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A Microfluidic Approach to Investigate Changes in Functional Properties of Nociceptive Axons Underlying Inflammatory Pain States

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***A Microfluidic Approach to Investigate Changes in
Functional Properties of Nociceptive Axons
Underlying Inflammatory Pain States***

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

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2014-2017

Declaration

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

Natasha R. Rangwani

November 2017

Abstract

The lack of analgesics available for the management of chronic pain is attributed to our limited understanding of the molecular mechanisms responsible for this debilitating condition. Our current understanding of pain mechanisms is primarily based on extrapolation from observations of the dorsal root ganglia (DRG) cell body. However, our knowledge of the functional properties of nociceptive nerve terminals and axons, where the pain response is initiated, is still lacking.

To address this gap in our understanding, I established an *in vitro* microfluidic (MFC)-based model to investigate functional properties of nociceptive axons in isolation from the cell soma. This platform made it possible to model peripheral sensitisation in response to a combination of inflammatory mediators (IM) including prostaglandin E2 (PGE2), bradykinin, histamine and serotonin. Two types of functional sensitisation were identified in isolated IM-treated nociceptive axons using this model: 1. enhanced magnitude of axonal responses, and 2. increased number of responders were observed in response to chemical stimuli, compared to vehicle-treated groups. We propose this MFC-based *in vitro* platform as a novel model to study peripheral inflammatory sensitisation, delivering an improved capacity to understand the major axonal molecular players of inflammatory pain conditions.

PGE2 is a widely-known pain mediator, and the most abundantly produced eicosanoid lipid in inflamed tissue. Despite the pivotal role of PGE2 in the genesis of neuropathic and inflammatory pain, the underlying molecular mechanisms are yet unclear. The developed MFC-based model was exploited to decipher axonal mechanisms responsible for changes in nociceptor function induced by PGE2. PGE2 directly depolarised the axonal membrane resulting in persistent neuronal activity, observed even after removal of the PGE2 stimulus. This points towards the basis of clinical ongoing pain patients experience, which persists despite resolution of inflammation. Using pharmacological strategies, we identify Nav1.8 channels and the cAMP/PKA signalling pathway as important mediators of PGE2-induced persistent neuronal activity.

To further investigate key players underlying nociceptor sensitisation, we hypothesized that axonal HCN2 channels are required for nociceptor sensitisation and activation in response to inflammatory mediators. The role of axonal HCN2 was explored using both pharmacological tools as well as HCN2 knockout mice. However, based on the data presented in this study, it remains inconclusive whether axonal HCN2 channels play an important role in inflammation-induced nociceptor sensitisation and PGE2-induced persistent axonal activity.

Next, I sought to extend the use of the MFC-based assay to human-induced pluripotent stem cells (hiPSC)-derived nociceptive neurons with the aim of developing a more clinically relevant *in vitro* model of peripheral sensitisation. We established optimal conditions to culture these cells in MFC devices and characterised the different populations of neurons present at 4 weeks *in vitro*. We also reveal preliminary data suggesting the presence of TRPV1-independent mechanisms underlying peripheral sensitisation as well as GDNF-dependent capsaicin sensitivity in hiPSC-derived sensory neurons.

This study describes the use of MFC platform as a novel and robust tool to study axonal mechanisms of pain in both primary rodent and human stem cell-derived neurons. Moreover, the work presented expands our knowledge of axonal molecular mechanisms contributing towards peripheral sensitisation and particularly, persistent neuronal activity observed during inflammatory pain states.

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At the beginning of my PhD, I started with a blank canvas, which I have now – three years later - managed to fill with unique knowledge. However, this work would have not been possible without the help of a number of people, and here I would like to express my gratitude towards all these individuals.

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Abbreviations

5HT- 5-hydroxytryptamine Receptor or Serotonin

AP – Action Potential

ASIC – Acid Sensing Ion Channel

ATP – Adenosine Triphosphate

AUC – Area under the Curve

BDNF – Brain derived Neurotrophic Factor

BK – Bradykinin

BSA – Bovine Serum Albumin

cAMP – cyclic Adenosine Monophosphate

Rp- cAMPS – (*R*)-Adenosine, cyclic 3', 5'-(hydrogenphosphorothioate)

triethylammonium

CCI – Chronic Constriction Injury

CFA – Complete Freud's Adjuvant

cGMP – cyclic Guanosine Monophosphate

CGRP – Calcitonin Gene Related Peptide

CMOS – complementary metal-oxide-semiconductor

CNG – cyclic nucleotide-gated channels

CNS – Central Nervous System

DAG – diacylglycerol

Dil – 1,1'-Dioctadecyl-3,3,3',3'-Tetramethylindocarbocyanine Perchlorate

DIV – Days *in vitro*

DMSO – Dimethylsulfoxide

DRG – Dorsal Root Ganglia

ET-1 – Endothelin 1

FITC – Fluorescein Isothiocyanate

FoV – Field of view

GDNF – Glial cell derived Neurotrophic Factor

GPCR – G-protein Coupled Receptor

HBSS – Hank's Balanced Salt Solution

HCN – Hyperpolarisation-activated Cyclic Nucleotide-gated

HEPES – *4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid*

hiPSC – human induced pluripotent stem cell

IM – Inflammatory Mediators

IP3 – inositol 1, 4,5-trisphosphate

KCl – Potassium Chloride

KO – Knock out

MAPK – Mitogen-activated Protein Kinase

MFC – Microfluidic Cultures

NB-A – Neurobasal A

NMDA – N-methyl-D-aspartate

NGF – Nerve Growth Factor

NK1 – Neurokinin Receptor 1

NT3 – Neurotrophin-3

PDMS – Polydimethylsiloxane

PBS – Phosphate Buffered Saline

P2X – Purinergic 2X

PGE2 – Prostaglandin E2

PIK3 – Phosphoinositide 3-Kinase

PIP2 – Phosphatidylinositol 4, 5-bisphosphate

PKA – Protein Kinase A

PKC – Protein Kinase C

PLC – Phospholipase C

PLL – Poly-L-Lysine

PNS – Peripheral Nervous System

ROI – Region of Interest

RT – Room Temperature

SND – Standard Neuron Device

SNL – Sciatic Nerve Ligation

SP – Substance P

TCND – Triple Chamber Neuron Device

TNF α – Tumor Necrosis Factor-alpha

TRKA – Tropomyosin Receptor Kinase A

TRPV1 – Transient Receptor Potential Vanilloid 1

TTX – Tetrodotoxin

TTX-R – Tetrodotoxin-Resistant

TTX-S – Tetrodotoxin- Sensitive

VGCC – Voltage-gated Calcium Channels

VGSC – Voltage-gated Sodium Channels

WT – Wild type

1. GENERAL INTRODUCTION

Inflammatory pain remains to be a significant clinical challenge, with a large number of patients receiving inadequate pain relief. Despite significant advances in our understanding of mechanisms underlying the pathophysiology of inflammatory pain, most findings from preclinical research have failed to translate into successful therapies in the clinical setting. Known molecular mechanisms underlying pain sensation are based primarily on traditional *in vitro* preparations which are restricted to one component of nociceptors: the cell body. However, our knowledge of the pharmacology of peripheral fine nerve terminals, where the nociceptor is exposed to the inflammatory insult, is yet primitive. Recent evidence points towards distinct mechanisms taking place in the subcellular compartments of nociceptors, underlying painful conditions. Thus, the role of axonal molecular mechanisms contributing to changes in sensory neuron function during inflammatory pain states will be the main focus of this thesis.

This introduction provides an overview of what pain is, the different types of pain and its pathophysiology, including both central and peripheral mechanisms. More specifically, the available literature on mechanisms associated with known key players involved in inflammatory pain mechanisms including inflammatory mediators (IM), second messenger pathways and ion channels will be discussed. Finally, this chapter will evaluate the translatability of current *in vivo* and *in vitro* models, and will describe the DRG axon as a better model to study molecular mechanisms of nociceptive nerve terminals underpinning painful conditions.

1.1. Introduction to Pain

Pain is defined as “an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage” by the International Association for the study of Pain (IASP).

According to Global Burden of Disease (GBD) study 2013, published in the Lancet, a number of both acute and chronic pain conditions are major contributors to the overall global burden of disease. This study reported that within the category of acute conditions, tooth pain was the 5th most common condition contributing to GBD and within the category of chronic conditions, six of the most common conditions contributing to GBD included migraines, lower back pain, chronic neck pain, pain-related to musculoskeletal conditions, osteoarthritis and urolithiasis (Rice et al., 2016). According to a recent meta-analysis, chronic pain conditions affect between one-third and one-half of the population in the United Kingdom and this is likely to increase over the next decade due to the ageing population (Fayaz et al., 2016). These findings highlight the importance of pain conditions in their contribution towards the overall global burden of disease and calls upon the recognition of pain as a clinical entity as opposed to a symptom of other conditions.

Opioids and non-steroidal anti-inflammatory drugs (NSAIDs) remain as the first-line classes of analgesics, since the discovery of morphine and the synthesis of aspirin in the 1800s. Opioids are the most potent analgesics that are routinely used for cases of acute severe pain, as well as chronic pain in terminal diseases. However, these drugs are associated with a number of life-threatening side effects including tolerance, addiction and respiratory depression, owing to which the use of opioids for

the management of pain has become highly controversial. A meta-analysis of 67 reports suggested that opioid analgesic therapy in chronic pain patients is associated with addiction and illicit substance abuse (Fishbain et al., 2008). Similarly, while NSAIDs are effective in a wide range of patients, the use of these drugs is associated with serious gastrointestinal side effects. A population study reported that NSAIDs cause 3500 hospitalisations for and 400 deaths from ulcer bleeding per year in the UK, in patients aged over 60 (Langman, 2001). NSAIDs act via inhibition of cyclooxygenase (COX) enzymes, which are responsible for the production of inflammatory prostaglandins. Two distinct isoforms of the COX enzyme (COX-1 and COX-2) were discovered in the 1990's (Tazawa et al., 1994). Initially, it was thought that the undesirable effects of NSAIDs were associated with inhibition of COX-1, which is constitutively present and responsible for homeostatic functions of prostaglandins, whereas the therapeutic effect of NSAIDs was owed to COX-2 inhibition, considered to be induced only during inflammatory conditions. Based on this, COX-2 selective inhibitors were developed with the aim of reducing the number of side-effects associated with NSAIDs. Although drug companies were successful in developing selective COX-2 inhibitors (i.e. rofecoxib, celecoxib) which showed an improved gastric safety profile, their long-term use has been associated with increased risk of heart attack and stroke (Bresalier et al., 2005). Due to this, rofecoxib was withdrawn from the market in 2004 and the cardiovascular risk of COX-2 selective inhibitors is currently being reassessed (Varga et al., 2017). It is now thought that COX-2 is not strictly inducible and may be involved in physiological functions of the body, as it has been found to be expressed constitutively in the brain, kidney and reproductive tract (Ferrerri et al., 1999; Kniss, 1999; Yamagata et al., 1993). Despite decades of research, drug development companies have failed to

design pain-killers with the broad range of analgesic efficacy or potency of opioids and NSAIDs, without their associated adverse effects.

1.2. Types of Pain

Pain can be classified into different categories based on its duration and pathophysiology. Acute pain is characterized by a sudden onset, with a duration of hours to days. In this case, the cause of pain is known (*i.e.* illness, trauma or surgery) and sensation of pain disappears when the underlying cause is treated. By contrast, chronic pain is thought to initiate in the same manner as acute pain, but its duration is much longer, as the pain persists despite the resolution of the underlying cause.

Chronic pain is generally divided into two main categories; inflammatory pain and neuropathic pain. Inflammatory pain is associated with tissue damage, which results in an ongoing inflammatory response. Neuropathic pain is defined as pain caused by lesion or disease affecting the somatosensory nervous system.

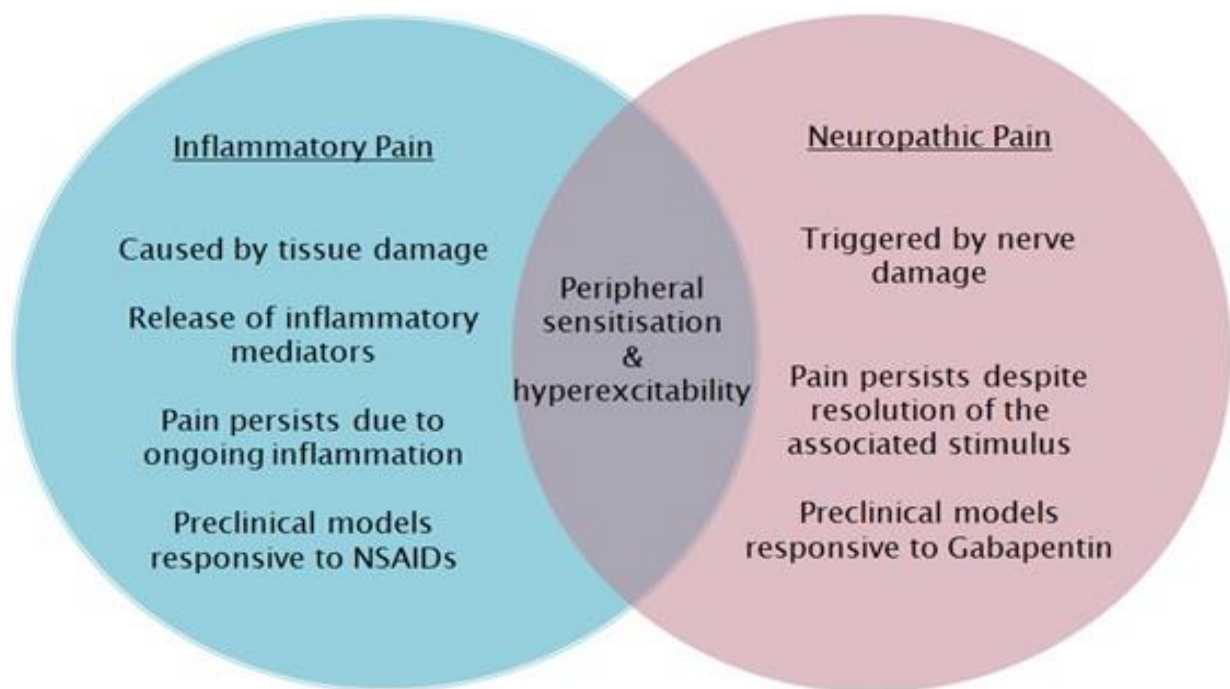


Figure 1. Key characteristics of chronic inflammatory and neuropathic pain.

Common aspect of peripheral sensitisation and hyperexcitability in both major types of chronic pain is highlighted.

However, it is important to note that there is significant overlap between these two categories. Sensory fibres can be damaged or sensitised as a result of long-term inflammation and neuronal damage can result in an ongoing inflammatory reaction such as in chronic inflammatory demyelinating neuropathy. Moreover, despite their distinct etiologies, both types of pain have a common feature: sensitisation of peripheral afferent neurons (**Figure 1**). The sensitisation of peripheral nociceptive neurons involves hyperresponsiveness, reduced activation thresholds and spontaneous activity which contribute towards common pain symptoms such as hyperalgesia, allodynia and spontaneous pain are observed in both types of chronic pain.

1.2.1. Neuropathic pain

Neuropathic pain is defined as pain arising as a direct consequence of a nerve lesion or a disease affecting the somatosensory system. This definition covers a variety of painful neuropathic disorders ranging from diabetic neuropathy to lower back pain (Treede et al., 2008). Neuropathic pain affects 1 in 5 adults world-wide and the long-term pain (> 6 months) results in a low quality of life, high morbidity and significant costs in healthcare (Andrew et al., 2014).

Both peripheral lesions such as mechanical trauma, metabolic disease or infections and nerve damage of the central processes (i.e. spinal cord injury, stroke or multiple sclerosis) can lead to chronic neuropathic pain, but its pathophysiology is not well understood. However, both peripheral and central nervous systems are thought to be involved and this complicates its clinical diagnosis, and as a consequence leads to difficulties in choosing appropriate treatment for patients.

Neuropathic pain induced-changes can vary between patients depending on the type and extent of the injury. Removal of debris, recruitment of anti-apoptotic survival factors, induction of axonal growth and collateral sprouting, synaptic remodelling and remyelination are some of the changes that are potentially adaptive and are aimed at the rebuilding and growth of the peripheral nerves as well as restoring normal axonal target contacts (Cafferty et al., 2008). However, there are a number of alterations that may occur in the nervous system after a neural insult which are clearly maladaptive and contribute to the development of neuropathic pain. These include both central and peripheral changes in the activation threshold of different ion channels, spontaneous action potential firing, reduced GABAergic signalling,

inadequate connectivity and neuronal loss which contribute to the hyperexcitable state of the nervous system (Woolf, 2011). There is evidence of a phenotypic switch of large diameter A β fibres. Under physiological conditions, these neurons respond to low-threshold input and generate innocuous sensation. However, following a neural insult, they undergo alterations and produce pain (Witting et al., 2001). For instance, a study showed that after an intraplantar injection with an inflammatory stimulus, A β fibres adopt the capacity to express substance P and enhance dorsal horn excitability resulting in an exaggerated response to innocuous stimuli (Neumann et al., 1996). Ectopic impulse generation is also an important aspect of neuropathic pain, as several patients report experiencing spontaneous pain or ongoing pain in absence of a stimulus (Campero et al., 1998). A significant amount of evidence points towards the fact that spontaneous activity originates in the periphery, from neuromas and cell bodies of injured DRG neurons (Amir et al., 2005; Wu et al., 2002).

1.2.2. Inflammatory pain

Pain is a common symptom underlying several chronic inflammatory conditions including different forms of arthritis, inflammatory bowel diseases and periodontitis to name a few. Arthritis alone affects approximately 10 million people in the UK, with a cost of 161million on community prescriptions for arthritis conditions in England in 2015 (According to Arthritis Research UK). These chronic inflammatory diseases are extremely debilitating as they deteriorate quality of life significantly and are associated with high costs of therapy and care as well as high mortality (Straub and Schradin, 2016). Pain is a characterizing symptom of arthritic conditions, as it is considered the primary reason patients seek rheumatic care. Moreover, pain syndromes such as fibromyalgia are strongly associated with rheumatoid arthritis

patients, which complicates disease management significantly (Coury et al., 2009). Traditional treatments such as disease modifying anti-rheumatic drugs (DMARD), focus on reducing inflammation though there has been a greater emphasis on treating the pain itself (Lee, 2013).

The cardinal signs of inflammation include *rubor* (redness), *tumor* (swelling), *dolor* (pain) and *calor* (heat) which occur due to vasodilation, oedema and altered neural signals (Donaldson, 2014). The inflammatory response is an extremely complex phenomenon, involving the simultaneous interplay of a number of different cell types including immune, epithelial and neuronal cells. Following tissue or nerve damage, a wide variety of mediators are released by damaged tissue, recruited immune cells as well as surrounding sensory fibres. These include prostaglandins, bradykinin, Substance P, histamine, adenosine triphosphate (ATP) and serotonin, amongst others (Dray, 1995). Ongoing inflammation presents a huge challenge for both researchers and clinicians as it is a primary cause of chronic painful conditions, organ dysfunction, and death. Mechanisms of inflammatory pain will be discussed in further detail in sections 1.4 and 1.5.

1.3. Pathophysiology of Pain

Pain is a protective response of our body, which is crucial for avoiding tissue damage, as it alarms the body of potentially damaging stimuli by triggering avoidance behaviours. Specialised sensory neurons or nociceptors are the cellular components of the painful response. Nociceptors have a number of receptors, including ion channels and GPCRs, that are responsible for transducing mechanical, thermal or chemical stimuli leading to depolarized membrane potential

(Basbaum et al., 2009; Gold and Gebhart, 2010). An intense enough change in membrane potential can result in the generation of electrical signals defined as action potentials which are transmitted to central relay stations of the pain pathway (Figure 2).

1.3.1. Different Components of Nociceptors

Nociceptors are pseudo-unipolar primary afferent neurons, where a single axon protrudes from the cell body of the dorsal root or trigeminal neuron and is split into one peripheral branch, sending the axon to innervate the skin and one axon to synapse on second order neurons of the dorsal horn of spinal cord. The subcellular components of sensory neurons include the nerve terminal, the axon, and the cell body which will be described further below in the context of nociception.

1.3.1.1. The Nerve Terminal

The peripheral terminals are responsible for the detection and transduction of external stimuli into electrical messages or pain signals (*i.e.* action potentials). These peripheral terminals or nerve endings express a variety of ionotropic and metabotropic receptors that are finely tuned to respond to specific thermal, mechanical or chemical stimuli. Moreover, the differential expression and distribution of ion channels defines specific pain modalities (*i.e.* type of sensation such as heat, cold or mechanical stimuli), conferring the rich functional heterogeneity observed between subpopulations of sensory neurons (Dubin and Patapoutian, 2010). For instance, noxious heat, activates nociceptors which express the transient receptor potential (TRP) vanilloid 1 (TRPV1), also known as the receptor for capsaicin, the active ingredient of chilli peppers (Caterina et al., 1997). Conversely, noxious cold, activates nociceptors expressing TRPM8, also known for its sensitivity to menthol

(Bautista et al., 2007). Sensors of mechanical stimuli are less well defined and characterized. However, studies have suggested potential candidates including some TRPV2 and TRPV4, acid-sensitive ion channels (ASIC1, 2, and 3), potassium channels as well as Piezo channels (Delmas et al., 2011; Patapoutian et al., 2009a). Finally, chemical stimuli can act on metabotropic receptors such as GPCRs located at the nerve endings, and modulate ion channels indirectly through the activation of signal transduction mechanisms. Indirect modulation of ion channels also results in altered nociceptor excitability.

1.3.1.2. The Axon

Supra-threshold stimuli results in activation of nociceptors, resulting in depolarization and subsequent generation of action potentials (AP). Axons are responsible for the conduction or transmission of these AP to the cell body. Voltage-gated ion channels along the axonal membrane are activated by the change in membrane potential and play crucial role in nociception: channels selective for sodium and potassium ions are critical for the generation of action potentials, whereas calcium channels serve in the release of neurotransmitters from central or peripheral terminals as depicted in **Figure 2** (Basbaum et al., 2009).

1.3.1.3. The cell body

The cell body, or the nucleus containing component of the neuron, responds to the arriving action potentials through transcriptional and post-translational modifications, which ultimately modulate gene expression of important molecular players of nociception resulting in altered function, localization or structure of a given protein (Woolf and Costigan, 1999). For instance, studies have shown that an injection of carrageenan, commonly used to induce an inflammatory response in animal models,

led to increased transcription and translation of the Nav1.8 ion channel, which contributes towards increased excitability of nociceptors (Coggeshall et al., 2004).

1.3.1.4. Higher central centres

Finally, induced action potentials reaches the central terminals, which synapse on second order neurons located in defined areas of the spinal cord dorsal horn (Woolf and Ma, 2007). The spinal cord is the first relay station, and is involved in modulation of the signals by inhibitory interneurons. These signals then travel supraspinally to the thalamus, where further processing of the nociceptive signal occurs. Nociceptive signals are interpreted and perceived as pain sensation in somatosensory and prefrontal cortices in the brain (Tracey and Mantyh, 2007). Of note, a descending feedback pathway modulates ascending information, and thus is responsible for the regulation of nociceptor activity (Millan, 2002). The limbic system, specifically the amygdala also plays an important role in the emotional evaluation of sensory information as well as pain-enhancing and pain-inhibiting functions, serving as an important modulator of the experience of pain (Strobel et al., 2014). To conclude, nociceptors are the cellular substrate for two pivotal aspects of nociception: the transduction and transmission of noxious stimuli from the periphery to the CNS.

1.3.2. Classification of nociceptors

Nociceptors are generally classified into two major categories, thinly myelinated A δ - and slow conducting or unmyelinated C- fibres (Meyer et al., 2006). Both A δ - and C- fibres are polymodal, as they are capable of detecting and transducing a variety of noxious stimuli, including thermal, mechanical and chemical. However, they differ in their anatomical and molecular characteristics which confer their distinctive response properties.

A δ fibres are medium-diameter myelinated afferents, responsible for mediating fast nociception. These are subdivided in two subpopulations: type I, and type II. Type I A δ - fibres are sensitive to mechanical and chemical stimuli, but have a relatively low sensitivity for heat. On the other hand, type II A δ - fibres are sensitive to heat but have a high threshold for mechanical stimuli (Basbaum et al., 2009; Lawson, 2002). C-fibres are small-diameter unmyelinated afferents that mediate slow nociception. C-fibres can be further classified in two subpopulations: peptidergic and non-peptidergic fibres. Peptidergic neurons express the receptor for nerve growth factor (NGF), TrkA, and release neuropeptides such as substance P (SP) and calcitonin gene-related peptide (CGRP). Peptidergic neurons project primarily to lamina I and outer lamina II. Non-peptidergic neurons express the receptor for glial-derived neurotrophic factor (GDNF), c-RET, and bind the IB4 isolectin. These neurons project primarily to inner lamina II (Basbaum et al., 2009; Lawson, 2002).

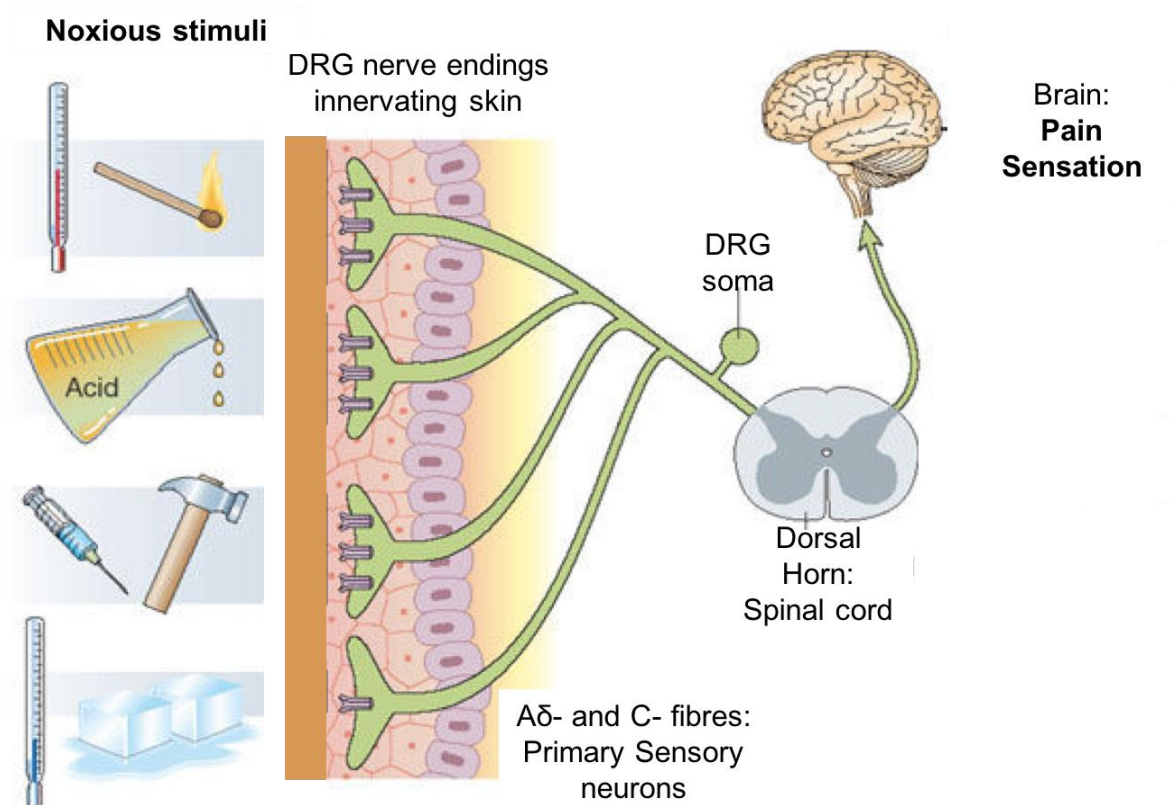


Figure 2. Schematic showing pain pathways of the nervous system.

Exposure to noxious stimulus in the periphery is detected by nerve terminals of primary sensory neurons, such as small diameter Aδ- and C- fibres. Nociceptive signal is sent to the dorsal horn of the spinal cord. The signal is modulated at this stage, and subsequently sent to higher centres in the brain via secondary neurons, where the signal is interpreted and perceived as pain sensation (Adapted from (Scholz and Woolf, 2002)).

1.4. Peripheral vs. Central Mechanisms of Sensitisation

The pain response is essential to protect our body from damaging stimuli, however under certain conditions this response is dysregulated and no longer has a beneficial function for the body (Dubin and Patapoutian, 2010). In these cases, nociceptors undergo a process of sensitisation to the persistent stimuli. Nociceptor sensitisation results in altered electrophysiological properties, including a reduction in activation threshold as well as higher magnitude of response or higher number of APs are triggered in response to noxious stimulation (Bessou and Perl, 1969). From a

behavioral point of view, these cellular changes are manifested as hyperalgesia, where stimulation of nociceptors causes an exaggerated response. In some cases, peripheral nociceptors fire action potentials in the absence of any type of stimulus, resulting in spontaneous pain (Djouhri et al., 2006). Although peripheral mechanisms of pain are the primary focus of my thesis and will be discussed in further detail, central mechanisms underlying pain pathophysiology will be discussed briefly in the following section.

1.4.1. Central Sensitisation

Central sensitization is an amplification of signaling within the CNS that elicits pain hypersensitivity. Evidence for the augmentation of sensory input in the CNS associated with pain hypersensitivity was first shown in 1983, when a preclinical study showed that injury induced firing in the nerve endings resulted in long-lasting increase in the excitability of spinal cord neurons (Woolf, 1983). Central sensitization is thought to involve several mechanisms including an increase in synaptic strength due to augmented excitatory transmitter release or increased post-synaptic response to the transmitter as well as a reduction in inhibitory pathways, which manifests as allodynia where pain sensation is evoked to innocuous stimuli (Takazawa and MacDermott, 2010; Woolf et al., 1994). Changes in functional properties of dorsal horn neurons have also been reported, where increased membrane excitability leads to recruitment of subthreshold synaptic input, contributing towards suprathreshold action potentials (Rivera-Arconada and Lopez-Garcia, 2010). As a consequence, an increase in synaptic strength develops involving changes in neurotransmitter synthesis and calcium channel density (Hendrich et al., 2008). Moreover, astrocytes, gap junctions, epigenetic mechanisms and microglia have also been linked to central sensitization mechanisms (Chacur et al., 2009; Chiang et al., 2010; Gao et al., 2009;

Hathway et al., 2009). Structural changes in the amygdala, and prefrontal cortex have been associated with the emotional aspects of pain and conditioned fear (Pedersen et al., 2007). Overall, these changes result in innocuous inputs resulting in painful sensations due to abnormal sensory processing within the central nervous system.

1.4.2. Peripheral Sensitisation

Peripheral sensitisation involves alterations in activation threshold, and membrane excitability of peripheral ends of nociceptors. Noxious stimuli can cause excitation of a nociceptor via activation of receptors such as ion channels, and an influx of cations resulting in a localised depolarisation. A large enough depolarisation, results in the opening of voltage-gated channels which are responsible for the generation of action potentials, or “pain signals” that travel to the DRG cell soma from the site of injury. The afferent functions of peripheral nerve fibres involve the transmission of pain signals from the skin to the CNS via A δ - fibres and C-fibres. The efferent functions involve the release of neuropeptides such as calcitonin gene-related peptide (CGRP) and SP from their nerve endings. Neuropeptides act by triggering a series of pro-inflammatory mechanisms including vasodilation, plasma extravasation, oedema formation, and recruitment of inflammatory cells. The antidromic release of neuropeptides in the peripheral tissue causing vasodilation, increased vascular permeability and alteration in immune cell biology is also termed neurogenic inflammation. Following injury and inflammation, a nociceptor is sensitised due to changes in the functional properties of ion channels found along the nociceptor. IMs for instance, can act on metabotropic receptors, triggering second messenger systems which then modulate ion channel function (Hucho and Levine, 2007; Linley et al., 2010b). Sensitisation can occur within minutes, involving post-translational

modification such as phosphorylation of ion channels. However, long term sensitization is also thought to involve alteration in the expression of ion channels due to transcriptional changes. Addition of phosphate groups can significantly alter properties of peripheral ion channels, which may potentially explain changes in excitability of neurons in painful conditions. Known mechanisms of peripheral sensitisation have been reviewed extensively (Basbaum et al., 2009; Dubin and Patapoutian, 2010; Gangadharan and Kuner, 2013; Schaible et al., 2011). The known players and mechanisms underlying peripheral sensitization will be discussed in further detail in sections 1.5-1.7.

In summary, both peripheral and central mechanisms contribute to the generation of pain after nerve injury and/or inflammatory insult. Collectively, these mechanisms contribute to rapid modulation in signal transduction pathways and long-term alterations in mediator and receptor systems. However, despite the importance of central changes taking place at the CNS which contribute to the amplification of the pain response during chronic pain conditions, this thesis will focus primarily on peripheral mechanisms underlying painful conditions. The following sections will discuss the available evidence with regards to key players in the context of inflammatory pain.

1.5. Key players of inflammatory pain states

Pro-inflammatory agents are responsible for enhancing the excitability of the nociceptor and amplifying the inflammatory response via direct or indirect modulation of several ion channels. There is evidence demonstrating that the action of IMs released during the inflammation response leads to functional modulation of a wide

variety of other ion channels such as, TRPV1, voltage-gated ion channels, ASIC and HCN channels amongst others, which leads to altered nociceptor excitability (**Figure 3**).

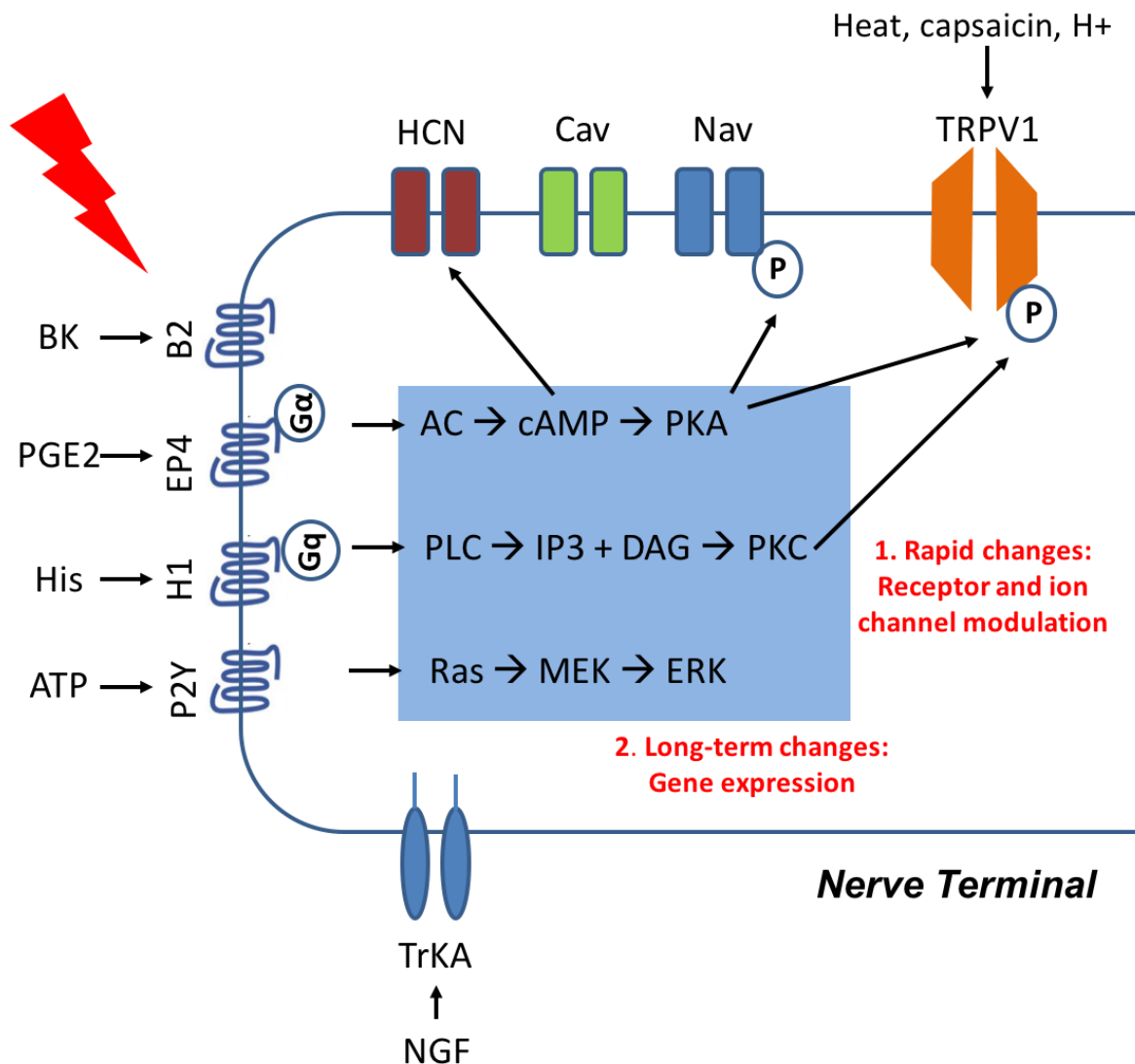


Figure 3. Peripheral mechanisms of sensitisation.

Tissue damage induces the synthesis and release of a cocktail of inflammatory mediators (IM). Prostaglandin E2, bradykinin, NGF, ATP and protons bind to their receptors on the peripheral terminals of nociceptors. This results in activation of signaling cascades including the cyclic-adenosine monophosphate/protein kinase A (cAMP/PKA) pathway, mitogen-activated protein kinase (MAPK) pathway, and phospholipase C/inositol 1,4,5-triphosphate/diacylglycerol (PLC/IP3/DAG) pathway leading to altered ion channel function as well as their expression/distribution at the nerve terminal. Collectively, these changes collectively contribute to peripheral sensitisation.

Thus, in the following sections, the key players involved in peripheral sensitisation in the context of inflammatory pain will be discussed in detail, including the main IM, signalling cascades that are activated as a result of mediator-GPCR receptor interactions as well as key ion channels that are modulated as a direct or indirect effect of IM.

1.5.1. IMs associated with pain

Nociceptor sensitization is a consequence of tissue damage and inflammation, which is driven primarily by the recruitment and activation of immune and tissue resident cells at the site of injury, and subsequent release of IM. It has been recognized that key players involved in the inflammatory response interact with the sensory nervous system and contribute to persistent pain states (Ji et al., 2014). IMs that are known contributors of pain will be discussed in detail in the following sections.

1.5.1.1. Prostaglandins

Prostaglandins are ubiquitously expressed lipid molecules that play an important role in the generation and development of the inflammatory response. They are produced when phospholipase A (PLA) releases arachidonic acid (AA), which is in turn metabolized by cyclooxygenase 1 and 2 (COX-1 and COX-2) enzymes to produce prostaglandin E₂ (PGE₂), prostacyclin (PGI₂), prostaglandin D₂ (PGD₂), and prostaglandin F₂ α (PGF₂ α) (**Figure 4**). The production of prostaglandins is maintained at low levels under normal conditions. Prostaglandins are responsible for housekeeping functions such as regulation of vascular tone, water homeostasis and pulmonary vascular remodelling (Lundequist, 2010). However, in inflammation,

production of prostaglandins is dramatically increased. The time-dependent increase of COX-2 mRNA levels have been demonstrated in both animal models of inflammation as well as in human oral mucosa following the onset of inflammation (Anderson et al., 1996; Khan et al., 2007). Moreover, a 10 to 80-fold increase in COX-2 expression has been observed in immune and non-neuronal cells present at the site of injury such as monocytes, macrophages, chondrocytes, fibroblasts and epithelial cells, leading to increased levels of prostaglandins (Crofford, 1997). The importance of prostaglandins in the context of pain is highlighted by the successful clinical use NSAIDS and selective COX-2 inhibitors, as analgesics for acute pain conditions. The role of prostaglandin E2 in peripheral sensitisation will be reviewed in further detail in Chapter 5.

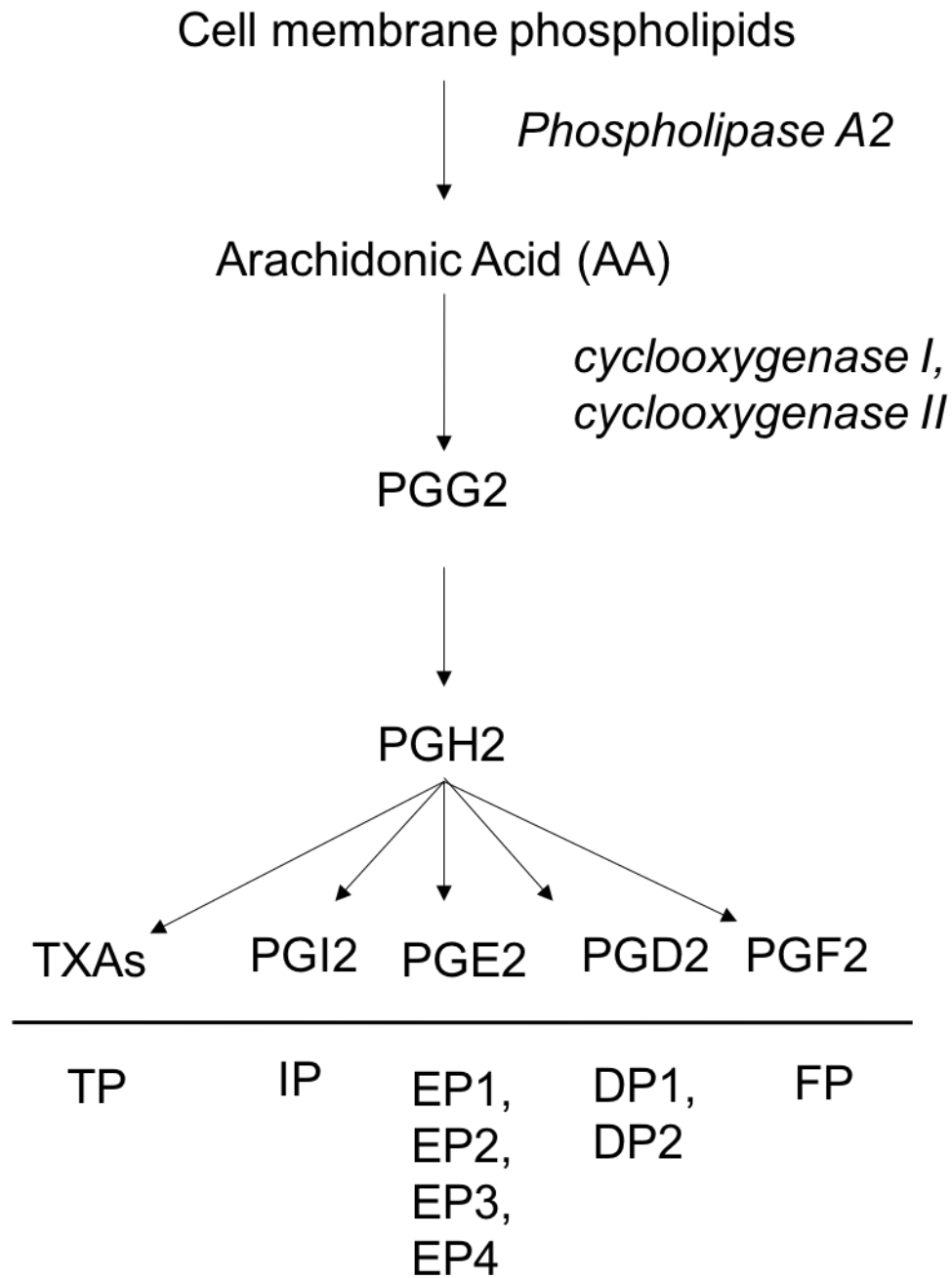


Figure 4. Biosynthetic pathway of prostaglandins.

Prostaglandins are synthesized in the cell from fatty acids which are converted to arachidonic acid(AA) via phospholipase A2 (PLA2). AA is broken down by cyclooxygenases (COX-1, COX-2) to a precursor prostaglandin H2 (PGH2) which, in turn, is broken down by different enzymes to produce thromboxanes (TXA2), prostaglandin E2 (PGE2), prostaglandin D2 (PGD2), and prostacyclin (PGI2), respectively.

1.5.1.2. *Bradykinin*

The kinin peptide, bradykinin, was first identified as an algogenic substance in human inflammatory exudates such as in fluids from arthritic joints (Armstrong et al., 1957). Bradykinin is a nonapeptide, endogenously released from kininogen precursors in plasma and tissues by the action of kallikrein-kinin enzyme. Its release is triggered in response to tissue damage or inflammation (**Figure 5**).

Bradykinin acts on two known GPCR receptors with 36% sequence homology; B1(B1R) and B2(B2R) (Menke et al., 1994). B2 is thought to be constitutively expressed on both primary afferent neurons, post-ganglionic sympathetic fibres, endothelium and macrophages under physiological conditions, whereas B1 receptors are primarily inducible following tissue injury and/or inflammation, as a result of increased transcription and subsequent protein synthesis (Ahluwalia and Perretti, 1999; Couture et al., 2001; Marceau and Bachvarov, 1998; Walker et al., 1995). The role of the B1 and B2 receptor in inflammatory pain has been demonstrated over the past decade by several knockout studies as well as the use of B1 and B2 selective antagonists in rodents (Ferreira et al., 2002; Mizumura et al., 2009; Pesquero et al., 2000). Signalling mechanisms downstream of kinin receptor activation have also been elucidated. Intramuscular formalin injection has been found to increase the expression of B1 and B2 receptors, which contributes to mechanical hyperalgesia via the PKC and p38/MAPK signaling pathways (Meotti et al., 2012). Human studies have confirmed the role of bradykinin as a pain-inducing mediator. Moreover, local anaesthetics such as lidocaine have been shown to suppress the inflammatory action of bradykinin significantly (Dias et al., 2008). The mechanism underlying this remains to be elucidated however, it was postulated that

local anaesthetics may inhibit signalling of Gq-coupled GPCRs. In support of this, bupivacaine, a local anaesthetic, has been shown to inhibit PGE2-EP1 downstream signalling via direct interaction with the G protein or GPCR (Honemann et al., 2001). Moreover, a study showed that administration of BK via injection or iontophoresis results in dose-dependent pain and pruritus. The same study reported that B2R signaling is predominant during physiological conditions, whereas potentiated B1R-mediated effects are observed following inflammatory insult using ultraviolet B (UVB) radiation. Another interesting observation from this study was that the sensitising effects of bradykinin are independent of mast cell degranulation and histamine release, at low doses at least (Paterson et al., 2013). Thus, it could be possible that the sensitizing effects of bradykinin are dependent on its direct action on B1R and B2Rs at the nerve terminals, leading to modulation of ion channel function and/or expression.

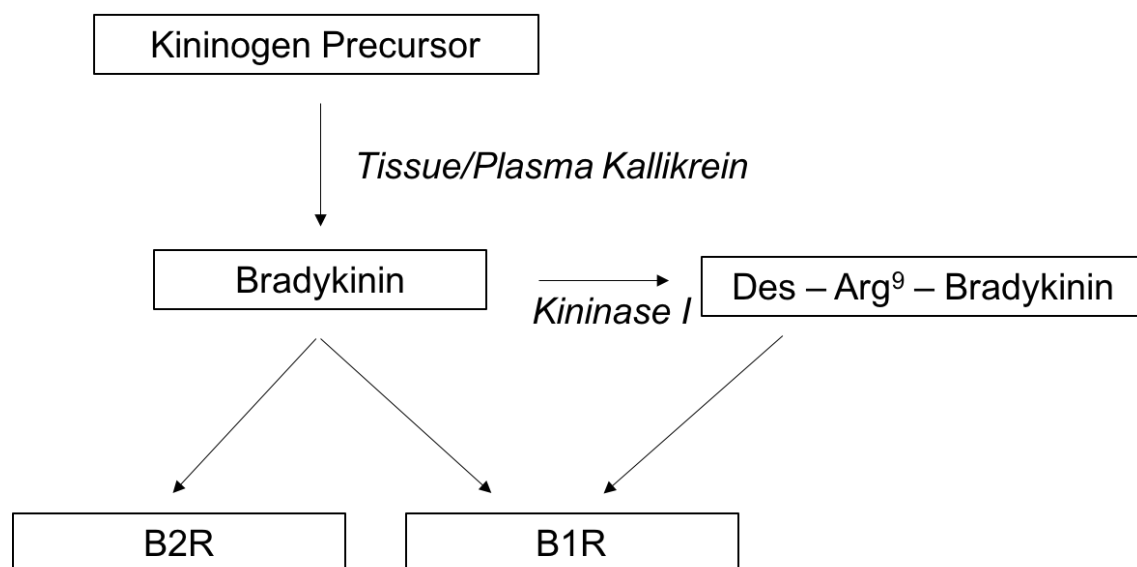


Figure 5. Biosynthetic pathway for bradykinin.

Kininogen precursors are cleaved by the protease kallikrein found in tissue and plasma to produce Bradykinin. Bradykinin acts on GPCR receptors B1R and B2R,

and can be metabolized by Kininase I to produce Des-Arg9-Bradykinin, which is more selective for B1R, than for B2R.

1.5.1.3. Histamine

Histamine is a multifunctional molecule which is known to be involved in inflammation, neurotransmission and immune modulation. It is synthesized from the decarboxylation of L-histidine and it is stored in cytoplasmic granules of mast cells, basophils, and histaminergic neurons (Jutel et al., 2006). Following tissue damage, activation of mast cells and basophils results in degranulation or stimulus exocytosis through which histamine is released at the site of injury. Histamine mediates its functions via action on 4 histamine receptors subtypes; H1, H2, H3 and H4. H1R is a GPCR associated with Gq/11 protein; therefore, its activation initiates activation of the PLC signaling pathway. By contrast, H3R and H4R activation instigate the MAPK pathways (Ichikawa and Tanaka, 2012).

Expression of H1, H3 and H4 was detected in peripheral sensory neurons innervating the skin in a recent study (Rossbach et al., 2011). All four histamine receptor antagonists have been suggested to display anti-nociceptive efficacy by reducing allodynia and hyperalgesia in rodent models of inflammatory and neuropathic pain (Hsieh et al., 2010; Medhurst et al., 2007; Medhurst et al., 2008; Mobarakeh et al., 2006). Efficacy of antihistamines for specific human painful conditions, such as IBS and severe bone pain have also been reported. A clinical study showed that treatment with H1 receptor antagonist reduced visceral hypersensitivity and abdominal pain in rectal biopsies from IBS patients (Wouters et al., 2016). By contrast, loratadine, an H1R antagonist failed to reduce bone pain in a randomized phase II study (Moukharskaya et al., 2016). Clinical studies assessing

the analgesic action of histamine antagonists have been equivocal. Therefore, further clinical evidence is required for antihistamines to be considered as analgesics for the management of pain. Notwithstanding, taken together, these studies confirm a role of histamine as a key mediator of the inflammatory response.

1.5.1.4. Serotonin

Serotonin, is a monoamine neurotransmitter synthesized by decarboxylation of tryptophan, its precursor molecule. Serotonin is involved in a number of physiological functions in the central nervous system such as the regulation of mood, appetite, sleep, and vasoconstriction. However, its role in the PNS is becoming increasingly apparent and has been established as an inflammatory modulator of acute and chronic pain (Bardin, 2011). Abundant levels of 5HT are found in peripheral tissue and endogenous 5HT levels have shown to be increased after inflammatory insult in humans and inflammatory rodent models (Ernberg et al., 2000; Kopp, 1998; Sasaki et al., 2006).

Serotonin receptors are classified into 7 isoforms (5HT1-7), with 5HT1 and 5HT2 further divided into subtypes 5HT1A, 5HT1B, 5HT1D and 5HT2A-C, respectively. All of these receptors are GPCRs, except 5HT3 which is a ligand-gated ion channel. There has been evidence for 5HT1B, 5HT1D, 5HT2A and 5HT3 expression in rat DRG (Van Steenwinckel et al., 2009; Zeitz et al., 2002). However, the lack of antibodies for the different 5HT receptor subtypes make it difficult to understand peripheral expression patterns of these receptors. Distinct serotonin receptors are responsible for different mechanisms underlying pain and inflammation. For instance, 5HT2A receptors are GPCRs, which trigger the $G_{\alpha q}$ signalling pathway resulting in phosphorylation of TRPV1 via PLC/PKC. Obata et al., showed that

peripheral 5HT_{2A} receptors play a role in mediating formalin-induced hyperalgesia in rats (Obata et al., 2000; Sasaki et al., 2006). Whereas, 5HT_{1B/1D} receptors are GPCRs coupled to the G_i/G_o which reduces cAMP signalling, resulting in decreased neurotransmission. The only 5HT selective agonist clinically used is 5HT_{1B/1D}, sumatriptan, which is prescribed for patients suffering from migraines and cluster headaches. A recent randomized phase III clinical trial also tested the efficacy and safety of duloxetine, a selective 5HT- norepinephrine reuptake inhibitor, in fibromyalgia patients. The study reports that the drug was well tolerated and reduced pain scores in patients given duloxetine compared to placebo (Murakami et al., 2015). Future strategies aim towards targeting the peripheral structures only.

1.5.1.5. Adenosine Triphosphate (ATP)

It was not until the early 70s that the ubiquitously expressed metabolite responsible for energetic metabolism, ATP, was found to evoke pain sensation (Blehen & Keele, 1977; Tominaga et al., 2001). It is now widely accepted that extracellular ATP is also a neurotransmitter and a neuromodulator. A neurotransmitter is a chemical messenger released at the end of a nerve fibre by the arrival of a nerve impulse and through diffusion across a synapse or junction, effects the transfer of the nerve impulse to another nerve or muscle fibre or another effector cell. A neuromodulator is a neurotransmitter that is not taken up by the pre-synaptic neuron or broken down into a metabolite, as its receptor is present on the post-synaptic neuron or effector cell. Peripheral administration of ATP or P2X agonists evoke nociceptive behaviours and are responsible for sensitisation of sensory neurons in both rodents and human models (Hamilton et al., 1999; Hamilton et al., 2000; Jarvis, 2003).

ATP acts as an agonist for 2 classes of purinergic receptors: ligand gated-ion channel P2X receptors and metabotropic GPCR P2Y receptors (Obata et al., 2000). Eight isoforms of the P2Y receptor, and seven subtypes of the P2X receptor have been identified (Khakh et al., 2001; Sak and Webb, 2002). P2X3 (homomultimer) and P2X2/3 (heteromultimer) receptors have been found to be selectively expressed on small-diameter sensory neurons in DRG and have been shown to be responsible for ATP-induced neuronal excitability (Chen et al., 1995; Lewis et al., 1995). Receptors for ATP have been considered as therapeutic targets for inflammatory pain. Clinical studies testing the efficacy of AF-219 compound, the only P2X3 antagonists known to have advanced to clinical trials as an anti-tussive, caused significant taste disturbance in patients (Abdulqawi et al., 2015). Moreover, activation of P2X7 receptors found on human macrophages cells triggers the release of several cytokines, which is why it has been considered as a therapeutic target for inflammatory pain. However, the P2X7 antagonists failed in phase II clinical trials when their efficacy was tested in patients with rheumatoid arthritis (Keystone et al., 2012; Stock et al., 2012).

1.5.1.6. Nerve Growth Factor (NGF)

NGF was the first molecule from the family of growth factors to be discovered back in 1948. It was first recognized for its role in the survival and development of sensory neurons during their embryonic phase. As neurons evolve into the adult phase they become less dependent on NGF (Easton et al., 1997).

Two main receptors for NGF are found on C- fibre nociceptors; low affinity p75 neurotrophin receptor and high affinity Tyrosine Kinase receptor (TrkA). NGF is considered an important component of the chemical milieu of inflammation at the site

of tissue damage. It is released by both, neuronal and non-neuronal cells at the site of injury. Early studies carried out in both rodents and humans proved that a single application of exogenous NGF can produce a long-lasting hyperalgesic phenotype (Dyck et al., 1997; Lewin et al., 1993).

NGF is responsible for mediating rapid functional changes as well as transcriptional alterations of pain-mediating proteins. For instance, NGF has shown to be responsible for potentiated gene expression of substance P, TRPV1 and Nav1.8 (Chao, 2003). Moreover, it has also been demonstrated that the polymodal TRPV1 channel plays an important role contributing towards NGF-induced sensitisation of nociceptors *in vitro* and NGF-induced hyperalgesia in rodents (Chuang et al., 2001; Eskander et al., 2015). Moreover, an autosomal recessive, loss of function mutation of the gene encoding for TrKA (NTRK1) and NGF β results in congenital insensitivity to pain (CIP) disorders in humans, characterised by absence of reaction to noxious stimuli, inability to sweat, and mental retardation (Carvalho et al., 2011; Einarsdottir et al., 2004). Based on all the evidence supporting the role of NGF as an important pain mediator, a humanised monoclonal antibody which selectively binds and targets NGF, Tanezumab, is currently being tested in a global, phase 3 clinical trial in patients with chronic lower back pain, osteoarthritis or cancer pain who have not received pain relief or cannot tolerate currently available analgesics.

1.5.2. Main Signalling Pathways activated by IM

A number of ion channels and their downstream signalling pathways have been highlighted as important mediators of inflammatory pain conditions. The first signalling cascade identified to play a role in the nociceptive activity of inflammatory mediators was the cAMP/PKA pathway (Taiwo et al., 1989). The cAMP/PKA

pathway is triggered downstream of the activation of GPCR receptors. Activation of GPCR with G_s type $G\alpha$ subunit results in activation of adenylate cyclase, which converts ATP to cAMP. Increase in intracellular cAMP levels then results in activation of protein kinase A. For instance, some prostaglandin receptors (EP2, EP4) and 5HT receptors (5HT4, 5HT6 and 5HT7) are GPCRs with a G_s type $G\alpha$ subunit and therefore trigger the cAMP/PKA pathway after activation (Meves, 2006).

Another important signalling cascade is the G_q /PLC pathway, which leads to activation of protein kinase C and has been associated with sensitisation of several ion channels (Patapoutian et al., 2009b). As examples, histamine receptors, bradykinin B2 receptor as well as the prostaglandin receptor EP1 are coupled to the G_q type $G\alpha$ subunit. Activation of these receptors results in stimulation of PLC, which in turn causes the hydrolysis of PIP₂, into DAG and IP₃ (Couture et al., 2001; Kühn et al., 1996; Meves, 2006). DAG causes activation of PKC whereas IP₃ induces an increase in intracellular Ca^{2+} concentration thereby triggering a number of Ca^{2+} -dependent processes. Both pathways have been reported to play an important role in inflammatory pain mechanisms, and are downstream effectors of a number of ion channels. For instance, bradykinin-induced acute sensitization of TRPA1 occurs via PLC signalling pathways (Wang et al., 2008). Cang and co-workers also suggested that the potentiation of $Na_v1.8$ current via NK1 activation is dependent on the PKC signaling pathway (Cang et al., 2009).

Other signaling pathways associated with inflammatory pain include MAPK and PI3K, which are activated downstream of receptors for growth factor receptors such as TrkA. These pathways have been involved in the sensitization (Zhu and Oxford,

2007) and insertion of TRPV1 channel into the plasma membrane of primary afferent neurons (Constantin et al., 2008; Tsantoulas et al., 2013; Zhang et al., 2005). Further, Binshtok and colleagues demonstrated that Interleukin-1 β (IL-1 β) enhanced TTX-R currents near threshold via the MAPK signaling pathway (Binshtok et al., 2008). Similarly, tumor necrosis factor (TNF) has been shown to be implicated in modulation of TTX-resistant currents, which was reversed by a p38-MAPK inhibitor (Jin and Gereau, 2006). These signalling pathways act synergistically *in vivo*, secondary to inflammatory mediator release at the site of injury. Collectively, all four major signalling pathways mentioned above contribute towards altered expression and function of different receptors and ion channels found along DRG neurons as described in the examples above, which ultimately leads to hyperexcitability of nociceptors during inflammatory pain conditions.

1.5.3. Ion channels as Peripheral Targets of IM

Binding pro-inflammatory substances to ion channels directly or indirect activation of ion channels via GPCR receptor activation, triggers signaling cascades leading to production of cyclic-adenosine monophosphate (cAMP), PKA, PKC or MAPK known to alter functional properties of ion channels via several mechanisms which will be discussed extensively in the following sections. Understanding the mechanisms underlying modulation of ion channels, by inflammatory mediators and their downstream effectors would not only aid our understanding of key players of peripheral sensitisation but also holds a great benefit from a therapeutic point of view. Modulation of functional properties of ion channels at the nerve endings could attenuate unwanted effects mediated by pro-inflammatory mediators, without deteriorating the immune system. In the sections below, ion channels which have

been identified and characterized at nerve terminals and have been linked to key inflammatory mediator-mediated mechanisms will be discussed.

1.5.3.1. Transient Receptor Potential Vanilloid 1 (TRPV1) channel

An important group of channels in the context of inflammatory pain are TRP channels which have been discovered and characterized on primary afferents, as important sensors of temperature. Although there are several TRP channels known to play a role in inflammatory pain (e.g. TRPA1), I will focus on TRPV1 as a prototypical example of TRP channel sensitisation in the context of inflammatory pain. TRPV1 is a non-selective cation channel which can also be activated by noxious temperature, low pH and monoacylglycerols (Caterina et al., 2000; Zygmunt et al., 2013). TRPV1 channels are polymodal, and therefore are activated by a number of stimuli (Tominaga et al., 1998). A number of groups have reported that pro-inflammatory agents such as protons and anandemides (Zygmunt et al., 1999) act on TRPV1 channels indirectly, via the activation of second messenger pathways, to cause pain. Several preclinical models of inflammation give evidence for the activation, up-regulated expression and increased phosphorylation of TRPV1 channels by disease associated inflammatory mediators cytokines such as IL-1 and IL-6, as well as eicosanoids (Zhang et al. 2005).

TRPV1 channels can be modulated by inflammatory mediators via rapid changes involving the activation of signalling cascades and subsequent phosphorylation of the ion channel, ultimately resulting in an amplification of the TRPV1-response. Additionally, the effects of TRPV1 can also be amplified due to increased expression levels of TRPV1 at the nociceptor. Long term exposure to inflammatory mediators result in transcriptional and translational alterations, which contribute towards an

increase in TRPV1 protein levels at the cell membrane. Both types of changes will be discussed further providing evidence using relevant examples. Positive modulation of TRPV1 can occur via G protein-mediated signalling pathways involving phosphorylation by PKC and PKA (Zhang and Oppenheim, 2005). For instance, bradykinin and NGF have been shown to act via B2 receptor and TRK A receptor, respectively, to activate intracellular PLC/phosphoinositide pathway in sensory neurons. This leads to phosphorylation of TRPV1 resulting in both positive modulation and rapid translocation of this channel to the membrane via exocytosis (De Petrocellis and Di Marzo, 2005). Other pro-inflammatory mediators such as IL-6 and IL-1 have been linked to TRPV1 sensitisation, and are thought to modulate its functional properties via phosphorylation by PKC (Obreja et al., 2002). Phosphorylation of the channel leads to a positive shift in temperature and ligand-concentration dependency of the ion channel.

Increased expression and transport of TRPV1 has been observed in response to TNF and NGF exposure in culture, resulting in an augmentation of proton and heat sensitivity (Amaya et al., 2004; White et al., 2010). Different signalling pathways have been shown to be responsible for these transcriptional and translational changes. NGF-induced increase in TRPV1 protein levels are mediated by PI3K and Src-mediated signaling pathways, whereas TNF-induced sensitisation of the receptor has been thought to be mediated by the p38/MAPK pathway (Constantin et al., 2008; Ji et al., 2002; Stein et al., 2006). The TRPV1 receptor has not only been shown to play a role in the context of inflammation, but also inflammatory conditions which are strongly associated with pain, such as in rheumatoid arthritis and inflammatory bowel disease (Akbar et al., 2010; Engler et al., 2007). In addition, TRPV1 channels have

been highlighted as important mediators of primary afferent sensitization in visceral distention and colitis models (Jones et al., 2005; Miranda et al., 2007).

Taken together, these studies point towards the pivotal role of these channels in integrating both chemical and noxious stimuli in the peripheral nervous system during inflammatory conditions (Caterina, 2000).

1.5.3.2. Hyperpolarisation cyclic nucleotide-gated (HCN) channels

Hyperpolarisation-activated cyclic nucleotide-gated cation channels (HCN) have been traditionally known for their role in the heart and were first described as the “funny channels” in the 1970s. Cloning of HCN channels in the 1990s aided the understanding of their contribution towards spontaneous activity of pacemaker cells in the heart (Baruscotti et al. 2010). They belong to a superfamily of cyclic nucleotide gated (CNG) channels and voltage-dependent potassium (K_v), with a similar structural arrangement as the latter. They are permeable to both Na^+ and K^+ ions, and conduct an inward current called I_f current in cardiac cells or I_h current in neurons. HCN channels can be dually activated, by both a hyperpolarising membrane potential (-60mV and -90mV) as well as by intracellular cAMP at their C-terminal, which is what makes them unique (Baruscotti et al., 2010; DiFrancesco, 1999). There are four isoforms that belong to this class of channels (HCN1-4). These 4 isoforms vary their biophysical properties, including their sensitivity to cAMP, and activation and deactivation kinetics, as well as their expression profiles. These will be discussed in further detail in Chapter 5.

There have been varied reported findings with regards to the role of HCN2 in the context of inflammatory pain, depending on the chosen model and readout. A study carried out by Schorr and colleagues showed that cAMP analogue administration did not lead to development of inflammatory pain behaviour in absence of the HCN2 gene, using a HCN2 mutant *snsHCN2KO* (Schnorr et al., 2014). However, in PGE2-induced inflammation both thermal and mechanical hyperalgesia were evident. Another group demonstrated that nociceptive phenotype was dramatically decreased in conditional *Nav1.8/HCN2 KO* mice in both, the carrageenan and the PGE2-induced inflammation *in vivo* models (Emery et al., 2011a; Emery et al., 2012). Thus, these data present an interesting hypothesis suggesting an important role for HCN2 channels in inflammatory pain. However, further investigation is required to confirm the role of HCN2 in peripheral inflammatory pain mechanisms. This idea will be explored and discussed in further detail in Chapter 5.

1.5.3.3. *Voltage-gated Sodium Channels (VGSC)*

VGSCs are imperative for the generation and propagation of action potentials in nociceptors. The *Nav* channel family includes nine mammalian isoforms that have been identified including *Nav1.1* to *Nav1.9* (Ertel et al., 2000). There are a number of natural toxins such as tetrodotoxin (TTX) that block these channels extracellularly. Of note, the different subtypes are classified into two classes, based on their sensitivity to TTX. *Nav1.1*- *Nav1.4*, *Nav1.6* and *Nav1.7* belong to the TTX-sensitive (TTX-S) category, with an $IC_{50} < 30nM$ of TTX whereas, *Nav1.5*, *Nav1.8* and *Nav1.9* belong to the TTX-resistant (TTX-R) category with an $IC_{50} > 30nM$ of TTX (Payandeh et al., 2012).

The importance of voltage-gated sodium channels in pain sensation is highlighted by the well-established and regular clinical use of non-selective sodium channels as local anaesthetics and analgesics (e.g. lidocaine and procaine). In particular, specific isoforms such as Nav1.8, Nav1.9, Nav1.7 have attracted most attention of academics and pharmaceutical companies. In fact, the discovery of a dozen of human mutations linked to these ion channels alongside *in vivo* experiments performed over the past decade confirm the critical role of these channels in inflammatory and neuropathic pain (Cummins et al., 2004). Novel mechanisms underlying inflammation-induced nociceptor sensitisation, such as the potentiation of sodium currents and their associated signaling pathways, are being discovered every year and will be discussed in this section.

Lack of hyperalgesia in mice knock out for the Nav1.9 channel stimulated with intraplantar administration of bradykinin, IL-1 β , P2Y agonists and prostaglandin E2 confirmed the role of Nav1.9 in inflammatory mechanisms (Amaya et al., 2006). This was further supported by another study where DRG neurons exposed to TNF on its own or a cocktail of inflammatory mediators displayed potentiated Nav1.9 channel-mediated currents (Jin and Gereau, 2006; Maingret et al., 2008).

The importance of Nav1.8 channels in inflammatory mechanisms have also been highlighted by knockout studies where deletion of Nav1.8-positive neurons resulted in reduced hyperalgesia in the commonly used, formalin and CFA-induced inflammatory animal models (Abrahamsen et al., 2008). Sensitisation of Nav1.8-positive nociceptors by pro-inflammatory and proalgesic agents, and their underlying mechanisms were first documented back in the 90s. Reports showed evidence for

PGE2, NGF and 5-HT inducing sensory neuron excitability via a shift in voltage dependence for activation and increase in the magnitude of Nav1.8 currents (Gold et al., 1998b). More recently, it was demonstrated that direct phosphorylation of two residues in loop 1 of Nav1.8 activates p38 MAPK pathway resulting in increased Nav1.8 current density (Hudmon et al., 2008). Other groups have supported this idea and reported that PGE2, NGF and endothelin-1 (ET-1) increase Nav current TTX density, and a reduction in activation threshold whereas TNF induces an increase in current density, without altering activation threshold of the channel (Zhang et al. 2002; Jin & Gereau 2006).

The critical role of Nav1.7 was initially revealed by the finding that a mutation in SCN9A gene encoding for Nav1.7 ion channel leads to pain hypersensitivity in patients affected by inherited erythromelalgia (EM) or “man on fire” syndrome occurred due to a mutation in SCN9A gene encoding for Nav1.7 ion channel, leading to pain hypersensitivity in these patients (Dib-Hajj et al., 2010). Later on, it was also found that a mutation of the same gene, resulting in a non-functional protein, is responsible for the lack of pain sensation, also known as congenital insensitivity to pain (CIP) in a family based in Pakistan. Over the past years, a number of other channelopathies associated with this particular ion channels were identified as a cause of a variety of human pain disorders (Dib-Hajj et al., 2010). Rodent genetic studies have also demonstrated a direct role of Nav1.7 in inflammatory pain by using a Nav1.7 knockdown approach, where deletion of this ion channel resulted in the absence of inflammatory pain (Nassar et al. 2004; Yeomans et al., 2005). Moreover, it has been shown that Nav1.7 function is modulated during inflammatory conditions. In the case of carrageneenan- induced inflammation, an increase TTX-S currents

was observed and was associated with an upregulation of Nav1.7 channels (Black et al., 2004). Therefore, it is not surprising that researchers and drug companies have invested vast amount of resources and efforts towards the development of Nav1.7 blockers as a new generation of analgesics over the past few years. As yet, specific pharmacological agents targeting Nav1.7 have failed to demonstrate analgesia in the clinical setting. However, there are still several ongoing Phase I clinical trials testing Nav1.7 selective blockers from which results have not yet been released (Emery et al., 2016; Lee et al., 2014b).

1.5.3.4. Voltage-gated Calcium Channels (VGCC)

Voltage-gated calcium channels are found on all excitable cells and are primarily responsible for regulating neuronal excitability, neurotransmitter release as well as regulation of transcriptional changes (Wheeler et al., 1995). Nine different subtypes have been discovered and characterized in the mammalian nervous system and they are classified into two main categories: low voltage activated (LVA) and high-voltage activated (HVA). The HVA category include L-type or Cav1.1-1.3, P/Q-type or Cav2.1, N-type or Cav2.2, R-type or Cav2.3, whereas the LVA category encompass T-type channel subtypes or Cav3.1-Cav3.3 (Yusaf et al., 2001).

The importance of VGCCs in chronic pain mechanisms is confirmed by the common use of gabapentin and pregabalin, the only current treatments for patients suffering from neuropathic pain conditions. In particular, gabapentinoids mechanism of action involves binding to auxiliary VGCC subunit $\alpha 2\delta$ -1 (Patel et al., 2013). However, it has been proposed that their analgesic effects involve inhibition of the increase in axonal trafficking of the subunit $\alpha 2\delta$ -1 observed after nerve damage (Bauer et al., 2009).

In addition to their role in neuropathic pain conditions, there has been a large body of evidence suggesting the contribution of Ca_v channels to inflammatory pain. A recent study reported that treatment with pregabalin caused a notable reduction in capsaicin-induced facial grooming, an attenuation in the second phase of the formalin response as well as a decrease in carrageenan-induced heat hyperalgesia, thus suggesting pregabalin may be effective in inflammatory pain as well (Hummig et al., 2014). In line with this, it has also been shown that chronic inflammation induces an alteration in VGCC distribution in rodent cutaneous DRG *in vitro* (Lu et al., 2010). Based on these findings, Ca_v -channels have been explored as a therapeutic target for inflammatory pain conditions. Within the N-type channels a state-dependent N-type blocker, TROX-1, with a selectivity similar for all three subtypes ($\text{Ca}_v2.1$, 2.2 and 2.3) has been developed. TROX-1 was shown to reverse behavioral hypersensitivity in the iodoacetate model of osteoarthritis, equivalent to effects of NSAIDs in rats (Abbadie et al., 2010). Further, this compound has been proposed as a potential treatment for osteoarthritis with neuropathic pain features (Patel et al., 2017). Several studies have suggested the role of T-type channels in inflammatory pain mechanisms (Lee et al., 2010). For instance, it was shown that T-type channel blockers reduces histamine-induced paw inflammation in rodents (Bilici et al., 2001). Out of the T-type calcium channels, $\text{Ca}_v3.2$ is of particular interest as it is expressed primarily in the nerve endings of DRGs. TTA-A2, a pan Ca_v3 blocker, was found to produce a dose-dependent reduction in hyperalgesia in the IBS model. The same study showed that TTA-A2 was ineffective in $\text{Ca}_v3.2$ knockout mice, suggesting that its effects are mediated primarily via the $\text{Ca}_v3.2$ channel (Francois et al., 2013). The critical role of these channels in neurotransmitter release, the effectiveness of VGCC blockers in hypersensitivity *in vivo*, as well as the effectiveness of calcium channel

blockers in reducing inflammation parameters in hypertensive patients argues that they could serve as good targets for chronic pain conditions (Farah, 2013; Patel et al., 2017).

Taken together, there is a substantial amount of literature suggesting that all the pro-inflammatory mediators and ion channels described above play an important role in inflammatory pain conditions. The effectiveness of strategies targeting these players has been demonstrated in a number of preclinical models, however the translatability of preclinical findings could be improved through the development of better models. The lack of translatability within pain research will be discussed in the following section with a focus on challenges and limitations of both *in vitro* and *in vivo* inflammatory pain models.

1.5. *The translatability of animal models used to understand pain mechanisms*

1.6.1. Animal Models of Inflammatory Pain

Routinely used inflammatory pain animal models commonly involve the administration of an irritant that induces quantifiable pain-related behaviours. These include the formalin test, complete Freund's adjuvant (CFA) test, carrageenan test or the zymozan test, and are used to model conditions with both acute and chronic inflammation (Beecher, 1957; Le Bars et al., 2001). Another method of modelling inflammatory pain responses is via an injection of individual pro-algesic substances known to sensitise nerve fibres such as prostaglandins, kinins, serotonin and cytokines. These are used to model specific stimulus- evoked behavioral pain responses *in vivo* (Rang et al., 1991). In contrast, there are more disease-specific

animal models which mimic key characteristics of chronic pain-associated human conditions. For instance, an insult to the bowel using 2,4,6-trinitrobenzene (TNBS) is commonly used as a model of colitis. Similarly, the intra-articular injection of monosodium iodoacetate is a common model of arthritis, along with other adjuvant induced poly- and mono-arthritic models (Neugebauer et al., 2007).

Despite the regular use of the animal models mentioned above, a number of limitations which may account for the lack of translatability of the findings from preclinical research. The limitations of animal models of inflammatory pain in terms of the validity of the models, and the parameters evaluated will be discussed in this section. Well-established animal models are commonly validated with a clinically effective and commonly used drug treatment. In the case of inflammatory pain states, models are often validated against NSAIDs. However, it should be taken into account that NSAIDs are only effective in a subpopulation of arthritic patients and are only recommended to be given at the lowest doses, for the shortest time required in high risk OA patients or patients with co-morbid conditions (Crofford, 2013; Laine et al., 2008). This is due to the risks associated with chronic administration of NSAIDs including renal, gastric and cardiovascular adverse effects (Marcum and Hanlon, 2010). Thus, validation of animal models with NSAIDs could introduce a bias towards specific mechanisms or readouts when assessing drug effects.

Lack of “pain fingerprints” or biomarkers for pain has forced researchers to rely on human questionnaires or quantifiable animal behaviour readouts, such as simple reflexes or the innate responses (*i.e.* licking, grooming, grimace scales). Hypersensitivity and drug effects are commonly quantified by observational rating of

mechanical or thermal stimulus-induced paw withdrawal responses or stimulus evoked changes in paw pressure against the floor while walking. Typical readouts include responses to von Frey filaments (Chaplan et al., 1994), deep pressure (Santos-Nogueira et al., 2012) or heat stimuli also known as the Hargreaves test (Hargreaves et al., 1988). Undoubtedly, observational rating is likely to introduce a certain degree of subjectivity and variability in results. Efforts have been made to reduce subjectivity of these models by ensuring blinding of experimenter whilst carrying out procedures. Moreover, the rapid advancement of technology has given rise to the creation of more automated systems. Novel methods such as video-tracking systems and video-based behavioral algorithms are being developed to assess hypersensitivity over longer periods of rodents in their home cages (Roughan et al., 2009). These methods would minimize confounds associated with handling, housing conditions and social behavioral factors, which have shown to have an impact on the hyperalgesic phenotype in rodents (Balcombe et al., 2004; Raber and Devor, 2002; Robinson et al., 2004).

Efficacy outcomes used in animal studies differ significantly from those used in humans. For instance, clinical trials data is analysed as a change from baseline within each individual, whereas in animal studies treated groups are often compared to naïve or vehicle groups (Quessy, 2009). Moreover, the predominant complaint of chronic pain patients is ongoing pain. Consequently, the primary efficacy outcome of clinical studies is ongoing pain, whereas currently available animal models' readouts focus predominantly on measuring hyperalgesia induced by thermal or mechanical stimulation and it has been difficult to model ongoing pain *in vivo*. In support of this, only 10% of published articles in the journal of Pain reported a measure of

spontaneous pain, whereas 42% to 48% published hypersensitivity data during a ten-year period (Mogil et al., 2009). In efforts to tackle this, the use of spontaneous flinching, licking and guarding of the paw as readouts for ongoing pain has been suggested (Tappe-Theodor and Kuner, 2014). Moreover, a recent study evaluating the effects of a dopamine receptor antagonist in the CFA-induced arthritis model showed efficacy of this drug in reducing spontaneous pain-like behaviours such as spontaneous flinching and guarding of the paw (Robledo-Gonzalez et al., 2017).

Taken together, there are a number of limitations that contribute to the lack of translatability of animal models. The use of more clinically relevant controls for the validation of these models as well as appropriate choice of outcomes/measured parameters is imperative. Using measures of ongoing pain, in addition to evoked pain will guide clinical usefulness of treatment in question.

1.6.2. *In vitro* Models used to study Pain

Unquestionably, *in vivo* experiments are essential for our understanding of the mechanisms taking place in the intact, living organism. However, in order to decipher mechanisms at a cellular level, researchers opt for *in vitro* or *ex vivo* preparations. Commonly used techniques in pain research include cell culture, patch clamp, and calcium imaging, which allow the study of electrophysiological properties of nociceptors. Advantages of these techniques include the ability to exploit key aspects of ion channel functionality such as alteration in membrane potential and neuronal firing in a controlled environment. Moreover, increased accessibility to human DRG cells from different disease patients has opened up new exciting avenues for *in vitro* scientists, which will be discussed in further detail in chapter 6. However, the recording techniques for studying electrophysiological properties of

nociceptors have remained the same since the days of Zotterman's fundamental discoveries in the 1950s. Importantly, these techniques focus primarily only one component of the nociceptor: the cell body or soma as they cannot be applied to nociceptive terminals. The difficulty of recording from thin, and tiny structures of small diameter axons has been the reason for our inability to distinguish between somatic, axonal and sensory terminal mediated mechanisms of chemosensitivity and nerve fibre sensitisation. Further, the development of *in vitro* models that better represent the biology of pain signalling would not only improve our understanding of cell-autonomous mechanisms responsible for painful conditions but would also have a positive impact on the reduction, refinement and replacement (3Rs) of animal usage for pain research, by significantly reducing the number of animals required for the study of pain signalling.

1.6.2.1. The need for alternative *in vitro* models

Despite the dramatic progress in our understanding of nociceptor excitability thanks to available *in vitro* techniques, the cell-autonomous mechanisms responsible for sensitisation of nociceptors remain to be fully elucidated. In recent years the study of axonal physiology and pathophysiology has gained momentum in the field of neuroscience, with the aim of shedding light on axonally-exclusive mechanisms responsible for axonal dysfunction- a common feature of neurodegenerative diseases. The availability of novel compartmentalized cell culture systems such as the microfluidic-based platforms allow the study of axonal-based mechanisms, independent of the cell body at a molecular level (Park et al., 2006a; Taylor et al., 2005). Latest generation microdevices not only provide a controlled and isolated environment to grow axons, but also enable the investigation of the electrophysiological properties of axons *in vitro*, for the first time. Microfluidic

technology enables the ability accurately model and/or control changes in neuronal microenvironments more accurately. The design and applicability of this tool will be discussed in further detail in Chapter 3.

1.7. Why study axons?

Traditionally the role of axons has thought to be limited to the transportation or conduction of signals from nerve terminals to higher centres of the nervous system. However, axonal malfunction is a prominent common factor of a wide range of neurological diseases. Currently, a large portion of our knowledge of molecular and cellular changes responsible for nociceptor sensitisation are based on *in vitro* studies carried out at the DRG soma, which has long been used as a model of primary afferents. Undoubtedly, the DRG soma expresses molecular markers similar to those found in the axon and nerve terminals. However, there have been some reports highlighting significant shortcomings of nociceptive soma as a model of the nerve endings. St Pierre and co-workers reported lack of tachyphylaxis, or rapidly diminishing response, to repeated applications of low pH and showed evidence for differences between proton sensitivity of cultured DRG cell bodies compared to that of DRG nerve endings (St Pierre et al., 2009). Further evidence of the presence of completely different mechanisms at the soma and nerve terminals was shown in a novel model of hyperalgesic priming, mimicking the transition from acute to chronic pain in the rat *in vivo*. This model involves “priming” of sensory neurons via the injection of a cAMP agonist, 8-bromo-cAMP. Once, the acute hyperalgesia caused by this injection is resolved, the intradermal injection of PGE2 induces a long-lasting (up to 24 hours) hyperalgesia in primed neurons, compared to a short-term hyperalgesia (up to 1 hour) observed in non-primed animals. Priming induced by intraganglionic injection of 8-bromo-cAMP was reversed by the antisense of the

transcription factor for CREB, and by pharmacological block of the translocation of activated CREB into the nucleus. Conversely, pharmacological block of protein translation at the peripheral terminals, not at the ganglion, reversed priming of nociceptors. They suggest that hyperalgesic priming involves gene transcription changes occur at the soma, whereas translation occurring at peripheral nerve endings during the chronification of pain (Ferrari et al., 2015). The same study showed that different agents induced priming when injected in the cell body or at the peripheral terminals. Whilst injection of 8-bromo-cAMP in the ganglion caused priming, injection of the same agent in the peripheral terminals did not induce priming and the opposite was true for other priming agents tested including NGF and $\text{TNF}\alpha$. These differences stress the importance for validating findings from experiments focusing on somal mechanisms using preparations allowing the study of peripheral components of the neuron, the axons and nerve terminals.

Furthermore, there is a large body of work reporting that messenger RNA (mRNA) are transported and locally translated in mammalian dendrites and axons (Andreassi et al., 2010). Reports have shown that the regenerative ability of sensory axons, including those with removed cell bodies, is dependent on local protein synthesis and degradation after axotomy (Verma et al., 2005). Axonal protein translation has also been investigated in the context of nociception. A study reported local protein synthesis is essential for nociceptor function in a subset of sensory fibres (Jiménez-Díaz et al., 2008). A more recent study showed, for the first time, the presence of inflammation and immune-related mRNAs in adult DRG axons and not in embryonic DRG axons (Gumy et al., 2011). Further, it has been suggested that local axonal mRNA machinery is responsible for the regulation of local expression of ion channels

and signal transduction molecules (Jung et al. 2012). Supporting this, a study reported an accumulation of Nav1.8 mRNA in peripheral axons of DRG neurons after unilateral sciatic nerve entrapment injury in rats (Thakor et al., 2009). Similarly, an accumulation of Nav1.8 channels in regenerating axons *in vitro* axotomy has also been reported (Tsantoulas et al., 2013). Taken together, these studies provide evidence for the existence of axonally-exclusive mechanisms which may contribute towards the genesis and maintenance of painful conditions.

In recent years, there has been significant evidence in favour of the idea that peripheral mechanisms are key drivers of pain. Of note, the arrival of pain signals to central structures is dependent on the activation of peripheral ion channels found along the nociceptor nerve terminals. In support of this theory, a topical application of lidocaine results in reduced spontaneous discharge, and attenuated mechanical hyperalgesia in animal models of inflammatory pain (Xiao and Bennett, 2008). Moreover, clinical evidence suggests that the lidocaine patch, a targeted peripheral analgesic, has shown to provide significant pain relief in cases of episodic erythromelalgia (Davis and Sandroni, 2005; Devers and Galer, 2000). These studies suggest that aberrant pain signals initiated in the nerve endings and axons contribute to pathological pain. Thus, there has been a push towards developing peripherally targeted drugs to not only target pain at its source, but also reduce the number of side effects associated with systemic delivery of analgesics (McDougall, 2011). Better understanding of peripheral mechanisms involved in sensitisation of the nervous system would allow us to find better targets for therapeutic intervention.

In conclusion, our knowledge regarding cell-autonomous mechanisms underlying changes in excitability of nociceptive terminals is primitive. Identification the role of various ion channels in determining membrane threshold changes, as well as changes in expression and distribution following injury and/or inflammation would provide important insights to understanding of peripheral hyperexcitability, a key aspect of all chronic pain conditions. Hence, the overarching aim of this thesis is to describe and utilize an *in vitro* model that recapitulates key aspects of nociceptor sensitisation, in order to delineate axonal mechanisms involved in inflammatory pain conditions.

1.8. Aims of the Work

As discussed above, the changes in excitability of nociceptive axons at the site of injury and inflammation in painful conditions is a significant contributor to pain originating at the periphery. We hypothesised that following inflammation and/or nerve damage, nociceptor sensitisation and spontaneous activity observed arises from the modulation of ion channels and hyperexcitability at the nociceptive nerve terminals and axons. With increasing evidence regarding the contribution of many ion channels to the sensation of nociceptors during painful conditions, it is likely that a combination of ion channels contributes towards peripheral hyperexcitability at the nerve terminals. Currently our knowledge of the mechanisms underlying these changes in excitability as well as the role of various ion channels that determine these threshold changes at the nerve terminals and axons is extrapolated from *in vitro* models focusing on the cell bodies or *in vivo* models where multicellular responses complicate the identification of mechanisms intrinsic to peripheral nerve terminals. Thus, there is a need for the development of *in vitro* models that enable

investigation of axonal mechanisms, and how these mechanisms are altered by different stimuli during painful conditions. Better understanding of key axonal players will lead to improved translatability of preclinical findings and aid the development of better analgesics for pain management. Therefore, this PhD thesis set out four aims:

Objective 1:

To develop and validate an *in vitro* model of peripheral sensitisation of nociceptive axons using a microfluidic-based culture platform: Firstly, I aimed to demonstrate axonal isolation in MFCs using adult DRG sensory neurons. Next, I aimed to validate the use of calcium imaging at the cell soma as a readout to measure axonal function and characterise axonal and somal responses to chemical stimulation in MFCs. Combining the MFC-based platform and the calcium imaging set-up, I aimed to measure functional changes in DRG axons following localized inflammatory insult as a model of peripheral inflammatory sensitisation *in vivo*. This is crucial as it recapitulates peripheral injury and inflammation in humans, where the cell bodies are not directly exposed to inflammatory mediators.

Objective 2:

To identify key axonal players underlying the effects of PGE₂, a potent inflammatory and pain mediator, on nerve terminals and axons: The aim was to study the changes in axonal excitability in response to PGE₂ only. In addition, I aimed to identify prominent axonal players contributing towards PGE₂-induced changes in axonal excitability using pharmacological tools.

Objective 3:

To determine the role of axonal HCN2 channels as a mediator of inflammation-induced nociceptor sensitisation and activation: I aimed to investigate whether axonal HCN2 channels are important mediators of the functional changes induced by a combination of inflammatory mediators, as well as PGE2 only. Pharmacological and genetic approaches were used to understand the role these ion channels following inflammatory insult and/or injury.

Objective 4:

To develop a microfluidic-based *in vitro* assay using human inducible pluripotent stem cell (hiPSC)- derived sensory neurons: I aimed to establish optimal culture conditions to readily maintain and isolate hiPSC-derived sensory neuronal axons in MFCs. Next, I aimed to characterise the different populations of neurons present at 4 weeks *in vitro*. Additionally, our objective was to characterise axonal responses of hiPSC-derived sensory neurons to chemical stimuli, and develop an MFC- based model of peripheral sensitisation to investigate functional changes in human nociceptive axons during inflammatory pain conditions.

2. MATERIALS & METHODS

2.1. Reagents

Stock solutions of PGE2 (Sigma), PF-01247324 (Sigma), Lidocaine (Sigma), Zatebradine (Tocris) and in-house developed HCN2 selective antagonist were dissolved in DMSO. Bradykinin (Sigma) was dissolved in 0.1 acetic acid, as a stock solution and diluted to desired final concentration in Hank's Balanced Salt Solution (HBSS, Invitrogen). Histamine (Sigma), 5-HT (Sigma), Rp-cAMPS (Tocris) were all dissolved in deionised water (dH₂O). HCN2 selective antagonist developed by McNaughton Lab, King's College London.

2.2. Sensory neuron culture

Dissociated primary DRG cultures were prepared from adult mice (8 weeks, C57BL/6, Charles Rivers UK). Mice were culled via Schedule 1 method of cervical dislocation. An incision was made through the dorsal skin along the spine, until the spine was exposed. The vertebral column was excised and was held by a pair of large forceps while a hemisection was carried out through the middle of the column. The spinal cord was removed carefully from either side, exposing the DRG capsules. DRGs from thoracic, cervical and lumbar spinal levels were aseptically excised and kept in HBSS on ice. Approximately, 25-30 ganglia were collected per mouse. DRGs were then microdissected to remove attached roots from the DRG capsule, and placed in a petri dish containing fresh HBSS solution. DRGs were then digested using an enzyme mixture containing 0.125% collagenase type XI (Sigma) and 10mg/ml dispase (Gibco), which was filter sterilised using a 2mL syringe and 0.22 μ m filter. DRGs were incubated in filter-sterilised enzyme mix for 50 min at 37°C and 5%

CO₂ in a humidified incubator. Enzyme mix was then removed, and DRGs were washed in 1 ml of Neurobasal growth medium-A (NB-A, Invitrogen) supplemented with 2% B27 supplement (Invitrogen), 1% Glutamax, followed by gentle mechanical dissociation using a fire-polished Pasteur glass pipette. Triturated cells were pelleted by centrifugation (900rpm approx. for 10 min) through a 10% bovine serum albumin (BSA) cushion, prepared by mixing 1mL of HBSS with 0.5 ml 30% BSA Fraction V solution (Sigma). 1mL of cell suspension was added to BSA solution in dropwise fashion to avoid mixture of two layers and formation of bubbles. Supernatant was removed and pellet was washed in 1ml of NB-A medium and spun down again at 900rpm for 10 minutes. Supernatant was discarded and cells were resuspended in appropriate volume of NB-A medium to achieve optimum cell density. Protocol used in this study for sensory neuron dissociation was adapted from previously published DRG dissociation protocols (Malin et al., 2007).

2.3. Preparation of microfluidic chamber (MFC) devices

MFCs were prepared using a modification of published protocols (Tsantoulas et al., 2013). Glass dishes (GWst-5040, WillCo Wells) were sonicated in dH₂O for 50 min, sterilised in 70% ethanol for 5-10 min, rinsed in dH₂O three times and left to dry for 2-3 hours. Sterilised glass dishes were then coated overnight at 37°C with 0.8mg/ml Poly-L-Lysine. Microfluidic devices (Xona Microfluidics, LLC) were sterilized in 70% ethanol for 5 min and left to dry out overnight.

The following day, glass dishes were rinsed 3 times with dH₂O and left to dry for a minimum of 3 hours. Through a series of optimizations, we found that the 3 hour-drying period was critical for optimal attachment of PDMS MFC to glass plate. As

depicted in **Figure 6**, two types of MFC configurations were used for experiments. The 2-channel MFCs enables separation of DRG neurons into 2 compartments (DRG soma and DRG axon), and is useful for the interrogation of axonal responses to different stimuli such as depolarising stimuli (*i.e.* KCl), or inflammatory mediators. The 3-channel MFC, enables separation of DRG neurons into 3 components: DRG soma, the proximal axon and the distal axon. The benefit of using this configuration, is that it facilitates the use of pharmacological agents to assess how these can alter signals being generated in the distal axons, and the propagation of these signals to the cell soma. For instance, a depolarising stimulus such as KCl could be applied to the *distal axon compartment*, which then generates a signal that is propagated to the cell body. The propagation of this signal can be blocked by application of pharmacological agents to the *proximal axon compartment*. Thus, further dividing the axons into 2 compartments using the 3-channel MFC enables a sophisticated method to identify important axonal players responsible for generation and propagation of signals in peripheral axons during physiological and pathological conditions.

For attachment of the device to the glass dish, the devices were placed on the coated glass bottom with the imprinted (microgroove) side down and gentle but firm pressure was applied with forceps to form a seal. 200 μ l of 20mg/ml laminin was added to the top left (somal side). The laminin was then forced through the channel towards the interconnecting well by pipetting at the channel entrance, taking care to avoid bubble formation in the device. The chamber was equilibrated at RT for 10 min to establish liquid flow through the microgrooves. Once microgrooves were filled up, 100 μ l of 20mg/ml laminin was added to the top right well (axonal side), in the same

manner as the soma side allowing it to flow through the chamber to the interconnecting well. Optimal attachment also allows maximised flow rate within the chambers. Laminin-coated plates attached to MFC chambers were allowed to incubate at 37°C for overnight.

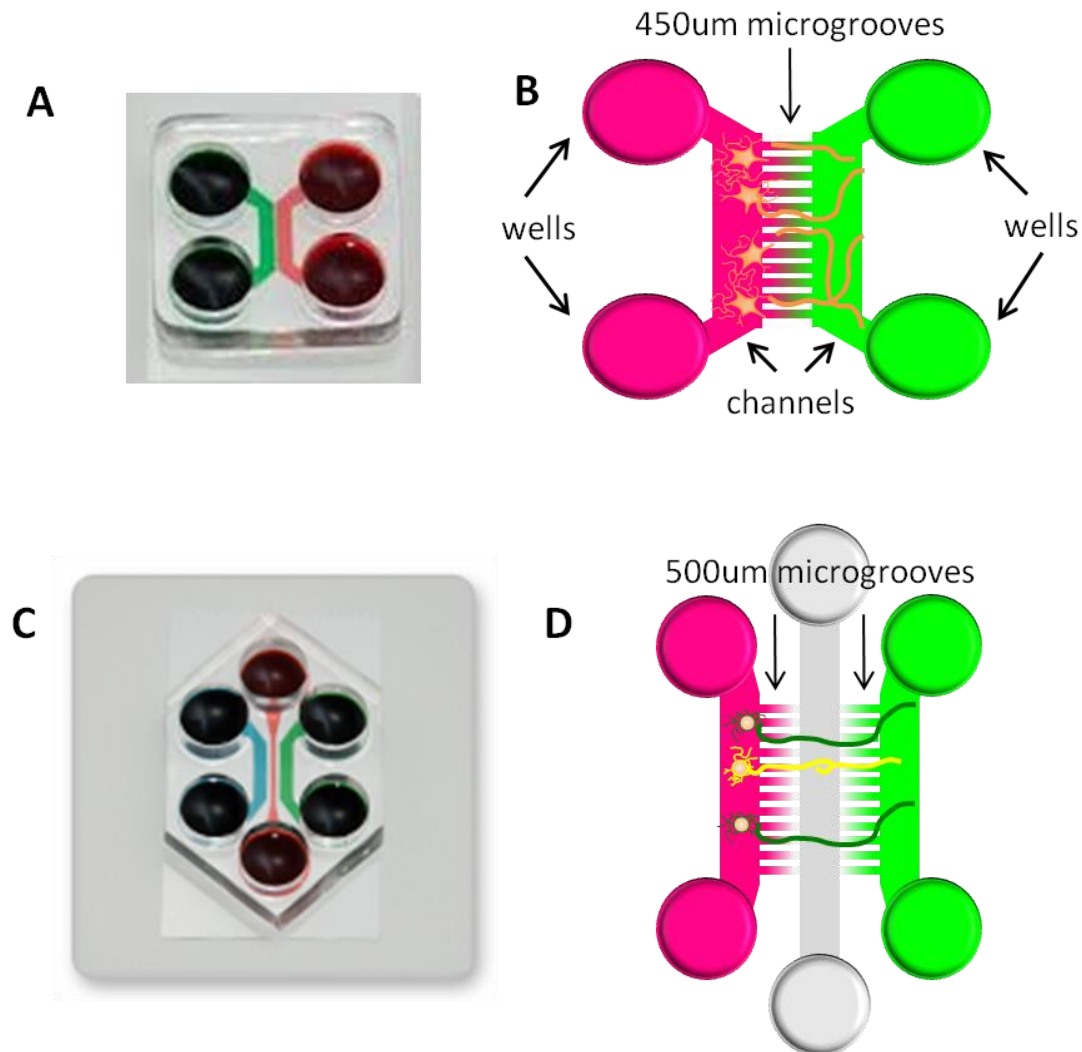


Figure 6. Different components of microfluidic culture devices.

A and C) SND450 and TCND500 devices with 450µm and 500µm length of microgrooves, respectively, mounted on glass coverslips. **B)** 2-channel MFC device, indicating 4 wells, 2 channels and 450µm length of microgrooves. **D)** 3-channel MFC device, with 6 wells, 3 channels and 500µm length of microgrooves.

For cell loading, laminin was removed from all wells using a suction glass pipette and 5µl of cell suspension at the appropriate dilution was added at the entrance of the

somal channel. Cells would typically enter the channel and slowly move towards the exit to the bottom well; when movement was restricted due to high cell density/debris or suboptimal flow, then gentle suction was applied to the exit to assist homogenous cell dispersion throughout the channel. Chambers should be positioned with a tilt for approximately one minute in order for cells to settle close to the microgrooves. This procedure was adopted to maximise the number of axonal crossings to the other compartment. Next, chambers should be placed on a flat surface for 10 minutes to allow cells to attach and settle. Any movement causes cells to lift off the plate at this stage, and flow back out of the chambers into the wells. Next, the MFC were placed in the incubator at 37°C for 1-1.5 hours to allow cell attachment. Each well of the somal compartment was then supplemented with 200 μ l of 50ng/ml NGF supplemented media, which was added taking care to keep the volumes as equilibrated as possible to minimize liquid flow through somal channel which would disturb cells. The axonal compartment wells were filled with 100 μ l of 100ng/ml NGF supplemented media. NGF was supplemented to facilitate axonal growth through microgrooves. Media supplemented with NGF was refreshed every 48 hours, to ensure optimal health of cells. Initially two configurations of the MFC device were tested: SND150 (microgroove length 150 μ m) and SND450 (microgroove length 450 μ m). However, in the case of SND150, it was observed that a higher number non-neuronal cells crossed over to the axonal compartment. We therefore concluded that SND450 would be used for most experiments unless specified otherwise.

2.4. Dil Tracing of cell bodies with axons traversing the microgrooves

After 5 days in culture, the neurons with axons that had traversed through the microgrooves and into the axonal compartment were stained with a fluorescent

tracer, Dil (Invitrogen, 1:200). The Dil tracer is a fluorescent lipophilic cationic indocarbocyanine dye usually made as a perchlorate salt, which is commonly used for *in vivo* tracing of neurons (Perrin and Stoeckli, 2000). Dil tracer was loaded to the axonal compartment, and was then retrogradely transported to the cell bodies in the somal compartment overnight via lateral diffusion in the plasma membrane. This property enables tracing axonal projections over long distances, and which can be maintained over long periods (Godement et al., 1987).

More specifically, media was removed from axonal wells, and 200 µl of 1:200 Dil was added to the top axonal well to allow dye to flow through chamber to the bottom axonal well. 200 µl of Dil was then added to the bottom axonal well. Microfluidic chambers were incubated with Dil for 1 hour. Dil solution was then removed, and NB-A media was added to the axonal compartment. Cell bodies in the somal compartment stained with the dye were visualized 24 hours after Dil-staining of axonal compartment using RITC filter set on a microscope with epifluorescence optics (Nikon Eclipse TE200).

The use of the Dil tracer in this system was crucial to enable the following:

1. Identification of the DRG cell bodies in somal compartment with axons that had traversed to the axonal compartment by Div 5, from those cell bodies with axons that had not crossed over. Only the Dil-stained cell bodies (indicative of axonal crossing to axonal compartment) were chosen for further analysis.
2. Ensured that further analysis such as calcium imaging recordings as detailed in the next section, was carried out in neuronal cell bodies as

opposed to non-neuronal cells present in the somal compartment, as the Dil diffuses from the axons and can only diffuse laterally *within* the neuronal cell.

2.5. Calcium Imaging and Analysis

Neurons in the somal compartment were loaded with 2 μ M of the calcium indicator dye Fura-2-AM (Invitrogen) and 1mM probenecid (Sigma) in imaging buffer for 45 min at 37°C. Imaging buffer consisting of Ca^{2+} and Mg^{2+} -free HBSS, (Gibco) containing 5.3mM KCl, 0.44mM KH_2PO_4 , 4.1mM NaOHCO_3 , 137mM NaCl, 0.33mM Na_2PHO_4 , 5.55mM D-Glucose and 0.03mM Phenol Red, was supplemented with 1 mM MgCl_2 , 2 mM CaCl_2 , 10 mM HEPES (Gibco). Both MFC compartments were washed twice with imaging buffer before the assay commenced. Microfluidic cultures were mounted on an inverted microscope connected to an EasyRatioPro imaging system (Photon Technology International, UK), comprising of an ORCA 4.2 sCMOS camera (Hamamatsu, Japan) and analysis software (EasyRatio Pro). First, bright field and Dil staining were visualised and *only neurons exhibiting staining in the soma (indicative of axonal crossing) were chosen for further analysis*. Areas of interest (ROI) were drawn around the corresponding cell soma and fluorescent images at 510nm were collected at 3.3Hz using 340 and 380 nm excitation wavelengths produced by a monochromator (Photon Technology International, UK).

Of note, KCl solutions were made up using Ca^{2+} imaging buffer containing 5.3mM KCl and therefore, making up 15mM KCl, 30mM and 60mM KCl in Ca^{2+} imaging buffer, resulted in a final bath concentration of 20mM, 35mM KCl, and 65mM, respectively. Axonal responses were always acquired first, while somal stimulation was applied at the end of the assay to avoid desensitization or apoptotic cell death

due to larger Ca^{2+} influx upon direct somal stimulation. Each drug application was separated by two washes in imaging buffer followed by a 10-minute rest period. Throughout the assay, caution was taken to retain the fluidic isolation between somal and axonal compartments (i.e. for axonal stimulation volume was higher on the somal compartment and this was reversed for somal stimulation). Protocol for calcium imaging in MFC chambers was adapted from a report previously published by our lab (Tsantoulas et al., 2013).

2.6. Treatment of Nociceptive Axons with Inflammatory Mediators

On day 6, the axonal responses were assayed. Baseline calcium responses of Dil-positive cell bodies was recorded as a readout for baseline axonal excitability with a short 20 sec stimulation with 20mM added to the axonal compartment. Following this baseline recording, the imaging buffer in the axonal compartment was then replaced with media containing a cocktail of inflammatory mediators (IM) consisting of 10mM PGE2, 10mM Histamine, 10mM Bradykinin and 10mM Serotonin and incubated at 37°C for 2 hours. These particular IMs and concentrations were chosen based on published studies in the literature, showing that exposure of this combination of IM to neuronal cultures *in vitro* results in a significant sensitisation of neuronal cell bodies and in single nerve-fibre preparations *in vivo* (Grossmann et al., 2009; Ma et al., 2006). Wells on the soma side were topped up with media to maintain a difference in volume of 100µL between compartments ensuring an adequate pressure gradient and fluidic isolation was sustained throughout experiments. Media was removed from both wells on the axonal side, and 150 µl of IM was added to the axonal compartment. Chambers were incubated at 37°C with IM for 2 hours. Finally, the

same field of cells and ROIs used for the baseline recordings were used to repeat the axonal stimulation with 20mM KCl added after IM treatment (**Figure 7**).

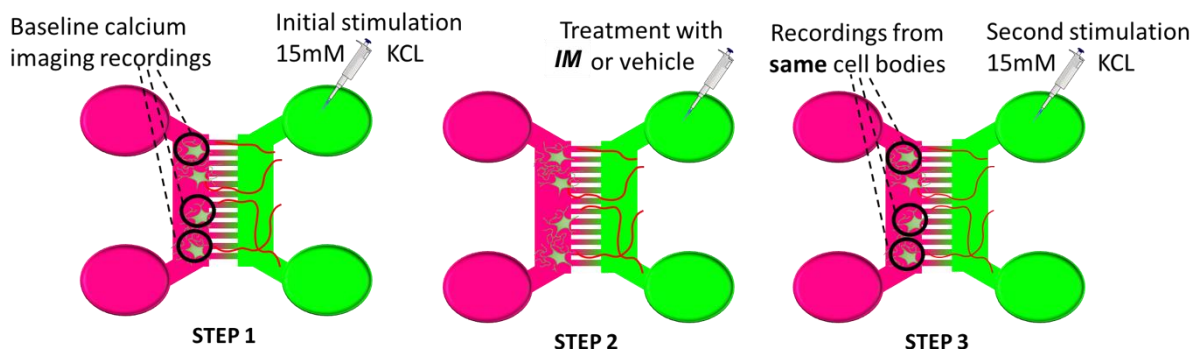


Figure 7. Protocol used for assessing changes in axonal excitability before and after IM treatment.

Protocol used is divided into three main steps. Step 1: Axonal responses were recorded to initial 20 second stimulus with 20mM KCl. Step 2: Axonal compartment was incubated with IM treatment for 2 hours. Step 3: Same FoV was found, and same ROIs were chosen as in step 1 were selected. Axonal responses were recorded after IM treatment, to 20 second stimulus with 20mM KCl.

2.7. Voltage Imaging and Analysis

For voltage imaging experiments, the voltage indicator Fluovolt (purchased from Life technologies) was used. Fluovolt voltage sensor is able to detect changes in voltage by modulation of photo-induced electron transfer (PeT) from an electron donor through a membrane-spanning synthetic molecular wire to a fluorophore or fluorescent reporter. Briefly, when a cell is hyperpolarised, the electric field is aligned opposite to the direction the electron transfer which results in effective PeT, and quenching of fluorescence. However, at depolarising potentials, the electric field is aligned parallel to the transfer of electrons resulting in reduced electron transfer, and thereby increasing fluorescence (Miller et al., 2012). DRG axons in the axonal compartment were loaded with the voltage indicator Fluovolt (Life Technologies),

and PowerLoad Concentrate (Life Technologies) in imaging buffer for 15 min at 37°C according to the manufacturer's manual for Fluovolt Membrane Potential Kit (Life Technologies). Following dye loading, the MFCs were mounted on an inverted microscope connected to an EasyRatioPro imaging system (Photon Technology International, UK), comprising of a CMOS camera (Hamamatsu, Japan) and analysis software (EasyRatio Pro) capable of acquiring digitized images every 20 ms upon illumination with the appropriate light source. Standard FITC filters were used for visualization and imaging Fluovolt. Fluorescent images at 388nm excitation were collected at 50 Hz using a 535nm emission.

2.8. Immunocytochemistry

For optimal incubation of DRG cells with any antibody solution or wash buffer, the following steps were carried out: solution was added to the somal top well (A) and allowed 30 seconds to flow to the somal bottom well (B). Following this, both A and B wells were topped up and allowed 1 minute for solution to flow across microgrooves. Finally, solution was added to the axonal top well (C) and allowed 30 seconds to flow through to the axonal bottom well (D) (**Figure 8**).

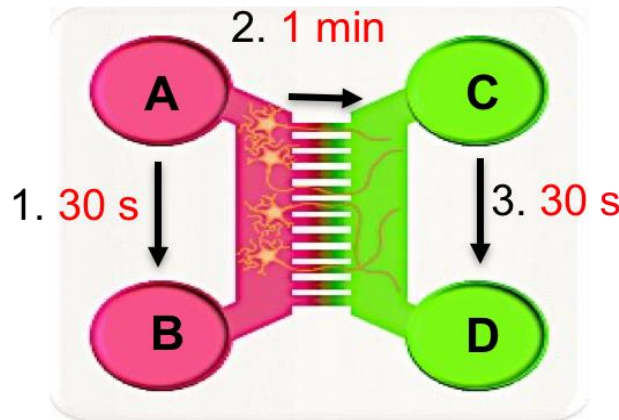


Figure 8. Steps followed for optimal antibody incubations and washes in MFC system.

Solution should be added to Well A, and allowed 30 seconds to flow through the channel to fill up Well B, a change in colour should be noted as the solution flows through. Next, top up both Well A and Well B, and allow 1 minute for solution to flow through microgrooves. Finally, add solution to Well C and allow 30 seconds for solution to flow through to Well D.

Media was removed from all MFC wells and washed with D-PBS once. Cells were fixed with 4% PFA at RT for 15 minutes. Next, cells were washed 3 times with D-PBS for 5 minutes.

D-PBS was removed and cells were incubated with blocking solution (10% normal donkey serum in 0.2% PBS-Tritan X, with 0.2% Sodium Azide) for 60 minutes. Blocking solution was then removed and primary antibody solution containing mouse Nav1.8 (1:500, Neuromab) and rabbit EP4 C-terminal (1:500, Cayman chemicals) primary antibodies in 10% normal donkey serum in 0.2% PBS-Tritan X, with 0.2% Sodium Azide. Cells were incubated with primary antibody overnight at 4°C, in the dark. On the following day, primary antibody solution was removed and washed 4 times with D-PBS. Secondary antibody solutions Alexa 488 anti-mouse and Alexa 546 anti-rabbit antibodies in blocking buffer were added. Cells were incubated with secondary antibody solution in the dark for 60 minutes at room temperature (RT).

Negative controls received secondary antibody solution only. Finally, cells were washed 4 times with D-PBS.

During the last wash with D-PBS, MFC devices were kept at 4°C for 5 minutes to allow detachment of PDMS device glass bottom plate. Using forceps, the PDMS device was gently lifted off Willco glass plate. Finally, coverslips were mounted with fluorescence mounting medium containing 4,6-diamidino-2-phenylindole (DAPI) and placed on the area of the glass with the MFC print. Cells were kept at 4°C in the dark before visualization.

2.9. HCN2 knockout mice

All animals were housed in a designated facility, maintained in accordance with Home Office regulations. Animals were kept in a 12hr light-dark cycle and fed *ad libitum*. Animals were sacrificed using Schedule 1 method of cervical dislocation. Globally deficient HCN2 mice were generated as published previously in supplementary methods section by Emery et al., 2011. Briefly, heterozygotes (HCN^{+/-}) were bred and HCN^{+/+}, HCN^{+/-} and HCN^{-/-} were obtained in Mendelian ratios. Ear snips were taken from each animal at Day 21 by animal facility staff and tail snips were taken after dissection of DRGs to cross-check genotype.

DNeasy Blood and Tissue Kit, (Quiagen) was used for purification and extraction of genomic DNA. Resulting total DNA was quantified by spectrophotometric absorbance at 260/280nm on a NanoDrop system. Master mix for PCR was made containing 5µL of GoTaq Green Mastermix (Promega), 1µL of common forward primer, 1µL of HCN2 KO reverse primer, 1µL of HCN2 WT reverse primer (

Table 1), and 1 µL of RNase-free H₂O per reaction. 9 µL of Master Mix was then added in Eppendorf PCR tubes (Sigma), after which 1 µL of DNA sample was added to each tube.

	Primer Pairs
HCN KO	FW: AAGCCTTCTCTGCGGTCTGG Rev: GCTGGCAGGCATCAGATACC
HCN WT	FW: AAGCCTTCTCTGCGGTCTGG Rev: AGAACCTGGAGAGAGGCAGG

Table 1. List of primer pairs used for genotyping of HCN KO and WT mice.

Sealed Eppendorf PCR tubes were placed in a thermal cycler (GeneAmp PCR System 9700, Thermo Fisher Scientific), used for amplification of DNA. The PCR protocol comprised of the following: pre-incubation at 94°C for 5 minutes, denaturation at 94°C for 30 seconds, annealing at 57°C for 30 seconds and extension reaction at 72°C for 40 seconds, followed by a final extension for 7 minutes. 40 repeat cycles were performed and tubes were held at 4°C at the end of the protocol. PCR products were resolved on 0.8% agarose gel, made up in TBE buffer (45mM Tris-Borate and 1mM EDTA). PCR products were loaded onto wells and gel was run at 95V for 30 minutes.

Pairing of the common forward primer (FWD), a sequence complementary to the intronic sequence upstream of exon 2, with the HCN2 WT primer (REV WT), a sequence that binds to exon 2, gives a product of 415bp, indicative of the presence of the HCN2 gene. Whereas, pairing of the common forward primer and the HCN2 KO reverse primer, a sequence complementary to the intronic sequence downstream of exon three, giving a product of 800bp indicative of HCN2 gene deletion (**Figure 9**).

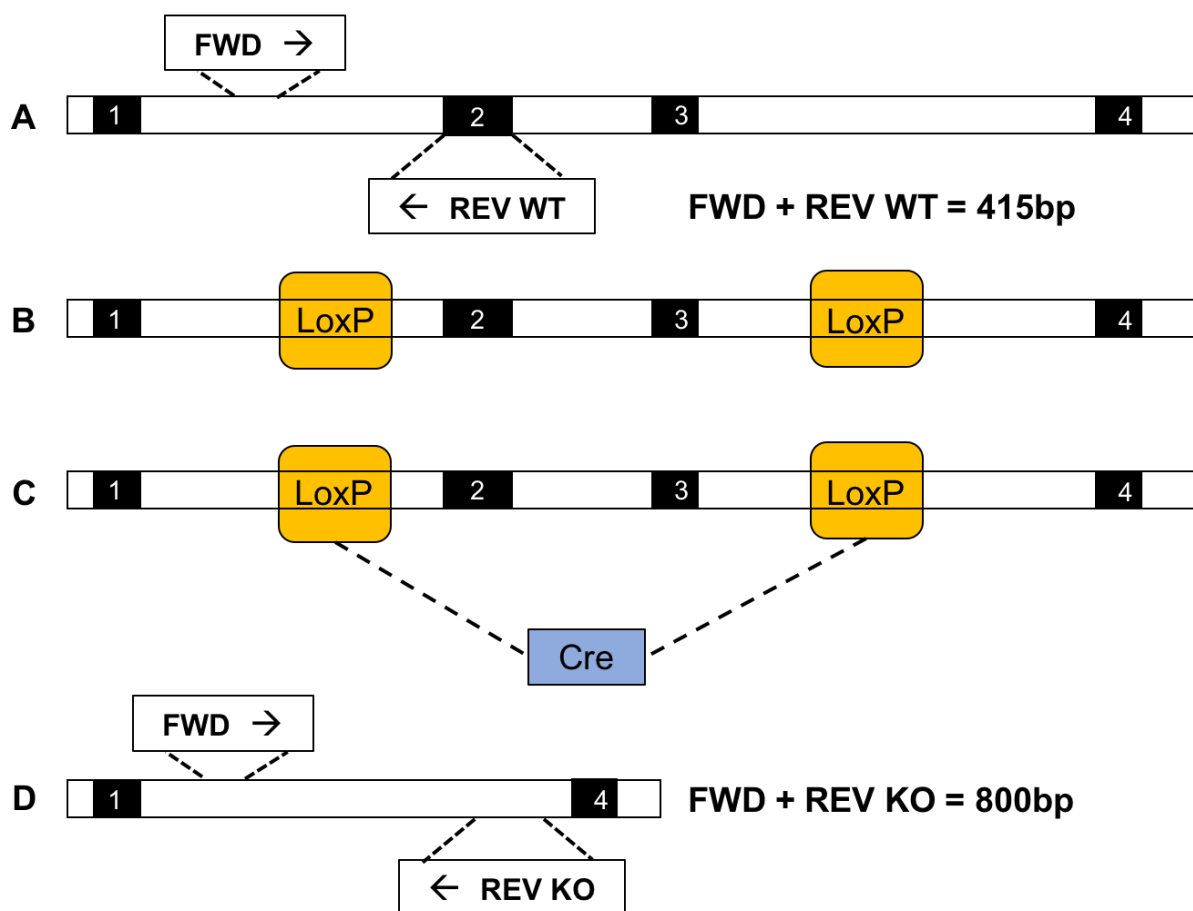


Figure 9. Genotyping of HCN2 WT and KO mice.

A) Common forward primer (Fwd) is complementary to the intronic sequence upstream of Exon 2. The reverse WT primer (Rev WT) binds within Exon 2. Pairing of Fwd and Rev WT, gives a product 415bp, verifying the presence of the HCN2 gene. **B)** Exons 2 and 3, which code for 5 of the 6 transmembrane regions of the HCN2 channel, were flanked with LoxP sites. Mice with HCN2 gene flanked with loxP sites, floxed, were provided by MSD Pharmaceutical, Scotland. **C)** Global HCN2 KO mice were generated by pairing homozygous floxed HCN2 mice with mice with globally expressed Cre, resulting in excision of Exons 2 and 3. **D)** Pairing of Fwd

with reverse KO primer (Rev KO), results in a product of 800bp, verifying the deletion of the HCN2 gene. (Adapted from (Emery et al., 2011b).

Heterozygous HCN2^{+/-} mice were bred and produced wild type, HCN^{+/-} and HCN^{-/-} offspring in Mendelian ratios. Cre/LoxP system was used to delete gene encoding HCN2. Exons 2 and 3, coding for 5 and 6 transmembrane segments of the channel, were deleted to produce global HCN KO^{-/-}. HCN KO and WT mice were genotyped. Two bands were identified for HCN^{+/-}, one band of 415bp was observed for HCN WT mice and 800bp band was observed for HCN KO mice (**Figure 10**). Phenotypical differences were also notable, as HCN2 KO mice appeared also to be smaller size compared to their WT littermates and HCN heterozygotes.



Figure 10. PCR of genomic DNA from DRG of HCN^{+/+}, HCN^{-/-} and HCN^{+/-}

All primers were mixed together prior to amplification. Forward common primer + WT reverse primer yields one band of 415bp, indicative of the presence of exon 2 and the intact HCN2 gene (415 bp). Forward common primer + KO reverse primer yields one band of 800bp, indicative of the excised HCN2 gene without exons 2 and 3. Two bands (800bp and 415bp) were observed in the case of heterozygote mice (HCN^{+/-}).

2.10. iPSC-derived human nociceptive neurons

Human-derived induced pluripotent stem cells were provided by Axol Bioscience, UK. Cells were thawed according to the manufacturer's protocols. Briefly, thawed cell suspension was transferred into a 15mL falcon conical and resuspended in 9mL

of Neural-XF Plating Medium in a slow, drop-wise manner. Cells were then centrifuged at 200rpm for 5 minutes at room temperature. Supernatant was discarded and cells were resuspended in 1mL of Neural-XF plating Medium to achieve single cell suspension. Cells were resuspended in appropriate volume of Neural-XF plating medium to achieve optimal cell loading and density for coverslips or MFCs. The day after plating, Neural-XF plating medium was replaced with Sensory Neuron Maintenance Medium supplemented with growth factors (25ng/ml GDNF, 25ng/ml NGF, 10ng/ml BDNF and 10ng/ml NT-3). In some cultures, as stated in chapter 6, GDNF concentration was increased to 125ng/ml. In order to prevent growth of non-neuronal cells in culture, 3 days after plating all cultures treated with Sensory Neuron Maintenance medium containing 3µg/ml Mitomycin-C. Cells were incubated for 1 hour at 37°C. After incubation period, cells were washed gently twice with Ca²⁺ and Mg²⁺ -free D-PBS (1x) and cells were supplemented with Sensory Maintenance Medium supplemented with growth factors.

To ensure optimal culture conditions, half the volume of medium was replaced with pre-warmed medium every 3 days. Neurons were maintained in culture for 4 weeks, to allow growth and maturation of neurons. Endpoint assays were carried out 4 weeks after plating.

2.10.1. Preparation of coverslips for hIPSCs

Glass coverslips were sonicated in dH₂O for 40 min, sterilised in 70% ethanol for 5-10 min, rinsed in dH₂O three times and left to dry for 2-3 hours. Sterilised glass dishes were then coated for different durations at 37°C with ReadySet (Axol Biosciences, USA) to establish optimal coating conditions as discussed further in

chapter 6. An equal volume of dH₂O was used to wash the glass plates, 4 times for a duration of 5 minutes each.

2.10.2. Preparation of MFCs for hIPSCs

Microfluidic devices (Xona Microfluidics, LLC) were sterilized in 70% ethanol for 5 minutes and left to dry out overnight. The TCND500 design (triple compartment chamber, with microgroove length of 500µm) was used for these experiments.

For attachment of the device to the glass dish, the MFC chamber was placed on the dry, coated glass bottom with the imprinted (microgroove) side down and gentle but firm pressure was applied with forceps to form a seal. SureBond (Axol Biosciences, USA) diluted in D-PBS was used, for second coating stage. Surebond solution was added to the MFC chamber following the steps illustrated in **Figure 8** for optimal and even coating across microgrooves and all channels. Chambers were allowed to incubate at 37°C for 1 hour.

For MFC loading approximately 70,000 were loaded per chamber. SureBond was removed from all wells using a suction glass pipette and 5µl of cell suspension in the appropriate dilution was added at the entrance of the somal channel. Next, the chambers were placed in the incubator at 37°C for 90 minutes to allow cell attachment. Following this, each well of the somal compartment was supplemented with 200µl of Neural- XF plating Medium (Axol Bioscience, UK), taking care to keep the volumes as equilibrated as possible to minimize liquid flow through somal

channel which would disturb cells. The axonal compartment wells were filled with 100µl of Neural- XF Plating Medium.

2.11. Data Analysis

For Ca^{2+} imaging experiments, Fura-2AM dye was used to quantify changes in intracellular Ca^{2+} levels by calculating the ratio of emission at 340nm and 380nm excitation wavelengths, expressed as fluorescence intensity F_{340}/F_{380} ratio (F_{ratio}). F_{ratio} was plotted against time (seconds) for each Dil-stained cell body, selected as a region of interest (ROI). Fluorescence intensity measurements from all ROIs were imported into Clampfit 9.0 software for calculation of maximum response (ΔF_{ratio}), calculated as difference between the maximum response and baseline (**Figure 11**), as well as area under the curve (AUC). Percentage of responders out of Dil-stained neurons were counted manually, through visual inspection of all ratio plots. For neuronal IM-induced sensitisation experiments, changes in the amplitude of response were expressed as fold change, measured as changes in maximum response to KCl stimulus after IM-treatment over maximum response to KCl stimulus before IM-treatment.

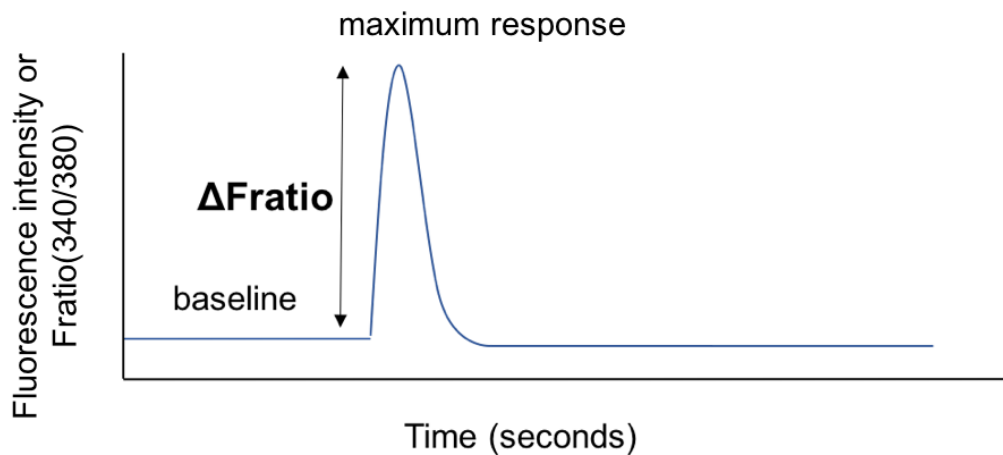


Figure 11. Measure of fluorescence intensity in calcium imaging experiments

Illustration of representative trace of fluorescence intensity or F340/F380 (Fratio) measured over time. Change in fluorescence intensity using Fluo-4 dye and Fratio using Fura-2 dye were measured as the difference between the baseline and maximum response.

For analysis of voltage signals, scripts were created for automated analysis of images compatible with Fiji software (Schindelin et al., 2012). The first 200 images (before application of stimulus) were averaged to calculate the mean baseline image. The calculated average baseline image was then subtracted from all images to create $\Delta F/F$ images, highlighting the responding areas along the DRG axon. Fiji particle analysis tool was used to select responding areas. Sequence of images were visually inspected to discard artefacts of movement. Fiji software's particle analysis tool was used to select responding areas as ROIs across all $\Delta F/F$ images. A positive response was assigned to a neuron when a maximum increase in ΔF_{ratio} greater than 3 times the standard deviation above baseline (calculated at least 5s prior to drug application), following drug application. All positive responses and maximum responses were confirmed by visual inspection of the ratio plots.

Statistical testing was performed using Microsoft Excel and GraphPad Prism software (Version 5.02, 2008, GraphPad Software Inc.). Two-tailed student's t-test, was used to determine statistical significance between two groups, whereas one-way analysis of variance (ANOVA) was used for experiments with 2 or more groups. Tukey's post-hoc test was used to analyse the data further for multiple comparisons. For normalised data, non-parametric statistical tests were used. Man-Whitney U test was used for comparison between two groups, and the Kruskal-Wallis test was used for comparison of more than two groups, followed by a Dunn's test for multiple comparisons.

3. Development of MFC-based *in vitro* assay to model peripheral sensitisation

3.1. Introduction

3.1.1. Current models of inflammatory peripheral sensitization

There are a number of animal models that have been used over the years for the study of inflammatory pain sensitisation. Compounds can be injected into the joint, muscle or plantar surface of the hind paw to produce sensitisation of peripheral sensory fibres. CFA, carrageenan, capsaicin, formalin, zymosan, and the mustard oil models are the most commonly used (Dobretsov et al., 2011). These models have been essential in the discovery of inflammatory pain mechanisms, yet significant gaps in our understanding of evoked and ongoing pain in human pain conditions remain. Technical difficulties often preclude the use of *in vivo* models to test

hypotheses at a molecular level. Further, multicellular responses *in vivo* complicate interpretation of the data when trying to elucidate mechanisms intrinsic to a particular cell-type. Hence, there is a need for *in vitro* models that allow interrogation of the molecular underpinning of inflammatory pain mechanisms, specific to peripheral neurons. However, currently, *in vitro* models focus primarily on IM-induced functional and expression changes occurring only at the neuronal cell bodies, side-lining other important structural components of peripheral sensory fibres. Moreover, during an inflammatory response *in vivo*, IMs are released at the site of injury and act on peripheral terminals whereas DRG cell bodies are not exposed to IMs directly.

The need for peripherally targeted therapeutic agents with reduced unwanted side effects has focused researchers' attention towards understanding how IM induced-changes in ion channel function and expression at the nerve terminals contribute to altered nociceptor function, as these findings could form the basis of new therapeutic strategies. It is assumed that ion channel properties and expression patterns are similar at the level of the cell body, axon, and nerve terminals despite the clear structural differences between the different sub-compartments of the neuron. In order to gain knowledge of the mechanisms underlying the interaction of IMs with ion channels found along the nerve endings, new models need to be developed.

3.1.2. Currently available methods to study functional properties of primary afferents

Both in vivo and ex vivo methodologies, such as the skin-nerve preparation, have enabled single unit recordings from nerve fibres have been critical for revealing the different types of nociceptor activated by noxious stimuli (Lawson, 2002;

Zimmermann et al., 2009). However, this technique is notoriously hard to maintain for multiple hours, producing low signal-to-noise ratio data that is difficult to interpret.

Campenot introduced *in vitro* systems that allow compartmentalization of the neuron into two separate areas containing either the cell body or neurite (Campenot, 1994). These first generation microdevices comprised of a Petri dish attached to a Teflon divider with silicon grease, which provided separation of the cell bodies and axons. Over the past decade, several adaptations of the compartmentalized cell culture system have been reported. Usoskin and colleagues reported the use of a gel-cushion axonal compartment, with a microenvironment that mimics the *in vivo* environment of nerve endings (Usoskin et al., 2010). However, important caveats of this technique include lack of reproducibility between devices, leakiness and significant challenges in assembly of the device which have been reported.

Recent publications have reported the use of porous filters for isolation of axons from sensory neurons, where axons pass through pores and grow along the bottom surface of the filter whilst neuronal cell bodies remain isolated on top. This technique results in the isolation of a population of pure axons, without contamination from non-neuronal cells. A major disadvantage of this technique however is its reliance on the use of still-developing embryonic DRG, as mature DRG neurons are not able to survive in this system (Unsain et al., 2014).

3.1.3. Use of microfluidic chambers to study axonal mechanisms

“Microfluidics” refers to a system that allows manipulation of extremely small (10^{-9} L or 10nL) volumes of fluids through a network of channels and chambers ranging from tens to hundreds of micrometres in size (Gross et al., 2007). High resistance

microchannels between both compartments allow a slow and sustained flow, which provides a barrier to the diffusion of molecules between the compartments (Taylor and Jeon, 2010). Compartmentalised microfluidic-based systems use polydimethylsiloxane (PDMS) soft lithography to produce devices enabling fluidic isolation of the two compartments of a neuron: an “axonal” and cell body or “somal” compartment. The advantages of this system include low reagent volumes required, better control of cellular microenvironments and highly sequential experimentation (Kim et al., 2012). This enables isolated treatment of both compartments, facilitating the investigation of axonal properties that are independent of the cell body.

Microfluidic devices have gained popularity and have been used for a number of applications such as the delivery of biochemical molecules, flow cytometric cell counting, and cell sorting (Huh et al., 2005; Kartalov and Quake, 2004; Kartalov et al., 2006; Sackmann et al., 2014). Recently, microfluidics has been adapted for use in neurobiology as peripheral axons can be isolated and grown in a particular direction, in the presence or absence of neurotrophins, providing an extremely versatile and adaptable system to model many aspects of neuronal sensitisation and injury (Park et al., 2006b).

3.2. Rationale and Aims

The objective of the initial part of my project, as described in this chapter, was to use a microfluidic based platform to develop a model enabling the study of mechanisms intrinsic to peripheral nerve fibres contributing towards nociceptor sensitisation, during inflammatory environments.

My objective was to develop and validate a microfluidic-based *in vitro* assay of peripheral sensitisation using adult DRG neurons in MFCs. This was achieved by isolating adult DRG axons in different configurations of MFC devices, to be able to distinguish between different mechanisms occurring in different components of DRG neurons. Next, we characterised distinct responses to locally applied chemical stimuli, in the axonal and somal neuronal compartments and aimed to exploit the developed MFC system as a tool to model peripheral sensitization more accurately allowing localized treatment of inflammatory mediators of isolated axons. Development of this model would enable better understanding of axonal

mechanisms underlying nociceptor hyperexcitability associated with painful conditions.

3.3. Results

3.3.1. Culturing adult DRG neurons in MFCs

Tsantoulas and co-workers had previously developed a MFC-based platform for the use of embryonic DRG neurons, in which case successful crossing of embryonic DRG axons through the microgrooves and into the axonal compartment was achieved by setting up a NGF gradient across the different compartments, with the somal compartment containing medium supplemented with 50ng/ml NGF, and the axonal compartment with medium supplemented with 100ng/ml NGF (Tsantoulas et al., 2013). Studies have demonstrated that NGF acts as a chemoattractant for embryonic sensory neurons, and is required for target innervation (Gundersen and Barrett, 1979; Paves and Saarma, 1997). However, it has been shown that NGF dependence of DRG neurons is only prominent during embryonic stages, as both rodent and human adult DRG neurons are able to survive and conserve their sensory modalities in the absence of NGF (Dodge et al., 2002; Ruit et al., 1992; Sosa et al., 1998). In support of this, a study demonstrated that using an NGF

gradient failed to direct growth of adult naïve DRG axons *in vitro* (Webber et al., 2008). Thus, we aimed to assess whether growth of axons of adult DRG neurons could be directed towards the microgrooves and the axonal compartment of the MFC device, and whether axonal isolation could be achieved using adult DRG neurons.

Here, we demonstrate that adult DRG axons do traverse the microgrooves and we were able to obtain axonal isolation in MFCs by DIV 5 (**Figure 12B-C**). To visualize the cell bodies with axons that have crossed the microchannels, Dil tracer was applied to the axonal compartment. Dil was incorporated into the cell membrane of the axons, before retrograde transportation to the cell bodies allowing for visualisation 24 hours post-staining. Positive Dil staining indicates cell bodies with axons that have crossed over to the axonal compartment (**Figure 12D-E**). Cell bodies closer to the microgrooves typically showed a stronger signal, as those neurons are most likely to cross over.

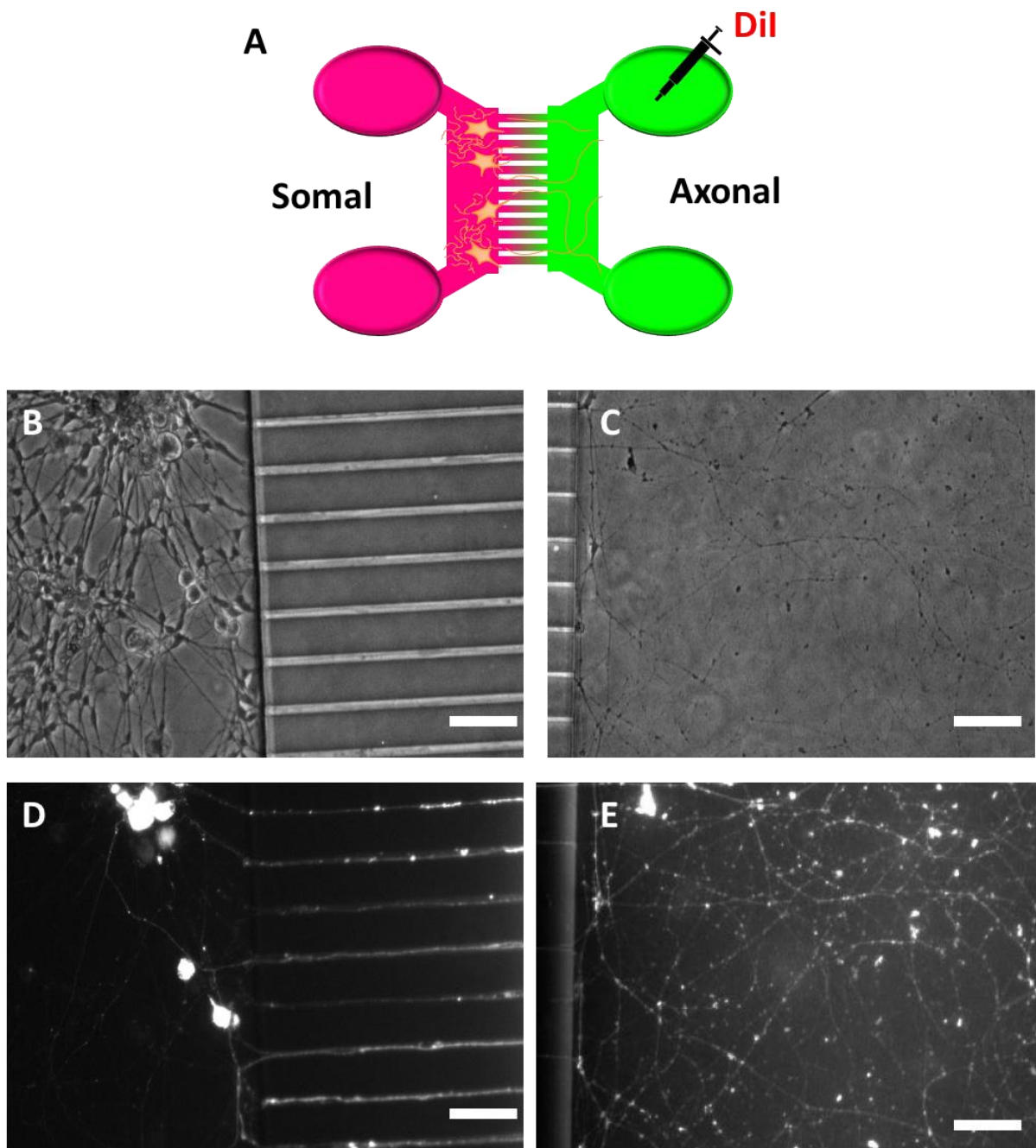


Figure 12. Dil stain indicative of DRG cell bodies with axons that have traversed to the axonal compartment

Representative images of adult DRG neurons at DIV 7 in MFCs. **A)** Schematic showing diagram of MFC device, highlighting somal and axonal compartments and indicating application of Dil to the axonal compartment. **B)** Bright field (BF) image of somal compartment of MFC, showing total number of cell bodies in a given field. **C)** BF image of axons shown in axonal compartment of MFC. **D)** Dil-stained soma in the somal compartment are observed, indicative of cell bodies that have crossed over to the axonal compartment. **E)** Dil-stained axons in the axonal compartment (n=3 mice, 8 MFCs). Scale bars, 100 μ m.

We next aimed to see whether axons of adult DRG neurons can be fluidically isolated and readily maintained in the 3-channel MFC device. Using the 3-channel MFC would enable more specific pharmacological investigation for future experimentation, as it divides DRG neurons into *three* subcellular components: the distal axons, proximal axons and cell bodies. Our lab had previously published the use of this configuration of the MFC device using embryonic mice (Tsantoulas et al., 2013). In our hands, we found that fluidic isolation adult DRG axons was achieved in 3-channel MFC device (**Figure 13E-H**), in a similar manner to that observed in the 2-channel MFC device (**Figure 13A-C**). Dil was added to the distal axonal compartment (**Figure 13H**) before being retrogradely transported to the cell bodies in the somal compartment. Adult DRG neurons were able to cross over to the distal axonal compartment by *DIV* 7 in 3-channel device. Percentage of Dil-positive cell bodies (or neurons with axons that cross over to distal axonal compartment), and percentage of cell bodies that respond to KCl stimulation of distal axons in the 3-channel device. Firstly, it was found that not all the cell bodies had axons that had traversed through the microgrooves. In the 2-channel MFC device (**Figure 13D**), approximately, 60% of the cell bodies out of the total number of DRG soma found in the somal compartment were positive for Dil, indicating that only 60% of the total number of cell bodies' axons had reached the axonal compartment by *DIV* 5. Surprisingly, we also found that only 40% of the Dil- stained neurons are responsive to axonal stimulation with 20mM KCl. The exact reason for this is not known, however we speculate that lack of maturity of axons at *DIV* 5 may explain why some cell bodies don't exhibit responses to stimulation of axons with 20mM KCl. In the 3-channel MFC device (**Figure 13I**), an average of 32% of the total number of cell

bodies' axons reached the distal axonal compartment 5 days after plating and only a 24% of the Dil- stained neurons are responsive to axonal stimulation with 20mM KCl. Although the 3-channel MFC device enables more detailed interrogation of subcellular components of adult DRG neurons, the 2-channel MFC device is a more high-throughput system enabling the study of axonal function in a greater number of neurons per experiment.

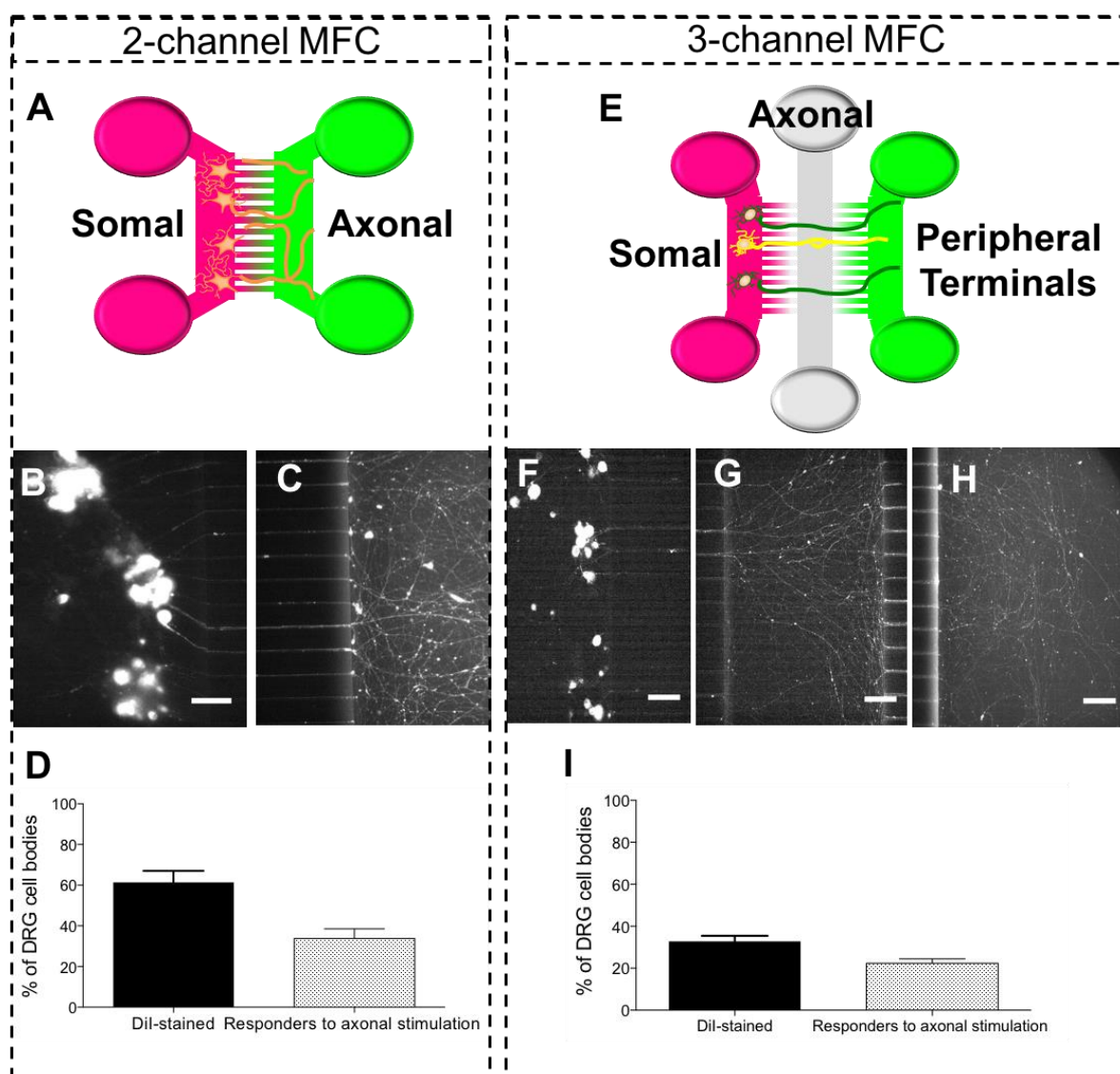


Figure 13. Adult DRG neurons in 2-channel and 3-channel MFC devices

A) Schematic of 2-channel MFC **B)** Dil-stained cell bodies in somal compartment of 2-channel MFC. **C)** Dil-stained axons traversing across microgrooves into axonal

compartment of 2-channel MFC. **D)** Bar graph illustrating number of Dil-stained cell bodies, and axonal responders to 20mM KCl in 2-channel MFC device. **E)** Schematic of 3-channel MFC. **F)** Dil-stained cell bodies in somal compartment of 3-channel MFC. **G)** Dil-stained axons traversing across microgrooves into axonal compartment of 3-channel MFC. **H)** Dil-stained axons in peripheral terminal compartment of 3-channel MFC. **I)** Bar graph illustrating number of Dil-stained cell bodies, and axonal responders to 20mM KCl in 3-channel MFC device. Bar graphs illustrating mean $\pm$ S.E.M, where bars represent the % of cell bodies per field of view, including the total number of cell bodies, Dil- stained cell bodies (indicative of axonal crossing), and the responders to axonal 20mM KCl stimulation (n=8 mice, 14 MFCs, 56-60 DRG neurons). Scale bars, 100 μ m.

Next, we set out to establish a robust and reproducible method to measure axonal function *in vitro* taking advantage of the MFC platform. Fluidically isolated axons were stimulated by application of increasing concentrations of KCl in the axonal compartment. Ca^{2+} responses were recorded from the neuronal cell bodies in the somal compartment as a readout for axonal excitability (**Figure 14A**). We aimed to establish the optimal concentration required to depolarise all axons and to produce a detectable signal at the cell body. Application of 20mM, 35mM and 65mM KCl solution to the axonal compartment resulted in a rapid increase in fluorescence signal in neurons with axons that had crossed into the axonal compartment (Dil-positive) at DIV 5 (**Figure 14B-F**). Axonal stimulation with KCl resulted in a transient response, characterised by a rapid onset and return to baseline before wash-out of KCl, as reported previously by (Tsantoulas et al., 2013). Percentage of responders after axonal and somal stimulation with KCl were also quantified. Axonal stimulation with 20mM, 35mM and 65mM KCl showed $51.5\% \pm 6.5$, $50\% \pm 2.5$ and $48\% \pm 7$ responders in a given field, respectively at DIV 7 (**Figure 14G**). The percentage responders did not linearly correlate with increasing concentrations of KCl (20mM, 35mM and 65mM, respectively). Using a lower concentration of KCl could perhaps provide a graded response in terms of the number of responders, this could be

tested in future experiments. However, these data suggest that application of 20mM KCl is sufficient to depolarize all the axons that have crossed through the microgrooves. If stimulation with 20mM was subthreshold, it would be expected that stimulation with a higher concentration of KCl (in this case 35mM and 65mM) would result in larger percentage of responders.

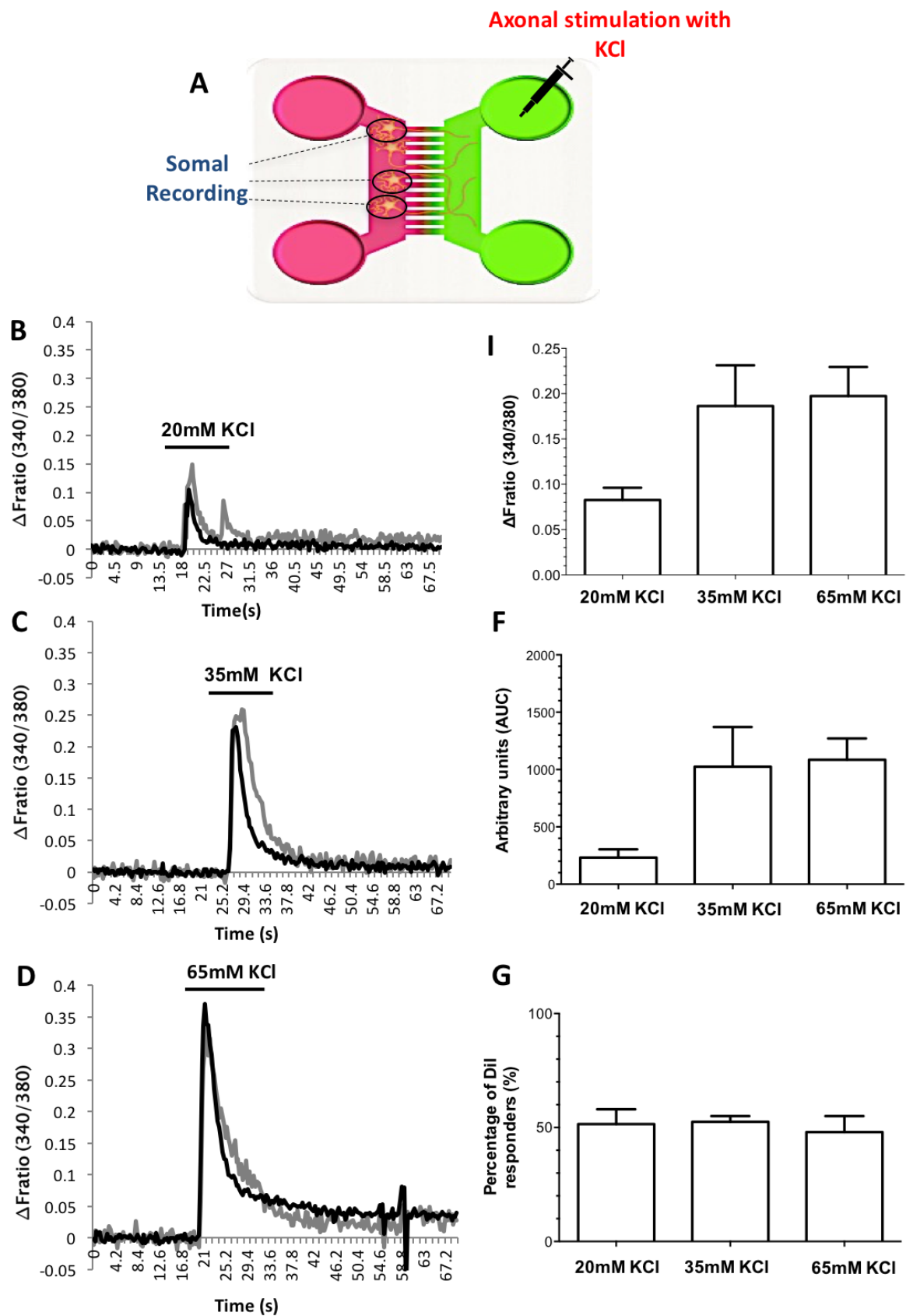


Figure 14. Characterisation of axonal responses to KCl in the MFC system.

Representative traces showing changes in Ca^{2+} concentration at the soma in response to KCl stimulation in the axonal compartment. **A)** Experimental set up: axonal compartment was stimulated with a given chemical stimulus and changes in $[\text{Ca}^{2+}]_{\text{in}}$ in the cell bodies were measured. **B)** Changes in $[\text{Ca}^{2+}]_{\text{in}}$ are measured as ΔF_{ratio} in response to axonal stimulation with added 20mM KCl. **C)** Changes in $[\text{Ca}^{2+}]_{\text{in}}$ in response to axonal stimulation with 35mM KCl. **D)** Changes in $[\text{Ca}^{2+}]_{\text{in}}$ in response to axonal stimulation with added 65mM KCl. **E)** Quantification of maximum responses to added 20mM, 35mM and 65mM KCl. **F)** Quantification of AUC of responses to added 20mM, 35mM and 65mM KCl. **G)** Percentage of responders from the Dil-stained cell bodies in a given field. Bar graphs showing data as mean $\pm$ S.E.M, where white bars represent response to axonal stimulation and black bars represent response to somal stimulation with KCl ($n=3$ mice, 5 MFCs, 20-25 DRG neurons).

In order to validate our method of measuring axonal function (stimulating axons and recording at the cell bodies), we stimulated cell bodies directly with increasing concentrations of KCl. We found that stimulation of cell bodies in somal compartment exhibited markedly different kinetics compared to the Ca^{2+} responses obtained from axonal stimulation with 35mM and 65mM KCl (**Figure 15A-C**). Maximal responses and AUC of responses to axonal stimulation with KCl were also quantified (**Figure 15D-E**). Responses to somal stimulation displayed a slower onset, with a return to baseline only after the wash-out of KCl. Somal stimulation with KCl produced a 100% response rate at all concentrations, whereas approximately 50% of neurons were responsive axonal stimulation with KCl (**Figure 15F**). The clear difference in the type of responses when stimulating the axonal compartment compared to when we stimulated the somal compartment with KCl, provides evidence validating our method as a robust and reproducible technique to measure axonal function *in vitro*. Moreover, somal stimulation with KCl served as a positive control in future experiments as an indicator of health of the neurons, optimal culture conditions and that adult neurons in MFCs are capable of responding to depolarizing stimuli.

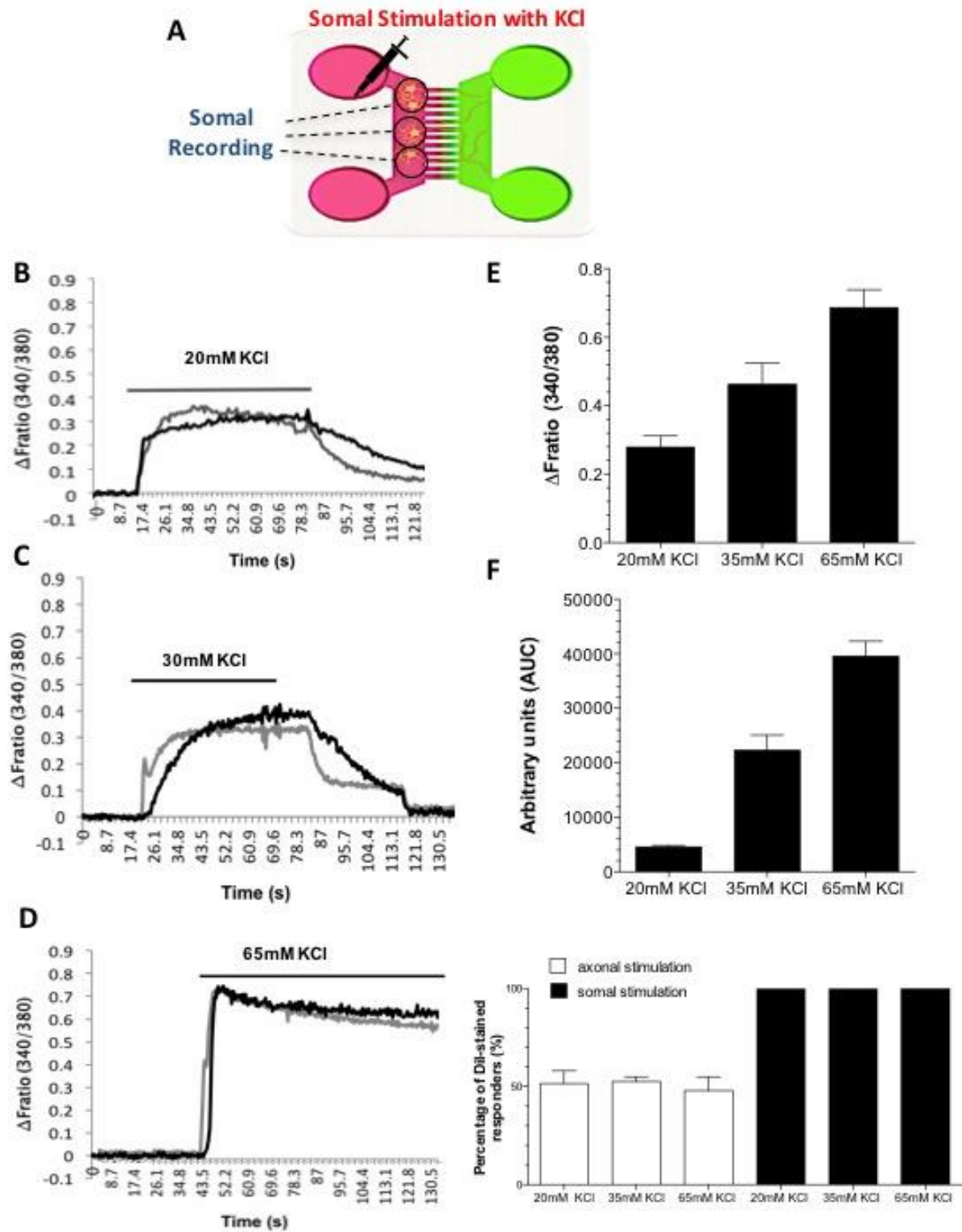


Figure 15. Characterisation of somal responses to KCl in the MFC system.

A) Experimental set up: somal compartment was stimulated with a given chemical stimulus and changes in $[\text{Ca}^{2+}]_{\text{in}}$ in the cell bodies were measured. **B)** Changes in $[\text{Ca}^{2+}]_{\text{in}}$ at the soma measured as ΔFratio in response to stimulation with 35mM KCl. **C)** Changes in $[\text{Ca}^{2+}]_{\text{in}}$ at the soma measured as ΔFratio in response to stimulation

*with 65mM KCl in the somal compartment. **D)** Quantification of maximum responses to 35mM and 65mM KCl. **E)** Quantification of AUC of responses to 35mM and 65mM KCl. **F)** Percentage of responders as determined by Dil-stained cell bodies in a given field. Bar graphs showing data as mean $\pm$ S.E.M, where white bars represent response to axonal stimulation and black bars represent response to somal stimulation with KCl (n=3 mice, 5 MFCs, 20-25 DRG neurons).*

Using the 3-channel MFC, Tsantoulas and co-workers demonstrated that electrical stimulation in the distal axonal compartment results in AP arriving at the DRG cell soma, which can be blocked by perfusion of lidocaine to the middle axonal compartment (Tsantoulas et al., 2013). The reported findings and the notably distinct kinetics of KCl responses after axonal and somal stimulation observed in this study, suggest that distinct mechanisms are responsible for the generation of Ca^{2+} signals at the DRG cell body compared to the DRG axon. We propose that stimulation of DRG axons with KCl results in the depolarisation of axons and generation of bursts of Aps that propagate towards the cell soma, resulting in a transient Ca^{2+} signal. On the other hand, stimulation of the DRG cell bodies with KCl results in depolarisation of the somal membrane, and the direct opening of voltage-gated Ca^{2+} channels at the somal membrane resulting in an influx somal Ca^{2+} which is maintained until the washout of KCl (**Figure 16**). In future experiments, it would be interesting to test this hypothesis by assessing whether application of a known voltage-gated calcium channel blocker to the somal compartment inhibits the somal Ca^{2+} response to KCl. Nonetheless, taken together, these experiments demonstrate that nociceptive axons from adult mouse DRG neurons in MFCs respond to stimulation in isolation from the soma, and their response to stimulation can be assayed by measuring a local increase in soma Ca^{2+} concentrations.

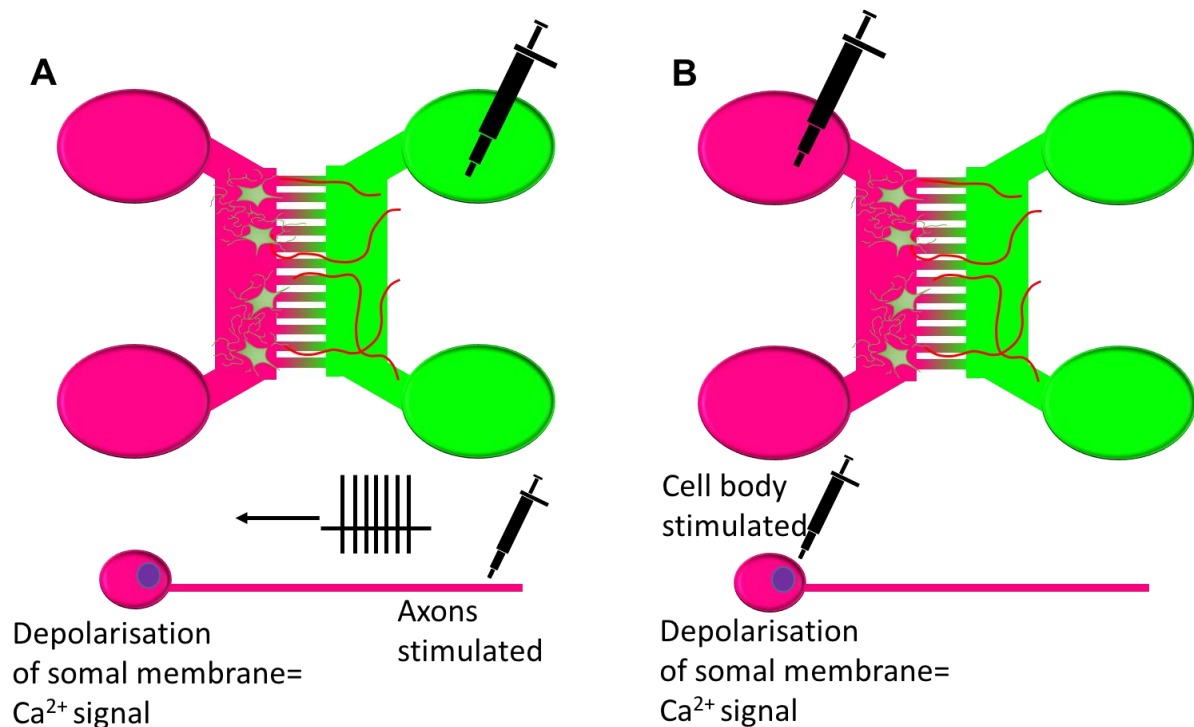


Figure 16. Distinct mechanisms underlying somal and axonal stimulation in DRG neurons.

A) Axonal stimulation leads to generation of a volley of action potentials travelling to the soma, which then depolarize the somal membrane. **B)** Somal stimulation results in direct depolarization of the somal membrane.

3.3.2. Development of a MFC-based *in vitro* assay of peripheral sensitisation

We next sought to understand whether treatment of nociceptive axons with inflammatory mediators in isolation from the soma, can sensitise axonal responses to stimulation *in vitro*. Acute peripheral sensitisation has been explored using patch clamp electrophysiology, where it has been demonstrated that application of Ims composed of histamine, bradykinin, serotonin, and PGE_2 , for two hours induced changes in neuronal excitability (Grossmann et al., 2009; Ma et al., 2006).

In preliminary experiments, we explored whether acute (2-hour) treatment with the same cocktail of Ims could induce a significant increase in neuronal responses detectable using Ca^{2+} imaging at the DRG soma using conventional cultures. At DIV 5, coverslips of DRG neurons were treated with Ims for 2 hours and control coverslips were treated with vehicle only (**Figure 17A**). Responses to KCl were measured using Ca^{2+} imaging and we observed a significant increase in Ca^{2+} signal expressed as maximum response and AUC, in IM-treated DRGs in comparison to control groups. Average maximum responses to 20mM and 35mM KCl were significantly increased in the IM-treated groups, compared to vehicle treated neurons, displaying a 1.45-fold increase (from 0.39 ± 0.03 to 0.57 ± 0.19) and 1.3-fold increase (from 0.35 ± 0.03 to 0.46 ± 0.04), respectively (**Figure 17B and D**). Thus, we concluded that this combination of Ims induces a significant change in neuronal responses to KCl stimulation, which are detectable measuring changes in Ca^{2+} concentration in the cell bodies of DRG neurons.

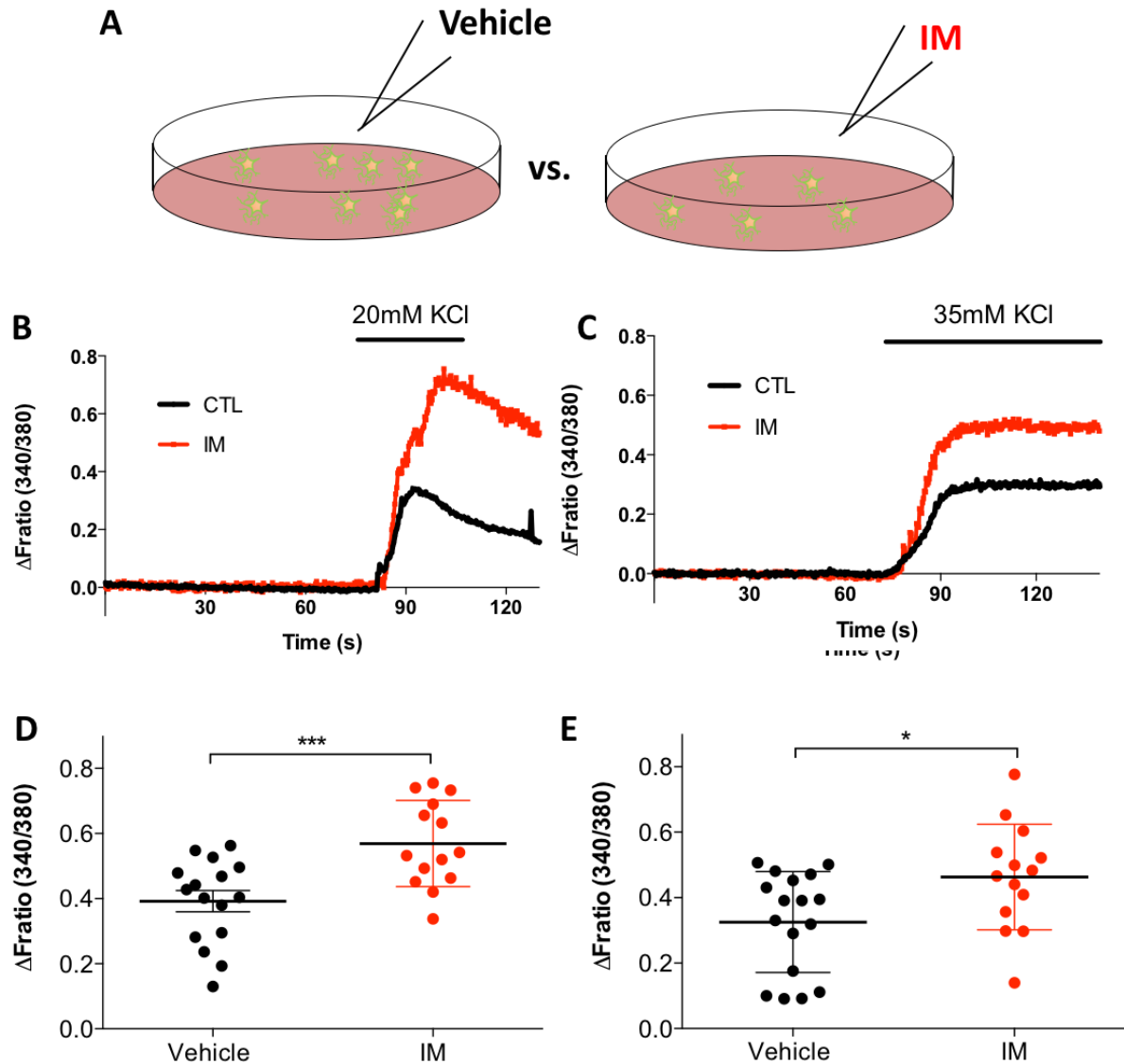


Figure 17. Changes in excitability in DRG neurons after sensitisation with IM.

A) Schematic of experimental set up: DRG neuron cultured in conventional coverslips were exposed to vehicle or IM-containing media for 2 hours at Div 5 post-plating. Following the 2-hour incubation, DRG neurons were loaded with fura-2 and Ca^{2+} responses to 25mM (**B**) and 30mM KCl (**C**) were recorded using calcium imaging. **B)** IM-treated DRG neurons showed a significantly higher maximum response after stimulation with 20mM KCl, in comparison to untreated DRG neurons, where $***p < 0.001$. **C)** IM-treated DRG neurons showed a significantly higher maximum response after stimulation with 35mM KCl, in comparison to untreated DRG neurons, where $*p < 0.05$, respectively. Data expressed as mean $\pm$ S.E.M., black dots representing untreated DRG and red dots representing DRG treated with IM. (Unpaired t-test, $n=2$ mice, 20 DRG neurons).

In the case of conventional culture systems, which are used extensively in cellular studies testing the effects of IMs, the observed sensitisation is likely to be due to a number of synergistic factors including changes in the excitability of the soma, changes in the excitability of the axon as well as the potentiation of VGCCs leading to enhanced Ca^{2+} influx in response to KCl stimulation. Thus, we sought to develop a model of *peripheral* inflammatory sensitisation that is more relevant to *in vivo* models, where the DRG cell body is not exposed to IMs directly. Therefore, the impact of a 2-hour IM-treatment, localised to the axonal compartment of DRG MFC cultures, on axonal responses to depolarizing stimulation with KCl was investigated **(Figure 18)**. Axons treated with IMs showed a significant increase in Ca^{2+} influx at the soma in response to stimulation of the axons with KCl, following IM-treatment. Using 20mM KCl, a 7.4-fold increase (from 0.009 ± 0.001 to 0.067 ± 0.02) was observed in IM-treated groups compared to vehicle treated, whereas using 35mM KCl, a 3.7-fold increase in average maximum response (from 0.02 ± 0.005 to 0.08 ± 0.02) was observed in average maximum response of Ca^{2+} responses of neurons with IM-treated DRG axons compared to vehicle treated **(Figure 18B)**.

Stimulation of axons with 20mM KCl resulted in a greater fold increase in average maximum response to that observed with 35mM KCl. Taken together, we conclude that 20mM KCl is not only sufficient to depolarise axons, but also the optimal concentration to detect significant changes in KCl response at the soma following axonal IM-treatment and hence, 20mM concentration was chosen for subsequent experiments. These results indicate that 2 hours of axonal treatment with IM results in a robust increase in excitability of nociceptive axons and that the MFC cultures are an appropriate model to study axonal sensitisation.

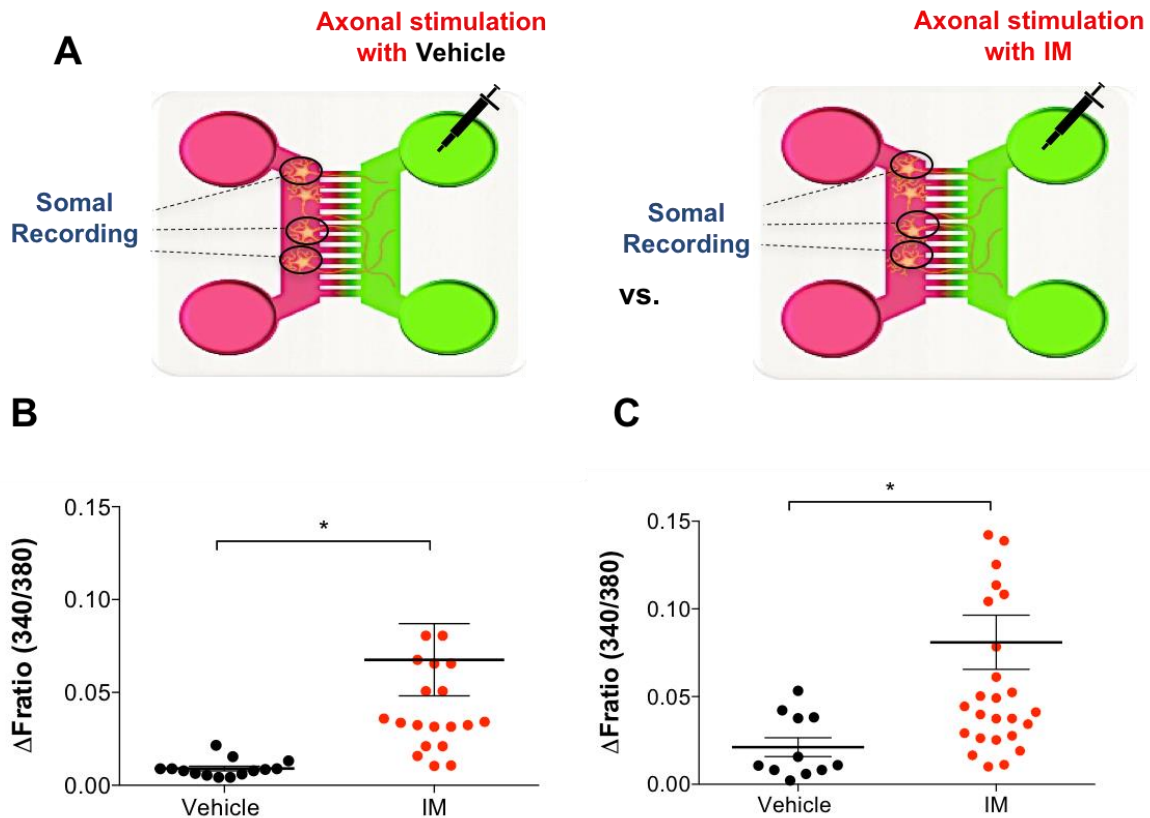


Figure 18. Changes in axonal excitability detected after localised sensitisation with IM using the MFC system.

Axonal treatment with IM resulted in increased excitability in comparison to untreated axons. **A)** Schematic of experimental set up: DRG neurons were cultured in MFCs, Dil was applied to the axonal compartment on Div 4. 24-hours following axonal Dil staining (Div 5), Dil stained cell bodies were identified. IM or vehicle containing media was applied to the axonal compartment for 2 hours. Following the 2-hour incubation of DRG axons with IM or vehicle, the somal compartment was loaded with Fura-2 and Ca^{2+} responses at the soma were recorded to stimulation of axons with 25mM KCl (**B**) and 30mM KCl (**C**) using calcium imaging. **B)** IM-treated DRG axons showed a significant increase in maximum response to 20mM KCl, in comparison to vehicle-treated DRG neurons, where $*p < 0.05$. **C)** IM-treated DRG axons showed a higher maximum response after stimulation with 35mM KCl, in comparison to vehicle DRG neurons, where $*p < 0.05$. Data expressed as mean $\pm$ S.E.M., black dots representing untreated DRG and red dots representing DRG treated with IM. (Unpaired t-test, $n=3$ mice, 10-24 DRG neurons).

At the end of every experiment, the DRG soma were stimulated with maximal KCl, as a positive control indicative of the health of cells as well as optimal culture conditions. We found that responses of the DRG soma varied greatly between

coverslips/MFCs. This suggested that differences between culture conditions and health of cells could be a confounding factor when interpreting differences between coverslips/MFCs as these variants are not taken into account. Using this protocol, it was difficult to conclude whether the observed differences in responses to KCl between coverslips were attributed to the given treatments (in this case IM or vehicle) or other variants such as the number of cells per FoV, health of cells and culture conditions. Therefore, we sought to develop a more robust method in order to reduce variability observed between coverslips, and enable more accurate interpretation of data.

With the objective of optimising our experimental method, we used a protocol which enabled the investigation of changes in axonal excitability before and after inflammatory insult in individual neurons, where each cell is its own control. Briefly, I recorded Ca^{2+} influx in DiI-stained DRG soma as a result of stimulation of DRG axons with 20mM KCl as a baseline response. Next, the axonal compartment was perfused with medium containing IM for 2 hours. Following IM-treatment, changes in Ca^{2+} influx at the DRG soma in response to stimulation of axons with 20mM KCl of the *same cells selected for the baseline responses* were recorded (**Figure 7**). Two types of axonal sensitisation after IM-treatment were identified by using this protocol: a decrease in activation threshold and a potentiation in Ca^{2+} response at the soma. Axons that were unresponsive to 20mM KCl stimulation, became responsive to the same concentration of KCl after 2-hour treatment with IM (**Figure 19A**). Moreover, axons that displayed a response to 20mM KCl, had an enhanced response to the same stimulus after IM-treatment (**Figure 19B**). A significant increase in maximum response was observed in cells treated with IM, compared to those exposed to 2-

hour vehicle treatment displaying a 2.3-fold increase (from 1.9 ± 0.2 to 0.86 ± 0.1) **(Figure 19C)**. Furthermore, a significant increase in the average percentage of responders within the Dil- stained cell bodies was also observed after IM treatment, with a 3.7-fold increase in percentage responders (from $18.7 \pm 3.2\%$ to $70.5 \pm 6.6\%$) **(Figure 19D)**. This protocol displayed more robust and reproducible results, allowing us to identify DRG neurons with axons that are readily sensitized after localized IM-treatment, and show enhanced magnitude of responses as well as an increased number of responders as a result.

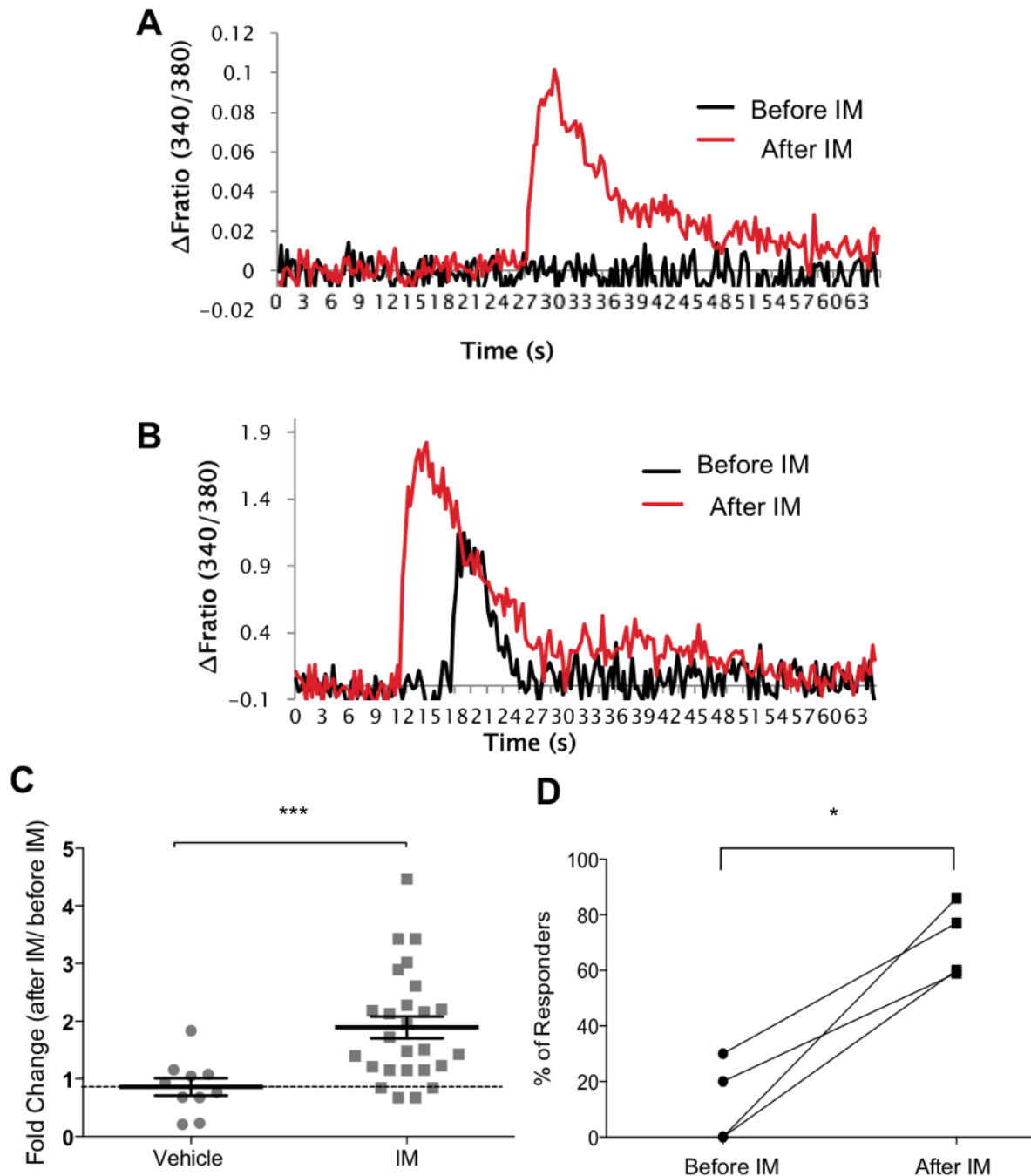


Figure 19. Axonal sensitisation in response to localised 2 hour IM-treatment.

After localised two-hour axonal treatment with IM, resulted in two types of axonal sensitisation. **A-B**) Representative responses of DRG axons to 20mM KCl before and after IM treatment; black trace represents axons before treatment, and red trace represents axons after treatment **A**) Representative trace of unresponsive (black) vs. responsive (red) axons **B**) Representative trace of responsive neuron (black), and comparably enhanced response (red). **C**) Data expressed as the mean $\pm$ S.E.M, showing an increase in fold change of the maximum response to 20mM KCl before and after axonal treatment with vehicle or IM. IM- treated neurons showed a significantly increased fold change to KCl stimulation after IM-treatment compared to

vehicle-treated neurons ($***p < 0.0007$, two-tailed, Mann-Whitney U test, $n = 3$ mice, 15-25 DRG neurons). **D)** Data expressed as the mean percentage of responders KCl per MFC. Graph showing an increase in the number of DRG responders after IM treatment compared to those given vehicle treatment ($*p < 0.02$, two-tailed Mann-Whitney U test, $n = 3$ mice, 15-25 DRG neurons).

Using the 2-hour IM treatment protocol we were able to detect a significant sensitisation of DRG axons, however this particular time-point was chosen based on the literature, where it has been shown that 2-hour treatment with IMs induces sensitisation of the DRG cell bodies resulted in a significant reduction of rheobase (Lee et al., 2014a). However, we hypothesised that a shorter exposure to IM (in minutes) would be sufficient to trigger sensitisation at the DRG axons. Moreover, it is well documented that nociceptors are not only sensitised, but can also be activated by inflammatory stimuli. A study showed that serotonin, bradykinin and PGE2 have been shown to evoke calcium influx in rat DRG soma (Linhart et al., 2003). At the primary afferent level, a study using canine testis-spermatic nerve preparation showed histamine and bradykinin were able to induce excitation of nociceptors (Koda and Mizumura, 2002b). Thus, we set out to investigate whether DRG axons can be sensitised after exposure to IM-treatment for 2 minutes, and whether application of IM could activate nociceptive axons directly.

To answer these questions, we used a protocol that enabled both 1. to test for sensitisation of axonal responses by measuring changes somal Ca^{2+} signals to KCl-stimulation of axons before and after 2-minute IM- treatment (localised to the axonal compartment) and 2. to test for nociceptor activation by monitoring changes in Ca^{2+} signal at the DRG soma throughout the duration of the experiment (including the duration of the IM-treatment). We found that axonal responses to 20mM KCl were

potentiated after 2-minute IM treatment, compared to baseline axonal response to 20mM KCl (**Figure 20A**). A significant increase in maximum response to KCl stimulation after IM-treatment was observed when compared to responses to baseline KCl stimulation before treatment, displaying a 3.9-fold increase (from 0.86 ± 0.03 to 3.37 ± 0.5) between groups (**Figure 20B**). There was a significant increase in percentage responders out of Dil-stained cell bodies after treatment with IM, with a 2.5-fold increase compared to the percentage of responders to baseline KCl stimulation (from $31 \pm 8.4\%$ to $81 \pm 6.3\%$) (**Figure 20C**). Of note, as mentioned previously, not all Dil-stained cell bodies respond to KCl stimulation of axons.

We found that axonal responses to KCl are potentiated after treatment with a cocktail of IMs containing PGE2, histamine, bradykinin and serotonin for a duration of both 2 hours and as little as 2 minutes. The MFC model allows us to identify two types of sensitisation at the axonal level, increased responsiveness as well as recruitment of otherwise non-responsive neurons, which we were able to model robustly and reproducibly using this protocol. Furthermore, we were also show direct activation of nociceptive axons following exogenous application of IMs (**Figure 21**).

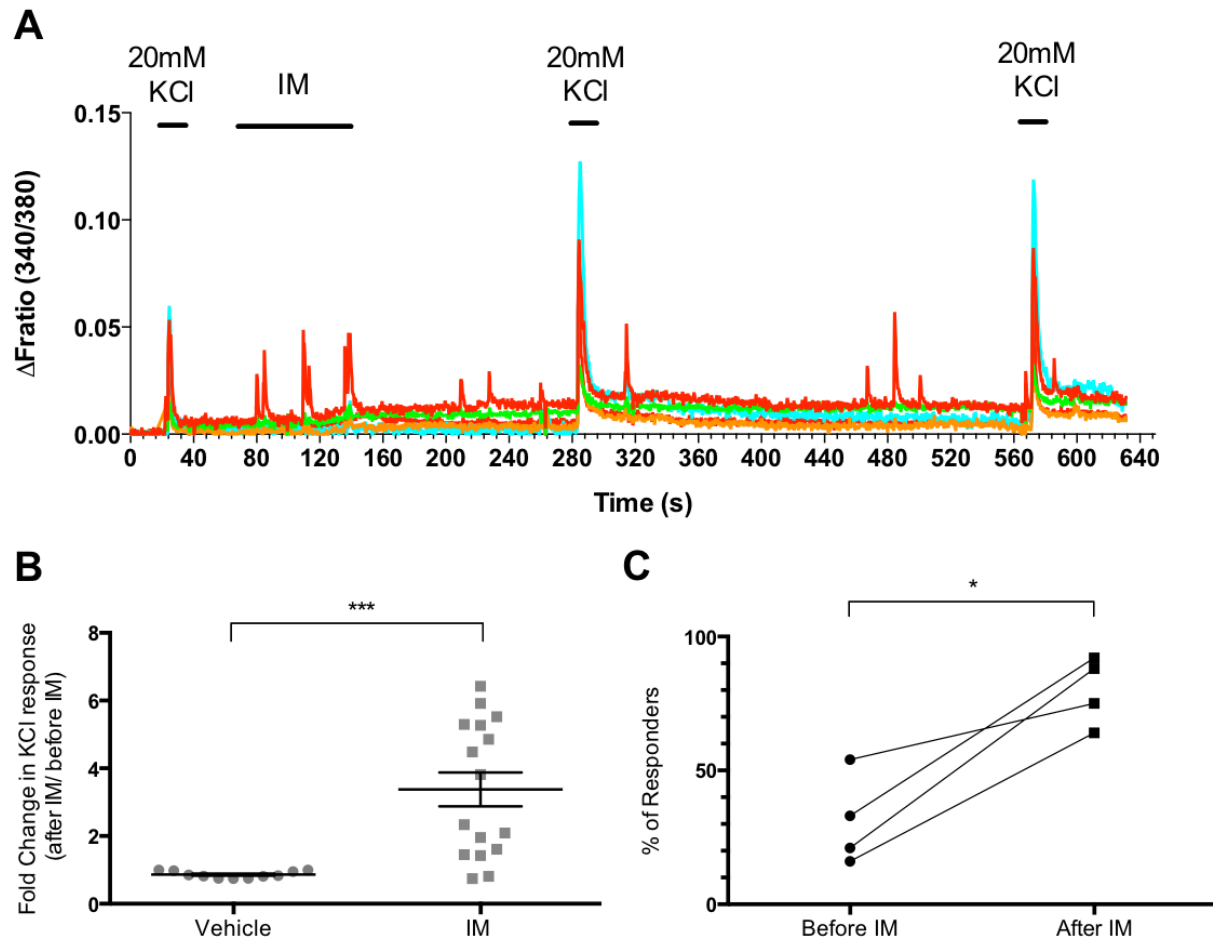


Figure 20. Axonal sensitisation in response to localised 2 minute IM-treatment.

Acute localised axonal treatment with inflammatory soup resulted in two types of sensitisation of the axon. **A)** Representative trace showing axonal responses to first KCl stimulation (20 second), IM stimulation (2 minutes) and a potentiated response to the second KCl stimulation (20 seconds), which is maintained in the third response to KCl stimulation (20 seconds) after a 10-minute wash period. **B)** Data expressed as mean $\pm$ S.E.M, where graph displays fold change in KCl response after vehicle treatment, compared to IM treatment. IM- treated neurons showed a significantly higher maximum response to KCl stimulation after treatment compared to vehicle treated neurons ($***p < 0.0002$, two-tailed, Mann-Whitney U t-test, $n = 18-20$ DRG, 4 mice). **C)** Data expressed as the mean percentage of responders to KCl per MFC. Graph showing a significant increase in the number of DRG responders to KCl stimulation after two-minute IM treatment compared to KCl stimulation before IM treatment ($*p < 0.01$, Mann-Whitney test U, 16-20 DRG neurons, $n = 4$ mice).

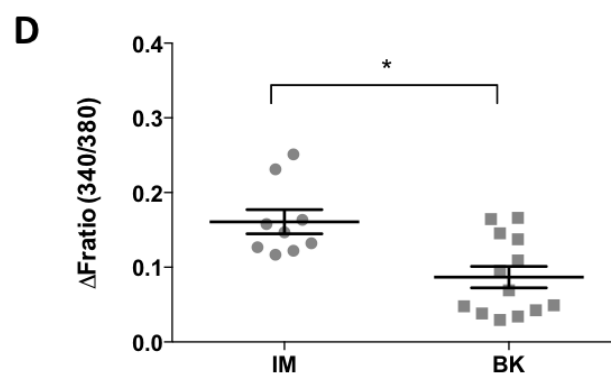
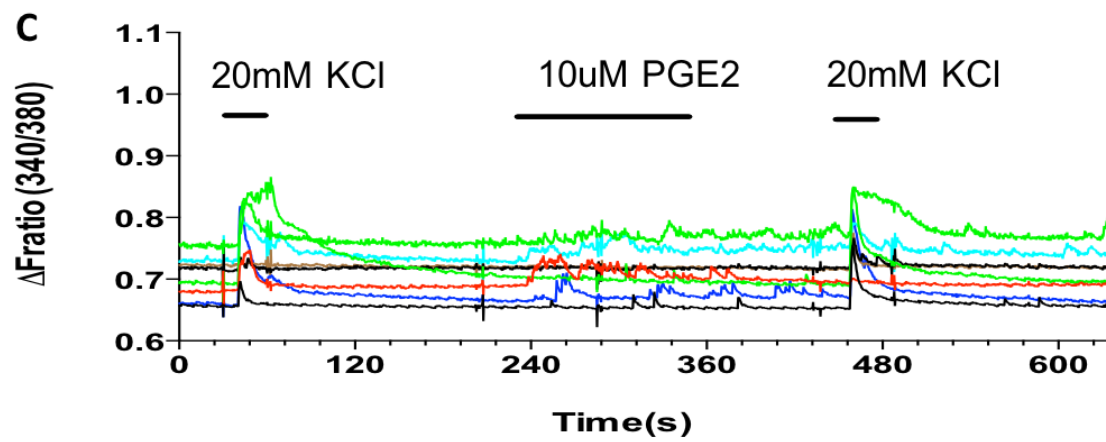
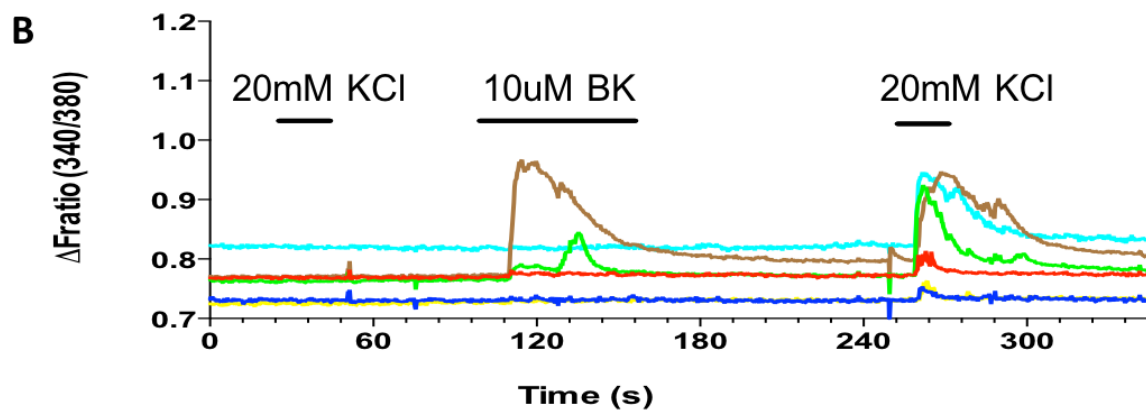
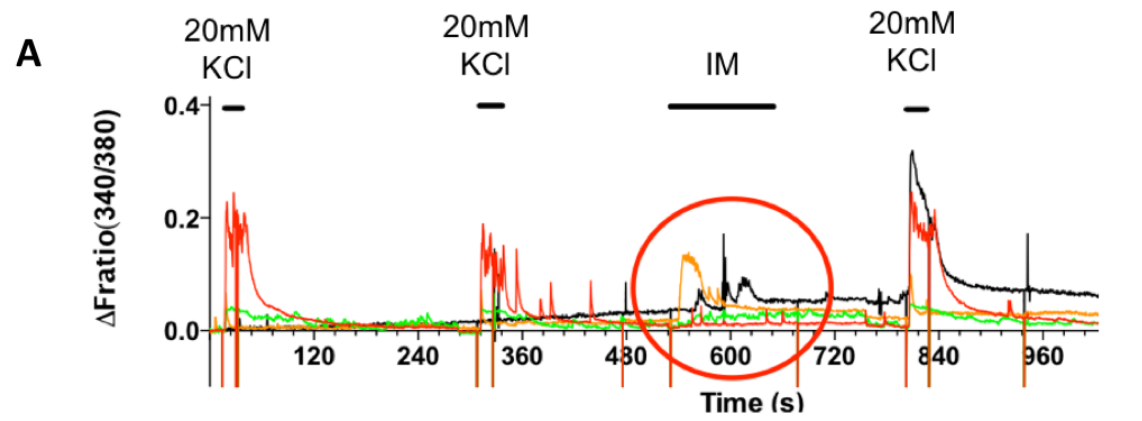


Figure 21. Direct responses of axons to IMs, PGE2 only and BK alone in the MFC system

A) Representative trace highlighting direct activation of a subpopulation of neurons, with axons that are responsive to inflammatory mediators. Traces demonstrate changes in Ca^{2+} concentration at the soma in response to application of the following to the axonal compartment: KCl stimulation (20 second), second KCl stimulation (20 seconds), IM stimulation (2 minutes) and a potentiated response to the second KCl stimulation (20 seconds) with 5-minute washout periods between each stimulation. Red circle highlights a subpopulation of neurons in which application of IM to the axonal compartment results in a Ca^{2+} transient at the soma. **B)** Representative trace showing direct transient responses of axons to $10\mu\text{M}$ bradykinin (1-minute application), as well as potentiation of KCl responses after treatment with bradykinin. **C)** Representative trace showing direct responses of axons to $10\mu\text{M}$ PGE2 (2-minute application), displaying short, repetitive bursts of activity in response to treatment with PGE2 only. **D)** Maximum peak of direct responses of DRG axons to IM compared to BK alone ($*p<0.05$, two-tailed, unpaired t -test, $n=9-13$ DRG neurons, $n=3$ mice, 9 MFCs)

In order to be able to tease out the molecular mechanisms underlying sensitising and excitatory effects of different IMs, we aimed to study the effects of individual IMs on peripheral nociceptors. Taking advantage of the MFC model, we studied the effects of bradykinin, PGE2 and histamine on DRG axons and found that axons respond differentially to exogenous application of these mediators. Treatment with $10\mu\text{M}$ bradykinin (1-minute application) displayed both sensitising and excitatory effects, resulting in increased number of responders to KCl, as well as evoking a transient Ca^{2+} signal similar to that seen with KCl (**Figure 21B**). No axonal responses were observed to $10\mu\text{M}$ histamine (data not shown). We found that treatment with $10\mu\text{M}$ PGE2 (2-minute application) resulted in a different type of axonal response. Short and repetitive Ca^{2+} transients were detected at the soma in response to a localized, short treatment of axons with PGE2 (**Figure 21C**). The direct responses to IM were significantly higher compared to direct responses to BK only, suggesting that other

mediators used in this assay other than BK are likely to contribute towards the direct response of axons to IMs (**Figure 21D**).

Interestingly, the different types of responses observed at the cell bodies in response to treatment of axons with BK and PGE2 point towards important mechanistic differences underlying the effects of both mediators. The ability of BK to activate nociceptors, and mechanisms underlying its excitatory action been studied extensively (Banik et al., 2001; Jęftinija, 1994; Kano et al., 1994; Mizumura et al., 2009; Wu and Pan, 2007). Moreover, responses to BK in single-fibre recordings and have been typically associated with significant tachyphylaxis, which may explain why responses to BK were transient in nature and did not result in repetitive activity (Banik et al., 2001). Conversely, ion channels and second messenger pathways involved in PGE2-induced neuronal discharge have not been studied in detail as the excitatory effects of PGE2 remain to be a contentious subject matter as reviewed in Section 4.1.4. Hence, in the next chapter we will aim to further understand the axonal mechanisms underlying PGE2-induced nociceptor activation.

3.4. Summary

In order to develop an *in vitro* assay to study changes in axonal excitability an MFC platform was used allowing isolation of subcellular components of DRG neurons. We show that adult DRG neurons are readily maintained in MFC chambers and their DRG axons are able to traverse through the microgrooves to a fluidically isolated chamber. We also demonstrate how Dil staining can be used to identify neurons with axons that had traversed to the axonal compartment. Responses of axons to depolarization were measured using changes Ca^{2+} concentration at the soma, resulting from APs generated at the axons, as a readout (Tsantoulas et al., 2013). This technique provides a robust and reproducible measure of axonal function due to specific stimulation of the axonal compartment of the neuron. Next, we took advantage of the MFC platform to model inflammation-induced peripheral sensitisation of nerve fibres *in vitro* and showed that nociceptive axons are sensitised in response to a cocktail of IMs. We observed two types of axonal sensitization using our assay. Fluidically isolated axons treated with IMs exhibited increased responsiveness to stimulation, and a reduction in nociceptor activation threshold. This was observed after both, 2-hour localized axonal treatment with IM as well as with a shorter treatment of 2 minutes. We also found that localized axonal IM-treatment not only results detectable sensitization, but also direct activation of a subpopulation of neurons. Exogenous application of inflammatory mediators resulted in responses with different kinetic properties. In comparison to the well-studied excitatory action of bradykinin on nociceptors, the ability of PGE2 to activate nociceptors directly and the underlying mechanisms remain elusive. Hence, we set out to further investigate molecular players responsible for PGE2-induced persistent activity at the primary afferent level in the next chapter.

4. Investigating axonal mechanisms underlying PGE2-induced spontaneous neuronal activity

4.1. Introduction

4.1.1. The role of Prostaglandin E2 in health and pathology

Prostaglandin E2 is responsible for homeostatic responses in several human systems including reproductive, gastrointestinal, and neuroendocrine (Dey et al., 2006; Duffy and Stouffer, 2001). However, the production of PGE2 increases dramatically during an inflammatory response and thus, is thought to mediate pathological features of inflammation (Kawabata, 2011). The accumulation of PGE2 at the site of injury causes PGE2-induced arterial dilatation and increased microvascular permeability resulting in redness and oedema (Omori et al., 2014). PGE2 also plays an important role in febrile responses and their action on key sites of the pain pathways such as peripheral sensory neurons, the spinal cord and the brain leads to the experience of somatic pain (Dinarello et al., 1984; Funk, 2001).

4.1.2. EP receptors and downstream signalling pathways

PGE2 acts via EP receptors (EP1, EP2, EP3 and EP4), which belong to the rhodopsin-like G-protein coupled receptors (GPCRs) family, comprised of seven transmembrane domains and intracellularly coupled to different G proteins. All four isoforms are coupled to distinct signal transduction pathways. EP2 and EP4, for instance, are coupled to Gs protein through which they activate adenylate cyclase (AC), leading to an increase in intracellular cAMP levels (Fujino et al., 2005; Regan, 2003). EP2 and EP4 have similar functions however, EP4 has higher affinity for PGE2 whereas, EP2 has a lower affinity for PGE2. EP1 is coupled to Gq protein,

triggering formation of inositol phosphate with the mobilization of intracellular free calcium, whereas EP3 couples with Gi/o resulting in a reduction in intracellular cAMP levels (Ji et al., 2010; Sugimoto et al., 1992). EP1-4 have all been found to be expressed on sensory neurons (Narumiya, 2009). The importance of EP4 receptors in pain has been highlighted by studies showing how a number of EP4 antagonists show analgesic efficacy in inflammation-induced behavioral hyperalgesia in both, the CFA and carrageenan rodent models (Murase et al., 2008). Evidence has also suggested an important role for PGE2/EP4 molecular pathway in animal models of rheumatoid arthritis and osteoarthritis (Clark et al., 2008; McCoy et al., 2002).

4.1.3. Peripheral sensitisation by PGE2

The sensitising effects of PGE2 to chemical, heat and mechanical stimuli has been supported by several groups. PGE2 was recognized as a potent sensitising agent since the 1990's, where pain responses elicited in healthy human subjects after injection of bradykinin into an isolated hand vein segment, were potentiated after pre-treatment with PGE2 (Kindgen-Milles, 1995). PGE2 has been shown to increase discharge activity of C-fibres to capsaicin and adenosine in anaesthetized rats, as well as enhance 5HT-induced responses in the rat skin-nerve preparation (Ho et al., 2000; Lang et al., 1990). PGE2 has been suggested to sensitise nerve endings to other pain-inducing molecules such as ATP and TRPA1 (Bang et al., 2007; Wang et al., 2007; Zhang et al., 2008).

PGE2 has been shown to potentiate heat-induced neuronal discharge at the peripheral terminals in isolated rat-skin nerve preparations and canine testicular nociceptors, *in vitro* (Derow et al., 2007; Kumazawa et al., 1996). Similarly, stable analogues of cAMP enhanced heat responses in rat cutaneous nociceptors (Kress et

al., 1996). Consistent with these reports, *in vivo* studies carried out in rats and rhesus monkeys have demonstrated the ability of PGE₂ to induce heat hyperalgesia after interplantar and subcutaneous tail injection of PGE₂, respectively (Negus et al., 2004; Schuligoi et al., 1994). Further, an intradermal injection of PGE₂ was reported to reduce heat thresholds in humans (Rukwied et al., 2007).

There is a vast amount of studies suggesting potential molecular players responsible for PGE₂-induced heat sensitisation. A study used an isolated sciatic nerve axon model to understand mechanisms underlying heat-induced sensitisation, and showed that PGE₂ sensitised heat responses in peripheral nerve axons (Fischer and Reeh, 2007). The same study demonstrated that effect was mimicked by application of forskolin, which raises intracellular cAMP levels, and was inhibited by application of H89, a PKA inhibitor. Fischer and Reeh also revealed application of a combination of PGE₂ and BK did not sensitise heat responses of axons from TRPV1 KO mice. Furthermore, studies have reported that PGE₂-induced heat hyperalgesia, which was measured using paw withdrawal latency as a readout, was reduced in EP1 KO, TRPV1 KO mice, and Nav1.9 KO mice suggesting their involvement in thermal sensitisation caused by PGE₂ (Amaya et al., 2006; Johansson et al., 2011; Moriyama et al., 2005). *In vitro* studies have also suggested the involvement of Nav1.8 channels by using Nav1.8 selective blockers that inhibited the sensitising effects of PGE₂ (Payne et al., 2015a). However, in Nav1.8 KO mice PGE₂-induced thermal hyperalgesia was unaffected, suggesting that Nav1.8 channels do not play a prominent role in PGE₂-induced heat hyperalgesia (Kerr et al., 2001).

Mechanical sensitising effects of PGE2 *in vitro* have been debated as different studies have reported opposing findings. Using single-fibre recording experiments, studies have reported a decrease in activation threshold of nociceptors to mechanical stimuli and an increase in neuronal spikes, mediated by the cAMP/PKA pathway (Koda and Mizumura, 2002b; Wang et al., 1996). A similar effect was observed when stable analogues of cAMP were applied to the rat skin-nerve preparation (Kress et al., 1996). Conversely, other studies have used the same preparation and reported that both PGE2 and IM did not alter mechanical von Frey thresholds or enhance the number of spikes observed (Lang et al., 1990; Schlegel et al., 2004). However, *in vivo* studies have consistently shown that interplantar injection of PGE2 results in pronounced mechanical hyperalgesia, which is mediated by PKA-cAMP pathway in the first 30 minutes and PKC is required for the long-lasting effects at 90 minutes (Sachs et al., 2009). Others have suggested the role of Nav1.9 and chemokine receptor 2 (CXCR2) in PGE2-induced mechanical hyperalgesia *in vivo* (Amaya et al., 2006; Manjavachi et al., 2010). More recent studies have shown that interplantar injection of PGE2 induces a significant mechanical allodynia, which can be reduced by cAMP, PKA, PKC, EP1 and EP4 inhibitors as well as IL-6 neutralising antibodies pointing towards important molecular players underlying PGE2-induced mechanical hyperalgesia (St-Jacques and Ma, 2014).

Although there is ample evidence for role of PGE2 as a potent sensitiser of nociceptors, its ability to activate nociceptors or induce pain has been controversial. Undoubtedly, COX blockers provide analgesia and prostaglandin inhibitors have been shown to reverse capsaicin-induced pain responses (Schmelz and Kress,

1996; Steen et al., 2000; Steen et al., 1995). However, application of PGE₂ via intradermal microdialysis membranes in humans produced only weak itch and pain at high concentrations (Neisius et al., 2002; Schmelz et al., 2003). Thus, there is an inconsistency in the effects of augmenting PGE₂ and reducing PGE₂ levels in humans. The evidence regarding the ability of PGE₂ to cause pain and excite nociceptors directly will be discussed further in the following section.

4.1.4. PGE₂-induced nociceptor activation

Over the years there has been conflicting data regarding the excitatory action of exogenously applied PGE₂ on DRG somata *in vitro* as well as in nerve-fibre preparations. At the primary afferent level, older studies have reported that PGE₂ fails to evoke spike discharge in visceral afferents and skin-saphenous nerve preparations (Brunsden and Grundy, 1999; Lang et al., 1990; Mizumura et al., 1993; Mizumura et al., 1987). On the other hand, PGE₂ has been reported to induce spike discharge in joint afferents (Schaible and Schmidt, 1988). More recent studies studying the excitatory action of PGE₂ on sensory neurons focus on the cell soma, 10 μ M PGE₂ was shown to evoke Ca²⁺ influx in dissociated rat DRG neurons (Linhart et al., 2003). Whereas others have reported that exogenous application of the same concentration of PGE₂ did not elicit an increase in intracellular Ca²⁺ levels in DRG cell bodies (Nicolson et al., 2007). Thus, further research is required to address whether or not PGE₂ exerts an excitatory effect directly on nociceptors, and the ion channels and signal transduction pathways underlying this phenomenon remain to be elucidated.

4.2. Aims

Using conventional *in vitro* preparations have enabled the mechanistic understanding of PGE₂-induced sensitising effects at level of the DRG cell body, however to our knowledge, the ion channels and signal transduction mechanisms underlying PGE₂-induced activation of primary afferent neurons remains limited. Given the prominent role of PGE₂ as an inflammatory mediator, which is abundantly produced in inflamed tissue, we investigated the changes in axonal excitability in response to PGE₂. Taking advantage of the MFC platform to study the function of sensory axons, the aim was to further characterise the modulation of axonal responses to chemical stimuli such as PGE₂.

4.3. Results

We first assessed the sensitising effects of PGE2 in our assay. Using the 2-channel chamber, we tested whether PGE2 on its own is sufficient to sensitise nociceptive axons and result in enhanced responses as well as increased number of responders to KCl stimulation, similar to that observed with treatment of a combination of IMs. We examined the magnitude of responses as well as the number of responders observed at the cell body to depolarisation of axons with KCl after treatment with PGE2 only, compared to before treatment. The magnitude of Ca^{2+} signals were increased after two-minute treatment with $10\mu\text{M}$ PGE2, compared to KCl responses before PGE2 treatment, displaying a 2.7 -fold increase, from 0.88 ± 0.03 to 2.38 ± 0.12 (**Figure 22A-B**). Percentage of Dil-stained neuronal cell bodies that responded to axonal stimulation were also quantified. Although not significant, there was an upward trend in the number of responders after localized axonal treatment with PGE2 in comparison to vehicle treated DRGs (**Figure 22C**). Thus, PGE2 on its own is able to cause a significant sensitisation of DRG axons, which is comparable to that observed with the combination of IMs.

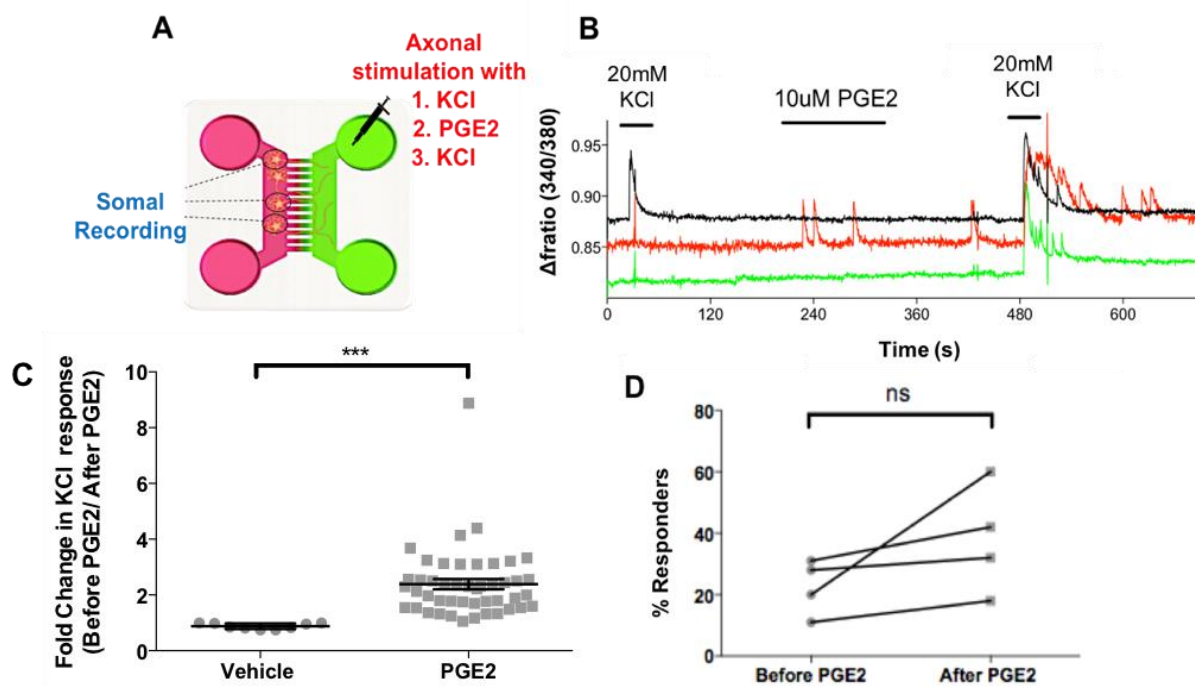


Figure 22. Axonal sensitisation to KCl responses by acute, localised PGE2 treatment.

A) Schematic of experimental set up. **B)** Representative trace showing potentiation of somal response to axonal stimulation with 20mM KCl, after 2-minute treatment with 10μM PGE2. **C)** Data expressed as the mean $\pm$ S.E.M; PGE2- treated neurons showed a significantly increased response to KCl stimulation after treatment compared to vehicle treated neurons, *** $p < 0.0001$ (Mann-Whitney U test, $n = 15-30$ DRG, $n = 4$ mice). **D)** Percentage of Dil- positive neurons responding to KCl showing an upward trend after PGE2 treatment compared to neurons with vehicle-treated axons, where $p = 0.154$ (Chi-square test, $n = 15-30$ DRG, $n = 4$ mice).

Next, we tested whether localised application of PGE2 can excite nociceptors directly by resulting in the generation of APs that are propagated to the DRG soma. We tested this hypothesis using the 3-channel MFC device, which would enable us to assess whether the propagation of axonal signals to the DRG cell body can be blocked by perfusion of pharmacological agents to the middle axonal chamber.

We first tested if application of PGE2 in the distal axonal compartment resulted in persistent Ca^{2+} transients detected at the soma (**Figure 23A**). We observed that 2-

minute application of PGE2 resulted in axonal activity that persisted after wash-out of PGE2 application (**Figure 23B and D**). We then tested whether the application of lidocaine to the middle axonal chamber blocked the propagation of signals generated by PGE2 application. To answer this question, PGE2 was applied to the distal axonal compartment (**Figure 23C**, green), after which lidocaine was applied to the middle axonal compartment (**Figure 23C**, white) which resulted in a complete block of PGE2-induced axonal signals arriving at the cell bodies (**Figure 23B-C**). Throughout the experiment, PGE2-induced axonal activity was monitored by recording from the cell soma and PGE2-induced ongoing activity was quantified as the number of transients observed per 3 minutes after application of PGE2. Application of 10 μ M PGE2 to the distal axons resulted in significantly increased number of transients/3 minutes compared to baseline (**Figure 23D**). Transients were considered a positive response when the maximum peak in ΔF_{ratio} was greater than 3 times the standard deviation above the baseline, which was calculated from at least 10 seconds before application of stimulus. No Ca²⁺ transients were observed with the application of DMSO vehicle (data not shown) whereas, PGE2 induced Ca²⁺ transients at an average rate of 6.12 ± 0.63 , per 3-minute after PGE2 application. Treatment with 5mM lidocaine completely abolished transients arriving at the cell soma. This experiment demonstrates that PGE2 on its own can excite DRG axons directly and that PGE2-induced Ca²⁺ transients at the cell body are arriving from the DRG axons.

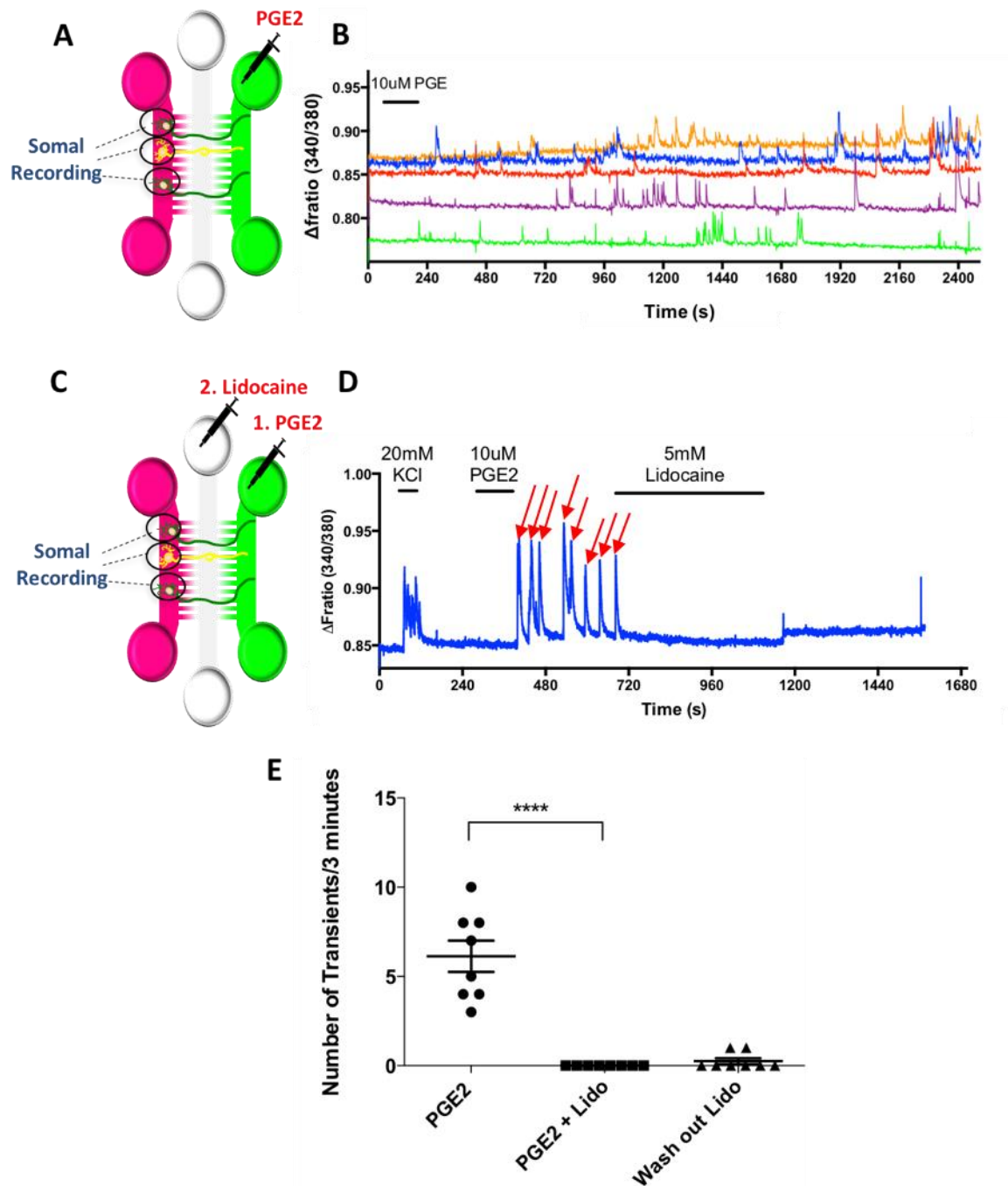


Figure 23. PGE2-induced ongoing neuronal activity is abolished by lidocaine

A) Schematic of experimental set up used for data shown in **B**, 3-channel MFC is depicted with the somal compartment in pink, and middle axonal compartment in grey and distal axonal compartment in green. PGE2 was applied to the distal axonal compartment. **B)** Representative trace showing Ca^{2+} transients recorded at the soma in response to distal axons treated with PGE2. Perfusion of $10\mu\text{M}$ PGE2 for 2 minutes results in ongoing axonal activity detected at the soma, which persists after washout of PGE2. **C)** Schematic of experimental set up used for data shown in **D** and **E**, 3-channel MFC was used. KCl and PGE2 were applied to the distal axonal

compartment. After application of PGE2, 5mM lidocaine was applied to the middle axonal compartment. Throughout experiment Ca^{2+} signals were recorded from the cell bodies in the somal compartment. **D)** Representative trace showing response to 20mM KCl axonal stimulation, and ongoing activity induced by PGE2 which is blocked after application of 5mM lidocaine to the middle axonal compartment. Red arrows indicating calcium transients. **E)** Quantification of the number of transients observed after application of stimulus, for a duration of 3 minutes. Data expressed as mean $\pm$ S.E.M, showing a significant increase in the number of transients after application of PGE2 compared to baseline ($***p<0.006$), and a significant decrease in number of transients after application of lidocaine ($****p<0.0001$). (One-way ANOVA with Tukey's multiple comparisons test was used, $n=10$ DRG, $n=4$ mice).

Next, we aimed to establish whether the application of PGE2 to the distal axons results in direct depolarisation of the axonal membrane. In order to answer this question, we sought to set up a simple method that enables direct measurement of the axonal transmembrane potential, which would give us more accurate information of the timing and location of subthreshold events taking place. For this purpose, the Fluovolt voltage sensor was used, which is a fast voltage-responding fluorescent dye that takes advantage of photo-induced electron transfer (PET) technology to detect changes in membrane potential (Miller et al., 2012). We first tested whether we could observe detectable changes in fluorescence signal (expressed as $\Delta F/F$ ratio) along DRG axons after stimulation with KCl (**Figure 24A**). We found that application of 20mM KCl to the axonal compartment resulted in depolarisation of the axonal membrane, which was maintained until washout of KCl (**Figure 24C-E**). This experiment served as a good positive control, demonstrating that using fluovolt dye we are able to detect changes in axonal membrane potential in DRG neurons.

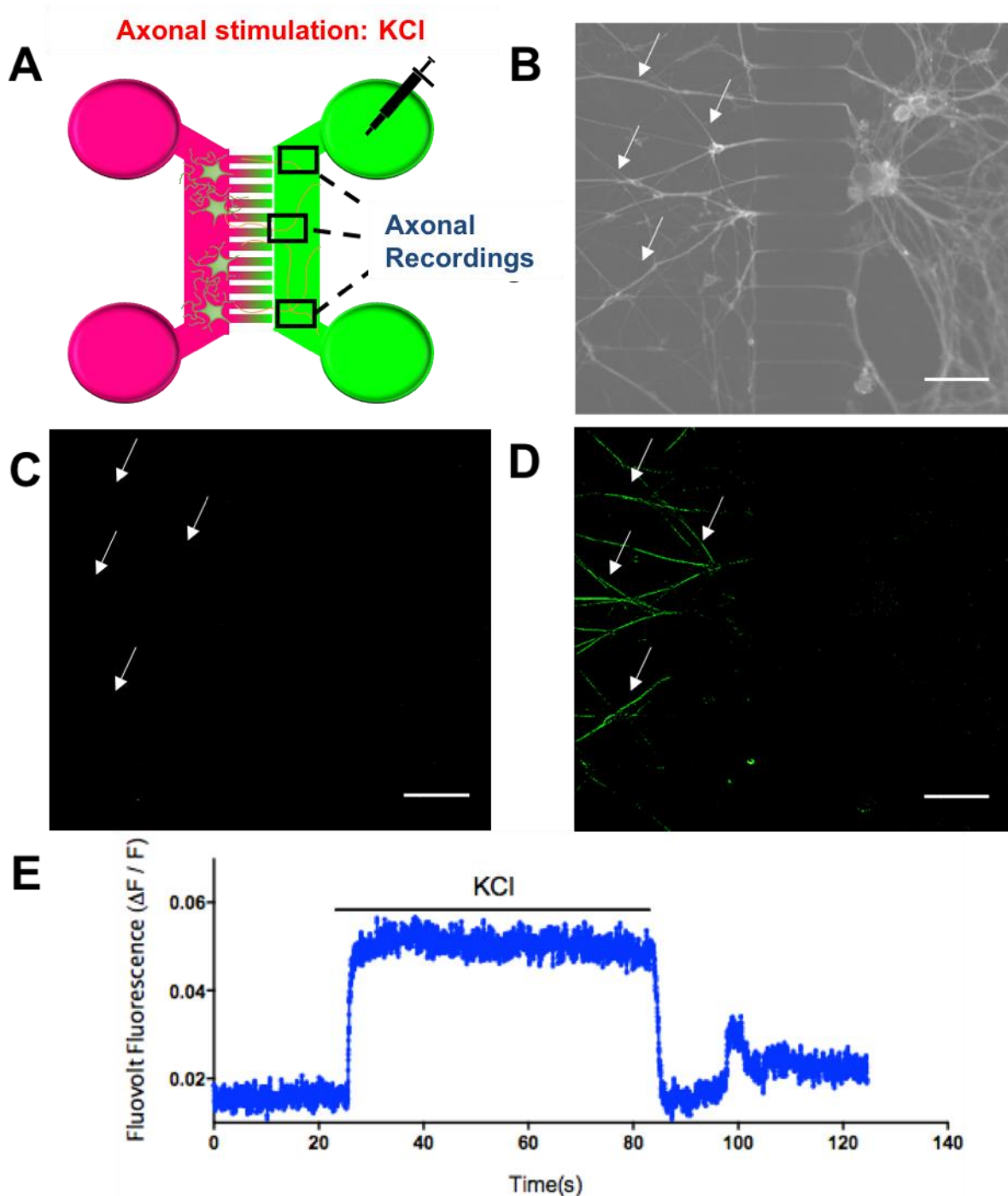


Figure 24. Measuring changes in axonal membrane potential to KCl using Fluovolt dye.

A) Schematic of experimental set up used for data shown in B-E, two-chamber compartment model MFC was used, with cell body compartment in pink, and axonal compartment in green. 20mM KCl was applied for 1-minute duration to the axonal compartment, and changes in fluorescence were recorded from the axonal compartment. **B)** Bright field image showing chosen field of view (FoV) in the axonal compartment of the MFC. **C)** Background subtracted $\Delta F/F$ image of the same FoV as B, before application of KCl. **D)** $\Delta F/F$ image showing same FoV after KCl application to the axonal compartment. White arrows highlighting examples of axons where

changes in fluorescence intensity increased in response to stimulation with KCl. E) Representative trace illustrating changes in fluorescence expressed as $\Delta F/F$, of one section of an axon in response to KCl application. Change in fluorescence returns to baseline after wash-out of KCl stimulation. Scale bars, 100 μ m.

We next tested whether application of PGE₂, using the same concentration and duration which induced generation of ongoing activity in DRG axons, could result in depolarisation of the axonal membrane. 10 μ M PGE₂ was perfused to the axonal compartment for 2 minutes, and changes in fluorescence signal were recorded from selected ROIs encompassing the axons (**Figure 25A**). We found that PGE₂ resulted in a significant change in fluorescence signal as shown in the $\Delta F/F$ image (**Figure 25D-F**), indicating that direct depolarization of axonal membrane occurs in response to PGE₂ application. In contrast to axonal depolarisation observed with KCl, where the fluorescent signal intensity returned to baseline after washout of KCl, the fluovolt fluorescence intensity in axon segments responding to PGE₂ did not return to baseline after washout of PGE₂ indicating a persistent depolarisation of axonal segments following removal of PGE₂ stimulus. Thus, the sustained depolarisation induced by 10 μ M PGE₂ is the likely mechanism for the generation of persistent axonal activity. Of note, calibration experiments of the fluovolt dye response in cochlear epithelial cells as well as HEK cells have reported an average voltage sensitivity of 22-25% increase in fluorescence per 100mV depolarisation (Ceriani and Mammano, 2013; Miller et al., 2012). However, for an accurate measure of the change in axonal membrane potential, a calibration study of the dye response in DRG axons in our experimental settings (i.e. light source, camera, objective) would be required.

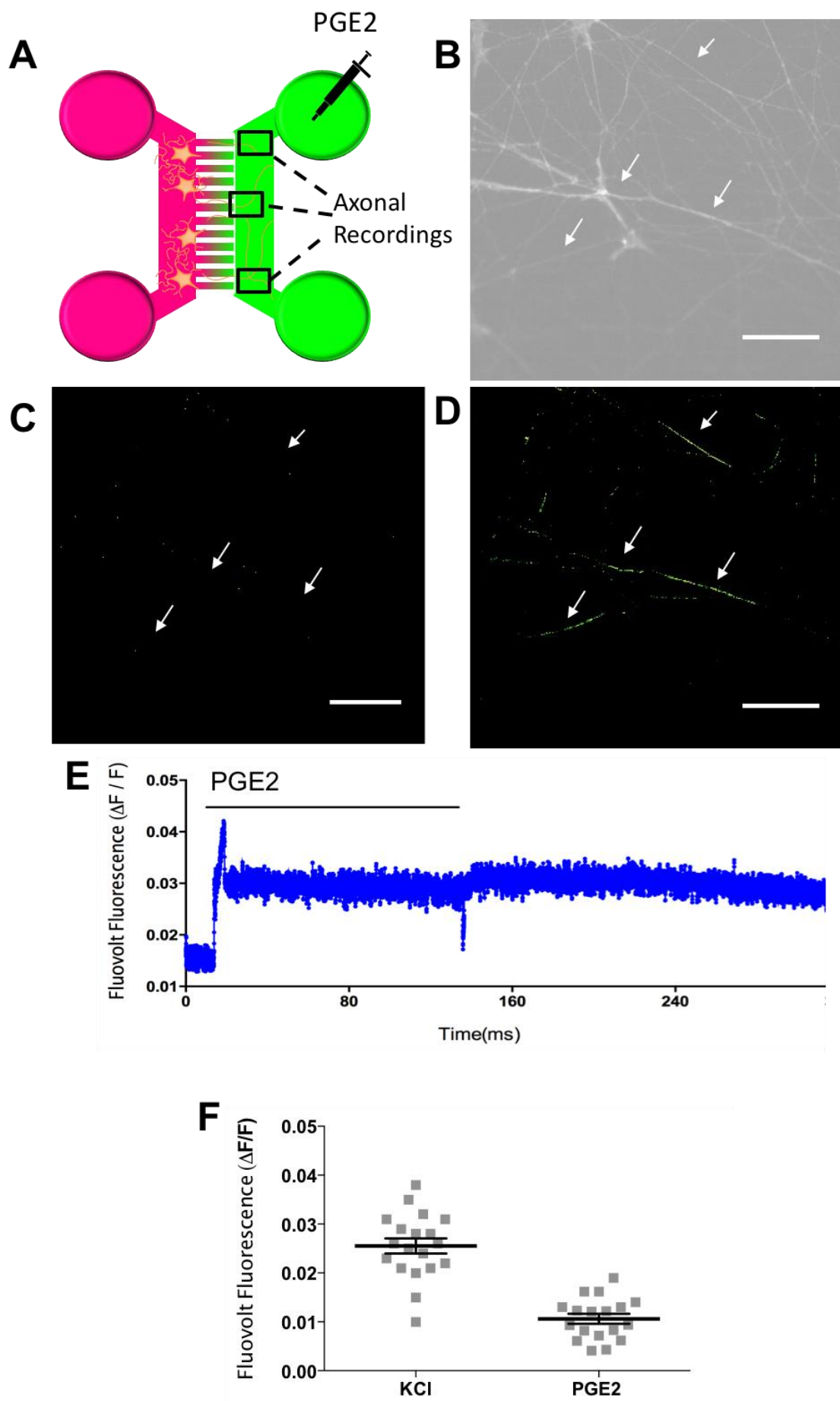


Figure 25. PGE2 induces a sustained depolarisation of the axonal membrane.

A) Schematic of experimental set up used for data shown in B-E, 2-channel MFC was used, with cell body compartment in pink, and axonal compartment in green. 10 μ M PGE2 was applied for 2-minute duration to the axonal compartment, and changes in fluorescence were recorded from the axonal compartment. **B)** Bright field image showing chosen FoV in the axonal compartment of the MFC. **C)** $\Delta F/F$ image of the same FoV as shown in B, before application of PGE2. **D)** $\Delta F/F$ image showing FoV after PGE2 application. White arrows highlighting axonal areas responding to stimulation with PGE2. **E)** Representative trace illustrating changes in Fluovolt Fluorescence ($\Delta F/F$), of an axon segment in response to PGE2 application. Change in fluorescence is maintained after wash-out of PGE2. **F)** Quantification of maximum response of fluovolt fluorescence signal ($\Delta F/F$) in response to 20mM KCl and 10 μ M PGE2 (n=19-20 DRG, 8 MFCs, 3 mice). Scale bars, 100 μ m.

Binding of PGE2 to EP2 or EP4, which are both coupled to Gs protein, results in an increase in intracellular cAMP levels (Fujino et al., 2005; Regan, 2003). We found that PGE2-induced depolarisation of the axonal membrane lasts beyond wash-out of PGE2 application. Moreover, the maintained depolarization observed could explain ongoing persistent activity observed in the DRG soma in response to treatment of axons with PGE2 (**Figure 26**). We hypothesized that second messenger pathways may be involved in the long-lasting effects of PGE2. Therefore, we assessed whether the cAMP/PKA pathway is an important player responsible for PGE2-induced ongoing activity. The cAMP analogue, Rp-cAMPS was chosen to test this hypothesis as its mechanism is well understood and has been routinely used to establish the role of the cAMP/PKA pathway *in vitro* and *in vivo* (Aley and Levine, 1999; Song et al., 2006). In addition, it has a better selectivity profile in comparison to another routinely used PKA inhibitor, H89, which has been shown to act on 8 other kinases displaying PKA-independent effects (Lochner and Moolman, 2006). Moreover, a study demonstrated tested increasing concentrations (ranging from 5 to

500 μ M) Rp-cAMPS, and showed a dose-dependent reduction in discharge rate of spontaneous activity in teased dorsal root filaments from chronically compressed DRG neurons (Hu et al., 2001). In our assay, we found that the localised axonal ongoing activity is reversibly blocked by 20 μ M of Rp-cAMPS, a cAMP analogue and inhibitor of PKA activation (**Figure 26A**). The number of transients/3 minutes recorded at the soma after application of PGE2 to the axonal compartment were significantly reduced by Rp-cAMPS. Interestingly, the axonal ongoing activity returned after washout of Rp-cAMPS in a subpopulation of neurons (**Figure 26B-C**). Although the 3-channel compartment MFC was set up for experiments involving pharmacological intervention, the following two studies were carried out in the 2-channel compartment, which is less technically challenging and enables more high throughput data, due to time constraints whilst carrying out these set of experiments.

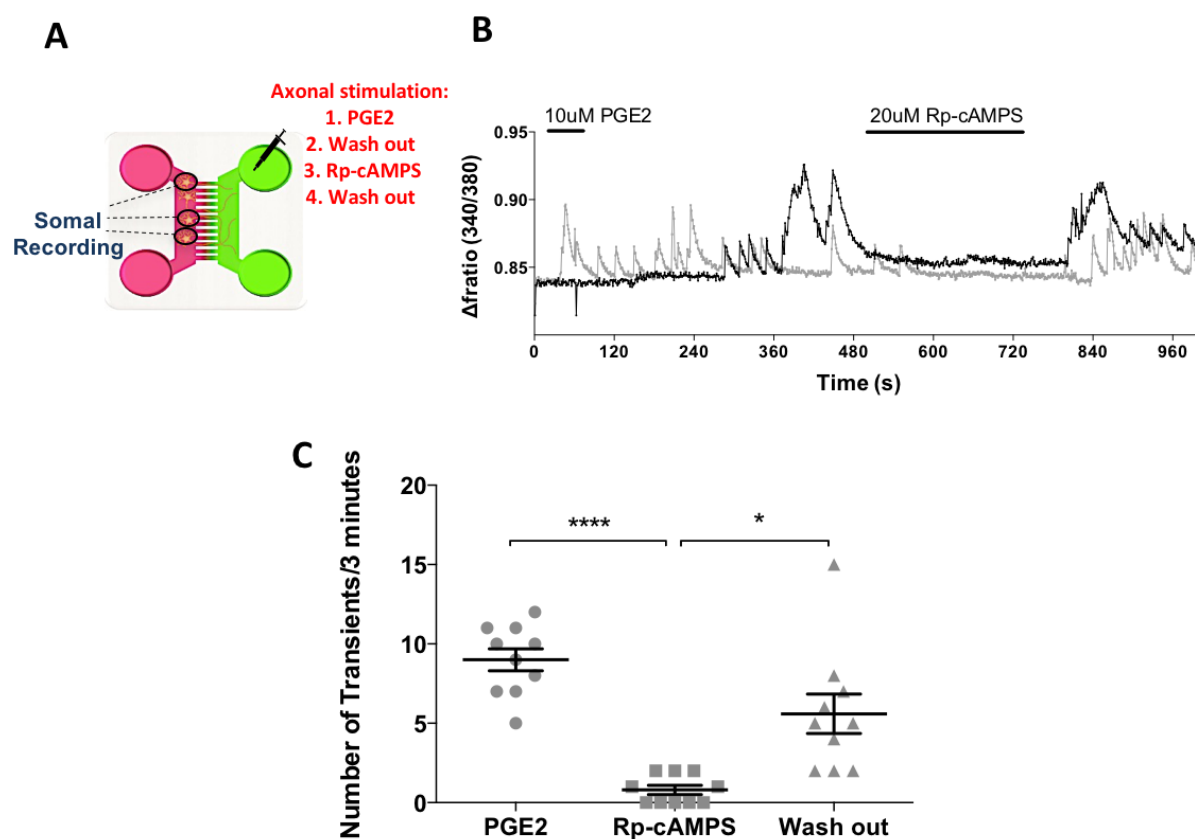


Figure 26. PGE2-induced ongoing activity is dependent on the cAMP/PKA pathway at the axonal level.

A) Schematic of experimental protocol: After overnight Dil staining of axons, cell bodies were labelled with Fura-2 and only Dil-stained cell bodies were selected for calcium imaging analysis. Axonal chamber was stimulated with 10 μ M PGE2, followed by a 5-minute wash-out, after which the Rp-cAMPS was applied to the axonal chamber to test its effect on the number of transients induced by PGE2 for 3 minutes, which was subsequently washed out. **B)** Representative trace showing ongoing activity recorded at the soma in response to 1-minute second axonal stimulation with PGE2, which is reversibly blocked with the application of 20 μ M Rp-cAMPS (cAMP-induced PKA activation inhibitor) for 3 minutes. **C)** Quantification of the Ca²⁺ transients observed after application of stimulus. Data expressed as mean $\pm$ S.E.M, showing a significant decrease in the number of PGE2-induced transients after application of Rp-cAMPS (**** $p < 0.0001$). The activity partially recovered following washout of Rp-cAMPS, where * $p < 0.05$ (One-way ANOVA with Tukey's multiple comparisons test was used, $n = 11$ DRG, $n = 3$ mice).

To identify what ion channels are responsible for PGE2-induced ongoing axonal activity, we investigated whether VSGC had a role to play in PGE2-induced ongoing activity. We found that ongoing axonal activity was unaffected by application of 500nM TTX, applied for 3 minutes (**Figure 27A**). This concentration of TTX was chosen as it has been used to both block TTX-S currents, as well as test the specificity of Nav1.8 blockers on TTX-R currents present in DRG neurons after exposure to 500nM TTX (Tan et al., 2014). There was no significant difference in the number of transients after application of the TTX, suggesting that TTX-S channels are not directly involved in PGE2-evoked ongoing activity (**Figure 27B**). Lidocaine is a non-selective VSGC blocker, thus blocks both TTX-S and TTX-R currents. The fact that lidocaine completely blocks PGE2-induced calcium transients (**Figure 23D**) but TTX did not have a significant effect (**Figure 27**) suggests that TTX-R channels may play a more important role.

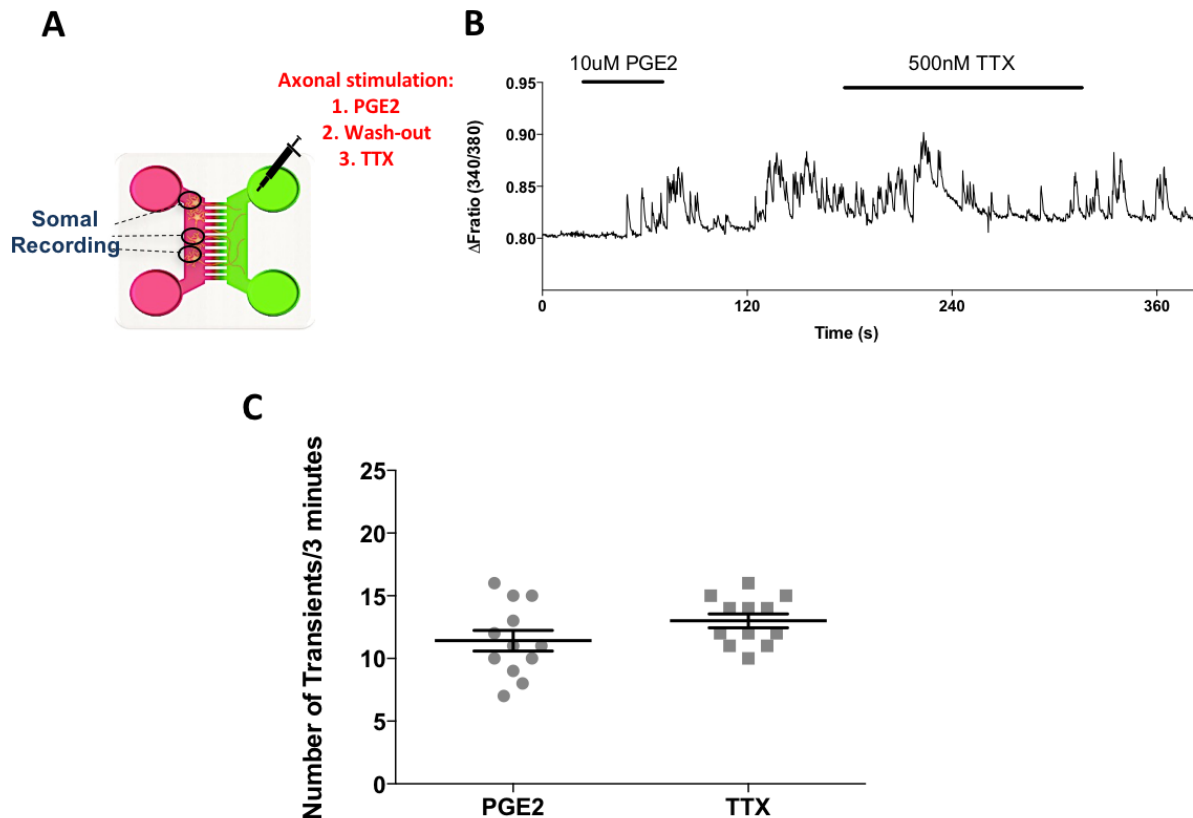


Figure 27. PGE2-induced ongoing activity is not dependent on TTX-S VGSCs at the axonal level.

A) Schematic of experimental set up: After overnight Dil staining of axons, cell bodies were labelled with Fura-2 and only Dil-stained cell bodies were selected for calcium imaging analysis. Axonal chamber was stimulated with 10uM PGE2, followed by a 5-minute wash-out, after which the TTX was applied to the axonal chamber. **B)** Representative trace showing ongoing activity recorded at the soma in response 1-minute axonal stimulation with PGE2, which is maintained despite treatment with 500nM TTX for 3 minutes. **C)** Quantification of the number of transients/3 minutes observed after application of stimulus. Data expressed as mean $\pm$ S.E.M, showing no significant change in the number of PGE2-induced transients after application of 500nM TTX, where $p=0.1261$. (One-way ANOVA with Tukey's multiple comparisons test, $n=13$ DRG, $n=3$ mice)

To test whether specific TTX-R channels are responsible for PGE2-induced ongoing activity we used more specific blockers. A number of publications have linked Nav1.8 channels with the effects of PGE2 at the DRG (Liu et al., 2010). A novel Nav1.8 selective channel blocker, PF- 01247324, was shown to reduce the potentiation of

neuronal excitability caused by PGE2 at the cell body at 1 μ M (Payne et al., 2015b). The same study showed that at 3 μ M PF- 01247324 begins to have non-specific effects as it acts on other Navs including Nav1.4, Nav1.6, Nav1.5; thus the concentration of 2 μ M was chosen to test whether Nav1.8 channels play a role in PGE2-induced activity in DRG axons. It was found that the localised ongoing axonal activity is significantly blocked by 2 μ M of PF- 01247324, a selective Nav1.8 blocker (Figure 28A-B). The number of transients/3 minutes were significantly reduced after application of PF- 01247324 (Figure 28C).

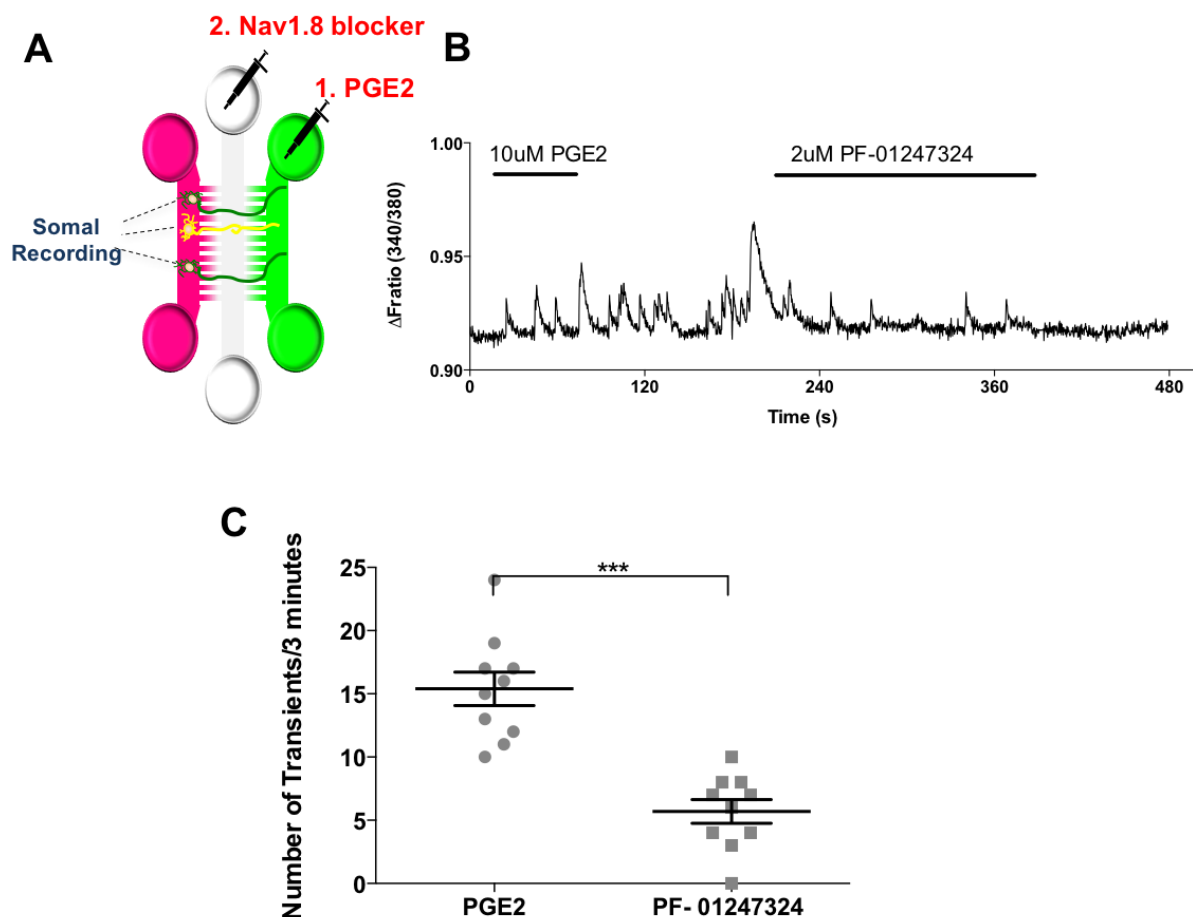


Figure 28. PGE2-induced ongoing activity is mediated by axonal Nav1.8 channels.

A) Schematic of experimental set up: After overnight Dil staining of axons, cell bodies were labelled with Fura-2 and only Dil-stained cell bodies in the somal compartment were selected for calcium imaging analysis. Distal axonal chamber was

*stimulated with 10 μ M PGE2, followed by a 5-minute wash-out, after which Nav1.8 selective blocker was applied to the middle axonal chamber. **B)** Representative trace showing repetitive ongoing activity recorded at the soma in response to 1-minute axonal stimulation with PGE2, which is partially blocked with the application of 2 μ M PF-01247324. **C)** Quantification of the number of transients observed after application of stimulus. Data expressed as mean $\pm$ S.E.M, showing a significant decrease in the number of PGE2-induced transients after application of PF-01247324, where **** $p < 0.0001$ (Paired, two tailed, Student's *t*-test was used, $n=10$ DRG, $n=3$ mice).*

Given the involvement of Nav1.8 channels in PGE2-induced ongoing activity observed in our hands, and the importance of EP4 receptors which has been suggested by several groups as reviewed in Section **4.1.2**, we carried out immunocytochemistry to investigate the expression and distribution of EP4 and Nav1.8 along the axonal membrane. In a set of preliminary experiments, we demonstrate the expression of EP4 receptors along the axons demarcated with β -3-tubulin providing evidence for the expression of EP4 receptors along the axons of adult mouse DRG neurons (**Figure 29A-D**). We also studied the co-expression patterns of EP4 receptors, and Nav1.8 channel and found that both EP4 receptors and Nav1.8 channels are co-expressed along adult DRG axons (**Figure 30A-B**). EP4 staining was evident in a subset of Nav1.8 positive axons (**Figure 30C-E**).

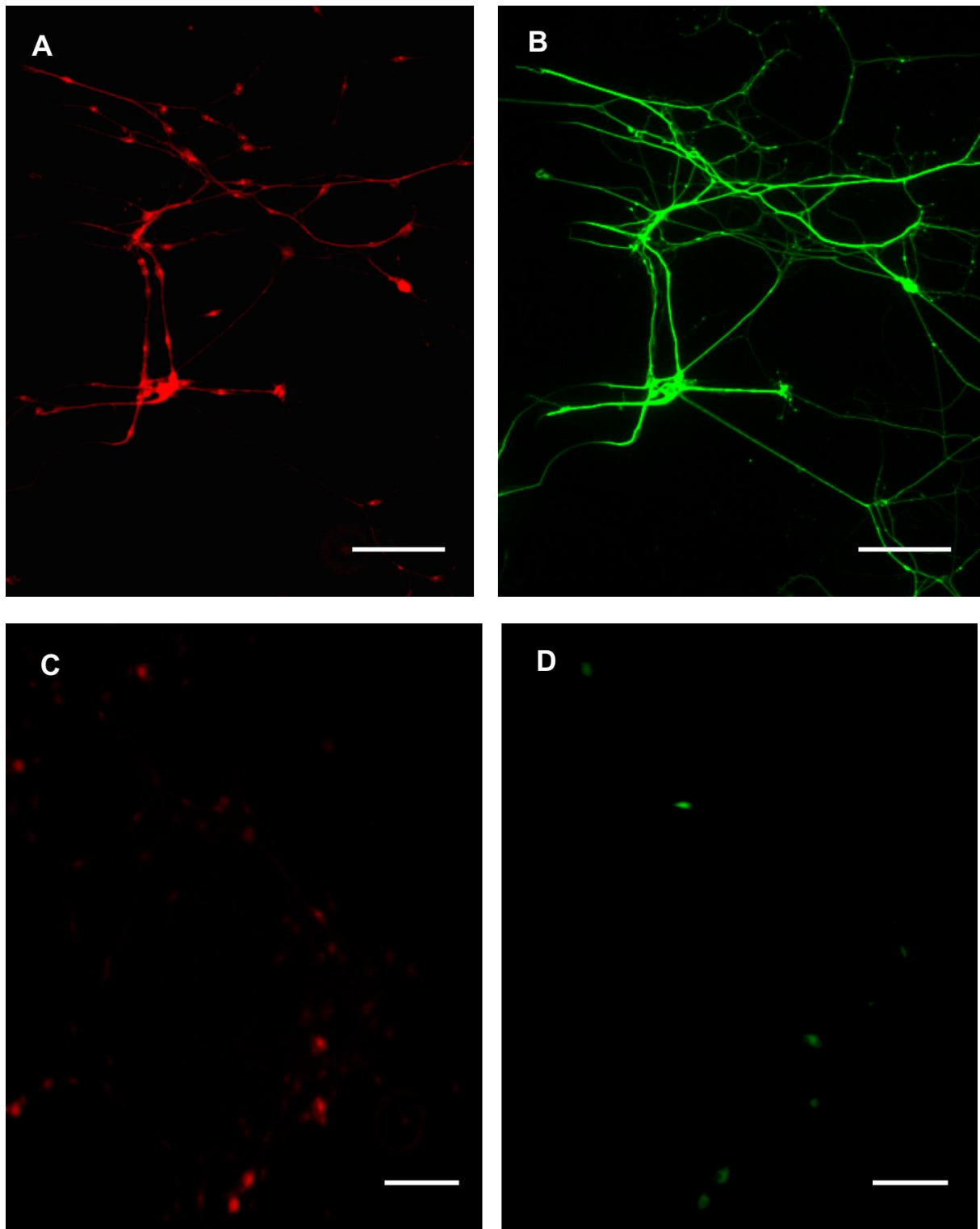


Figure 29. EP4 receptor expression along isolated adult DRG axons.

A) Preliminary data: representative image of axonal compartment showing expression and distribution pattern of EP4 receptor along DRG axons. **B)** Representative image of axonal compartment showing counter staining with β -3-tubulin along DRG axons. **C and D)** Secondary alone controls. Scale bars, 100 μ m

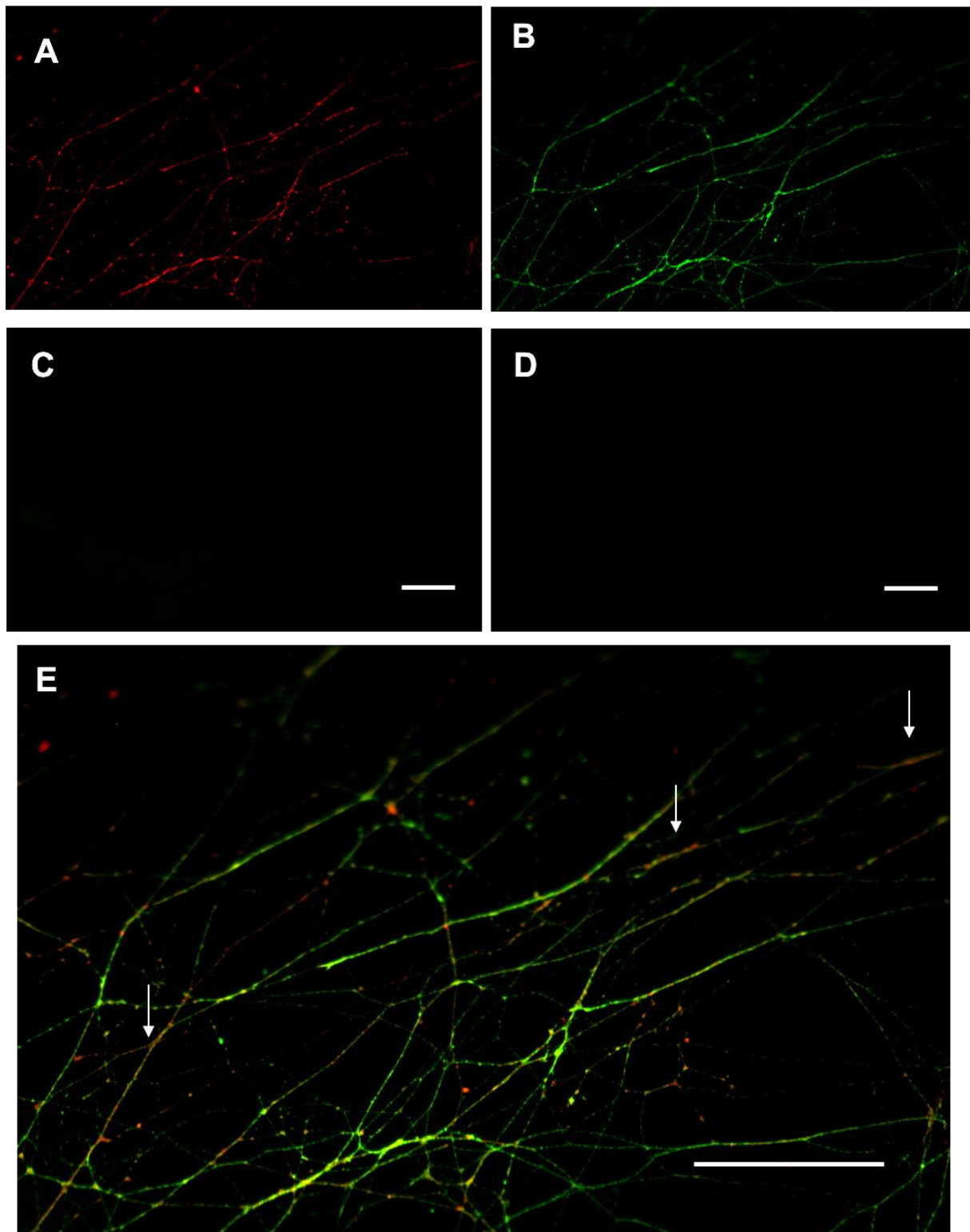


Figure 30. Co-localisation of EP4 receptors and Nav1.8 channels along the axonal membrane.

Preliminary data: Representative images of adult DRG axons taken at DIV 5. A) Expression of EP4 in DRG axons in the MFC axonal compartment B) Nav1.8 staining

in same FoV as B. **C)** DRG axons in distal axonal compartment showing image of control MFC treated with secondary antibody alone for EP4 stain. **D)** DRG axons in peripheral terminal compartment showing image of control MFC treated with secondary antibody alone for Nav1.8 stain. **E)** An overlay of EP4 (red) and Nav1.8(green) staining from **A** and **B**, respectively showing co-localisation of EP4 receptors and Nav1.8 channels. Scale bars, 100 μ m.

4.4. Summary

In this chapter, we take advantage of the MFC-based *in vitro* system to provide evidence to show that PGE2 can not only sensitise nerve endings but also activate nociceptors directly. Using novel voltage imaging techniques, we show that local application of PGE2 depolarized the axonal membrane. Using calcium imaging, we demonstrate that application of PGE2 to the distal axons resulted in repetitive Ca^{2+} transients at the soma, which persisted for 30 minutes after wash-out of PGE2. Moreover, the perfusion of lidocaine to the middle axonal compartment blocked the propagation of PGE2-induced axonal signals from the distal axons to the DRG cell bodies. Mechanistically, we show that PGE2-evoked persistent axonal activity is dependent on the cAMP/PKA pathway. We also provide evidence suggesting that this persistent activity generated in the distal axons is not mediated by TTX-S channels, but can be attenuated through inhibition of $\text{Na}_v1.8$ channels.

Taken together, we provide evidence suggesting that PGE2 has a long-lasting excitatory effect on nociceptive axons, as the application of PGE2 induces a sustained depolarisation of the axonal membrane resulting in the generation and propagation of persistent axonal activity arriving at the DRG cell bodies. Although PGE2-evoked depolarization of nociceptive axons could potentially present a novel mechanism for ongoing pain associated with inflammatory disorders, further work is required to understand how this depolarisation comes about. We also show that PGE2-induced ongoing activity is dependent on local second messenger cAMP/PKA pathway and is mediated, at least in part, by axonal $\text{Na}_v1.8$ channels. This mechanism potentially contributes to our knowledge of peripheral mechanisms underlying ongoing pain during inflammatory conditions.

5. Investigating the role of axonal HCN channels in inflammatory pain

5.1. Introduction

5.1.1. HCN channels

As discussed in Chapter 1, HCN channels are activated by both hyperpolarization of the membrane potential ($<-60\text{mV}$), and the presence of intracellular cAMP (DiFrancesco, 1999). An interesting characteristic of these channels is their response to increased intracellular levels of cAMP. Binding of cAMP binds to the cyclic-nucleotide binding domain, results in a positive shift in its activation threshold, towards more depolarised membrane potentials (Emery et al., 2012), which causes a greater inward current and also reduces the latency of the next action potential, thereby increasing the frequency of action potential firing (**Figure 31**). This mechanism allows HCN channels to play an important role in regulating spontaneous neuronal activity and frequency control of cellular excitability (Emery et al., 2011a).

5.1.2. HCN Isoforms

The main characteristic used to differentiate between all four HCN isoforms is their activation kinetic profiles; HCN1 being the fastest activating channel, HCN4 displaying the slowest activation kinetics and HCN2 and HCN3 show an intermediate speed of activation. The HCN isoforms also differ in terms of their modulation by intracellular cAMP, HCN2 and HCN4 are the most sensitive to changes in cAMP whereas HCN1 is only partially sensitive and HCN3 is not modulated by cyclic nucleotides (Wainger et al., 2001). Evidence suggests that HCN channel activation

can be shifted to a more positive potential, through a similar mechanism as with cAMP, by phosphatidylinositol-4,5-bisphosphate (PIP₂), with all isoforms being equally sensitive to modulation by PIP₂ (Pian et al., 2006; Zolles et al., 2006). Furthermore, channel activation kinetics of HCN2 and HCN4, but not HCN1 can be regulated by other modifications such as Src Kinase-induced tyrosine phosphorylation of the tyrosine residue in the C-linker region between transmembrane domain 6 and cyclic-nucleotide binding domain (Li et al., 2009).

There has been conflicting evidence published with regards to differential expression profiles of HCN isoforms. It was originally thought that only HCN4 was primarily found in the heart's sino-atrial node, however recent evidence suggests that HCN1, HCN2 are also expressed; indicating that all three isoforms contribute to I_f current, an inward current activated by hyperpolarisation in cardiac cells, also known as I_h in neurons (Herrmann et al., 2011). In the central nervous system, all four isoforms are differentially expressed in different regions. HCN2 is thought to be ubiquitously expressed, HCN3 is mainly found in the hypothalamus, HCN4 in the thalamus and HCN1 in the neocortex, hippocampus and cerebellum (Notomi and Shigemoto, 2004). In the PNS, HCN1, HCN2 and HCN4 are mainly expressed in small diameter neurons, whereas HCN1 is not thought to be functionally active in small neurons as its deletion had no effect aspects of pain in mouse models (Momin et al. 2008). The expression of HCN3 in small diameter neurons has been reported to be low, which has been supported by both electrophysiological and knock out studies (Emery et al., 2011a; Gao et al., 2012; Momin et al., 2008a). The HCN2 channel is thought to be responsible for the I_h current in small diameter neurons, as genetic deletion of HCN2 reduces the cAMP-dependent component of I_h significantly (Momin et al., 2008b).

5.1.3. Role of HCN Channel in Pain

There has been ample evidence to suggest that increased activation of the I_h current is associated with peripheral hyperexcitability or increased excitability of nociceptors in both inflammatory and neuropathic pain (Luo et al., 2007; Momin et al., 2008a). Moreover, the widespread use of Ivabradine in the clinic for the treatment of heart failure, with a relatively safe side effect profile makes these channels an attractive target for different indications such as pain, given the involvement of HCN channels in neuronal excitability (Psotka and Teerlink, 2016; Tse and Mazzola, 2015). Two groups have reported evidence for the involvement of I_h current in both neuropathic, SNL and PNL models as well as inflammatory associated sensitisation in the formalin test (Luo et al., 2007; Takasu et al., 2010). Further, an accumulation and redistribution of axonal HCN channels was demonstrated by immunocytochemistry and western blotting in nerve injury models *in vivo* (Jiang et al., 2008; Smith et al., 2015). The role of the HCN2 isoform in neuropathic pain was revealed as the deletion of this channel resulted in a significant reduction in mechanical and thermal hypersensitivity in both, CFA- and carrageenan-induced inflammatory rodent pain models (Emery et al. 2012). Another study suggested that heat hyperalgesia associated with inflammatory pain requires the expression of HCN2 in higher centres, not peripheral HCN2 channels (Schnorr et al., 2014).

5.1.4. Role of HCN2 Channel in PGE2-induced axonal ongoing activity

It has been demonstrated that inflammatory mediators, such as PGE2 act on GPCRs to increase intracellular cAMP levels, ultimately resulting in an increased rate of action potential firing (Momin et al., 2008b). Given the role of cAMP in modulating HCN channels and their role in regulating action potential frequency, these studies suggested the role of HCN channels as an important candidate responsible for

increased action potential frequency in response to IM at the DRG soma. Moreover, deletion of the HCN2 gene abolished increased neuronal firing induced by PGE2 or forskolin, a rapid activator of adenylate cyclase, in isolated neurons (Emery et al., 2011a). Taken together, these findings propose an interesting hypothesis suggesting an important role of HCN channels in painful conditions. Moreover, these studies suggest the HCN2 channel are the primary downstream candidate of PGE2 – induced increased excitability of nociceptors.

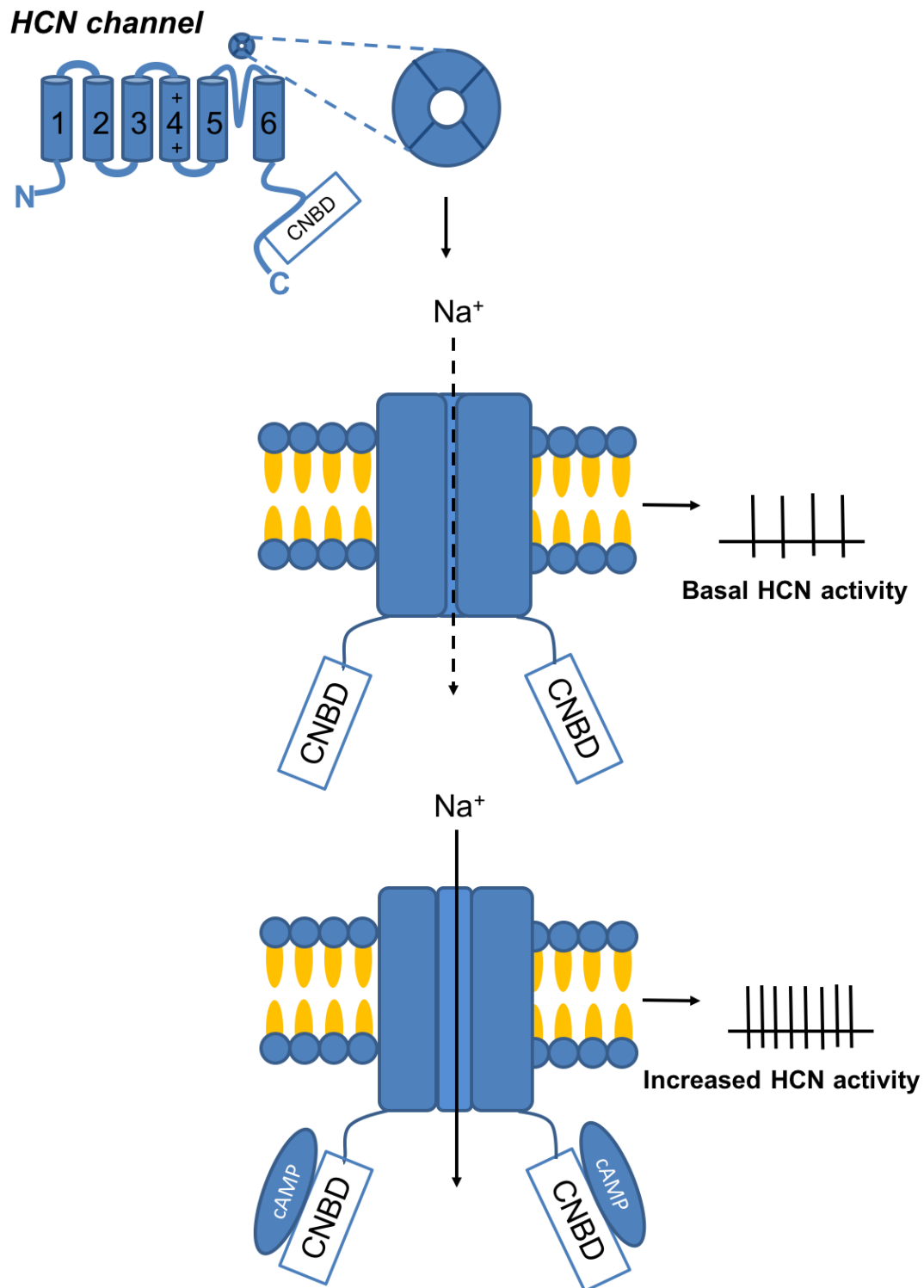


Figure 31. Activation of HCN channels and impact on neuronal firing.

Binding of cAMP to cyclic nucleotide-binding domain (CNBD) the C-terminal of HCN channel results in a positive shift in activation threshold. This causes the channel to reduce its latency between action potentials, as it is activated at more positive membrane potentials, thereby explaining an increase in action potential firing frequency by a rapid repolarization of the membrane potential.

5.2. *Rationale and Aims*

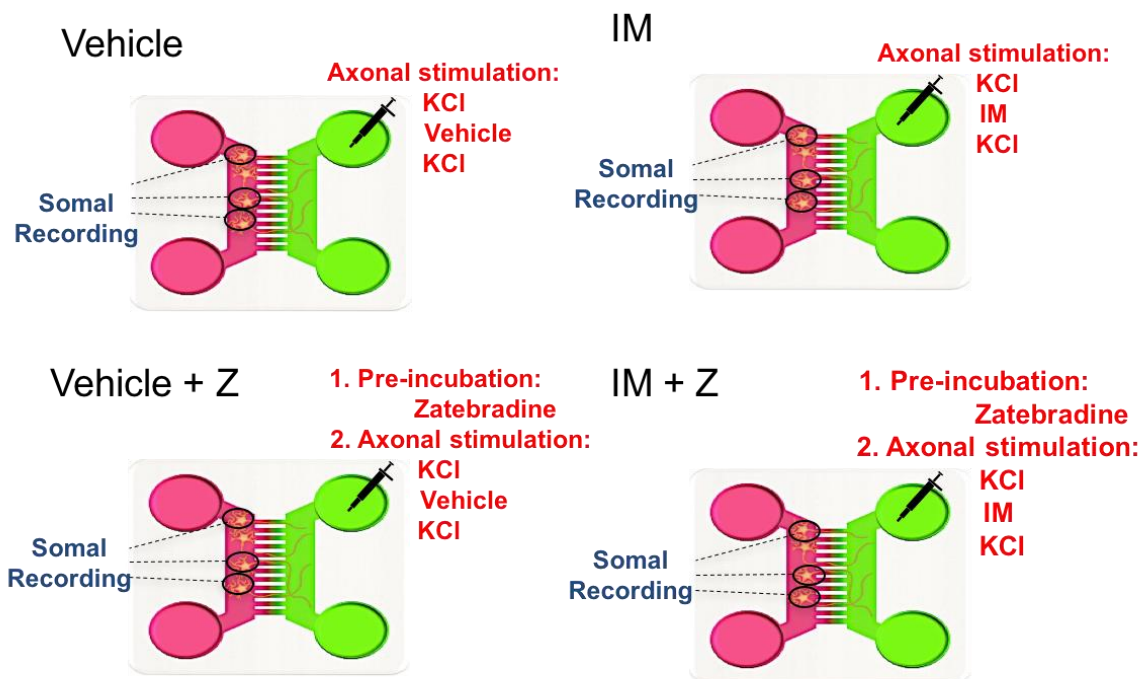
One of the interesting aspects of axonal response to PGE₂ sensitisation we observed was the direct depolarization of the axons and persistent activity in the axons after termination of the signal. Given the lack of inflammatory pain sensitisation in HCN2 KO mice, as well as the fact that HCN channels are heavily modulated by cAMP, which is a second messenger activated by PGE₂, HCN channels appear to be good candidates as downstream effectors of PGE₂-mediated neuronal sensitisation and activation. Initial reports using DRG neurons in conventional culture configuration showed that forskolin evoked changes in neuronal function at the cell soma, which is abolished in HCN2 KO neurons (Emery et al., 2011a). We hypothesized that axonal HCN2 channels might respond to cAMP elevation downstream of PGE₂-EP receptor activation, leading to subsequent depolarization of axons. The aim of the experiments in this chapter is to understand whether HCN2 channels are involved downstream of PGE₂ sensitisation and activation of nociceptive axons. Using pharmacological blockers of HCN channels and HCN2 knockout mice, the contribution to axonal activity recorded at the soma following PGE₂ application to axons was assessed.

5.3. Results

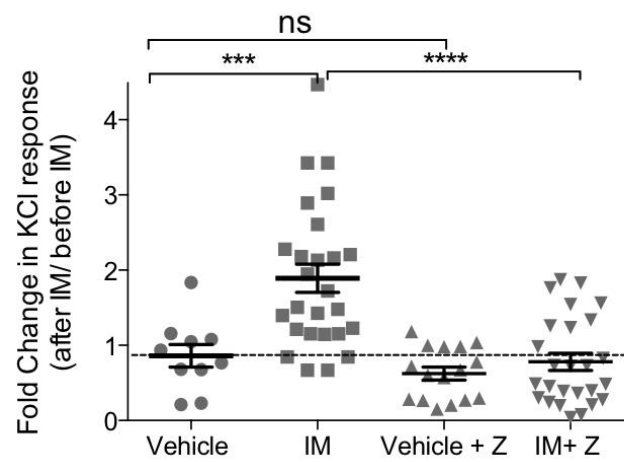
Firstly, we tested to see whether HCN channels play an important role in inflammatory sensitisation of DRG axons. For this initial experiment, we used a non-selective HCN channel blocker, Zatebradine, which has a similar chemical structure to Ivabradine. Changes in KCl response in DRG cell bodies before and after IM or vehicle treatment to isolated DRG axons were measured. This experiment was then repeated in the presence of Zatebradine or its vehicle control, pre-incubated in the axonal chamber for 10 minutes prior to the start of the experiment. Axonal sensitisation assessed using two measurements, changes in the intensity of the maximum response of Ca^{2+} signal (ΔF_{ratio}) and the changes in the number of axonal responders to 20mM KCl.

A significant increase in fold change of the maximum response in cell bodies of IM-treated axons compared to vehicle group (**Figure 32A**). However, following a 10-minute pre-incubation with 10 μM Zatebradine, the potentiation in maximum response induced by IM was abolished. This concentration of Zatebradine was chosen based on published studies showing its effectiveness in inhibiting Ih current at 10 μM (Gill et al., 2004; Van Bogaert and Pittoors, 2003). Pre-incubation with Zatebradine, resulted in no significant increase in the number of responders was observed, despite treatment with IM for two hours (**Figure 32B**). This data suggested that HCN channels in peripheral terminals are important mediators of axonal sensitisation following inflammatory insult.

A



B



C

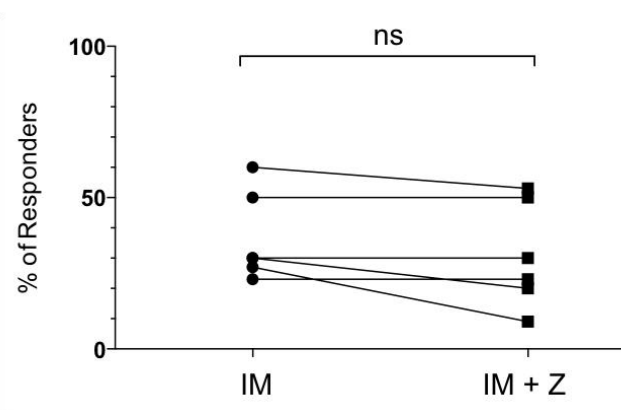


Figure 32. Zatebradine, a non-selective HCN blocker, reverses IM-induced axonal sensitisation.

A) Schematic of experimental set up and protocol used for different groups: KCl responses were recorded from cell bodies before and after IM or vehicle treatment of DRG axons, this was then repeated in MFCs where the axonal chamber was pre-incubated with Zatebradine or vehicle. **B)** Data expressed as the mean $\pm$ S.E.M, showing an increase in the maximum responses to 20mM KCl after axonal treatment with IM compared to vehicle (* $p < 0.05$). IM-induced potentiation of second KCl response was significantly reduced in neurons whose axons were pre-treated with 10 μ M Zatebradine, compared to those treated with IM only (**** $p < 0.0001$, Kruskal-Wallis test with Dunn's test for multiple comparisons, $n = 12-28$ DRG neurons, $n = 3-8$ mice/group). **B)** Mean percentage responders to KCl. No significant changes were observed in number of responders in groups treated with Zatebradine. (Mann-Whitney U test, $n = 12-28$ DRG neurons, $n = 3-8$ mice/group)

We further investigated the role of HCN2 isoform in inflammatory axonal sensitisation. Taking advantage of a KO mouse model of HCN2 ablation from the McNaughton lab, the contribution of this channel to the sensitisation of axons was assessed. HCN KO mice developed ataxia after 4 weeks and did not survive past 5 weeks. Thus, for the experiments described in this chapter, all HCN2 KO/WT mice were used by week 4 unless stated otherwise.

Unexpectedly, it was found that both in HCN2 WT and KO mice, axonal responses to 20mM KCl were not sensitised after IM-treatment (**Figure 33A-C**). No difference was observed between maximum response before and after IM-treatment in both HCN2 KO and WT mice (**Figure 33D**). Based on this data, we postulated that IM-induced axonal sensitisation may be an age-dependent effect, and is therefore only present in older mice. To address this question, this experiment was repeated in young, C57bl/6 mice at 4-5 weeks of age, using the same experimental protocol and same batch of IM stock solution.

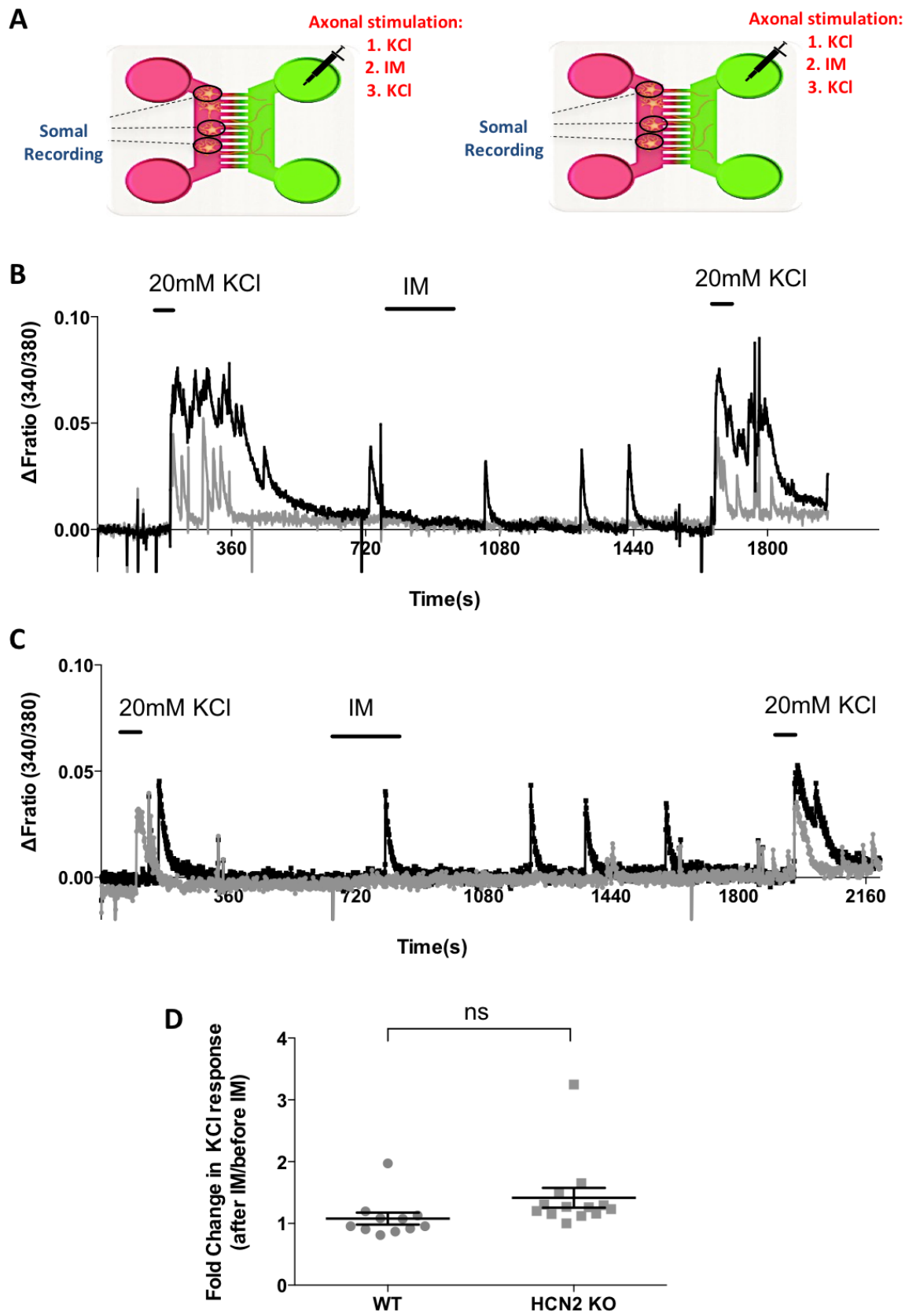


Figure 33. HCN WT DRG axons are not sensitised after IM-treatment.

A) Schematic of experimental set up and protocol used for different groups: KCl responses were recorded from cell bodies before and after IM or vehicle treatment of DRG axons from HCN2 KO and HCN2 WT mice. **B)** Representative trace showing no potentiation of KCl response after exposure to IM for 3 minutes in HCN2 WT DRG neurons. **C)** Representative trace showing no potentiation of KCl response after exposure to IM for 3 minutes in HCN2 KO DRG neurons. **D)** Data expressed as the mean $\pm$ S.E.M, showing no difference in maximum response to 20mM KCl after axonal treatment with IM was observed in either HCN2 KO and WT. (Mann-Whitney U, $p < 0.1898$, $n = 11-12$ DRG, $n = 6$ mice)

However, we found that IM-induced axonal sensitization to 20 mM KCl stimulation was present in 5-week old C57bl/6 mice (**Figure 34CA-B**). Maximum responses to 20mM KCl after IM- treatment were significantly increased, with a 6.1-fold increase from 0.132 ± 0.01 to 0.801 ± 0.01 (**Figure 34C**). We also observed a significant increase in the number of percentage responders to KCl after IM treatment in comparison to baseline responses before treatment (**Figure 34D**). Therefore, we concluded that IM-induced axonal sensitisation is present in younger mice, at 4 weeks. The genetic background is potentially a contributing factor; although, we have observed sensitisation of axons by IM both in WT C57BL/6 as well as CD-1 outbred mice (data not shown). Therefore, it is unclear why the strain of HCN2 KO mice are refractory to axonal sensitisation by IM.

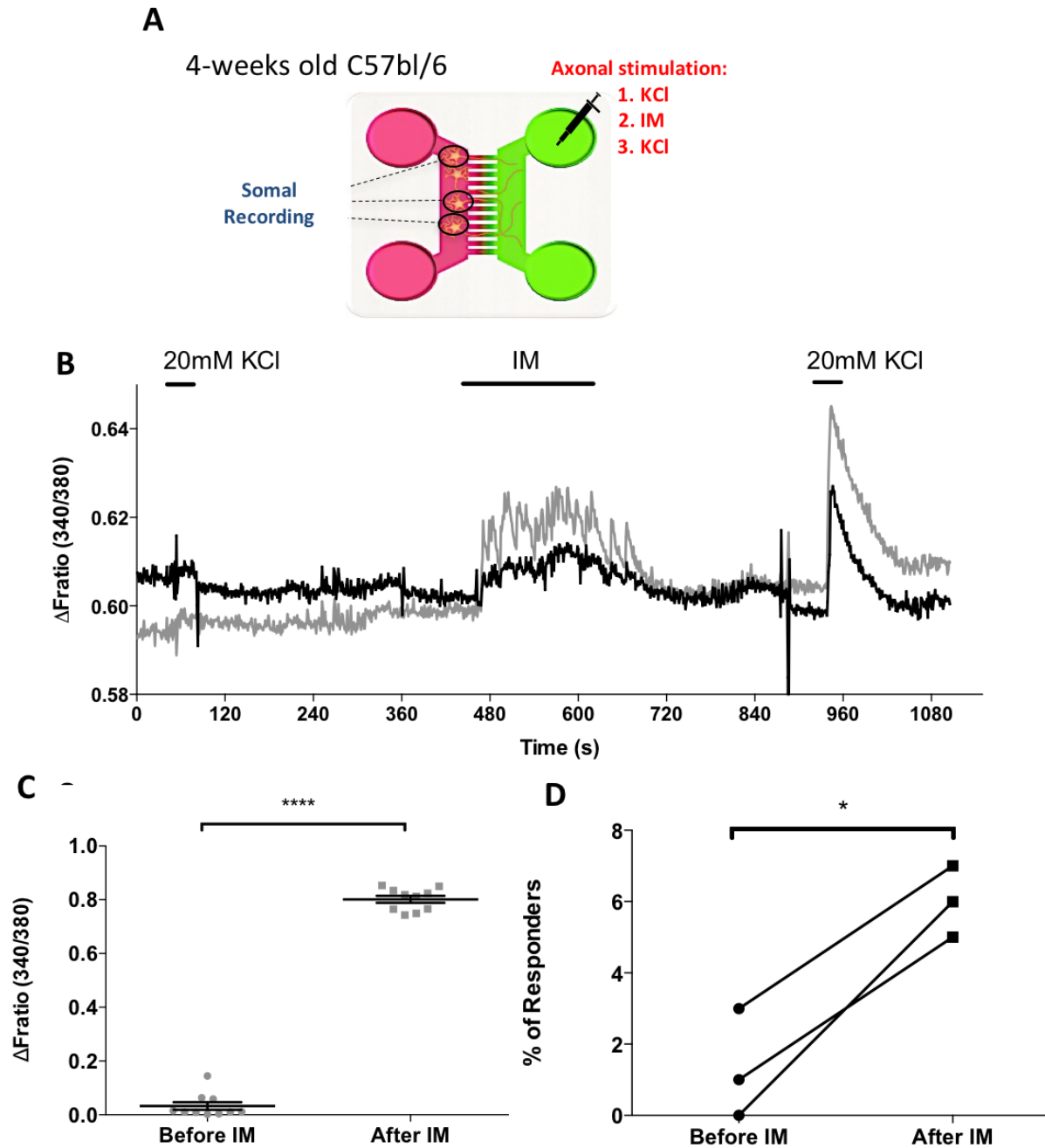


Figure 34. IM-induced axonal sensitisation is not age-dependent.

A) Schematic of experimental set up: KCl responses were recorded from cell bodies before and after IM or vehicle treatment of DRG axons in C57bl/6 mice of the same age as the HCN2 KO and WT used (4 weeks). **B)** Representative trace showing potentiation of KCl response after exposure to IM for 3 minutes in DRG neurons from 4-week old C57bl/6 mice. **C)** Data expressed as mean $\pm$ S.E.M, where graph displays quantification of maximum responses to 20mM KCl before and after axonal treatment with IM. IM- treated neurons showed a significantly higher maximum response to KCl stimulation after treatment compared to vehicle treated neurons,

where $**p<0.01$ (Two-tailed, paired *t*-test, $**p<0.01$ $n=10-15$ DRG, $n=3$ mice). **D)** Mean percentage of responders to 20mM KCl significantly increased after IM treatment ($*p<0.05$, Chi-square test, $n=10-15$ DRG, $n=3$ mice).

Next, we tested whether the HCN2 isoform is a downstream effector of PGE₂, and whether it is responsible for PGE₂-mediated ongoing axonal activity. In order to test this hypothesis, a selective HCN2 blocker was provided by courtesy of the McNaughton Lab which was demonstrated to be 30x more selective for HCN2 over Nav1.3, Nav1.7, and Nav1.8, as well as Cav1.2, Cav1.3, and Cav2.2 (**Table 2**, provided by McNaughton Lab).

Compound	HCN2 IC ₅₀ (μM)	Na _v 1.3 IC ₅₀ (μM)	Na _v 1.7 IC ₅₀ (μM)	Na _v 1.8/β3 IC ₅₀ (μM)
HCN2 selective blocker	1.6	>30	>30	>30
Compound	HCN2 IC ₅₀ (μM)	Ca _v 1.2 IC ₅₀ (μM)	Ca _v 1.3 IC ₅₀ (μM)	Ca _v 2.2 IC ₅₀ (μM)
HCN2 selective blocker	1.6	>30	>30	24.1

Table 2. Summary of HCN2 antagonist selectivity data.

5μM of the HCN2 selective compound silenced PGE₂- induced ongoing axonal activity (**Figure 35A-B**). The number of Ca²⁺ transients/5 minutes measured at the soma after treatment of axons with 10μM PGE₂ for 1 minute, were significantly reduced after application of the HCN2 selective inhibitor. The effects of the HCN2 selective compound appeared to be irreversible, as the Ca²⁺ transients did not return despite wash-out of compound (**Figure 35C**).

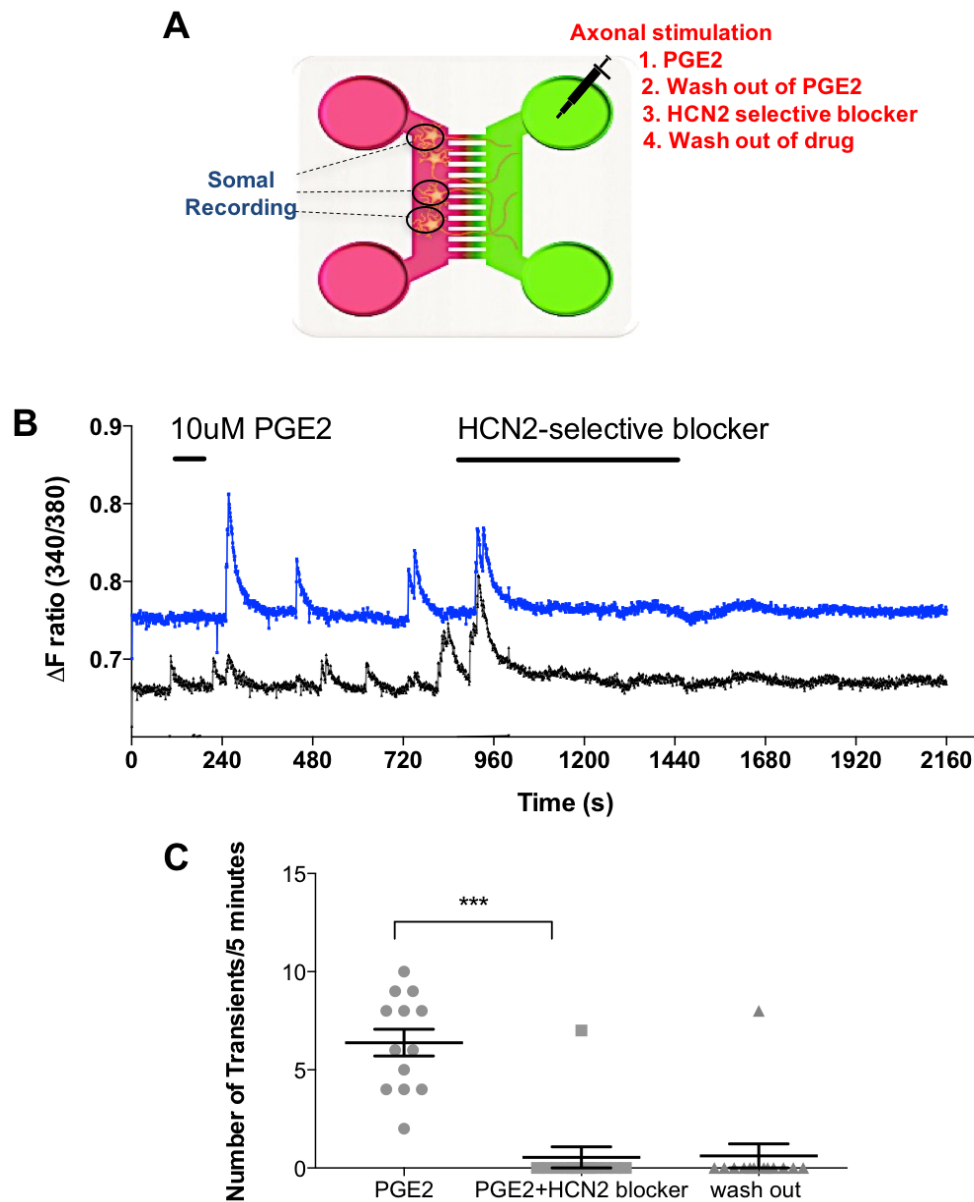


Figure 35. HCN2 selective blocker silences PGE2-induced ongoing activity.

A) Schematic of experimental set up and protocol: Only Dil-stained cell bodies were selected for calcium imaging analysis. Axonal chamber was stimulated with 10uM PGE2, followed by a wash-out, after which the selective HCN2 blocker was applied to the axonal chamber to test its effect on the number of transients induced by PGE2 for 5 minutes. **B)** Representative trace showing PGE2-induced axonal ongoing activity irreversibly silenced by 5 μ M HCN2 selective compound. **B)** Quantification of the number of transients/5 minutes observed after application of stimulus. Data expressed as mean $\pm$ S.E.M, showing a significant decrease in the number of PGE2-induced spikes after application of HCN2 selective inhibitor. (One-way ANOVA with Tukey's multiple comparisons test, *** $p < 0.001$, $n = 12-15$ DRG, $n = 4$ mice)

We further addressed this question by using HCN2 KO and WT mice, and postulated that PGE2-induced ongoing activity would be absent in HCN2 KO mice. We found that the absence of HCN2 channels had no effect on PGE2-induced ongoing axonal activity. PGE2-induced persistent activity was present in DRG axons of both HCN2 KO and their WT littermates with no significant difference in number of transients/5 minutes between both groups (**Figure 36A-D**). These data would suggest that HCN2 is not strictly required for PGE2 mediated nociceptor activation and persistent activity in DRG axons.

Taken together, the results of these experiments provide some support for involvement of HCN channels in IM-induced sensitisation of sensory axons. However, it remains unclear whether HCN2 channels are responsible for PGE2-induced ongoing activity observed at the axonal level.

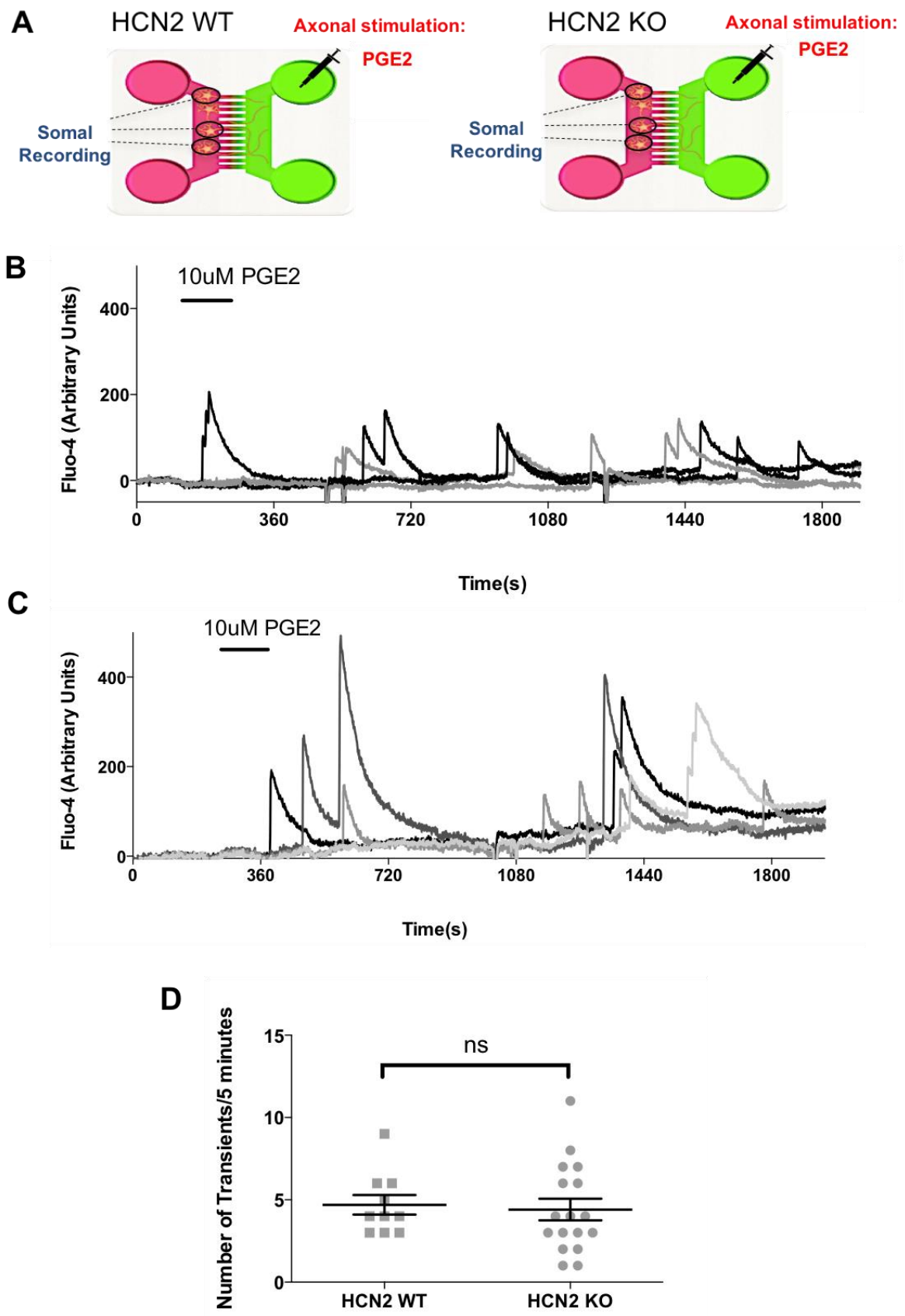


Figure 36. PGE2-induced ongoing activity present in both HCN2 WT and KO DRG axons.

A) Schematic of experimental set up and protocol used for different groups: the number of Ca^{2+} transients induced by 10uM PGE2 were measured in DRG axons from HCN2 KO mice compared to HCN2 WT mice. **B)** Representative trace showing PGE2-induced ongoing activity at the axonal level in HCN2 WT DRG neurons. **C)** Representative trace showing PGE2-induced ongoing activity at the axonal level in HCN2 KO DRG neurons. **D)** Data expressed as the mean $\pm$ S.E.M, showing no significant difference in number of transients/5 minutes induced by PGE2, between HCN2 KO and WT neurons. (Two tailed, unpaired t-test, $p < 0.77$, $n = 15-20$ DRG, 6-12 mice).

5.4. Summary

Based on available literature, HCN channels appear to be good candidates as downstream mediators of PGE₂-induced nociceptor activation and persistent activity in DRG axons. Thus, we assessed whether HCN channels, with a particular focus on the HCN2 subtype, play an important role in axonal activation and sensitisation.

A non-selective HCN blocker, Zatebradine, was used which reversed IM-induced axonal sensitization pointing towards the idea that HCN channels are important mediators of IM-induced sensitisation in DRG axons. Further, we investigated the contribution of the HCN2 isoform to IM- induced sensitization, and found that DRG axons of both HCN2 WT and KO could not be sensitised following inflammatory insult. Therefore, it remains unclear whether HCN2 is a key axonal player responsible for IM-induced sensitisation of peripheral axons.

We also tested to see whether HCN2 acts a downstream mediator of PGE₂- induced persistent axonal ongoing activity. Using a HCN2 selective antagonist, we were able to detect complete silencing of PGE₂-induced ongoing activity. However, again this was not supported when using HCN2 KO DRG neurons, as genetic deletion of HCN2 did not have an effect on PGE₂-induced persistent activity at the axonal level. Hence, it is unlikely that HCN2 channels are responsible for PGE₂-mediated persistent activity observed at the axonal level.

6. RESULTS IV: Development and characterisation of hIPSC-derived sensory neurons in MFCs

6.1. Introduction

6.1.1. Are rodent models predictive of human disease?

Undoubtedly, animal models have been pivotal in our understanding of mechanisms underlying disease pathology. However, their predictive value for the development of clinically effective therapeutic agents remains to be a contentious subject matter. A systematic review reported that out of the chosen highly cited publications of animal studies, one third are contradicted by randomized human trials, one third remain untested and only one third are actually replicated as randomized human trials (Hackam and Redelmeier, 2006). A clear example of this, within the field of pain, is the history of NK1 receptor antagonists; despite their ability to block behavioral responses to noxious stimuli in rodents, they failed to demonstrate efficacy in clinical trials of a number of chronic pain states (Hill, 2000). Although blockade of NK1 receptors is sufficient to attenuate responses to stressors in humans in a similar manner to what is observed in preclinical studies, it is not sufficient to provide clinical pain relief.

Furthermore, a recent detailed study highlighted several, previously underestimated, differences between the transcriptome of human and mice, which are likely to account for important differences in physiology. They claimed that gene expression was more similar between different tissues of the same species as opposed to comparable tissue of different species (Lin et al., 2014).

6.1.2. Currently available tools to study disease pathology in human models

The failure of a number of clinical trials over the past decade, has driven the shift towards developing alternative approaches to the traditional animal models used in the field of neuroscience, with many research groups turning towards the possibility of experimentation using human cells and tissues. The increasing availability and access to human DRG from organ donor networks, has enabled research using human tissue in the field of pain over the years. Studies have highlighted several differences in functional properties of ion channels when compared to rodent models. For instance, a TTX-resistant current was discovered in human DRG, which had not been previously identified in rodents (Dib-Hajj et al., 1999). Moreover, long-term viability of healthy human DRG neurons in culture for up to almost a month has also been reported recently (Enright et al., 2016). Human DRGs have become increasingly popular and have been put forward as preclinical vehicles for the target validation of phase of the drug development process (Copits et al.; Davidson et al., 2014; Davidson et al., 2016).

However, human tissue or cellular material is generally limited and very expensive to acquire. Therefore, there has been a focus on using stem cells which can be differentiated into any cell type of the body. Furthermore, advances in stem cell technology have enabled the development of protocols for the generation of sensory neurons from adult human induced pluripotent stem cells (hiPSCs) (Cai et al., 2016; Park et al., 2008). Human iPSC-derived neurons have become an increasingly popular model for neurodegenerative disease studies and drug discovery efforts.

The ability to differentiate cells from patients suffering from pain syndromes could provide a unique opportunity to understand the cellular pathophysiology of human pain conditions.

6.1.3. In vitro disease modelling using hIPSC-derived neurons

The pluripotency of embryonic stem cells (ESC) and the use of ESC-derived cell lines for experimental research was first suggested in 1998 (Thomson et al., 1998). The ethical concerns associated with using embryonic stem cells from human embryos have hampered the progress within this particular field (de Miguel-Berriain, 2015). In order to circumvent this issue, there had been interest in developing human inducible pluripotent stem cells (hIPSC), which are derived from adult somatic cells and can be reprogrammed by retroviral transduction of transcription factors such as Oct3/4, Sox2, Klf4, and c-Myc (Takahashi et al., 2007). The exact mechanism through which these four transcription factors induce pluripotency is yet unclear, however, iPSCs can be derived from any tissue such as neural cells, keratinocytes, adipose-derived cells and blood cells amongst others, and can differentiate into any somatic cell type (Carey et al., 2009; Soldner et al., 2009). Human iPSCs show similar properties to human embryonic cells in morphology, proliferation, cell surface markers, gene expression amongst others (Daley et al., 2009). These characteristics make iPSCs a cost-effective alternative to primary human neurons for studying molecular mechanisms of human diseases as well as drug screening.

The expression profile of hIPSC-derived sensory neurons has been shown to follow a similar pattern *in vitro* when compared to human DRG samples *in vivo*. It was shown that expression of neuronal markers appeared around 9 to 16 days after plating and increased gradually until the end of the differentiation protocol. The

confirmed the functionality of GABA_A receptors, HCN channels, ASIC channels and voltage gated potassium channels. Moreover, the differentiated cells expressed 141 out of 168 ion channel genes found in adult human DRG samples (Young et al., 2014b). Young and co-workers suggest that hiPSC-derived sensory neurons serve alternative and more physiologically relevant model to rodent DRGs for pain research, as they provide evidence for molecular and functional similarities between inducible sensory neurons and primary human DRG. Moreover, another study showed that inducible sensory neurons derived from human fibroblasts not only exhibited responses to TRP agonists, demonstrating functional properties of nociceptive neurons, but also showed a marked sensitisation of capsaicin responses after treatment with either PGE₂ or oxaliplatin, a commonly used chemotherapeutic drug, recapitulating key mechanisms of inflammatory pain hypersensitivity and peripheral chemotherapy-induced neuropathy (Wainger et al., 2015).

The availability of hiPSCs has significantly increased the usage of *in vitro* disease modelling. Reports have been published for the first set of drug candidates to be tested in clinical trials based on findings obtained from iPSC-derived neurons (Mullard, 2015). Furthermore, hiPSCs are being used for models of pain, such as chemotherapy induced peripheral neuropathy (Morrison et al., 2016; Wainger et al., 2015). The use of *in vitro*-differentiated sensory neurons have the potential to accelerate our understanding of pain-related signalling pathways and functional properties of receptors more accurately.

6.2. Rationale and Aims

The use of hIPSC-derived neurons in conventional cultures is now routinely performed however, culturing these neurons in MFC will enable the study of axonal function in human nociceptive neurons as a more physiological model of human pain. The aim of this chapter was to assess whether it would be feasible to maintain commercially sourced hIPSC-derived neurons in MFCs and the use of this platform to investigate axonal function.

In this chapter I aimed to establish *in vitro* conditions for maintaining healthy hIPSC-derived sensory neurons on glass coverslips as well as maintaining hIPSC-derived sensory neuronal axons in the 3-channel MFC system for 4 weeks. In addition, I aimed to characterise and identify different cell populations present in these *in vitro* cultures at 4 weeks post-plating, using pharmacological tools. Finally, to characterise axonal responses of hIPSC-derived sensory neurons to chemical stimulation.

6.3. Results

Human iPSC- sensory neuron progenitors were provided by Axol Biosciences, which had been derived from iPSCs of healthy male donors and had been differentiated to sensory neurons progenitors using small molecule inhibitors. Data from other labs using human iPSC-derived neuron progenitors provided by Axol Biosciences had shown expression of important pain markers such as Nav1.7, Nav1.8 and Nav1.9 at 4 weeks post-plating (Axol Biosciences, personal communication, 2017). Thus, we aimed to establish optimal *in vitro* culture conditions with adequate cell adherence for at least 4 weeks post-plating on both conventional cultures and in MFC system. In order to achieve this, different combinations of coating substrates were tested. Coating substrates kindly provided by Axol Biosciences, including ReadySet and Surebond, which were tested at different durations as well as against matrigel, which has been reported as the coating substrate of choice in several reports characterising hPSC-derived neurons (Young et al., 2014; Cai et al., 2016).

The combination of 24-hour incubation ReadySet and 1-hour Surebond was first tested, which resulted in extensive clumping of cell bodies in somal compartment by week 2 (**Figure 37B**). Clumping of cell bodies presents a significant issue for carrying out calcium imaging or patch clamping experiments, as it makes analysis of signals single-cell signals difficult. Axonal degeneration was also observed by week 2, in both axonal and peripheral terminal compartment of the MFC system (**Figure 37C-D**). This protocol was therefore not optimal for cell attachment of hPSC-derived sensory neurons in the 3-channel MFC system. To address the source of this issue, we aimed to identify the optimal coating substrate on regular glass coverslips first. We compared the combination of 1-hour ReadySet + 1-hour Surebond to coating for

2 hours with a thin layer of 1:40 diluted matrigel on regular glass coverslips. No differences were observed between groups during the first 2 weeks *in vitro*, however extensive somal clumping was apparent by week 4 with matrigel-coating coverslips **(Figure 38C-D)**, compared to coating with 1-hour ReadySet + 1-hour Surebond **(Figure 38A-B)**. Finally, we tested whether the 1-hour ReadySet + 1-hour Surebond combination would enable optimal cell adherence in the 3-channel MFC system. This combination enabled optimal maintenance of hPSC-derived sensory neurons in MFCs for 4 weeks, with reduced somal clumping and no axonal blebbing **(Figure 39B-C)**. Therefore, this coating protocol was chosen for future experiments. **Table 3** provides a summary of the different coating combinations used and their outcomes.

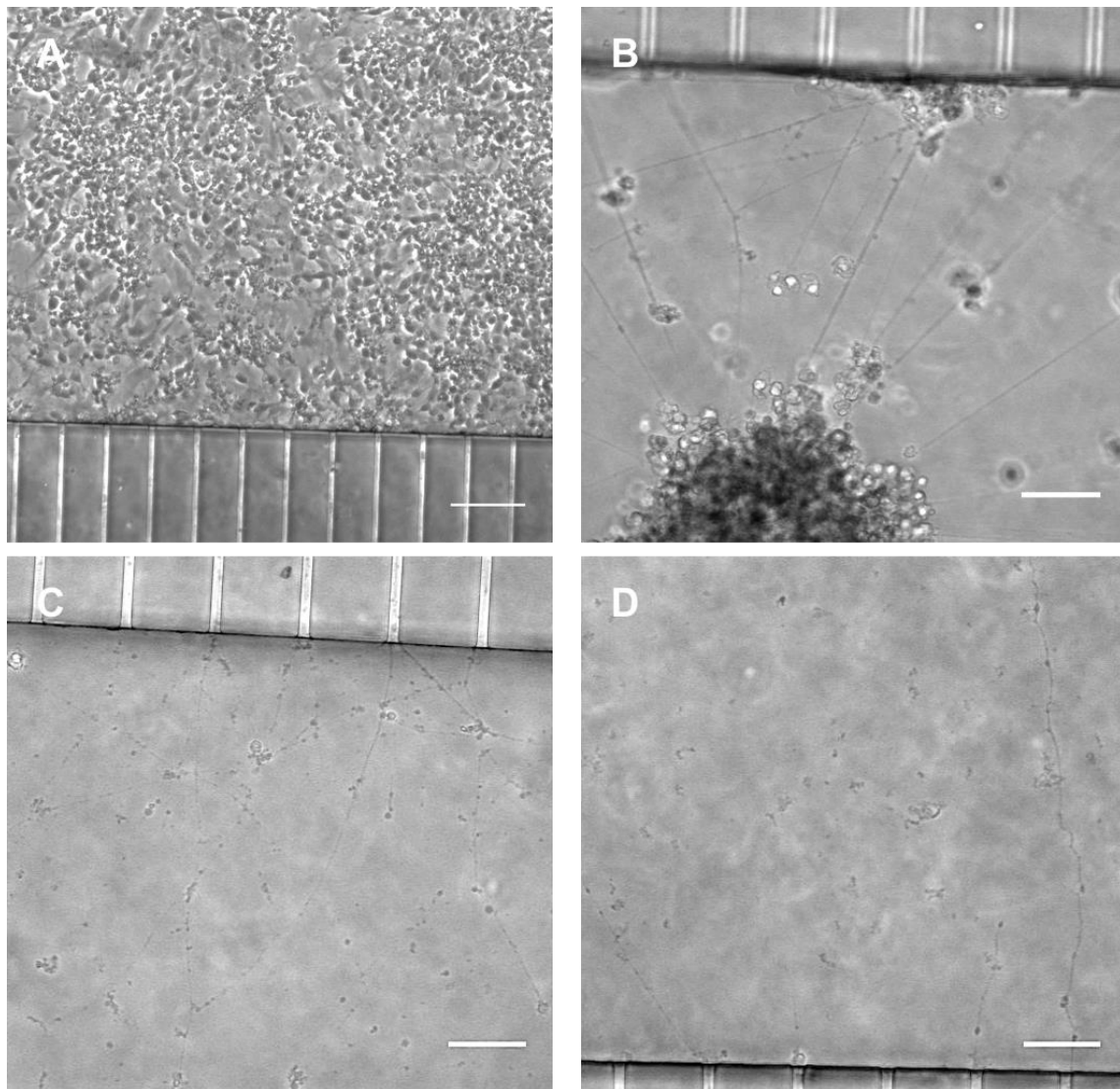


Figure 37. 24-hour ReadySet + 1-hour Surebond coating combination in MFCs.

*Representative images of different compartments 1-day post plating and 2 weeks post plating. **A)** hIPSC-derived sensory cell bodies in somal compartment 1 day post-plating. **B)** hIPSC-derived sensory neurons in somal compartment showing clumping of cell bodies 2 weeks post-plating. **C)** hIPSC-derived sensory axons in proximal axonal compartment showing axonal degeneration 2 weeks post-plating. **D)** hIPSC-derived sensory axons in distal axonal compartment showing axonal degeneration 2 weeks post-plating. Scale bars, 100 μ m.*

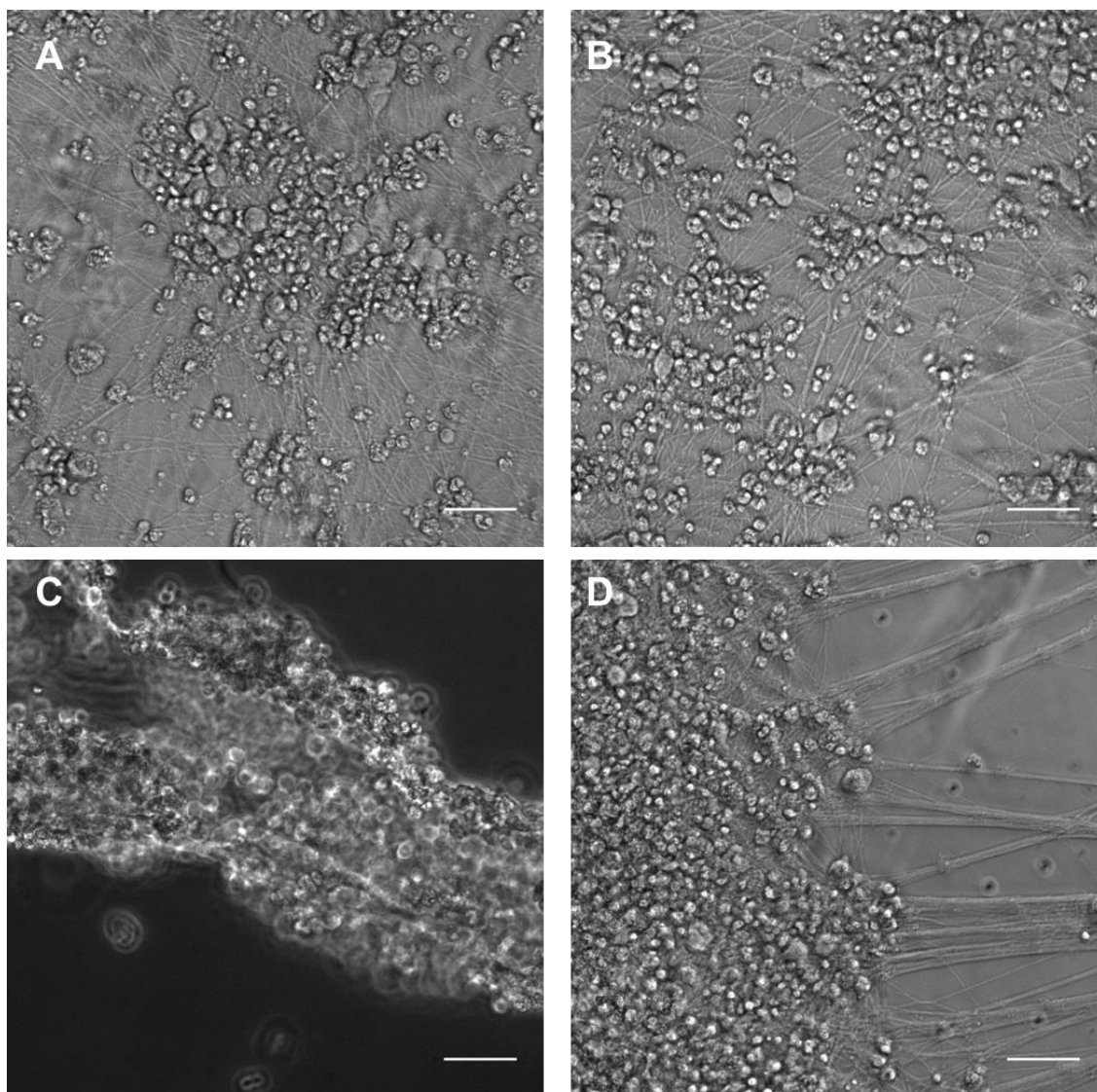


Figure 38. 1-hour ReadySet + 1-hour Surebond combination vs. matrigel coating on glass coverslips.

Representative images at 4-weeks post plating. **A-B)** hIPSC-derived sensory neurons on glass coverslips coated with ReadySet + Surebond combination. **C-D)** hIPSC-derived sensory neurons on glass coverslips coated with 1:40 matrigel for 2 hours showing clumping of cells. Scale bars, 100 μ m.

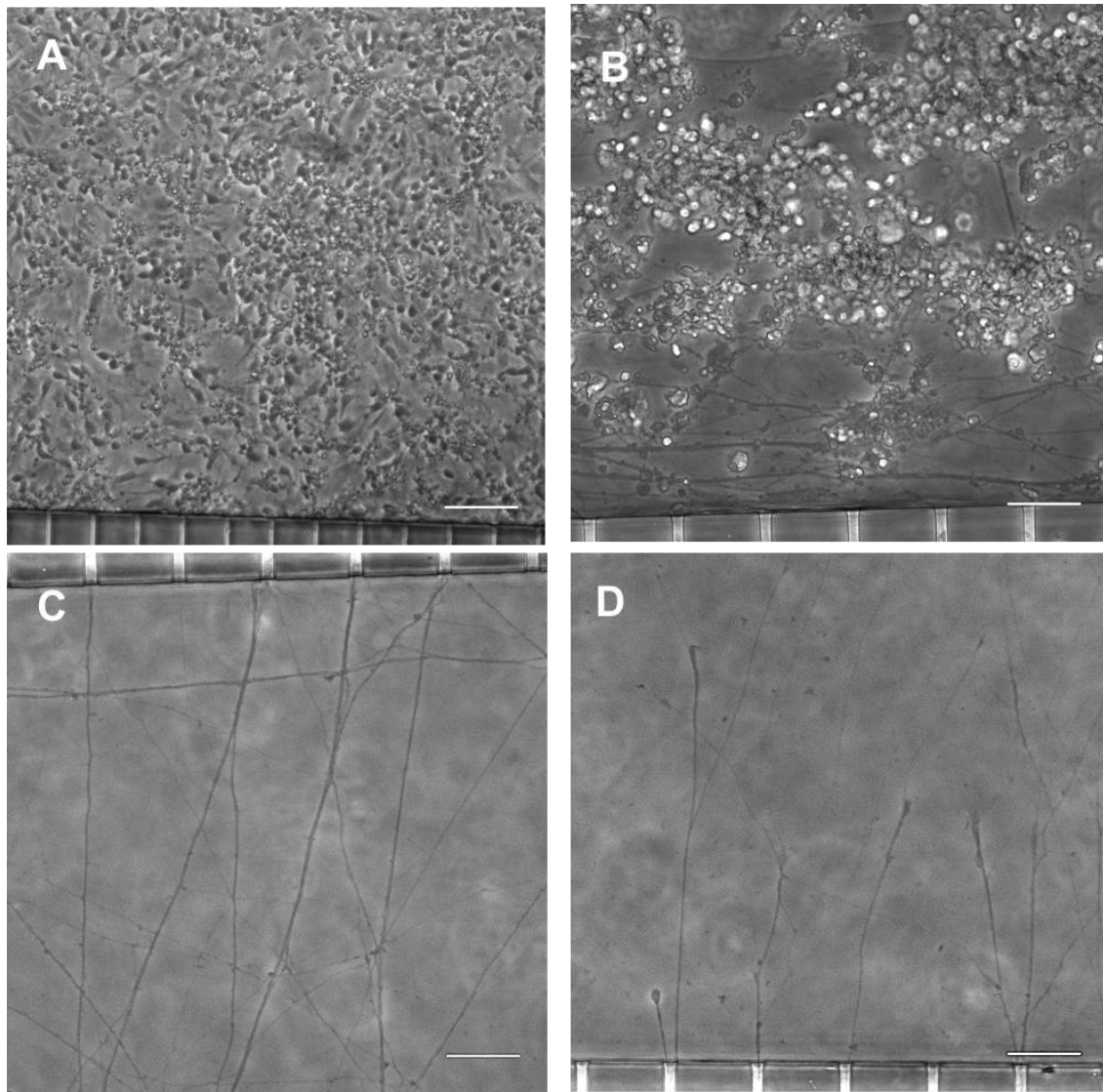


Figure 39. 1-hour ReadySet + 1-hour Surebond coating combination in MFCs.

*Representative images of different compartments 1-day post-plating and 4 weeks post-plating. **A)** hIPSC-derived sensory cell bodies in somal compartment 1 day post-plating. **B)** hIPSC-derived sensory neurons in somal compartment with no clumping of cell bodies observed 4 weeks post-plating. **C)** hIPSC-derived sensory axons in proximal axonal compartment with no axonal degeneration observed 4 weeks post-plating. **D)** hIPSC-derived sensory axons in distal axonal compartment with no blebbing of axons observed 4 weeks post-plating. Scale bars, 100 μ m.*

	24hrReadySet + 1hr Surebond	1:40 Matrigel 2 hours	1hrReadySet + 1hr Surebond
Glass coverslips		×	✓
MFC system	×		✓

Table 3. Summary of different coating combinations tested for optimal adhesion of hIPSC-derived sensory neurons.

To characterise the populations of the cells being produced using the current protocol, we next assessed the functional properties of the different populations of cells present in our hIPSC-derived sensory neuron cultures based on their responses to TRP channel agonists including icilin, AITC, and capsaicin.

In the first set of experiments where functional properties of neurons on glass coverslips were tested. We found that hIPSC-derived sensory neurons were responsive to 20mM KCl, 1 μ M icilin and 65mM KCl (**Figure 40A, B and D**). No responses to AITC were observed (**Figure 40C**). The magnitude of responses to 20mM KCl, 100 μ M AITC and 1 μ M icilin were quantified as maximum responses normalised to responses to 65mM KCl (**Figure 40E**). Interestingly, we found that 33.3% of neurons were responsive to icilin and 100% of neurons responded to 20mM KCl, whereas no response to AITC or capsaicin was detected in cultures 4 weeks post-plating (**Figure 40F**).

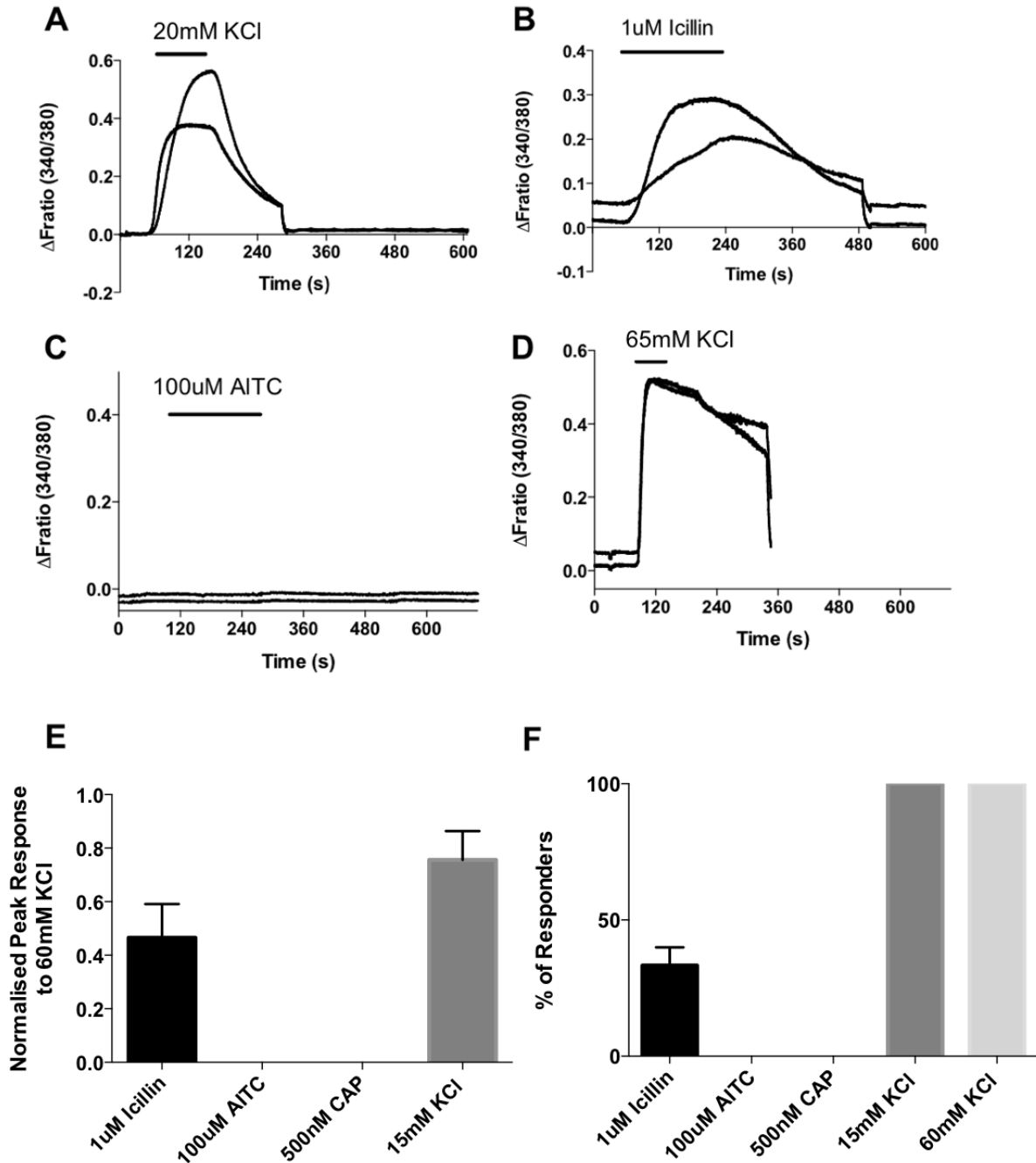


Figure 40. *hIPSC- derived sensory neurons on glass coverslips were responsive to different chemical stimuli, at 4 weeks in vitro*

Representative trace of responses, where each trace represents one DRG soma. **A)** Responses to 20mM KCl (1-minute application) **B)** Response to 1μM Icillin (2.5 min application) **C)** Representative trace showing responses to 100μM AITC (3 min application). **D)** Responses to 65mM KCl (1 min application). **E)** Bar graph expressed as mean ± S.E.M. showing average maximum responses to 1μM icillin, 100μM AITC, 20mM KCl and 500nM Capsaicin, normalized to maximal or 65mM KCl response of DRG neurons. **F)** Bar graph expressed as mean ± S.E.M. showing

average % responders to 1 μ M icilin, 100 μ M AITC, 20mM KCl and 500nM capsaicin out of total responders to 65mM KCl per coverslip. (n=4 independent cultures, 120-150 neurons)

As mentioned above, using the original protocol for maintenance of hPSC-derived sensory neurons, where media containing 25ng/ml GDNF, 25ng/ml NGF, 10ng/ml BDNF and 10ng/ml NT-3 was refreshed every 3 days, we observed no capsaicin-sensitivity was observed in hPSC-derived sensory neuronal cultures (**Figure 41A**). However, serendipitously we discovered that in the presence of 125ng/ml GDNF, neurons developed capsaicin sensitivity (**Figure 41B**). No capsaicin responsiveness was recorded in cultures maintained in media containing 25ng/ml GDNF (**Figure 41C**), whereas an average of 66% of neurons were responsive to 500nM capsaicin when cells were maintained in media containing 125ng/ml GDNF (**Figure 41D**). On average, 70% of neurons were responsive to 20mM KCl out of the total number of responders to 65mM KCl (**Figure 41C-D**). This suggests that higher concentrations of GDNF may be required for TRPV1 expression in developing hPSC-derived neurons. It could be that a higher concentration of GDNF is required to drive the neuronal differentiation process from progenitor cells to sensory neuronal populations expressing TRPV1 channels.

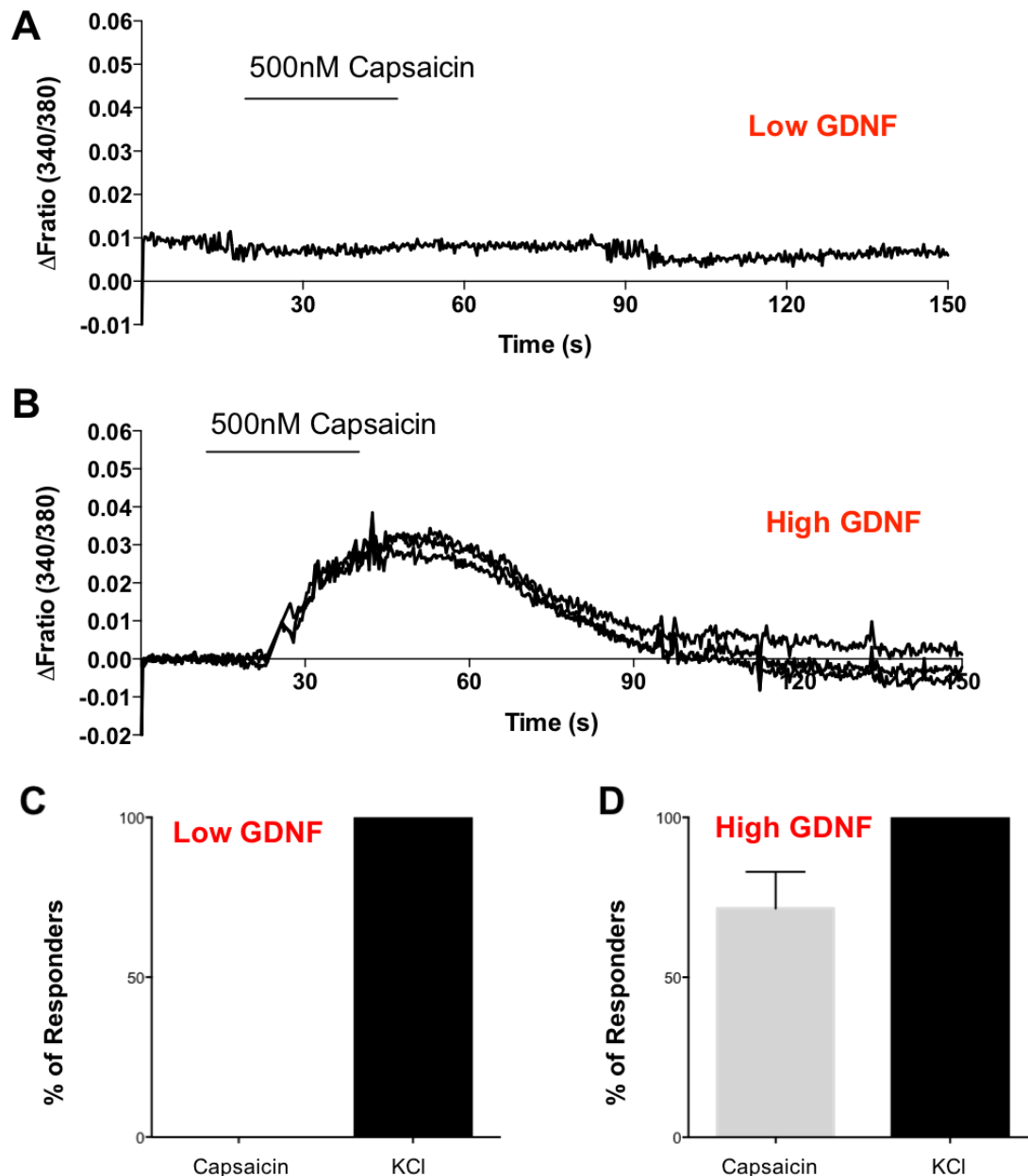


Figure 41. Development of capsaicin sensitivity in hIPSC- derived neurons depends on GDNF concentration.

A) Representative traces of responses to 500nM capsaicin at the soma, when treated with 25ng/ml GDNF. **B)** Representative traces of responses to 500nM capsaicin at the soma, when treated with 125ng/ml GDNF. **C)** Bars representing mean $\pm$ S.E.M. showing average percentage of responders to 500nM capsaicin in presence of 25ng/ml GDNF, out of total number of responders to 65mM KCl. **D)** Bars representing mean $\pm$ S.E.M. showing average percentage of responders to 500nM capsaicin in presence of 125ng/ml GDNF, out of total number of responders to 60mM KCl. (n=3 independent cultures, 60-130 neurons/per culture)

Next, we assessed whether hPSC-derived sensory neurons could be sensitised by inflammatory mediators. We found that despite lack of capsaicin sensitivity observed in these cultures containing recommended levels of growth factors (according to protocol provided by Axol Biosciences), somal responses to 20mM KCl were potentiated after IM treatment, compared to baseline somal responses to 20mM KCl (**Figure 42A**). KCl responses at the soma were potentiated 1.3-fold after treatment with IM, from 0.157 ± 0.007 to 0.215 ± 0.01 (**Figure 42B**).

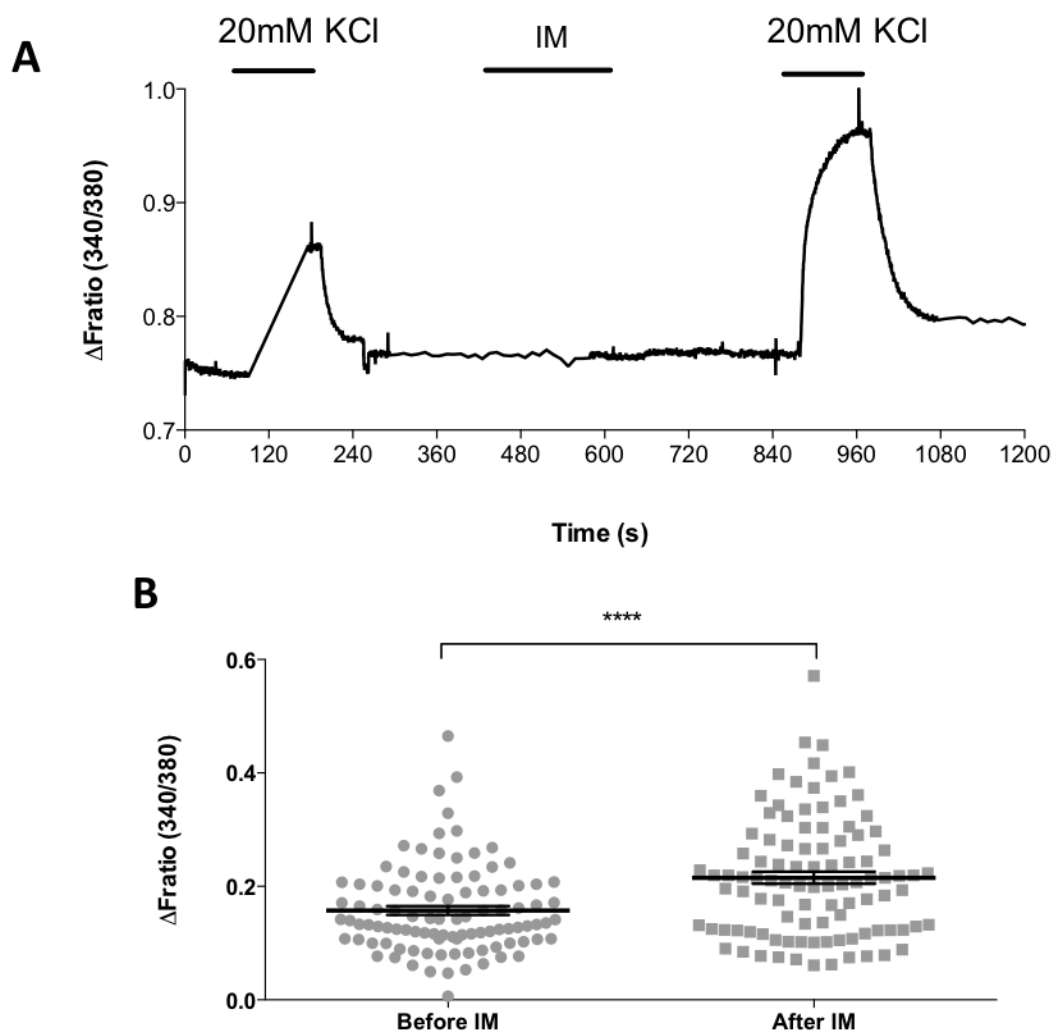


Figure 42. hPSC- derived sensory neuron cell bodies are sensitised after IM-treatment, despite capsaicin insensitivity in MFCs.

A) Representative trace showing responses to 20mM KCl, IM, and potentiation of second response to 20mM KCl. Each sweep represents one DRG neuron. **B)** Bar

graph expressed as mean $\pm$ S.E.M. showing average maximum responses to 20mM KCl response before and after IM-treatment. Significant increase in maximum response observed to 20mM KCl, where **** $p < 0.0001$. (Two-tailed, paired student's t -test, $n=79$ DRG neurons, $n=3$ batches of hIPSCs)

Next, we examined hIPSC-derived neurons' axonal responses to depolarisation with KCl and their ability to propagate signals to the cell body in the MFC model. Preliminary data suggested that a small population of fluidically isolated axons were responsive to increasing concentrations to KCl (**Figure 43**). We found that many axons did not respond to KCl stimulation, despite a robust signal in the cell soma. Given the variability observed in detecting axonal function in hIPSC-derived neurons between all six batches provided by Axol Biosciences, we were not able to test the impact of IM on axonal function.

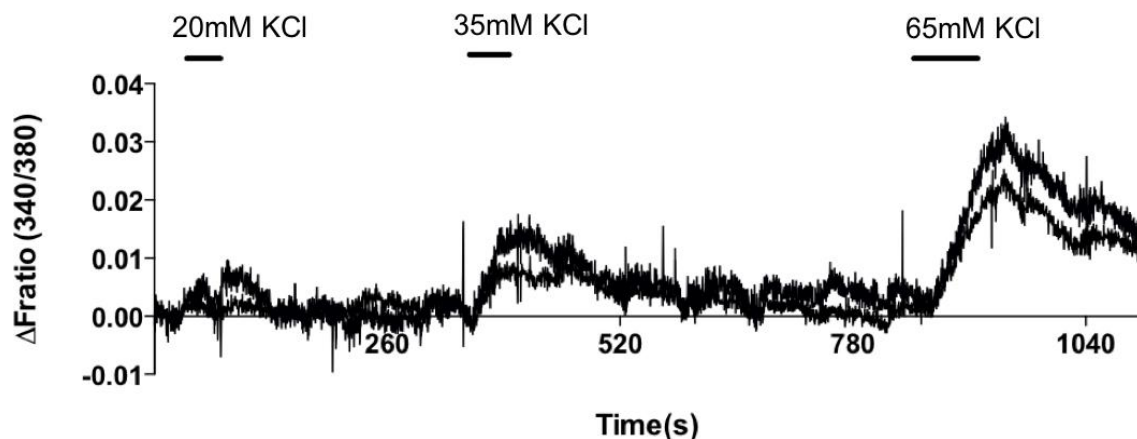


Figure 43. Preliminary result: Isolated hIPSC-derived sensory neuronal axons appear to be electrically active at week 4 in vitro in MFCs.

Representative traces, each sweep representing one neuron, showing axonal Ca^{2+} responses to 20mM KCl, 35mM KCl and 65mM KCl ($n=1$ culture).

6.4. Summary

One of the difficulties in using conventional cultures of iPSC-derived neurons for cell physiological measurement is the clumping of the neurons and lack of sustained adhesion to the substrate. Here we optimized a protocol that ensures adequate cell adhesion and maintenance of hIPSC-derived sensory neurons for up to 4 weeks post-plating in MFC devices. It was established that a combination of 1-hour ReadySet and 1-hour Surebond, coating substrates provided by Axol Biosciences, were optimal for growth and cell adhesion.

We characterized the phenotype of cells present at 4 weeks *in vitro*, and demonstrate responsiveness to icillin and KCl. Capsaicin sensitivity in hIPSC-derived sensory neurons appeared to be dependent on GDNF concentration in the media, despite similar levels of KCl sensitivity. Further, we found that hIPSC-derived sensory neuronal cell bodies can be sensitized using a cocktail of inflammatory mediators, despite the lack of capsaicin sensitivity observed in these cultures. Finally, fluidically isolated axons of hIPSC-derived neurons responded to KCl, although, this property varied significantly between all six batches of hIPSC-derived neurons tested. We postulate that this may be due to lack of maturity of hIPSC-derived neuronal axons at 4 weeks post-plating. Although further optimization is required, we present proof of concept that microfluidic cultures of hIPSC-derived sensory neurons can become a viable model of human nociceptive pain and sensitization.

7. DISCUSSION

7.1. *Using microfluidic-based platforms to study peripheral sensitisation in DRG neurons*

With increasing evidence pointing towards peripheral mechanisms of sensitisation as key drivers of pain, the study of axonal mechanisms at a molecular level would provide novel insights into important aspects of both inflammatory and neuropathic pain. However, the study of peripherally-restricted mechanisms required a robust system that enables interrogating DRG axons in isolation of the DRG cell soma. To date, there has been a lack of adequate *in vitro* models that enable the study of functional properties of axons, which motivated this work focusing on the development of a cell culture-based assay to test axonal function. In this study, we successfully set up a microfluidic-based *in vitro* assay in order to study axonal properties under inflammatory conditions.

7.1.1. Optimisation of the MFC system for the use of adult DRG mice

Previously, a MFC-based *in vitro* system was successfully optimized and characterised for the study of axonal hyperexcitability using postnatal (p1) rat pups (Tsantoulas et al., 2013). Conversely, in this thesis, DRG cells from adult mice were used with the aim to make this system compatible for use of transgenic mice, to enable the investigation of the role of key players responsible for mediating significant changes in nociceptor function during inflammatory conditions in this system (as discussed in Chapter 5). An additional benefit of using adult mice is that it serves as a more clinically relevant model in comparison to postnatal or embryonic rodents, as chronic pain states are more common in the elderly population (Fayaz et

al., 2016). Several modifications of different parameters were made in order to grow and maintain adult DRG neurons successfully in the MFC system, as adult DRG neurons are notoriously difficult to maintain in culture over long periods of time. MFC devices required strong adhesive surfaces in order to achieve optimal flow rate across channels and microgrooves. In our case, we found out that increasing the PLL concentration improved adherence of PDMS to glass plate maximizing flow rates and reduced clumping of cell bodies, indicative of better adhesion to glass coated surfaces. Although we established different NGF concentrations in the two compartments, it is likely that the main parameter driving axons to cross through the microgrooves had been the height differential as a result of a differences in volume between the MFC compartments. Interestingly, Araci and colleagues reported similar findings for retinal ganglion neurons in microfluidic cultures (Araci et al., 2014). We did not formally test this hypothesis, however, the constant flow from the soma compartment to the axonal compartment would not have resulted in an effective NGF gradient. Moreover, trophic factors are known to regulate expression profiles of different subpopulations of neurons. In future experiments, it would be interesting to consider the effects of GDNF, NT3 in either compartments on crossing of axons through the microgrooves. Although, we found that the conditions were suitable to promote crossing of sufficient number of axons, an increase in the number of axons would lead to increased number of neurons examined in each MFC. Of note, Phenotypic characterisation of the sensory neurons present in the MFC system of those cell bodies with axonal crossings has been carried out previously demonstrating approximately 36% CGRP-positive neurons, 32% IB4-positive neurons and 17% neurofilament-200 positive neurons (Tsantoulas et al., 2013).

7.1.2. Validation of the use of Ca^{2+} imaging as a readout for axonal function

Through the work presented in this thesis we demonstrate that Ca^{2+} imaging serves as an excellent measure of axonal activity. Tsantoulas and colleagues elegantly used patch clamp electrophysiology in combination with the 3-channel MFC system to demonstrate that fluidically isolated axons stimulated with KCl lead to the generation of APs that propagate towards the soma, causing a change in somal Ca^{2+} concentration (Tsantoulas et al., 2013). The same study showed that the propagation of APs from the distal axonal compartment to the soma was blocked by application of lidocaine to the middle axonal compartment of a 3-channel MFC chamber, whereas responses to somal stimulation were not affected. This experiment served as important proof of principle study indicating that the change in Ca^{2+} signal detected at the cell body in response to axonal stimulation with KCl was independent of the DRG cell body.

With the aim of setting up a higher-throughput preparation, we set up the MFC system using Ca^{2+} imaging as a readout for axonal function. This enabled sampling of multiple neurons simultaneously, which is advantageous due to the inherent heterogeneity of DRG sensory neurons. The clear difference in the kinetics of Ca^{2+} signals observed in response to somal and axonal stimulation with the same concentration and duration of KCl, is indicative of different mechanisms of Ca^{2+} influx at the soma in response to stimulation of different subcellular compartments of DRG neurons. KCl induces a sustained response at the DRG soma as a result of direct depolarisation of the somal membrane leading to opening of VGCCs, resulting in sustained influx of Ca^{2+} until washout of KCl stimulus. We propose that the Ca^{2+} transient observed in response to treatment of axons with KCl is a result of

generation and propagation of bursts of APs arriving at the soma. Thus, using this model we were able to measure somal Ca^{2+} concentration as a readout for AP generation and propagation at an axonal level.

In terms of the frequency of this phenomenon, quantified as percentage of responders, we observed that somal stimulation lead to 100% responders (out of total number of Dil- stained cell bodies in the field), as opposed to approximately 50% when stimulating the axons with KCl. These responses to somal stimulation therefore served as positive controls and were used as a test for viability of cells. Using Ca^{2+} imaging enables the study of populations of neurons, serving as a more high-throughput method in comparison to patch clamp electrophysiology. Further, through characterisation of somal and axonal stimulation responses, we confirm that imaging Ca^{2+} in the soma is a robust and sensitive assay of axonal function in MFC cultures. The combination of the MFC system with Ca^{2+} imaging enables the study of axonal mechanisms as a model of *in vivo* nerve ending stimulation. Of note, although it would be possible to image Ca^{2+} signals in the axons themselves, the benefits of using the DRG soma as a readout include: 1. It has been shown that Ca^{2+} signals at the soma are a result of AP generated and propagating from the axons, and 2. the reliability and convenience of measuring signals at the soma, as they display a better signal-to-noise ratio.

7.1.3. Inflammatory mediators sensitise DRG axons

The transition from high-threshold baseline nociception to low-threshold clinical pain hypersensitivity is thought to involve peripheral sensitisation of nociceptors. Hyperexcitability of peripheral neurons through sensitisation is an important mechanism contributing to behavioral changes and the perception of pain

in the presence of inflammatory mediators, manifested as a reduction in activation threshold, and hyperresponsiveness to noxious stimuli in sensory neurons.

In conventional cell culture models, cell bodies are exposed to inflammatory mediators and somal changes in responsiveness to various stimuli are determined. However, our model enables the study of axonally-restricted manipulations, and recapitulates physiological and anatomical features of the peripheral pain system more closely, particularly since the DRG cell bodies are not directly exposed to IMs during inflammatory conditions. Our current understanding is that peripheral nerve terminals are sensitised in response to a combination of IMs which are released locally, at the site of injury. In line with this, we demonstrate that localised treatment of axons with PGE₂, histamine, bradykinin and serotonin can lead to reduced activation thresholds, and show significantly enhanced KCl-induced responses of nociceptive axons. However, we show that responses to KCl at the soma remained the same, confirming that the effect observed does not require the soma of the neuron and is localized to the axons. Thus, it is likely, that the combination of IMs used in this study activate local axonal pathways leading to nociceptor hyperexcitability. Moreover, this model enabled us to compare changes in excitability before and after treatment of individual neurons, reducing variability inherent in heterogeneous neuronal populations in culture. In addition, we consistently saw an increase in number of cells responding to treatment of axons with IMs or PGE₂ only in this model, suggesting a reduction in activation threshold, where axons previously unresponsive to KCl stimulation became responsive due to lower thresholds. Thus, we provide evidence of different subtypes including silent nociceptors in our assay.

Chemically-induced hyperexcitability of peripheral nerve fibres have been studied previously using *ex vivo* preparations. For instance, it has been shown that protons cause an a long lasting sensitisation to mechanical stimuli in C-fibres in the rat skin-saphenous nerve preparation (Steen et al., 1992). Another study used single fibre recordings in canine testis-spermatic nerve preparations to assess the sensitising effects of pro-inflammatory agents to mechanical stimuli, measuring single fibre recordings from canine testis-spermatic nerve preparations (Koda and Mizumura, 2002b). This report suggested that histamine and PGE2 sensitise the mechanical response via cAMP and PKC signalling pathways at the level of the primary afferents, supporting the idea the peripheral nociceptor sensitisation is an important mechanism contributing to hyperalgesia to mechanical stimuli in inflammatory pain states (Koda and Mizumura, 2002b). Further, our study revealed that individual inflammatory mediators are able to sensitise responses to KCl. We found that a subpopulation of neurons responds directly to application PGE2 and BK. The excitatory effects of BK have been studied in testicular nociceptors *in vitro*, Koda and co-workers also demonstrated that application of 1nM BK alone resulted in nociceptor excitation (Koda and Mizumura, 2002a). Similarly, using the skin-saphenous nerve preparation it was found that application BK induces excitation at primary afferent level in a concentration-dependent manner (Banik et al., 2001). Whilst the excitatory effects of bradykinin have been extensively studied, whether PGE2 induces nociceptor activation at the axonal level has is still a contentious subject matter.

7.1.4. Potential mechanisms of IM-induced sensitisation of axons

Interestingly, we found that reducing the duration of IM-treatment had no effect on the observed sensitisation as there was no differences between groups treated with

IM for 2 minutes, and those treated for 2 hours (**Figure 19 and Figure 20**). IM-treatment with a duration of two minutes resulted in altered excitability of axons, displaying hyperresponsiveness to stimulus and reduced activation thresholds similar to that observed with a 2-hour treatment. This suggests that changes in axonal excitability are likely to be due to rapid, local changes, such as modulation of ion channels via phosphorylation induced by activation of local signalling pathways or due to insertion of pre-synthesized ion channels to the axonal membrane as suggested by other groups (Cardenas et al., 2001; Ma et al., 2017). There is a vast amount of evidence for the role of protein kinases in the alteration of nociceptor function through phosphorylation of ion channels found along peripheral nociceptors as reviewed by several groups (Basbaum et al., 2009; Linley et al., 2010a). Reports have also demonstrated the contribution of second messenger pathways underlying the sensitising effects of histamine, PGE₂ and bradykinin (Mizumura et al. 2000; England et al. 1996). Moreover, it was reported that ATP and BK-induced increase in the number of low voltage activated-positive neurons in DRG neurons was abolished after treatment with a PLC-inhibitor, pointing towards the involvement of PLC second messenger pathway downstream of these pro-inflammatory agents (Huang et al., 2016). Further, increased externalization of EP₄ receptors has been suggested to be responsible for the sensitising effects of PGE₂ (Ma & St Jacques, 2013). There is also a vast amount of literature linking modulation of VGSCs via cAMP/PKA/PKC pathways to the sensitizing effects of 5HT, BK and PGE₂ (Cardenas et al., 2001; Evans et al., 1999; Gold et al., 1998a). However, these studies focus primarily on mechanisms taking place at the DRG cell bodies *in vitro*. It would be interesting to see whether these mechanisms are conserved at the axonal level.

7.1.5. Limitations of MFC-system

An important caveat of this system is that axons and nerve terminals are taken away from their physiological conditions within a living organism. Although isolating nerve endings enables the study of intrinsic properties of nociceptors, isolation of nerve endings from their biological milieu in itself may alter their properties. For instance, the dissociation process in itself involves an axotomy of the dorsal roots, and has been reported to cause hyperexcitability in DRG neurons (Zheng et al., 2007). We aim to overcome this important caveat by using a warmed, perfused physiological solution to mimic the *in vivo* conditions as closely as possible, and demonstrate that DRG axons in MFCs exhibit key aspects of nociceptors such as responsiveness to different chemical stimuli as well as sensitisation. Thus, we propose that studying axonal based mechanisms serves as an important complement, to the traditionally-used DRG cell bodies, as it serves as a better model of *in vivo* free nerve endings. Furthermore, using changes in KCl responses as a measure of sensitisation is not representative of sensitisation to all the different modalities of pain (i.e. thermal, mechanical). However, our assay robustly demonstrates sensitisation to chemical stimuli and enables reproducible and sequential experimentation in MFC cultures without causing desensitisation of neurons, which typically occurs with the use of capsaicin. NGF is known for its potent sensitising effects, and has been reported to cause sprouting of neurons (Mearow and Kril, 1995), therefore growing the axons in the presence of media containing NGF for 5-6 days could potentially sensitise neurons. In efforts to avoid this confounding factor when interpreting the data, our assay enables comparisons of changes in response to IM compared to a baseline response with the aim of ensuring that the changes observed are due to the IM treatment itself. In future, it would be interesting to test whether it is possible for adult

DRG axons to be readily maintained and cross over to the axonal compartment in the MFC system, without the presence of NGF in media. Finally, another important limitation of this study does not evaluate the recovery to IM- induced sensitisation which could also provide valuable insights to understanding nociceptor sensitisation at the axonal level.

In summary, our model allows the study of cell-autonomous mechanisms at an axonal level using the combination of the MFC system with calcium imaging, as an extremely powerful tool for studying functional properties of DRG axons, across populations of neurons. Furthermore, it recapitulates key characteristics of human chronic pain conditions, such as sensitisation of primary afferents by inflammatory mediators. Combining the developed MFC-based *in vitro* axonal hypersensitivity model with genetic, and pharmacological strategies, it would be possible to interrogate nociceptive axons to elucidate molecular mechanisms underpinning peripheral sensitisation during painful conditions.

7.2. Identifying axonal-based mechanisms underlying PGE2-induced nociceptor sensitisation and activation

In chapter 4, we exploit the *in vitro* MFC system to study the effects of PGE2 on axonal excitability *in vitro*. Firstly, we demonstrate that treatment with PGE2 on its own can sensitise as well as excite DRG axons. We made two intriguing observations 1) PGE2 stimulus induced the generation of axonal signals, recorded at the cell body as repetitive Ca^{2+} transients, 2) PGE2 induced- axonal activity persisted for 40 minutes even after the wash out of PGE2. Further, we demonstrate that axonal ongoing activity can be blocked by the perfusion of lidocaine to the middle axonal compartment (**Figure 23**). This strongly suggests that the treatment of distal axons with PGE2 results in the generation and propagation of repetitive bursts of action potentials in sensitised axons which arrive at the cell bodies, which we were able to detect as persistent Ca^{2+} transients. It also provides further evidence in favour of the idea that peripheral mechanisms are key drivers of ongoing pain. In line with this, the lidocaine patch 5% has also been shown to reduce the intensity of pain significantly in patients with moderate-to-severe osteoarthritis (Gammaitoni et al., 2004). Moreover, the observation that PGE2 induces persistent activity in nociceptive axons suggests the possibility of such mechanisms to underpin clinical ongoing pain, where patients experience significant ongoing pain despite the resolution of the initiating cause of the pain response. For instance, there is increasing evidence suggesting that a large subpopulation of RA patients in remission experience significant persistent pain and inflammatory disease activity does not correlate with elevated pain (Lee et al., 2011). Similarly, a significant number of IBD patients experience persistent pain despite the resolution of mucosal

inflammation (Bielefeldt et al., 2009). Further experiments will shed light on the connection if any. Nevertheless, we conclude that our MFC platform recapitulated salient features of inflammatory pain sensitisation and serves as a useful tool to further understand molecular mechanisms underlying peripheral sensitisation in inflammatory pain conditions.

7.2.1. Molecular mechanisms of PGE2 mediated axonal activity

The study of electrophysiological properties of axons and detailed understanding of voltage-gated axonal ion channels would require patch clamping onto a section of an extremely thin axonal structure. These techniques are extremely difficult, requiring a lot of training and expertise and do not allow the study of axonal mechanisms in isolation of the cell body. Recently, a new generation of voltage sensitive dyes have been developed to be able to monitor subthreshold events at multiple areas along the axonal membrane, across a population of neurons (Miller et al., 2012). To our knowledge, the ability of inflammatory mediators to induce localised changes in membrane potential along the DRG axon have not been demonstrated previously. By using voltage imaging dyes in our *in vitro* MFC system, we were able to detect changes in membrane potential of fluidically isolated DRG axons in response to treatment with KCl as well as IMs. Our results show for the first time that PGE2 induces a localized membrane depolarization of the axonal membrane. In agreement with our Ca^{2+} imaging recordings demonstrating persistent activity arriving at the soma long after PGE2 application, the depolarisation induced by PGE2 persisted despite washout of PGE2. This suggests that the sustained depolarisation induced by a short PGE2 treatment is responsible for the repetitive Ca^{2+} transients detected at the DRG soma.

In order to understand the mechanism underlying this phenomenon we investigated the potential role of second messenger signalling pathways. As mentioned earlier, PGE2 exerts a multitude of effector functions via the activation of second messenger signalling pathways (Park et al., 2004; Qian et al., 2011; Steinert et al., 2009; Su et al., 2008). Several lines of evidence have suggested the role of cAMP/PKA in ion channel modification, underlying the sensitizing effects of PGE2 in DRG cell bodies *in vitro* (Evans et al., 1999; Gu et al., 2015; Gu et al., 2003). Thus, we investigated whether cAMP/PKA pathway had a role to play in ongoing activity induced by PGE2 at the axonal level in our assay. We used Rp-cAMPS which is an analogue of the naturally occurring cAMP that competitively binds to cyclic nucleotide binding domains on PKA and prevents the dissociation and subsequent activation of PKA (Schwede et al., 2000). We found that administration of Rp-cAMPS reversibly blocked ongoing Ca²⁺ transients induced by PGE2 at an axonal level, suggesting the involvement of cAMP/PKA second messenger pathways. It would be interesting to confirm these results by using specific PKA blockers with a distinct mechanism of action, and testing to see whether inhibition of axonal activity occurs similar to that observed with Rp-cAMPS.

In addition, we investigated the key axonal ion channels responsible for the observed PGE2-induced persistent axonal activity. We found that TTX had no effect on PGE2-induced repetitive transients suggesting that TTX-sensitive channels including Nav1.7 do not play a role in PGE2-induced axonal activity. In contrast to our data, Nav1.7 global rodent KOs display reduced C-fibre spiking activity contributing to loss of inflammatory pain phenotype (Gingras et al., 2014). However, there are increasing reports arguing that the observed insensitivity to noxious stimuli observed in Nav1.7

KOs is due to compensatory mechanisms such as increased endogenous opioids, and decreased serotonergic signalling (Isensee et al., 2017; Minett et al., 2015). We also assessed the involvement of axonal Nav1.8 channels in the local effects of PGE2. Using PF-01247324, a Nav1.8 selective blocker, we demonstrated that Nav1.8 channels play an essential role in PGE2-induced ongoing activity at the axonal level, as the propagation of PGE2-induced axonal signals was blocked after perfusion of the Nav1.8 blocker to the middle axonal compartment. The role of Nav1.8 has been investigated using KO mice, where studies have shown that the development of thermal hyperalgesia induced by carrageenan was significantly delayed in Nav1.8 KO mice (Akopian et al., 1999). Conversely, it has been shown that there was no effect of the genetic deletion on PGE2-induced thermal hyperalgesia (Kerr et al., 2001). However, the latter has been considered to be largely attributed to compensatory mechanisms taking place in the Nav1.8 knockout mice, exhibiting an upregulation of other TTX-R channels (Akopian et al., 1999). In line with our findings, PGE2-induced hyperalgesia was shown to be reduced by antisense Nav1.8 in rats (Khasar et al., 1998). Moreover, PF-01247324 acts as a selective antagonist for Nav1.8 and in both rat and human DRG neurons, and application of the drug results in reduced repetitive firing induced by current injection (Payne et al., 2015a). The same study used current clamp recordings to show that in presence of 1uM PGE2, the percentage inhibition of AP number following application of PF-01247324 was reduced, suggesting a strong role for Nav1.8 underlying the sensitizing effects of PGE2 at the DRG cell body (Payne et al., 2015b). Our data provide further evidence in favour of the idea that PGE2-mediated persistent axonal activity occurs in Nav1.8-positive neurons and that these channels may play an important role in ongoing pain associated with inflammatory pain conditions. A

limitation of the work presented however, is the lack of vehicle controls used to demonstrate that the addition of vehicle solution only does not have an effect on PGE2-induced ongoing activity. Further, for future experiments, it would be interesting to test whether PGE2-induced long-lasting axonal activity is still present after the genetic deletion of Nav1.8. Moreover, to gain further insight into the molecular players responsible for PGE2-induced depolarisation, the effects of different blockers targeting Nav1.8 or the cAMP/PKA pathway on the changes in axonal membrane potential induced by PGE2, using voltage imaging techniques with Fluovolt dye could be tested.

Further, we demonstrate preliminary data showing the expression and distribution pattern of EP4 receptor on isolated nociceptive axons *in vitro*. All four PGE2 receptor subtypes (EP1-4) are known to be expressed by DRGs, whereas the contribution of EP4 receptors to inflammatory pain hypersensitivity has been highlighted by several groups. For instance, the upregulation of EP4 receptor expression in DRG cell bodies, and not of EP1-3 receptors, following CFA-induced inflammation has been reported (Lin et al., 2006). In line with this, another group suggested axonal anterograde trafficking of EP4 receptors to the nerve terminals as a potential mechanism underlying PGE2-induced nociceptor sensitisation. The same group also demonstrated increased synthesis and axonal trafficking of EP4 receptors in the sciatic nerve using western blots (St-Jacques and Ma, 2014). The pilot study also showed co-localisation of EP4 receptors and Nav1.8 ion channels at the axonal level. The anatomical proximity of these receptors along DRG axons points towards the activation of local signalling pathways downstream the effects of EP4 receptors, which can lead to modulation of Nav1.8 channel function during inflammatory pain

states. Whether the expression of channels along the end of peripheral axons accurately represents nerve endings *in vivo* is yet to be demonstrated. Further experimentation and quantification of data would be required to provide concluding evidence demonstrating co-localisation of Nav1.8 and EP4 receptors in the peripheral axons and whether this is comparable to what is observed in nerve terminals *in vivo*.

In summary, as illustrated in **Figure 44**, we propose that PGE2 acts on EP4 receptors found along the DRG axon, which leads to the activation of cAMP/PKA second messenger pathway. We also show that PGE2 application depolarises the axonal membrane leading to bursts of action potentials arriving at the cell bodies long after washout of PGE2 stimulus. As our data demonstrates, PKA activation and Nav1.8 are two important downstream mediators of PGE2 effect. The activation of PKA can lead to potentiation of Nav1.8 channels, it is unclear however, if this potentiation is responsible for the observed depolarization of the membrane. We hypothesize that PKA functionally modulates axonal Nav1.8 channels which are responsible for repetitive Ca²⁺ transients arriving at the soma (**Figure 44**). HCN2 channels are also likely candidates activated by cAMP downstream of EP4 receptor activation. However, as discussed in the following section in detail, the data from HCN2 KO mice did not agree with the pharmacological blockade of the channels making it difficult to formulate conclusions with regards to the role of HCN2 in PGE2-induced depolarisation of DRG axons. Therefore, further experiments will be required to determine the mediators of the depolarisation induced by PGE2.

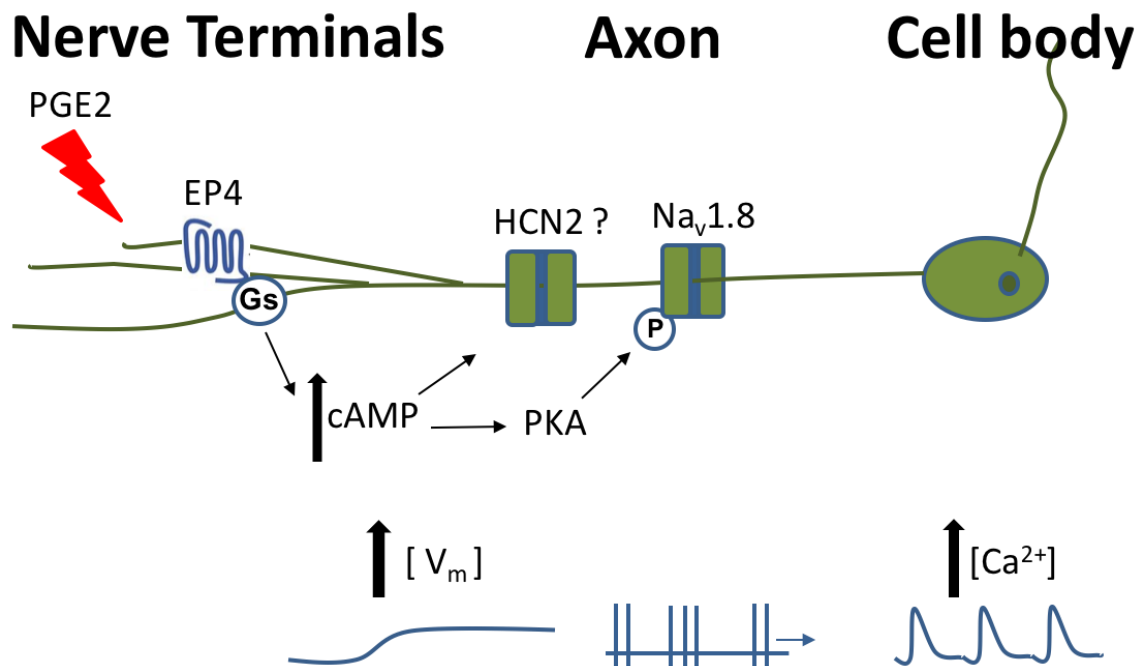


Figure 44. Proposed mechanism underlying PGE2-induced ongoing axonal activity.

PGE2 acts on EP4 receptor, which stimulates local cAMP/PKA secondary messenger pathway. We postulate that PKA activation results in phosphorylation of Nav1.8 channel leading to the opening of Nav1.8 channel which is partially responsible for persistent volleys of APs propagating to the cell body, detected as repetitive Ca^{2+} transients. However, the mechanism by which PGE2 causes a sustained depolarisation at the axonal membrane is yet unclear.

7.3. *Evaluating the role of HCN2 in peripheral inflammatory sensitisation*

7.3.1. Role of HCN2 channels in peripheral inflammatory sensitisation

The McNaughton group published a study in 2011 suggesting an important role for HCN2 channels in both inflammatory and neuropathic pain (Emery et al., 2011b). Behavioral data suggested that PGE₂-induced heat and mechanical hyperalgesia was abolished in conditional Nav1.8/ HCN2^{-/-} mice. Similarly, in a commonly used model of neuropathic pain, the CCI model, heat and mechanical hyperalgesia as well as cold allodynia were abolished in conditional HCN2 knockout mice. This led to the hypothesis that HCN2 channels in Nav1.8 expressing neurons are pivotal for originating neuropathic pain, as well as for certain aspects of inflammatory pain (Emery et al., 2011). The role of HCN2 in neuropathic pain was confirmed using another model and the snsHCN2KO mutant mouse (Schnorr et al., 2014). Thus, we decided to explore these findings using our model of IM-induced axonal hypersensitivity. In agreement with these findings, non-specific block of HCN channels with Zatebradine reversed IM-mediated axonal sensitization. Zatebradine is structurally, very closely related to Ivabradine, which is clinically used as a treatment for chronic stable angina and heart failure. The antagonists only differ in their binding sites to HCN channels (Bucchi et al., 2002).

The compatibility of our model with the use of transgenic lines, enabled us to validate these results using genetic manipulations. HCN2^{-/-} global KO were used in this study. HCN2^{-/-} KO mice however, are ataxic and rarely live past 5 weeks. We found that IM-induced axonal sensitization observed in both adult and younger mice of C57bl/6 strain, was not reproducible in this HCN2^{+/+} WT littermates. We concluded that the

genetic background of transgenic mice may explain the discrepancy between findings, as IM-induced axonal sensitization was not an age-dependent effect as sensitisation of axons was clear in DRG from younger mice. Other groups have reported similar findings, where they demonstrate that peripheral inflammation alters electrophysiological properties of neonatal rat dorsal horn neurons (Torsney & Fitzgerald, 2001). Moreover, a study compared nociceptive behaviour in 12-week old mice and 6-week old in two behavioral models and showed that aged mice presented a reduced nociceptive response to zymozan and formalin (King-Himmelreich et al., 2015). Based on both pharmacological and genetic strategies used, it is difficult to conclude whether HCN2 channels play an important role in IM-induced sensitization of nociceptors at the axonal level.

7.3.2. Role of HCN2 channels as a downstream mediator of PGE2-induced ongoing activity

Emery et al., (2011) hypothesized that release of the inflammatory mediator PGE2 at the site of injury and/or inflammation results in activation of adenylate cyclase, leading to increased cAMP levels and modulation of HCN2 channels promoting repetitive action potential firing activity. In *vitro* electrophysiology studies carried out in the DRG somata, demonstrated that forskolin or PGE2 was responsible for accelerated action potential firing in response to current injection, which was absent in conditional Nav1.8/ HCN2^{-/-} mice.

We explored this further and hypothesized that activation of HCN2 channels downstream of PGE2 could be responsible for depolarisation of the axonal membrane, subsequently leading to ongoing axonal activity observed in our assay. We used a selective HCN2 blocker, developed by the McNaughton lab, and found that blocking axonal HCN2 channels with this drug silenced PGE2- induced axonal

ongoing activity. This result supported the idea proposed by Emery and co-workers, pointing towards the role of HCN2 channels as downstream effector of PGE2 pathway via modulation by increased intracellular cAMP levels.

Following the pharmacological results, we further tested the involvement of HCN2 channels in ongoing activity generated in DRG axons using HCN2 global knockout mice, and hypothesized that PGE2-induced ongoing axonal activity, observed as repetitive Ca²⁺ transients at the soma would be abolished in with a global HCN2 KO DRG neurons. However, we found that PGE2-induced repetitive Ca²⁺ transients were present in both WT and KO mice, suggesting that ongoing activity induced by localized PGE2 does not require HCN2 channels.

Two possible reasons may provide an explanation for these findings. Although there is no direct evidence for this, we speculate that the global deletion of HCN2 could have led to compensatory mechanisms in these mice, resulting in compensatory upregulation of other HCN subtypes in the HCN2 global knockouts which could be responsible for mediating PGE2-induced effects observed in the HCN2 KO DRG axons. Another plausible explanation could be that a combination of ion channels contributes towards spontaneous activity induced by PGE2 at the axonal level. PGE2 increases intracellular cAMP concentrations, and has also been linked to activation of PKA. PKA has been demonstrated to phosphorylate a number of channels such as TRPV1 and TRPA1 (Brackley et al., 2017). For instance, A-kinase anchoring protein (AKAP), a regulatory protein of PKA was shown to facilitate PKA-mediated phosphorylation of peripheral TRPV1 and TRPA1 channels in *ex vivo* preparations of DRG neurons. Moreover, Nav1.8 channels have also been linked to PGE2-mediated effects as discussed and demonstrated in the previous section (Liu

et al., 2010; Payne et al., 2015a). Therefore, PGE₂-induced increase in cAMP, and subsequent activation of PKA is likely to act on a number of ion channels, which collectively contribute to hyperexcitability and peripheral sensitisation in inflammatory pain states.

We conclude that further research is required to establish the role of axonal HCN2 channels in IM-induced sensitisation of nociceptive axons. Moreover, it is likely that HCN2 channels are not solely responsible for PGE₂ mediated effects at an axonal level. A combination of other channels including TRPV1, TRPA1 and Nav1.8 could be potential candidates collectively responsible for PGE₂-mediated persistent axonal activity observed in our assays.

7.4. Development of an *in vitro* model of pain signalling using microfluidic-based cultures of hIPSC-derived sensory neurons

IPSCs have become key tools for disease modelling, particularly in the case of cell types such as sensory neurons which are difficult and expensive to obtain from donors. In chapter 6, we extend the use of the MFC system to IPSC-derived sensory neurons with the aim of developing an *in vitro* platform enabling the study of the functional properties of human sensory axons from human IPSC-derived neurons.

Human primary DRG neurons have been used for *in vitro* modelling of pain in conventional cultures. Although primary human DRG neurons are a potentially better model for understanding molecular mechanisms, their availability is limited. Additionally, primary DRG neurons need to be excised in order to be plated in culture. Using hIPSC-derived sensory neurons enables the study of naïve and intact sensory neurons whereas both rodent and humans is that dissociation, and dissociation-induced stress has been shown to alter electrophysiological properties of neurons, making neurons hyperexcitable to many mediators such as cAMP- and cGMP. Hyperexcitability of DRG neurons caused by dissociation was similar to that observed in DRGs from chronic compression treatment (Zheng et al., 2007). Thus, not only does the use of hIPSC-derived sensory neurons serve as a more clinically relevant system for disease modelling but also allows the study of naïve axons, in comparison to those exposed to injury or inflammatory insult.

7.4.1. Optimisation of culture conditions for adequate cell attachment of hIPSC-derived sensory neurons

We establish that 1-hour incubation with both ReadySet and 1-hour incubation with Surebond (coating substrates provided by Axol Biosciences) are optimal for attachment of PDMS to Willco glass plates, and for subsequent attachment of iPSC-derived sensory neurons to glass surface within MFC devices. Matrigel coating lead to inadequate cell adherence resulting in clumping of cell bodies and eventual detachment from plates by 2 weeks post-plating. Hence, matrigel was not used for future experiments despite being the coating substrate of choice used by other groups previously (Young et al., 2014b). 24-hour incubation with ReadySet results in axonal degeneration, and eventual death. We speculated that ReadySet may potentially have toxic effects on neurons themselves (Personal communication, Axol Biosciences). Therefore, thorough washing is required after 1 hour incubation to ensure optimal attachment and growth of axons into the peripheral terminal and axonal compartments of 3-channel MFC devices.

7.4.2. Characterisation of the functional properties of hIPSC-derived neurons at 4 weeks *in vitro*

Concerns have been raised with regards to variability reported between hIPSC-derived cell lines from different donors due to epigenetic memory maintained from different somatic cell types, in addition to variation in genetic and epigenetic factors due to inefficient reprogramming and prolonged periods in culture (Bar-Nur et al., 2011; Lister et al., 2011; Newman and Cooper, 2010). In agreement with this view, a study reported that cell lines derived from different cell types of the same donor are show less genetic heterogeneity than cell line derived from same tissue type but different donors (Rouhani et al., 2014). Moreover, some differences in epigenetic markers and transcriptomes when testing 16 iPSC derived cell lines from donors of

different sex, age and health status were also reported (Boulting et al., 2011). However, the extent to which these impacted the subsequent terminally differentiated cells was not clear. Thus, these studies point towards the importance of evaluation and characterization of phenotypic markers between different batches and cells derived from different donors.

The expression of important sensory neuronal markers in hiPSC-derived sensory neurons was validated in a recent study, giving evidence for the presence of Kv channels, HCN and ASIC channels by day 9-16 *in vitro* (Young et al., 2014a). In our study, we investigated the functional properties of these cultures at 4 weeks post-plating (DIV 30) in the presence of 25ng/ml NGF, GDNF, NT-3 and BDNF. We found that cell bodies are responsive to KCl, demonstrating similar responses to chemical stimuli, as rodent DRGs. Additionally, at 4 weeks post-plating we were able to detect axonal responses to KCl. We observed axonal responses to 35mM and 65mM KCl, recorded with calcium imaging at the soma similar to those observed in our rodent MFC system. However, this was highly variable between coverslips as in some cases axons were unresponsive, even to high KCl concentrations. Therefore, further optimisation is required to be able to study functional properties of hiPSC-derived sensory neuronal axons. We hypothesize, that 30 days *in vitro* may not be sufficient for maturation of human axons and expression of relevant ion channels in axons. For instance, longer differentiation period may allow ion channels to be transported from the cell bodies to the distal segments of the axons.

The expression of TRP channels, which are markers of mature sensory neurons and play an important role in peripheral sensitisation, has not been well characterised in

iPSC-derived sensory neurons. Surprisingly, in our hands, we found no responders to capsaicin or AITC, indicative of a lack of TRPV1- and TRPA1-positive in hIPSC-derived sensory neurons. Responses to icillin were detected pointing towards the presence of TRPM8-positive neurons, with 33% of average responders which is higher than what is usually observed in mouse DRG cultures where approximately 20% of cell bodies respond to icillin (Bautista et al., 2007). Another interesting observation from these preliminary findings was that cell bodies of hIPSC-derived sensory neurons can be sensitised after inflammatory insult despite the lack of capsaicin sensitivity observed. This points towards the idea that that human-derived sensory neurons can be sensitised by inflammatory mediators in the absence of functional TRPV1 in these neurons.

Furthermore, we found that capsaicin sensitivity appeared to be dependent on the level of GDNF concentration. In the presence of low GDNF concentration (25ng/ml), no capsaicin sensitivity was observed, whereas by increasing the level of GDNF by 5-fold in these cultures cell bodies became capsaicin-responsive. In the presence of 125ng/ml GDNF, hIPSC-derived neuronal cell bodies displayed 30% capsaicin sensitivity. Using immunohistochemistry, it has been previously demonstrated that GDNF and NGF facilitate an increase TRPV1 expression in IB4-positive and trkA neurons, respectively, in rodent DRGs. Whereas NGF-mediated increase in TRPV1 expression is rapid and transient, GDNF-mediated effects on TRPV1 appeared to be gradual lasting approximately one week (Amaya et al., 2004). There has been contrasting evidence with regards to GDNF-induced TRPV1 expression in murine DRGs. On study showed that transgenic mice with overexpressed GDNF did not display increased TRPV1 expression, and did not show potentiated responses to

noxious heat *in vivo* (Zwick et al., 2002). Moreover, GDNF overexpressing transgenic mice displayed a decrease in ganglionic TRPV1 mRNA levels (Albers et al., 2006). In contrast, adult mice DRG cultures showed potentiated capsaicin responses as well as capsaicin-induced release of CGRP from nerve terminals, after exposure to GDNF (Schmutzler et al., 2009). In our study, exposure to high GDNF concentrations led to higher capsaicin sensitivity which was absent in the presence of low GDNF concentrations. However, it is unclear whether higher GDNF concentrations drove the neuronal differentiation process towards TRPV1-positive populations or whether exposure to high levels of GDNF, sensitised iPSC-derived sensory neurons resulting in increased capsaicin sensitivity.

iPSCs are an invaluable tool for both a more accurate mechanistic understanding of diseases as well as for more efficient drug screening. However, our preliminary data suggest that current variability in the cultures and batches may limit their application beyond testing of analgesic compounds. For future use and the compatibility of iPSCs with the MFC system, further optimization of culture conditions is required to yield a more representative population of neurons maintained for longer periods. Maintaining hiPSC-derived sensory neurons in culture for longer periods would ensure their maturity, and expression of canonical markers in the neuronal cell bodies as well as at an axonal level.

7.5. Conclusions and Future Directions

We have developed a robust *in-vitro* model that mimics key aspects of peripheral pain circuitry and allows the study of peripheral sensitisation in inflammatory pain. We have shown that using microfluidic cultures provides a convenient platform for axonal molecular mechanisms to be studied in detail in the context of pain as well as other neurological disorders. Combining other recording techniques such as patch clamp electrophysiology or microelectrode arrays (MEA) with MFC culture platform would enable gathering more detailed information on ionic mechanisms responsible for changes in axonal function during painful conditions.

Non-neuronal cells play an important role in modulating pain responses. Hence, it would be interesting to study the influence of non-neuronal cells on IM-induced axonal sensitisation. The influence of non-neuronal cells on IM-induced effects could be investigated by testing whether IM-induced effects are altered in purified DRG neuronal cultures, using a magnetic assisted cell sorting (MACS), which isolates predominantly small-diameter neurons (Thakur et al., 2014). Understanding the role of non-neuronal cells in axonal sensitisation *in vitro* would help us gain understanding of the extent to which non-neuronal cells influence neuronal function during health and disease. Moreover, understanding the cross-talk between the immune and nociceptive systems is an attractive area of research. Although mast cells and macrophages have been pointed out as important players responsible for the release of inflammatory mediators leading to subsequent sensitisation of nociceptors, the impact of other immune cells is yet to be characterised. The use of

microfluidic platforms can be extended to the use of co-cultures in order to understand interactions between specific immune cells and nociceptors which could shed light on mechanisms underlying the modulation of nociceptors by the immune system and vice versa.

Here, we propose an axonal-based mechanism underlying ongoing pain in during inflammatory pain conditions. We show for the first time that inflammatory mediators can cause direct depolarisation of axons, resulting in a volley of action potentials arriving at the cell soma and as such, demonstrate the importance of voltage changes in the peripheral terminals and axons in the context of inflammatory pain. We suggest that PGE₂-induced repetitive axonal activity is caused by a local depolarisation, which persists beyond PGE₂ application. We also show evidence for the cAMP/PKA pathway, and Nav1.8 channel as important mediators of ongoing axonal activity induced by PGE₂. In future, understanding of key mediators underlying inflammation induced ongoing activity could lead to therapeutic approaches for the management of ongoing pain observed in arthritis or IBD patients.

Finally, we optimised and characterise the functional properties of hiSPC-derived sensory neurons in the MFC system. Optimal coating conditions were established to readily maintain induced sensory neurons in culture for 4 weeks. We also provide proof of concept for the idea that hiSPC-derived sensory neurons can be used in MFC platform as an *in vitro* model of human nociceptor sensitisation. We also show that the cell bodies can be sensitised by inflammatory mediators in absence of TRPV1 channels, and that TRPV1 expression in these neurons is GDNF-dependent.

Further optimisation and characterisation of this model using hIPSC-derived nociceptive neurons will deliver a versatile platform to model peripheral pain signalling *in vitro*.

The combination of changes taking place in the peripheral nerve terminals, spinal cord and brain collectively contribute towards altered pain processing responsible for the transition from acute to chronic pain. Using the MFC platform, we were able to model one of these key features by demonstrating sensitisation of peripheral nociceptors. Our model does not account for sensitisation to different pain modalities such as thermal and mechanical stimuli, however we do demonstrate key characteristics of sensitisation to chemical stimuli: decreased activation threshold and increased magnitude of responses. The main players responsible for these phenomena however, are yet to be elucidated. Alterations in the expression and activity of channels such as TRPV1 following tissue injury and inflammation suggest that important peripheral changes do take place and other such changes may contribute towards altered processing of pain signals originating in response to noxious stimuli in the peripheral nerve endings.

Moreover, the use of microfluidic platforms is extremely useful to provide a detailed profile of the differences between mechanisms taking place in different subcellular components as well as between different cell-types. The present work describes the compatibility of the use of hIPSC cell lines with the MFC system, however primary human DRG neurons could also be used to evaluate whether mechanisms of nociceptor sensitisation in these are comparable to results observed using rodent and IPSC-derived DRG axons. Elucidating peripherally restricted mechanisms in

human DRG neurons could provide useful information for future drug therapies aimed to target the periphery, thereby avoiding systemic side effects.

8. REFERENCES

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