DH neurons. For this purpose, we used the lipophilic dyes DiI and DiO that were applied at the peripherally sprouted and DH sprouted axons, respectively (see Methods). The axonal application of the dyes retrogradely stained the cell bodies of DRG neurons in the middle compartment (**Figure 4.2A**). Therefore, we got a clear indication of which cells bodies had only sprouted towards the peripheral compartment, which had only sprouted towards the DH compartment and which had sprouted bidirectionally towards both compartments.

In 3 devices, we found that $43.6\% \pm 2.8\%$ of stained DRG cell bodies were positive for DiI, hence showing that about half of the sprouted axons cross towards the periphery (**Figure 4.2B**). On the other hand, $56.4\% \pm 4.9\%$ of DRG cell bodies were DiO positive and their correspondent axons crossed towards the DH compartment (**Figure 4.2C**). Of the pool of neurons stained by either DiI or DiO, an overlapping percentage of $27.5\% \pm 1.2\%$ DRG cell bodies were found to have axons that crossed into the other two compartments. This means that those bilaterally crossing neurons can tentatively recapitulate the connectivity between sensory and DH neurons (**Figure 4.2D**).

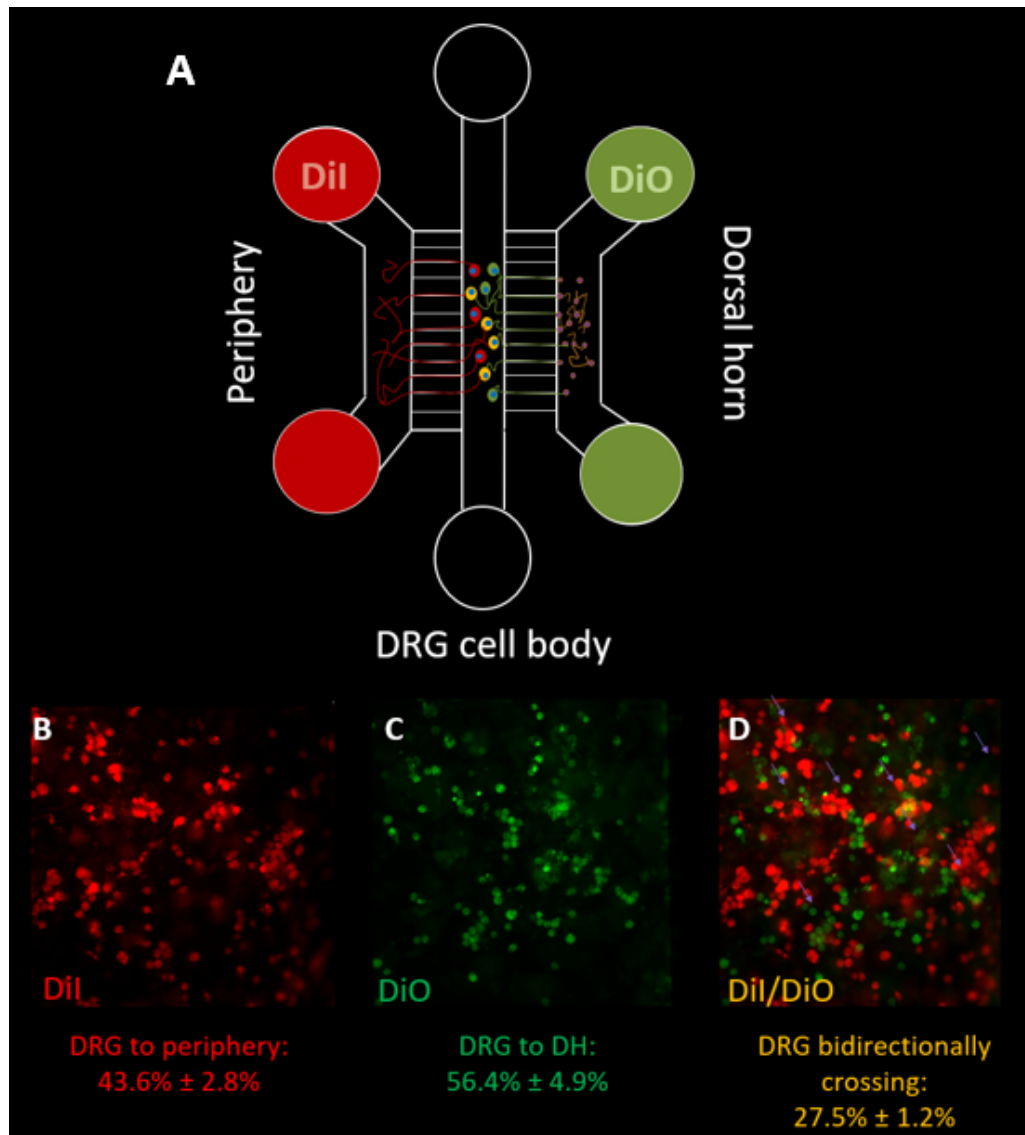


Figure 4.2 Quantification of DRG axons crossing to the periphery and DH compartments. DiI was applied to the peripheral axonal compartment and DiO was applied to the DH compartment. The dyes retrogradely traversed from the axons to the cell bodies of DRG neurons and the percentage of peripherally crossed, DH crossed, and bilaterally crossed axons was quantified in the same field of view (n=3 devices and 3 cultures). **A)** Schematic of the staining experiment. **B)** DiI staining of DRG cell bodies after application of the dye in peripheral axons. Of the total number of stained DRG cell bodies, 43.6% were DiI positive. **C)** DiO staining of DRG cell bodies after application of the dye in the DH axonal compartment. Of the total number of stained DRG cell bodies, 56.4% were DiO positive. **D)** DiI/DiO overlap of DRG cell bodies after application of the dyes in both corner sides. From the total number of stained DRG cell bodies, 27.5% were DiI/DiO double stained, indicating axonal crossings into both compartments.

4.2.3 Characterization of the microfluidic co-culture model: DH compartment

Next, we focused on the DH compartment and sought to characterize through immunocytochemistry the properties of the cells cultured in that compartment. Because of the gelatinous nature of the spinal cord of mice when performing the P2-P4 dissections, it is sometimes difficult to accurately isolate the dorsal horn. To characterize the central neurons cultured in the DH compartment, we used a microtubule-associated protein 2 (MAP2) antibody, a common neuronal marker of cell bodies and dendrites (De Camilli et al,1984). The confirmation that the cultured cells in the DH compartment were indeed neuronal was important because the survival of spinal cord neurons in culture is technically challenging (Figure 4.3B). We wanted to further confirm that the neurons obtained were not only central, but also glutamatergic, a proof that would corroborate that the cultured neurons are indeed dorsal horn neurons. We hence co-stained for MAP2 in combination with VGLUT2, a marker of glutamatergic neurons which is predominantly expressed in the laminae I and II of the dorsal horn and which nociceptors innervate with their central terminals (Todd et al,2003) (Punnakal et al,2009). However, as VGLUT2 is also expressed in peripheral sensory neurons (Scherrer et al,2010), only an overlap between MAP2 and VGLUT2 could provide evidence for the central and glutamatergic nature of these neurons. A further potential caveat in this approach is that while inhibitory interneurons of the cord can be omitted as a contingency, it cannot be known if any of the MAP2/VGlu2 double-positive neurons may include excitatory interneurons. However, because the excitatory interneurons are predominantly located in laminae III and IV (Todd & Wang,2018), we do not expect them to be stained by VGLUT2 which is predominantly expressed in laminae I and II. Nevertheless, we observed a clear overlap between MAP2 and VGLUT2 indicating that most of the neurons were glutamatergic spinal cord neurons.

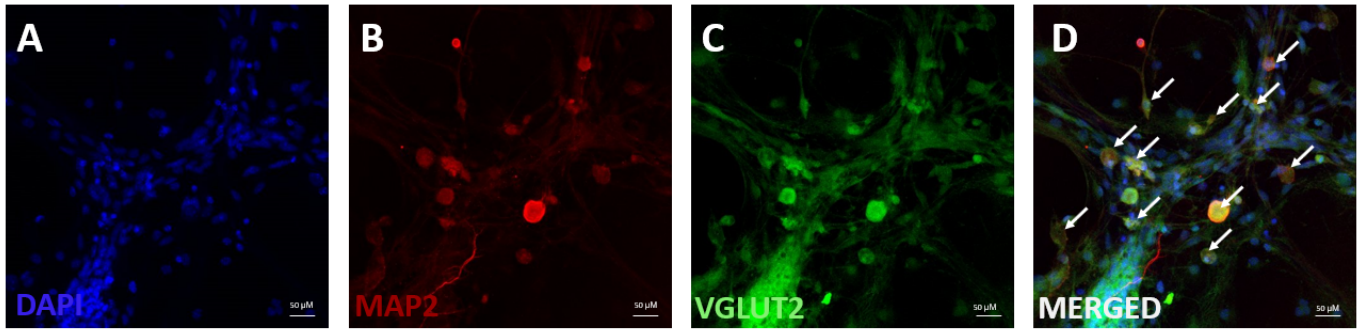


Figure 4.3 Expression of MAP2 and VGLUT2 in the DH compartment. MAP2, a neuronal marker was co-stained with VGLUT2, a marker of glutamatergic neurons predominantly expressed in laminae I and II of the dorsal horn. The overlap shows that the cultured cells from the mouse spinal cord in the DH compartment are neurons and specifically glutamatergic ones. **A)** DAPI-stained confocal image (blue) of the DH compartment. **B)** MAP2-stained confocal image (red) of the DH compartment showing central neurons. **C)** VGLUT2-stained confocal image (green) of the DH compartment showing glutamatergic neurons. **D)** Merged image of DAPI (blue), MAP2 (red), VGLUT2 (green) highlighting an overlap between MAP2 and VGLUT2 positive neurons. The overlap confirms that the neurons stained are central and are glutamatergic.

We next sought to investigate whether synapse formation was occurring within the microfluidic DH compartment. We hence employed again the MAP2 staining of central neurons in combination with a synaptic marker, Synapsin1. Synapsin1, is a neuronally-expressed phosphoprotein that is localized pre-synaptically in synaptic vesicles and plays a regulatory role in neurotransmitter release (**Sudhof et al,1989**). The puncta of Synapsin1 in combination with the stained MAP2 neurons show the active synaptic sites formed between the incoming axons from the periphery and the DH neurons. We observed an abundance of Synapsin1 puncta co-localized with the MAP2 in the DH compartment (**Figure 4.4D**) suggesting active synapse formation in the DH compartment. The observation that MAP2 is receiving synaptic input is important for dual reasons: first, it further provides evidence for the healthy maintenance of the DH neurons and their susceptibility to form functional synapses. Second, we observed puncta between DH sprouted axons suggesting that axo-axonic synapses were formed, hence confirming a faithful recapitulation of the spinal cord neuron network formation observed *in vivo*.

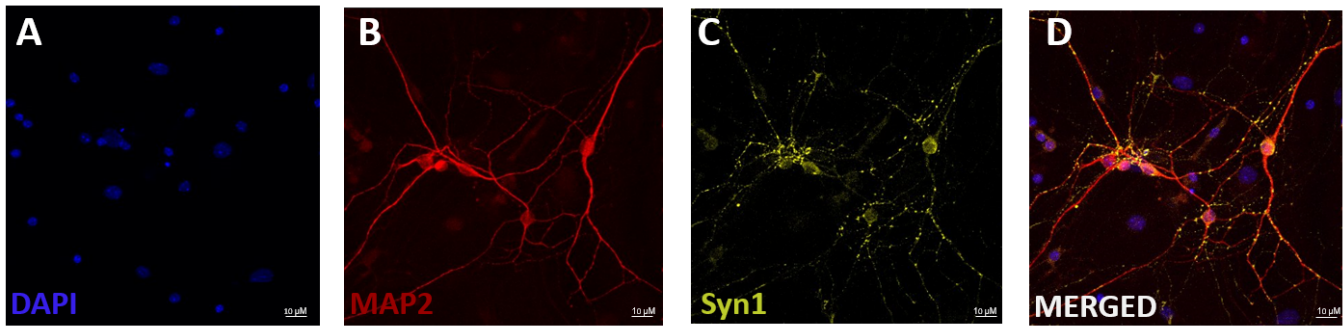


Figure 4.4 Synapse formation in the DH compartment. MAP2 was co-stained with Synapsin1, a synaptic vesicle marker expressed in presynaptic membranes. The overlap between the Synapsin1 puncta (yellow) and the MAP2-positive neurons (red) shows the synapse formation and confirms the synaptic input that the DH neurons receive from the sensory axons traversing through the compartment **A)** DAPI-stained confocal image (blue) of the DH compartment. **B)** MAP2-staining (red) of the DH compartment shows the spinal cord central neurons in the DH compartment. **C)** Synapsin1 expression (yellow) in the DH compartment showing synaptic puncta. **D)** Merged image of DAPI (blue), MAP2 (red), Synapsin1 (yellow) evidence of putative synapses on DH neurons.

Taken together, these results provide an immunocytochemical characterization of the DH compartment in the microfluidic co-cultures. The MAP2 staining of spinal cord neurons in the DH compartment in combination with the VGLUT2 expression indicate that the cultured neurons are glutamatergic, resembling those normally found in laminae I and II of the dorsal horn. However, the finding is surprising as any features of the somatotopic alignment between sensory neurons and corresponding dorsal horn laminae projections are not expected to be maintained in *in vitro* preparations of dissociated neuronal cultures. A second interesting observation is that the glutamatergic dorsal horn neurons are putatively forming functional synapses with other DH neurons and most importantly they receive synaptic input from the sensory axons that traverse from the DRG compartment to the DH compartment. The potential formation of axo-axonic synapses in the DH is a salient feature of spinal cord networks, while it might explain potential latencies picked up from certain DH neuron responses.

4.2.4 Activation of DRG cell bodies and peripheral axons in microfluidic cultures by electrical stimulation

Based on the immunocytochemical evidence for active synapses in the DH compartment, we then sought to investigate the presence of functional synaptic contacts between incoming sensory axons and DH neurons in the DH compartment of the microfluidic co-cultures. The desideratum of this model is the *in vitro* replication of peripheral nerve stimulation and the recording of DH neuron activation, thus replicating the periphery-to-center circuit. We therefore first used an electrical stimulation protocol which we applied to the sensory neurons in the middle compartment to confirm their activation of DRG neurons in the 3-compartment configuration. For the electrical stimulation, we used a biphasic stimulation system (See Methods), where 8 biphasic pulses were given at a 4Hz frequency, of 2 ms duration at 4 μ A constant current amplitude. These parameters were set to establish a stimulation protocol which would depolarize the sensory neurons but would not desensitize them over repeated stimulations. First, the DRG cell bodies were stimulated and their activity was directly recorded Fluo-4, a calcium indicator (**Figure 4.5A**).

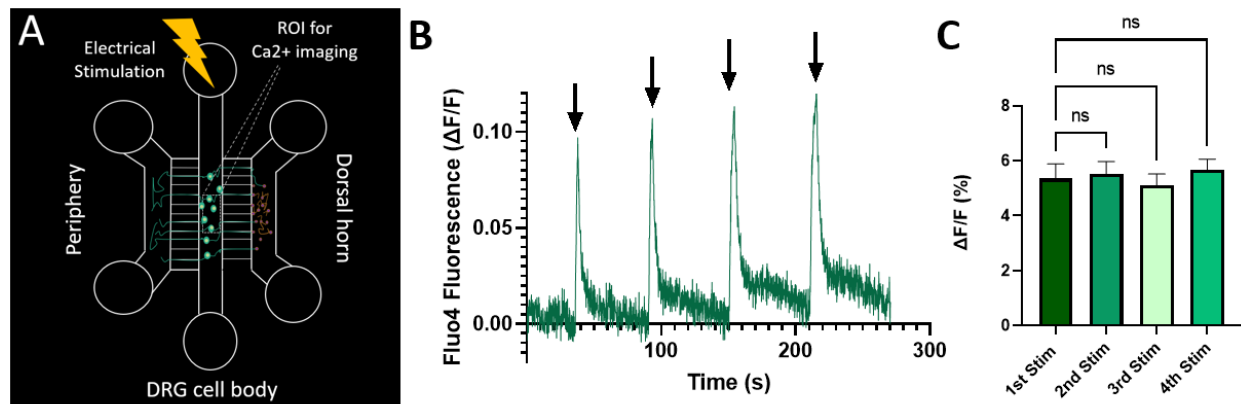


Figure 4.5 Electrical stimulation of DRG neuron cell bodies cultured in the middle compartment. A) Schematic of the experimental setup. Stimulation of the DRG somal side (middle) (8 biphasic pulses at 4Hz frequency. Each pulse lasted 2 ms 4 μ A) and recordings of calcium activity from the cell bodies using Fluo-4. **B)** Representative trace showing responses to consecutive applications of electrical stimulation in the DRG compartment. Between each stimulation, neurons were allowed 60-90 seconds to return to baseline Fluo4fluorescence before being stimulated again. The baseline-subtracted responses ($\Delta F/F$) are shown **C)** Comparison of the average amplitude of responses following 4 successive stimulations. Each response was of almost equal amplitude debunking any potential electrode fatigue or neuronal desensitization concerns (ns $p < 0.05$, One-way ANOVA with Tukey's multiple comparisons test, $n = 53$ neurons, $n =$ devices from 3 cultures).

We found that each electrical pulse led to an increase in the Fluo4 fluorescence as an indication of increase in $[Ca^{2+}]$ concentration in the soma (**Figure 4.5B**). An important concern in applying consecutive electrical stimulations was that either due to electrode fatigue or due to neuronal desensitization, the response to multiple stimulations might decline. However, with our protocol, we did not find any alteration in the average $\Delta F/F$ amplitudes after 4 consecutive stimulations (**Figure 4.5C**). Hence, the DRG neurons in the middle compartment are responsive to electrical stimulation and that repetitive stimulation does not alter the amplitude of responses.

After establishing that the DRG cell bodies in the middle compartment were responsive to electrical stimulation, we replicated the experiment, but this time we stimulated the axonal compartment and recorded from the DRG compartment in the middle (**Figure 4.6A**). In agreement with the previous stimulation protocol, we found that the DRG neuronal cell bodies responded to peripheral axonal stimulation using the same stimulation protocol (**Figure 4.6C**). Moreover, we observed that multiple consecutive axonal stimulations did not alter the average amplitude of successive responses of the DRG cell bodies. These results corroborate our observation and establish that the depolarization of the axonal compartment leads to an increase in calcium efflux to the DRG cell body compartment.

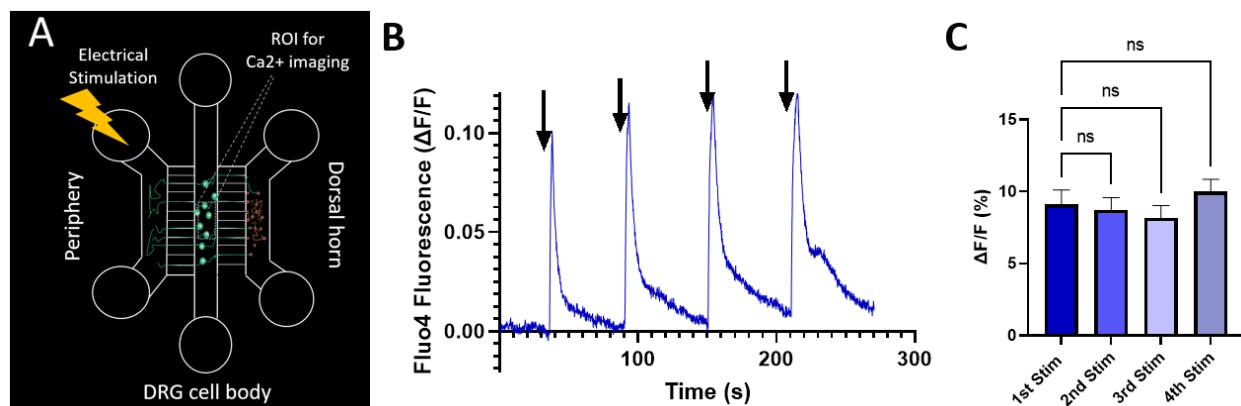


Figure 4.6 Electrical stimulation of sensory axons in the periphery activates DRG cell bodies in microfluidic co-cultures. **A)** Schematic of the experimental setup. Stimulation of sensory axons in the Periphery compartment (left) (8 biphasic pulses at 4Hz frequency, 2 ms duration at 4 μ A amplitude) with the DRG somal side (middle) as the readout site of calcium activity, using Fluo-4 **B)** Representative trace showing the responses at the cell bodies following 4 consecutive applications of electrical stimulation in the Periphery compartment. **C)** Comparison of the average amplitude of responses following 4 successive stimulations. Consecutive responses showed no significant changes in amplitude (ns $p < 0.05$, One-way ANOVA with Tukey's multiple comparisons test, $n = 44$ neurons, $n = 5$ devices from 3 cultures).

Taken together, these experiments provide evidence for the excitability of the DRG cell bodies and their axonal projections in the peripheral compartment. Not only do cell bodies of DRG neurons respond directly to the electrical stimulation, but they also receive input following axonal stimulation. Therefore, the latter experiment (**Figure 4.6**) advocates that the APs generated in the axons upon electrical stimulation are propagated towards the cell bodies of DRG neurons and results in their subsequent depolarization and the ensuing Ca response at the cell body.

4.2.5 Activation of dorsal horn neurons in microfluidic cultures by electrical stimulation

After ensuring that the DRG cell bodies and axons are responsive to electrical stimulation, we focused on the excitability of DH neurons. The DH compartment was previously shown to contain glutamatergic neurons (**Figure 4.3**) and that the DH neurons are receiving synaptic input, pinpointing towards the putative formation of functional synapses between each other (**Figure 4.4**). The DH neurons form a network which occasionally engages in spontaneous activity and crosstalk and while normally at rest and in the absence of stimulation, DH neurons may spontaneously fire repetitively (**Figure 4.7A**). We electrically stimulated the DH cell bodies and recorded the responses using calcium imaging to ensure that the DH neurons are excitable and amenable to neuronal excitation. The DH neurons responded to the electrical stimulation (8 biphasic pulses at a 4Hz frequency x 2 ms @4 μ A) and observed no significant fluctuations in the amplitude of their excitability (**Figures 4.7B&C**). The excitability of DH neurons following peripheral stimulation together with the immunocytochemical evidence from **Figures 4.3 and 4.4**, complete the isolated characterization of the DH compartment.

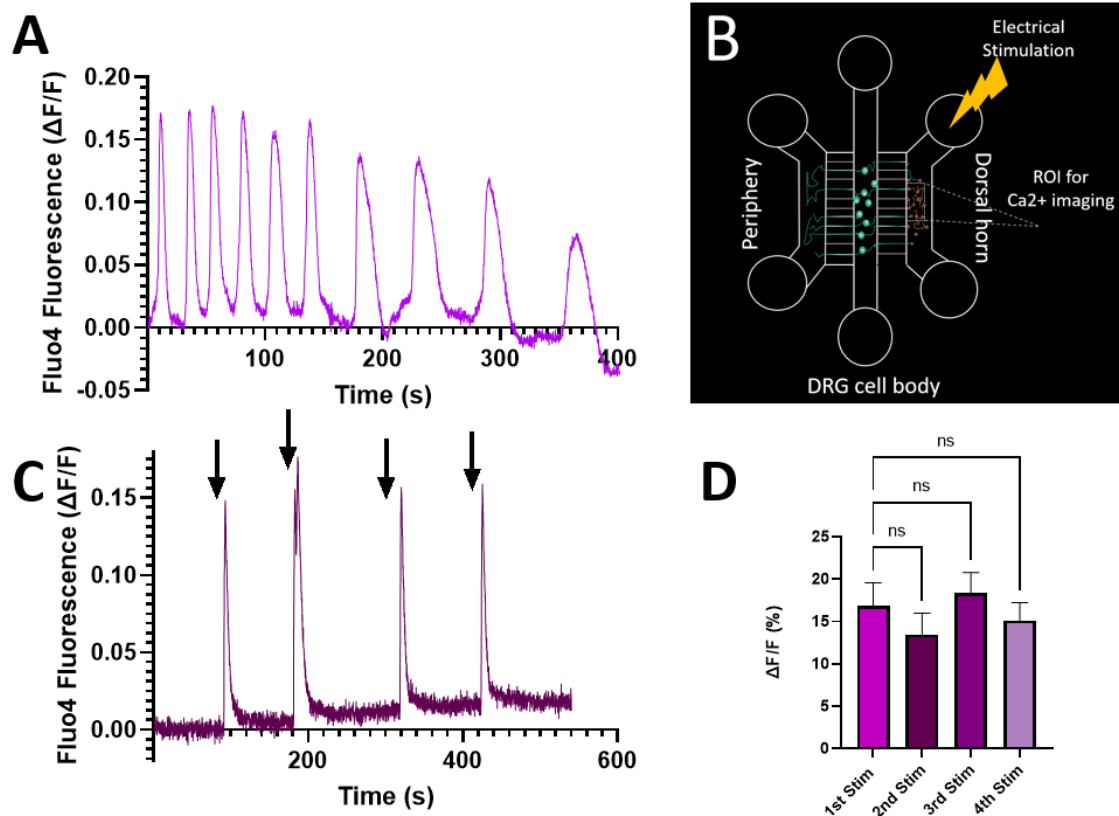


Figure 4.7 Excitability properties of DH cell bodies following electrical stimulation. **A)** Representative trace showing the occasional occurrence of spontaneous activity of a DH cell body in the absence of any stimulation **B)** Schematic of the experimental setup. Stimulation of DH neurons (right) (8 biphasic pulses at 4Hz frequency x 2 ms @4 μ A) with the DH compartment itself as the readout site of calcium activity. **C)** Representative trace showing changes in calcium influx of DH cell bodies following 4 consecutive applications of electrical stimulation directly to the DH compartment. **D)** Comparison of the average amplitude of responses following 4 successive stimulations. Responses had no significant changes in amplitude (ns $p < 0.05$, One-way ANOVA with Tukey's multiple comparisons test, $n = 10$ neurons, $n = 3$ devices from 3 cultures).

4.2.6 Activation of dorsal horn neurons following stimulation of the peripheral compartment

From the data obtained in the DiI/DiO experiment (**Figure 4.2**) 30% of total DRG cell bodies have axons crossed bidirectionally towards both the peripheral and DH compartments. Further, from the immunocytochemical experiments of co-staining MAP2 with VGLUT2 we have established that cultured neurons in the DH compartment are comprised of central neurons which express the glutamate transporter VGLUT2, hence are likely to be glutamatergic and align with the physiological *in vivo* profiles of DH neurons (**Figure 4.3**). Similarly, in co-staining of MAP2 with Synapsin1, we have evidence that those spinal cord glutamatergic neurons are also forming functional synapses and are amenable to receiving input from other axons (**Figure 4.4**). We have so far proved that DRG cell bodies respond to electrical stimulation generated both through direct stimulation of the middle compartment but also from peripheral stimulation (**Figure 4.5&4.6**) and that the DH neurons are activated following direct stimulation (**Figure 4.7**). It was therefore of interest to find out if peripheral axonal and DRG cell body stimulation will affect DH neuron excitability. This way, the characterization of the first sensory synapse will have been achieved in both immunocytochemical and functional/fluorometric terms.

Hence, the next step of this chapter is the examination of DH neuron activation following stimulation of peripheral neurons that had crossed bidirectionally (**Figure 4.2**). The logic is straightforward: if functional synapses are formed between sensory axons and DH neurons, then electrical stimulation generates APs. The APs are propagated across the axons and end their peripheral course via triggering neurotransmitter release that consequently activates dorsal horn neurons. Hence, we reprised the protocols of stimulating the DRG cell bodies and the peripheral axons but this time we used as a functional output the cell bodies of the characterized DH neurons in the DH compartment.

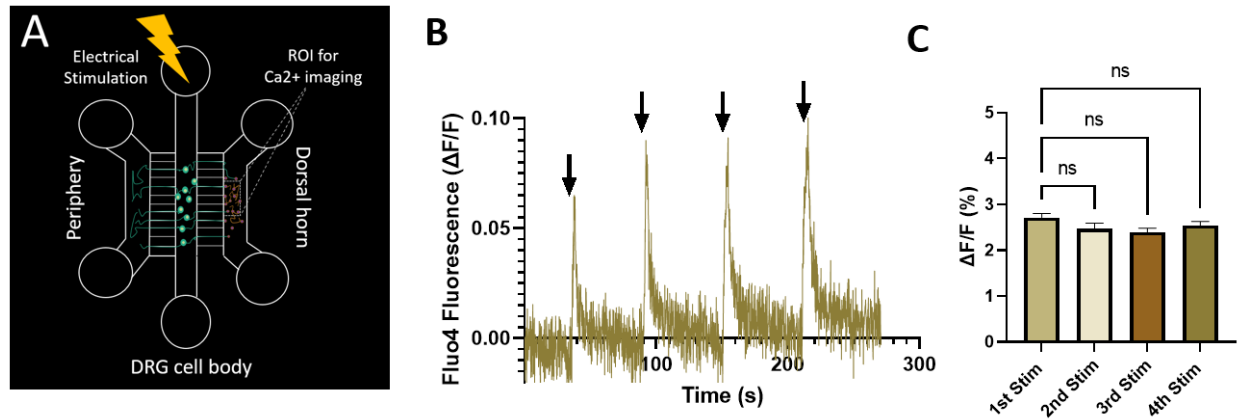


Figure 4.8 Electrical stimulation of DRG cell bodies leads to depolarization of DH neurons. A) Schematic of the experimental setup. Stimulation of the DRG neurons (middle) (8 biphasic pulses at 4Hz frequency x 2 ms at 4 μ A) elicits calcium responses at the DH compartment (right). Activity is recorded using the calcium indicator Fluo-4. **B)** Representative trace showing changes in calcium influx of DH cell bodies following 4 consecutive applications of electrical stimulation in the DRG cell body compartment. **C)** Comparison of the average amplitude of responses following 4 successive stimulations. Responses showed no significant changes in amplitude (ns $p < 0.05$, One-way ANOVA with Tukey's multiple comparisons test, $n = 92$ neurons, $n = 5$ devices from 3 cultures).

We found that electrical stimulation (8 biphasic pulses at a 4Hz frequency x 2 ms @4 μ A) induced to the DRG cell bodies in the middle compartment results in a depolarization of DH neurons, which substantiates functional synaptic input from the crossed DRG axons (**Figure 4.8A**).

The magnitude of depolarization of DH neurons following stimulation of the DRG cell bodies differs in amplitude to the elicited calcium responses in the experiments where DH cell bodies were stimulated and directly recorded from the DH (**Figures 4.8B&C**). The reason for the difference is that direct DH stimulation is mediated by voltage-gated ion channels while DH activity following DRG stimulation is dependent on a neurotransmitter release to elicit DH neuron depolarization. In the experiments where recordings of DRG stimulation were done in the DRG themselves, the depolarization induced by the electrical stimulation was mediated directly by changes in membrane voltage excitability. On the contrary, recordings of DH neurons following stimulation of stimulation of DRG neurons have a different mechanism. The excitability of those DH neurons is dependent on the neurotransmitters released from the pre-synaptic site of the DRG axons. Upon binding of the neurotransmitters to ligand-gated ion channels in the post-synaptic site of the DH neurons, it causes a generation of APs and a subsequent membrane depolarization. We

found that the consecutive applications of electrical stimulation did not alter DH-elicited calcium responses, implying that there was no runup or rundown in synaptic responses and hence the quantal neurotransmitter release mechanism was not compromised (**Figure 4.8C**). The observation is important for the integrity of the protocols in the next experiments.

Next to determine the completeness of peripheral nociceptive circuitry recapitulation, we stimulated the peripheral axons in the Peripheral compartment of the devices and recorded from the DH neuronal cell bodies in the DH compartment. The stimulation of peripheral axons would mean that the APs generated in the periphery traverse through the middle compartment containing the DRG cell bodies and from there to the proximal to the DH compartment axons (**Figure 4.9A**). Indeed, stimulation of peripheral axons resulted in calcium responses in the DH cell compartment (**Figure 9B**). Similar to previous experiments, consecutive axonal stimulations held the amplitudes of responses intact at the DH compartment.

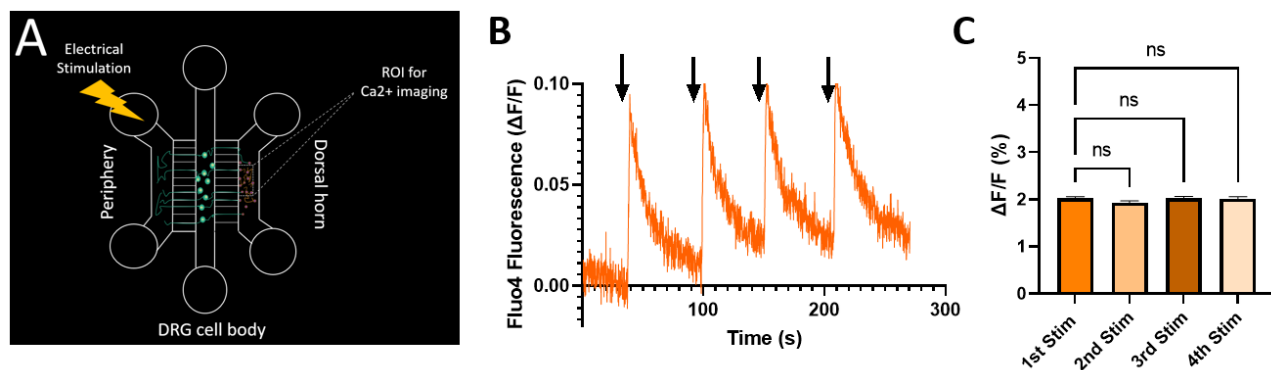


Figure 4.9 Electrical stimulation of peripheral axons leads to depolarization of DH neurons. A) Schematic of the experimental setup. Stimulation of the peripheral axons (left) (8 biphasic pulses at 4Hz frequency x 2 ms @4 μ A) depolarizes the cell bodies at the DH compartment (right) B) Representative trace showing changes in $[Ca^{2+}]$ of DH cell bodies following 4 consecutive applications of electrical stimulation in the axons of the periphery compartment. C) Comparison of the average amplitude of responses following 4 successive stimulations. Responses had no significant changes in amplitude (ns $p < 0.05$, One-way ANOVA with Tukey's multiple comparisons test, $n = 121$ neurons, $n = 3$ devices from 3 cultures).

4.2.7 Glutamatergic dependence in synaptic transmission between DRG and DH neurons

The confirmation of the formation of functional synapses between DRG and DH neurons was a crucial step towards the establishment of our model. It further allowed us in later chapters to investigate how the pharmacological blockade of certain ion channels affects the excitability of DH neurons following peripheral stimulation. A strength of the microfluidic cultures is that pharmacological manipulations in each of the fluidically isolated three compartments may provide spatial information on the mode of action of targeted molecules. We have established from the MAP2/Synapsin1 co-staining that the DH neurons are receiving synaptic input. We further showed by stimulating the peripheral axonal and DRG middle compartments that DH neurons elicit calcium responses, confirming the formation of functional synapses between the DRG and DH neurons in microfluidic co-cultures.

As mentioned previously, fast neurotransmission at the first sensory synapse is glutamatergic. The ligand-gated ion channels expressed predominantly at the post-synaptic site of DH neurons are NMDA and AMPA receptors. However, the NMDA receptors are also expressed and can increase neurotransmitter release in the pre-synaptic site of proximal DRG axons in intact slice preparations (**Bardoni et al,2004**) (**Xie et al,2022**). Given this previous evidence, we hypothesized that a glutamate receptor blockade of NMDA and AMPA receptors would block the trans-synaptic transmission to DH neurons. For this purpose, we applied at the DH compartment a competitive antagonist of AMPA receptors, CNQX (20 μ M) in concomitance with an NMDA receptor blocker, MK-801 (1 μ M) which is commercially known as dizocilpine. In the same experiment, we stimulated the DRG cell bodies twice in the absence of any glutamate blocker and then stimulated twice more in the presence of the glutamate blockers applied to DH compartment (**Figure 4.10A**). We found that the application of the glutamate receptor blockers largely attenuated the amplitude of the evoked signals in DH neurons (**Figures 4.10B&C**). The decay time, represented as a function of tau, signifies the area and width required for the traces to return to baseline. The repetitive stimulation in the presence (1st and 2nd stimulations) and absence (3rd and 4th stimulations) of CNQX and MK-801 did not alter the decay time of DH neuron calcium responses (**Figure 4.10D**).

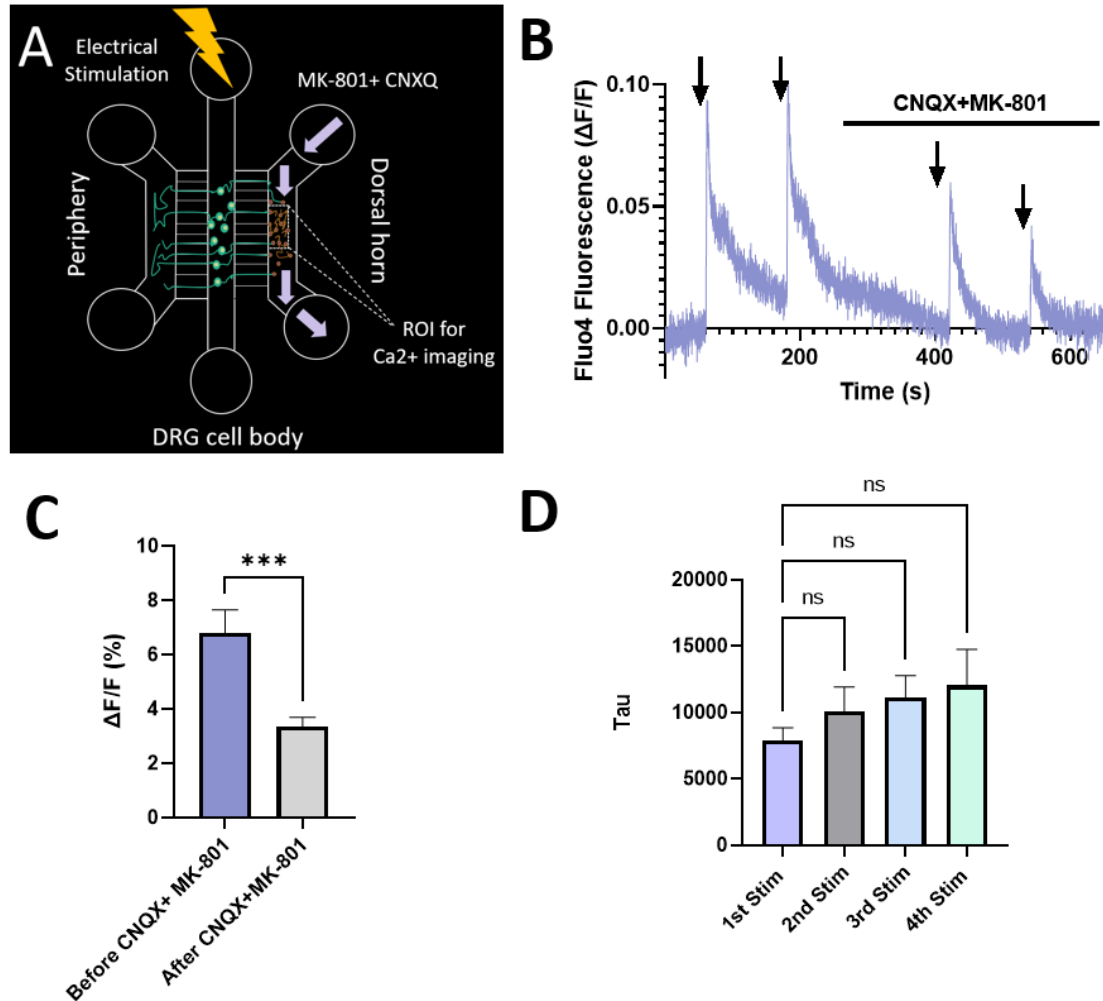


Figure 4.10 Dorsal horn responses to electrical stimulation in the DRG before and after glutamate receptor blockade **A)** Schematic of the experimental setup. Stimulation of the DRG cell bodies (middle) with the DH compartment (right) as the readout for calcium imaging. After two stimulations, the glutamate receptor blockers CNQX (20 μ M) and MK-801 (1 μ M) were applied and then two more stimulations under the presence of blockers were made. **B)** Representative trace showing changes in calcium influx of DH cell bodies following 2 applications of electrical stimulation in the absence and 2 applications of electrical stimulation in the presence of glutamate receptor blockers. **C)** Comparison of the average amplitude of responses before and after application of the glutamate blockers. The application of CNQX and MK-801 significantly reduces the amplitude of DH neuron responses to electrical stimulation (Welch's t-test, *** $p < 0.0001$, $n = 3$ devices from 3 cultures). **D)** Decay time for DH responses to return to baseline, represented as a function of tau. The addition of CNQX and MK-801 did not alter the decay time following electrical stimulation of the DRG cell bodies (ns $p < 0.05$, Brown-Forsythe and Welch ANOVA, $n = 40$ neurons, $n = 3$ devices from 3 cultures).

Similarly, electrical stimulation at the peripheral compartment was performed with the calcium responses at the DH cells bodies as the readout. As per previous sets of experiments, we applied electrical stimulation both to the DRG cell bodies and the peripheral axons to detect if there are any discrepancies in the DH responses following stimulation. The stimulation of peripheral axons in the presence of CNQX and MK-801 again produced a stark reduction in the amplitude of DH cell body responses (**Figures 4.11A-C**). Similarly, the decay time represented as a tau value remained unaltered in the presence of glutamatergic blockade (**Figure 4.11D**) The pharmacological blockade confirms the dependence of synaptic transmission on glutamate release and post-synaptic binding while it confirms the full recapitulation of a periphery-to-center functional circuit.

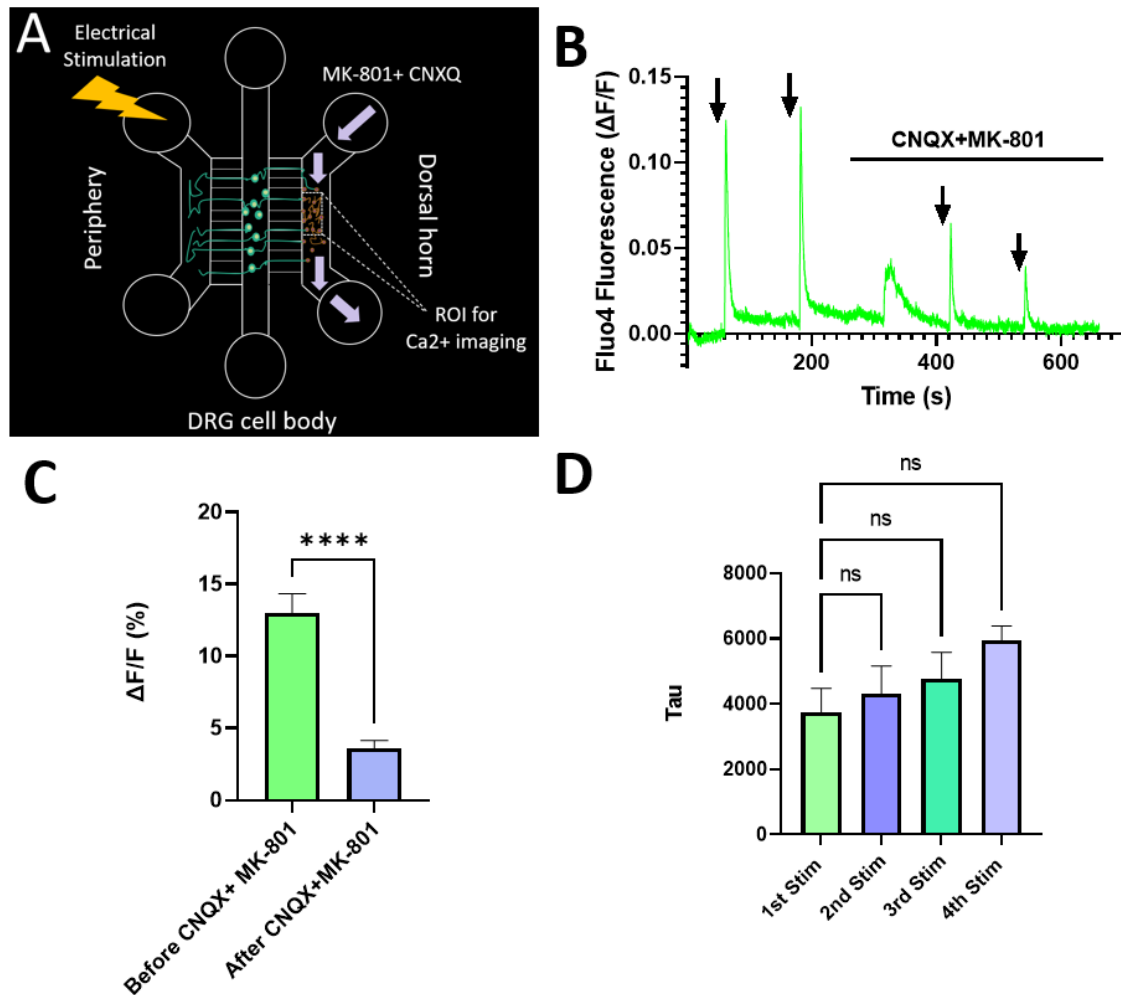


Figure 4.11 Dorsal horn neuron responses to electrical stimulation in peripheral axons before and after glutamate receptor blockers application **A)** Schematic of the experimental setup. Stimulation of the peripheral compartment (left) with the DH compartment (right) as the readout for calcium imaging. After two stimulations, the glutamate receptor blockers CNQX (20 μM) and MK-801 (1 μM) were applied and then two more stimulations under the presence of the blockers were made. **B)** Representative trace showing changes in calcium influx of DH cell bodies following 2 applications of electrical stimulation in the absence and 2 applications of electrical stimulation in the presence of glutamate receptor blockers. **C)** Comparison of the average amplitude of responses before and after application of the glutamate blockers (Welch's t-test, **** $p < 0.0001$, $n = 3$ devices from 3 cultures). **D)** Decay time for DH responses to return to baseline represented as tau. The addition of CNQX and MK-801 did not alter the decay time following electrical stimulation of the peripheral axons (ns $p > 0.05$, Brown-Forsythe and Welch ANOVA, $n = 10$ neurons, $n = 3$ devices from 3 cultures).

Last, we hypothesized that glutamate receptor blockade should not play a role in the direct excitability of the DH cell bodies. We applied electrical stimulation and recorded directly from the DH neurons in the presence and absence of the glutamate blockers (**Figure 4.12A**). As expected, changes in membrane excitability after direct depolarization are not affected by NMDA and AMPA receptors which induce excitability via ligand-gated mechanisms (**Figures 4.12B&C**). The only contingency where NMDA and AMPA receptor blockade could affect DH cell body responses, would be in axo-axonic DH synapses. However, very few DH neurons that responded to direct electrical stimulation were activated in axo-axonic synapses, hence they have not altered the lack of significance of the results. Last, the decay of calcium responses evoked by repetitive electrical stimulation of the DH neurons did not change (**Figure 4.12D**). Indeed, we did not observe any changes in the kinetics and amplitudes of electrical stimulation-elicited responses. The result also diminishes any potential doubt about artifactual responses in our previous experiments.

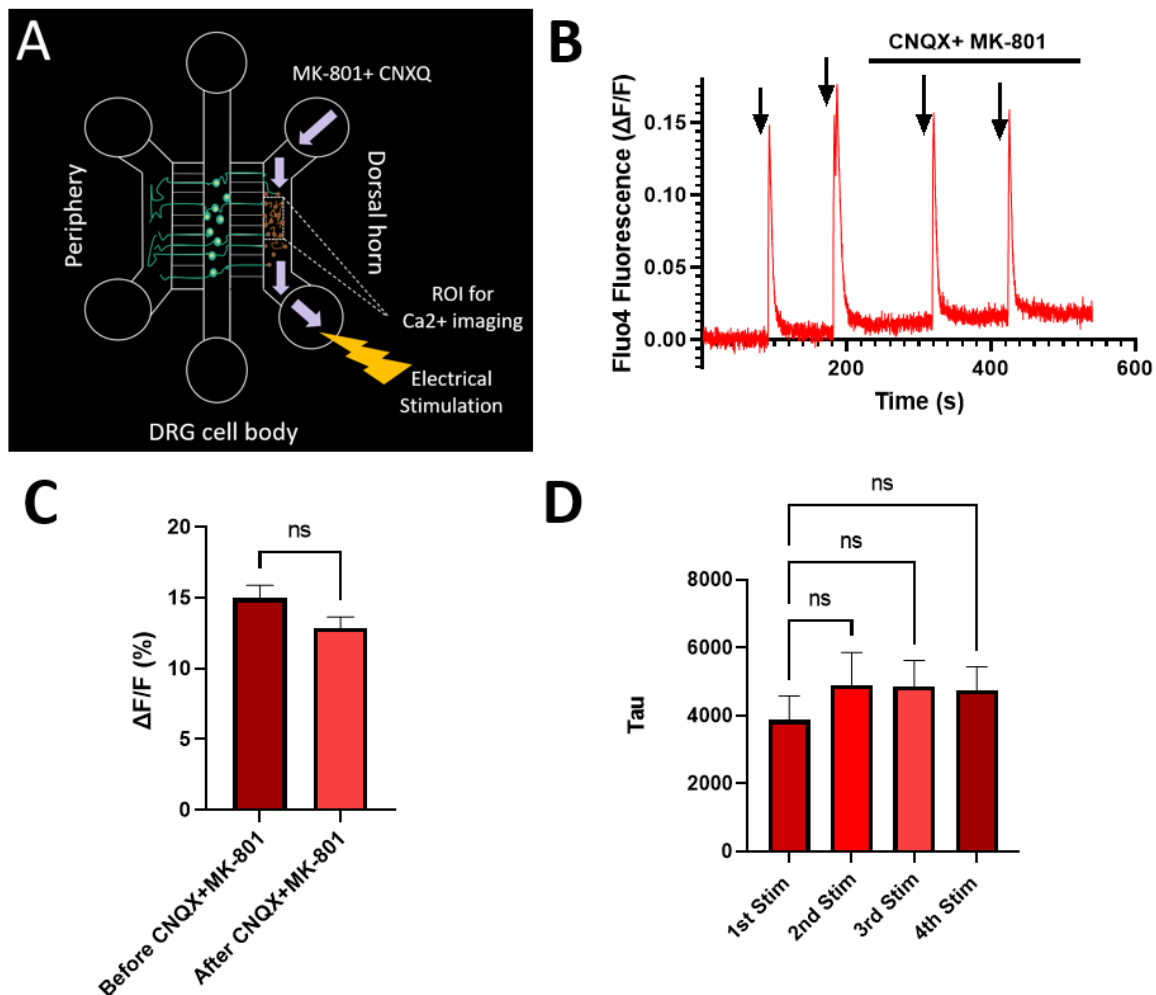


Figure 4.12 Dorsal horn responses to direct electrical stimulation before and after glutamate receptor blockade **A)** Schematic of the experimental setup. Stimulation of the DH compartment (right) combined with calcium imaging in the same compartment. Following two trains of stimulation, the glutamate receptor blockers CNQX (20 μ M) and MK-801 (1 μ M) were applied and then two more stimulations under the presence of the blockers were made. **B)** Representative trace showing changes in calcium influx of DH cell bodies following 2 applications of electrical stimulation in the absence and 2 applications of electrical stimulation in the presence of glutamate receptor blockers. **C)** Comparison of the average amplitude of responses before and after application of the glutamate blockers. The application of CNQX and MK-801 does not affect the amplitude of DH neuron responses to electrical stimulation in the same compartment (ns $p < 0.05$, Welch's t-test, $n = 2$ devices from 2 cultures). **D)** Decay time for DH responses to return to baseline represented as tau. (ns $p < 0.05$, Brown-Forsythe and Welch ANOVA, $n = 24$ neurons, $n =$ devices from 2 cultures).

Taken together, we consolidate the existence of glutamatergic signaling between DRG and DH neurons in our model system, a feature that signifies the accuracy of the recapitulation of the *in vivo* anatomical features of the peripheral nociceptive circuit and the conditions of the first sensory synapse. A caveat of the experimental design regards the separate application of the two blockers. Potential discrepancies between the reduction induced by CNQX and by MK-801 could provide insight on the respective magnitude of AMPA and NMDA receptor contribution to DH neuron excitation. More, the lack of complete silencing of DH responses following DRG or peripheral axonal stimulation, may point towards two contingencies: either that a higher dose of the compounds might be needed for complete silencing of DH neuron responses, or, that other molecules such as Substance P or CGRP are contributing towards DH excitability in this assay. Nevertheless, the evidence provided are robust and clearly showcase the glutamatergic component of DH neuron excitability.

4.2.8 Axotomy as a surrogate model of neuropathic pain in microfluidic co-cultures

Following a thorough characterization of the first sensory synapse using the microfluidic co-culture platform, we wondered which pain disease models can be examined using it. We showed in the previous chapter that we can model inflammatory pain mediator activity in sensory axons. In this chapter, we pursued to model peripheral nerve injury, a common etiology of neuropathic pain. As mentioned in the introduction, neuropathic pain is a complex pathology, where nerve lesion or damage results in alterations in several different sites of the pain pathway (**Campbell and Meyer, 2006**). The platform, by design, can discriminate and readily investigate between different anatomical sites of nociceptors. We chose to focus on how peripheral axotomy affects synaptic transmission and subsequently the excitability of DH neurons (**Figure 4.13**).

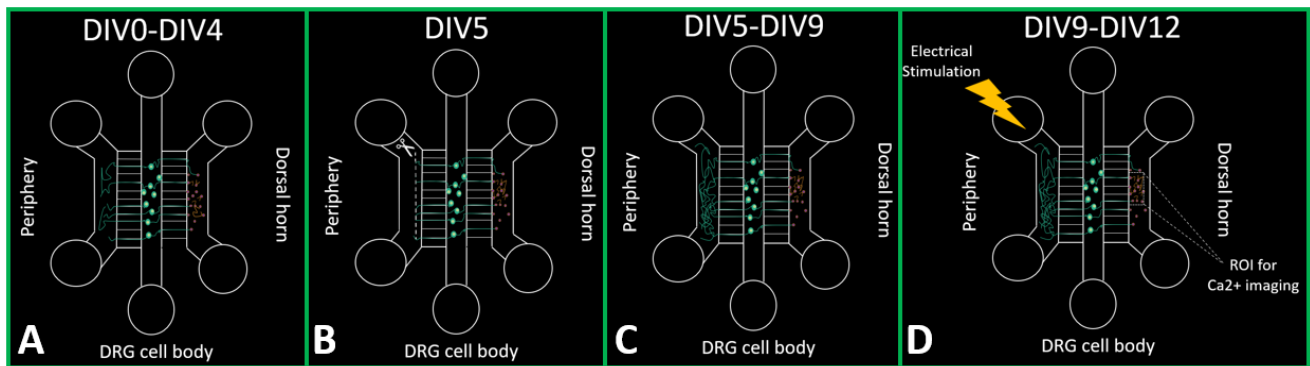


Figure 4.13 Schematic protocol of the axotomy model in microfluidic co-cultures between DRG and DH neurons **A**) From days in vitro (DIV) 0 to 4, the DRG neurons and DH neurons were allowed to grow neurites and sprout **B**) On DIV5, axotomy was induced in the axons that sprouted to the peripheral compartment (left) **C**) The axons of peripheral neurons were allowed 4 more days (DIV5-DIV9) to re-sprout following the axotomy. All other neuronal compartments have been left intact. **D**) Since the peripheral axons have re-sprouted, calcium imaging of the DH neuronal compartment was used between DIV9-DIV12 (right) to measure changes in excitability following electrical stimulation of the peripheral axons where axotomy had been induced (left).

We devised a protocol (see Methods), where upon co-culturing DRG and DH neurons and allowing the neurons to sprout neurites for 5 days (**Figures 4.13A**), we performed axotomy of the axons found in the peripheral compartment (**Figures 4.13B**). We then allowed them to re-grow for another 4 days (**Figures 4.13C**). After re-growth of the axotomized neurons, we reprised the protocol for calcium imaging by stimulating the peripheral axons and recording changes in ensued

excitability of DH neurons in the DH compartment (**Figures 4.13D**). Comparisons were then made in matching experiments between intact and axotomized cultures, where DH responses were recorded following stimulation of the axonal compartment.

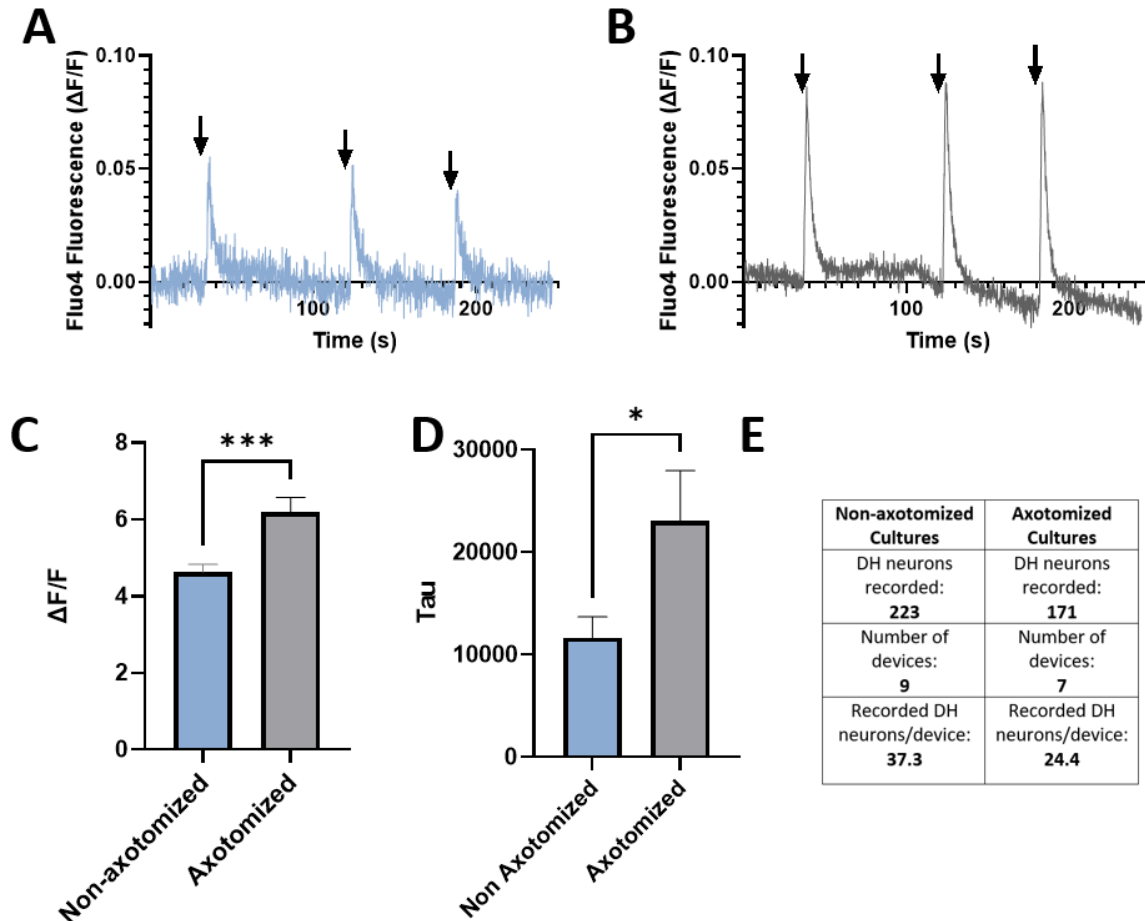


Figure 4.14 Comparison of DH responses to sensory axonal stimulation of axotomized vs non-axotomized cultures. **A)** Representative trace showing responses of DH cell bodies following electrical stimulation of sensory axons in non-axotomized cultures (8 biphasic pulses at a 4Hz frequency x 2 ms @4 μ A). **B)** Representative trace showing responses of DH cell bodies following electrical stimulation of sensory axons in axotomized cultures **C)** DH neurons in axotomized cultures display greater calcium responses following stimulation of peripheral axons. (Welch's t-test, *** $p < 0.0001$, $n = 9$ devices from 5 non-axotomized cultures and $n = 7$ devices from 4 axotomized cultures) **D)** Decay time of DH neuron responses represented as tau following stimulation sensory axons. The decay time of DH neuron responses following axotomy is significantly longer than the non-axotomized responses (Welch's t-test, * $p < 0.0347$, $n = 334$ neurons, $n = 9$ devices from 5 non-axotomized cultures and $n = 254$ neurons, $n = 7$ devices from 4 axotomized cultures) **E)** Table of the number of responder DH neurons between axotomized and non-

axotomized devices. The non-axotomized devices had more DH responder neurons than the axotomized ones.

We observed that axotomy at DIV5 induced a peripheral resprouting of axons, in agreement with previous observations (Seijffers et al,2007), (Vysokov et al,2019). Through electrical stimulation of the peripheral axons, we then compared the excitability of DH neurons in axotomized and non-axotomized devices. We found that, using the same stimulation parameters, DH neurons in devices receiving input from axotomized sensory axons evoked larger responses to electrical stimulation in comparison to the DH neurons that received input from non-axotomized peripheral compartment (**Figure 4.14C**). This result implies that potential changes in the excitability of sensory neurons due to axotomy results in enhanced synaptic transmission and excitability of DH neurons. Moreover, we quantified the decay time of the Ca responses in the activated DH neurons to return to baseline using tau, a time-constant for a mono-exponential decay function fit to the traces. We observed that the DH responses of axotomized cultures had a larger decay time in comparison to the non-axotomized ones, suggesting a longer and amplified DH activation duration (**Figure 4.14D**). Another compelling observation regarded the number of DH neurons that we recorded activity from. We observed on average 37.3 DH cell bodies per device in non-axotomized cultures, while recorded from 24.4 DH cell bodies per device in axotomized ones (**Figure 4.14E**). While this observation could be due to stochasticity in the quality of recorded devices, it might also imply a certain loss of DH neuron density when in contact with axotomized neurons. If true, the observation would corroborate that the peripherally induced hyperexcitability that results in enhanced DH responses, eventually triggers apoptosis in those DH neurons. Taken together, we find that axotomy induces larger in amplitude and longer in duration depolarization of DH neurons, revealing that ensued changes in the excitability of sensory axons influence synaptic transmission mechanisms across the first sensory synapse. The observation that peripheral nerve injury promotes a transition towards a hyperexcitable state of DH neurons may provide general insight into the development of neuropathic pain. It most importantly suggests that peripheral plasticity, probably exerted as a transcriptional re-organization of sprouted sensory axons can be the driver towards central nervous system sensitization.

4.3 Discussion

The establishment of a microfluidic culture model of the first sensory synapse is an essential contribution to our ability to study the pain pathway. While *in vivo* studies have provided insights into the properties of DRG or DH cell bodies in pathological pain models, there has been less knowledge about the pre-synaptic mechanisms of painful signaling transmission (**D'Mello & Dickenson,2008**). Given that the primary afferent neurons are perhaps the longest cells of the mammalian nervous system, it is important to investigate their properties with a more precise spatial resolution (**Clark et al,2008**). Other studies have focused on either the nerve endings of nociceptors (the distal axonal side), or central mechanisms in the dorsal horn. At the same time, conventional culture methods have focused predominantly on the cell bodies of nociceptors.

Until recently, there have been scarce studies of DH neuron properties *in vitro*, as these cells are challenging to survive in culture. Last, due to such survival issues, most of the *in vitro* work has been performed in embryonic rat slice cultures in microfluidic co-cultures (**Vysokov et al,2019**) and not postnatal mice. Previous efforts of co-culturing DRG and DH neurons were performed using a variation of the Campenot chambers. Those experiments were performed in a 2-compartment configuration in rats, where the stimulation of DRG cell bodies could not be discriminated from the sensory axons (**Vikman et al,2001**). Another previous study utilized calcium imaging to record fluorometric responses to noxious stimuli, but the DRG and DH neurons were co-cultured in the same compartment, hence there was limited precision in the pharmacological manipulations (**Ohsiro et a, 2007**).

The microfluidic model of the first sensory synapse has resolved several of these issues. We demonstrated that utilizing a 3-compartment configuration we can isolate and study the 3 different important anatomical sites, namely the distal axonal, the DRG cell body and the proximal site/DH central neuron sites. We showed that mouse DRG neurites bilaterally cross towards the two corner peripheral compartments of the microfluidic devices; a configuration that allows separation of the sensory axons in one compartment and the recapitulation of synaptic site with the co-cultured DH neurons in the other compartment. Through this configuration, it is possible to locally apply pharmacological agents, quantify evoked-responses and subsequently measure excitability using fluorometric techniques. Moreover, the use of postnatal mice opens the possibility of combining pharmacological studies of the first sensory synapse with the available genetic models.

Non-neuronal cells have been shown to modify the function of neurons in many contexts including pathological pain **(McMahon & Malcangio,2009) (Ji et al,2013)**. The examination of synaptic transmission between DRG and DH neurons in the absence of non-neuronal cells, was achieved with the application of the mitotic inhibitor, AraC. While this strategy is advantageous for purely studying neuronal properties of DRG and DH neurons, it also poses a double-edged sword. There are peripheral glial cells in the DRG compartment such as Schwann cells and satellite glial cells which are contributing to peripheral pain plasticity **(Abdo et al,2019) (Jager et al,2020) (Rinwa et al,2021)**. Similarly, there are resident microglial cells and astrocytes which have a long-established role in modulating spinal cord excitability **(Marchand et al,2005) (McMahon & Malcangio,2009)**. By eliminating these non-neuronal cells from our model, we are also missing their potential contributions following persistent stimulation or in our axotomy model variation. However, an advantage of the model is the potential to “add back” the missing non-neuronal cells. Another important caveat of the *in vitro* recapitulation of the first sensory synapse is the lack of somatotopic alignment between sensory neurons and DH neurons **(Todd, 2010)**. As mentioned, at the first relay point of the pain pathway, nociceptors innervate the central sites of distinct laminae of the dorsal horn, specifically laminae I and II. However, with the current *in vitro* techniques, this anatomical feature is not maintained, since both DRG and DH neurons are dissociated and randomly distributed in the devices.

Surprisingly, certain elements of the somatotopic alignment are present in these cultures. From a previous study, we have evidence in microfluidic DRG cultures for mainly the survival of small-diameter, unmyelinated sensory neurons **(Tsantoulas et al,2013)**. Concomitantly, the immunocytochemistry experiments at the DH compartment illuminated the identity of the cultured cells, as spinal cord dorsal horn neurons. The MAP2 staining confirmed that these neurons are indeed central and secondarily, through the VGLUT2 staining, they were identified as glutamatergic. The glutamatergic nature of sensory neurons has been well-established together with the excitatory nature of primary afferents synapsing at the dorsal horn **(Wall,1964) (Dale & Kandel,1993)**. Thirdly, via the expression of Synapsin1, we showed that these central, glutamatergic neurons form active synapses within that compartment. As mentioned, the VGLUT2-expressing dorsal horn neurons are predominantly localized in laminae I and II of the dorsal horn **(Todd et al,2003) (Moechars et al,2006)**. It is truly compelling how within a compartment consisted primarily of nociceptor axons, there are synapses formed between them

and glutamatergic DH neurons of their somatotopically correspondent laminae. Recently, a gene locus consisted of 22 γ -protocadherins has been identified as the regulators of synapse formation of touch (low-threshold mechanoreceptors or LMTRs) (Meltzer et al,2023). In neurons of the CNS, the pre-synaptically expressed Latrophilin 1 and its post-synaptically expressed ligand Lasso/Teneurin-2 guide synapse formation (Silva et al,2011). Previous studies have identified axonal guidance cues of peripheral sensory axons, such as the Slit protein (Wang et al,1999) and the Runx3 which is responsible for the guiding the dorso-ventral termination of cutaneous DRG axons into the spinal cord (Chen et al,2006). The *in vitro* laminar targeting of DRG axons to distinct DH neuronal bodies requires much further investigation. It elicits important questions about the temporal expression of such synapse formation cues throughout the postnatal life of sensory neurons both *in vivo* and *in vitro*. Complementary questions relevant to our model study of axotomy, regard the re-branching properties of nociceptors. It is evident yet not fully understood how the molecular (Renthall et al,2020) and anatomical (Gangadharan et al,2022) re-modelling of nociceptors following nerve injury can alter synapse formation and consequently central excitability.

Through the combination of electrical stimulation of different compartments with calcium imaging, we methodically established the functionality of our model. We found that the stimulation of sensory axons produced calcium responses of DRG cell neurons and that repetitive stimulations did not alter the amplitude of the responses elicited. We therefore showed that our protocol does not induce electrode fatigue or desensitization of DRG neurons. We further showed through stimulating the DH neurons directly, that they are healthy and amenable to electrical excitability. More importantly, we studied the synaptic transmission properties between the DRG and DH neurons. Unlike the cumbersome recording of DH activity using patch-clamp electrophysiology, the utilization of Fluo4 allowed the large-scale recording of DH cell body responses to DRG stimulation.

Stimulation of the axonal or DRG compartment elicited robust responses from the DH cell bodies confirming our observation of functional synapses between the two neuronal types. Antithetically to the concerns about electrode fatigue or desensitization confounding the DRG responses, in the DH compartment, the repeated stimulation could potentially confound our results due to progressive changes in excitability of DH neurons after successive stimulations. Since 1965

(Mendell and Wall,1965), it is known that the repetitive and high-frequency stimulation of nociceptors may amplify and prolong DH responses, a phenomenon that later was termed ‘wind-up’ (Dickenson & Sullivan,1987) (Davies & Lodge,1987). The cumulative depolarization of the postsynaptic DH neurons occurs due to the summation of slow excitatory post-synaptic potentials (EPSPs) which result in the windup (Sivillotti,1993). It has been shown that wind-up is dependent on glutamatergic transmission from nociceptors to wide-dynamic range neurons (WDRs) (Thompson et al,1990). The windup of DH neurons has been shown to be dependent on NMDA receptors (Dickenson & Sullivan,1987) (Davies & Lodge,1987). A more recent GWAS study in humans and confirmed with experimental mouse models, identified the sodium-calcium exchanger type 3 (NCX3) being responsible for regulating calcium efflux and underlying the DH neuron temporal summation manifested as wind-up (Trendafilova et al,2022). Through our stimulation strategy, we did not observe changes in the amplitude or the decay time of DH evoked responses, hence our study does not exhibit any wind-up features. Wind-up is a frequency dependent phenomenon and potentially with supramaximal frequencies, currents or more stimulus applications, the DH neurons could exhibit windup *in vitro*. In the microfluidic model, however, our aim was to establish a protocol where the baseline responses are not altered by the repetitive stimulation, as it may have confounded the interpretation of the experiments involving the pharmacological manipulation of DH neurons.

It is known that synaptic transmission between primary afferent fibers and DH neurons is dependent on glutamate release. Early evidence in explants of embryonic rat DRG and DH neurons showed that glutamate was the amino acid transmitter responsible for eliciting excitatory EPSPs in the synapses formed (Jahr & Jessell,1985) (Jessell, Yoshioka & Jahr,1986). While the authors of these studies did not observe that NMDA antagonism was effective towards EPSPs attenuation it was later found that the post-synaptic NMDA receptors are essential for ligand-binding of glutamate, along with a role for AMPA receptors (Chapman & Dickenson, 1992) (Kohno et al,2008). Therefore, we were compelled to test the dependence of DH responses to glutamate receptor binding and hence to demonstrate that the activation of DH neurons in response to stimulation of DRG axons is likely due to the activation of excitatory synapses We employed a selective AMPA receptor blocker, CNQX (Blake et al,1988) and a selective NMDA receptor blocker, MK-801 (Wong et al,1986). We found that the blockade largely attenuated the peripherally evoked responses, providing evidence for the glutamatergic transmission between

DRG and DH neurons in microfluidic cultures. While the DH-evoked responses were attenuated following glutamatergic blockade, they were not completely abolished. The observation may have several explanations: first, the concentrations used may not have been sufficient to fully block the receptors. Second, yet far-fetched, it is possible that certain neurons on the DH compartment forming synapses may have been between DRG axons and inhibitory GABA-ergic interneurons, which are not NMDA/AMPA dependent. Third, and perhaps quite compelling, would be the concomitant dependence of the DH neurons to more than one neurotransmitters acting together with glutamate, such as Substance P and CGRP (Oku et al,1987) (Lagerstrom et al,2011) or nitric oxide, although limited evidence exist for expression of nitric oxide synthase (NOS) expression in laminae I and II of dorsal horn neurons (Sardella et al,2011). Despite the attenuation in the amplitude of DH responses due to the glutamatergic blockade, we did not observe significant changes in the decay rate of responses. This implies that the DH neuron calcium efflux mechanism remains unaltered in the wild-type mouse model, perhaps in compliance with the recent finding of the sodium-calcium exchanger NCX3, which was found to regulate prolonged temporal responses of DH neurons in mice lacking it (Trendafilova et al,2022). In summary, the evidence suggests the presence of glutamatergic transmission between DRG and DH neurons in the microfluidic model of mouse first sensory synapse.

It has been discussed in the introduction, that neuropathic pain is caused by damage or disease that affects the somatosensory nervous system. Several *in vivo* animal models of neuropathic pain have been developed, including that of spared nerve injury (SNI) and spinal nerve ligation (SNL) (Jaggi et al,2011). The robust establishment and characterization of the microfluidic co-cultures has allowed us to further develop an *in vitro* model to study neuropathic pain mechanisms. We therefore devised an axotomy strategy where we compared DH responses where the input was received following stimulation of DRG axons in culture. The DH responses were thus compared between axotomized and non-axotomized devices containing peripheral neurons. It has been suggested that persistent NMDA receptor activation results to an apoptotic degradation of DH neurons following peripheral nerve injury, accounting for a transition to pain chronification (Inquimbert et al,2018). We recorded from less DH neurons per device in axotomized (24.4) vs non-axotomized cultures (37.3) (Figure 14E), which could reflect a potential loss of synapse-forming DH neurons 96 hours following axotomy. However, this observation does not necessarily

contradict or corroborate the aforementioned study where significant apoptotic evidence appeared after 7 days from the induction of the spared nerve injury (SNI).

We found that DH neurons receiving input from axotomized DRG neurons displayed larger responses and with greater duration in comparison to those receiving input from non-axotomized stimulated axons. This result is of great interest as it suggests that there has been a peripherally induced change which has facilitated the synaptic transmission and has modulated the excitability of DH neurons **(Nguyen et al,2009)**. Previous studies have suggested that long-term potentiation (LTP) mechanisms of spinal synapses account for the sensitization of the dorsal horn even in the presence of nerve injury **(Sandkuhler & Liu,1998)** **(Ikeda et al,2003)**. The induction of acute nerve injury on this occasion was the squeezing of the sural nerve which resembles more a reversible nerve crush rather than an axotomy **(Sandkuhler & Liu,1998)**. Nevertheless, the peripheral hyperexcitability of axotomized nociceptors may provide the basis of the induction of LTP in spinal cord dorsal horn neurons. Distal axotomy of pre-synaptic pyramidal neurons was found to increase glutamate release in CNS neurons **(Nagendran et al,2017)**. An elevated glutamatergic neurotransmission and saturated uptake by the metabotropic glutamate receptor 5 (mGluR5) has been shown in the laminae I and II of the dorsal horn during neuropathic pain **(Inquimbert et al,2012)** **(Osikowicz et al,2013)**. In a neuropathic pain model, it has been shown that uptake of glutamate by DH neurons leads to a large elevation of calcium responses **(Vincent et al,2016)**. Hence, it is plausible that axotomy in microfluidic co-cultures replicates salient features of the nerve damage-induced changes in the first sensory synapse, by undergoing pre- and post- synaptic modulation.

A phenomenon that is not captured by the acute stimulation of axons and subsequent fluorometric readout of DH neurons is the disinhibition of inhibitory interneurons in the dorsal horn. Evidence from neuropathic pain injury models have shown a loss of GABA-ergic inhibition in the superficial dorsal horn as well as a loss of glycinergic inhibition which is known to facilitate NMDA receptor activation **(Sivillotti & Woolf,1994)** **(Moore et al,2002)** **(Ahmadi et al,2003)** **(Harvey et al,2004)**. The spinal disinhibition has been attributed to loss of dorsal horn neurons **(Moore et al,2002)** **(Inquimbert et al,2018)**. At the same time, axotomy induced plastic changes in the primary sensory neurons, among them a downregulation of Substance P and CGRP **(Hokfelt et al,1994)**, along with a transcriptional reprogramming of the sensory neurons **(Renthal et al,2020)**.

Since there is a downregulation of essential pro-nociceptive transmitters, what could then account for the paradoxical phenomenon of the occurrence of painful responses when a nerve is damaged, instead of exhibiting hypoalgesia (**Koltzenburg,1998**)? The general framework would regard that the peripherally induced plasticity due to nerve injury may result in glutamate receptor activation and a temporary LTP of DH neuron excitability (**Ji et al,2003**). Concomitantly, while Substance P is downregulated in small axons, it is produced *de novo* in the large diameter A-fibers and in spinal cord neurons after axotomy (**Noguchi et al,1995**) (**Malcangio et al,2000**). The initial persistent activation of glutamate receptors and the influx of calcium elicited have been shown to induce apoptosis in DH neurons (**Scholz et al,2005**) (**Inquimbert et al,2018**) and this observation might provide the basis of a prolonged, central sensitization of the spinal cord (**Woolf,2011**). However, another group has suggested that it is microglia and not the DH neurons that undergo apoptosis (**Polgar et al,2005**). In a very reductionist demonstration, our experiment showed that axotomized neurons elicit larger and longer DH responses to electrical stimulation within 96 hours following axotomy. The increased neuronal activity as well as the prolonged calcium influx may prove detrimental for the survival of the DH neurons. This may provide a useful link between the temporary, peripheral neuron hyperexcitability after nerve injury and the chronic spinal cord sensitization. It perhaps highlights the importance of understanding the peripheral mechanisms that cause the increased synaptic transmission, an idea that will be examined more thoroughly in the next chapters of this thesis.

In conclusion, we have devised the first *in vitro* model of the first sensory synapse using mice. Our model reprises robustly most salient features of the *in vivo* environment between DRG and DH neurons. We show that the model is amenable to pharmacological manipulations to each distinct respective compartments while it can also become amenable to genetic manipulations. There are two important considerations for improvement: first, it would be important to address whether DH neurons receive monosynaptic or polysynaptic input from the DRG axons that have traversed. Second, it has been so far impossible to distinguish which DH neurons are “innervated” by which specific DRG axons. Viral strategies or the use of a photoconvertible fluorescent probe such as cAMPARI might prove useful strategies for future characterization experiments (**Moeyaert et al,2018**).

The platform can be used to mimic neuropathic pain as shown from our axotomy model. It can also be used for studies of inflammatory pain in a fashion of escalated complexity to the previous chapter where we studied the effects of PGE₂ on sensory axons. Further, it can be used to study developmental aspects of synapse formation and axonal guidance cues between DRG and DH neurons. Other studies using the existing platform could induce a further level of complexity by introducing triple cultures with peripheral non-neuronal cells such as Schwann cells, satellite glial cells, immune cells, or keratinocytes. Conversely, on the DH compartment, CNS glial cells such as microglia and astrocytes could be co-cultured.

Last, the microfluidic platform can be directly utilized as a screening assay of compounds targeting the peripheral nociceptors, where silencing of the DH compartment could be the desired functional output. Whether used as a surrogate model of pain pathology in pre-clinical studies or as a comparatively higher throughput method of drug screening by both academia and industry, the microfluidic model can provide valuable insight on the cellular and molecular physiology of the first sensory synapse.

5. The contribution of Voltage-Gated Sodium and TRP channels to transmission within the first sensory synapse

Aims and objectives.

Utilizing the DRG-DH microfluidic co-culture model, this chapter aimed to examine the pre-synaptic roles of certain ion channels which are widely expressed in sensory neurons. The investigation was separated into two conditions, where the pre-synaptic roles of the channels were compared in axotomized and non-axotomized cultures.

- 1) It was hypothesized whether Nav1.7, Nav1.8 and Nav1.6 channels contribute to synaptic transmission in intact and axotomized cultures.
- 2) The examination of concomitant sodium channel blockade was proposed to evaluate whether synaptic transmission is masked by non-selective sodium channel blockade.
- 3) It was hypothesized whether TRPV1, TRPA1 and TRPM8 channels contribute to synaptic transmission in intact and axotomized cultures.
- 4) The pre-synaptic plasticity of nociceptors in axotomized cultures was sought to be examined, advocating in favor or against the development of certain analgesic targets.

This chapter utilizes the DRG-DH microfluidic co-cultures to examine the pre-synaptic role of selected Nav and TRP channels as modulators of pre-synaptic excitability and facilitators of neurotransmitter release. The role of these channels is further examined under axotomized conditions to evaluate whether they contribute to synaptic plasticity when peripheral nerves are injured. The hypothesis is compelling as Nav channels have been mostly considered as contributors to the propagation of action potential across sensory axons while TRP channels have been traditionally considered as transducer molecules in the distal axonal sites of nociceptors.

5.1 Introduction

The electrogenic identity of sensory neurons has maintained an eminent position in the study of nociception and pain. The Sherringtonian notion that specialized free nerve endings called nociceptors, detect and process noxious stimuli (**Sherrington,1906**), was unequivocally demonstrated independently by Iggo in the UK Perl in the US and their colleagues in electrical stimulation studies of myelinated and unmyelinated fibers (**Iggo,1960**) (**Burgess & Perl,1967**) (**Bessou & Perl,1969**), expressing an advocacy towards a theory of pain specificity. In the late 1940's, the wife-and-husband team of Aurelia Jancsó-Gabor and Nicholas Jancsó had demonstrated that exposure to capsaicin, a pungent chemical product found in chili peppers, produces a desensitization of sensory nerve endings (**Jancsó, & Jancsó,1949**). The work by the Jancsós acted as a proof-of-concept confirmation that an exteroceptive noxious stimulus may alter the excitability of sensory neurons. Almost a century earlier, Albert Niemann had isolated an alkaloid extract from coca leaves which he aptly named cocaine (**Niemann,1860**). Two decades later, Vassily von Anrep first reported the anesthetic properties of cocaine after injecting it under his own skin (**Von Anrep,1880**). The Austrian ophthalmologist Karl Koller, having been prompted by the cocaine aficionado Sigmund Freud, performed the first operation under cocaine-induced local anesthesia to remove a glaucoma (**Koller, 1884**) (**Bezerra et al,2020**).

The 19th century physicians had no mechanistic knowledge on how local anesthesia was produced. It was around Jancsós' time, that Hodgkin and Huxley indirectly set the foundations for unravelling this phenomenon. They discovered in their pioneering voltage-clamp experiments the existence of sodium and potassium ionic contributions to AP propagation in the giant squid axon (**Hodgkin & Huxley,1952a**) (**Hodgkin & Huxley,1952b**). The electrophysiological observations of Hodgkin and Huxley which changed the course of neurophysiology, were taken up later by the somatosensory field, when it was hypothesized that the excitability of mammalian sensory nerves depended on such ionic mechanisms. It was only natural to postulate that the mediation of sodium and potassium ionic flows was occurring via specialized channels that would enable a selective cationic permeability (**Ritchie & Rogart,1977**) (**Waxman,1977**). As early as the 1970's, Hans Kosterlitz, the discoverer of endogenous opioid peptides, hypothesized that peripheral enkephalin effects may be modulated by sodium channels (**North & Hughes,2013**).

The molecular correlates which facilitate the above-described electrophysiological observations were revealed in the latter part of the 20th century, thanks to advances in cloning techniques and the molecular revolution (**Wood & Iseppon,2018**). The study of the encoding and expression of channels that allow the passage of ions across the otherwise impermeable lipid bilayers in nervous tissue has since blossomed and been studied extensively (**Hille,1978**). In a triumphant discovery, the first crystallographic structure of a potassium channel by the Mackinnon group demonstrated how an ion channel's architecture may underlie the physical principles of cationic conduction (**Doyle et al,1998**). In the most recent decades, the development of cryo-electron microscopy (Cryo-EM) has widely accelerated the determination of the macromolecular structures of ion channels and the acquisition of high-resolution structural snapshots of ion channel activation and inactivation states (**Rubinstein,2017**).

Fast forward to the present day and with all mentioned developments in mind, it is common knowledge amongst sensory neuroscientists that nociceptors detect chemical, thermal and pressure noxious stimuli. Responsible for the transduction of external stimuli such as capsaicin, are specialized ion channels localized in the free nerve endings of nociceptors. The transducer ion channels distinctly interact with diverse noxious stimuli and convert them to electrical signals (**Dubin & Patapoutian,2010**). It is also common knowledge nowadays that the propagation of APs and the flow of sodium ions is dependent on correspondent voltage-gated ion channels, expressed in the long axons of nociceptors. Local anesthetics such as cocaine or lidocaine render these voltage-gated channels in an inactivated state and disable the propagation of APs across the sensory nerve axons (**Scholz,2002**) (**Tikhonov & Zhorov,2017**).

The identification of ion channels that are responsible for the transduction of noxious modalities and the propagation of actions potentials in nociceptors has been a quest for several research groups. Among the transducer molecules expressed in sensory neurons, none other ion channel family has been as exhaustively studied as that of transient receptor potential (TRP) channels, following the discovery of the capsaicin receptor TRPV1 by Julius and Caterina (**Caterina et al,1997**). Similarly, the VGSCs have been the pursuit of immense research and pharmaceutical drug development, fostered by the landmark discovery that Nav1.7, a subtype widely expressed in sensory neurons, is responsible for the congenital insensitivity to pain manifested in humans (**Cox et al, 2006**). This chapter will examine specific spatial properties of these two ion channel families.

The pre-synaptic expression of the channels in the spinal cord has compelled us to examine if in addition to being nociceptive transducers (in the case of TRP channels) or involved in the propagation of pain signals (in the case of Nav channels), they also modulate synaptic transmission. A background is provided on the nomenclature, the structural and the functional details of these fascinating channels, with a focus on their role within the pain pathway and relevant pathological pain states.

5.1.1 The TRP channel family

The TRP channel superfamily is consisted of approximately 30 subtypes with distinct structures and functions (**Ramsay et al,2006**). The existence of TRP channels was identified in *Drosophila* fruit flies where they contribute towards phototransduction (**Cosens & Manning, 1969**) (**Scott & Zuker,1998**). The first TRP channel was cloned in *Drosophila* in 1985 (**Montell et al,1985**) and the first cloned mammalian TRP was a human homolog of that channel (**Wes et al,1992**). The mammalian TRP channels are separated into six protein families: the TRP canonical (TRPC), the TRP vanilloid (TRPV), the TRP ankyrin (TRPA), the TRP melastatin (TRPM), the TRP polycystin (TRPP) and the TRP mucolipin (TRPML) channels. The families share small homology and present considerable diversity in their cytoplasmic domain architecture. General structural similarities regard the formation of six-transmembrane polypeptides that exhibit a tetrameric assembly to form cation-permeable pores via a loop between spanning segments 5 and 6 (**Clapham,2003**).

The mammalian TRP channels exhibit tremendous versatility in their functions: they mediate phototransduction in human melanocytes (**Bellono et al,2013**) and enable infrared detection by bats (**Gracheva et al,2011**). Distinct TRP isoforms are expressed in the tongue to enable taste coding (**Chandrashekar et al,2016**) while they determine biological sex recognition via pheromonal cues (**Stowers et al,2002**). Several hibernating mammals exert adaptive changes to TRP channel function due to the thermoreceptive properties of the channels (**Hoffstaetter et al,2018**), while others survive under extreme conditions due to their TRP sensory adaptations (**Laursen et al,2016**).

Most relevant to the field of somatosensation, several TRP channel subtypes are responsible for detecting heat, cold and chemical irritation (**Patapoutian et al,2003**) (**Caterina & Tominaga,2004**). In 1988, Wood, Bevan and colleagues observed increases in calcium influx in response to direct application to capsaicin in sensory neurons (**Wood et al,1988**). Cesare and McNaughton discovered a heat-activated current in nociceptors which was sensitized by bradykinin in a PKC-dependent manner, paving the way for the cloning of the inflammatory mediator and heat-activated TRP channels (**Cesare & McNaughton,1996**). The Julius lab at UCSF eventually cloned TRPV1, then called VR1, the first discovery of a heat activated receptor expressed in sensory neurons (**Caterina et al,1997**). Colloquially referred to as “the capsaicin receptor”, TRPV1 responds to noxious heat stimulation ($>43^{\circ}\text{C}$) and protons, it is Ca^{2+} -permeable and its activity is enhanced by nerve growth factor and bradykinin, while it is inhibited by PIP_2 (**Tominaga et al,1998**) (**Chuang et al,2001**). Other heat sensing TRPV channels are TRPV2, TRPV3 and TRPV4. TRPV2, a homologue to TRPV1, has a higher heat activation threshold ($>52^{\circ}\text{C}$), it is widely expressed predominantly in large and medium myelinated fibers as well as in other tissues (**Caterina et al,1999**). There is increased temperature detection by TRPV3 ($>31^{\circ}\text{C}$) (**Smith et al,2002**) (**Xu et al,2002**) and TRPV4 ($>25^{\circ}\text{C}$) (**Guler et al,2002**) (**Vriens et al,2004**) which are both expressed in sensory neurons and other nervous tissue, with TRPV4 plays additional roles in adipocyte metabolism and inflammation (**Ye et al,2012**). The TRPM3 channel was later reported to respond to extreme heat conditions (**Vriens et al,2011**). Only the combination of multiple TRP channel blockade is sufficient to block all noxious heat responses, posing an important consideration for the use of knockout models for accurate behavioral readouts, as well as the susceptibility of genetic mutants to developmental compensation of function (**Vanderwauw et al,2018**). The TRPM2 channel from the melastatin family has been found to encode moderate heat responses between 23°C and 38°C (**Tan & McNaughton,2016**). Very recently, the TRPC5 channel was identified to be involved in inflammatory and mechanical behavioral responses following chemical irritation with lysophosphatidylcholine (LPC), yet it is not known which modality between heat, pressure, or chemical irritation it is responsive to at the single DRG neuron level (**Sadler et al,2021**).

Regarding the noxious cold molecular transduction, the TRPM8 channel was discovered to mediate the effects of menthol and cold temperatures ($<25^{\circ}\text{C}$) and is recognized as the bona fide noxious cold sensor channel (**McKemy et al,2002**) (**Peier et al,2002**) (**Bautista et al,2007**). The

TRPA1 channel was cloned and characterized in sensory neurons a year later and was initially proposed to mediate cold temperatures ($<13^{\circ}\text{C}$) (**Story et al,2003**). Further studies showed in fact that TRPA1 predominantly transduces noxious stimulation evoked by pungent isothiocyanate compounds such as mustard oils, cannabinoids and by the endogenously secreted inflammatory peptide bradykinin (**Bandell et al,2004**) (**Jordt et al,2004**). It is now established that TRPA1 is essential to mediating several environmental irritants of mechanical, and chemical nature together with inflammatory agents (**Kwan et al,2006**) although its role in detecting environmental cold stimuli is equivocal.

The thermoreceptive properties of TRP channels have been closely associated with nociception and pain sensation. Both external and endogenous stimulation may activate TRP channels to produce aberrant excitability and hyperalgesia in sensory neurons (**Basbaum et al,2009**). This was demonstrated early following the identification of TRPV1, where its genetic ablation showed a critical contribution of the channel to thermal hyperalgesia following tissue injury and acute thermal nociception (**Caterina et al,2000**). The gating of TRPV1 by both noxious heat and pungent natural products such as capsaicin and resiniferatoxin has elucidated the channel's role in mediating noxious thermal nociception (**Caterina et al,1999**). In a model of carrageenan-induced inflammation, TRPV1 knockout mice exhibited an absence of thermal hyperalgesia (**Davis et al,2000**). Screening identification of two selective TRPV1 antagonists attenuated neurogenic inflammation evoked by capsaicin but left mechanical responses intact (**Garcia-Martinez,2002**). In a perioperative pain model, TRPV1 blockade abolished heat but not mechanical hyperalgesia while the upregulation of the TRPV1 protein occurred following by the emergence of inflammation but not operative incision, implying that activation of the channel occurs due to inflammatory mediator secretion (**Pogatzki-Zahn,2005**). TRPV1 antagonists suggested for the treatment of inflammatory and neuropathic pain induce hypothermic effects by modulating sensory responses to the spinal cord (**Yue et al,2022**). Moreover, capsaicin administration on spinal cord slices results in the release of glutamate (**Ueda et al,1994**) and the neuropeptides CGRP and Substance P (**Saria et al,1986**). Paradoxically, intradermal injection of capsaicin in high concentrations causes desensitization and a loss of sensory sensation due to the ensuing internalization of the TRPV1, and concomitantly, Nav channels. This strategy is utilized to temporarily palliate acute or chronic pain through capsaicin cream topical application (**Luo et al,2019**) (**Rosenbaum et al,2022**). It has been shown that TRPV1 receptors are expressed both

pre-synaptic in the central terminals of sensory neurons and post-synaptically at the superficial laminae of the dorsal horn in rats (**Valtschanoff et al,2001**), yet their molecular role at the central sites has remained less understood.

Cold sensation would be intuitively considered antithetical to the thermal hyperalgesia induced by inflammation. Yet, cold temperatures at the noxious range evoke acute painful sensation too (**Peier et al,2003**). The TRPM8 knockout studies showed amelioration of behavioral responses to noxious cold, but do not fully account for cold avoidance, suggesting the involvement of other transducer molecules in low-threshold cold detection (**Bautista et al,2007**) (**Colburn et al,2007**). The largest prevalence in experiencing cold pain occurs in conditions such as diabetic neuropathy, chemotherapy-induced peripheral neuropathy (CIPN) and ciguatera poisoning (**Vetter et al,2012**) (**Yin et al,2015**). A “triple cold” syndrome of peripheral polyneuropathy has been reported where abolished A δ -fiber input shifts the mediation of cold pain to C-fiber nociceptors (**Ochoa & Yarnitsky,1994**). Also, long exposure to cold stimulation produces the so-called “trench foot” syndrome where it eventually leads to the development sensory neuropathy (**Vale et al,2017**). Only a subset of small diameter C-fibres expresses widely the TRPM8 channel, suggesting a defined neuronal subtype that processes cold input (**Usoskin et al,2015**), where 90% of the TRPM8-expressing subtype respond to cooling in the trigeminal ganglia (**Yarmolinsky et al,2016**). An epiphenomenal symptom of certain neuropathic pain forms is the development of cold allodynia, where patients experience colder temperatures in an amplified manner in comparison to healthy individuals (**Lolignier et al,2016**). While cold sensation is established to be sensory neuron-mediated, the potential mechanisms through which cold allodynia is conferred are more complex. The proposed mechanisms thus far are 3: one regards that threshold shifts occur due to potassium channel downregulation, another considers the transcriptional plasticity of cold-insensitive nociceptors to convey cooling responses and last that the sensitization to noxious cold input occurs exclusively at the central level with no alterations in peripheral detection and processing (**MacDonald et al,2020**).

The TRPA1 channel was initially reported to function as a cold-transducing channel (**Story et al,2003**). It is paradoxically associated with cold hypersensitivity following injury but not with acute cold sensation (**Knowlton et al,2010**). The TRPA1 channels are expressed in nociceptors at different anatomical locations such as the distal axons, the cell bodies and the central terminals.

The role of neuronal transduction of TRPA1 channels is well characterized. Evidence exists for the pre-synaptic expression of the channel in primary afferents where administration of AITC increases the amplitude and frequency of spontaneous EPSCs, thus also suggesting in synaptic transmission **(Kosugi et al,2007)**. The channels have been implicated in the contribution to inflammatory pain. In an isothiocyanate-inducing model of osteoarthritis pain in rats, a selective TRPA1 antagonist attenuated cold allodynic symptoms without reducing the cold sensation perception **(Chen et al,2011)**. However, TRPA1 is widely regarded as a versatile mediator of chemical nociception of exogenous or endogenous origin that mediate acute and inflammatory pain sensation **(Nassini et al,2014)**. Several environmental irritants and pro-algesic agents have been identified among them mustard oil, the air pollutants acrolein and formalin and the antifungal drugs clioquinol and pyrithione **(Andersson et al,2009) (Julius,2013)**. TRPA1 is activated also by endogenous inflammatory mediators, namely bradykinin, ATP and nitric oxide **(Bandell et al,2004) (Jordt et al,2004) (Miyamoto et al,2009)**. Synthetic cannabinoid agonists have been shown to inhibit the activation of TRPA1 and attenuate sensory neuron responses to capsaicin and mustard oils **(Akopian et al,2008) (Sagar et al,2009)**. It has been further demonstrated that acetaminophen (paracetamol) and Δ^9 -tetrahydrocannabinol target TRPA1 to promote a spinal antinociceptive effect **(Andersson et al,2011)**. A model of formalin induced pain has shown that pharmacological blockade or genetic ablation of TRPA1 ameliorates the evoked nociceptive responses **(McNamara et al,2007)**. Similarly, during CFA-induced inflammation, TRPA1 activation was found to enhance mechanical sensitivity and hyperalgesia, together with an upregulation in TRPA1 expression **(Obata et al,2005) (Lennertz et al,2012)**. A neuropathic injury model of spinal nerve ligation also highlighted a role for TRPA1-mediated sensitization **(Obata et al,2005)**. Again, in a sciatic nerve injury model **(Staaf et al,2009)** it was shown that TRPA1 mediates thermal and mechanical hyperalgesia. Several models of diabetic neuropathic pain and CIPN have also involved the channel in thermal and mechanical hyperalgesia **(Nassini et al,2014)**. Last, a human genetic role of the channel has been confirmed where a gain of function mutation of TRPA1 underlies an autosomal-dominant familial episodic pain syndrome condition (FEPS) **(Kremeyer et al,2010)**.

5.1.2 The voltage-gated sodium channel family

In contrast to the TRP channels, the family of voltage-gated sodium channels is encoded by only 9 genes, named *SCNA1-SCNA5* and *SCNA8-SCNA11*. The genes translate into respective functional α subunit subtypes (Nav1.1-Nav1.9) **(Bennett et al,2019)**. The exploitation of isolated neurotoxins of fish and bacterial origin such as saxitoxin (STX) and tetrodotoxin (TTX), allowed via pharmacological means to determine structural and molecular features of sodium channel gating **(Hartshorne & Catterall,1981)**. By copying cDNA from mRNA transcripts, Numa and Noda cloned and solved the primary structure of a VGSC expressed in electric eels **(Noda et al,1984)**. The group then reported the existence of multiple VGSC transcripts found in the rat brain, where they isolated them and expressed functional channels in xenopus oocytes **(Noda et al,1986)**. Crystallographic and Cryo-EM studies have provided detailed insights into the architecture of VGSCs **(Payandeh et al,2011)** **(Shen et al,2019)**. The VGSCs contain sequences of approximately 2000 amino acids which are organized in four domains and where each subdomain is consisted of 6 transmembrane segments (S1-S6). The S1-S4 transmembrane segment subdomain possesses voltage-sensitive properties while the S5-S6 subdomains are responsible for filtering ion selectivity **(Payandeh et al,2011)**.

Despite their comparatively smaller subtype diversity, VGSCs are extensively distributed across excitable tissues. Due to their principal role in the initiation and propagation of APs, they are involved in several physiological processes and their dysfunction is responsible for defined channelopathies. The coordination of heart contractility in cardiomyocytes is dependent on Nav1.5 channels and channel perturbation may cause arrhythmias and cardiomyopathy **(Rogart et al,1989)** while Nav1.8 channel ablation decreases the PR interval of the electrocardiogram and may increase the risk of heart block **(Chambers et al,2010)**. There are four VGSCs highly expressed in the mature brain, namely Nav1.1, Nav1.2, Nav1.3 and Nav1.6 and gene mutations induce epileptic syndromes **(Brunklaus et al,2022)**. A childhood epileptic disorder called the Dravet syndrome is induced by heterozygous, loss-of-function mutations of the Nav1.1 channel expressed in brain interneurons **(Catterall,2016)**. Forms of autism, intellectual disabilities and ataxias have been attributed to mutations induced in the brain-expressed VGSCs while musculoskeletal disorders are closely associated with mutations in the Nav1.4 channel subtype **(Mantegazza et al,2021)**. A synaptic integrative role for Nav1.7 has been demonstrated in hypothalamic regions that regulate body weight **(Branco et al,2016)**, while loss-of-function

mutations in the channel impair synaptic transmission from olfactory sensory neurons, resulting in anosmia **(Weiss et al,2011)**.

In the context of pain neurophysiology, the VGSC subtypes expressed in sensory DRG neurons are the Nav1.1, Nav1.3, Nav1.6, Nav1.7, Nav1.8 and Nav1.9 **(Bennett et al,2019)**. The Nav1.7 channel was cloned from a pheochromocytoma PC12 cell line **(Toledo-Aral et al,1997)** and due to its high expression levels in the growth cones of small diameter neurons, it was speculated that the channel might be important for nociception **(Nassar et al,2004)**. The Nav1.7 channel is expressed in all nociceptive neuronal subtypes, where 2/3rds of C-fibers and about 15% of myelinated A fibers express it. There is additional localization of the channel in peripheral unmyelinated axons and at the nodes of Ranvier of medium-sized myelinated neurons **(Bennett et al,2019)**. It is established that Nav1.7 has a significant role in the initiation and propagation of APs in nociceptor cell bodies and their axonal terminals **(Akin et al,2019)** **(Goodwin et al,2022)**. Due to the finding that the channel plays a role in neurotransmitter release in olfactory sensory neurons **(Weiss et al,2011)** it has been shown with pharmacological and knockout genetic evidence that pre-synaptic Nav1.7 is also involved in modulating neurotransmitter release across the first sensory synapse **(Alexandrou et al,2016)** **(MacDonald et al,2021)**. As mentioned earlier, a confirmation of the role of Nav1.7 channels in nociception arose from the identification of three homozygous nonsense mutations in the channel that result in a congenital inability to sense pain in humans **(Cox et al,2006)**. While loss-of-function mutations in Nav1.7 abolish nociception, gain-of-function mutations have been identified to cause primary erythromelalgia, an autosomal dominant condition which results in extreme pain sensation, redness and swelling of the extremities **(Yang et al,2004)**. Similarly, rectal, ocular and submandibular pain occurring in paroxysmal extreme pain disorder (PEPD) is induced due to mutations that cause fast inactivation of the Nav1.7 channel, enabling a persistent sodium current to flow **(Fertleman et al,2006)**.

Inflammatory pain induction by complete Freund's adjuvant (CFA) administration in conditional Nav1.7 KO failed to elicit thermal hyperalgesic or mechanical allodynic responses **(Nassar et al,2004)**. The Nav1.7 global-KO mice are neonatally lethal, yet nociceptor-specific ablation studies of Nav1.7 Cre-LoxP mice expressed in Nav1.8-positive neurons has shown a role for the channels in responses to acute noxious mechanical stimulation, where responses are largely attenuated but not abolished in a skin-saphenous nerve preparation **(Gingras et al,2014)**. Although

Nav1.7 expressed in C-fibers plays a role in acute noxious mechanical sensation (**Middleton et al,2022**), genetic deletion of the gene has no effect in the development of neuropathic pain (**Nassar et al,2005**).

The discovery of a TTX-resistant current in rat sensory neurons led to the identification and cloning of the Nav1.8 channel, then named sensory-neuron specific (SNS) or PN3 (**Akopian et al,1996**) (**Sangameswaran et al,1996**). Expression studies found that the channel is distributed almost universally among small diameter C-fibers, hence rendering the channel as a marker of nociceptors (**Djouhri et al,2003**). In biophysical terms, the channel contributes towards the inward current of the rising phase of the APs (**Renganathan et al,2001**). Null mutant mice of the channel were subsequently found to elicit reduced responses to noxious mechanical stimuli and displayed deficits in thermal hyperalgesia and acute noxious thermal stimulation (**Akopian et al,1999**). The expression of Nav1.8 is upregulated by trophic factors such as NGF, indicating that this upregulation occurs in the TrkA-expressing, peptidergic nociceptors (**Fjell et al,1999**), while GDNF upregulates the expression of the channel in non-peptidergic nociceptors (**Cummins et al,2000**). Nav1.8 channels play an important role in inflammatory pain, where carrageenan induced inflammation upregulates the mRNA transcripts of the channels found in nociceptors (**Tanaka et al,1998**). In behavioral pharmacology experiments, the induction of hyperalgesia following PGE₂-injection was attenuated by administering an antisense oligodeoxynucleotide of Nav1.8 (**Khasar et al, 1998**).

The release of inflammatory mediators upon tissue injury increases the activity of Nav1.8 channels through downstream signaling cascades. The inflammatory mediator PGE₂ has been shown to modulate the TTX-resistant sodium currents and specifically Nav1.8 channels expressed in sensory neuron cell bodies (**England et al,1996**) (**Gold et al,1998**) and axons (**Kimourtzis et al,2022**), via an EP4/cAMP/PKA cascade. Analogous activation of Nav1.8 via PKA and PKC phosphorylation following downstream cascades have been reported for interleukins, serotonin, adenosine and tumor necrosis factor- α (TNF- α) (**Gold et al,1996**) (**Jin & Gereau,2006**) (**Binshtok et al,2008**). The role of Nav1.8 channels in neuropathic pain has been more contentious. It has been reported that genetic ablation of Nav1.8 does not contribute to the development of neuropathic pain (**Nassar et al,2005**). In fact, axotomy of sensory neurons results in a downregulation of mRNA transcripts of Nav1.8 (**Dib-Hajj et al,1996**) justifying the potential lack

of role in neuropathic pain. However, administration of a selective Nav1.8 blocker was found to attenuate neuropathic pain in rats (**Jarvis et al,2007**). Further, the optogenetic silencing of Nav1.8-positive nociceptors has been reported to attenuate neuropathic pain (**Daou et al, 2016**), a neuronal subset which however encompasses almost all nociceptors and is not necessarily indicative of specific Nav1.8 involvement.

Less studied are the TTX-S channels Nav1.1, Nav1.3 and Nav1.6, and the TTX-R, Nav1.9. The Nav1.9 channel is the second TTX-R VGSC channel expressed in sensory neurons together with Nav1.8 (**Tate et al,1998**). Similar to Nav1.8, the Nav1.9 channel is modulated by inflammatory mediators via second messenger cascades (**Rush & Waxman, 2004**) and it is downregulated following axotomy (**Dib-Hajj et al,1998**). There are no pharmacological studies for the channel due to the lack of development of a selective blocker. The Nav1.1 channel has been implicated in the mediation of mechanical pain induced by spider toxins in modality-specific myelinated nociceptors (**Osteen et al,2016**). The Nav1.3 channel lacks expression in adult DRG neurons under physiological conditions, but it is re-expressed following nerve injury (**Waxman et al,1994**). Despite its expression post-axotomy, mice lacking Nav1.3 develop allodynia as well as acute and inflammatory pain behavior normally (**Nassar et al,2006**). The Nav1.6 channel is expressed predominantly in myelinated neurons where it is localized at the nodes of Ranvier and synapses (**Caldwell et al, 2000**) while it is also found in the axons of nociceptors (**Black et al,2002**). An emerging role for Nav1.6 channels has been observed in neuropathic pain where it regulates the excitability of mechanosensitive neurons (**Israel et al,2019**). The conditional-KO of Nav1.6 partially attenuates mechanical allodynia following nerve injury (**Chen et al, 2018**), while the channel has been shown to be the major contributor among Nav channels to synaptic transmission following axotomy (**Vysokov et al,2019**). In chemotherapy-induced peripheral neuropathy models induced by oxaliplatin (**Sittl et al,2012**) and vincristine (**Chen et al,2020**) a role for Nav1.6 has been demonstrated, while Nav1.6 activation elicits spontaneous pain along with mechanical and cold allodynia (**Deuis et al,2013**).

Together, TRPV1 (heat), TRPA1 (chemical irritation) and TRPM8 (cold) form the most investigated TRP channel triad in the context of pain, where most studies have focused on the exteroceptive transduction properties of the channels. Much less is known about the expression and role of the channels found in the central site of nociceptors and whether they facilitate other

functions than transducing noxious modalities. Analogously, VGSCs such as Nav1.6, Nav1.7 and Nav1.8 are traditionally perceived as contributors to the initiation and propagation of APs across nociceptor axons, but they have not been thoroughly examined in the context of synaptic transmission.

In this chapter, we utilize the microfluidic co-culture model of DRG and DH neurons described in the previous chapter, to investigate the role of these six important TRP (TRPV1, TRPA1, TRPM8) and VGSC (Nav1.6, Nav1.7, Nav1.8) channels in synaptic transmission. The fluidic isolation of the three distinct compartments in our model, has allowed us to locally apply pharmacological manipulations and quantify contributions of these channels to synaptic transmission. The axotomy of peripheral sensory neurons is also used as a surrogate model for neuropathic pain conditions. It provides a measure of comparison between DH cell body responses to sensory input from intact and axotomized sensory neurons. The results suggest novel, localized contribution of a subset of TRP and VGSCs to synaptic transmission and underscore the plasticity of their contribution following axonal injury in sensory neurons.

5.2 Results

The microfluidic co-culture model of DRG and DH neurons was employed to recapitulate the first sensory synapse *in vitro*. A protocol established that between DIV9-12, the cultured postnatal mouse neurons reached maturity and enabled the performance of calcium imaging experiments (**Figure 5.1A**). Electrical stimulation was applied to the peripheral axons (8 biphasic pulses at 4Hz frequency x 2 ms @4 μ A) with the DH cell bodies being the readout site of calcium responses using Fluo4. To evaluate the contribution of TRP and VGSCs in synaptic transmission, pharmacological manipulations were applied at the DH compartment. To study the effect of these channels in synaptic transmission following nerve injury, we also employed the axotomy model established in the previous chapter (**Figure 5.1B**) and performed matching experiments between intact and axotomized microfluidic devices. For each experiment, there were 1 or 2 stimulations in the absence of pharmacological blockade and following the addition of blockers, 2 additional stimulations were given (**Figure 5.1C**). The amplitude of the responses was then quantified and compared to examine whether TRP or Nav channel blockade affects synaptic transmission.

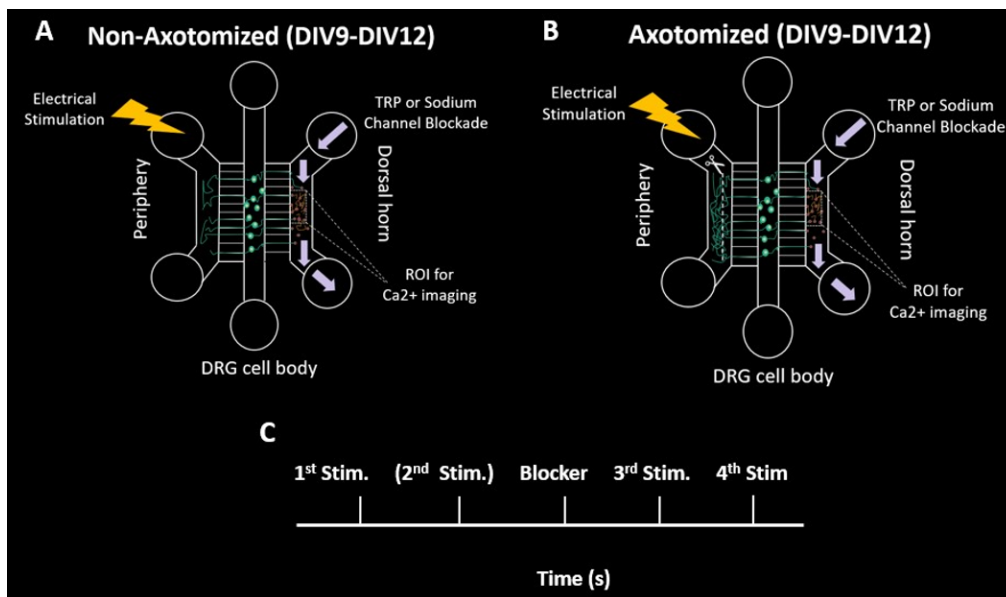


Figure 5.1 Schematic protocol of the microfluidic co-culture model of DRG and DH neurons in intact and axotomized configurations. A) Between days in vitro (DIV) 9 to 12, calcium imaging recordings of DH neurons in response to stimulation of non-axotomized peripheral axons were taken. **B)** Between days in vitro (DIV) 9 to 12, calcium imaging recordings of DH neurons in response to stimulation of axotomized peripheral axons were taken. **C)** Timeline of events record in a calcium imaging experiment.

5.2.1 The contribution of Nav channels to synaptic transmission

5.2.1.1 Nav1.7 channel contribution to synaptic transmission of non-axotomized cultures

The contribution of Nav1.7 to synaptic transmission was first examined in intact, non-axotomized cultures. It has been hypothesized that Nav1.7 channels expressed at both the pre- and post-synaptic sites may influence the excitability of DH neurons (Alexandrou et al,2016) (Alles et al,2020). For this reason, the selective Nav1.7 blocker PF-05089771 (8nM) was applied to the DH compartment containing the pre-synaptic terminals of DRG axons and DH neurons (Figure 5.2A). Changes in the excitability of DH neurons were then recorded. Comparisons between the amplitude of Ca^{2+} responses evoked by electrical stimulation (8 biphasic pulses at 4Hz frequency x 2 ms @4 μA) before and after the addition of the Nav1.7 blocker revealed that the blockade significantly attenuates the amplitude of DH responses (Figure 5.2B&C). Thus, under physiological conditions it is suggested that Nav1.7 channels expressed pre-synaptically contribute to synaptic transmission and neurotransmitter release.

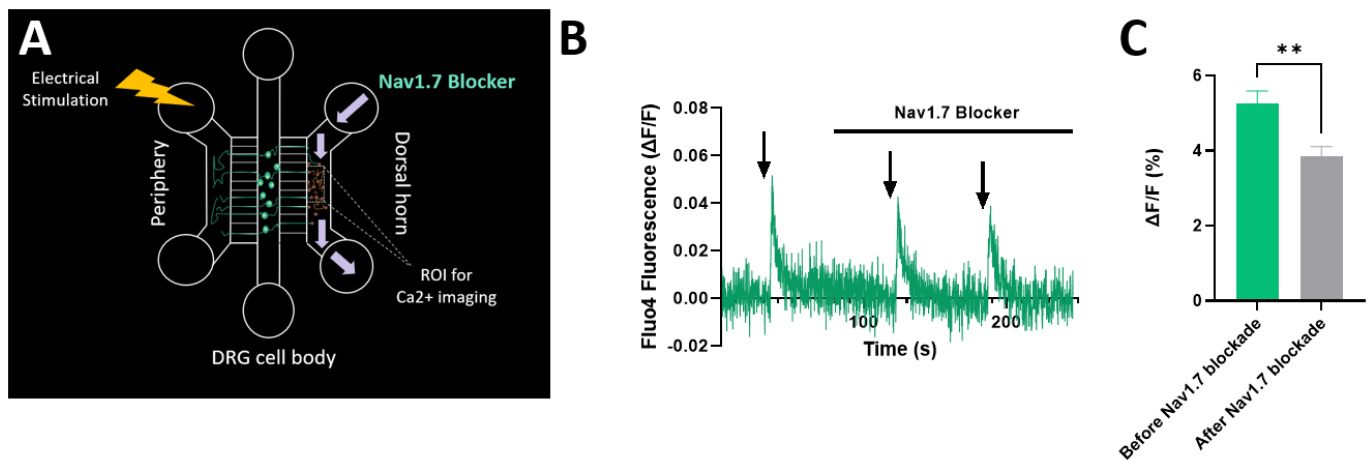


Figure 5.2 A) Schematic setup of the experiment with the addition of a selective Nav1.7 blocker PF-05089771 (8nM) in intact, non-axotomized cultures. B) Representative trace showing changes in calcium influx of DH cell bodies following applications of electrical stimulation in the axons of the periphery compartment, before and after Nav1.7 blockade. C) Comparison of the average amplitude of responses before and after the addition of the Nav1.7 blocker (Welch's t-test, ** $p=0.0013$, $n=143$ neurons, $n=4$ devices from 4 cultures).

5.2.1.2 Nav1.7 channel contribution to synaptic transmission of axotomized cultures

Then, the role of Nav1.7 channels in synaptic transmission following axotomy was examined. It has been proposed that the channels do not contribute to the development of neuropathic pain in Nav1.7-KO mice (Nassar et al,2005). Using a pharmacological approach instead of genetics, we sought to determine whether blockade of the channel impairs transmission across the DRG-DH synapse. The same stimulation parameters were used (8 biphasic pulses at 4Hz frequency x 2 ms @4 μ A) as well as the concentration of the Nav1.7 blocker (PF-05089771, 8nM) for this experiment (**Figure 5.3A**). Interestingly, the addition of the Nav1.7 blocker in the DH compartment did not alter the amplitude of DH cell body responses revealing that the channels no longer play a role in synaptic transmission in axotomized cultures (**Figure 5.3B&C**). The result demonstrates that following axotomy in mouse DRG neurons, Nav1.7 channels no longer contribute to synaptic transmission at the DRG-DH synapses. This suggests that peripheral downregulation or trafficking of the channel in axonal injury might be responsible for this effect and that Nav1.7 blockers may indeed not be efficacious targets for neuropathic pain.

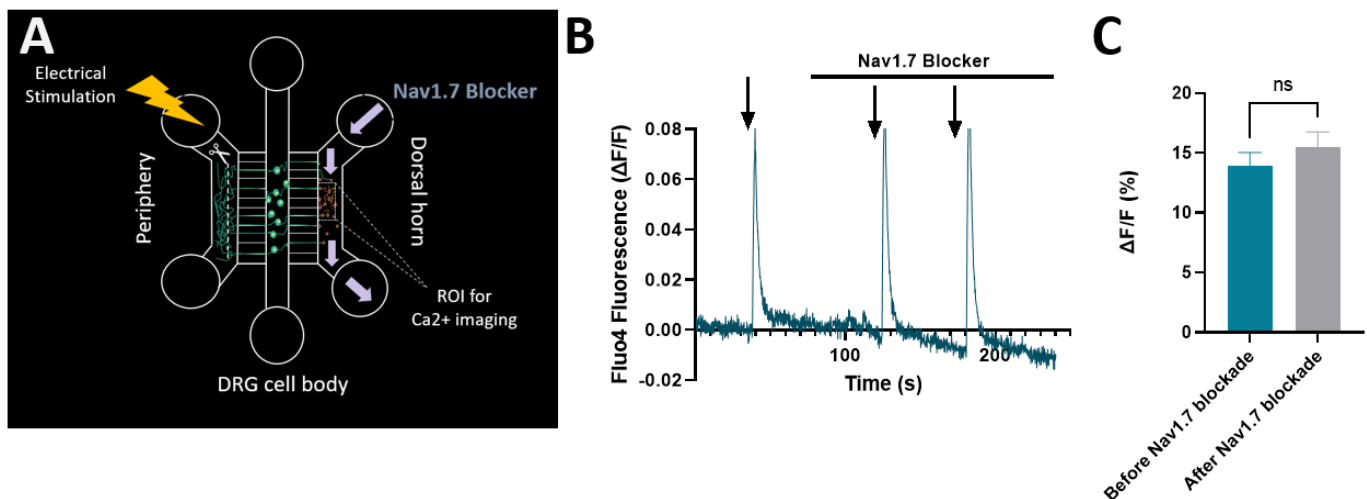


Figure 5.3 **A)** Schematic setup of the experiment with the addition of a selective Nav1.7 blocker PF-05089771 (8nM) in axotomized cultures. **B)** Representative trace showing changes in calcium influx of DH cell bodies following applications of electrical stimulation in the axons of the periphery compartment, before and after Nav1.7 blockade. **C)** Comparison of the average amplitude of responses before and after the addition of the Nav1.7 blocker (ns $p < 0.05$, Welch's t-test, $n = 48$ neurons, $n = 3$ devices from 3 cultures).

5.2.1.3 Nav1.7 channel contribution to DH neuron excitability

It is difficult to discern using the pharmacological blockade in our model system whether the effect of Nav1.7 channels in synaptic transmission occurs pre-synaptically, post-synaptically or in concomitance between the two sites. To exclude contingencies by process of elimination, DH neurons alone were cultured, and the blocker was applied directly in the absence of sensory neuron input to measure changes in amplitude following electrical stimulation (8 biphasic pulses at 4Hz frequency x 2 ms @4 μ A) (**Figure 5.4A**). While direct stimulation of the DH cell bodies does not directly reveal or conceal a role for Nav1.7 in synaptic transmission, it provides a helpful indication for the dependence of DH neuron excitability to these channels. The addition of the Nav1.7 blocker (PF-05089771, 8nM) did not attenuate the responses following direct stimulation of the DH compartment (**Figures 5.4B&C**). Given the lack of effect of Nav1.7 channels to block DH neuron excitability and taken together with the previous results of **Figure 5 2**, the hypothesis for a post-synaptic contribution of Nav1.7 channels to the attenuation of DH responses is considered improbable. In fact, the data suggest that the effect of Nav1.7 channels on synaptic transmission and neurotransmitter release is pre-synaptic and a transcriptional modification of the channels' expression in proximal sensory axons might be responsible for the lack of efficacy of the selective blocker in the axotomized neurons (**Figure 5.3C**).

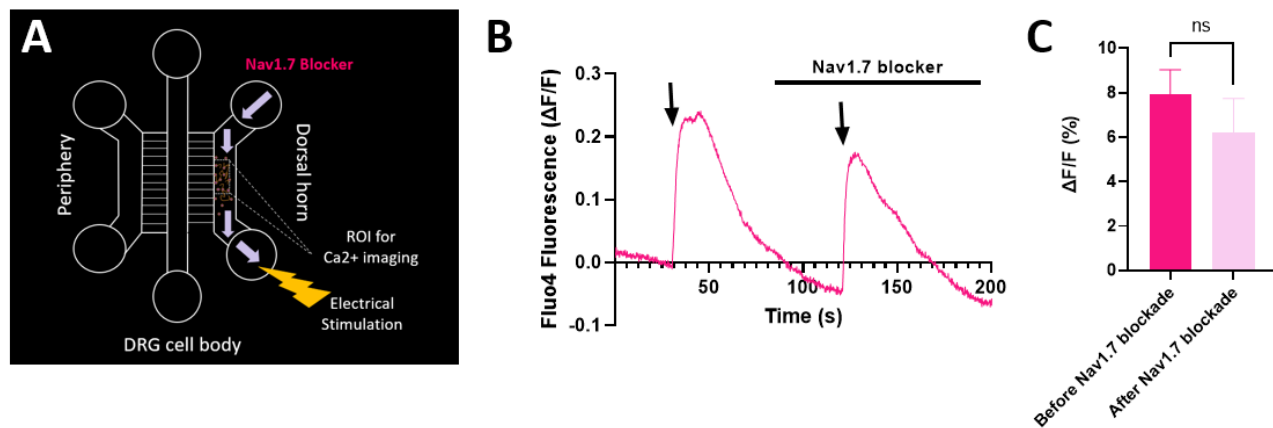


Figure 5.4 **A)** Schematic setup of the experiment with the addition of a selective Nav1.7 blocker PF-05089771 (8nM) directly in DH neurons. **B)** Representative trace showing changes in calcium influx of DH cell bodies following applications of electrical stimulation in DH cell bodies, before and after Nav1.7 blockade. **C)** Comparison of the average amplitude of responses before and after the addition of the Nav1.7 blocker in the DH (ns $p < 0.05$, Welch's t-test, $n = 13$ neurons, $n =$ devices from 3 cultures).

5.2.1.4 Nav1.8 channel contribution to synaptic transmission of non-axotomized cultures

Given that the TTX-S sensitive Nav1.7 channels showcased an important role in synaptic transmission which was lost following axotomy, we next sought to examine the synaptic role of the other VGSCs which are abundantly expressed in nociceptors, the TTX-resistant Nav1.8. The Nav1.8 channels are crucial for inflammatory pain and the sensitization of nociceptive neurons. The experimental setup was not altered (stimulation parameters: 8 biphasic pulses at 4Hz frequency x 2 ms @4 μ A) and amplitude of DH responses was measured following responses evoked in the peripheral axonal compartment using Fluo4. A selective Nav1.8 blocker was employed (A-803467, 10nM) and applied to the DH compartment to record changes in the excitability of DH neurons following stimulation of intact, non-axotomized peripheral axons (**Figure 5.5A**). Addition of the Nav1.8 blocker did not produce significant alterations in the responses of DH neurons to axonal stimulation, suggesting a lack of a role for the channel in synaptic transmission under these conditions (**Figures 5.5B&C**).

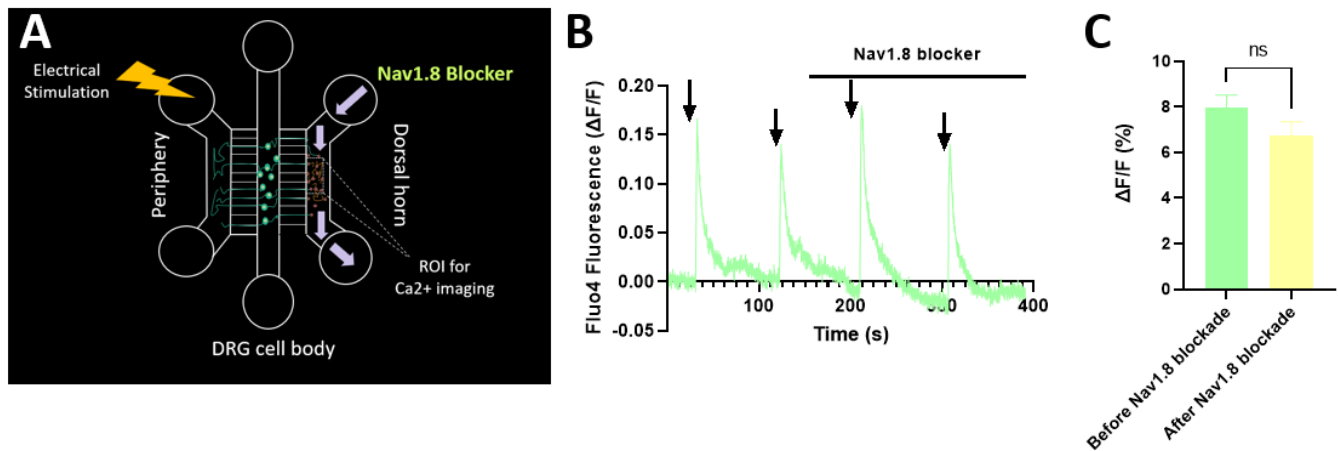


Figure 5.5 **A)** Schematic setup of the experiment with the addition of a selective Nav1.8 blocker A-803467 (10 nM) in intact, non-axotomized cultures. **B)** Representative trace showing changes in calcium influx of DH cell bodies following applications of electrical stimulation of peripheral sensory axons, before and after Nav1.8 blockade. **C)** Comparison of the average amplitude of responses before and after the addition of the Nav1.8 blocker in the DH (ns $p < 0.05$, Welch's t-test, $n = 54$ neurons, $n = 3$ devices from 3 cultures).

5.2.1.5 Nav1.8 channel contribution to synaptic transmission of axotomized cultures

In the introduction, it was reported from previous studies Nav1.8 mRNA expression is downregulated in sensory neurons following axotomy (Dib-Hajj et al,1996). The hypothesis was tested again in our experiments, using pharmacological means in a spatially precise manner. The axotomy model was once employed to quantify the contribution of Nav1.8 channels to synaptic transmission between DRG and DH neurons. As previously reported, DH responses to axonal sensory neuron electrical stimulation were recorded in the presence or absence of the selective Nav1.8 blocker (Figure 5.6A). There were no significant changes in DH responses after stimulation of axotomized neurons while in the presence of a Nav1.8 blocker (Figures 5.6B&C). The results confirm the lack of Nav1.8 involvement in nerve injury at least at the pre-synaptic site, although no applications in the DRG cell body or peripheral axon compartment were attempted. Taken together with the Figure 5.5 results, the data exclude a pre-synaptic role for the channel both under physiological and neuropathic pain conditions.

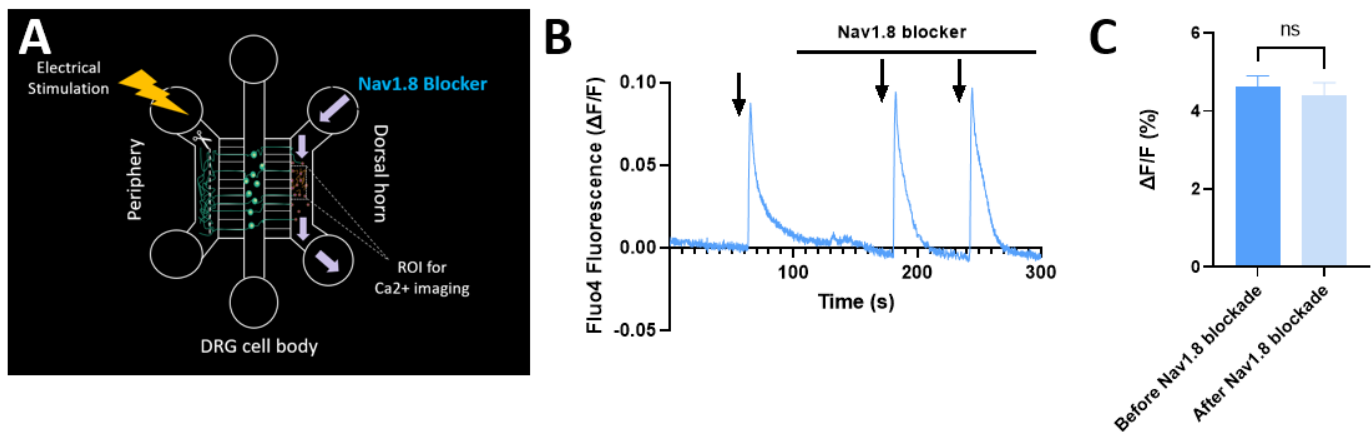


Figure 5.6 A) Schematic setup of the experiment with the addition of a selective Nav1.8 blocker A-803467 (10 nM) in axotomized cultures. B) Representative trace showing changes in calcium influx of DH cell bodies following applications of electrical stimulation of peripheral sensory axons, before and after Nav1.8 blockade. C) Comparison of the average amplitude of responses before and after the addition of the Nav1.8 blocker in the DH (ns $p < 0.05$, Welch's t-test, $n = 70$ neurons, $n = 3$ devices from 3 cultures).

5.2.1.6 Nav1.7 and Nav1.8 channel contribution to synaptic transmission of non-axotomized cultures

Since a role for Nav1.8 channels was excluded in facilitating synaptic transmission, it was hypothesized that a dual blockade of Nav1.7 and Nav1.8 channels would produce the same effect that Nav1.7 channels produced alone, perhaps through a synergistic mechanism. The responses of DH cell bodies were recorded following stimulation of intact, non-axotomized peripheral axons, before and after dual addition of the Nav1.7 (PF-05089771, 8nM) and Nav1.8 (A-803467, 10 nM) selective blockers (**Figure 5.7A**). The concomitant addition of the blockers significantly reduced the amplitude of DH responses (**Figure 5.7C**). From this data, it is concluded that only Nav1.7 channels play a pre-synaptic role which can partially yet significantly attenuate the DH responses. At the same time, given that Nav1.7 and Nav1.8 are the most abundantly expressed VGSCs in nociceptors, the fact that their pre-synaptic blockade does not abolish completely DH responses may possibly mean that the channels are significant yet not principal contributors in synaptic transmission. Rather, either individually or synergistically, previously proposed roles of Nav1.7 and Nav1.8 in the initiation and propagation of APs provide more plausible explanations for the analgesic effects of their blockade in conditions of acute and inflammatory pain.

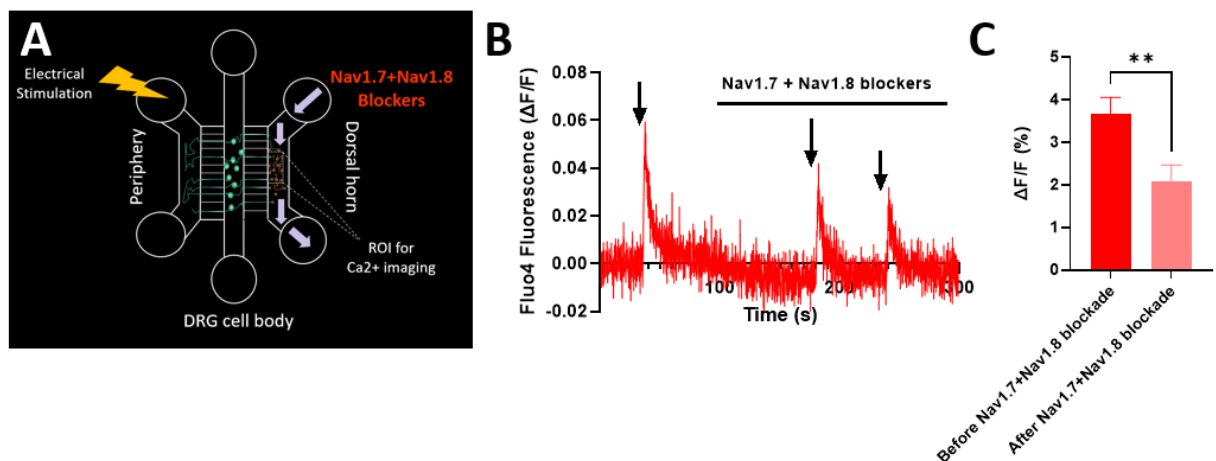


Figure 5.7 **A)** Schematic setup of the experiment with the concomitant addition of the selective Nav1.7 blocker PF-05089771 (8nM) and the Nav1.8 blocker A-803467 (10 nM) in intact, non-axotomized cultures. **B)** Representative trace showing changes in calcium influx of DH cell bodies following applications of electrical stimulation of peripheral sensory axons, before and after dual Nav1.7 and Nav1.8 blockade. **C)** Comparison of the average amplitude of responses before and after the addition of the blockers in the DH (Welch's t-test, **p=0.0218, n =81 neurons, n=3 devices from 3 cultures).

5.2.1.7 Nav1.7 and Nav1.8 channel contribution to synaptic transmission of axotomized cultures

The dual Nav1.7 and Nav1.8 blockade at the central site produced a significant reduction of DH responses after stimulation of non-axotomized peripheral axons. Conversely, given the responses of the individual blockade of either Nav1.7 or Nav1.8 after stimulation of axotomized peripheral axons, it was expected that a dual blockade of the channels would not produce any significant effect. Indeed, addition of the Nav1.7 (PF-05089771, 8nM) and Nav1.8 (A-803467, 10 nM) blockers before and after stimulation of the axotomized sensory axons (**Figure 5.8B**) did not produce an effect in the excitability of DH cell bodies (**Figure 5.8C**). Thus, whether blocked individually or together, the results further corroborate that these channels do not cause any changes in synaptic transmission following axotomy. The finding provides a justification for the lack of analgesic efficacy of selective Nav1.7 and Nav1.8 blockers for the treatment of neuropathic pain and is in agreement with the finding that the channels are not involved in the development of neuropathic pain (Nassar et al, 2005).

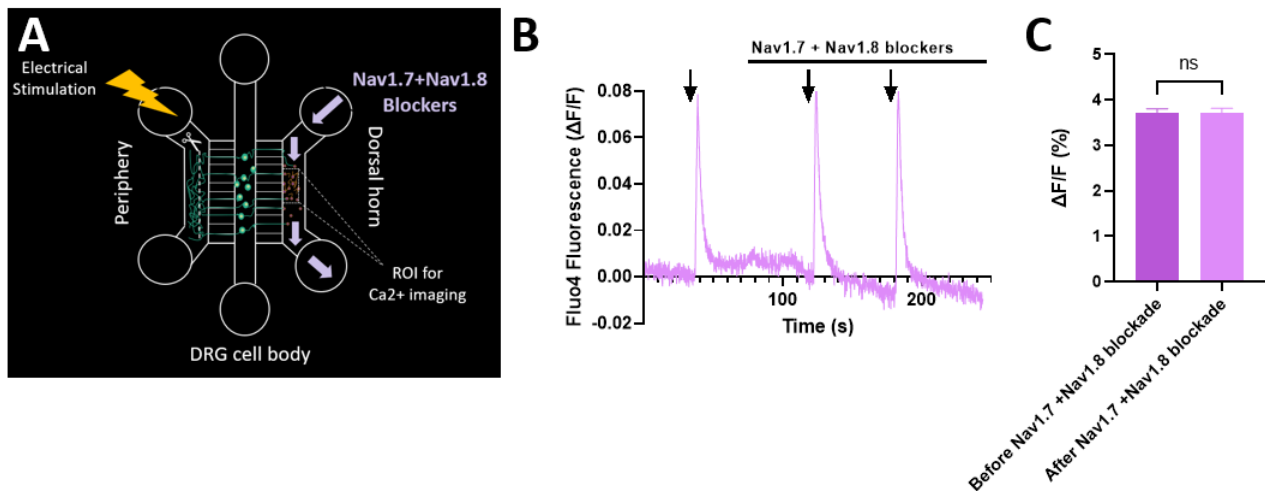


Figure 5.8 A) Schematic setup of the experiment with the concomitant addition of the selective Nav1.7 blocker PF-05089771 (8nM) and the Nav1.8 blocker A-803467 (10 nM) in axotomized cultures. B) Representative trace showing changes in calcium influx of DH cell bodies following applications of electrical stimulation of peripheral sensory axons, before and after dual Nav1.7 and Nav1.8 blockade. C) Comparison of the average amplitude of responses before and after the addition of the blockers in the DH (ns $p < 0.05$, Welch's t-test, $n = 17$ neurons, $n = 3$ devices from 3 cultures).

5.2.1.8 Nav1.6 channel contribution to synaptic transmission of non-axotomized cultures

The loss of a functional role of the two principal nociceptor VGSCs, Nav1.7 and Nav1.8 observed in the axotomy model, highlighted the profound plasticity at the DRG-DH synapses following injury to the sensory axons. A previous pharmacological study from the lab (Vysokov et al,2019) using rat embryonic tissues showed that Nav1.6 channels become prominent contributors to synaptic transmission following axotomy. As well as a behavioral study from the Waxman/Dib-Hajj lab using conditional-KO mice (Chen et al,2018) showed that Nav1.6 channels acquire a role in neuropathic pain. To confirm whether this observation is a cross species phenomenon and to support or debunk the behavioral observations, we tested the role of the Nav1.6 in synaptic transmission using a selective blocker, 4,9-Anhydrotetrodotoxin (AHTTX, 500nM) (Vysokov et al,2019) (Figure 5.9A). In non-axotomized neurons, addition of AHTTX did not change the amplitude of DH responses following stimulation of peripheral axons (Figure 5.9C). The result confirms in mice that Nav1.6 channels, which are normally expressed in the nodes of Ranvier of myelinated sensory axons (Caldwell et al,2000), do not play a role in synaptic transmission in intact DRG and DH neurons.

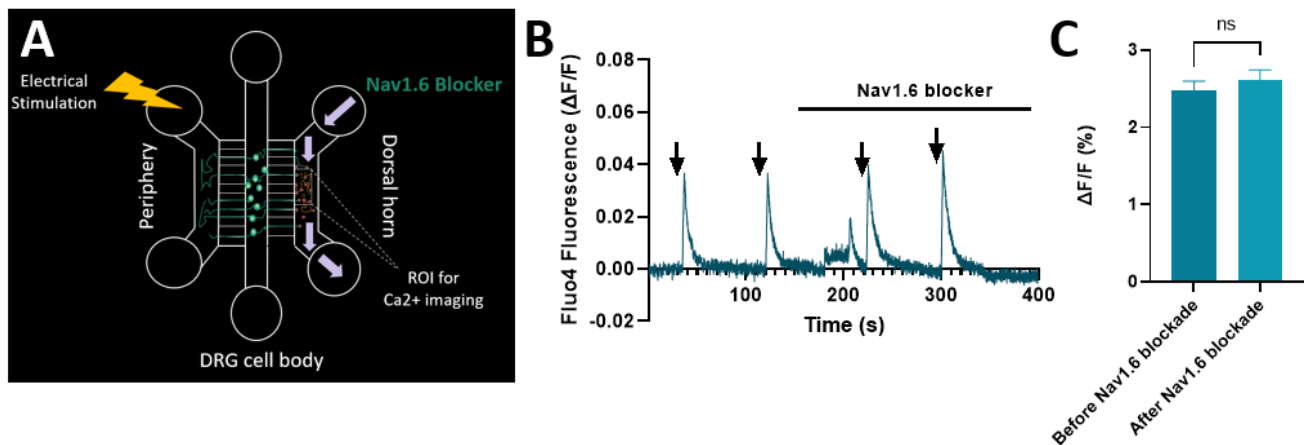


Figure 5.9 A) Schematic setup of the experiment with the addition of a selective Nav1.6 blocker AHTTX (500 nM) in intact, non-axotomized cultures. B) Representative trace showing changes in calcium influx of DH cell bodies following applications of electrical stimulation of peripheral sensory axons, before and after Nav1.6 blockade. C) Comparison of the average amplitude of responses before and after the addition of the Nav1.6 blocker in the DH (ns $p < 0.05$, Welch's t-test, $n = 85$ neurons, $n = 3$ devices from 3 cultures).

5.2.1.9 Nav1.6 channel contribution to synaptic transmission of axotomized cultures

In contrast to the absence of a role of Nav1.6 in synaptic transmission under physiological states, the channel has been shown to acquire a role following axotomy in rats (Vysokov et al,2019). Hence, the responses of DH neurons were recorded following stimulation of axotomized peripheral axons in the presence or absence of the Nav1.6 blocker, AHTTX (500nM) (**Figure 5.10A**). The blockade of Nav1.6 channels within the first sensory synapse attenuates DH responses in axotomized neurons following stimulation of peripheral axons (**Figure 5.10C**). The result demonstrates the cross-species identity of the phenomenon that in both mouse and rat co-cultures, axotomy modulates the cohort of Nav channels that contribute to synaptic transmission between DRG and DH neurons. Further, it reveals that the plasticity of sensory neurons following axotomy generates a previously unsuspected role for Nav1.6. This change might be due to an upregulation of the expression of Nav1.6 after axotomy but also due to compensatory mechanisms in the absence of Nav1.7.

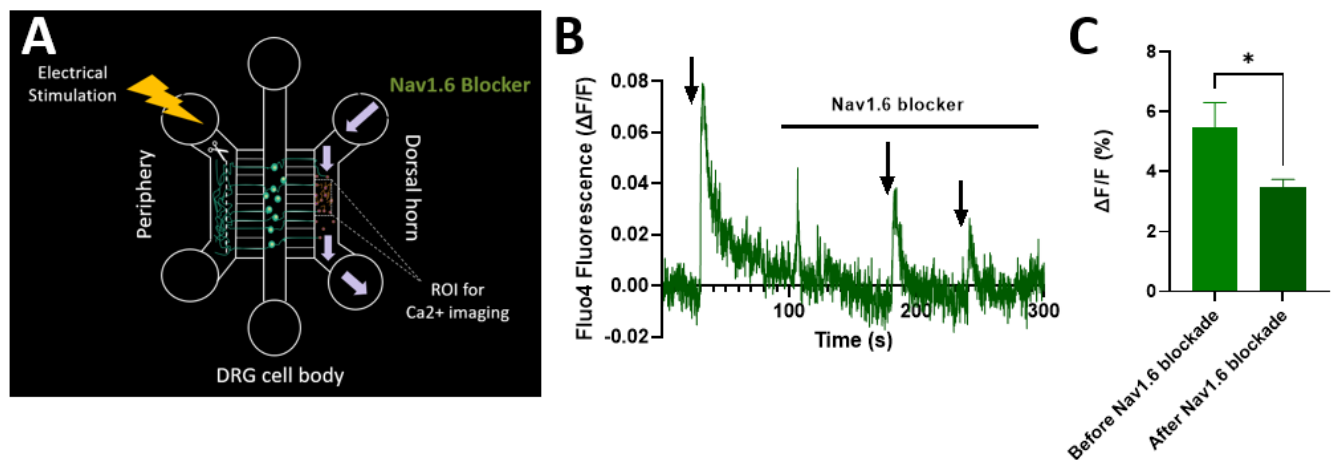


Figure 5.10 **A)** Schematic setup of the experiment with the addition of a selective Nav1.6 blocker AHTTX (500 nM) in axotomized cultures. **B)** Representative trace showing changes in calcium influx of DH cell bodies following applications of electrical stimulation of peripheral sensory axons, before and after Nav1.6 blockade. **C)** Comparison of the average amplitude of responses before and after the addition of the Nav1.6 blocker in the DH (Welch's t-test, * $p=0.0251$, $n=58$ neurons, $n=4$ devices from 3 cultures).

5.2.1.10 Nav1.6 channel contribution to DH neuron excitability

In the case of Nav1.7, blockade of the channels at the synaptic site attenuated the responses of DH neurons under physiological conditions following sensory axon stimulation (**Figure 5.2C**). However, the evidence was provided that the effect is pre-synaptic and that Nav1.7 are not contributing to the direct excitability of DH neurons (**Figure 5.4C**). On the contrary, Nav1.6 channels emerge as novel modulators of DH responses only following axotomy, implying that a sensory neuron transcriptional adaptation has altered their expression. Thus, by stimulating and recording directly from DH neurons in the absence or presence of the Nav1.6 channel, we sought to determine whether Nav1.6 plays a role in DH neuron excitability without any input received from sensory neurons (**Figure 5.11A**). The addition of AHTTX (500nM), did not attenuate DH responses, suggesting that these channels are not critical mediators of DH neuron excitability (**Figure 5.11C**). Given that Nav1.6 channels potentially do not contribute to DH neuron excitability, the result pinpoints further towards a sensory neuron-specific, pre-synaptic role for the channel that occurs after axotomy.

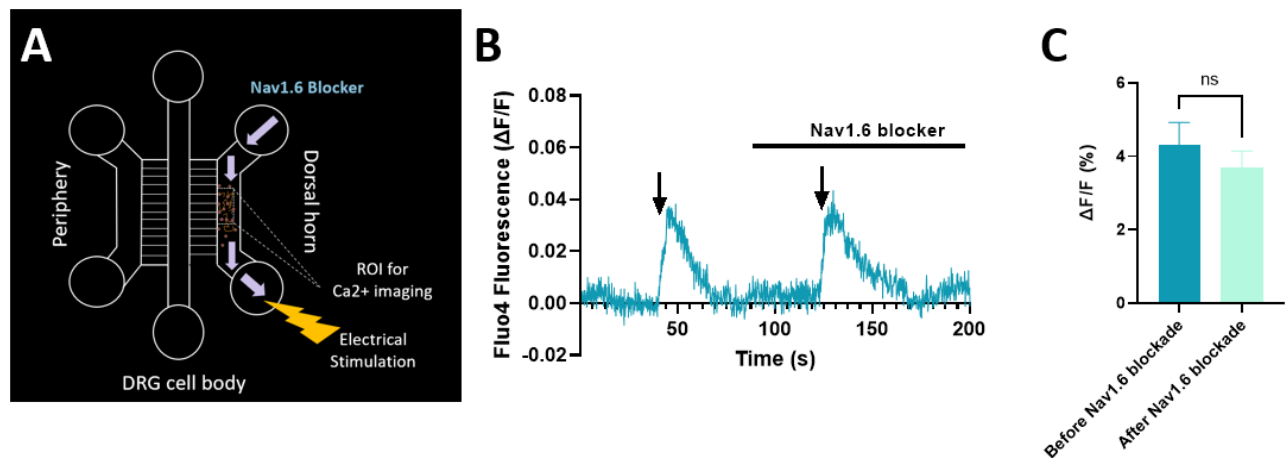


Figure 5.11 A) Schematic setup of the experiment with the addition of a selective Nav1.6 blocker AHTTX (500nM) directly in DH neurons. B) Representative trace showing changes in calcium influx of DH cell bodies following applications of electrical stimulation in DH cell bodies, before and after Nav1.6 blockade. C) Comparison of the average amplitude of responses before and after the addition of the Nav1.6 blocker in the DH (ns $p < 0.05$, Welch's t-test, $n = 29$ neurons, $n = 4$ devices from 3 cultures).

5.2.1.11 Nav1.6, Nav1.7 and Nav1.8 channel contribution to synaptic transmission of non-axotomized cultures

The Nav1.7 channels play a pre-synaptic role in modulating synaptic transmission under intact conditions. The Nav1.6 channels acquire a pre-synaptic role after nerve injury, while the Nav1.8 channels do not participate in either condition. It was hypothesized whether a blockade of all three channels might produce a near complete reduction in synaptic transmission, and presumably that it would only be Nav1.7 driven until physiological conditions. Hence, we sought to determine the effects that a combination of all 3 Nav1.6, Nav1.7 and Nav1.8 blockers would exert. The blockers AHTTX (500nM), PF-05089771 (8nM) and A-803467 (10 nM) were concomitantly administered at the DH compartment and recordings of DH neuron fluorescence were made following stimulation of peripheral axons in the absence or presence of the Nav blockade (**Figure 5.12A**). The blockade of all 3 channels significantly attenuated the fluorometric responses of DH cell bodies (**Figure 5.12C**). However, given that Nav1.6 and Nav1.8 channels were previously shown to not affect synaptic transmission, it is strongly suggested that under physiological conditions, the reduction of DH responses is solely driven by pre-synaptic Nav1.7 channels.

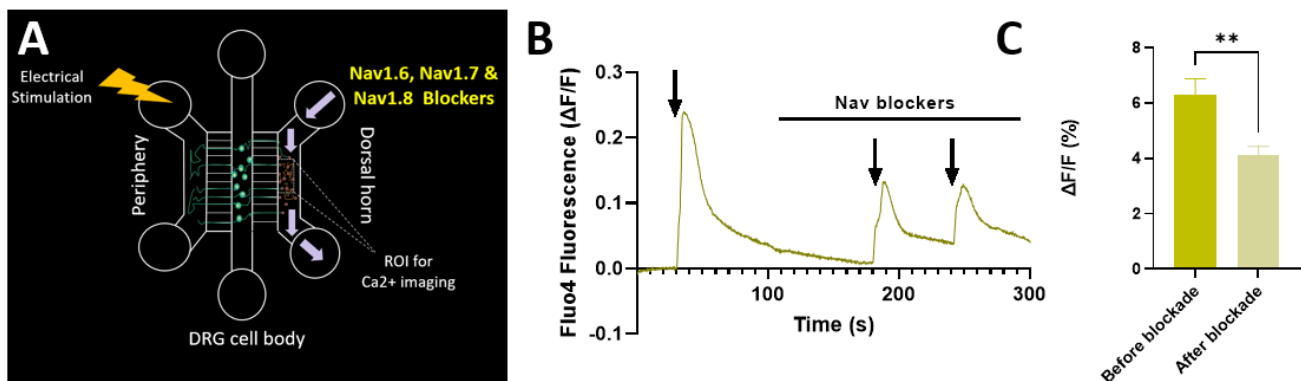


Figure 5.12 **A)** Schematic setup of the experiment with the concomitant addition of the selective Nav1.6 blocker AHTTX (500 nM), Nav1.7 blocker PF-05089771 (8nM) and the Nav1.8 blocker A-803467 (10 nM) in intact, non-axotomized cultures. **B)** Representative trace showing changes in calcium influx of DH cell bodies following applications of electrical stimulation of peripheral sensory axons, before and after triple Nav channel blockade. **C)** Comparison of the average amplitude of responses before and after the addition of the blockers in the DH (Welch's t-test, ** $p=0.0014$, $n=64$ neurons, $n=4$ devices from 4 cultures).

5.2.1.12 Nav1.6, Nav1.7 and Nav1.8 channel contribution to synaptic transmission of non-axotomized cultures

Next, the addition of all 3 blockers at the synaptic site was used to determine changes in the excitability of DH neurons following stimulation of axotomized peripheral axons (**Figure 5.13A**). It was previously shown that in axotomized cultures, Nav1.7 and Nav1.8 channels do not play a role in synaptic transmission, while Nav1.6 channels emerge as modulators of DH synaptic excitability. The addition of all 3 blockers significantly attenuated DH responses in axotomized cultures (**Figure 5.13C**). Given that Nav1.6 channels were shown in our previous experiments (**Figure 5.10**) to attenuate DH responses in axotomized cultures and that the other two channels lack a similar role, it is concluded that Nav1.6 is the sole driver of this DH response attenuation. The finding is compelling because it provides an explanation for the effective blockade of non-selective sodium channel blockers such as local anesthetics in both intact and nerve injury cases. Hence, non-selective blockers that do not discriminate the separate effects of the channels are unable to unmask their respective contributions. Taken together with **Figure 5.12**, the results show that triple blockade of Nav1.6, Nav1.7 and Nav1.8 channels exerts a significant attenuation of DH mediated responses under both intact and axotomized conditions. However, on each conditions the VGSCs that mediate this attenuation differ.

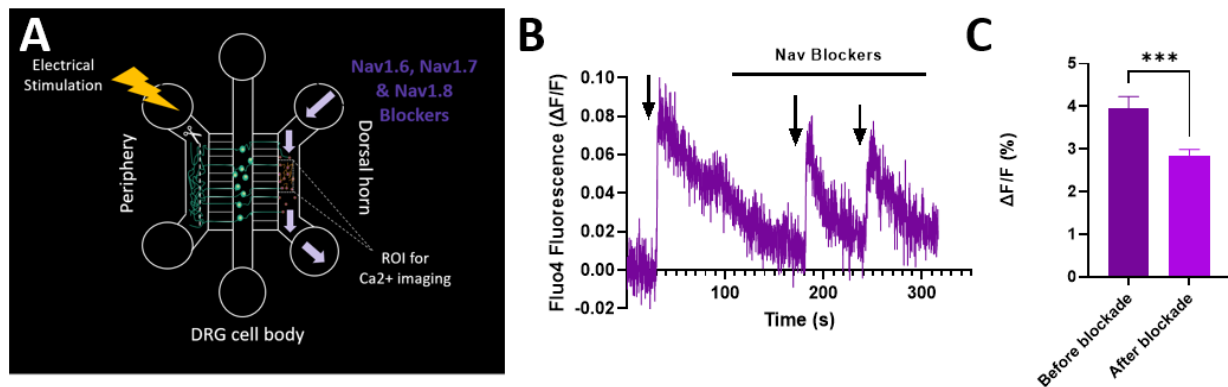


Figure 5.13 A) Schematic setup of the experiment with the concomitant addition of the selective Nav1.6 blocker AHTTX (500 nM), Nav1.7 blocker PF-05089771 (8nM) and the Nav1.8 blocker A-803467 (10 nM) in axotomized cultures. **B)** Representative trace showing changes in calcium influx of DH cell bodies following applications of electrical stimulation of peripheral sensory axons, before and after triple Nav channel blockade. **C)** Comparison of the average amplitude of responses before and after the addition of the blockers in the DH (Welch's t-test, ***p=0.0081, n =68 neurons, n=4 devices from 3 cultures).

5.2.1.13 Nav1.6, Nav1.7 and Nav1.8 channel contribution to DH neuron excitability

In previous individual applications of Nav1.7 and Nav1.6 blockade directly in the DH compartment to evaluate responses to peripheral axonal stimulation, blocking the channels did not produce any significant attenuation of DH excitability (**Figure 5.4** and **Figure 5.11**, respectively). To determine if the modulation of the synaptic transmission is significantly affected by synergistic effect of post-synaptic Nav1.6, Nav1.7 and Nav1.8 channels, all three channels were blocked by addition of their respective blockers (**Figure 5.14A**). The addition of the blockers did not change the excitability of DH responses following direct stimulation of DH neurons (**Figure 5.14C**). It is important to note that while in the co-culture experiments the excitability of DH neurons occurs due to neurotransmitter/neuropeptide release, in the single DH culture experiment the excitability is induced due to a direct depolarization through the opening of voltage-gated ion channels. Hence, in direct stimulation experiments, the excitability of DH neurons might be dependent on other ion channels, and it might conceal any Nav.-mediated effects. Nevertheless, due to this lack of dependence of DH neurons on Nav1.6 and Nav1.7 channels, as mentioned, it is most probable that the effect of both these channels is indeed pre-synaptic.

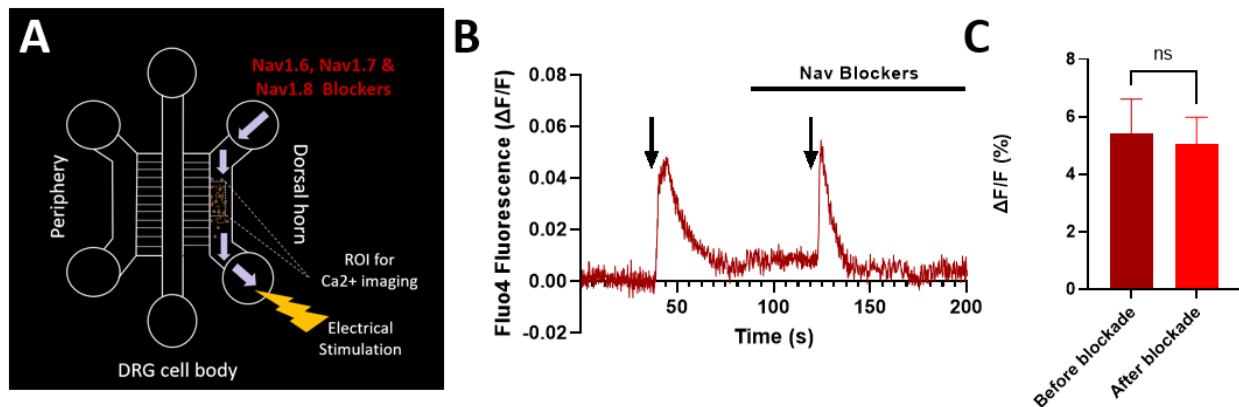


Figure 5.14 **A)** Schematic setup of the experiment with the addition of the Nav1.6 blocker AHTTX (500 nM), the Nav1.7 blocker PF-05089771 (8nM) and the Nav1.8 blocker A-803467 (10 nM) directly in DH neurons. **B)** Representative trace showing changes in calcium influx of DH cell bodies following applications of electrical stimulation to DH compartment, before and after triple VGSC blockade. **C)** Comparison of the average amplitude of responses before and after the addition of the blockers in the DH (ns $p < 0.05$, Welch's t-test, $n = 28$ neurons, $n = 3$ devices from 3 cultures).

5.2.2 The contribution of TRP channels to synaptic transmission

5.2.2.1 TRPA1 channel contribution to synaptic transmission of non-axotomized cultures

Once concluded with the examination of VGSC modulation of synaptic transmission, the respective role of TRP channels was investigated. Initially, the contribution of TRPA1 channels was tested by employing an analogous combination of pharmacological fluorometric readout strategy that was used for the VGSC experiments. A selective TRPA1 blocker, A-967079 (2 μ M) (Zhao et al,2020) was applied in the DH compartment to evaluate the role of TRPA1 channels in synaptic transmission of intact, non-axotomized cultures. As per previously used protocol, the electrical stimulation of peripheral axons (8 biphasic pulses at 4Hz frequency x 2 ms @4 μ A) was used to determine the excitability of DH neuron in the absence or presence of the TRPA1 blocker (Figure 5.15A). Interestingly, the blockade of TRPA1 channels produces a significant reduction in the amplitude of DH responses (Figure 5.15C), suggesting a central role for these channels. The channels are traditionally considered to mediate the transduction of pungent chemicals and endogenous inflammatory mediators at the nerve endings of nociceptors (Bautista et al,2013). Here, in the absence of inflammatory mediator input, the results suggest a putative role for TRPA1 in facilitating synaptic transmission and neurotransmitter release. The observation is also surprising since TRPA1 is expressed only in certain nociceptor subsets.

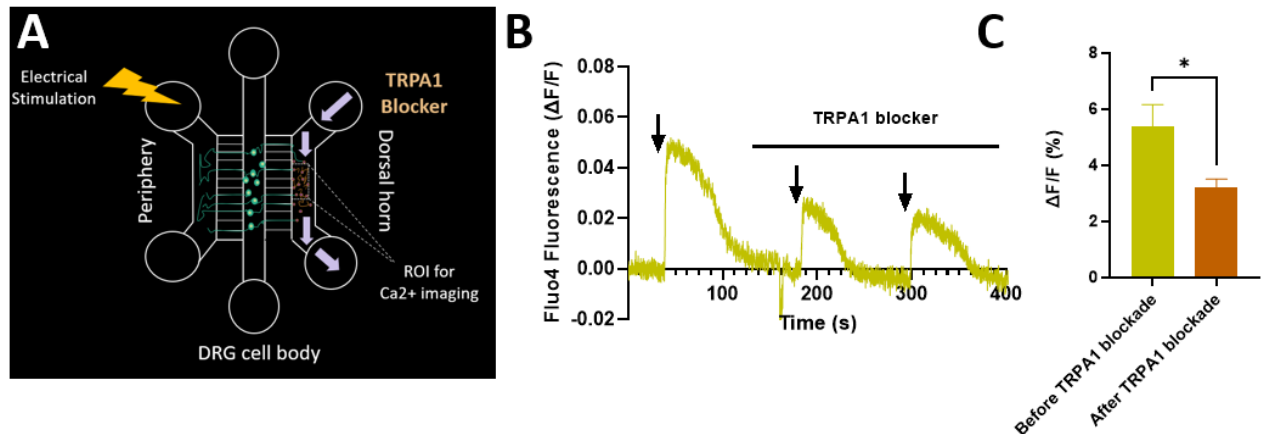


Figure 5.15 A) Schematic setup of the experiment with the addition of the selective TRPA1 blocker A-967079 (2 μ M) in non-axotomized cultures. B) Representative trace showing changes in calcium influx of DH cell bodies following applications of electrical stimulation of peripheral sensory axons, before and after TRPA1 channel blockade. C) Comparison of the average amplitude of responses before and after the addition of the blockers in the DH (Welch's t-test, *p=0.0118, n=22 neurons, n=4 devices from 3 cultures).

5.2.2.2 TRPA1 channel contribution to synaptic transmission of axotomized cultures

The observation that TRPA1 blockade at the central terminal of intact microfluidic cultures reduced the excitability of DH neurons, was succeeded by a follow up experimental repetition of the protocol in axotomized cultures. Stimulation of the peripheral axons with the DH cell bodies as the readout was performed in the absence or presence of the TRPA1 blocker A-967079 (2 μ M) (**Figure 5.16A**). In contrast to the role of TRPA1 in synaptic transmission in intact DRG neuron conditions, the blockade fails to produce a significant attenuation of DH responses (**Figures 5.16 B&C**). Despite a seeming reduction trend, the channel loses its role following axotomy of sensory axons. In analogous fashion to Nav1.7 channels, transcriptional downregulation, or trafficking of the channel after nerve injury might be responsible for lack of an effect. While studies implicate TRPA1 as a target for the inflammatory component suppression during neuropathic pain conditions, it is imperative to consider whether the channel has a physiological relevance in nerve injury.

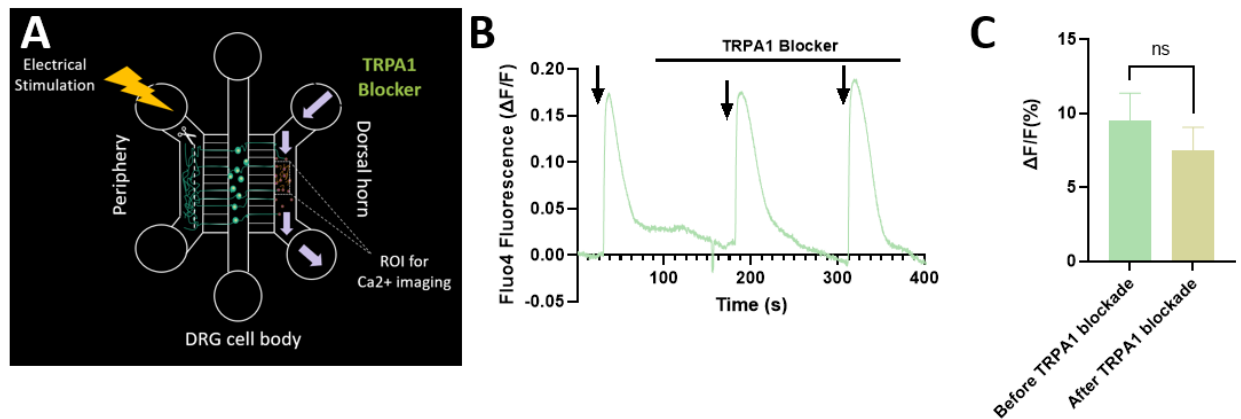


Figure 5.16 **A)** Schematic setup of the experiment with the addition of the selective TRPA1 blocker A-967079 (2 μ M) in axotomized cultures. **B)** Representative trace showing changes in calcium influx of DH cell bodies following applications of electrical stimulation of peripheral sensory axons, before and after TRPA1 channel blockade in the axotomized cultures condition. **C)** Comparison of the average amplitude of responses before and after the addition of the blockers in the DH (ns $p < 0.05$, Welch's t-test, $n = 21$ neurons, $n = 4$ devices from 3 cultures).

5.2.2.3 TRPV1 channel contribution to synaptic transmission of non-axotomized cultures

The finding of the TRPA1 contribution in synaptic transmission compelled us to further investigate the role of other TRP channels expressed primarily in nociceptors. Unlike TRPA1, which is expressed in a smaller subset of nociceptors, the TRPV1 channel is abundantly expressed among nociceptor subtypes (Usoskin et al,2015). As in the case of TRPA1, the TRPV1 channels are also calcium permeable and may exert a potential role other than transduction of heat stimuli. In accordance with the consistently used strategy, the stimulation of peripheral axons with the DH cell bodies as the fluorometric readout was employed in the absence or presence of the selective TRPV1 blocker AMG517 (200nM) in intact, non-axotomized cultures (Figure 5.17A). The addition of the TRPV1 blocker produces a notable attenuation of DH responses, suggesting that the channels play an important part in modulating transmission across the first sensory synapse (Figure 5.17B&C). The observation provides novel evidence for a role of TRPV1 in facilitating synaptic input into the DH. The potential implications of this observation are wide in the context of nociceptor physiology and are analytically described in the discussion section.

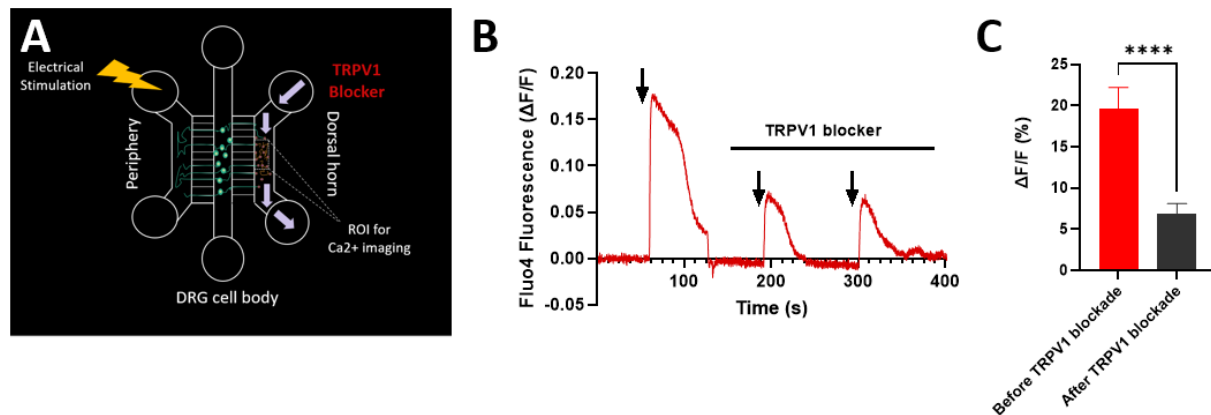


Figure 5.17 A) Schematic setup of the experiment with the addition of the selective TRPV1 blocker AMG517 (100 nM) in non-axotomized cultures. B) Representative trace showing changes in calcium influx of DH cell bodies following applications of electrical stimulation of peripheral sensory axons, before and after TRPV1 channel blockade in the axotomized cultures condition. C) Comparison of the average amplitude of responses before and after the addition of the TRPV1 blocker in the DH (Welch's t-test, **** $p < 0.0001$, $n = 23$ neurons, $n = 5$ devices from 4 cultures).

5.2.2.4 TRPV1 channel contribution to synaptic transmission of axotomized cultures

The remarkable attenuation of DH responses by TRPV1 blockade under physiological conditions compelled us further to investigate whether TRPV1 exerts this effect in axotomized cultures as well. An experiment with identical stimulation and recording parameters was performed on the axotomized cultures, using the same selective TRPV1 blocker, AMG517 (200nM) (Yue et al,2022) (Figure 5.18A). Surprisingly, the TRPV1 blockade did not attenuate the responses of DH neurons following stimulation of the peripheral axons (Figures 5.18B&C). In a similar fashion to Nav1.7 and TRPA1, the TRPV1 channel loses its role in synaptic transmission upon nerve injury. As in the case of Nav1.7 and TRPA1, the channel exerts a functional role in synaptic transmission under normal conditions, which is lost in the axotomy condition. The result highlights the notable plasticity that sensory neurons may exert following nerve injury and provides an explanation why TRPV1 blockade may not provide a promising therapeutic target for neuropathic pain relief.

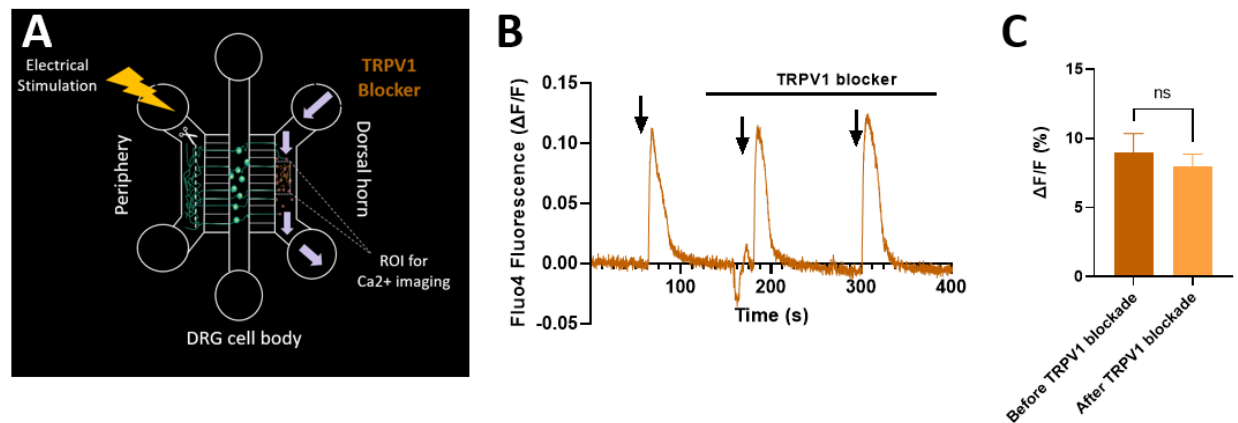


Figure 5.18 **A)** Schematic setup of the experiment with the addition of the selective TRPV1 blocker AMG517 (100 nM) in axotomized cultures. **B)** Representative trace showing changes in calcium influx of DH cell bodies following applications of electrical stimulation of peripheral sensory axons, before and after TRPV1 channel blockade in the axotomized cultures condition. **C)** Comparison of the average amplitude of responses before and after the addition of the TRPV1 blocker in the DH (ns $p < 0.05$, Welch's t-test, $n = 22$ neurons, $n = 4$ devices from 3 cultures).

5.2.2.5 TRPM8 channel contribution to synaptic transmission of non-axotomized cultures

After the examination of the heat- and pungent chemical- sensitive TRPV1 and TRPA1 channels, the contribution of the cold transducer ion channel TRPM8 was evaluated in synaptic transmission. Intact, non-axotomized microfluidic co-cultures were employed, consistent with all previous experiments. For the blockade of TRPM8, a selective blocker (M8-B hydrochloride ,50 nM) (Almeida et al,2012) was applied in the DH compartment, and the responses of DH neurons to peripheral stimulation were examined before and after the addition of the blocker (**Figure 5.19A**). Application of the TRPM8 blocker failed to attenuate DH responses after stimulation of the peripheral compartment, revealing a potential lack of contribution for the channel in transmission at the central site (**Figures 5.19 B&C**). However, given that TRPM8 is expressed only by a subpopulation of DRG neurons (Usoskin et al,2015) (Zeisel et al,2018), it is possible that most sensory neurons which were electrically stimulated belonged to TRPM8-negative nociceptor subtypes. Hence, any potential reduced responses would not be unmasked by stimulating the entire axonal compartment in an indiscriminating manner. To determine whether the channel has a role in synaptic transmission in the TRPM8-positive subpopulation, the TRPM8-expressing sensory neurons should be isolated and studied separately, a challenging technical consideration for primary co-cultures of sensory and DH neurons.

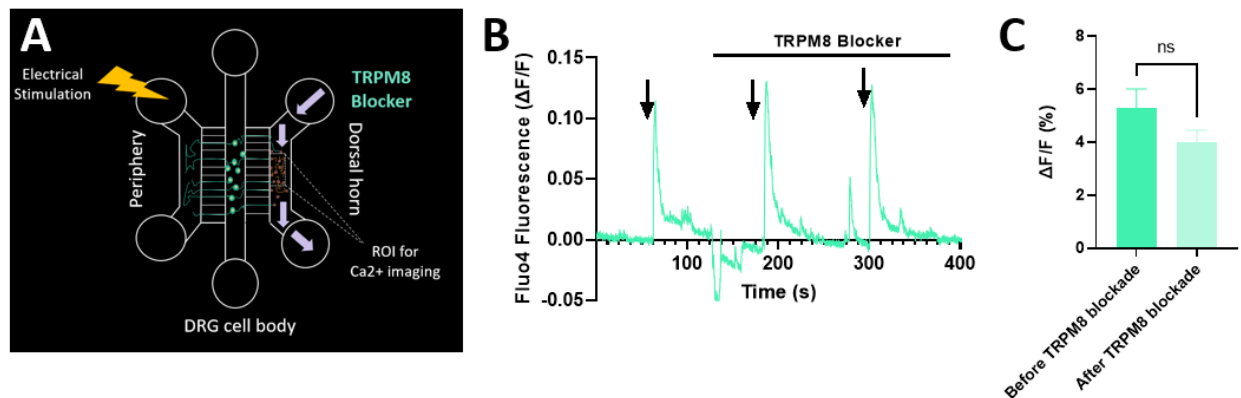


Figure 5.19 **A)** Schematic setup of the experiment with the addition of the selective TRPM8 blocker M8-B hydrochloride (50 nM) in non-axotomized cultures. **B)** Representative trace showing changes in calcium influx of DH cell bodies following applications of electrical stimulation of peripheral sensory axons, before and after TRPM8 channel blockade in the non-axotomized cultures condition. **C)** Comparison of the average amplitude of responses before and after the addition of the TRPM8 blocker in the DH (ns $p < 0.05$, Welch's t-test, $n = 16$ neurons, $n = 3$ devices from 3 cultures).

5.2.2.6 TRPM8 channel contribution to synaptic transmission of axotomized cultures

Last, the contribution of TRPM8 channels was investigated in axotomized cultures. Nerve injury frequently results in the development of cold allodynia and TRPM8 channels have been implicated in this phenomenon (Xing et al,2007). Therefore, the effect of TRPM8 channels in synaptic transmission is important in neuropathic pain. The TRPM8 blocker, M8-B hydrochloride (50nM) was used again to evaluate whether the channels play a role in synaptic transmission after axotomy (Figure 5.20A). The results indicate that M8-B hydrochloride does not reduce the responses of DH neurons following peripheral stimulation (Figures 5.20B&C). However, the same technical caveat of the *in vitro* primary cultures may hinder the unmasking of the effects of TRPM8-positive neurons. Despite the expression of TRPM8 channels in distinct nociceptor subtypes, an interesting observation would have been the *de novo* upregulation of TRPM8 following injury in all nociceptor subtypes (Yarmolinsky et al,2016), in similar fashion that Nav1.6 acquired a role after axotomy. The lack of DH response reduction after TRPM8 blockade suggests that the channels are not modulated at the central site and do not contribute to synaptic transmission under both physiological and axotomy conditions, at least in the general, stochastic population sample of diverse nociceptor subtypes existing in the microfluidic cultures.

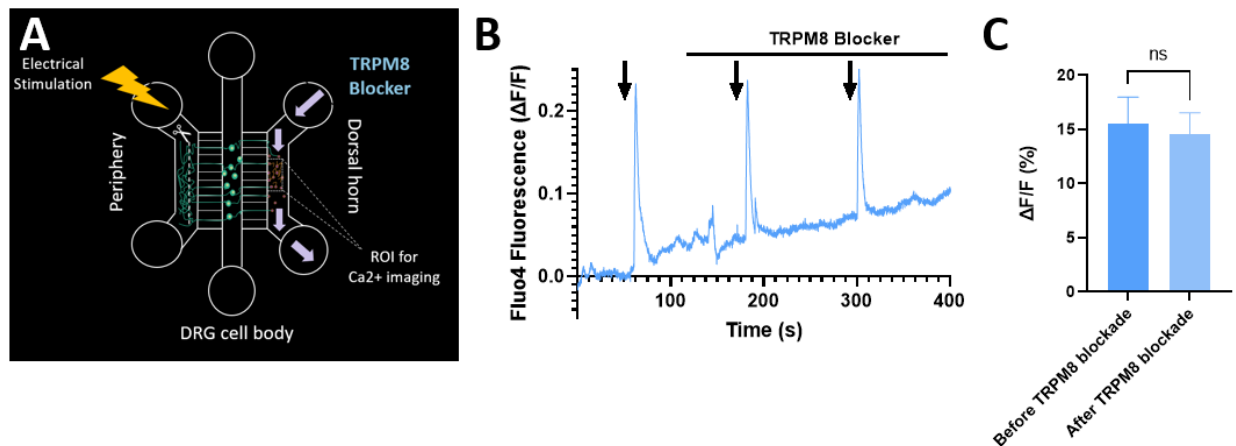


Figure 5.20 A) Schematic setup of the experiment with the addition of the selective TRPM8 blocker M8-B hydrochloride (50 nM) in axotomized cultures. B) Representative trace showing changes in calcium influx of DH cell bodies following applications of electrical stimulation of peripheral sensory axons, before and after TRPM8 channel blockade in the axotomized cultures condition. C) Comparison of the average amplitude of responses before and after the addition of the TRPM8 blocker in the DH (ns $p < 0.05$, Welch's t-test, $n = 13$ neurons, $n =$ devices from 3 cultures).

5.3 Discussion

The present chapter has delved into the modulation of synaptic transmission by ion channels expressed in nociceptors and has provided evidence for their role in both physiological and axon injury to sensory neurons. The results have highlighted certain unexpected roles for the channels which taken in a broader context, may underlie plausible explanations for certain pain manifestations. Based on the work described in the previous chapter, the microfluidic based model between mouse DRG and DH neurons was employed as the functional assay of studying transmission properties. The recapitulation of the periphery-to-center pain pathway has allowed us to investigate distinctly the spatial properties of nociceptors that can influence synaptic transmission to the DH. Further, the induction of a physical lesion in the form of an axotomy has provided a further condition that can be examined as a surrogate model of neuropathic pain (Tsantoulas et al,2013) (Vysokov et al,2019).

There are several *in vivo* models of neuropathic pain that involve the lesioning of the peripheral nerves such as the spared nerve injury model (Decosterd & Woolf, 2000) and spinal nerve injury (Kim & Chung, 1992). While these models have been combined with *in vivo* pharmacology to monitor behavioral responses to neuropathic injury, it is impossible to localize the pharmacokinetic properties of the administered drugs or pinpoint topographic information on the anatomical site of action of the drug on the nociceptors. In addition, according to current ethical regulations on substance administration to laboratory animals, intrathecal injections of substances can only be performed once per animal (Turner et al,2011). To minimize the attrition rate of pharmacological targets that are effective for analgesic pain relief both under physiological and neuropathic conditions, the microfluidic cultures allow a larger-scale testing of several substances per animal, as 4-5 microfluidic devices can be prepared per mouse. In the previous chapter, it was shown that the *in vitro* recapitulation of the first sensory synapse resolves the spatially controlled administration of drugs, instead of conducting intrathecal injections. Hence, pharmacological tools can be used effectively in microfluidic co-cultures to study nociceptor plasticity in the form of protein trafficking or local transcriptional changes.

Due to the previous amenability of monitoring changes *in vivo* and *in vitro* in the cell body and peripheral axons of nociceptors following axotomy, there is much more information about the plasticity of these sites in nerve injury. It has been less studied how peripheral injury affects pre-

and post- synaptic signaling at the DRG-DH relay point. Therefore, three principal ion channels widely expressed in nociceptors from each of the TRP and Nav families were examined to determine how they affect synaptic transmission. Much interest has been recently generated around the role of ion channels at the central site of nociceptors. It has been proposed that Nav1.7-KO mice produce congenital insensitivity to pain by impairing synaptic transmission and by potentiating peripheral opioid receptor function in sensory neurons **(Isensee et al,2017)** **(MacDonald et al,2021)**. However, another study contradicts the recent results that Nav1.7 constitutive activity is dependent on enkephalin overexpression by arguing that the phenomenon occurs only in cLTMR fibers responsible for touch and insists on the traditional viewpoint that the channel is required for the initiation of APs **(Deng et al,2023)**. The finding of Nav1.7 dependence on opioidergic signaling is counterintuitive in the context of neuropathic pain. Given previous evidence that TTX-sensitive channels such as Nav1.7 are downregulated after nerve injury **(Cummins & Waxman, 1997)** **(Renthall et al,2020)**, one would expect according to the argument lied above, that partial loss of Nav1.7 would produce an enhanced opioidergic tone, hence resulting in analgesia. However, that is not the case in neuropathic pain, where nerve injury results in both hyperalgesia during the inflamed phase of the injury and allodynia in later stages **(Colloca et al,2017)**. Further, genetic studies of Nav1.7 deletion argue against a role for the channel in the development of neuropathic pain **(Nassar et al,2005)**.

Our results in the microfluidic model of the first sensory synapse suggest a compelling role for Nav1.7 in modulating synaptic transmission at the central site under normal conditions. The blockade of Nav1.7 channels at the central site significantly reduces the amplitudes of DH neurons after stimulation. Moreover, to determine whether the effects of Nav1.7 attenuation of synaptic transmission are pre-synaptic, post-synaptic or concomitant, we found that direct Nav1.7 blockade of isolated DH neurons does not reduce DH responses. Hence, the evidence from our experiments eliminates the probability of the Nav1.7-mediated effects to be post-synaptic and suggests a putatively exclusive pre-synaptic contribution. However, a caveat of comparing responses from direct stimulation of DH neurons with responses of DH neurons receiving sensory input is that the former are dependent on the depolarization by voltage-gating of ions following stimulation while the latter are depolarized by neurotransmitter binding to neurotransmitter-gated ion channels.

The finding does not exclude other functions that have been previously attributed to Nav1.7, such as the contribution of the channel in the initiation and propagation of APs originating at the nerve endings of nociceptors following noxious stimulation. Epitope tagging of Nav1.7 has found a widespread localization of the channel in axons, cell bodies, axon terminals and nodes of Ranvier in nociceptors (**McDermott et al,2019**). It would be interesting in follow-up studies to pharmacologically block the cell body and the peripheral axonal compartment respectively to determine the contribution of each anatomical site to DH responses. The emergence of spatially resolved transcriptomic and translomic technologies at the single neuron level (**Liu et al,2020**) (**Zeng et al,2023**) together with microfluidic cultures (**Deng et al,2023**), could provide a topographic map of mRNA/protein expression in each anatomical part of sensory neurons and could illuminate the sites where ion channels such as Nav1.7 are likely to exert their function. The finding of DH response attenuation to central Nav1.7 blockade does not clarify whether the channels contribute directly towards synaptic transmission or whether they pre-synaptically facilitate the propagation of APs. For instance, pre-synaptically expressed calcium channels detecting intracellular Ca^{2+} ions act as the conduits for the trigger of synaptic vesicle exocytosis to force neurotransmitter release (**Catterall and Few,2008**). Such a role has not been demonstrated for VGSCs in sensory neurons, and most likely the pre-synaptic function of these channels is to facilitate the local propagation of generated APs and contribute to the depolarization of the presynaptic terminal, hence modulating neurotransmitter release.

Although Nav1.7 channels are required for acute and inflammatory pain, their role in neuropathic pain is contested. An early study had shown that application of TTX can reduce neuropathic pain behaviors in a spinal nerve ligation model (**Lyu et al,2000**). However, it was later shown that Nav1.7 expressed in sensory neurons did not influence the development of neuropathic pain (**Nassar et al,2005**) and it was later suggested that Nav1.7-KO mice exert attenuated neuropathic pain symptoms due to a loss in sympathetic rather than sensory neuron activity (**Minett et al,2012**). Our results showed that while Nav1.7 channels are important contributors to synaptic transmission, the channels lose their pre-synaptic role following axotomy. The result agrees with the downregulation of Nav1.7 channels in nerve injury which has been demonstrated via electrophysiology (**Cummins & Waxman, 1997**), single-cell RNA sequencing (**Renthal et al,2020**) and qPCR (**Vysokov et al,2019**). The pre-synaptic loss-of-function for Nav1.7 following axotomy, together with the data in the previous chapter that DH neurons receiving sensory input

from axotomized axons produce enhanced responses (**Figure 4.14**), suggest plasticity in the contribution of Nav1.7 channels in modulating synaptic transmission. The downregulation of the channel at the pre-synaptic site, either through trafficking or other mechanisms, could be an explanation for other ion channels taking over as the drivers of pre-synaptic excitability in sensory neurons.

Hence, the role of Nav1.8 channels was examined in synaptic transmission. No evidence has been previously reported for a Nav1.8 involvement in neurotransmitter release, a result which was confirmed in both physiological and axotomy conditions in this chapter. Sporadic evidence for a role of Nav1.8 has been proposed in neuropathic pain, especially in a gain-of-function human study of small-fiber neuropathies (**Faber et al,2012**). An accumulation of Nav1.8 channels together with other TTX-S and TTX-R channels has been reported in painful human neuromas (**Black et al,2008**). A study from the lab had shown that concomitant blockade of pre-synaptic Nav1.7 and Nav1.8 channels ameliorates completely the responses of embryonic rat DH neurons (**Vysokov et al,2019**). This chapter's work corroborates the finding that dual blockade of Nav1.7 and Nav1.8 impairs the synaptic transmission of postnatal mice neurons under physiological conditions, thus revealing a cross species similarity with the embryonic rat study. A difference between the two studies is that dual Nav1.7 and Nav1.8 blockade abolishes DH responses in rats, yet in mice there is a significant attenuation but not complete silencing. A step further was taken to discern the individual contribution of Nav1.7 and Nav1.8 channels to synaptic transmission, as in the previous study experiments had been performed only during dual blockade. Considering the results of these experiments together, it was found that at least in the postnatal mice co-cultures, the contribution to synaptic transmission is only Nav1.7 and not Nav1.8-dependent.

In lieu of Nav1.7 and Nav1.8 channels, it was examined whether other VGSCs may emerge as contributors to synaptic transmission following axotomy. The expression of Nav1.6 has been shown to be upregulated following axotomy (**Vysokov et al,2019**) while a conditional KO study has shown that deletion of the channel in nociceptors ameliorates neuropathic pain symptoms (**Chen et al,2018**). Our results suggested that under physiological conditions, Nav1.6 does not play a role in synaptic transmission. However, Nav1.6 blockade attenuates DH responses following axotomy, suggesting an acquired role for the channel in synaptic transmission. For the observation that Nav1.6 channels obtain a novel role only following nerve injury, two contingencies may arise:

the highly plausible one is that sensory neurons are transcriptionally reprogrammed to upregulate pre-synaptic Nav1.6 expression after axotomy. A second, far-fetched possibility would be that the increased DH activity induces post-synaptic transcriptional modifications to change Nav1.6 expression. However, given that Nav1.6 channels are not mediating DH excitability under intact conditions and given that the direct stimulation of DH neurons in the presence of Nav1.6 blockade did not produce a significant effect, it is highly improbable that there is a post-synaptic component to this phenomenon. A compelling pursuit is to determine and compare the extent to which existing Nav1.6 channels compensate for the loss of function of Nav1.7 and Nav1.8 or whether *de novo* expression of Nav1.6 channels alters the excitability of nociceptors. A potential way to partially address this is the use of conditional Nav1.6-KO mice in the co-cultures between axotomized and non-axotomized cultures.

Another interesting consideration is to decipher whether there is a teleological adaptation to this manifested plasticity. Very recently, in an impressive longitudinal study of neuropathic pain, it was observed that nociceptors aberrantly reinnervate the tactile end organs following SNI, while low-threshold activated afferents do not, providing a novel mechanism for the development of allodynia (**Gangadharan et al,2022**). The study demonstrated persuasively the structural and anatomical reorganization of nociceptors, however the molecular correlates of the abnormal miswiring and aberrant excitability remain to be determined with spatially resolved transcriptomic studies in the future. Returning to the upregulation of Nav1.6 channels, it remains elusive how a channel normally expressed in the nodes of Ranvier (**Caldwell et al,2000**), which exhibits rapid activation and fast inactivation properties while it contributes to resurgent conduction in myelinated and unmyelinated fibers (**Black et al,2002**) is upregulated in nociceptors after axotomy. Our results demonstrated that DH neurons receiving input from the axotomized fibers produce larger responses, implying that axotomized nociceptors elicit greater DH responses and that this is at least partially Nav1.6-mediated at the pre-synaptic site. Given that nociceptor hyperexcitability is a driver of persistent nociceptor hyperactivity, it would be interesting to associate the emergence of Nav1.6, a rapidly activating channel as one of possibly several molecular correlates of evolutionary maladaptation to nerve injury in mammals (**Walters et al,2023**). As neuropathic pain resembles “an alarm that has gone off” (**Woolf,2010**), it is plausible that upon nerve injury, nociceptors molecularly adapt via transcriptional changes to become more rapidly hyperexcitable and concomitantly innervate wider surfaces through their nerve endings.

Such adaptations may culminate in accelerated alerts towards the CNS, enhanced protective responses and the promotion of organismal survival despite a henceforth absence of potential danger after healing.

A last observation of both physiological and clinical relevance in respect to the contribution of Nav channels to synaptic transmission, is the differential effects each channel exerts under physiological or neuropathic conditions. Although the triple blockade of Nav1.6, Nav1.7 and Nav1.8 channels significantly attenuated the responses of DH neurons in both conditions, the individual blockade of each channel revealed condition-specific effects. The Nav1.7 blockade is the driver of DH response attenuation under physiological conditions while Nav1.6 channels drive DH response attenuation only after axotomy. However, this physiological subtlety is masked during the application of non-selective VGSC blockers such as local anesthetics that appear to be effective in both healthy and neuropathic individuals **(Mao & Chen,2000)**. Similarly, TTX application may have analgesic properties in neuropathic pain models **(Lyu et al,2000)** but does not discriminate between TTX-sensitive VGSC subtypes. Due to the off-target effects of non-selective VGSC blockers, their chronic administration in neuropathic pain patients is not a desirable strategy. Our observation provides a basis for the ineffectiveness of selective Nav1.7 and Nav1.8 blockade in neuropathic pain and counter highlights Nav1.6 and the contingency of other upregulated ion channels as more effective targets for selective analgesic drug development to silence nociceptors.

Even more enigmatic is the role of pre-synaptic TRP channels. Early evidence found a dense localization of TRPV1 in synaptic terminals at the dorsal horn site **(Tominaga et al,1998)**. It has been shown that TRPM8-positive fibers project to lamina I of the dorsal horn **(Dhaka et al,2008)**, while in a co-culture model DRG and DH neurons, application of menthol increased the miniature EPSCs hinting towards a pre-synaptic localization for TRPM8 channels **(Tsuzuki et al,2004)**. Several studies support the pre-synaptic localization of TRPA1 channels **(Kosugi et al,2007)** **(Wringley et al,2009)** **(Kim et al,2010)**. Traditionally considered as transducer molecules that convert thermal and cold stimuli into electrical signals at the distal axons of nociceptors, the TRP channels may exert other functions depending on their expression site. Our data from the microfluidic cultures showed that under physiological conditions, mostly TRPV1 but also TRPA1 blockade may reduce synaptic transmission, suggesting a localization for the channels at the

central terminals. There has been evidence for both the pre- and post- synaptic expression of TRPV1 in the dorsal horn lamina II (**Valtschanoff et al,2001**) and for TRPA1 in the superficial laminae of the dorsal horn (**Kim et al,2010**). The pharmacological experiments of the selective blockade with the selective TRPV1 blocker AMG517 and the TRPA1 blocker A-967079 at the DH compartment do not discriminate between a pre- and post-synaptic synaptic role for the channels but underscore their involvement in modulating the excitability of DH responses from sensory neuron input.

The involvement of TRPV1 and TRPA1 is demonstrated at the “central” terminals of DRG in our co-culture model yet there are two outstanding questions regarding the physiological role of the channels at central terminals and the endogenous mode of activation of these channels. While both TRPV1 and TRPA1 are calcium permeable and a facilitatory role in synaptic transmission may be suspected (**Caterina et al,1997**) (**Tominaga et al,1998**), the opening of the channels is dependent on the presence of an endogenous ligands, but their identities remain elusive. The TRPV1 channels have been shown to be endogenously activated by the cannabinoid receptor agonist anandamide (AEA) (**Zygmunt et al,1999**) (**Tognetto et al,2001**), N-arachidonoyl-dopamine (NADA) which however is found in low concentrations in the DRG (**Huang et al,2002**) and by two linoleic acid derivatives, 9-HODE and 13-HODE, that have been shown to activate TRPV1 in DRG cell bodies (**Patwardhan et al,2009**) but their role inflammatory pain has been contested (**Alsalem et al,2013**). Other evidence has suggested that nitric oxide (NO) generated by nitric oxide synthases (NOS) and tetrahydrobiopterin (BH4) metabolism causes an S-nitrosylation of TRP channels to activate them (**Yoshida et al,2006**). The presence of NO both in the DRG and DH activates TRPV1 and TRPA1 to produce nociceptive effects (**Miyamoto et al,2009**). It has been proposed that intracellular $[Ca^{2+}]$ may directly activate human TRPA1 channels expressed in HEK293 cells (**Zurborg et al,2007**) suggesting a further physiological role for the channels. Other candidate endogenous ligands for TRPA1 are thiol-reactive molecules such as hydrogen peroxide, alkenyl aldehydes and prostaglandin J₂ (**Andersson et al,2008**) as well as bradykinin (**Bandell et al,2004**). The inflammatory mediators and subsequent metabolites may be excluded from the endogenous ligand candidates that activate TRPV1 and TRPA1 channels in our experimental model. There is an artificial mitigation of immune cell presence due to the treatment with the mitotic inhibitor AraC but also due to the *in vitro* nature of the assay used, the resident immune cells may no longer be present. The NO and endocannabinoid/endovanilloid candidates concentrate the highest

probability of activating the channels upon stimulation to mediate nociception. A distinct or synergistic effect of the endogenous compounds may be responsible for the pre-synaptic function of these channels. While a functional role remains to be determined, it is possible that they possess a thermoregulatory and nociceptive role under physiological conditions. Last, it has been proposed that TRPA1 channel may control the constitutive activity of KOR receptors, and that inhibition of the channel produces analgesia (**Semizoglou et al,2022**), a finding that might implicate pre-synaptic TRPA1 channels in mediating analgesia to the spinal cord.

On the contrary, both TRPA1 and more impressively TRPV1 blockade is no longer effective in attenuating DH responses after stimulation of axotomized sensory neurons. First, this observation corroborates that TRPA1 and TRPV1 may modulate synaptic transmission through their pre-synaptic, sensory neuron expression. Otherwise, in the case of a post-synaptic contribution of TRPA1 and TRPV1, both in intact and axotomized cultures at least a partial attenuation of DH responses would have been observed. Second, the results point towards a downregulation of trafficking of the channels following axotomy. A study of traumatic and diabetic neuropathy in human tissues has reported a reduction in the expression of TRPV1 in neuropathic nerves (**Facer et al,2007**). There has been no evidence for the involvement of neuronal TRPA1 and TRPV1 in neuropathic pain, unless regarding the sensitization of sensory neurons by mediators during the inflammation phase following nerve injury (**Bandell et al,2004**) (**Trevisan et al,2016**). There is clinical relevance to this observation, due to a paradoxical effect of persistent capsaicin application to the skin (**Anand & Bley,2011**). Topical capsaicin application in the form of capsaicin patches is used as a first line of treatment for acute pain. Although the mechanism of action of this effect has not been demonstrated yet, there is merit to the idea that prolonged capsaicin application may desensitize the primary afferent nociceptors and saturate neurotransmitter release and neuropeptide secretion to the dorsal horn, ultimately producing an analgesic effect. In that scenario, the TRPV1 and TRPA1 channels may be important modulators of pre-synaptic excitability and facilitators of neurotransmitter release. A contingency would regard both TRPV1 and TRPA1 blockade to augment the depolarization at the presynaptic site. However, this strategy might not be effective for long term neuropathic pain relief due the observed downregulation of the channels following axotomy.

Under the same rationale that capsaicin can produce short-term analgesic effects, menthol has been proposed to provide a cooling mechanism via TRPM8 for transiently attenuating inflammatory and acute pain (**Liu et al,2013**). However, our experiments demonstrated that the blockade of TRPM8 channels did not significantly attenuate DH responses to sensory axon input. There is a conjecture for the lack of role of TRPM8 in mediating synaptic transmission: the specialized expression of TRPM8 channels in a specific C-fiber subtype (**Usoskin et al,2015**) (**Zeisel et al,2018**) may be masking any reduced responses in TRPM8-expressing nociceptors, where the sum of the rest of nociceptors which are TRPM8-negative remains unaffected. For the observed effect of menthol causing producing analgesia, there might be a concomitant transduction of the compound by both TRPM8 and the more widely expressed TRPA1 channels which have also been shown to respond to menthol (**Karashima et al,2007**). Nevertheless, our results show that TRPM8 is not a significant or at least widespread contributor to synaptic transmission between the DRG and DH.

There is controversial evidence for the role of TRPM8 in neuropathic pain. In two chronic constriction injury (CCI) models of the sciatic nerve, a hypersensitivity to cold and menthol was produced, however the expression of TRPM8 is not altered in sensory neurons suggesting a lack of involvement for the channel in the manifestation of cold allodynia (**Caspani et al,2009**) (**Su et al,2011**). Application of icilin, a TRPM8 and TRPA1 agonist, increased intracellular $[Ca^{2+}]$ in sensory neurons and modulated the sensitivity of dorsal horn neurons to mechanical stimulation (**Brignell et al,2008**). The activation of the channel has been proposed to counteract the painful responses of sensory neurons in a neuropathic pain context (**Proudfoot et al,2006**) as well as in acute and inflammatory pain (**Liu et al,2013**) supporting a hypothesis that TRPM8 agonism by agents like menthol produce analgesia, hence ameliorating the increased thermal sensitivity in animals and humans (**Wasner et al,2004**). Given recent single-cell RNA sequencing data which identify a specialized cold-sensing subpopulation of sensory neurons (**Usoskin et al,2015**) (**Sharma et al,2020**), it is possible that the activation of the TRPM8-expressing sensory neuron subtype antagonizes the noxious input from the inflicted sensory neurons, facilitating a peripheral gate control of sensory transmission to the spinal cord (**Fuller et al,2023**).

Our results show that TRPM8 channels do not play a pre-synaptic role in mediating transmission between the DRG and DH after axotomy. It is probable that this occurs again due to the lack of

discrimination between nociceptors subtypes in culture, where TRPM8-positive neurons that respond to the blockade are masked. Another possibility is that channel expression remains stable after axotomy, hence continuing to not play role in neuropathic pain. On the contrary, it has been reported that nociceptors produce a *de novo* upregulation of TRPM8 channels following injury (Yarmolinsky et al,2016). A pathological consequence of neuropathic injury is the development of cold allodynia which has a TRPM8-dependent component (Xing et al,2007). It is a compelling hypothesis that the molecular reorganization of thermoregulatory elements in nociceptors may thus contribute towards the occurrence of cold allodynia and the maladaptive processing of temperature stimuli in neuropathic pain patients. Given the abundance of TRPV1 and TRPA1 expression in nociceptors (Usoskin et al,2015) (Sharma et al,2020) and their mRNA downregulation following nerve injury (Renthall et al,2020) it is possible that they might be involved in the development of cold allodynia. The pharmacological inhibition of TRPA1 reduced symptoms of cold allodynia after SNI (del Camino et al,2010). It has been shown that in human nerve injured tissues there is a reduced expression of TRPV1 channels (Facer et al,2007). It has also been recently shown that TRPV1 antagonists act on sensory neurons to alter the core body temperature to cause hypothermia in mice, a symptom that also occurs in patients (Yue et al,2022). In contrast, the ablation of TRPV1 lineage nociceptors produces only latent and weak development of cold allodynia (Cobos et al,2018), however other heat sensitive TRP channels may compensate for the genetic deletion. A study using silencer RNA has in contrast shown that TRPV1 knockdown markedly reduces cold allodynia in neuropathic models (Christoph et al, 2006). Taken together, these results raise the possibility that upon nerve injury, the loss of thermal sensor TRPV1 and TRPA1 expression may account for changes in synaptic transmission but also may contribute towards the development of cold allodynia, while silent TRPM8-positive silent nociceptors concomitantly emerge as an acquired cellular substrate for this phenomenon (Yarmolinsky et al,2016).

In summary, this chapter provides evidence for the physiological properties of six important pre-synaptic TRP and Nav channels in both physiological and neuropathic pain-mimicking conditions. At the basic science level, the findings advocate for a role of Nav1.7 channels in neurotransmitter release which is lost following injury. While Nav1.8 channels do not contribute to the synaptic transmission between DRG and DH neurons, Nav1.6 channels emerge as significant modulators following axotomy. In regard to TRP channels, TRPV1 and TRPA1 play an eminent role in modulating synaptic responses to peripheral stimulation which is lost after injury. While TRPM8

channels putatively do not play a role in synaptic transmission across all nociceptor subtypes, the loss of TRPV1 and TRPA1 function after axotomy may be responsible for the thermostatic reorganization of sensory neurons that contributes towards cold allodynia occurring after nerve injury. At a translational level, these results advocate against the development of selective Nav1.7 and Nav1.8 for the treatment of neuropathic pain, while they highlight Nav1.6 and other post-injury upregulated ion channels as more effective drug targets. For TRP channels, it is shown that TRPV1 agonism with capsaicin may be an ineffective treatment against neuropathic pain while menthol induced analgesia might not be exclusively TRPM8-positive nociceptor mediated. Overall, the described observations provide explanations for the lack of efficacy of certain selective drugs in pathological pain conditions, such as Nav1.7 blockers that do not cross the blood-brain barrier, while they underscore a role for other therapeutic targets for neuropathic pain. Moving forward, further *in vivo* behavioral and electrophysiological, molecular and immunohistochemical work is necessary to evaluate these electrophysiological observations and confirm whether these explanations define the broader physiological interpretations discussed here.

6. General conclusions

The introductory chapter stated that the tenet of this thesis is to provide a platform where the properties and plasticity of sensory neurons can be robustly investigated. To explore the plasticity and excitability of nociceptors at different, isolated, anatomical compartments (axons and soma) and under different conditions, we have developed a novel *in vitro* microfluidic platform. The *in vivo* studies that employ electrophysiological and behavioral approaches lack the spatial resolution of investigating properties at different anatomical locations across nociceptors. Moreover, while the conduction velocity or the diameter size properties of nociceptors can be used as a measure of sensory neuron subtype identification, the molecular classification of these neurons is yet impossible in *in vivo* experiments. In *in vitro* preparations such as the skin-saphenous nerve preparation, there is limited ability for pharmacological or genetic manipulations accompanied by the requirement of a manual dexterity that can prove very time-consuming. The conventional primary DRG neuron cultures are amenable to pharmacological investigations but have focused on the excitability of DRG cell bodies which most often do not represent the sites of injury or inflammation.

In contrast, microfluidic cultures resolve many of these technical difficulties. They incorporate the positive attributes of conventional cultures but allow the investigation of different anatomical properties of nociceptors such as the axons, cell bodies and pre-synaptic terminals. Moreover, the isolation of the compartments allows via pharmacological manipulations the determination of spatially precise properties of nociceptors. The use of mice in both DRG axonal investigations and DRG-DH co-culture examination of synaptic transmission properties has unraveled the opportunity to introduce transgenic animal models together with the pharmacological studies.

This thesis opens (or at times closes) several new avenues for analgesic drug development. The third chapter highlights that PGE₂ does not only sensitize the responses of sensory axons to depolarizing stimuli, but it also directly and persistently activates sensory axons. The relevance of this finding regards acute and persistent inflammatory pain conditions where the presence of PGE₂ may directly elicit painful sensation. Given that the attenuation of PGE₂ production by COX-inhibitors may result to adverse effects, we delineated the downstream pathway upon which PGE₂ exerts this depolarizing effect. The mediation of the EP₄/cAMP/PKA pathway ultimately results

in the activation of Nav1.8 and ANO1 channels. The observation points towards the exploration of Nav1.8, ANO1 or even synergistic blockade of the channels for the attenuation of PGE2-mediated inflammatory pain. More *in vivo* behavioral and electrophysiological, as well as *in vitro* patch-clamp electrophysiological experiments will further delineate the contribution of these channels to inflammatory pain.

The fourth chapter provides an *in vitro* recapitulation of the synaptic connections between DRG and DH neurons. It demonstrates the formation of functional synapses in the co-culture between postnatal mouse DRG and DH neurons, resolving a previous technical challenge regarding the survival of mouse DH neurons in culture. The establishment of this platform recapitulated salient features of the *in vivo* first sensory synapse and enabled the investigation of molecules that contribute to synaptic transmission. The glutamatergic nature of the synapse was confirmed via immunocytochemical and calcium imaging experiments combined with pharmacology. This platform can be used to delineate further topographical features of the molecular physiology of nociceptors, as every compartment (DRG axonal, DRG cell body, DH) is isolated. At the same time, the platform has an industrial utility, as it can also be readily used to screen blockers of nociceptor excitability and determine whether the blockade is sufficient to silence DH activity. Last, an axotomy model is introduced to mimic the *in vivo* models of nerve transection. It is shown that stimulation of axotomized neurons results in enhanced DH neuron activity in contrast to non-axotomized neurons, pinpointing to enhanced excitability and modulated neurotransmitter release mechanisms following axotomy. This evidence supports that the enhanced excitability of DH neurons responses following nerve injury *in vivo* as a contributor to neuropathic pain.

The fifth chapter utilizes the DRG-DH co-culture model to unravel novel roles for the contribution of six nociceptor-expressed TRP and Nav channels in synaptic transmission. The physiological role of the nociceptor expressed TRP channels had been perceived to be the transduction of thermal and pungent chemical stimuli, while Nav channels had been considered to mediate the generation and propagation of APs across the nociceptor membrane. Our results show a pre-synaptic role for Nav1.7 channels in uninjured axons. Performing an axotomy on DRG neurons results in a loss of this synaptic function. Conversely, Nav1.6 channels are not contributing to synaptic transmission in uninjured axons but emerge as the predominantly contributing VGSC to synaptic transmission following axotomy. The results underscore that VGSC may exert pre-synaptic roles that modulate

neurotransmitter release mechanisms in the pre-synaptic zone, while they advocate against the development of Nav1.7 and Nav1.8 blockers for neuropathic pain conditions. Regarding TRP channels, TRPV1 and TRPA1 blockade surprisingly revealed an important contribution for these channels to synaptic transmission which is lost after axotomy. The existence of these channels pre-synaptically elicits novel questions about their physiological role while also points to the need for further experiments to re-evaluate the effectiveness of TRPV1 and TRPA1 blockers for neuropathic pain.

As with every technique, there are ensuing caveats that require improvement. The first one is the use of NGF, a pro-nociceptive and sensitizing agent, for the establishment of a gradient that enables axonal crossing within the microfluidic corridors. As it could be considered that the presence of NGF “primes” nociceptors to be more excitable, it would perhaps be interesting to investigate whether the gradient can be maintained under lower NGF concentrations or substituted with another neurotrophic agent. Second, in contrast to *ex vivo* spinal cord slices, the recapitulation of the center-to-periphery circuitry with the DRG-DH co-cultures does not maintain the somatotopic alignment observed *in vivo*. The reason is simply because the DRG and DH neurons are collected and enzymatically digested separately in order to be loaded to different compartments of microfluidic devices. There are however at least two strengths of the model: first, the platform maintains salient features of the DRG-DH physiology such as the formation of functional synapses between sensory axon terminals and glutamatergic DH neurons. Second, the full periphery-to-center circuit is recapitulated, ranging from the sensory axons that represent the free nerve endings of nociceptors all the way to the proximal site of the DRG-DH synapse. A third, also unresolvable caveat regards the axotomy model of DRG neurons. It can be validly argued that the removal of DRG neurons through their dissection for primary cultures, is a form of axotomy itself. Hence, it may be considered that the DRG neurons are undergoing a second axotomy within our model. While these technical limitations can be optimized but not completely resolved *per se*, the validation of the *in vitro* culture observations may be corroborated by nerve explants, slice recordings and with *in vivo* imaging and behavioral studies.

An important improvement in the DRG-DH co-culture model would regard the precise delineation of which sensory neuron directly provides input to an activated DH neuron following stimulation. Thus far, the activation of DH neurons is recorded after the stimulation of sensory neurons,

however the identity of the DRG neurons that formed functional connections could not be pinpointed. Partially, this occurs due to the convoluted sprouting of axons which does not allow a 1:1 correspondence with their synapse-forming DH neurons. A potential way to resolve this technical consideration is the use of photoconvertible fluorescent probes such as CaMPARI **(Fosque et al,2015)**, a calcium indicator that labels and traces the activated sensory neurons and their respondent DH neurons. A second improvement regards the use of pharmacological tools to characterize the molecular properties of nociceptors. The use of pharmacology is limited to the delineation of mechanisms of only known molecular targets. The reason is simply because since selective agonists/antagonists of these molecules have already been developed, it also means that the molecules have already been cloned and characterized by previous efforts. If one is searching for novel, largely uncharted molecular mechanisms, then large-scale molecular genetic techniques are needed. The emergence of spatial transcriptomics **(Ortiz et al, 2021 for review)** is revolutionizing molecular biology and is rapidly involving into spatiotemporal transcriptomics **(Ren et al,2023)** and spatial translomics **(Zeng et al,2023)**, where the expression and topography of several molecules will be detected at single cell resolution in the future. It may be possible one day to characterize the entire transcriptome of each nociceptor subtype with topographical precision, where the axonal expression of a particular molecule could be immediately quantified and compared to the cell body expression of that molecule. Moreover, the optimization of super-resolution imaging techniques such as that of STORM **(Rust et al,2006)** and MINIFLUX **(Eilers et al,2018)** will allow the localization of molecules in nociceptors and may even resolve debates about the trafficking or *de novo* localized mRNA translation across them. The subtlety, expertise, financial costs and geographical restriction of these technological advancements are prohibitive for the time being, especially outside of the US.

Overall, this thesis provides novel insights about the cellular, molecular and synaptic properties of nociceptors. It corroborates the tenet that effective therapeutic strategies can be devised at the level of peripheral sensory neurons that exhibit tremendous plasticity. The microfluidic platform is versatile and can be readily exploited using different tissues such as mouse, rat, human and hiPSC neurons. It can also be used for the study of isolated cellular and molecular interactions where DRG neurons can be co-cultured with skin, joint, immune, visceral and glial cells. The platform can also assist in disease modelling, of inflammatory and neuropathic pain, diabetic neuropathy, chemotherapy-induced peripheral neuropathy (CIPN), regeneration, motor neuron diseases and

more. Perhaps more than anything, it highlights how a technical advancement can allow us to revisit questions that were previously considered more elusive and hopefully in the future it can display an exponential potential in its utilities, along with the ones demonstrated here.

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