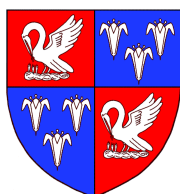


Proton-sensing G protein-coupled receptor signalling in inflammation

This thesis is submitted for the degree of Doctor of Philosophy



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i. Preface

This thesis is the result of my own work and includes nothing which is the outcome of work done in collaboration except as declared in the Preface and specified in the text. I further state that no substantial part of my thesis has already been submitted, or, is being concurrently submitted for any such degree, diploma or other qualification of the University of Cambridge or any other University or similar institution except as declared in the Preface and specific in the text. It does not exceed the prescribed word limit for the Faculty of Biology Degree Committee.

Some of the work discussed here has been published (or is available as a pre-print):

Pattison, L.A., Callejo, G. and Smith, E.St.J., 2019. Evolution of acid nociception: ion channels and receptors for detecting acid. *Phil. Trans. R. Soc. B* **374**:20190291.

Pattison L.A., Krock E., Svensson C.I. and Smith E.St.J., 2021. Cell-cell interactions in joint pain: rheumatoid arthritis and osteoarthritis. *PAIN*. **162**(3), 714-717.

*Bohic M., *Pattison L.A., Jhumka Z.A., Rossi H., Thackray J.K., Ricci M., Foster W., Arnold J., Mossazghi N., Tischfield M.A., Yttri E.A., Smith E.St.J., Abdus-Saboor I. and Abaira V.E., 2021. Mapping the signatures of inflammatory pain and its relief. *bioRxiv*. doi: 10.1101/2021.06.16.448689
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Work contributed to during doctoral studies, relevant to the overall theme of this thesis, but not discussed here includes:

Chakrabarti S., Pattison L.A., Singhal K., Hockley J.R.F., Callejo G., and Smith E.St.J., 2018. Acute inflammation sensitizes knee-innervating sensory neurons and decreases mouse digging behavior in a TRPV1-dependent manner. *Neuropharmacology*. **143**, 49–62.

*Lee M.C., *Nahorski M.S., *Hockley J.R.F., *Lu V.B., Ison G., Pattison L.A., Callejo G., Stouffer K., Fletcher E., Brown C., Drissi I., Wheeler D., Ernors P., Menon D., Reimann F., Smith E.St.J., and Woods C.G., 2020. Human Labor Pain Is Influenced by the Voltage-Gated Potassium Channel KV6.4 Subunit. *Cell Rep*. **32**, 107941.
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Callejo G., Pattison L.A., Greenhalgh J. C., Chakrabarti S., Andreopoulou E., Hockley J.R.F., Smith E. St.J., and Rahman T., 2020. In silico screening of GMQ-like compounds reveals guanabenz and sephin1 as new allosteric modulators of acid-sensing ion channel 3. *Biochem. Pharmacol*. **174**, 113834.

Chakrabarti S., Hore Z., Pattison L.A., Lalnunhlmi S., Bhebhe C.N., Callejo G., Bulmer D.C., Taams L.S., Denk F., and Smith E.St.J., 2020. Sensitization of knee-innervating sensory neurons by tumor necrosis factor- α activated fibroblast-like synoviocytes: an in vitro, co-culture model of inflammatory pain. *PAIN*. **161**(9), 2129-2141.

Chakrabarti S., Pattison L. A., Doleschall B., Rickman R.H., Blake H., Callejo G., Heppenstall P.A., and Smith E.St.J., 2020. Intra-articular AAV-PHP.S mediated chemogenetic targeting of knee-innervating dorsal root ganglion neurons alleviates inflammatory pain in mice. *Arthritis Rheumatol*. **72**, 1749-1758.

Signed [Signature redacted]

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ii. Summary

Proton-sensing G protein-coupled receptor signalling in inflammation

Luke A. Pattison

Inflammation, the body's response to tissue damage or infection, involves a number of cell types and is often associated with localised tissue acidosis. The resulting decrease in extracellular pH can exacerbate inflammation, additionally protons may directly depolarise or sensitise sensory neurones to drive the heightened nociception associated with chronic inflammatory diseases such as rheumatoid arthritis and inflammatory bowel disease. Extracellular protons can coordinate cellular responses through a number of proton-sensitive receptors including both ion channels and G protein-coupled receptors, many of which are expressed by immune cells and sensory neurones, the principal drivers of inflammation and nociception respectively. While proton-sensitive ion channels, such as the acid-sensing ion channel family have established roles in inflammatory pain, the contribution of proton-sensing G protein-coupled receptors (PS-GPCRs) is less clear. The overarching aim of this work was to further basic pharmacological understanding of the PS-GPCR family and assess their role in inflammatory processes and nociception.

The pain associated with chronic inflammation is severely debilitating and represents major unmet clinical need worldwide, there is thus considerable interest in elucidating the molecular mechanisms underlying inflammatory pain. Animal studies represent a valuable tool for studying the complexities of inflammation, which involves a concert of cell types. However, the interplay between changes at the cellular level and the manifestation of hypersensitive states in rodent models is often poorly assessed. Here, combining evoked and spontaneous behavioural assays, target innervating electrophysiology and gene expression studies, a comprehensive characterisation of sensory neurones and pain behaviour was made for the intraplantar carrageenan model of inflammatory pain in mice. Results highlighted a dynamic relationship between physical inflammation, changes in sensory neurone function and rodent pain experience, and suggested a potential role of PS-GPCRs in the observed pain.

To further explore the possibility that PS-GPCRs might mediate proton-induced pain, attention was turned to the naked mole-rat (*Heterocephalus glaber*), a species which does not perceive acid as painful. Cloning and characterisation of PS-GPCRs from naked mole-rats and mice revealed differences in the abilities of protons to coordinate signalling at PS-GPCRs between the two species, furthering the idea that PS-GPCRs might contribute to proton-induced pain.

The lack of pharmacological and physiological understanding of PS-GPCRs may be attributed to the fact that protons activate a number of other receptors, as well as technical difficulties arising from the high sensitivity of many pharmacological assays to perturbations in pH. Using a uniform cellular background, the signalling and intracellular trafficking of the PS-GPCR GPR65 was interrogated in response to stimulation with protons as well as two other reported agonists. These studies highlighted that a synthetic agonist (BTB09089), with higher selectivity for GPR65 over other PS-GPCRs, was able to recapitulate many of the proton-induced signalling responses of GPR65. Injecting BTB09089 into the knee joint of mice, resulted in joint swelling and a concomitant increase in nociceptive behaviours. Electrophysiological studies of knee-innervating neurones isolated from mice injected with BTB09089 revealed higher excitability, offering some explanation to the observed pain. However, acute stimulation of naïve neurones with BTB09089 alone could not reproduce the changes in excitability, suggesting the involvement of another cell type. Fibroblast-like synoviocytes, non-neuronal cells resident in the joint, were shown to express GPR65 and secrete pro-inflammatory mediators in response to BTB09089 stimulation, which might be responsible for the sensory neurone sensitisation observed.

Taken together data presented here support a central hypothesis that PS-GPCRs, in particular GPR65, are critical integrators of neuroimmune responses to tissue acidosis in inflammation

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Table of Contents

i. Preface.....	1
ii. Summary	2
iii. Acknowledgements.....	4
iv. List of abbreviations	9
v. List of figures.....	11
vi. List of tables	13
1. Introduction	14
1.1. Inflammation and pain.....	14
1.2. Inflammation and acidosis.....	17
1.3. Chronic inflammatory diseases and arthritic pain	18
1.4. Proton-sensitive receptors (ASICs, K2Ps and TRPs)	23
1.5. Proton-sensitive G protein-coupled receptors.....	28
1.6. Roles of PS-GPCRs in normal physiology	31
1.7. Proton-sensing GPCRs in inflammation.....	33
1.8. Aims and scope of thesis	39
2. Materials and Methods	41
2.1. Agonists, activators and inhibitors.....	41
2.2. Animals	41
2.2.1. Mice	41
2.2.2. Naked mole-rats	42
2.3. Intra-articular injections	42
2.4. Intra-plantar injections.....	42
2.5. Mouse behavioural studies	42
2.5.1. Digging behavioural paradigm.....	43
2.5.2. Dynamic weight bearing	43
2.5.3. Rotarod.....	43
2.5.4. Pressure application measurement.....	44
2.6. Isolation and culture of mice dorsal root ganglia (DRG)	44
2.7. Isolation and culture of mouse fibroblast like synoviocytes (FLS).....	45
2.8. Cell line culture and generation of stable cell lines	45
2.8.1. Chinese hamster ovary (CHO) cells	45
2.8.2. Flp-In Chinese hamster ovary (Flp-In CHO) cells.....	45
2.8.3. Generation of CHO cell lines stably expressing PS-GPCRs	46
2.9. RNA extraction and reverse transcription	46
2.9.1. RNA extraction from tissue.....	46
2.9.2. RNA extraction from cultured cells	46
2.9.3. Reverse transcription.....	47
2.10. Molecular cloning of PS-GPCR constructs	47
2.10.1. Site-directed mutagenesis	47
2.11. RT-PCR.....	47

2.12.	qPCR.....	48
2.12.1.	qPCR analysis to determine relative expression	50
2.12.2.	qPCR analysis to determine change in expression	51
2.13.	Immunocytochemistry	51
2.13.1.	Staining of cultured CHO cells.....	51
2.13.2.	Staining of FLS cells.....	51
2.14.	Immunohistochemistry	52
2.15.	Antibody array to assess inflammatory mediator release by FLS cells.....	52
2.16.	BRET β -arrestin recruitment assays	53
2.17.	BRET intracellular trafficking assays.....	53
2.18.	cAMP accumulation assays	54
2.19.	Ca ²⁺ mobilisation assays.....	54
2.20.	ERK phosphorylation assays	55
2.21.	DRG Electrophysiology	56
2.22.	Data analysis and statistics.....	57
3.	Temporal resolution of behaviour and nociceptor states following carrageenan-induced inflammation.	58
3.1.	Authorship and publication status statement	58
3.2.	Chapter Summary	58
3.3.	Introduction	59
3.4.	Methods	60
3.4.1.	Hargreaves test	60
3.5.	Results	61
		65
3.6.	Discussion.....	70
4.	Expression and activity of PS-GPCR homologs from mice and naked mole-rats.....	74
4.1.	Chapter Summary	74
4.2.	Introduction	74
4.3.	Methods	76
4.4.	Results	76
4.5.	Discussion.....	80
5.	Signalling and trafficking bias at the PS-GPCR GPR65.....	84
5.1.	Chapter Summary	84
5.2.	Introduction	84
5.3.	Methods	86
5.4.	Results	86
5.5.	Discussion.....	99
6.	A BTB09089-GPR65 signalling axis in inflammatory joint pain.	104
6.1.	Authorship statement	104
6.2.	Chapter Summary	104

6.3.	Introduction	105
6.4.	Methods	108
6.5.	Results	108
6.6.	Discussion	122
7.	Conclusions and Future Directions	126
8.	References	130

iv. List of abbreviations

ASIC	Acid-sensing ion channel	GI	Gastrointestinal
AUC	Area under the curve	GIRK	G-protein-activated inwardly rectifying potassium channel
BASIC	Bile acid-sensitive ion channel	H ⁺	Proton(s)
BRET	Bioluminescence resonance energy transfer	HBSS	Hanks' balanced salt solution
BSA	Bovine serum albumin	Hg	<i>Heterocephalus glaber</i>
BtB	BTB09089	IBD	Inflammatory bowel disease
Ca _v	Voltage-gated Ca ²⁺ channel	IL	Interleukin
CCL	Chemokine (C-C motif) ligand	iNOS	Inducible nitric oxide synthase
cDNA	Complementary DNA	IP ₃	Inositol trisphosphate
CFA	Complete Freund's adjuvant	Ipsi.	Ipsilateral
CGRP	Calcitonin gene-related peptide	K2P	Two-pore potassium channel
CHO	Chinese hamster ovary	KC	Keratinocyte chemoattractant
CoH	Coelenterazine H	LAMP1	Lysosome-associated membrane glycoprotein 1
Contra.	Contralateral	MCH-R1	Melanin-concentrating hormone receptor 1
COX	Cyclooxygenase	Mm	<i>Mus musculus</i>
CRE	cAMP response element	Na _v	Voltage-gated Na ⁺ channel
CXCL	Chemokine (C-X-C) ligand	NGF	Nerve growth factor
DAPI	4',6-diamidino-2-phenylindole	NMR	Naked mole-rat
DRG	Dorsal root ganglia	NSAID	Nonsteroidal anti-inflammatory drug
DSS	Dextran sulphate sodium	OA	Osteoarthritis
FLS	Fibroblast like synoviocyte	PDBu	Phorbol 12, 13-Dibutyrate
FRET	Fluorescence resonance energy transfer	PFA	Paraformaldehyde
FSK	Forskolin	PG	Prostaglandin

PS-GPCR	Proton-sensing G protein-coupled receptor	SNP	Single nucleotide polymorphism
PS2	PitStop2	SRE	Serum response element
PSY	Psychosine	TLRs	Toll-like receptors
qPCR	Quantitative polymerase chain reaction	TNF	Tumour necrosis factor
RA	Rheumatoid arthritis	VEGF	Vascular endothelial growth factor
RT-PCR	Reverse-transcription polymerase chain reaction	YAP	Yes-associated protein
SD	Standard deviation	YFP	Yellow fluorescent protein
SEM	Standard error of the mean		

v. List of figures

- Figure 1.1 Pathophysiological aspects of RA and OA.
- Figure 1.2 Membrane topologies of proton-sensitive receptor subunits.
- Figure 1.3 Proton-sensing at the peripheral terminal.
- Figure 1.4 Mechanisms of PS-GPCR activation.
- Figure 2.1 Annotation of parameters analysed from action potentials generated in current clamp mode.
- Figure 3.1 Unilateral injection of carrageenan into the hind paw of mice results in swelling, thermal hypersensitivity and weight bearing deficits.
- Figure 3.2 Sensory neurones innervating carrageenan injected hind paws are more excitable than those innervating non-carrageenan injected hind paws during early inflammation.
- Figure 3.3 Changes in sensory neurone macroscopic voltage-gated currents during inflammation.
- Figure 3.4 Expression changes within hind paw innervating sensory neurones 24-hours post-inflammation.
- Figure 4.1 CHO cells are a suitable cellular background for studying PS-GPCR function.
- Figure 4.2 Most NMR PS-GPCR homologs are proton insensitive.
- Figure 4.3 Mice and NMRs show similar expression profiles of PS-GPCRs in different organs.
- Figure 5.1 Modulation of cAMP levels by GPR65 agonists.
- Figure 5.2 Mobilisation of Ca^{2+} following GPR65 stimulation with various agonists.
- Figure 5.3 GPR65-mediated activation of ERK kinases.
- Figure 5.4 β -arrestin recruitment to GPR65 following receptor activation with protons, BTB or psychosine.
- Figure 5.5 Mutation of histidine residues in extracellular portions of MmGPR65 reduces receptor sensitivity to agonists in cAMP assays.
- Figure 5.6 GPR65 internalises to early endosomes following activation.
- Figure 5.7 Assessing the molecular mechanisms behind agonist-induced internalisation of GPR65.
- Figure 5.8 BTB and psychosine are specific for GPR65 and induce distinct intracellular signalling cascades.
- Figure 6.1 Single-cell RNA-seq of colonic sensory neurons in mouse.

- Figure 6.2 Unilateral injection of CFA into the knee joint of mice results in swelling and pain-like behavioural defects.
- Figure 6.3 Unilateral injection of BTB into the knee joint of mice evokes swelling and pain-like behavioural deficits.
- Figure 6.4 Overnight stimulation of naïve dissociated DRG sensory neurones with BTB does not affect neuronal excitability or voltage-gated currents.
- Figure 6.5 Intra-articular injection of BTB increases the excitability of sensory neurones innervating the knee joint 24-hours post-injection.
- Figure 6.6 Following stimulation with BTB the conditioned media of FLS increases the excitability of sensory neurones.
- Figure 6.7 Stimulation of FLS with BTB causes secretion of inflammatory mediators.

vi. List of tables

Table 2.1	Primers designed to amplify short sections of the acid-sensitive receptors or the housekeeping gene <i>Gapdh</i> based on the reference genome for Chinese hamster (<i>Cricetulus griseus</i>).
Table 2.2	TaqMan mouse expression assay IDs.
Table 2.3	Primers were designed to amplify short sections of the acid-sensitive receptors or the housekeeping gene β -actin based on the reference genome for mice (<i>Mus musculus</i>) and naked mole-rats (<i>Heterocephalus glaber</i>).
Table 3.1	Intrinsic and active properties of hind paw innervating dorsal root ganglion neurones from the carrageenan injected side (Ipsi.) and contralateral (Contra.) side.
Table 6.1	Intrinsic and active properties of dorsal root ganglion neurones cultured overnight in either control medium, or medium containing with 100 μ M BTB or 0.1% (v/v) DMSO.
Table 6.2	Intrinsic and active properties of knee innervating sensory neurones from the BTB-injected side (Ipsi.) and contralateral (Contra.) side.
Table 6.3	Intrinsic and active properties of dorsal root ganglion neurones cultured overnight in either control FLS medium, or the media of FLS stimulated with 100 μ M BTB or 0.1% (v/v) DMSO.

1. Introduction

1.1. Inflammation and pain

Inflammation is the body's natural response to infection, injury or irritation (Medzhitov, 2008). Induction of inflammatory programmes serves to resolve the underlying cause of the inflammation and promote repair of the afflicted area and presents with several well-established clinical symptoms, first described in the 1st century by Celsus, who defined inflammation by its redness (*rubor*), swelling (*tumor*), heat (*calor*) and pain (*dolor*) (Rocha e Silva, 1994). These features remain characteristic of most cases of inflammation, although a greater understanding of the underlying physiological phenomena has been attained: Inflamed tissue appears swollen, as a result of increased permeability of the surrounding vasculature, which permits increased infiltration of immune cells, necessary to clear pathogens and/or begin to remediate the cause of injury or irritation and gives rise to the observed oedema. Additionally, the inflammation-induced vasodilation can give inflamed tissue a 'red' apparel. The heat characteristic of inflammation is generated by increased blood flow to the site of inflammation and metabolic activity of the orchestrating immune cells. Lastly, heightened sensitivity to noxious stimuli, such as mechanical pressure and temperature, also occurs and is responsible for the pain associated with inflammation. Detection of noxious stimuli in the periphery is coordinated by a specialised subset of sensory neurones, termed nociceptors, whose cell bodies reside in the dorsal root ganglia (DRG) for the body and trigeminal ganglia for the head and neck. The ability to detect painful stimuli has persisted through evolution, highlighting the importance of pain sensation (Smith and Lewin, 2009; Walters and Williams, 2019). Indeed, inflammation-induced pain is a good thing, serving a protective role, such that the enhanced sensitivity of the afflicted area serves to prevent any further damage and thus aids recovery. However, pain becomes a problem for diseases associated with chronic inflammation in which resolution of inflammation fails to occur, such diseases include: rheumatoid arthritis (RA), osteoarthritis (OA), and inflammatory bowel disease (IBD), as well as certain cancers and diabetes mellitus; as the enhanced nociception takes hold and establishes a chronic state of pain (discussed more in section 1.2).

As alluded to above, inflammation is a complex process reliant on multiple cell types acting in concert. The importance of communication between cells of the immune system is thus evident and is predominantly achieved through molecular messengers, which are often considered together in families based on their function. A brief background of immune mediators follows, key groups will be addressed in roughly the order in which they act during the onset of inflammation, although obviously this can differ depending upon the initial

stimulus. Among the mediators responsible for first initiating inflammation is histamine: histamine is released upon degranulation of mast cells and basophils, triggered by the detection of pathogens, irritants or general damage. Histamine triggers vasodilation as well as the production of cytokines (Thangam et al., 2018). Bradykinin can be produced during inflammation by the action of the protease kallikrein on the plasma protein kininogen, kallikrein is secreted in response to the detection of pathogens, or as a consequence of tissue damage (Kaplan et al., 2002). Bradykinin contributes to inflammation through its two receptors: B2 receptors are constitutively expressed and function to drive vasodilation upon the binding of bradykinin. B1 receptor expression is induced in a variety of cells during inflammation, induction and activation of this receptor by endothelial cells has been shown to contribute to immune cell infiltration through production of chemokines, such as C-X-C Motif Chemokine Ligand 5 (CXCL5) (Duchene et al., 2007). A number of lipid-based mediators are also produced during active inflammation, these are mostly derived from arachidonic acid which is cleaved from the plasma membrane by phospholipase A2 and further converted to various mediators including prostaglandins and leukotrienes, which serve similar roles to histamine and bradykinin, causing vasodilation and cytokine production (Hata and Breyer, 2004). Invading pathogens and molecules released by damaged cells are able to activate a number of pattern recognition receptors expressed by cells of the immune system, such as the toll-like receptors (TLRs), activation of these receptors also triggers the production of cytokines (Li and Wu, 2021).

Once recruited to the site of inflammation, immune cells secrete their own mediators, including cytokines, which act to reinforce immune cell driven inflammation. Cytokines are among the largest family of immune mediators and may be further broken down into subfamilies. Chemokines, are one such cytokine subfamily and are predominantly responsible for the activation and recruitment of other immune cells, serving to reinforce processes started by histamine and bradykinin, through the establishment of a chemotactic gradient. The largest subgroup of cytokines are the interleukins (IL), which coordinate changes in gene expression, resulting in immune cell activation, proliferation, and differentiation, and serve to further drive the release of inflammatory mediators. However, not all ILs are pro-inflammatory, many also exerting anti-inflammatory effects and thus certain ILs are also important in the natural resolution of inflammation (Feghali and Wright, 1997).

As well as acting on other immune cells, the inflammatory mediators described above can also coordinate changes on non-immune cells that drive inflammatory processes, for example the induction of increased expression of adhesion molecules by vascular smooth muscles cells which facilitates the increased infiltration of immune cells (Read et al., 1997). Sensory

neurons are also strongly modulated by immune mediators, particularly the nociceptive population, which express receptors activated by noxious, or painful stimuli, such as harsh chemicals, temperature and mechanical force, but are also responsive to cytokines and prostaglandins (Zeisel et al., 2018). Activation of receptors for immune mediators on nociceptors allows the engagement of intracellular signalling cascades that serve to sensitise these neurons and permit lower thresholds for activation, which may give rise to both hyperalgesia (greater nociceptor depolarisation brought about by noxious stimuli) and allodynia (previously innocuous stimuli can now depolarise/activate nociceptors and are thus perceived as noxious) and offers some explanation to the enhanced nociception experienced during inflammation (Pinho-Ribeiro et al., 2017).

Indeed, this has been recreated *in vitro* whereby electrophysiology studies, assessing the properties of sensory neurons, have reported reduced thresholds for their activation following exposure to a variety of inflammatory mediators including bradykinin, prostaglandin E2 (PGE2) and IL-1 β (Binstok et al., 2008; Davidson et al., 2014). *Ex vivo* electrophysiology performed on nerves innervating visceral organs has also reported an ability of purified inflammatory mediators to increase nerve activity (Dmitrieva and McMahon, 1996; Grundy et al., 2020). Electrophysiology recordings obtained *in vivo* from afferents innervating the knee joints of rats have shown a similar phenomenon, for example injection of recombinant rat IL-17A into the knee potentiates afferent activity evoked by noxious rotation of the joint (Richter et al., 2012). Additionally, stimulation of fibroblast-like synoviocytes (FLS) isolated from the knee joint of mice with tumour necrosis factor α (TNF- α) has been shown to induce the secretion of inflammatory mediators, with the resulting conditioned media capable of sensitising sensory neurons (Chakrabarti et al., 2020a). These observations have usually been mechanistically linked to the modulatory effects and/or changes in expression of ion channels capable of contributing to neuronal depolarisation and/or orchestrating action potentials. Reports of direct administration of mediators such as TNF- α , IL6 and IL17 to the hind-paw of rodents being sufficient to evoke nociceptive-like behaviours (lower withdrawal thresholds to mechanical or thermal stimulation), further support the role of immune mediators in establishing pain (Cunha et al., 1992; Kim and Moalem-Taylor, 2011).

Animal models of inflammatory joint pain evoked by the administration of immunogenic substances, such as complete Freund's adjuvant (CFA) or carrageenan have also been shown to have similar, sensitising effects and cause pain-like behaviour in rodents (Chakrabarti et al., 2018; Bohic et al., 2021). Similarly arthritis can be modelled by priming the immune system in the joint environment with antigen- or antibody-induced models of arthritis

that give rise to behavioural hyperalgesia (Bas et al., 2012; Jochmann et al., 2015). The mechanical and thermal hypersensitivities experienced by rodents undergoing these models have been shown to be decreased following administration of non-steroidal anti-inflammatory drugs (NSAIDs) or neutralisation of inflammatory mediators with antibodies, further more advanced analysis of spontaneous behaviours resolved differences in treated and untreated groups (Boettger et al., 2008; Bohic et al. 2021). Such findings heavily implicate the action of mediators produced during inflammation on sensory neurones, resulting in their increased excitability as an underlying mechanism of inflammatory pain, this is widely recognised among the field and usually termed peripheral sensitisation.

Nociceptors lie at the start of the 'pain pathway', as such peripheral sensitisation is one of the earliest acting mechanisms of inflammatory pain. Upon detection of noxious stimuli nociceptors feed into the central nervous system and ultimately the brain, where signals are interpreted as pain. The importance of both the peripheral and central components of pain are highlighted by the International Association for the Study of Pain definition of pain: An unpleasant sensory and emotional experience associated with, or resembling that associated with, actual or potential tissue damage (Raja et al., 2020). Spinal mechanisms and changes in brain circuitry can also contribute to the heightened pain experienced during inflammation, changes in the central processing of pain are referred to as central sensitisation (Latremoliere and Woolf, 2009); central sensitisation mechanisms are not the focus of this Thesis and as such will not be discussed further. Targeting peripheral sensitisation i.e. pain at the source, is thought to hold promise for the development of novel analgesics. It is generally thought that such approaches might avoid potential complications associated with targeting central mechanisms given the brain's critical role in the central nervous system. Thus, furthering the study of sensitising mechanisms initiated by inflammation might lead to the identification of novel drug targets for treating pain. Just as central sensitisation is an important consideration in understanding pain, a number of other factors exert large influences on pain that are also beyond the scope of this thesis, these include sex (Rosen et al., 2017), genetics (Mogil, 2012), epigenetics (Géranton, 2019) and psychosocial influences (Edwards et al., 2016).

1.2. Inflammation and acidosis

As well as the generation of lipid and protein based immune mediators, inflammation results in increases in the local concentration of protons, purines (e.g. adenosine triphosphate, ATP) and nucleosides. These may be released via regulated processes or during the death of cells present at the site of inflammation (Borregaard et al., 1984; Dosch et al., 2018; van Zwieten

et al., 1981). The local concentration of protons is further increased by the infiltration of immune cells, which establishes a crowded hypoxic environment causing a widespread switch to anaerobic metabolism. The production and excretion of lactate by cells undergoing anaerobic respiration further increases the extracellular concentration of protons at inflamed sites. In cases of inflammation induced by infection, certain bacterial species have also been shown to secrete macromolecules that can reduce local pH (Niederman et al., 1997). Together these phenomena culminate in the establishment of localised acidosis (Falchuk et al., 1970; Harrison et al., 1986). Indeed, tissue acidosis is well documented in chronic inflammatory conditions, such as RA, IBD, asthma and certain forms of cancer (Andersson et al., 1999; Hunt et al., 2000; Sasaki et al., 1997; Yoneda et al., 2015). The resulting decrease in extracellular pH can exacerbate inflammation: under acidic conditions immune cells produce and secrete more pro-inflammatory mediators (Riemann et al., 2016). Additionally, protons may directly activate or sensitise sensory neurones and thus contribute to the hyperalgesia associated with inflammation (Gregory et al., 2015; Steen et al., 1995). The proton-sensitive receptors, which presumably drive such phenomena thus represent an interesting avenue of investigation for further understanding inflammation and associated pain (see sections 1.3 and 1.6 for more detail).

1.3. Chronic inflammatory diseases and arthritic pain

A number of diseases are associated with long lasting inflammation, including certain cancers, diabetes mellitus, neurodegeneration, IBD, RA and OA (Coussens and Werb, 2002; Glass et al., 2010; Lontchi-Yimagou et al., 2013; Shen et al., 2017; Spel and Martinon, 2020; Zhang and Li, 2014). According to the 2017 Global Burden of Disease Study RA had a prevalence of ~20 million, while OA is estimated to affect ~300 million individuals (Safiri et al., 2019; 2020). Individuals with chronic inflammatory disease often report pain as their major complaint and find that this is ineffectively managed by currently available therapeutic options (Breivik et al., 2006; Lee, 2013). Moreover, inflammatory pain has a severe defect on quality of life of patients, with many additional implications on wider society (Enns et al., 2018).

The pharmacological agents currently approved for treating the pain associated with inflammatory diseases include NSAIDs, paracetamol and opioid-based analgesics. Of these, NSAIDs, are the most targeted to inflammation and facilitate analgesia in the periphery by inhibiting the cyclooxygenase (COX) enzymes responsible for the synthesis of prostaglandins from arachidonic acid. The therapeutic benefit of NSAIDs results from reduced production of prostaglandins, which principally drive inflammation and can sensitise sensory neurones

(Bindu et al., 2020). The therapeutic use of NSAIDs is limited however by their potential to trigger adverse effects on the gastrointestinal (GI) tract, cardiovascular and renal systems, which often means dosing regimens are restricted to prevent complications that in turn has the effect of lessening the therapeutic benefit (da Costa et al., 2021). The mechanism of action of paracetamol is not fully resolved, although is thought to include stimulation of the endocannabinoid system and descending serotonergic neuronal pathways (Jóźwiak-Bebenista and Nowak, 2014). Therapeutic use of paracetamol is even more limited when compared to NSAIDs due to issues surrounding ineffective pain relief and safety concerns (Brune et al., 2015; Moore and Moore, 2016).

Prescription of opioid-based medicines remains the mainstay treatment option for individuals with chronic pain; opioid-based therapeutics stimulate the endogenous opioid system: four inhibitory G protein-coupled receptors with widespread expression in the peripheral and central nervous systems (Corder et al., 2018). Mediation of opioid analgesia in the periphery predominantly arises from a blanket toning down of neuronal activity achieved by the inhibition of voltage-gated calcium channels (Cavs) and activation of G-protein-activated inwardly rectifying potassium (GIRK) channels, the former inhibiting neurotransmitter release, the latter serving to hyperpolarise sensory neurones and thus reduces excitability (Nockemann et al., 2013; Stötzner et al., 2018; Su et al., 1998). Combined, these effects reduce nociceptor discharge and ability of exciting secondary neurones in the spinal cord, thus preventing the generation of pain signals. Given the widespread expression of opioid receptors, opioid analgesics also often give rise to a number of undesirable side-effects which can be traced to similar actions on autonomic neurones innervating the GI tract, resulting in opioid-induced constipation (Streicher and Bilsky, 2018). Another side-effect of opioid treatment is the paradoxical ability of opioids to induce hyperalgesia following sustained long term use, this has been shown to be a result of direct effects on nociceptors in the periphery (Ferrari et al., 2019). Opioids also act centrally on the spinal cord and brain to alter pain perception (Corder et al., 2018). A complex relationship between opioid dependence, the development of tolerance and effects on reward/reinforcement centres of the brain have resulted in many issues concerning opioid misuse (Schaefer et al., 2017). This can be summarised as follows: patients develop tolerance to their current dose of opioid, but the doses prescribed must be capped to prevent the side effects on the autonomic nervous system becoming serious problems. This sees patients seek to satisfy their reliance on opioids by turning to illicit (and thus unregulated) opioids, such as heroin and fentanyl, produced and/or obtained illegally often involving increased risk-taking behaviour and too frequently resulting in overdose and loss of life. This has led to the establishment of the opioid crisis, currently endemic in the United States, but also becoming more of a problem in the United Kingdom where opioid

prescriptions are on the rise (Jani et al., 2020; Rudd et al., 2016). The opioid crisis has sparked interest in developing synthetic opioid agonists that can selectively discriminate between intracellular signalling cascades, a concept known as biased agonism. This is due to reports that β -arrestin signalling may mediate the unwanted side-effects triggered by activation of opioid receptors, which is distinct from the signalling that gives rise to Ca_v inhibition and GIRK activation and mediates pain relief (Bohn et al., 1999). However the effectiveness of this as a strategy has recently come into question with new reports that knocking out β -arrestins does not prevent opioid-induced respiratory depression (Bachmutsky et al., 2021), the development of non-opioid analgesics with novel mechanisms of action is thus of great importance.

As discussed above, inflammatory pain is predominantly driven by peripheral sensitisation, the action of immune mediators and substances released during inflammation initiating cascades within nociceptive neurones giving rise to hyperalgesia. Understanding of immune mediator release and the subsequent effects on nociception in chronic pain conditions is key for the development of more targeted therapeutic options. Differences in pathologies, mean that such diseases need to be considered separately. Emphasis will now focus on RA and OA which are major causes of musculoskeletal pain and are known to have strong inflammatory components (Blyth et al., 2019).

RA is an autoimmune disease typically affecting smaller joints (i.e. fingers and toes) but can progress to larger joints, whereas OA involves biomechanical and inflammatory processes typically affecting the knee and hip joints and ultimately lead to structural deterioration. Joint pathology in RA is characterised by synovial inflammation (synovitis), cartilage degradation, bone erosion and bone marrow lesions. Synoviocytes, resident in the joint, develop a proinflammatory phenotype, while numerous immune cells infiltrate the synovium and synovial fluid where they produce proinflammatory mediators and catabolic factors to further recruit immune cells and drive tissue damage (Lin et al., 2020, Croft et al., 2019). In OA, joint changes include alterations to chondrocyte phenotype, density and activity – cartilage erosion is followed by chondrocyte hypertrophy and generation of matrix degradation products, proinflammatory mediators, infiltration of mast cells, macrophages and lymphocytes (Hunter and Bierma-Zeinstra, 2019).

The importance of cell-cell interactions in RA and OA mediated by release of soluble factors is demonstrated by the observations that synovial fluid obtained from OA patients can sensitise mouse sensory neurones, reducing the threshold for action potential discharge (Chakrabarti et al., 2020b). This thus suggests that the targeting of mediators known to be

upregulated may represent novel points of therapeutic intervention. Studies of human patients and animal models of RA and OA have led to the identification of a factors shown to be either increased and/or directly participate in establishing the pathology of each disease. Some factors are common to both diseases, for example TNF- α , nerve growth factor (NGF), PGE₂, IL-1, IL-6 and calcitonin gene-related peptide (CGRP) have been shown to contribute to both diseases (Attur et al., 2008; Kay and Calabrese, 2004; Manara and Sinigaglia, 2015; McCoy et al., 2002; Seidel et al., 2010; Walsh et al., 2015; Wojdasiewicz et al., 2014; Yoshida and Tanaka, 2014). A number of interactions more specific to each disease have also been identified as causal of the associated pain, including activation of toll-like receptor 2 (TLR2) by a 32-amino-acid aggrecan found in OA synovial fluid (Miller et al., 2018). OA has also been shown to be driven by vascular endothelial growth factor (VEGF), with increased levels of VEGF correlating with disease progression (Hamilton et al., 2016). RA-specific interactions include HMGB1 activation of toll-like receptor 4 (TLR4, Agalave et al., 2014) and excitation of neuronal Fc γ receptor I by complexes of autoantibodies and cartilage proteins (Bersellini Farinotti et al., 2019). The pathological features of each disease and some of the mediators shown to be release by certain cells that act to establish pain via sensory neurones are outlined in Figure 1.1.

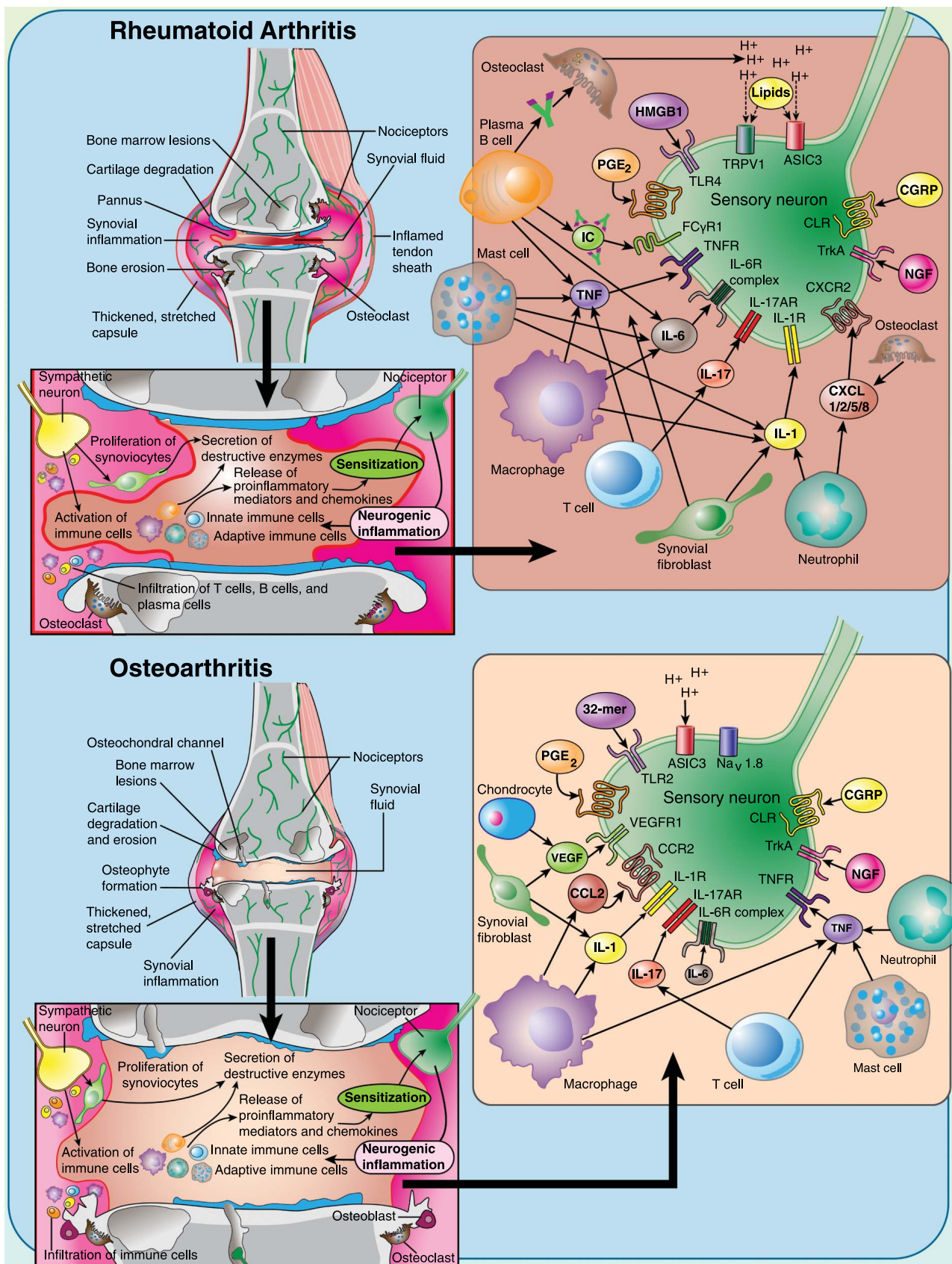


Figure 1.1. Pathophysiological aspects of RA and OA. Schematic representation of gross histological changes characteristic of RA and OA, and a look at how these diseases affect the joint environment on a cellular and molecular level. This figure was developed with graphic designers working for The International Association for the Study of Pain's scientific journal PAIN as part of a PAIN pictured article (Pattison et al. 2021).

Blockade of the signalling cascades initiated by these immune mediators is an active area of research in the hunt for novel drugs to treat both forms of arthritis. A popular approach is the use of monoclonal antibodies to either sequester elevated levels of mediators or prevent their activation of their cognate receptors, with many making it through clinical trials and available as prescription medicines: Anti-TNF- α (e.g. adalimumab) and anti-IL-6 receptor (e.g. tocilizumab) therapies have shown promise for RA (Choy et al., 2002; Keystone et al., 2004). Although anti-NGF antibodies (e.g. tanezumab) have proved to be effective at reducing the pain experienced by individuals with OA (Berenbaum et al., 2020), the European Medicines Agency has recently refused authorisation for tanezumab to be sold as a therapeutic for OA. This decision was based on safety concerns, regarding tanezumab treatment leading to a higher risk of rapid progressive osteoarthritis and the need for joint replacement surgery, as well as minimal analgesic benefit over currently available NSAIDs.

A common feature between RA and OA is the raised concentration of protons in the inflamed environment, which makes synovial fluid of RA and OA more acidic than healthy controls (Andersson et al., 1999). Given that administration of acid through the skin is perceived as painful (Jones et al., 2004; Ugawa et al., 2002), it is thus believed this acidification may also contribute to the pain of RA and OA. Indeed, extracellular acidosis has also been reported for IBD, cancer and chronic obstructive pulmonary disease (Hunt et al., 2000; Sasaki et al., 1997; Yoneda et al., 2015), which further implicates the receptors which detect protons as potential drug targets. As such, known proton-sensitive receptors will now be discussed.

1.4. Proton-sensitive receptors (ASICs, K2Ps and TRPs)

Among the receptors for inflammatory mediators, sensory neurones and immune cells express a range of proton-sensitive receptors. These receptors fall into four families: the acid-sensing ion channels (ASICs), certain members of the transient receptor potential channel (TRP) family, some two-pore potassium channels (K2P) and proton-sensing G protein-coupled receptors (PS-GPCR), and permit detection of the elevated level of protons brought about by inflammation. The basic structure and list of proton-sensitive members of each family are summarised in Figure 1.2.

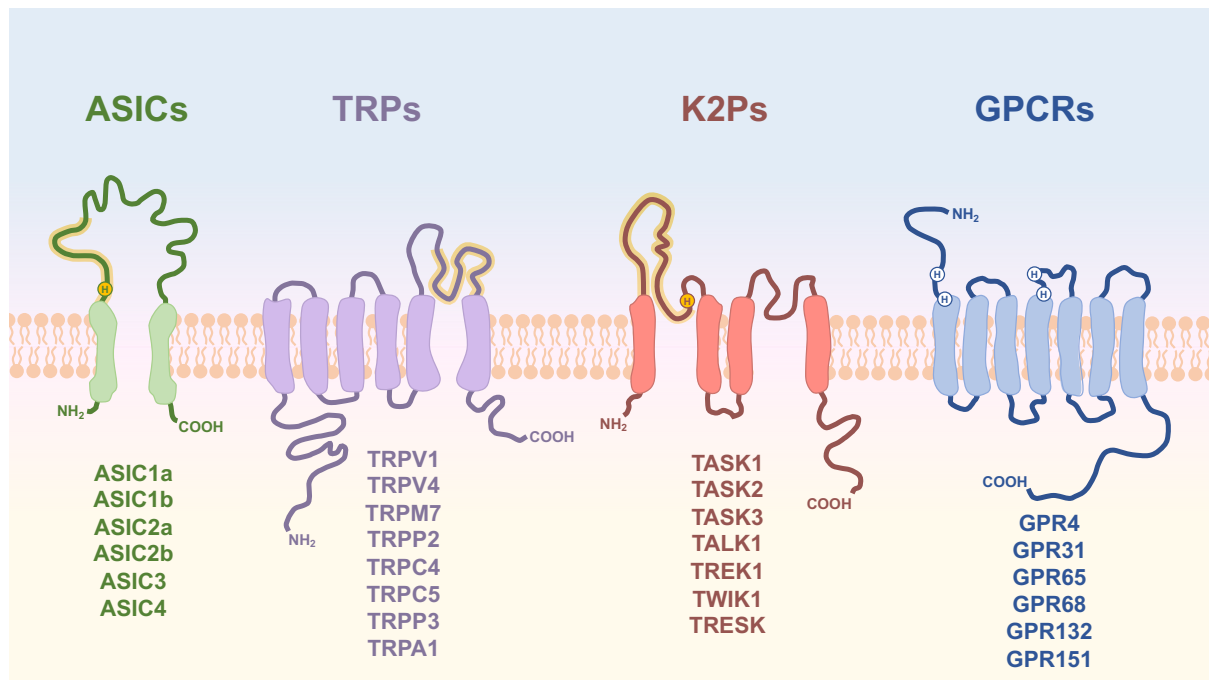


Figure 1.2. Membrane topologies of proton-sensitive receptor subunits. Schematic diagram of the basic structure of proton-sensitive receptors, with residues (H, histidine) or regions important for proton sensitivity annotated (yellow – highly conserved among family members; white – less conserved or important for some, but not all, family members). Functional ASICs, K2Ps and TRPs are multimeric, but for simplicity only one subunit of each receptor is shown.

A brief background of ASICs, TRPs and K2Ps and summary of their involvement in inflammatory pain follows. Given PS-GPCRs are the subject of this thesis they will be addressed more thoroughly in sections 1.4, 1.5 and 1.6.

Six ASIC subunits have been identified in mammals: ASIC1a, ASIC1b, ASIC2a, ASIC2b, ASIC3 and ASIC4 (Akopian et al., 2000; Bässler et al., 2001; Chen et al., 1998; Gründer et al., 2000; Lingueglia et al., 1997; Price et al., 1996; Waldmann et al., 1997a; 1997b). These subunits assemble as hetero- or homotrimeric channels, however not all permutations are proton-sensitive: homomeric ASIC2b and ASIC4 channels are proton-insensitive (Hesselager et al., 2004). The bile acid-sensitive ion channel (BASIC) is considered a member of the same ion channel superfamily as the ASICs due to high sequence homology and has been referred to as ASIC5. Like ASICs, BASIC is sensitive to inhibition by amiloride, however does not appear to be sensitive to activation by protons (Schaefer et al., 2000). ASICs are expressed throughout the sensory nervous system, although ASIC4 expression is comparatively lower (Schuhmacher and Smith, 2016; Zeisel et al., 2018). When activated by acidic shifts in the extracellular pH, ASICs mediate cation influx, which for nociceptors can result in depolarisation, action potential discharge and thus the sensation of pain (Deval et al., 2008; Steen et al., 1992). This has been demonstrated by findings that the inhibition of ASIC1a/b with toxins purified from *Psalmopoeus cambridgei* and *Dendroaspis polylepis*, reduced

inflammation-induced defects in mechanical and thermal sensitivity (Diocot et al., 2012; Mazzuca et al., 2007). Overall, there is more evidence implicating ASIC3 as a critical integrator of acidosis, coordinating pain in the periphery, compared to other ASIC subunits. For example, the ASIC3 inhibitor APETx2 has been shown to prevent acid-induced action potential discharge of sensory neurones (Callejo et al., 2015). Furthermore administration of APETx2 reduced the pain experienced by rats following induction of inflammation with CFA, less thermal hyperalgesia was also observed following intrathecal delivery of siRNA to knock down ASIC3 (Deval et al., 2008). Compared to wildtype littermates, ASIC3^{-/-} mice also fared better when mechanical sensitivity was assessed following induction of inflammation with carrageenan or intramuscular injection of acid (Price et al., 2001). Further to this, chronic hyperalgesia cannot be achieved in mice lacking ASIC3 following repeated intramuscular injections of acid (Sluka et al., 2003). Additionally a peptide isolated from the venom of *Conus textile* has been shown to potentiate ASIC3 activity and enhance acid-evoked hyperalgesia (Reimers et al., 2017). Further supporting a role for ASIC3 in inflammatory pain, arachidic acid, produced during inflammation, has also been shown to potentiate acid-induced inward currents of sensory neurones, via both ASIC1a and ASIC3 (Smith et al., 2007) and enhance acid-mediated nociception (Deval et al., 2008).

TRPs are generally considered to function as homotetramers, each subunit comprises intracellular N- and C- termini and 6 transmembrane domains, a re-entrant loop between the fifth and sixth transmembrane domains forms the channel pore (Figure 1.2, Hellmich and Gaudet, 2014). Variations in motifs and modalities present within the receptors permit them to respond to a diverse range of stimuli including both chemicals and physical properties such as pressure, light and temperature; TRPs are generally cation permeable and thus also permit neuronal depolarisation (Damann et al., 2008). The ability of TRPs to respond to protons was first demonstrated with TRPV1 when acidic pH was shown to potentiate capsaicin-induced inward currents (Caterina et al., 1997), protons were later shown to be capable of directly activating the receptor (Tominaga et al., 1998) with different amino acids being involved in each process (Jordt et al., 2000). Since these findings, other members of the TRP family have been shown to be activated or positively modulated by extracellular pH, including TRPV4, TRPM7, TRPP2, TRPC4, TRPC5, TRPP3 and TRPA1 (Chang et al., 2010; Jiang et al., 2005; la Roche et al., 2013; Semtner et al., 2007; Suzuki et al., 2003). Examples of TRPs inhibited by extracellular acidosis (Semtner et al., 2007) or activated by intracellular acidosis (Cao et al., 2012) have also been described. Avoidance of acidic environments by *C. elegans* suggests an ability of the nematode to sense extracellular pH, with acidic areas perceived as unpleasant and potentially painful. This has been shown to be mediated by *osm-9*, a proposed homolog of mammalian TRPV channels, as reduced acid-avoidance behaviour was observed

in *osm-9* mutants and following treatment of wildtype nematodes with the broad-spectrum TRP inhibitor ruthenium red (Sambongi et al., 2000). Evidence explicitly linking proton-induced TRP signalling to nociception is relatively scarce for higher-order organisms, perhaps due to the promiscuous nature of TRP activation. However, a large body of evidence exists that implicates TRPs in nociception, particularly in the context of thermal hyperalgesia, see (Dai, 2016) for a comprehensive review of the role of TRPs in both inflammatory and neuropathic pain. Indeed, studies of knockout mice suggest that TRPV1, as well as ASIC3, are relatively redundant in the development of pain states during the acute stages of inflammation, but significantly contribute to hypersensitivity as pathologies become more chronic (Hsieh et al., 2017). Given the prominence of acidosis in many conditions associated with pain, particularly at chronic stages following mass influx of immune cells and the establishment of a hypoxic environment (Harrison et al., 1986; Roiniotis et al., 2009), and the high expression of proton-sensitive TRPs by nociceptors (Mickle et al., 2016) it is widely accepted that acidosis likely potentiates TRPs resulting in hyperalgesia. Although this is yet to be proved *in vivo*, it is well described in *in vitro* systems (la Roche et al., 2013; Semtner et al., 2007; Tominaga et al., 1998). In addition to TRP potentiation leading to more frequent action potential discharge, activation of TRPs in sensory neurones has been shown to coordinate release of the neuropeptides substance P and CGRP, which can in turn prime other neurones leading to hyperalgesia, as well as contribute to central sensitisation (Engel et al., 2011; Meng et al., 2009; Nakamura et al., 2012). Inhibition of the proton-sensitive TRPs, TRPA1 and TRPV1 has been explored as means of relieving the pain induced by subcutaneous injection of pH 4.3 solution to human volunteers, however neither antagonism of TRPV1 nor TRPA1 provided any analgesia. Antagonism of TRPV1 has however, been shown to reduce the pain evoked by milder stimulation with pH 6. Amiloride, a pan-ASIC inhibitor also failed to remedy pain induced by either pH (Heber et al., 2020; Schwarz et al., 2017), however, other studies have shown a role of ASICs using other methods (Jones et al., 2004; Ugawa et al., 2002).

The K2P ion channels contribute to the background K⁺ current observed in both excitable and non-excitable cells, and thus impart a large influence on the resting membrane potential of neurones (Mathie and Veale, 2015). Functional K2P channels are dimers of two subunits comprising four transmembrane domains and two pore-forming domains (Figure 1.1). There are 15 different K2P subunits, which may assemble as homo- or heterodimers, and thus a great deal of diversity is possible, which permits integration of a number of extracellular and intracellular signals including pH, temperature and membrane stretch (Enyedi and Czirják, 2010). Among K2Ps, TASK1, TASK2, TASK3, TWIK1, TREK1 and TALK1 have been shown to be sensitive to extracellular acidification, lower pH inhibiting their activity (Cohen et al., 2009; Duprat et al., 1997; Kang and Kim, 2004; Rajan et al., 2000; 2005; Reyes et al., 1998).

Additionally the mouse TRESK K2P has been shown to be inhibited by acid, but the human homolog is unaffected by extracellular protons due to the loss of a key histidine residue (Dobler et al., 2007). Many of these K2Ps are expressed by DRG neurones and thus, extracellular acidification, caused by inflammation, may result in proton-induced inhibition of K2Ps, reducing constitutive K^+ efflux. In the case of nociceptors this could result in membrane depolarisation and thus decrease the threshold needed for action potential discharge, hence the importance of K2Ps as potential analgesic targets is evident (Gada and Plant, 2019). Specific inhibitors of K2Ps are not widely available yet, however, studies of a TRESK mutant identified from a single nucleotide polymorphism (SNP) known to correlate with increased incidence of migraine have shown reduced activity (Liu et al., 2013b), furthering the idea that inhibition of proton-sensitive K2Ps within sensory neurones could have a depolarising effect and cause pain. Taking the opposite approach, overexpression of TRESK has also been shown to decrease trigeminal neurone excitability (Guo and Cao, 2014). Another genetic association with migraine, involves a frameshift mutation-induced alternative translation initiation of TRESK, resulting in a defective protein product which can combine with TREK1 and TREK2, resulting in reduced activity, causing higher excitability of trigeminal ganglia neurones and propagating facial mechanical hypersensitivity in rodents (Royal et al., 2019). Further supportive of a role of K2Ps in inflammatory pain are reports of decreased expression of TASK1 and TASK3 in DRG following induction of inflammation with CFA, correlations between the reduction of both channels and the incidence of spontaneous pain were also reported (Marsh et al., 2012). TREK1^{-/-} mice have also been shown to have higher baseline sensitivities to painful heat and mechanical pressure and experience greater hyperalgesia following the induction of inflammation with carrageenan (Alloui et al., 2006). Knockout of TASK1 and TASK3 in mice also impacts the thermosensitivity of animals, such that TASK1^{-/-} experience pain after lower thermal stimulation compared to wild-type controls and TASK3^{-/-} animals are hypersensitive to cold temperatures (Linden et al., 2006; Morenilla-Palao et al., 2014). Finally, the inflammatory mediators arachidonic acid and lysophosphatidic acid have been shown to inhibit TRESK and TREK1 channels respectively, with extracellular acidification having an additive effect on the inhibitions observed (Callejo et al., 2013; Cohen et al., 2009).

In summary, extracellular acidification can directly influence sensory neurone excitability through a number of proton-sensitive channels which have been implicated in inflammatory pain, the mechanisms behind this are summarised in Figure 1.3.

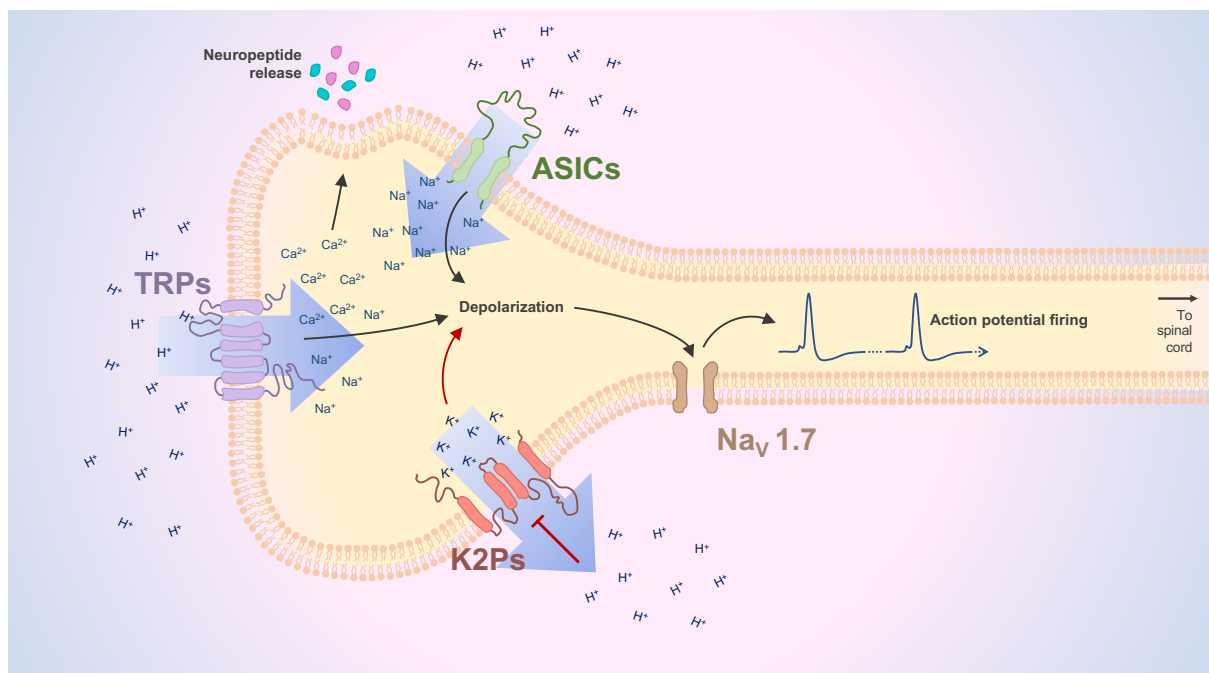


Figure 1.3. Proton-sensing at the peripheral terminal. Following localised acidosis the increased extracellular concentration of protons is sensed by several families of receptors which act in concert to increase neuronal excitability and release mediators which may sensitise other neurones. Increased extracellular proton concentration induces the activation of proton-sensitive depolarising channels (ASICs and TRPs) causing cation influx and membrane depolarisation. Simultaneously, proton-induced inhibition of K2P channels reduces constitutive K⁺ efflux facilitating further membrane depolarisation. Altogether, nociceptor depolarisation activates Na_v subunits resulting in the generation of action potentials that transmit nociceptive signals to the spinal cord.

1.5. Proton-sensitive G protein-coupled receptors

G protein-coupled receptors (GPCRs) comprise the largest family of transmembrane proteins, transducing external signals to the cell interior and contribute to most physiological processes. Unsurprisingly they are thus the target of approximately a third of drugs approved for human use, which may act to enhance or inhibit GPCR signaling (Sriram and Insel, 2018). For the most part, the mechanism of GPCR activation and signal transduction is not that dissimilar from one receptor to another: binding of an extracellular ligand induces a conformational change in the receptor permitting interaction with heterotrimeric G-proteins. Within humans there are over 800 genes encoding GPCRs permitting the diversity of signals necessary for these receptors to conduct a multitude of processes. Several members of the GPCR mega family exhibit atypical activation mechanisms including rhodopsins, which are activated by photons of light, and a family of four receptors activated by proteolytic cleavage, the protease-activated receptors (Smith, 2010; Zhao et al., 2014). Activation of GPCRs by mechanical stress and membrane depolarization has also been described (Ben-Chaim et al., 2006;

Chachisvilis et al., 2006). Another atypical group of receptors is the proton-sensing GPCRs (PS-GPCRs).

PS-GPCRs are activated in response to acidic shifts in the extracellular environment; although activation of some members by lipids has also been described. The ability of protons to activate GPCRs was first demonstrated with GPR68 (also known as the ovarian cancer gene related 1 receptor, OGR1), for which cells expressing the receptor accumulated inositol phosphate as the pH of the assay buffer was decreased. Based on sequence homology a further three GPCRs which respond to acid were identified: GPR4, GPR65 (also known as the T-cell death-associated gene 8 receptor, TDAG8) and GPR132 (also known as the G2 accumulation receptor, G2A) (Ludwig et al., 2003; Murakami et al., 2004; Wang et al., 2004). More recently, two further GPCRs were proposed to be proton sensitive: GPR31 and GPR151 bind GTP as extracellular pH decreases and can coordinate gene transcription based on reporter gene assays; interestingly these new PS-GPCRs appear to be active at more extreme pH than the better characterized PS-GPCR GPR4 (Mashiko et al., 2019). Proton-induced activation of PS-GPCRs has been shown to be dependent on histidine residues within extracellular portions of the receptors, with site-directed mutagenesis of certain histidines to uncharged phenylalanine rendering GPR4, GPR65, GPR68 and GPR132 insensitive to protons (Liu et al., 2010; Ludwig et al., 2003; Murakami et al., 2004; Wang et al., 2004). The histidines implicated in conferring proton sensitivity to the four original PS-GPCRs are generally conserved, as shown in Figure 1.4A. Neither GPR31 nor GPR151 exhibit homologous histidines and the molecular basis of the proton sensitivities of these receptors remains to be determined, however, both receptors exhibit histidines within their extracellular portions. The exact mechanism by which protons activate PS-GPCRs remains to be empirically proven, however, Ludwig and colleagues (2003) propose that hydrogen bonding between histidines may stabilize the receptors in inactive conformations, with increases in the local concentration of protons leading to protonation of the histidines and disruption of such bonds, allowing the receptors to adopt an active conformation, couple to heterotrimeric G proteins and coordinate intracellular signaling cascades. Recent experiments with a GPR68 activation biosensor, also suggest that challenge of the receptor with protons induces a conformational change (Ozkan et al., 2021). Sato and colleagues (2020) have also recently described the loss of proton-induced activation of GPR68 by a naturally occurring missense mutation which changes a leucine residue within the second transmembrane helix to a proline. They observed normal expression of the mutant receptor at the membrane and thus attributed the loss of proton-induced signaling to an inability of the receptor to change its conformation following protonation. The inclusion of Zn^{2+} and Cu^{2+} ions in assay buffers has been shown to prevent proton-induced activation of PS-GPCRs, which has been suggested to be due to

the technical difficulties associated with working with lipids, differences in the cellular backgrounds and assays employed in studies and an omission of certain signaling responses from studies. GPR68 has also been shown to be activated by mechanical force and an ability of benzodiazepines to positively modulate proton-activation of GPR68 has been described (Huang et al., 2015b; Xu et al., 2018). Several synthetic agonists of the PS-GPCRs have also been reported, including BTB09089, Isx-1 and ONC212, which are believed to be specific for GPR65, GPR68 and GPR132 respectively (Nii et al., 2019; Onozawa et al., 2012; Russell et al., 2012). GPR4 antagonists have also been developed from high throughput screens (Miltz et al., 2017; Velicky et al., 2017). These synthetic compounds represent valuable tools to interrogate the pharmacology and contributions of individual PS-GPCRs to health and disease in more complex systems, tissues or even live animals without engaging the many other proton-sensitive receptors (Pattison et al., 2019). However, in most cases a full characterization of these tools is lacking, which thus limits their usefulness.

1.6. Roles of PS-GPCRs in normal physiology

Expression of PS-GPCRs is widespread, vastly different tissues expressing multiple members of the family, suggesting there may be some redundancy between members, although the differing pH sensitivities may allow for the mounting of graded responses, as well as the potential role of differential lipid activation (Okajima, 2013). The physiological roles of PS-GPCRs have been inferred from studies of mice which have had the genes encoding the receptors knocked out, these animals do not present with any major phenotype, strengthening the notion of a potential redundancy across the family.

Loss of GPR4, GPR68 or GPR132 has been shown to result in developmental defects. For example, although GPR4^{-/-} mice are viable and present no gross phenotypic difference, higher perinatal mortality due to haemorrhaging arising from poorly formed blood vessels was observed (Yang et al., 2007). Ren et al. (2016) reported that GPR4 was crucial for expression of Notch1 by endothelial cells, with Notch1 mediating tube formation. Higher expression of GPR4 has been reported for cardiac progenitor cells compared to other stem cells, suggesting they may also play a role in the differentiation of cardiac tissues. Endothelial progenitor cells have also been shown to express GPR68, but engagement of the receptor by extracellular protons was shown to reduce proliferation, tube formation and migration of the cells (Ding et al., 2019; Torán et al., 2019). GPR132 was also shown to suppress proliferation of cardiac smooth muscle cells following loss of the transcription factor yes-associated protein (YAP), it was shown that in normal circumstances YAP exerts epigenetic silencing of GPR132

permitting proliferation of cardiac smooth muscle (Wang et al., 2014). As well as regulating development of the cardiovascular system, several roles of PS-GPCRs in cardiology have been reported including that GPR4 may contribute to blood pressure through influencing the renin-angiotensin system and that GPR68, through its role as a mechanotransducer, facilitates remodelling of small diameter blood vessels (Sun et al., 2016; Xu et al., 2018).

Further developmental contributions of GPR68 have been reported in remodelling of the skeletal system, such as GPR68 being shown to drive the differentiation of macrophages to osteoclasts and facilitating osteoclast survival at acidic pH (Pereverzev et al., 2008; Yang et al., 2006). Mutations in *GPR68* in humans have also been linked to poor development of dentition (Parry et al., 2016; Sato et al., 2020). As well as being expressed by osteoclasts which absorb bone, GPR68 is expressed by osteoblasts, the cells responsible for secreting bone minerals (Ludwig et al., 2003). It is thus evident that extracellular acidosis and GPR68 may have a large influence on bone development, indeed, loss of the receptor led mice to develop increased bone density (Krieger et al., 2016).

Changes in pH can have dire consequences on the structure of proteins and electrochemical gradients, which has knock-on consequences on enzymatic kinetics and biochemistry, adeptly, homeostatic roles of PS-GPCRs have been described. For example, GPR4 is expressed by neurons of the retrotrapezoid nucleus, which are involved in adapting respiratory output in response to changes in CO₂ and H⁺ concentrations. Verifying the role of GPR4 in this process was the finding that GPR4^{-/-} mice exhibited reduced ventilation following exposure to air with elevated CO₂ levels, a higher occurrence of spontaneous apnoea, but normal response to hypoxia. It was shown that GPR4 and the acid sensitive K2P channel, TASK-2, independently conferred pH and CO₂ sensitivity to retrotrapezoid nucleus neurons, permitting neuronal activation and appropriate regulation of ventilation to adjust CO₂ excretion and pH homeostasis (Kumar et al., 2015). Along with GPR68, GPR4 has also been shown to be capable of initiating the endoplasmic reticulum stress pathway, which enhances cell survival during spells of localized acidosis (Dong et al., 2017; Maeyashiki et al., 2020). Similarly, GPR68 promotes cell survival during acidosis by regulation of proton transporters, such as sodium-proton exchangers, in response to extracellular acidification (Mohebbi et al., 2012). While many cells adapt their behaviour to cope during acidosis, PS-GPCRs expressed within the kidneys are activated and coordinate pathways to counteract the increased concentration of protons by increasing extrusion of acid (Sun et al., 2010; 2015).

The PS-GPCRs may also regulate endocrine functions. For example, GPR65 has been identified as a marker of vagal sensory neurons innervating the gastrointestinal mucosa, these

neurons are responsive to serotonin and were shown to modulate gut motility, although the specific contributions of GPR65 to such processes remain unknown (Egerod et al., 2018; Williams et al., 2016). GPR68 has also been shown to affect the activity of the anterior pituitary gland, increasing IL-6 expression which is proposed to negatively regulate proopiomelanocortin expression, and stimulating production of luteinizing hormone by gonadotropic cells in response to extracellular acidification (Horiguchi et al., 2014; Kojima et al., 2019). Depression, which can be in part be explained by hormonal imbalances, may also be linked to GPR65 (Vollmer et al., 2015).

The roles of PS-GPCRs are certainly far-reaching and the receptors appear to play important roles from early development to everyday physiology. The potential for the dysregulation of PS-GPCRs to causes disease is thus apparent: their roles in acute inflammation and chronic inflammatory disease states will now be discussed.

1.7. Proton-sensing GPCRs in inflammation

Inflammation is a physiological response to diverse stimuli, involving a number of cell types including immune cells, epithelia/endothelia and fibroblasts. Infection, tissue damage and a number of diseases are associated with inflammation and very often inflammation coincides with localized tissue acidosis owing to the recruitment of immune cells, which creates a hypoxic environment, as well as regulated or necrosis-induced release of protons and/or acidic molecules (described in Section 1.2). The more general links between inflammation, acidosis and PS-GPCRs will be discussed first.

Cytokines are the master regulators of the immune system, being implicated in the proliferation, activation, recruitment and inhibition of the body's arsenal of immune cells and are attractive drug targets for diseases of inflammation in their own right. A number of cells are able to produce and secrete cytokines and thus contribute to the inflammatory process and acidosis has been reported to enhance cytokine levels for a variety of cell types. Fibroblasts are present all over the body and their contributions to various diseases is becoming ever more apparent. Fibroblasts are thus likely to experience the acidosis associated with local inflammation. Indeed, acidosis has been shown to increase expression of TNF- α , COX-2 and inducible nitric oxide synthase (iNOS) by fibroblasts. Although Riemann and colleagues (2015) did not explicitly link this increased expression of pro-inflammatory mediators to PS-GPCRs, they demonstrate expression of GPR4, GPR65 and GPR132 by fibroblasts, and that cAMP and mitogen-activated kinases were part of the mechanism,

indicative of an involvement of PS-GPCRs. Whereas GPR68 was shown to coordinate expression of the anti-inflammatory PGI₂ in aortic smooth muscle cells, acidosis increased COX-2 expression by osteoblastic cells, with a concomitant rise in the production of pro-inflammatory PGE₂ shown to be mediated by the actions of protons on GPR68 (Liu et al., 2010; Tomura et al., 2005; 2008; Wang et al., 2012). Interestingly, 12-S-HETE, a reported high-affinity lipid agonist of GPR31, is synthesized from arachidonic acid released during inflammation, thus the PS-GPCRs may also contribute to inflammation via their non-proton agonists (Black et al., 1985; Guo et al., 2011).

As well as orchestrating the synthesis and secretion of inflammatory mediators, the PS-GPCRs have also been shown to have more direct effects on immune cells. Depending on the environment and inflammatory timeline, macrophages may adopt either pro- or anti-inflammatory phenotypes, classed M1-like and M2-like respectively. GPR132 has been shown to have a role in this polarization process with loss of the receptor shown to prevent accumulation of macrophages at the site of inflammation and reducing mechanical hyperalgesia (Kern et al., 2018). Attenuation of the development of autoimmune inflammation has been observed following depletion of GPR68, which was mainly attributed to an impaired ability to generate antigen-specific T-cells (D'Souza et al., 2016). A role for GPR68 in B-lymphocyte differentiation has also been suggested, with GPR68^{-/-} mice having lower levels of circulating B-lymphocytes and non-proton agonists of the receptor increasing cell numbers for wild-type mice (He et al., 2020). On the other hand, Wirasinha and colleagues (2018) reported exacerbated autoimmune inflammation when GPR65 was knocked out.

The endothelial cells which line blood vessels play an important role in the inflammatory response, they too secrete interleukins and chemokines to stimulate and recruit immune cells, but must also increase expression of adhesion molecules, permitting the 'capture' of immune cells at the site of infection or damage. Increased expression of IL-8, vascular cell adhesion protein 1 and E-selectin by endothelial cells has been shown to be caused by extracellular acidification, which activates NFκB via GPR4 (Dong et al., 2013). Following the recruitment of immune cells to sites of inflammation, extravasation occurs allowing the immune cells access to the inflammatory insult, this relies on increased vascular permeability and is responsible for the swelling associated with the inflammatory response. GPR4 has also been implicated in increasing the permeability of vascular endothelial cells via engagement of Gα_{12/13}, with GPR4^{-/-} mice experiencing less oedema and inflammation compared to wild-type animals following an acute loss of blood supply to the hindlimb (Krewson et al., 2020).

The inflammatory process is subject to tight regulation, which enables it to serve its function in aiding repair and preventing further damage, but not persist for too long so that it causes further problems, however, persistent inflammation that fails to resolve occurs in some chronic conditions. Interestingly, anti-inflammatory roles of the PS-GPCRs, mainly involving GPR65, have also been described. Extracellular acidification of macrophages has been shown to reduce their ability to produce TNF- α and IL-6 in a GPR65/PKA-dependent manner (Mogi et al., 2009). Similar findings of reduced secretion of IL-1 β following exposure of microglia to an acidic environment were reported and stimulation of immune cells with the synthetic GPR65 agonist BTB09089 reduced production of pro-inflammatory TNF- α and IL-6, but increased the level of anti-inflammatory IL-10 (Jin et al., 2014; Onozawa et al., 2012). Moreover, the glucocorticoid dexamethasone has been shown to increase expression of GPR65 by macrophages, which, given ability of GPR65 to negatively regulate pro-inflammatory cytokines, may in part explain the anti-inflammatory properties of dexamethasone (He et al., 2011). The anti-inflammatory role of GPR65 has also been proven *in vivo*, Onozawa and colleagues (2011) reported earlier onset and more severe progression of anti-collagen antibody-induced arthritis in GPR65^{-/-} mice; they also reported a higher degree of immune cell hypersensitivity following loss of the receptor. By contrast, loss of GPR65 in mice was only shown to reduce eosinophilia following intestinal inflammation, with similar cytokine levels, antigen sensitization and pathological responses seen in wild-type animals (Zhu et al., 2014). The finding that dexamethasone-induced expression of GPR65 also enhances immune cell death via apoptosis may offer some explanation for the disparity on the role of GPR65 in inflammation: perhaps the hypoxia and acidosis, which only arise following recruitment of immune cells, is intended to induce GPR65 expression and start the resolution of the immune response via the generation of anti-inflammatory mediators and pruning of immune cells (Malone et al., 2004).

It is clear that the development and maintenance of inflammation hangs in delicate balance with both pro- and anti-inflammatory actions being orchestrated by various PS-GPCRs. The roles of PS-GPCRs in more chronic inflammation and associated pain will now be discussed. Given the role of PS-GPCRs in general inflammatory programs (discussed above), it is perhaps unsurprising that genetic associations and/or increased expression of the PS-GPCRs have been reported for asthma, RA, ulcerative colitis and Crohn's disease, all chronic inflammatory diseases, which are often accompanied by varying levels of acidosis (Andersson et al., 1999; Aoki et al., 2013; Chen et al., 2009; de Vallière et al., 2015; Hardin et al., 2014; Hunt et al., 2000; Sasaki et al., 1997; Wang et al., 2018).

The airway is exposed to many pathogens and irritants which may rouse the immune system, triggering inflammation and respiratory issues. GPR68 is the most highly expressed PS-GPCR in airway smooth muscle cells and has been shown to be responsible for coordinating the production of IL-6 in response to acidic challenge (Ichimonji et al., 2010). Hypersecretion of inflammatory mucin5AC and enhanced production of connective tissue growth factor by airway smooth muscle cells have also been attributed to acid-induced GPR68 signaling (Liu et al., 2013a; Matsuzaki et al., 2011). Extracellular acidification and GPR68 have also been implicated in contraction of airway smooth muscle cells, thus GPR68 represents a key driver of inflammatory pulmonary disorders (Saxena et al., 2012). Additionally, GPR68 expressing dendritic cells have been shown to regulate airway inflammation in an asthma model with less airway hyperresponsiveness and eosinophilia following adoptive transfer of dendritic cells from GPR68^{-/-} mice. The less severe response in the asthma model could be explained by reduced migration of dendritic cells due to loss of GPR68-mediated expression of the chemotaxis receptor CCR7 (Aoki et al., 2013). The role of GPR65 is less clear, bioinformatic analyses of chronic obstructive pulmonary disorder patients showed elevated expression of the receptor by those suffering from severe emphysema compared to those with mild emphysema (Hu et al., 2018). GPR65 expression also increased during a murine model of asthma and in human asthmatic patients, the receptor being shown to increase the viability of eosinophils, the predominant immune cells mediating asthma pathology, under acidic conditions (Kotlyan et al., 2009). Conversely, Tsurumaki et al (2015) report that GPR65 plays a protective role in lipopolysaccharide-induced lung injury, they observed increased expression of IL-6 and keratinocyte chemoattractant (KC) following acute lung injury in knockout mice, which coincided with higher recruitment of neutrophils to the airway and greater lung injury.

Acidosis has been observed for patients with inflamed joints, who report persistent pain. Given sensory neurones express PS-GPCRs, an acid-nociceptor signaling axis likely contributes to the pain reported by those with chronic inflammatory conditions (Huang et al., 2007). Indeed, genetic associations between PS-GPCRs and pain experienced have been reported by human patients including, increased GPR132 expression by patients experiencing persistent pain following breast cancer surgery and GPR65 associations with ankylosing spondylitis (Kringel et al., 2019; Yuan et al., 2019). Furthermore, GPR65, along with the proton-sensitive ion channels ASIC3 and TRPV1, was shown to contribute to the mechanical hyperalgesia of an experimental RA model in mice, with knockout of ASIC3 or TRPV1 improving pain scores during the later chronic stage of the model, whereas loss of GPR65 ameliorated hyperalgesia in both the acute and chronic stages of the model (Hsieh et al., 2017). Knockdown of GPR65 has also been shown to reduce pain phenotypes in models of inflammatory pain, as well as

an acid-induced hyperalgesia model and the pain produced by administration of the synthetic GPR65 agonist BTB09089. Use of GPR65^{-/-} mice has allowed the contribution of GPR65 to the pain experienced during chronic stages of joint inflammation to be explored. Following CFA-induced inflammation of the knee joint knockout mice took longer to experience hyperalgesia, the peak sensitivity and duration that this persisted was also reduced compared to wildtype counterparts: GPR65^{-/-} mechanical sensitivity had returned to basal levels 35 days after inflammatory insult with wildtype mice taking ~56 days. siRNA mediated knockdown of GPR65 for wild-type animals prevented chronic mechanical hyperalgesia following intraplantar injection of pH 5 solution, similar results were also observed following injection of the GPR65 agonist BTB (Dai et al., 2017). Further probing the role of GPR65 in joint pain, Dai and colleagues (2020) reported reduced numbers of satellite glial cells and macrophages in GPR65^{-/-} mice versus wild-type following CFA-induced inflammation of the knee joint, which was associated with a modest reduction in the acute phase of inflammatory pain, but was sufficient to completely prevent establishment of chronic pain. A switch from PKA driven signalling to PKC ϵ activity has been shown to mediate sustained hyperalgesia during the transition from acute to chronic inflammation, following induction with CFA or acidic solutions. Furthermore, challenge of sensory neurones with acid permitted translocation of both kinases to the plasma membrane, the predominant site of activity for these enzymes, and inhibition of PKC ϵ prevented upregulation of GPR65, further supporting the role of GPR65 in driving nociceptor priming (Huang et al., 2015a). More recently, (Kung et al., 2020) identified three cytokines for which expression peaked in either the acute, transition or maintenance stage of a RA model. A novel compound capable of preventing expression of and functionally inhibiting GPR65 was shown to reduce the increased expression of these cytokines in their respective disease stage, as well as the observed switch in macrophage polarisation. Although pre-treatment of mice with this compound did not affect arthritis scores (based on visual examination of swelling), a reduction in mechanical hypersensitivity and better histological scores were observed. Such findings implicate GPR65 in peripheral sensitization and indeed a role of GPR65 in mediating sensitization of TRPV1 has also been demonstrated, which may provide mechanistic understanding of the thermal hyperalgesia observed in the RA model and has been proven to contribute to the pruritoception associated with intradermal acid application (Lin et al., 2017). Antagonism of GPR4 has also recently been shown to reduce secretion of proinflammatory cytokines and matrix metalloproteinases by human chondrocytes, and thus GPR4 signalling may also contribute to OA pathogenesis (Liu et al., 2020). Further perpetuating the involvement of PS-GPCRs in inflammatory pain is the finding that a recently discovered antagonist of GPR4 was proven to be effective at reducing swelling and hyperalgesia produced by an antigen-induced arthritis model (Miltz et al., 2017).

As well as contributing to enhanced nociception associated with joint inflammation, GPR65 activity within the central nervous system has been shown to confer bone cancer pain in a PKA-dependent manner and GPR68 has been proposed to mediate the pain of heartburn (Hang et al., 2012; Ru et al., 2015). Furthermore, GPR151 expression in trigeminal ganglia has been shown to increase in a model of neuropathic pain, with roles of the receptor in the production of pro-inflammatory mediators responsible for neuroinflammation and modulation of the purine-activated ion channel P2X3 being reported (Jiang et al., 2021; Xia et al., 2021). However, it appears that not all of the PS-GPCRs act to enhance nociception, for example, knockdown of GPR132 led to increased immune cell recruitment and greater sensitivity to mechanical stimuli following induction of inflammation. The opposite was observed when GPR132 was overexpressed: reduced immune cell recruitment and hyperalgesia, which was attributed to $G\alpha_i$ -mediated signalling, as pertussis toxin could reverse the effect (Su et al., 2018). However, it has also been reported that loss of GPR132 reduced macrophage migration following nerve injury, which had the effect of reducing levels of inflammatory mediators at the site of injury and that GPR132 is responsible for driving the production of IL17, CXCL1 and CXCL2 in a model of allergic inflammation; the behavioural implications of these findings however were not studied and responses have been suggested to be driven by lipid activation of the receptor as opposed to protons (Li et al., 2021; Osthues et al., 2020; Song et al., 2021). Huang et al. (2015a) also found that inhibition of PKA, shown to be responsible for driving the acute stages of inflammatory pain, enhanced GPR132 expression which may offer some explanation as to why PKA inhibition soon after an inflammatory insult prevented development of enhanced nociception.

Both GPR4 and GPR68 play pro-inflammatory roles in IBD, expression of these receptors in intestinal samples from human patients with ulcerative colitis and Crohn's disease being elevated; additionally, exposure of colonic tissue or monocytic cells to hypoxic conditions further increased GPR68 expression (de Vallière et al., 2015; Wang et al., 2018). Furthermore, exposure of macrophages to acidic environments increased the expression of pro-inflammatory and adhesion factors in a GPR68-dependent manner and loss of GPR68 expression was shown to delay and reduce the severity of the spontaneous colitis experienced by IL-10^{-/-} mice. Loss of GPR4 in mice has also been shown to ameliorate dextran sulphate sodium- (DSS) induced colitis and spontaneous colonic inflammation that occurs in IL-10^{-/-} mice; as with GPR68, this has mostly been attributed to a reduction in immune cell recruitment and production of pro-inflammatory signaling molecules (de Vallière et al., 2015; 2016; Sanderlin et al., 2017; Wang et al., 2018). The role of GPR65 is less clear, Tcymbarevich and colleagues (2019), reported increased expression of GPR65 by T cells and macrophages during acute DSS-induced colitis. Although expression of pro-inflammatory cytokines was

higher in GPR65^{-/-}, the knock-out mice did not fare any worse in terms of disease severity during acute experimental colitis. Increased infiltration of T cells and macrophages was observed in a chronic DSS model, which coincided with increases in pro-inflammatory mediators, but again histological assessments showed no difference between wild-type and GPR65^{-/-} mice during chronic disease. No obvious differences were reported between GPR65^{-/-} and wild-type mice for an adoptive transfer model nor development of spontaneous colitis by IL-10^{-/-} mice. On the other hand GPR65 has been reported to be important for autophagy-based bacterial clearance and GPR65^{-/-} mice have been shown to be more susceptible to bacterial-induced colitis, thus although GPR65 may act to lessen the severity of inflammation during colitis it may in fact be a contributing factor to disease development (Lassen et al., 2016). Interestingly, an association between carriers of a SNP in *GPR65* and severity of ulcerative colitis has been reported for a Swiss population. The same study reports a modest impairment of acid-induced RhoA activation by monocytes isolated from IBD patients compared to healthy controls, however, the relevance of this to disease progression remains unknown (Tcymbarevich et al., 2019). While the studies described above implicate PS-GPCRs in IBD pathology, investigations have focussed on their role in orchestrating IBD-associated colonic nociceptor hyperexcitability and the pain experienced remains unknown.

As well as their direct effects on immune cells, expression of the PS-GPCRs by other cell types and tissues allows them to contribute to the ongoing inflammation associated with conditions such as arthritis and IBD. However, whether engagement of these receptors serves to maintain the inflammation and/or associated enhanced nociception, or if they exert negative regulation during chronic inflammation is not always clear, and it may also depend on what is activating the receptor. GPR132, for example, has been reported to reduce immune cell recruitment and mechanical hypersensitivity following CFA-induced inflammation (Su et al., 2018). Nonetheless, it has also been reported that GPR132 drives hypersensitivity following oxaliplatin treatment through sensitizing TRPV1 channels, however, this was shown to be due to the activation of the receptor by lipids, which increased in concentration as a result of oxaliplatin administration (Hohmann et al., 2017).

1.8. Aims and scope of thesis

It is clear that there are many links between localised inflammation driven acidosis and pain. This is further reinforced by the fact that direct application of acid is perceived as painful. While ion channels that are activated by protons, such as ASICs and TRPV1, can directly alter sensory neurone physiology and hence drive the enhanced nociception common during

inflammation, the involvement of PS-GPCRs is less clear. Genetic associations and studies of mice lacking PS-GPCRs strongly suggest PS-GPCRs play a role in inflammatory processes and pain, and might thus represent novel targets for therapeutic drugs. Interestingly, a machine learning algorithm designed to identify novel pain targets from scientific literature has also recently independently identified the PS-GPCRs as potential targets for analgesics (Jeon et al., 2021). A major hurdle to developing drugs active at the PS-GPCRs however, is the lack of mechanistic detail, both in terms of basic receptor pharmacology and their contribution to health and disease. The aims of this Thesis were thus twofold: to increase understanding of PS-GPCR pharmacology and investigate the involvement of PS-GPCRs in inflammatory joint pain, working on the hypothesis that these receptors are expressed by nociceptors and may integrate the acidosis of inflammation to coordinate nociception and drive inflammatory pain. More specifically, experimental work was designed to address the following research questions:

1. How does the time course of inflammation correspond with sensory neurone activity and pain-like behaviours? Are PS-GPCRs involved in commonly used models of inflammatory pain?
2. Does PS-GPCR expression and function differ in the naked mole-rat, a species that does not perceive acid as noxious?
3. How do the signalling and trafficking signatures elicited by reported agonists of the PS-GPCR GPR65 compare to one and other?
4. Can a surrogate GPR65 agonist alter nociception and sensory neurone activity in rodents?

2. Materials and Methods

2.1. Agonists, activators and inhibitors

The GPR65 agonist, BTB09089 (BTB) was synthesised by Otava Chemicals and reconstituted in DMSO (Sigma Aldrich), the highest stock concentration prepared was 10 mM. 5 mM stocks of psychosine, a sphingolipid also reported to activate GPR65 (PSY, Sigma Aldrich) were prepared in chloroform:methanol (1:1). The clathrin-mediated endocytosis inhibitor PitStop2 (PS2, Abcam) was prepared at 300 μ M in DMSO. Forskolin (FSK, Sigma Aldrich), an adenylyl cyclase activator, was prepared at 10 mM in DMSO. The phorbol ester, phorbol 12,13-dibutyrate (PDBu, Tocris), an activator of mitogen-activated kinases, was prepared at 10 mM in DMSO. 100 mM stocks of the Ca^{2+} ionophore ionomycin (Cayman Chemicals) were prepared in ethanol. 500 μ M stocks of the *Renilla* luciferase substrate coelenterazine H (CoH, Nanolight Technologies) were prepared in 100% methanol.

2.2. Animals

All animal work was regulated in accordance with the United Kingdom Animal (Scientific Procedures) Act 1986 Amendment Regulations 2012 and was approved by the University of Cambridge Animal Welfare Ethical Review Body. Throughout studies on animals undergoing inflammation models daily monitoring was performed: general welfare (signs of inappetence, coat condition and mobility) was observed and animal weight and joint inflammation were measured to ensure that the humane endpoints (weight loss of 15% within a 24-hour period; joint swelling that exceeds 35% pre-inflammation levels) were not exceeded.

2.2.1. Mice

C57BL/6J mice (Envigo) were housed in groups of up to five per cage with access to food and water *ad libitum*. The holding room was maintained at 21 °C and operated a 12-hour light/dark cycle. Mice aged 10-12 weeks were used. Sex is known to influence susceptibility to inflammatory pain and pain experiences measured in behavioural assays (Mogil, 2012), to prevent this impacting on observations, studies presented in this Thesis were limited to a single sex. The experiments described in Chapter 3 were performed as part of a collaboration for which data had already been gathered on male mice only. Female mice were used for experiments in Chapter 6, looking specifically at joint inflammation, due to the higher incidence of rheumatoid arthritis in female humans (Safiri et al., 2019). It would however, be interesting in the future to extend all studies to both sexes to resolve any sex specific effects.

2.2.2. Naked mole-rats

Naked mole-rats (*Heterocephalus glaber*, NMR) were bred in-house and maintained in an inter-connected network of cages in a humidified (~55%) environment held at 28 °C with red lighting (08:00 – 16:00). Animals had access to food *ad libitum*. Non-breeding NMRs of both sexes, aged 1-2 years, were used.

2.3. Intra-articular injections

Intra-articular injections of mice knee joints were performed under anaesthesia (ketamine, 100 mg/kg and xylazine, 10 mg/kg administered intra-peritoneally). After complete loss of consciousness injections were made through the patella tendon using a Hamilton syringe and 30 g needle. To label knee-innervating sensory neurones, 1.5 µl of the retrograde tracer Fast Blue (FB, 2% in saline, Polysciences) was injected. To induce inflammation 10 µl Complete Freund's adjuvant (CFA, 10 mg/ml, Chondrex) or 100 µM BTB was injected unilaterally (ipsilateral knee randomly determined) 5-7 days after labelling. Control cohorts were injected with 10 µl DMSO (Sigma Aldrich) at a comparable concentration. Inflammation of the knee joint was determined by measuring the joint diameter with digital Vernier callipers before and after injections at specified time points. The weights of animals were monitored daily after procedures to ensure weight loss did not exceed 15% body weight in a 24-hour period.

2.4. Intra-plantar injections

To label cutaneous afferents innervating the hind paw 3 x 1 µl injections of FB were made to the lateral, central and medial plantar aspects of paws under anaesthesia (ketamine, 100 mg/kg and xylazine, 10 mg/kg administered intra-peritoneally). Injection of 20 µl 3% (w/v) λ-carrageenan (prepared in sterile phosphate buffered saline (PBS), Sigma Aldrich) to the right hind paw was performed to induce inflammation 5-7 days after retrograde labelling. Injections of carrageenan were made under inhalation anaesthesia (2% isoflurane). The diameters of footpads and ankles were measured before and after injections with digital callipers to quantify inflammation.

2.5. Mouse behavioural studies

Before commencing any behavioural testing, mice were allowed to acclimatise to the testing room in the presence of experimenters for at least 30 minutes.

2.5.1. Digging behavioural paradigm

Assays were designed with reference to Wodarski et al. (2016). Mice were transferred to testing cages set up with ~3.5 cm of tightly packed fine-grain aspen midi 8/20 wood chip bedding (LBS Biotechnology). Mice were allowed 3 minutes to explore the testing cage without interference under video surveillance. Mock/training sessions were carried out the day before baseline digging was assessed, on these days mice were placed in the test cages twice, with a 30 minute break between sessions. On the rare occasions that a mouse did not dig for at least ~15 seconds during the first training session, the individual was placed in a test cage with a mouse that met this criterion before the second test dig. No further training sessions were performed during studies after a baseline test had been recorded. The number of visible burrows at the end of each testing period was recorded, burrowing behaviour was further analysed offline via video playback at the conclusion of each study following blinding of all videos. The time mice spent burrowing was measured from videos and was scored independently by two observers (Rebecca Rickman and Luke Pattison); since the scores of experiments correlated well ($R^2 = 0.934$, across 190 scorings) an average is reported as the time burrowing.

2.5.2. Dynamic weight bearing

The weight bearing of free-moving animals was assessed using a dynamic weight bearing apparatus comprising an open field area with a pressure sensitive floor (matrix of 2000 high precision force sensors) (Bioseb). Each test lasted 3 minutes, mice were naïve to the test device before baseline weight-bearing was assessed. The 2 highest confidence levels of automatic paw assignment by the accompanying software were taken forward for analyses; correct paw assignment was manually validated for at least 1 minute 30 seconds of each test. Outputs taken forward for analysis included % time animals spent rearing or immobile in the arena, the proportion of weight placed through individual rear paws and the ratio of weight placed through front paws relative to that placed through the rear paws.

2.5.3. Rotarod

A rotarod apparatus (Ugo Basile 7650) was used to assess the motor coordination of mice. Animals were allowed 1 minute to acclimatise to the apparatus on a slow setting (7 revolutions per minute), before an accelerating program (7-40 rpm in 5 minutes) was started, test runs were video-recorded. Mice were removed from the rotarod if they fell, following two consecutive passive rotations or after 6 minutes of the accelerating program, whichever

occurred first. Analysis of video recordings was performed at the conclusion of individual studies, videos were blinded and the latency to passive rotation or fall was scored.

2.5.4. Pressure application measurement

Mechanical sensitivity of the knee joint was assessed using a pressure application measurement device (Ugo Basile). Experiments were designed with reference to Miller et al. (2017). Briefly, one experimenter scruffed and presented animals to another experimenter, who used the force transducer to apply gradual force to each of the animals' knee joints, by squeezing the joint medially. The withdrawal threshold was recorded when an animal either: withdrew the limb being tested, vocalised, or after 450 g force was applied. Each animal was tested twice per day, with a short break between tests. For behavioural studies comparing mice injected with BTB or DMSO over 7-days, the experimenter applying force was blinded to the treatment group of animals by the experimenter presenting animals. Pressure application measurements were taken by a single experimenter for mice injected with BTB to be used for electrophysiology experiments 24-hours post injection, thus the scruffing and application of force was slightly different for these experiments, which were also unable to be blinded.

2.6. Isolation and culture of mice dorsal root ganglia (DRG)

Mice were sacrificed by cervical dislocation before the lumbar DRG (L2 – L5) were collected in dissociation medium (L-15 + GlutaMAX growth media supplemented with 24 mM NaHCO₃; Life Technologies). For experiments on cells from mice which underwent unilateral injection of an inflammatory substance (carrageenan or BTB), DRG from the injected (ipsilateral, ipsi.) and non-injected (contralateral, contra.) sides were collected separately and underwent the rest of the culturing process independently. When naïve mice were used, lumbar DRG from both sides of each mouse were pooled and cultured together. Once dissected, DRG were incubated in dissociation media containing 1 mg/ml type 1A collagenase (Sigma-Aldrich) and 6 mg/ml BSA for 15 min at 37 °C, 5% CO₂, before a further 30 min incubation in dissociation media containing 1 mg/ml trypsin (Sigma-Aldrich) and 6 mg/ml BSA. DRG were then suspended in culture media (L-15 + GlutaMAX growth media supplemented with 10% (v/v) foetal bovine serum (FBS, Sigma Aldrich), 24 mM NaHCO₃ 38 mM glucose and 2% (v/v) penicillin/streptomycin (Life Technologies)) before several rounds of mechanical trituration and brief centrifugation (160g, 30 s). After sufficient trituration, dissociated cells were pelleted (160g, 5 min), resuspended in culture media and plated on poly-D-lysine/laminin coated glass

coverslips (BD Biosciences) or glass-bottomed dishes (MatTek) and incubated at 37 °C, 5% CO₂.

2.7. Isolation and culture of mouse fibroblast like synoviocytes (FLS)

The knee joints of mice were exposed by removing the skin, the quadriceps muscles were then resected in the middle and pulled in a distal direction to expose the patella. Patellae were collected in PBS before transfer to FLS media: Dulbecco's Modified Eagle Medium/F-12 Nutrient Mixture + GlutaMAX (Life Technologies) supplemented with 25% (v/v) FBS and 1% (v/v) penicillin/streptomycin in a 24-well plate and incubated at 37 °C, 5% CO₂. Media was refreshed daily. Once a substantial number of FLS had grown out of the patellae (~10 days), the tissue was discarded and remaining adherent cells trypsinised, cells from two patellae were combined in 6-well plates and maintained by replacing culture media every other day. FLS were used for experiments from passage 3 (P3), as from this point FLS markers have been shown to be enriched, with low endothelial and macrophage marker expression (Chakrabarti et al., 2020a). P3 FLS were stimulated with either 100 µM BTB or an equivalent concentration of DMSO prepared in unsupplemented Dulbecco's Modified Eagle Medium/F-12 Nutrient Mixture + GlutaMAX; a control condition of cells incubated solely in unsupplemented media was included for each experiment. 24-hours post-stimulation, culture media was collected and stored at -80 °C, RNA was extracted from control cells (cultured overnight in unsupplemented media) and cells from the other conditions were fixed for immunostaining.

2.8. Cell line culture and generation of stable cell lines

All cell lines were cultured at 37 °C in a humidified 5% CO₂ incubator.

2.8.1. Chinese hamster ovary (CHO) cells

Chinese hamster ovary (CHO, 85050302, Sigma Aldrich) cells were maintained in F12 media supplemented with 10% (v/v) FBS and 1% (v/v) penicillin/streptomycin.

2.8.2. Flp-In Chinese hamster ovary (Flp-In CHO) cells

Flp-In CHO cells (R75807, Thermo Fisher) were cultured in the same media as CHO cells also containing 100 µg/ml zeocin (InvivoGen).

2.8.3. Generation of CHO cell lines stably expressing PS-GPCRs

Cells stably expressing PS-GPCR constructs were generated by co-transfecting Flp-In CHO cells with the desired PS-GPCR construct (cloned into a pcDNA5/FRT/TO vector) and the Flp recombinase, pOG44, at a ratio of 1:9, PS-GPCR:pOG44 using lipofectamine LTX and in the absence of any antibiotics. 48-hours post-transfection, culture media was replaced with standard CHO media containing 550 µg/ml hygromycin-B (InvivoGen). After expansion of hygromycin-B resistant cells, expression of constructs was confirmed using reverse transcription PCR (RT-PCR) and immunocytochemistry.

2.9. RNA extraction and reverse transcription

2.9.1. RNA extraction from tissue

Tissues of interest were dissected, cut into small (~50 mm³) sections and washed twice in PBS before collection in RNA^{later}[®] solution (Sigma Aldrich). Tissues were either physically macerated or passed through a 21G needle several times before brief sonification. RNA was purified using a RNeasy kit (Qiagen) following the manufacturers' instructions.

Due to low yield, RNA extraction from DRG was performed using TRIzol reagent (Sigma Aldrich): DRG were suspended in 700 µl TRIzol and passed through a 21G needle several times, after a 5 min incubation 100 µl 1-bromo-3-chloropropane was added and samples were shaken vigorously before a further 3 minute incubation. Samples were then centrifuged at 13,000 rpm for 15 minutes at 4 °C to separate the different phases, the least dense, colourless phase, containing RNA, was collected and cleaned up using the spin columns and wash buffers supplied with Qiagen RNeasy kits.

After purification RNA yield and quality was assessed using a N50 nanovolume nanophotometer (Implen). Samples were only carried forward if absorbance ratios met certain criteria: $2.1 \geq A_{260}/A_{280} \geq 1.9$ and $1.7 \geq A_{260}/A_{230}$. Samples were stored at -80 °C until use.

2.9.2. RNA extraction from cultured cells

Once confluent in a 6-well plate, CHO cells or FLS were trypsinised and pelleted before RNA was extracted and purified using a Qiagen RNAeasy kit according to the manufacturer's instructions. Purified RNA was quantified, quality assessed and stored as above.

2.9.3. Reverse transcription

High-capacity reverse transcription reagents (Applied Biosystems) were used to synthesise complementary DNA (cDNA) from RNA samples using random hexamers to prime reactions. Reverse transcription reactions included 500 ng of RNA as a template, the efficiency of reverse transcription was assumed to be 100% (i.e. 500 ng RNA assumed to produce 500 ng cDNA). Controls where reverse transcriptase was omitted from the reaction mix were performed in parallel.

2.10. Molecular cloning of PS-GPCR constructs

Phusion high-fidelity DNA polymerase (Thermo Fisher) was used to amplify the open reading frame of genes of interest from cDNA libraries prepared from RNA extracted from an appropriate tissue (reported to express the gene of interest). Primers were designed to introduce flanking restriction sites and N- and C-terminal tags as necessary to permit sub-cloning into a variety of recipient plasmids following standard restriction cloning and/or subcloning practices. The vector pcDNA5/FRT/TO was kindly gifted by Dr Graham Ladds (University of Cambridge). Constructs encoding Lysosome-associated membrane glycoprotein 1 (LAMP1) fused to -eGFP and -mCherry at the C-terminus were kindly gifted by Prof. Colin Taylor (University of Cambridge). A construct encoding a protease-activated receptor-2 tagged with Renilla luciferase 8 (RLuc8) at the C-terminus was a kind gift from Prof. Nigel Bunnett (New York University). All constructs were verified by Sanger sequencing (Eurofins Genomics).

2.10.1. Site-directed mutagenesis

Primers and protocol for site-directed mutagenesis were designed with reference to Zheng et al. (2004). Following amplification of mutated products wildtype template plasmids were digested with DpnI (37 °C, 30 min, Thermo Fisher), 2 µl digested PCR product was then used to transform 10-β competent E. coli (New England Biolabs). Constructs were purified from one colony and verified by Sanger sequencing (Eurofins Genomics).

2.11. RT-PCR

PCR reactions were set up using 1.25 ng of cDNA and primers designed to amplify hamster PS-GPCRs, ASIC1 and GAPDH, the sequences of which are provided in Table 2.1.

Target (NCBI Ref Seq)	Sequence (5' → 3')	Amplicon length (bp)
Hamster <i>Gpr4</i> (XM_015987275.1)	<i>Fwd</i> GGGATACCACTCTGAAGC	147
	<i>Rev</i> TTGTGGGTAGAAGGAGAACT	
Hamster <i>Gpr31</i> (XM_027437727.1)	<i>Fwd</i> CCCTCTGGACCTTCTTTTAC	127
	<i>Rev</i> CCCTTCAGATAGAAGACAGC	
Hamster <i>Gpr65</i> (XM_003495977.1)	<i>Fwd</i> GAACGAGAAGATTTGCACTC	140
	<i>Rev</i> GTCATAACACAGGGTGAAGT	
Hamster <i>Gpr68</i> (XM_016979359.1)	<i>Fwd</i> CAAGTTCCTGAATAGGGCTT	187
	<i>Rev</i> CTCTGGTATCAGTGTCTTGG	
Hamster <i>Gpr132</i> (XM_007633001.2)	<i>Fwd</i> CAAGAAGTGTCCAGAATCCA	121
	<i>Rev</i> GGAAGGTGTAGCTGTTTGTGTA	
Hamster <i>Gpr151</i> (XM_027397991.1)	<i>Fwd</i> TACCTGCCATCTGACTCCAA	138
	<i>Rev</i> CATGGATGGCTTTCTTTTCCG	
Hamster <i>Asic1</i> (XM_003507844.3)	<i>Fwd</i> TTAAGCACCGTTTGTGTAGA	171
	<i>Rev</i> GATGGTGAGGTAGGATGTTG	
Hamster <i>Gapdh</i> (NM_001244854.2)	<i>Fwd</i> CCTGGTATGACAATGAATTTGG	157
	<i>Rev</i> ATGGAAACTGTGAGGGAGAT	

Table 2.1 Primers designed to amplify short sections of the acid-sensitive receptors or the housekeeping gene *Gapdh* based on the reference genome for Chinese hamster (*Cricetulus griseus*).

Control reactions using the no reverse transcriptase control from cDNA preparation were set up in parallel using the primers designed to amplify the housekeeping gene *Gapdh*. A standard thermocycling programme was run for 25 cycles: after a 2 minute initial degradation, reactions were cooled to 55 °C for 30 s primer annealing, a one minute extension phase at 72 °C was performed each cycle, samples were held at 72 °C for 5 minutes after all cycles were run for final extension. PCR products were resolved on 1.5% agarose gels stained with GelRed nucleic acid stain (Biotium) alongside Gene Ruler 100 bp DNA ladder (Thermo Fisher) and imaged with a GeneFlash Gel Documentation System (Syngene).

2.12. qPCR

cDNA samples were diluted to 1 ng/μl in ddH₂O. For studies only conducted on mouse samples, gene expression was assessed using TaqMan assays (Thermo Fisher) as per the manufacturer's guidelines. Thermocycling was performed using a StepOnePlus™ System (Thermo Fisher) and the following settings: 50 °C for 2 min, 95 °C for 10 min, 40 cycles of 95

°C for 15 sec and 60 °C for 1 min. The fluorescence intensity of samples was captured during the final minute of each cycle. Each sample of cDNA was assayed in duplicate. All reactions were performed in duplicate alongside appropriate negative controls where a combination of RNA samples was used as the template. For each cDNA sample the expression of the housekeeping gene *18SrRNA* was also analysed. All probes used are detailed in Table 2.2.

Target	Probe ID
<i>Gpr4</i>	Mm00558777_s1
<i>Gpr31</i>	Mm02391728_g1
<i>Gpr65</i>	Mm00433695_m1
<i>Gpr68</i>	Mm01335275_m1
<i>Gpr132</i>	Mm00490809_m1
<i>Gpr151</i>	Mm00808987_s1
<i>Trpv1</i>	mm01246302_m1
<i>Asic3</i>	Mm00805460_m1
<i>18SrRNA</i>	Mm03928990_g1
<i>Cdh-11</i>	Mm00515466_m1
<i>Cd-248</i>	Mm00547485_s1
<i>Cd-68</i>	Mm03047343_m1
<i>Cd-31</i>	Mm01242576_m1

Table 2.2 TaqMan mouse expression assay IDs.

To compare expression of PS-GPCRs between mice and naked mole-rats, reactions were prepared with SYBRgreen reagents (2 x qPCR master mix, PrimerDesign), 1 ng template cDNA and primers designed to amplify short segments within the open reading frames of genes of interest (sequences detailed in Table 2.3). Thermocycling was performed using a StepOnePlus™ System (Thermo Fisher) and the following settings: 95 °C for 10 min, 40 cycles of 95 °C for 15 sec and 60 °C for 1 min. The fluorescence intensity of samples was captured during the final minute of each cycle. Each sample of cDNA was assayed in duplicate. At the end of thermocycling a melt curve was performed to assess the specificity of primers, single peaks were obtained for all primer pairings.

Target (NCBI Ref Seq)	Sequence (5' → 3')	Amplicon length (bp)
Mouse <i>Gpr4</i> (NM_175668.4)	<i>Fwd</i> ATTCAGCACCGCTCTTCCAT	236
	<i>Rev</i> CAGGGCCAGACGTTTGATCT	

Mouse <i>Gpr31</i> (NM_001013832.2)	<i>Fwd</i> GTGTCCTGACCAGGGTTCTG	152
	<i>Rev</i> GGCTGGATTGGAGAAGCAGT	
Mouse <i>Gpr65</i> (NM_008152.3)	<i>Fwd</i> CAACATCGGATCTTTATGCG	195
	<i>Rev</i> ATGTAGGTGAAGAAAACGCT	
Mouse <i>Gpr68</i> (NM_001177673.1)	<i>Fwd</i> TTCTCCCTCCTCCTCACCAG	234
	<i>Rev</i> GGCTGAGTGGAGCTTGGTTA	
Mouse <i>Gpr132</i> (NM_019925.4)	<i>Fwd</i> CCACTACCTGCGTTTCACCT	161
	<i>Rev</i> CCAGGAAGATGGTGACGACC	
Mouse <i>Gpr151</i> (NM_181543.2)	<i>Fwd</i> TTTGGGATCTCGGCTGGTTC	236
	<i>Rev</i> GTGACGTCTGGTGGTGCTAA	
Mouse <i>β-Actin</i> (NM_007393.5)	<i>Fwd</i> CGATATCGCTGCGCTGGTC	243
	<i>Rev</i> TTCTCCATGTCGTCCCAGTTG	
Mole-rat <i>Gpr4</i> (XM_004867144.3)	<i>Fwd</i> TACCACGTGCTGCTGCTATC	230
	<i>Rev</i> ATCTCCTGGGGCTTGTCCT	
Mole-rat <i>Gpr65</i> (XM_004837096.2)	<i>Fwd</i> TGTAATCAGTGTCCCAGCCA	240
	<i>Rev</i> AGAAACGCTGTGCTGCTGTA	
Mole-rat <i>Gpr68</i> (XM_004837064.2)	<i>Fwd</i> TGCTTCGTCAGTGAGACCAC	198
	<i>Rev</i> GCTAGGGGTCTGAAAGGCTG	
Mole-rat <i>Gpr132</i> (XM_021243445.1)	<i>Fwd</i> GCTCTGCAATGCATCCTTCG	150
	<i>Rev</i> AACAGGTAGACAGCCAGCAC	
Mole-rat <i>Gpr151</i> (XM_004854504.2)	<i>Fwd</i> GGAGAGCTTACGGCCAATGT	166
	<i>Rev</i> GCCTTCAGGTGCCATATCCA	
Mole-rat <i>β-Actin</i> (XM_004848498.2)	<i>Fwd</i> ATATCGCTGCGCTCGTTGTC	190
	<i>Rev</i> AGGGTCAGGATGCCTCTCTT	

Table 2.3 Primers were designed to amplify short sections of the acid-sensitive receptors or the housekeeping gene β -actin based on the reference genome for mice (*Mus musculus*) and naked mole-rats (*Heterocephalus glaber*).

2.12.1. qPCR analysis to determine relative expression

To determine the relative expression of genes, the Ct values obtained for the housekeeping gene for each sample were subtracted from Ct values obtained for genes of interest, to yield a Δ Ct value. Relative expression was calculated as $2^{-\Delta\text{Ct}}$ assuming a 100% primer efficiency, however, this remains to be empirically confirmed from a dilution curve.

2.12.2. qPCR analysis to determine change in expression

Δ Ct values were calculated for each sample as described above. To assess inflammation-induced changes in gene expression the Δ Ct values for samples obtained from the inflamed (ipsi.) side of mice were subtracted from those of the contralateral (contra.), fold change was calculated from the resulting $\Delta\Delta$ Ct value by calculating $2^{-\Delta\Delta\text{Ct}}$ as described by Livak and Schmittgen (2001).

2.13. Immunocytochemistry

2.13.1. Staining of cultured CHO cells

Where necessary CHO cells were transfected with 1 μ g fluorescently tagged PS-GPCR constructs once ~80% confluent in 6-well plates 24-hours before cell plating. CHO cells were plated on poly-D-lysine coated 22 x 22 mm glass coverslips (15717582, Fisher Scientific) and cultured for 24 hours. Cells were fixed with 4% (w/v) paraformaldehyde (PFA) for 10 minutes at room temperature after which cells were washed twice with ice-cold PBS. Cells were then incubated with antibody dilutant (1 % (w/v) BSA, 5% (v/v) donkey serum and 0.02% (v/v) Triton-X-100 in PBS) at room temperature for 1 hour before overnight incubation at 4 °C with primary antibodies: anti-FLAG (DDDDK) tag (1:500; rabbit polyclonal, Abcam, ab1162). The following day slides were washed three times with PBS containing 0.001% (v/v) Tween-20 (PBS-Tween, Thermo Fisher Scientific) before incubation with donkey anti-rabbit IgG-AF594 (1:1000; donkey polyclonal, Invitrogen, A32754) for 2 hours at room temperature, cells were washed once with PBS-Tween before a 10 minute incubation with 4',6-diamidino-2-phenylindole (DAPI) nuclear dye (300 nM in PBS, Thermo Fisher) and a further two washes. Coverslips were then mounted on microscopy slides and later imaged with an Olympus BX51 microscope and Q-Imaging camera. Fluorescently tagged PS-GPCR constructs were imaged with Slides were imaged with a Leica SP5 laser-scanning confocal microscope and 63x oil objective.

2.13.2. Staining of FLS cells

FLS were fixed by exposure to ice cold 100% methanol for 10 mins at -20 °C, after which cells were washed with ice cold PBS three times. Cells were incubated in antibody dilutant for 1 hour, before overnight incubation with primary antibodies: CD248 (1:200, rabbit monoclonal, Cell Signalling Technology, 47948) or CDH-11 (1:100, rabbit polyclonal, Invitrogen, 71-7600) at 4 °C. The following day cells were washed with PBS-T three times before incubation with

donkey anti-rabbit Ig-AF488 (1:1000; donkey polyclonal, Invitrogen, A32790) for 2 hours at room temperature, followed by a further two washes, cells were left in PBS and imaged with a FLoid™ Cell Imaging station (Thermo Fisher).

2.14. Immunohistochemistry

Mice for immunohistochemistry were transcardially perfused with 4% (w/v) PFA under terminal anaesthesia (intraperitoneal delivery of 200 mg/kg sodium pentobarbital). Lumbar DRG (L3 – L4) were then collected from both the inflamed and non-injected sides and post-fixed in Zamboni's fixative (containing 4% (w/v) PFA) for 30 min, followed by overnight incubation in 30% (w/v) sucrose at 4 °C for cryoprotection. Individual DRG were then snap-frozen in Shandon M-1 Embedding Matrix (Thermo Fisher). 12 µm sections of each DRG were cut on a Leica CM3000 cryostat sequentially across 10 slides. After washing with PBS-Tween, slides were incubated with antibody dilutant at room temperature for 1 hour before overnight incubation at 4 °C with primary antibodies: TRPV1 (1:500; guinea-pig polyclonal; Alomone, AGP-118); P2X3 (1:1000, rabbit polyclonal, Alomone, APR-016). The following day slides were washed three times with PBS-Tween before incubation with donkey anti-guinea-pig IgG-AF488 (1:1000; donkey polyclonal, Jackson Immuno, 706-545-148) and donkey anti-rabbit IgG-AF594 (1:1000; donkey polyclonal, Invitrogen, A32754) for 2 hours at room temperature, followed by a further two washes and mounting. Images were acquired with an Olympus BX51 microscope and Q-Imaging camera. Two sections per DRG per animal were analysed. Briefly, each cell was selected as a region of interest and individual cells were considered positively stained if the background corrected intensity exceeded 2x standard deviation (SD) of the normalised intensity across all sections. Negative controls which were not exposed to any primary antibody showed no fluorescent staining.

2.15. Antibody array to assess inflammatory mediator release by FLS cells

The media collected from FLS after 24-hours of stimulation with 100 µM BTB or an equivalent concentration of DMSO was analysed with a Mouse Inflammation Antibody Array (Abcam, ab133999) following the manufacturer's guidelines. Media from three biological replicates were pooled and incubated (total volume 1.2 ml) with the array membranes overnight at 4 °C. Chemiluminescent signals were captured with an Odyssey XF imager (Li-Cor). Densitometry analysis was performed in ImageJ: for each array the intensities of targets was normalised to the average intensity of positive control spots to yield relative expression. Fold change was

obtained by dividing normalized intensities of the membrane of interest by the control membrane, analyte-by-analyte.

2.16. BRET β -arrestin recruitment assays

Flp-In CHO cells were plated in 100 mm dishes, once 80% confluent, cells were transiently transfected with 4 μ g β -arrestin1-YFP/ β -arrestin2-YFP/pcDNA3.1 and 1 μ g MmGPR65-RLuc8 using Lipofectamine LTX transfection reagent (3:1, LTX:cDNA). Tagged β -arrestin constructs were a kind gift from Prof. Nigel Bunnett (New York University). 24 hours post-transfection, cells were resuspended in media and seeded in poly-D-lysine coated white 96-well OptiPlates (PerkinElmer) at a density of 30,000 cells / well. The following day, culture media was replaced with 100 μ l HBSS containing 10 mM HEPES, 10 mM MES and 2 mg/ml BSA, pH 7.40. Cells were allowed to acclimatise for 30 minutes at 37 °C before the HBSS was replaced with 90 μ l stimulatory solutions containing various concentrations of agonist or pH buffered as necessary. Cells were incubated (37 °C) in stimulatory solutions for 10 minutes before the addition of 5 μ M coelenterazine-H. After a further 5 minutes incubation the intensities of RLuc8 and YFP luminescence were measured using a CLARIOstar plate reader (BMG LabTech) with bandwidth settings 475 ± 30 nm and 535 ± 30 nm respectively, each stimulation was assayed in duplicate. BRET ratios were calculated by dividing YFP emission by RLuc8 luminescence, ratios from cells expressing MmGPR65-RLuc8 and pcDNA3.1 were subtracted from those expressing YFP constructs to yield netBRET ratios.

2.17. BRET intracellular trafficking assays

CHO cells were plated in 100 mm dishes, once 80% confluent, cells were transiently transfected with 4 μ g Rab5a-Venus/RIT-Venus/pcDNA3.1 and 1 μ g MmGPR65-RLuc8 using Lipofectamine LTX transfection reagent (3:1, LTX:cDNA). To explore the role of dynamin in receptor endocytosis cells were transfected with 2.5 μ g Dynamin-HA WT/Dynamin-HA K44E/pcDNA3.1, 500 ng MmGPR65-RLuc8 and 2 μ g Rab5a-Venus/pcDNA3.1. Tagged dynamins and BRET acceptor constructs were a kind gift from Prof. Nigel Bunnett (New York University). 24 hours post-transfection, cells were resuspended in media and seeded in poly-D-lysine coated white 96-well OptiPlates (PerkinElmer) at a density of 30,000 cells / well. The following day, culture media was replaced with 100 μ l HBSS containing 10 mM HEPES, 10 mM MES and 2 mg/ml BSA, pH 7.40. Cells were allowed to acclimatise for 30 minutes at 37 °C before the HBSS was replaced with 90 μ l stimulatory solutions containing various concentrations of agonist or pH buffered as necessary and 5 μ M of the RLuc8 substrate

coelenterazine-H. RLuc8 luminescence (475 ± 30 nm) and Venus emission (535 ± 30 nm) were read every minute at 37 °C using a CLARIOstar microplate reader (BMG LabTech). BRET ratios (Venus intensity divided by RLuc8 intensity) were calculated and corrected to those obtained from cells transfected with RLuc8 and pcDNA3.1 in place of any BRET acceptor to yield netBRET ratios which were also baseline and vehicle corrected. Where used, the clathrin inhibitor PitStop2 was incubated with cells during the 30-minute acclimatisation before assays at a final concentration of 30 μ M and present throughout assays, control experiments were incubated with an equivalent concentration of DMSO.

2.18. cAMP accumulation assays

The day before experiments cells were seeded in standard 96-well plates at a density of 15,000 cells / well, the following day culture media was replaced with HBBS containing 10 mM HEPES, 10 mM MES and 2 mg/ml BSA, pH 7.40. Cells were allowed to acclimatise for 30 minutes at 37 °C before the HBSS was replaced with 100 μ l solutions of HBSS containing various concentrations of agonist or pH buffered as necessary. After 15 minutes of stimulation at 37 °C, plates were transferred to ice, stimulatory solutions were aspirated and 50 μ l lysis buffer (50 mM HEPES, 10 mM CaCl_2 , 0.35% Triton X-100; pH 7.40) was added to each well, the plate was incubated on ice with gentle agitation for 30 minutes to ensure full cells lysis. Each condition was assayed in duplicate.

The cAMP in lysates was quantified using LANCE *Ultra* cAMP detection reagents (Perkin Elmer). Briefly 10 μ l of cell lysate was incubated with europium (Eu) labelled cAMP tracer and cAMP-specific antibody labelled with *ULight*TM dye in white ProxiPlate 384-well plates (Perkin Elmer) for 1 hour in reduced lighting as per the manufacturer's guidelines. After the incubation, TR-FRET was measured using a Mithras LB 940 microplate reader (Berthold Technologies) with excitation at 340 nm and emission recorded at 665 nm.

2.19. Ca^{2+} mobilisation assays

The day before assays were conducted, cells were seeded in black 96-well ViewPlates (PerkinElmer) at a density of 50,000 cells / well. The following day, cells were loaded with the Ca^{2+} -sensitive dye Fluo4 (Thermo Fisher) by exchange of culture media for HBSS containing 10 mM HEPES, 10 mM MES, 2 mg/ml BSA, 250 mM probenecid and 2.5 μ M Fluo4-AM. Cells were incubated for 45 minutes at 37 °C to ensure good uptake of the dye, after which cells were washed with HBSS containing 10 mM HEPES, 10 mM MES, 2 mg/ml BSA and 250 mM probenecid twice, cells were left in 90 μ l of this assay buffer. Experiments were performed

using a CLARIOstar microplate reader (BMG LabTech) using the inbuilt incubator set at 37 °C. The intensity of Fluo4 was monitored every second through excitation at 483 ± 14 nm and emission recorded at 530 ± 30 nm. Each well was monitored individually, after recording a 15 second baseline, the automated injectors of the microplate reader were used to dispense 10 μ l of stimulatory solutions and measurements continued for a further 2 minutes, each stimulation was performed in duplicate. Stimulation-induced changes in Ca^{2+} mobilisation were observed after correcting Fluo4 intensity for the 15 second baseline of each recording, signals were then normalised to an F_{max} achieved by stimulating cells with 10 μ M ionomycin to yield F/F_{max} , the peak values of which for each stimulation were used to plot concentration-response curves. Stimulatory solutions containing agonists were made at a 10 X concentration, to permit the desired concentration once injected to the 90 μ l assay buffer. For proton-stimulation in Ca^{2+} experiments, the concentration of 1 ml HCl required to change the pH of 9 ml pH 7.40 assay buffer to the desired pH was determined empirically before each experiment, the same change in pH was assumed to take place following injection of 10 μ l HCl at the desired concentration to 90 μ l assay buffer pH 7.40.

2.20. ERK phosphorylation assays

Cells were plated at a density of 50,000 per well in standard 96-well plates, the following day culture media was replaced with serum-free Opti-MEM (Life Technologies). 16 hours later, cells were acclimatised in HBSS containing 10 mM HEPES, 10 mM MES, 2 mg/ml BSA, pH 7.40 for 30 minutes at 37 °C, after which assay buffer was exchanged for 100 μ l stimulatory solutions (assay buffer containing agonists or buffered to a certain pH), cells were stimulated for 5 minutes at 37 °C before being placed on ice and lysed (50 mM HEPES, 10 mM CaCl_2 , 0.35% Triton X-100; pH 7.40), plates were gently agitated for 30 minutes to ensure full cells lysis. All stimulations were performed in duplicate.

Phosphorylated ERK1/2 was quantified in lysates using Phosphorylated ERK1/2 (T202-Y204) LANCE *Ultra* detection reagents. 15 μ l of each lysate was combined with 0.5 nM Eu-labelled-anti-ERK1/2 (T202/Y204) antibody and 5 nM *ULight*-labelled-anti-ERK1/2 antibody in white ProxiPlate 384-well plates in reduced lighting. After 4 hours of incubation TR-FRET was measured with a Mithras Plate Reader with excitation at 340 nm and emission recorded at 615 nm and 665 nm. Emission at 665 nm was divided by emission at 615 nm and data normalised to the ERK activation achieved by 10 μ M PDBu.

2.21. DRG Electrophysiology

Electrophysiology recordings were performed the day after DRG cultures were prepared, using an EPC-10 amplifier (HEKA) and corresponding Patchmaster software. The extracellular solution contained (in mM): NaCl (140), KCl (4), MgCl_2 (1), CaCl_2 (2), glucose (4) and HEPES (10), pH 7.40. Patch pipettes were pulled from borosilicate glass capillaries (Hilgenberg) using a P-97 pipette puller (Sutter Instruments) with resistances of 4-8 $\text{M}\Omega$ and back-filled with intracellular solution containing (in mM): KCl (110), NaCl (10), MgCl_2 (1), EGTA (1), HEPES (10), Na_2ATP (2), Na_2GTP (0.5), pH 7.30. Whole cell currents or voltages were acquired at 20 kHz. Where necessary FB labelled neurones, identified by LED excitation at 365 nm (Cairn Research). Step-wise depolarisation ($\Delta 10$ pA, 50 ms) was used to determine the action potential threshold (rheobase) of cells. Only cells which fired action potentials and had a resting membrane potential less than or equal to -40 mV and were included in analyses. Action potential parameters were measured using Fitmaster software (HEKA) and IgorPro software (Wavemetrics) as annotated in Figure 2.1. The excitability of neurones was further assessed by applying a suprathreshold ($2\times$ action potential threshold) for 500 ms, the frequency of action potentials during this time was noted.

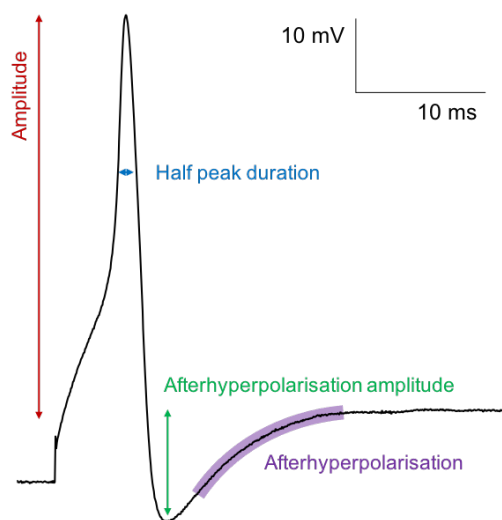


Figure 2.1. Annotation of parameters analysed from action potentials generated in current clamp mode. Close up of an evoked (rheobase = 310 pA) action potential from an acutely dissociated sensory neurone annotated to show how the amplitude, half peak duration, afterhyperpolarisation and afterhyperpolarisation amplitude parameters were measured.

The activity of macroscopic voltage-sensitive channels was assessed in voltage-clamp mode with appropriate compensation for series resistance. Cells were held at -120 mV for 150 ms before stepping to the test potential (-60 mV – 55 mV in 5 mV increments) for 40 ms and returning to a holding potential of -60 mV for 200 ms between steps. Peak inward and outward currents were normalised to cell size by dividing by cell capacitance. Peak inward current densities were then fit to a Boltzmann function to determine the reversal potential and half-activating potential of voltage-sensitive channels. Where necessary, the total conductance of

inward currents was determined using the equation, $G = I/(V_m - E_{rev})$, where G , total conductance; I , peak inward current; V_m , mV step to elicit I and E_{rev} , reversal potential and fit to a Boltzmann function. To compare macroscopic voltage-sensitive current densities between neurones isolated from the inflamed and non-inflamed sides or treated conditions, peak current densities and total conductance values were normalised to those obtained from the most appropriate control (the contralateral side or control media condition).

2.22. Data analysis and statistics

Data are presented as mean values, the number of biological replicates and definition of error bars is noted in individual figure legends. Before statistical analyses were made the distributions of data were assessed to select an appropriate test, details on the tests employed are provided in the body of results and appropriate figure legends. Statistical analyses were performed in Prism 8 software (GraphPad) and RStudio. Concentration response curves were fitted in Prism 8 using a three-parameter logistic equation.

3. Temporal resolution of behaviour and nociceptor states following carrageenan-induced inflammation.

3.1. Authorship and publication status statement

Manon Bohic, a postdoctoral fellow in the group of Victoria Abraira, Rutgers University, USA performed and analysed Hargreaves behavioural studies, data from these experiments have been included in this chapter with permission as indicated in the relevant figure legends. Some of the work presented in this chapter is currently under peer review (Bohic et al., 2021).

3.2. Chapter Summary

Rodent models of inflammation are a valuable tool for studying the complexities underlying and arising from inflammatory conditions, such as rheumatoid arthritis and inflammatory bowel disease, and the associated changes in nociception. Models of acute inflammation, such as local injection of carrageenan are also commonplace in preclinical assessment of novel analgesics. The end points of such studies, however, are often limited to the remedying of an evoked pain response at a particular time point and thus do not capture all of the pathophysiological facets which contribute to nociception and overall pain experience. This may in part explain the poor translation of from bench to bedside. This Chapter describes a thorough characterisation of the development of pain-like behaviours and changes at the level of the nociceptor in mice following the induction of inflammation with carrageenan. Distinct behavioural states were identified 4-hours post induction of inflammation, mice demonstrating thermal hypersensitivity and a deficit in weight-bearing. Whereas the abnormal gait of animals resolved alongside the physical inflammation, thermal hypersensitivity persisted at least 24-hours post-inflammation. Similarly, higher excitability of sensory neurones innervating the site of inflammation was only observed 4-hours post induction of inflammation, while the expression of receptors associated with nociceptive pathways was shown to be increased on both transcriptional and protein levels 24-hours post-inflammation. The results presented in this Chapter highlight the need for careful consideration of readouts and end-points in studies utilising inflammatory models and represent a move towards a comprehensive framework for profiling the pain status of rodents.

3.3. Introduction

Sensory neurones can be both heterogenous and polymodal, allowing them to integrate a plethora of stimuli (Zeisel et al., 2018). A specialised subset of sensory neurones termed nociceptors express various receptors which permit the detection of noxious stimuli such as high temperature, excessive force and harsh chemicals. Activation of these receptors usually converges on action potential discharge, nociceptors signal to the central nervous system where the signals manifest as pain, affecting the individual's mood, behaviour and motivations (Wang and Thyagarajan, 2020). As well as expressing receptors that can be activated by noxious stimuli, nociceptors express receptors for inflammatory mediators, such as cytokines and prostaglandins. The action of these mediators on their receptors usually serves to sensitise the neurones, making nociceptor discharge more likely and giving rise to inflammatory pain (Pinho-Ribeiro et al., 2017). The heightened nociception experienced during inflammatory pain is protective, serving to promote repair and recovery, however, excessive inflammation is a feature of many diseases which are also associated with chronic pain, such as RA (Walsh and McWilliams, 2014). Behaviourally, this results in hyperalgesia and general apathy, reducing the quality of life of those suffering from chronic inflammatory pain (Turk et al., 2016). There are also a number of further reaching social and economic burdens associated with chronic pain conditions, including social impacts on friends and family and economic hits from time missed at work and an inability to participate fully in society (Enns et al., 2018). Despite this, approved therapeutic options for treating the pain associated with inflammation are often inadequate, particularly at alleviating pain, or are accompanied by undesirable side-effects, there is thus unmet need to develop novel analgesics for treating conditions of chronic inflammatory pain (Ghouri and Conaghan, 2019; Woodcock, 2009). Development of novel therapeutics will rely on a greater understanding of the complexities underlying pain, from cellular and molecular changes within the sensory nociceptive system to the behavioural level.

Rodent models of inflammatory pain represent a valuable preclinical tool to identify molecular mechanisms which drive inflammation and pain. Local injection of carrageenan is a popular model of inflammatory pain. Intraplantar injection of carrageenan results in robust oedema of the hind paw, peaking within 4-hours, after which swelling subsides (Morris, 2003). However, heightened sensitivity to both thermal and mechanical stimulation has been documented to persist up to 24-hours post-induction (Buisseret et al., 2019; Hargreaves et al., 1988). To date studies characterising the carrageenan model and assessing the efficacy of therapeutic agents have relied on histological analyses and evoked-pain behavioural assays (Buisseret

et al., 2019; Miyake et al., 2019; Pritchard et al., 2016; Rock et al., 2018). Indeed, such approaches have demonstrated that independent administration of prostaglandin D2-glycerol, cannabinoids and ivabradine all alleviate evoked pain behaviours in rodents following induction of inflammation with carrageenan (Buisseret et al., 2019; Miyake et al., 2019; Rock et al., 2018). Although capturing common hallmarks of inflammatory pain, evoked measures of pain achieved via von Frey filaments (mechanosensitivity) and Hargreaves (heat sensitivity) are often criticized for not fully capturing the nature of pain in humans and their poor correlation with how pain is assessed in the clinic (Mao, 2012). These studies also neglect to address the impact of carrageenan-induced inflammation on the nociceptors which first encode noxious stimuli. The need for a multidisciplinary approach to capture the molecular, cellular and behavioural facets encompassing inflammatory pain is thus evident.

The aim of this work was to study how unilateral injection of carrageenan affects the behaviour and physiology of mice during inflammation. A combination of evoked and spontaneous behavioural assays was employed to gauge both thermal hyperalgesia and changes in weight-bearing. Following behavioural profiling, inflammation induced changes in the function of and expression pattern of nociceptive ion channels in sensory neurones were assessed. Use of the retrograde tracer, Fast Blue, to identify cells projecting to the afflicted area, allowed more specific elucidation of the effects of carrageenan on sensory neurones. When considered together, these data allow for a more comprehensive assessment of the pain status of mice over the inflammatory time course by assessing both evoked and spontaneous pain behaviours.

3.4. Methods

See Materials and Methods chapter for detailed descriptions of mouse hind paw injections (Section 2.4), assessment of dynamic weight-bearing (Section 2.5), DRG dissociation and culture (Section 2.8), whole cell patch clamp electrophysiology (Section 2.21), immunohistochemistry (Section 2.13) and qPCR (Sections 2.11 and 2.11.2).

Brief descriptions of methodology for experiments performed by others but discussed in this chapter follow:

3.4.1. Hargreaves test

To assess hind paw heat sensitivity, the Hargreaves test was conducted using a plantar test device (IITC Life Sciences). Mice were placed individually into Plexiglas chambers on an

elevated glass platform and allowed to acclimatise for at least 30 minutes before testing. A mobile radiant heat source of constant intensity was then applied to the glabrous surface of the paw through the glass plate and the latency to paw withdrawal measured. Paw withdrawal latency is reported as the mean of 3 measurements for both hind paws with at least 5 minutes recovery between measurements. A cut-off of 20 seconds was applied to avoid tissue damage.

3.5. Results

Consistent with previous reports, unilateral injection of carrageenan into the hind paw stimulated an inflammatory response and pronounced swelling of both the footpad (pre-carrageenan, 2.26 ± 0.19 mm, 4-hours, 3.56 ± 0.49 mm, $F = 38.02$, $p < 0.0001$, ANOVA with Bonferroni post-hoc, mean $\pm$ SD, $N = 13 - 17$, Figure 3.1A-B) and ankle (pre-carrageenan, 2.65 ± 0.20 mm, 4-hours, 3.28 ± 0.43 mm, $F = 12.26$, $p < 0.0001$, ANOVA with Bonferroni's post-hoc, mean $\pm$ SD, $N = 13 - 17$, Figure 3.1A, C) of the injected paw 4-hours after the induction of inflammation. No such swelling was observed for the contralateral hind paw (footpad: pre-carrageenan, 2.23 ± 0.16 mm, 4-hours post-carrageenan, 2.18 ± 0.11 mm; ankle; pre-carrageenan, 2.68 ± 0.19 mm, 4-hours post-carrageenan, 2.59 ± 0.17 mm, mean $\pm$ SD, $N = 13 - 17$, Figure 3.1A-C). Swelling of the injected footpad persisted at 24-hours (pre-carrageenan, 2.26 ± 0.19 mm, 24-hours, 2.92 ± 0.65 mm, $p < 0.0001$, ANOVA with Bonferroni post-hoc, mean $\pm$ SD, $N = 13 - 17$, Figure 3.1B), although was distinct from that seen at 4-hours suggesting inflammation had started to resolve by this time point ($p < 0.001$, ANOVA with Bonferroni post-hoc, Figure 3.1B). Ankle inflammation had mostly resolved by this time (pre-carrageenan, 2.65 ± 0.20 mm, 24-hours, 2.87 ± 0.37 mm, mean $\pm$ SD, Figure 3.1C).

Hypersensitivity to thermal stimuli, a hallmark of inflammatory pain, was assessed using the Hargreaves test. Thermal hyperalgesia was evident from the shorter latency to withdraw the inflamed paw from the heat source compared to pre-inflammation latencies at both the 4-hour and 24-hour post-injection time points (pre-carrageenan, 17.4 ± 1.94 seconds, 4-hours, 4.76 ± 1.52 seconds, 24-hours, 4.92 ± 0.88 s, $F = 118.3$, $p < 0.0001$, ANOVA with Bonferroni post-hoc, mean $\pm$ SD, $N = 10 - 20$, Figure 3.1D).

The dynamic weight-bearing of mice was also assessed as a readout of spontaneous pain-behaviour following the induction of inflammation with carrageenan. The observed swelling corresponded to reduced weight loading through the rear paw for the injected side after 4-hours (pre-carrageenan, $27.07 \pm 2.56\%$, 4-hours, $19.93 \pm 5.85\%$, $F = 4.071$, $p < 0.01$, ANOVA

with Bonferroni post-hoc, mean $\pm$ SD, N = 13 – 17, Figure 3.1E), but not 24-hours after carrageenan injection (pre-carrageenan, $27.07 \pm 2.56\%$, 24-hours, $27.13 \pm 8.71\%$, mean $\pm$ SD, Figure 3.1E). This finding coincided with mice placing more weight through their front paws relative to their rear paws at the 4-hour time point (pre-carrageenan, 0.13 ± 0.08 , 4-hours, 0.54 ± 0.46 , $F = 8.320$, $p < 0.001$, ANOVA with Bonferroni post-hoc, mean $\pm$ SD, N = 13 – 17, Figure 3.1F) and the distribution of weight returning more closely to pre-inflammation levels 24-hours after carrageenan injection (pre-carrageenan, 0.13 ± 0.08 , 24-hours, 0.21 ± 0.21 , mean $\pm$ SD, N = 13 – 17, Figure 3.1F). The proportion of time that animals spent rearing, an explorative stance relying on significant weight-bearing through the rear paws, was reduced 4-hours after injection of carrageenan (pre-carrageenan, $50.66 \pm 11.57\%$, 4-hours, $23.87 \pm 17.41\%$, $F = 10.40$, $p < 0.001$, ANOVA with Bonferroni post-hoc, mean $\pm$ SD, N = 13 – 17, Figure 3.1G). Again, the time animals spent rearing 24-hours after induction of inflammation was more comparable to pre-inflammation measures (pre-carrageenan, $50.66 \pm 11.57\%$, 24-hours, $50.59 \pm 22.61\%$, mean $\pm$ SD, N = 13 – 17, Figure 3.1G). These findings build upon previous reports of mechanical and thermal allodynia following induction of inflammation with carrageenan, demonstrating a weight-bearing deficit that correlates with the peak of physical inflammation, but longer-lasting thermal hypersensitivity.

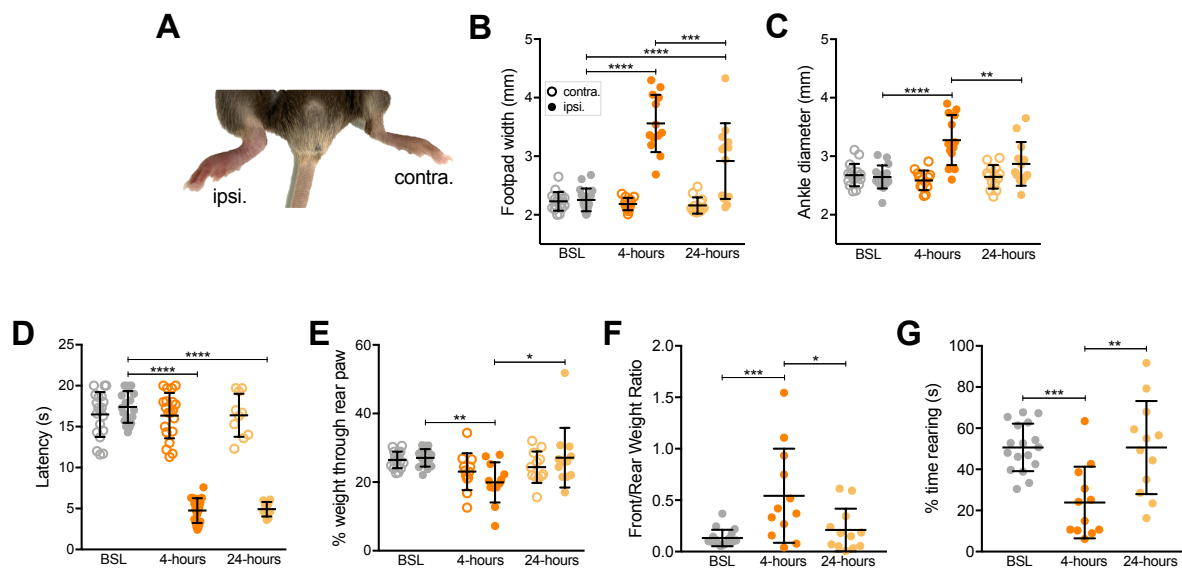


Figure 3.1. Unilateral injection of carrageenan into the hind paw of mice results in swelling, thermal hypersensitivity and weight bearing deficits. (A) Pronounced inflammation of the injected (ipsi.) hind paw was observed 4-hours post-injection with carrageenan compared to the non-injected hind paw (contra.). Swelling of both the (B) footpad and (C) ankle was quantified for both paws with digital callipers before, 4-hours post- and 24-hours post-induction of inflammation with carrageenan. (D) Hargreaves measurement of carrageenan-induced thermal hypersensitivity during inflammation (data kindly provided by Manon Bohic, Rutgers University, USA). (E) The weight through each hind paw, (F) ratio of weight through front paws compared to the rear and (G) time spent rearing by mice was assessed with dynamic weight bearing apparatus. Data are mean $\pm$ SD. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, one-way ANOVA followed by Bonferroni post-hoc. N = 17 (BSL), 13 (4-hours; 24-hours).

Having characterised the behaviour of mice following injection of carrageenan, focus was switched to study how the induction of inflammation affected primary afferent neurones innervating the site of inflammation. Sensory neurones innervating the hind paw were identified with the retrograde tracer Fast Blue (Figure 3.2A). Of acutely cultured lumbar DRG neurones (L2-L5) 4.46% were found to be Fast Blue positive (Figure 3.2B insert). The relative size distribution of the Fast Blue positive population closely did not significantly differ from that of the total lumbar DRG neuronal population measured (Total: Amplitude, 9.23, Mean, 28.64 μm , SD, 8.35; FB+: Amplitude, 9.32, Mean, 30.45 μm , SD, 8.80, $p = 0.2276$, two-sided Kolmogorov-Smirnov Test, Figure 3.2B), that is to say, using size as a marker, the hind paw is likely innervated by the full range of neuronal subtypes. Electrophysiological characterisation of Fast Blue positive neurones cultured from the inflamed side (ipsilateral, ipsi.) and non-injected side (contralateral, contra.) revealed a lower rheobase (current injection required for action potential discharge) of ipsilateral neurones compared to contralateral neurones when collected 4-hours post-carrageenan, suggesting the onset of peripheral sensitisation following exposure to carrageenan (contra., 272.1 ± 48.95 pA, ipsi., 125.1 ± 25.36 pA, $p < 0.001$, Mann Whitney test, mean $\pm$ SEM, $n = 20 - 39$, $N = 5$, Figure 3.2C). Given no inflammation was observed on the contralateral side and the relatively short timescale of the inflammatory model, cells of the contralateral side were deemed an appropriate control for comparisons, with the additional benefit of reducing use of animals. No significant difference in rheobase was observed between ipsilateral and contralateral neurones at the 24-hours post-inflammation time point (contra., 290.00 ± 61.61 pA, ipsi., 201.14 ± 37.74 pA, mean $\pm$ SEM, $n = 20 - 39$, $N = 5$, Figure 3.2C). After determining the rheobase, neurones were stimulated at suprathreshold (2x rheobase). Neurones isolated from the ipsilateral side after 4-hours of inflammation had a higher tendency, albeit non-significant, to fire multiple action potentials during this stimulation than those from the non-injected side (contra., 1.87 ± 0.47 , ipsi., 3.00 ± 0.42 , $p = 0.0881$, Mann Whitney test, mean $\pm$ SEM, $n = 20 - 39$, $N = 5$, Figure 3.2D), no such difference was observed 24-hours post-inflammation (contra., 2.92 ± 0.58 , ipsi., 2.65 ± 0.42 , mean $\pm$ SEM, $n = 20 - 39$, $N = 5$, Figure 3.2D). The neurones recorded from did not differ in any other intrinsic or active parameter measured for either time point, as summarised in Table 3.1.

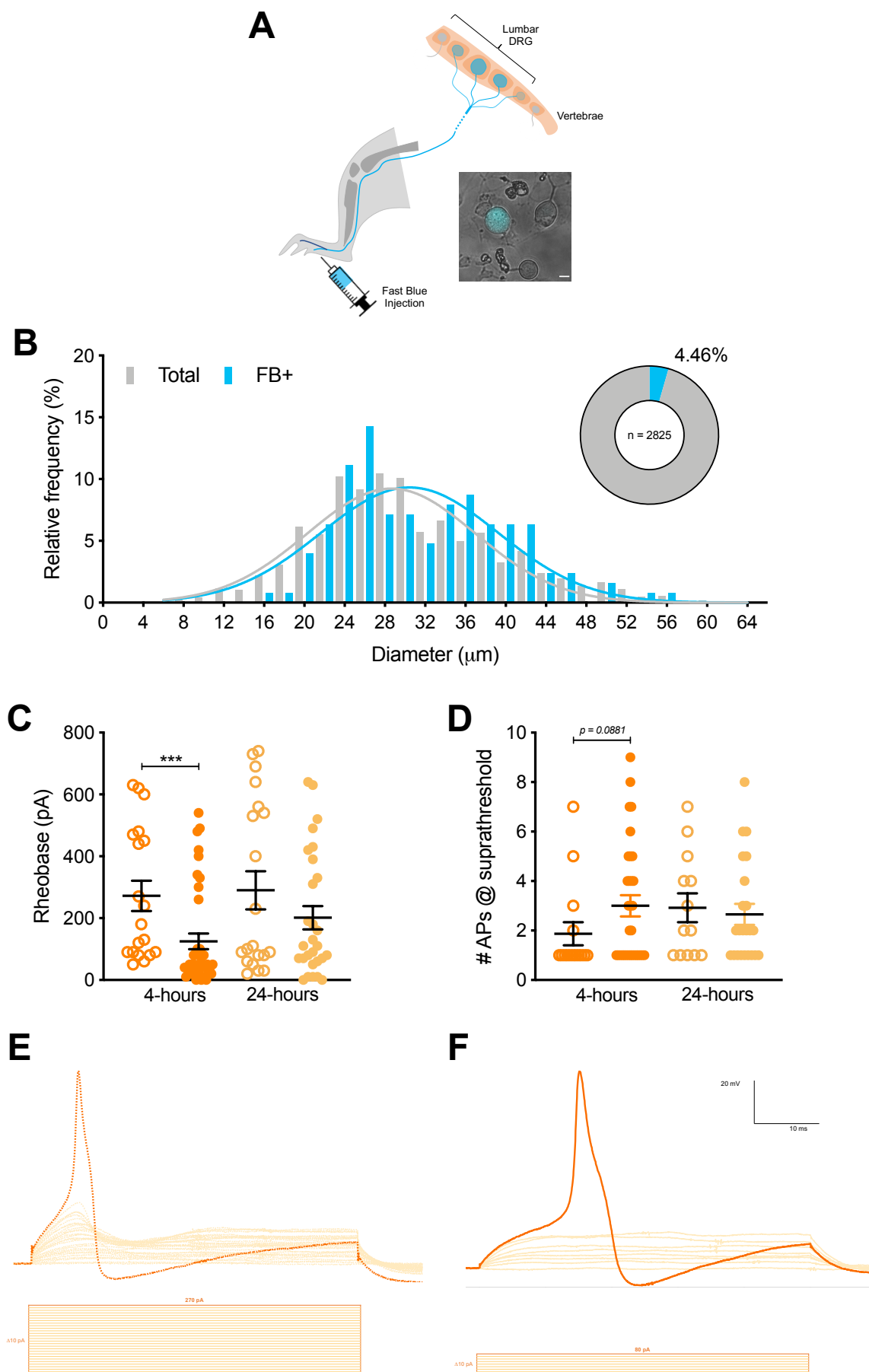


Figure 3.2. Sensory neurones innervating carrageenan injected hind paws are more excitable than those innervating non-carrageenan injected hind paws during early inflammation. (A) Schematic representation of retrograde labelling of hind paw innervating sensory neurones with Fast Blue, Insert: Fast Blue positive neurone (blue) following acute dissociation, scale = 50 μ m. (B) Relative frequency distributions of dissociated sensory neurone soma diameter from hind paw innervating and total lumbar populations, Insert: Proportion of Fast Blue positive cells from acutely dissociated cultures of lumbar DRG. (C) Step-wise current injections were used to determine the rheobase of hind paw innervating sensory neurones from the injected and non-injected sides 4- or 24-hours post-induction of inflammation with carrageenan. (D) The number of action potentials (APs) discharged following stimulation of cells with a suprathreshold (2x rheobase). Representative traces from step-wise current injections to determine the rheobase of sensory neurones from (E) contralateral and (F) ipsilateral sides 4-hours post-injection of carrageenan. Data are presented as mean $\pm$ SEM. ** $p < 0.01$, Mann Whitney test. $n = 20$ cells (4-hours contra.), 39 cells (4-hours ipsi.), 20 cells (24-hours contra.) and 28 cells (24-hours ipsi.) from $N = 5$ animals per time-point.

	Hours post-injection			
	4-hours (N = 5)		24-hours (N = 5)	
	Contra. (n = 20)	Ipsi. (n = 39)	Contra. (n = 20)	Ipsi. (n = 28)
Resting membrane potential (mV)	-48.20 $\pm$ 1.49	-46.05 $\pm$ 0.83	-49.00 $\pm$ 1.43	-45.64 $\pm$ 1.40
Capacitance (pF)	31.23 $\pm$ 2.69	25.90 $\pm$ 1.73	30.77 $\pm$ 4.44	33.17 $\pm$ 3.03
Rheobase (pA)	272.10 $\pm$ 48.95**	125.10 $\pm$ 25.36**	290.00 $\pm$ 61.61	201.40 $\pm$ 37.74
Action potential amplitude (mV)	80.33 $\pm$ 3.95	84.58 $\pm$ 2.17	85.45 $\pm$ 3.65	87.11 $\pm$ 2.74
Half peak duration (ms)	3.04 $\pm$ 0.26	3.50 $\pm$ 0.15	3.87 $\pm$ 0.35	3.37 $\pm$ 0.27
Afterhyperpolarization amplitude (mV)	15.43 $\pm$ 1.30	14.10 $\pm$ 0.76	16.61 $\pm$ 0.71	16.15 $\pm$ 0.84
Afterhyperpolarization duration (ms)	15.36 $\pm$ 1.55	17.82 $\pm$ 1.55	15.48 $\pm$ 1.17	16.45 $\pm$ 1.19
# Action potentials at suprathreshold	1.87 $\pm$ 0.47	3.00 $\pm$ 0.43	2.92 $\pm$ 0.58	2.65 $\pm$ 0.42

Table 3.1 Intrinsic and active properties of hind paw innervating dorsal root ganglion neurones from the carrageenan injected side (Ipsi) and contralateral (Contra) side. Data are mean $\pm$ SEM. *** $p < 0.001$, Mann Whitney test. $n =$ no. cells from condition, $N =$ no. animals from time point.

Next, the activity of voltage-gated channels was assessed in voltage clamp: macroscopic peak inward and outward currents were measured as the holding voltage was stepped in 5 mV steps from -60 mV to 55 mV. Total inward current was greater for ipsilateral neurones after 4-hours of inflammation relative to neurones from the non-injected side (peak normalised current: contra., -0.97 $\pm$ 0.47, ipsi., -1.29 $\pm$ 0.54, $p < 0.05$, unpaired t-test, mean $\pm$ SEM, $n = 20 - 39$, $N = 5$, Figure 3.1C). The cell conductance permitting inward current was also found to be higher (peak normalised total conductance: contra., 1.00 $\pm$ 0.67, ipsi., 1.43 $\pm$ 0.74, $p < 0.05$, unpaired t-test, mean $\pm$ SEM, $n = 20 - 39$, $N = 5$, Figure 3.1G). However no difference in the gating properties of channels was observed (V_{half} : contra., -38.73 $\pm$ 4.13 mV, ipsi., -40.07 $\pm$ 1.40 mV, mean $\pm$ SEM, $n = 20 - 39$, $N = 5$). By contrast, peak current, total cell conductance and gating properties were comparable at the 24-hour time point (peak normalised current: contra., -0.96 $\pm$ 0.40, ipsi., -0.95 $\pm$ 0.44; peak normalised total conductance: contra., 1.00 $\pm$ 0.47, ipsi., 0.96 $\pm$ 0.50; V_{half} : contra., -43.68 $\pm$ 1.78 mV, ipsi., -45.18 $\pm$ 1.09 mV, mean $\pm$ SEM, $n = 20 - 39$, $N = 5$, Figure 3.1A,D-E,H-I). Focusing on total

outward currents revealed no difference between neurones innervating the contralateral and ipsilateral sides after 4-hours of inflammation (contra., 1.00 ± 0.62 , ipsi., 1.20 ± 0.58 , mean $\pm$ SEM, $n = 20 - 39$, $N = 5$, Figure 3.1J-K) or 24-hours (contra., 1.00 ± 0.47 , ipsi., 0.90 ± 0.46 , mean $\pm$ SEM, $n = 20 - 39$, $N = 5$, Figure 3.1L-M).

A

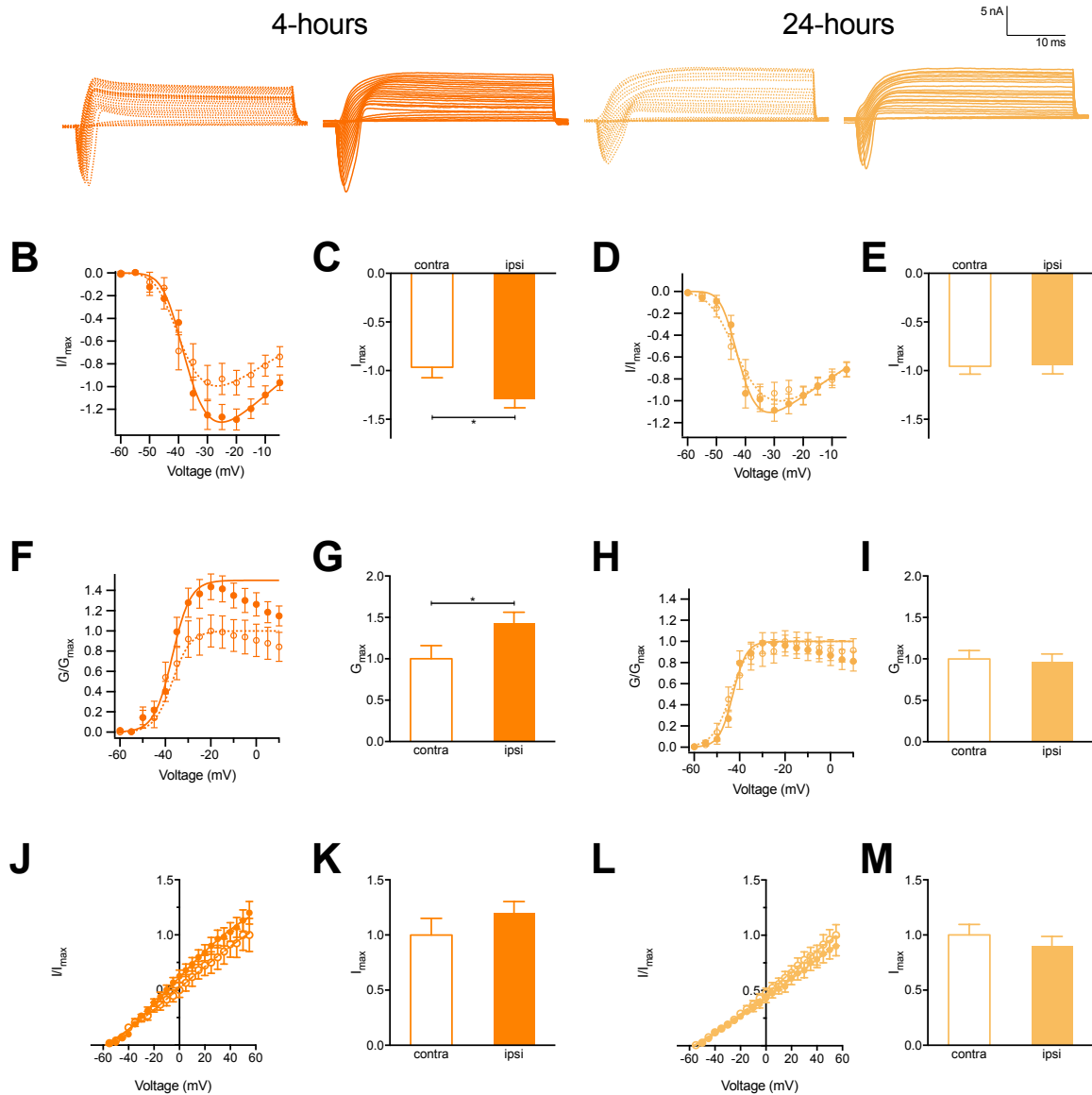


Figure 3.3. Changes in sensory neurone macroscopic voltage-gated currents during inflammation.

(A) Current traces from IV curve generation for neurones from both contralateral (dashed) and ipsilateral (solid) sides either 4- or 24-hours post-inflammation, representative traces from neurones of comparable capacitance. Inward macroscopic current densities normalised to the average peak inward current density of contralateral neurones either (B) 4- or (D) 24-hours post-inflammation. (C,E) Normalised peak inward current densities. (F,H) Calculated total conductance of inward voltage-gated currents normalised to average peak total conductance of contralateral neurones. (G,I) Normalised peak total conductance of inward voltage-gated currents. Outward macroscopic current densities normalised to the average peak outward current density of contralateral neurones either (J) 4- or (L) 24-hours post-inflammation. (K,M) Normalised peak outward current densities. Data are presented as mean $\pm$ SEM. * $p < 0.05$, unpaired t-test. $n = 20$ cells (4-hours contra.), 39 cells (4-hours ipsi.), 20 cells (24-hours contra.) and 28 cells (24-hours ipsi.) from $N = 5$ animals per time-point.

The higher excitability of ipsilateral sensory neurones observed at 4-hours post-injection of carrageenan correlates with the peak of physical inflammation, suggesting that mediators released during the inflammatory process may be responsible for sensitising the neurones. As inflammation subsides, the levels of inflammatory mediators decline, which corresponds with the degree of excitability from the ipsilateral side being more comparable to the contralateral side at the later, 24-hour post-carrageenan time point. Although the inflammatory peak has passed at 24-hours (Fig. 3.1B,C), changes in the expression of nociceptive channels, that were initiated during the height of inflammation, will manifest and influence behaviour at later time points. Thus qPCR was performed on bulk lumbar DRG to assess changes in gene expression following 24-hours of inflammation. Lumbar DRG were assessed due to the high proportion of cells that project to the hind paw, the site of inflammation (da Silva Serra et al., 2016). Given that inflammation often results in a hypoxic environment and local pH has been shown to decrease in many inflammatory conditions, the expression of proton-sensitive receptors was profiled. To address the hypothesis that mediators elevated during early inflammation might induce upregulation of proton-sensitive receptors and channels that may reinforce inflammation as a decrease in local extracellular pH ensues, the expression of the four well-established PS-GPCRs (GPR4, GPR65, GPR68 and GPR132), as well as the ion channels TRPV1 and ASIC3, was assessed. All of the receptors profiled, bar GPR132, were found to be expressed by DRG. Differential expression analyses revealed that the expression of GPR68 was unaffected by carrageenan-induced inflammation, while higher expression of GPR4, GPR65, TRPV1 and ASIC3 was observed for the ipsilateral side compared to contralateral DRG, however only the upregulation of GPR65 was statistically significant (contra., 25.35 ± 0.04 , ipsi., 24.83 ± 0.08 , $p < 0.01$, unpaired t-test, mean $\pm$ SEM, $N = 3$, Figure 3.4A).

Immunohistochemistry performed on DRG sections from animals that had received Fast Blue injections to the hind paw, allowed specific assessment of changes in protein expression within hind paw innervating sensory neurones following carrageenan induced inflammation. Unfortunately, this technique relies on the availability of validated antibodies against proteins of interest, so it was only possible to look at expression of the nociceptive channels TRPV1 and P2X3 (a purinoreceptor). Both channels were found to be expressed by a subset of hind paw innervating sensory neurones (Figures 3.4B,D). Greater immunoreactivity against TRPV1 (contra., $20.01 \pm 1.87\%$, ipsi., $29.88 \pm 3.80\%$, $p = 0.0571$, Mann Whitney test, mean $\pm$ SEM, $N = 4$, Figure 3.4C) and P2X3 (contra., $13.54 \pm 2.61\%$, ipsi., $26.74 \pm 3.73\%$, $p = 0.0571$, Mann Whitney test, mean $\pm$ SEM, $N = 4$, Figure 3.4D) was observed for sensory neurones innervating the inflamed hind paw compared to DRG isolated from the contralateral side, although the changes did not reach statistical significance. The proportion of Fast blue was

comparable between the two sides (contra., $16.12 \pm 3.42\%$, ipsi., $16.46 \pm 4.85\%$, mean $\pm$ SEM, N = 4). The finding that ipsilateral neurones have higher expression of TRPV1 24-hours post-inflammation offers some explanation to the earlier observation of thermal hypersensitivity persisting at the 24-hours post-inflammation time point (Fig 3.1D).

Taken together, these results suggest an adaptation of the sensory neurones in response to the initial inflammatory insult, which renders them more sensitive to noxious stimuli, after inflammation has begun to subside. In addition, since the electrical excitability of hind paw innervating sensory neurones returns to basal levels 24-hours post-inflammation, but certain pain behavioural signatures (thermal hypersensitivity) persist, this suggests that central sensitisation might drive pain states beyond the initial few hours.

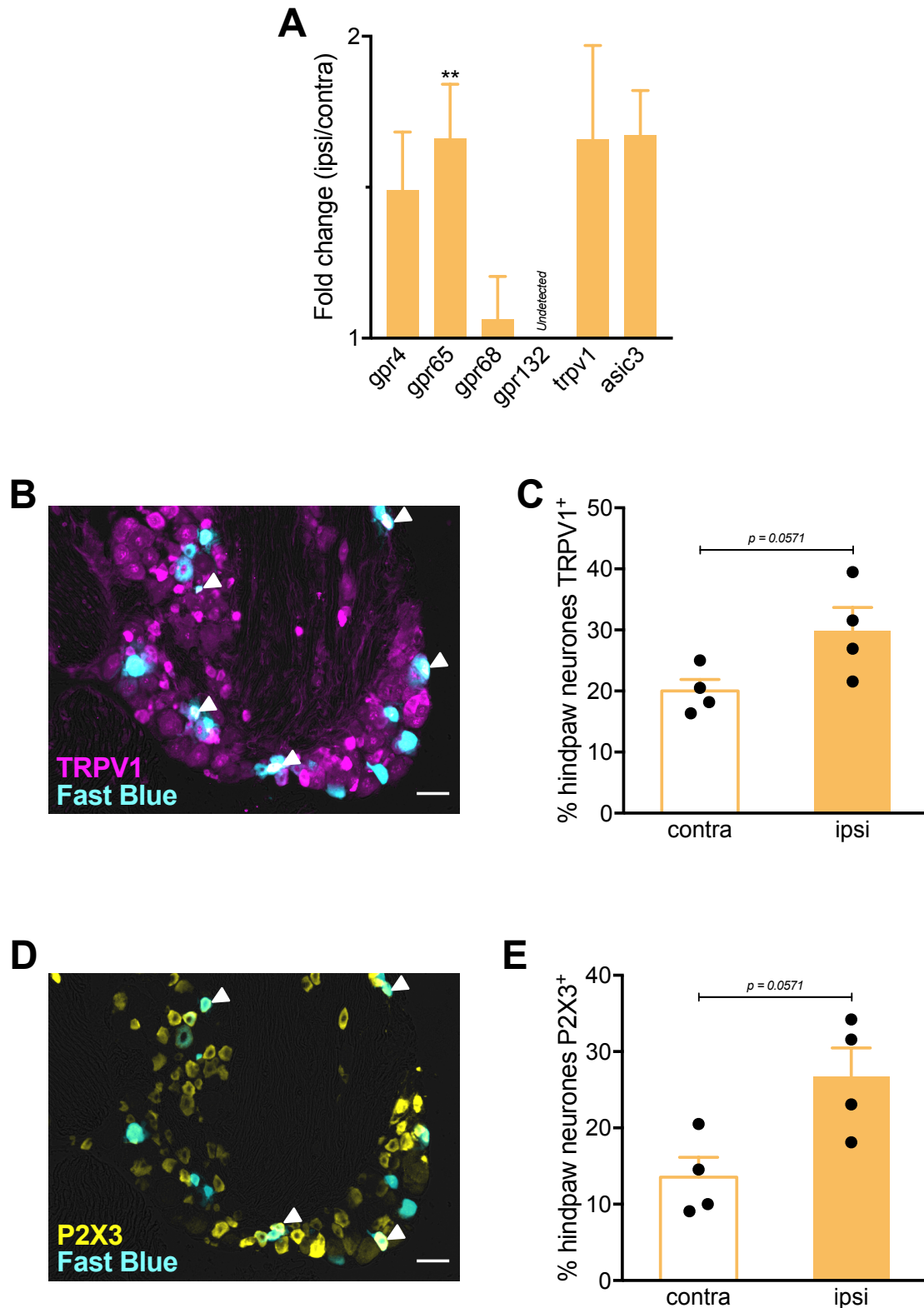


Figure 3.4. Expression changes within hind paw innervating sensory neurones 24-hours post-inflammation. (A) Fold change in expression of various proton-sensitive receptors as assessed by qPCR performed on cDNA prepared from total lumbar (L2-L5) DRG, data are presented on a log₂ scale. (B) A subset of hind paw innervating sensory neurones (blue) express the ion channel TRPV1 (magenta) as determined by immunohistochemistry, neurones positive for both Fast Blue and TRPV1 are identified by white arrowheads, scale = 50 μ m. (C) The proportion of hind paw innervating sensory neurones from either side expressing TRPV1 after 24-hours of inflammation. (D) A subset of hind paw innervating sensory neurones (blue) express the ion channel P2X3 (yellow) as determined by immunohistochemistry, neurones positive for both Fast Blue and P2X3 are identified by white arrowheads, scale = 50 μ m. (E) The proportion of hind paw innervating sensory neurones from either side expressing P2X3 after 24-hours of inflammation. Data are presented as mean $\pm$ SEM. (A) ** $p < 0.01$, unpaired t-test of Δ CT values. (C,E) Mann Whitney test. N = 3 (qPCR) and 4 (IHC) animals.

3.6. Discussion

Unilateral intraplantar injection of carrageenan has previously been shown to evoke both mechanical and thermal hypersensitivity which persist for at least 24-hours (Hargreaves et al. 1988; Buisseret et al. 2019). Here, extending behavioural characterisation to include dynamic weight bearing revealed a difference in the duration of evoked and spontaneous behaviours following exposure to carrageenan: weight bearing deficits, mostly driven by spontaneous pain, resolved alongside physical inflammation, but evoked thermal hypersensitivity persisted longer. Similarly, profiling sensory neurones directly innervating the site of inflammation revealed hyperexcitability after 4-hours of inflammation, which resolved to a basal level at the 24-hour time point. Moreover, expression of nociceptive ion channels and receptors was elevated at 24-hours post-inflammation suggesting a longer-lasting adaptation of nociceptors following the inflammatory insult might in part explain longer lasting evoked behaviours. Taken together, these data highlight the need for careful consideration of experimental read outs and the times at which these are taken when using models of inflammation to assess the pain status of rodents.

A single injection of carrageenan produces localised inflammation which begins to resolve within a day, but pain-like behaviour that can persist far longer. DRG neurones innervating the site of inflammation showed no signs of hyperexcitability 24-hours after inflammation, suggesting that persistent pain behaviours are likely driven by adaptations, such as changes in gene expression, in the nociceptive system initiated during active inflammation. Indeed an increase in the number of sensory neurones expressing TRPV1, P2X3 and GPR65 was observed 24-hours post-inflammation, although this was not assessed functionally here, this might be studied performing voltage-clamp electrophysiology experiments to quantify the proportion of cells responsive to capsaicin and α,β -methylene ATP, agonists of TRPV1 and P2X3 respectively, this would also allow an assessment of changes in current density to be addressed. It is also plausible that central sensitisation contributes to the persistent nociceptive behaviours observed. By following up a behavioural testing regimen capturing spontaneous and evoked measures of pain with site-specific electrophysiology, the overall pain status and physiological state of nociceptors can be considered in relation to one and other permitting comprehensive pain phenotyping. Using a multi-method approach, as described here, may thus offer a higher resolution of the development of pain, particularly the transition from acute pain to a more chronic state. It would be of interest to apply the experimental pipeline described here to characterise a more chronic model, such as intra-articular injection of monosodium iodoacetate, a model of OA (Haywood et al., 2018) to assess

this. Extending the experimental repertoire to address central sensitisation, by recording from spinal cord neurones to assess long-term potentiation (Ikeda et al., 2003) or using brain imaging techniques to study the extent of central circuitry evoked by noxious stimulation (Abaei et al., 2016) would also provide greater insight to the transition from acute to chronic inflammatory pain.

Using the retrograde tracer Fast Blue, higher excitability of sensory neurones innervating the inflamed hind paw was demonstrated after 4-hours of inflammation. Subsequent experiments exploring the activity of voltage-gated ion channels revealed a greater total conductance of inward current in response to depolarisation, which may offer some explanation of the hyperexcitable state observed, as action potential discharge can be more readily achieved. The rapid onset of this change suggests a post-translational modification might be responsible, indeed phosphorylation of voltage-gated sodium channels induced by mediators released during inflammation, including PGE2 and IL1 β , have been shown to increase inward currents (Binshtok et al., 2008; Gold et al., 1996). Experiments were performed on macroscopic currents though, so it is also plausible that a reduction in total outward currents, contributes to the apparent increased inward currents measured, further elucidation of the channels modulated by carrageenan-induced inflammation could be studied by isolated specific currents using a combination of pharmacological agents and altering the driving force of the solutions used to bath cells during experiments. Considering that peak inflammation coincides with the heightened conductance suggests a strong role of pro-inflammatory mediator-induced phosphorylation of voltage-gated sodium channels driving the hyperexcitability of peripheral sensory neurones and pain behaviours that manifest following induction of the carrageenan model. Furthering this, independent studies have reported induction of COX-2 (an enzyme that can initiate PGE2 synthesis) in the hind paw carrageenan model and less pain-like behaviour following induction of the model in mice deficient in the voltage-gated sodium channel Nav1.7 on nociceptors (Ibuki et al., 2003; Nassar et al., 2004). This hypothesis could be further explored by using the experimental pipeline described to profile mice following induction of inflammation with carrageenan and subsequent administration of an NSAID, as these agents act to prevent the production of prostaglandins, or an inhibitor of protein kinase A. The phosphorylation status of voltage-gated sodium channels might also be assessed by immunoblotting.

Using Fast Blue to identify the cell bodies of sensory neurones projecting to the inflamed hind paw, an apparent upregulation of TRPV1 and P2X3 was observed through immunohistochemistry after 24-hours of inflammation. Previous studies have reported that TRPV1 knock-out mice experience less mechanical hyperalgesia, compared to wild-type

animals, following carrageenan-induced inflammation (Watanabe et al., 2015) and administration of an antagonist of P2X3 has been shown to reduce carrageenan-induced nocifensive behaviour (Teixeira et al., 2010). Considering these findings alongside those reported in this chapter suggests an involvement of TRPV1 and P2X3 in the enhanced nociception observed during inflammation. Although higher levels of TRPV1 transcripts were observed for DRG isolated from the ipsilateral side compared to the contralateral side, the difference was deemed less likely to be significant than the increased TRPV1 immunoreactivity. TRPV1 has an established role in inflammatory pain and has been shown to increase in expression in human patients of inflammatory disease and rodent models of both OA and RA (Akbar et al., 2008; Chakrabarti et al., 2018; Hwang et al., 2021; Kelly et al., 2015), this increase can be explained by *de novo* synthesis, or increased trafficking of pre-formed receptors to the plasma membrane (Meng et al., 2016; Sheehan et al., 2019). The data presented here, suggest carrageenan-induced inflammation triggers upregulation of TRPV1 synthesis, evident from the increased immunoreactivity and transcript levels relative to the contralateral side. Although a higher level of *Trpv1* transcripts were detected for DRG isolated from the inflamed side, the increase was not significant, this however may be an artefact of qPCR being performed on total lumbar DRG (L2-L5), better resolution of the transcriptome of sensory neurones specifically innervating the hind paw would allow a more definite conclusion on the mechanism behind the observed thermal hypersensitivity.

The finding that GPR65 expression by sensory neurones increased following the induction of inflammation, agrees with similar studies (Chen et al., 2009) and furthers the central hypothesis of this Thesis that PS-GPCRs might play an orchestrating role in the process of inflammation and associated pain. However, the lack of pharmacological tools to inhibit GPR65 make it difficult to assess whether the increased levels of GPR65 induced by carrageenan injection contribute to the associated pain. Two potential options to further explore the role of GPR65 in carrageenan-induced inflammation would be to use GPR65^{-/-} mice or siRNA-directed knockdown of the receptor.

In this Chapter a very basic assessment of changes in gene and protein expression following induction of inflammation with carrageenan was performed: both qPCR and immunohistochemistry are guided by prior selection of probes/primers and antibodies respectively, and are thus directed by identifying genes/proteins that are already well-characterised in inflammatory processes or of interest to the researcher. Combining retrograde labelling with techniques such as single-cell RNA sequencing would allow a broader assessment of the molecular state of sensory neurones during inflammation and may

represent an opportunity to identify novel players in inflammatory pain networks at the level of the sensory neurone.

To summarise, this body of work highlights the complexities of inflammatory pain and provides a framework for comprehensive assessment of pain on multiple levels. Building on this work it would be interesting to screen the effects of known analgesics, such as NSAIDs to assess how they affect both peripheral sensory neurone activity and overall behaviour. Similarly, if single-cell transcriptomics were extended as suggested, and novel genes were identified as being differentially expressed following carrageenan-induced inflammation, the involvement of these in inflammatory pain could be explored using suitable antagonists or inhibitors, knock-downs or knock-outs where available to make a preclinical assessment of new targets before following up with human translatability.

4. Expression and activity of PS-GPCR homologs from mice and naked mole-rats.

4.1. Chapter Summary

The naked mole-rat (NMR, *Heterocephalus glaber*) is an unusual model organism, but well placed for studying acid nociception. NMRs do not perceive injection of acid as nocifensive, nor do protons sensitise cutaneous sensory afferents, presumed adaptations to life in a hypercapnic environment. Absence of acid nociception has been partly attributed to acid acting like a local anaesthetic through inhibition of voltage-gated sodium channel Nav1.7. However, NMR acid-sensing ion channel 3 (ASIC3) is also unable to be activated by protons suggesting further adaptation. Proton-sensing G protein-coupled receptors (PS-GPCR) are widely expressed, thus warranting exploration of NMR PS-GPCR acid-sensitivity. Using a uniform cellular background comparisons of mouse and NMR PS-GPCRs revealed a difference in the ability of protons to stimulate the accumulation of cAMP, with mouse homologs facilitating proton-induced cAMP generation and no response observed from cells expressing NMR PS-GPCRs. Interestingly, the PS-GPCRs suggested to couple to inhibitory G-proteins, and thus inhibit cAMP accumulation through inhibition of adenylyl cyclase, were functional for both mice and NMRs. Expression of PS-GPCRs in various organs was also compared between mice and NMRs, very similar levels of expression being found for both species. The results discussed here offer another intricacy in NMR biology and suggest the species as useful in future study of PS-GPCR driven physiology, due to an apparent inability of some members of the family to be activated by protons.

4.2. Introduction

Mammals express a number of receptors which allow them to detect and respond to changes in extracellular pH, among these are the PS-GPCRs (Pattison et al., 2019). Unlike conventional GPCRs, which typically bind endogenous peptide ligands, PS-GPCRs coordinate intracellular signalling cascades in response to a mild increase in the extracellular concentration of protons. To date, 6 PS-GPCRs have been identified: GPR4, GPR31, GPR65 (also referred to as the T cell death associated gene 8 receptor, TDAG8), GPR68 (also referred to as the ovarian cancer gene related 1 receptor, OGR1), GPR132 (also referred to as the G2 accumulation receptor, G2A) and GPR151. Proton-induced activation of these receptors leads to various signalling responses suggesting differences in the preferential coupling of each receptor to G α subunits (Ludwig et al., 2003; Mashiko et al., 2019; Murakami

et al., 2004; Wang et al., 2004). The proton sensitivities of GPR31 and GPR151 have only recently been described using radiolabelled-GTP γ S binding and reporter gene assays, as such the downstream signalling cascades have not been empirically explored, although it has been suggested that these receptors may couple to G $\alpha_{i/o}$ and/or G $\alpha_{12/13}$ (Mashiko et al., 2019). A number of the PS-GPCRs have also been described to be activated or antagonised by lysolipids, glycosphingolipids, lipoamines and oxidised free fatty acids (Foster et al., 2019; Guo et al., 2011; Mogi et al., 2005; Obinata et al., 2005). However, the specificity and nature of these interactions are a matter of contention in the field (Im, 2005). Additionally, GPR68 has also been shown to function as a mechanoreceptor (Xu et al., 2018).

Interestingly, the ability of protons to engage PS-GPCRs does not appear to be conserved across phyla: although GPR68 homologs cloned from *D. rerio*, *X. laevis*, *G. gallus*, *M. musculus*, *R. norvegicus*, *S. scrofa* and *H. sapiens* all responded to protons in a reporter gene assay, a response from GPR65 homologs cloned from *D. rerio* and *G. gallus* was not observed (Mochimaru et al., 2018; Musha et al., 2019). Thus, it is unclear whether these receptors first evolved to sense changes in extracellular pH or lipids and which of these modes of activation underlies their physiological roles.

The utility of the naked mole-rat (*Heterocephalus glaber*, NMR) in biomedical research is becoming more apparent due to the species' compelling biology (Buffenstein et al., 2021). Famed for a high resistance to cancer, NMRs are extraordinarily long-lived enjoying good health and minimal senescence far longer than other similarly sized rodents (Buffenstein, 2008; Liang et al., 2010). NMRs can also survive extended periods of hypoxia and have a unique arsenal of immune cells (Hilton et al., 2019; Park et al., 2017). Furthermore, the NMR shows no nocifensive behaviour in response to subcutaneous injection of acid or capsaicin, it has also been demonstrated that although acid provides a much weaker activation of NMR visceral afferents than those of mice, it does induce mechanical hypersensitivity (Hockley et al., 2020; Park et al., 2008). These observations can in part be attributed to the ability of acid to inhibit voltage-gated sodium channel 1.7 (Nav1.7) in nociceptors of the NMR, preventing action potential propagation along the neurones responsible for detecting painful stimuli (Smith et al., 2011); the response observed in visceral afferents aligns with data showing that Nav1.7 is critical for somatic, but not visceral pain (Hockley et al., 2017). It has also been shown that homomeric assemblies of NMR ASIC3 are non-responsive to protons, given these are cation-permeable channels and thus have the potential to depolarise nociceptors, this offers further explanation as to why NMRs do not perceive acid as noxious (Schuhmacher et al., 2018).

Within the peripheral nervous system PS-GPCRs are expressed by the peptidergic nociceptor population, they may therefore contribute to acid-induced nociception (Hockley et al., 2018; Huang et al., 2007). Indeed, GPR65^{-/-} mice experienced less mechanical hypersensitivity following intraplantar injection of acid (Dai et al., 2017). Given the NMR's lack of nocifensive response to acid and reported proton-insensitivity of NMR ASIC3, the proton sensitivities and expression of PS-GPCRs by NMRs were assessed and compared to those of mice, working on the hypothesis that NMR PS-GPCRs might also be insensitive to protons.

4.3. Methods

See Materials and Methods chapter for detailed descriptions of RNA extraction and cDNA synthesis (Section 2.9), molecular cloning (Section 2.10), generation and culture of cell lines (Section 2.8), RT-PCR (Section 2.11), cAMP accumulation (Section 2.18), immunostaining (2.13.1) and qPCR (Section 2.12 and 2.12.1).

4.4. Results

To investigate the function of PS-GPCRs a cellular background devoid of endogenous expression of any proton-sensitive receptor is needed, as upon introduction of cloned PS-GPCRs any signalling events observed following acidic challenge can be solely attributed to the activation of the ectopically expressed PS-GPCR. Several standard cell lines are known to express ASICs and other acid-sensitive receptors, but Chinese hamster ovary (CHO) cells are often used for studying ASICs due to a lack of endogenous expression (Smith et al., 2007). Whether CHO cells express PS-GPCRs is unknown, to address this RT-PCR was performed on cDNA prepared from CHO cells. CHO cells were not found to express any of the PS-GPCRs nor ASIC1a (Figure 4.1A) and are thus useful for studying PS-GPCR function. The open reading frames of the six reported PS-GPCRs were cloned from mouse and NMR cDNA libraries prepared from a combination of RNA from the spleen, brain and DRG of each species. Sequencing of cloned HgGPR31 found premature stop codons, which agreed with reference sequences (NCBI Reference Sequence XM_013076682.1), therefore, given it would be impossible to produce a fully formed GPCR from this, HgGPR31 was not studied any further.

CHO cells stably expressing each PS-GPCR construct were generated using FlpIN CHO cells. FlpIN CHO cells have been modified to permit the inclusion of transgenes cloned into the pcDNA5/FRT/TO vector between two Flp sites in the genome upon transfection with the transgene and Flp recombinase, they thus represent a way to achieve isogenic stable cell

lines with comparable expression when many different constructs are transfected independently allowing comparisons to be drawn (O'Gorman et al., 1991). Upon selection of stable cell lines, RT-PCR was repeated for each cell line to ensure intended PS-GPCR constructs were expressed at the transcriptional level. Like CHO cells, FlpIN CHO cells did not express endogenous PS-GPCRs, but the mRNA of the transgenes transfected in could be detected (representative gel for mouse GPR65 cell line shown in Figure 4.1B). During cloning a FLAG tag was appended to the N-terminus of PS-GPCRs and a HA tag was introduced after the C-terminus, these tags allowed protein expression to be assessed using validated commercial antibodies against the tags: cells were fixed and stained with an anti-FLAG tag antibody, staining could only be achieved in stable cell lines (Figure 4.1C), not the parental FlpIN CHO cells (Figure 4.1D). To further ensure parental FlpIN CHO cells did not express PS-GPCRs, cells were stimulated with protons and cAMP accumulation was quantified. Stimulation of the parental FlpIN CHO cell line with protons did not induce cAMP accumulation (Figure 4.1E) further confirming that FlpIN CHO cells are a suitable background to study PS-GPCR function.

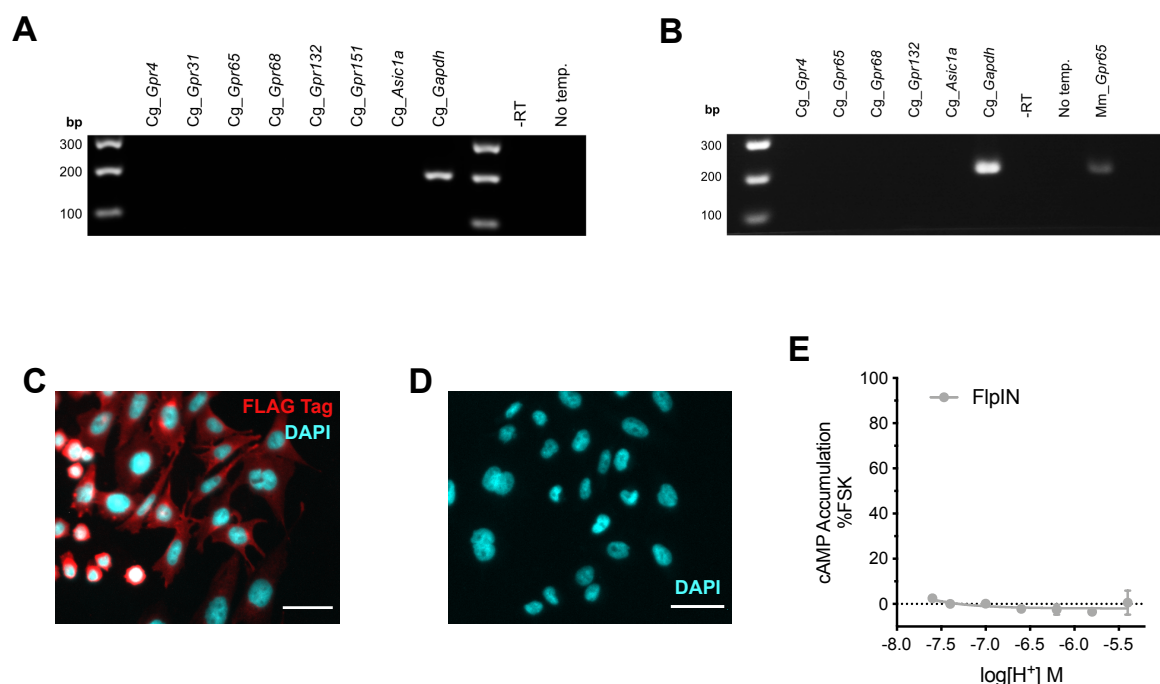


Figure 4.1. CHO cells are a suitable cellular background for studying PS-GPCR function. (A) CHO cells do not endogenously express any of the six hamster (*Cricetulus griseus*, Cg) PS-GPCRs or ASIC1a as confirmed by RT-PCR. (B) FlpIN CHO cells also do not express endogenous PS-GPCRs, but can be used to generate cell lines stably expressing PS-GPCRs cloned from other species, in this case mouse (*Mus musculus*, Mm) GPR65. Protein expression of PS-GPCRs was confirmed by immunocytochemistry. (C) FlpIN CHO cells transfected with FLAG-PS-GPCR-HA constructs stained positive when probed with an anti-FLAG tag antibody (red), (D) while the FLAG tag could not be detected for parental FlpIN CHO cells. The nucleus of cells was stained with 4',6-diamidino-2-phenylindole (DAPI, blue). Scale bar = 50 μ m. (E) Parental FlpIN CHO cells did not generate cAMP in response to proton (H^+) stimulation. Data are mean $\pm$ SEM from 3 independent experiments performed in duplicate.

reported for many of the PS-GPCRs (Huang et al., 2015; Ludwig et al., 2003; Wang et al., 2004), this was used to assess the sensitivity of PS-GPCRs from both species to protons. Cells expressing mouse (Mm) GPR4, GPR65, GPR68 and GPR132 all showed an increase in the accumulation of cAMP in response to proton stimulation (Figure 4.2A). The sensitivities of each receptor for protons varied considerably: cAMP accumulation peaked at ~ pH 6.6 for GPR4, GPR65 and GPR132, while GPR68 appeared to be more active at even more acidic pH with peak cAMP levels being achieved at pH 5.8. The efficacy of protons to elevate cAMP levels also differed between receptors, with proton-stimulation of GPR132 giving a maximum cAMP accumulation ~15% that of 100 μ M forskolin, whereas GPR65 achieved ~80%. Interestingly, proton stimulation of mouse GPR31 expressing cells caused no significant increase in cAMP accumulation, and in cells expressing MmGPR151 protons appeared to slightly reduce basal levels of cAMP compared to the neutral pH (Figure 4.2A), results suggesting that these receptors may couple to inhibitory G α subunits. To further explore the possibility that GPR151 might couple to inhibitory G proteins, cAMP levels were quantified upon co-stimulating cells with 1 μ M forskolin prepared at different pH; given that GPR31 has also been suggested to couple to inhibitory G proteins and protons failed to induce a clear cAMP accumulation, GPR31 was also assessed. Application of forskolin at lower pH reduced the amount of cAMP generated compared to the same treatment of FlpIN CHO cells (MmGPR31 IC₅₀ ~ pH 5.9, MmGPR151 IC₅₀ ~ pH 7.08; Figure 4.2B), confirming that GPR31 and GPR151 can inhibit adenylyl cyclase when activated with protons. Assaying NMR (Hg) PS-GPCRs showed an inability of protons to coordinate cAMP accumulation at any pH tested for all PS-GPCRs (Figure 4.2C). As for MmGPR151, proton-stimulation of HgGPR151 inhibited forskolin-induced cAMP accumulation and although the extent of inhibition was similar to that achieved with MmGPR151, HgGPR151 required more extreme pH (MmGPR151 IC₅₀ ~ 7.08, HgGPR151 IC₅₀ ~ pH 6.50; Figure 4.2D).

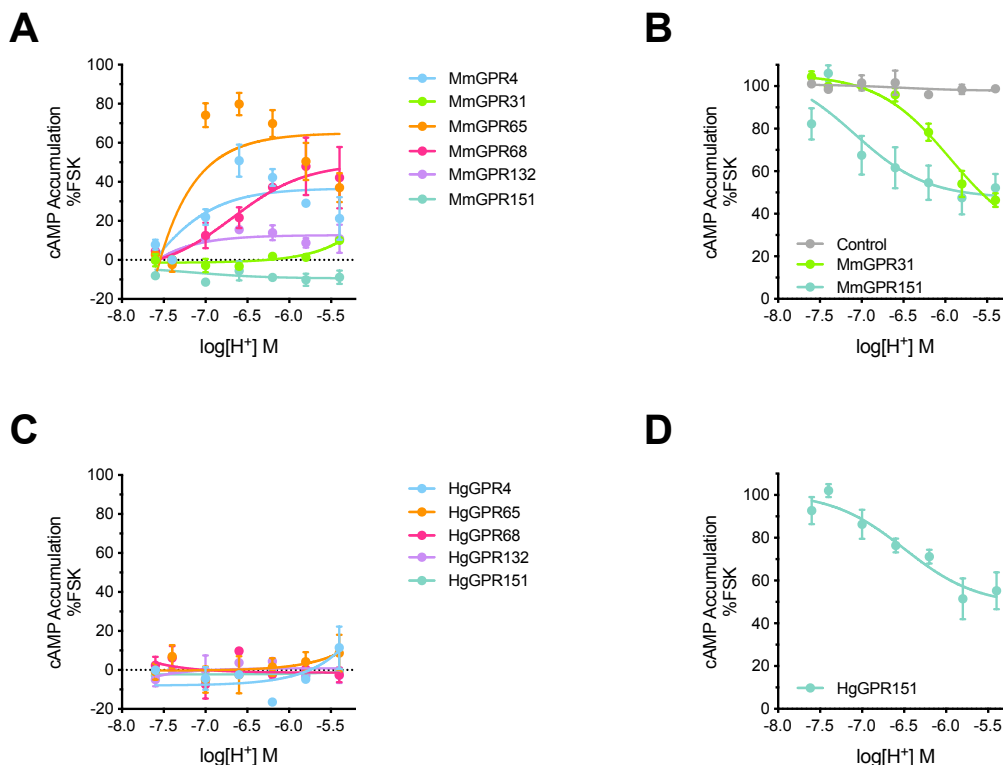


Figure 4.2. Most NMR PS-GPCR homologs are proton insensitive. (A) CHO cells stably expressing PS-GPCRs cloned from mouse (*Mus musculus*, Mm) were stimulated at different pH and the resulting cAMP accumulation was quantified. (B) The ability of protons to inhibit forskolin-induced cAMP accumulation was assessed for parental FlpIN CHO and in cells expressing mouse GPR31 or GPR151. Cells expressing NMR PS-GPCRs were also assessed for their ability to increase cAMP production (C), or inhibit forskolin-induced cAMP generation upon stimulation with protons (D). Data are expressed as mean $\pm$ SEM from at least 4 independent experiments where each condition was assayed in duplicate.

It thus appears that 4 out of 5 NMR PS-GPCRs are proton insensitive, however, the receptors could be cloned from NMR cDNA suggesting the receptors are not completely redundant. To begin to probe where PS-GPCRs might function in NMR, qPCR was performed to assess expression levels across distinct tissues. Similar expression was seen across tissues for NMRs and mice (Figure 4.3A-B). Expression of GPR151 was highest among the PS-GPCRs in the brains of both species, generally, lower PS-GPCR expression was observed among the central (brain) and peripheral (DRG) nervous systems of NMRs, compared to mice, most notably GPR4 expression in both tissues was $\sim 10\times$ less in NMRs. Fairly uniform expression of PS-GPCRs was observed in the kidneys of both animals, but as with the nervous system, reduced expression of PS-GPCRs was seen in NMR liver samples compared to those of mice, particularly in the case of GPR4 which was one of the most highly expressed PS-GPCRs in the mouse liver but among the lowest expressed by NMR liver. Strong expression of GPR65 was reported in the lung of both mice and NMRs. Lastly, GPR65 and GPR132 expression

dominated that of other PS-GPCRs in the spleen for both mice and NMRs, spleen being the only tissue studied in which GPR151 expression was not detected.

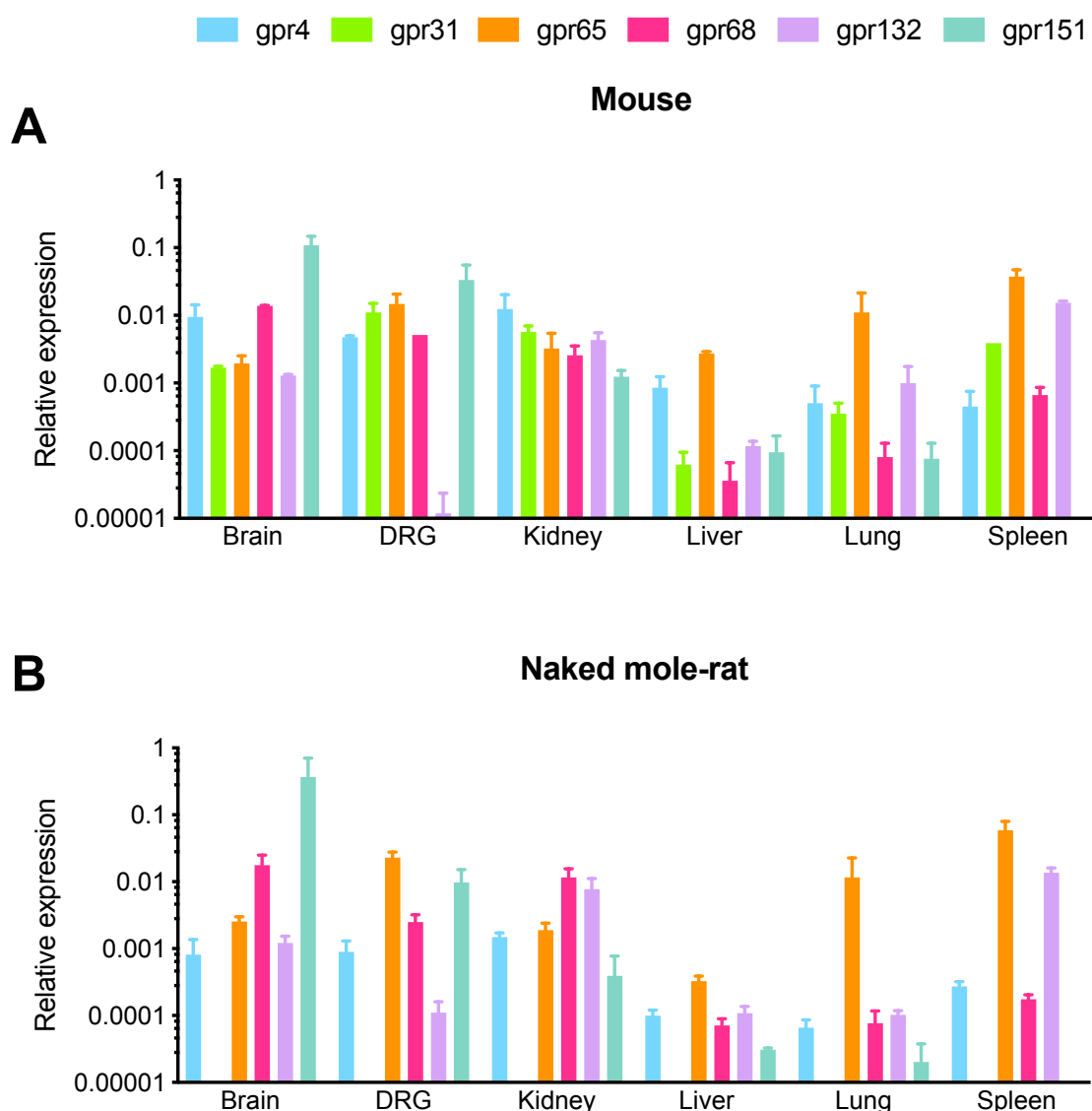


Figure 4.3. Mice and NMRs show similar expression profiles of PS-GPCRs in different organs. qPCR was performed on cDNA libraries of mouse and NMR organs, β -actin was used as the housekeeping gene for analysis of relative expression. Data are expressed as mean $\pm$ SEM from three animals of each species, each gene was analysed in duplicate for each biological sample.

4.5. Discussion

Here the proton-sensitivities and expression of PS-GPCRs were compared between mice and NMRs. While similar expression patterns of this family of receptors was documented across key tissues, NMR PS-GPCRs were found to be unable to coordinate the accumulation of cAMP in response to acidic challenge. Interestingly, protons coordinated an inhibition of

forskolin-induced cAMP accumulation in cells expressing GPR151 from both species, although HgGPR151 appears less sensitive, requiring stronger proton stimulation to inhibit forskolin-induced cAMP production. GPR31 cloned from mice, could also drive proton-induced inhibition of adenylyl cyclase, but such a comparison with HgGPR31 could not be made due to premature stop codons preventing cloning of a full functional GPCR.

Based upon data with the human proteins, GPR31 and GPR151 have only recently been proposed as PS-GPCRs, thus results presented here for mouse GPR31 and GPR151 confirm that both these receptors are able to coordinate intracellular cascades in response to changes in the extracellular concentration of protons. As previously suggested from GTP γ S binding experiments, it appears that both of these receptors preferentially engage inhibitory G-proteins in response to acidic shifts in extracellular pH (Mashiko et al., 2019). It would be well worth exploring precisely which G-proteins GPR31 and GPR151 can engage, as other PS-GPCRs have been suggested to reduce forskolin-induced cAMP accumulation via pertussis toxin insensitive G α_z (Im et al., 2001). G α_z subunits are far less studied than their fellow family members G $\alpha_{i/o}$ and if more are activated by PS-GPCRs this could be interesting line of enquiry. A first step to address G-protein coupling might involve using a bioluminescence resonance energy transfer (BRET) assay to monitor the dissociation of G $\beta\gamma$ subunits from various G α subunits in cells overexpressing the receptors of interest and individual G α subunits, as has recently been carried out for human PS-GPCRs (Rowe et al., 2021). Additionally, the ability of protons to coordinate other signalling cascades GPCRs are known to initiate should be explored, including Ca²⁺ mobilisation, activation of RhoGTPases and the possibility of G-protein independent signalling driven by the recruitment of β -arrestins. Once pharmacological understanding of GPR31 and GPR151 as proton-sensors is furthered, it would be interesting to assess their physiological roles.

As per pharmacological convention, dose response data were fitted to the Hill equation to assess the ability of protons to activate each receptor using cAMP as a readout, however, it appears proton-induced activation of PS-GPCRs does not follow standard agonist concentration dependence, evident from lower levels of cAMP accumulation being achieved upon stimulation of some receptors with more extreme pH. This may be due to the inherent sensitivity of all proteins to pH, which means that mild increments in the local concentration of protons can induce an active conformation of PS-GPCRs, but that this conformation (or downstream signalling proteins) may be compromised when there are too many protons, reducing the ability of PS-GPCRs to transduce signals. It is thus clear that extra consideration is needed to when analysing the pharmacology of PS-GPCRs due to the nature of their

activation by protons and that methods chosen should be driven by the biological question in mind. The importance of considering analysis and experimental set up is evident from the results presented here: In order to draw comparisons between the different receptors, cAMP accumulation upon proton-stimulation of PS-GPCRS was normalised to a maximum response, achieved by stimulation with 100 μ M forskolin and the cAMP produced upon stimulation with pH 7.6 was set as a baseline. Consequently, data reflect the change in cAMP accumulation induced by altering the pH of stimulation from pH 7.6 to the other proton concentrations assessed, which means that the values reported are relative and may not fully describe the activity of the receptor at the pH it is stimulated at. For example, Ludwig and colleagues (2003) have reported similar levels of cAMP being generated upon stimulation of cells expressing GPR4 with solutions at pH 7.4, 7.2, 7.0 and 6.8 when cAMP was quantified using radioactivity, while results here demonstrate greater differences in cAMP accumulation across that pH range. Moving forward it would be beneficial to assess relative cAMP accumulation using other pH as a reference, or quantifying standards containing a known amount of purified cAMP alongside the lysates of cells stimulated with protons to allow an absolute cAMP level to be interpolated.

In this study functional characterisation of PS-GPCRs was limited to the cAMP pathway, however, as mentioned above, GPCRs are notorious for their pleiotropic abilities to engage multiple signalling modalities, it thus remains to be explored whether all aspects of NMR PS-GPCR signalling is proton-insensitive. Although all of the better characterised PS-GPCRs have been shown to coordinate cAMP responses, it would be of interest to study the ability of protons to induce Ca^{2+} mobilisation, particularly for GPR68 as this has been reported to be the predominant coupling of human GPR68 (Ludwig et al., 2003). To reduce confounding factors surrounding transactivation of different signalling pathways, it may be more useful to first focus efforts on the first step in GPCR activation, engagement of $\text{G}\alpha\text{s}$, which may be assessed through BRET or $\text{GTP}\gamma\text{s}$ binding (Harrison and Traynor, 2003; Masuho et al., 2015). A fluorescence-based activation probe for GPR68 has recently been described (Ozkan et al., 2021), and this approach might also be expanded to other PS-GPCRs of different species to probe their agonist sensitivity.

Another potential caveat of the results here is the use of a third species in the cellular background. Although all stable cell lines were shown to produce mRNA of the PS-GPCR transfected in and stained positive when probed with an anti-FLAG antibody, post-translational modifications usually added to the PS-GPCRs when produced natively may not be possible in the CHO background, which has been shown to have a distinct repertoire of enzymes responsible for protein glycosylation, which might affect protein trafficking and function

(Noguchi et al., 1995). It would thus be interesting to study the PS-GPCRs of mice and NRMs in native cells, based on the qPCR data obtained in this study, splenocytes may be a good starting point for such studies. Expression of PS-GPCRs within the spleen of mice and NMR were most similar (Figure 4.3A-B), additionally splenocytes can be cultured relatively easily and a large number of cells harvested from individual animals (Huang et al., 1997). Of course, this would only allow a blanket assessment of the ability of protons to coordinate GPCR-driven signalling responses, such as cAMP accumulation and not assessment of individual PS-GPCRs due to the widespread expression.

If the studies described above further suggest that PS-GPCRs are redundant in their proton-sensing capabilities in NMR, it raises further questions on the evolution of PS-GPCRs as sensors of protons or lipids. Given the subterranean lifestyle of NRMs and their demonstrated ability to tolerate hypoxia and hypercapnia (Park et al., 2021), it certainly makes sense that they would be less sensitive to protons, particularly if the receptors activated by these drive processes such as inflammation and pain. Interestingly comparisons of the primary sequences of receptors from both species reveal that the histidine residues, shown to confer proton sensitivity to mouse and human PS-GPCRs (Liu et al., 2010; Ludwig et al., 2003; Murakami et al., 2004; Wang et al., 2004) are conserved by NMR homologs, suggesting other residues may impart a larger influence on proton-sensitivity of the receptors. Further studying differences between PS-GPCRs of NMR and mice may reveal more on the mechanism of proton-induced activation, likely candidate residues involved in proton-induced activation include glutamate and aspartate, due to their pKa values lying close to physiological pH, meaning they are more amenable to protonation following an increase in the local concentration of protons. It would also be worth assessing the sensitivity of NMR PS-GPCRs to lipids reported to activate mouse or human counterparts (e.g. psychosine for GPR65 (Im et al., 2001)) to gain a better understanding on mechanisms of activation of PS-GPCRs by these distinct classes of agonist.

While a lot remains to be determined regarding the function of NMR PS-GPCRs, the results described in this chapter reinforce the utility of the NMR as a model organism to study acid-induced signalling and the physiological consequences of this.

5. Signalling and trafficking bias at the PS-GPCR GPR65.

5.1. Chapter Summary

GPR65 has been reported to be activated by changes in extracellular pH as well as psychosine, a sphingolipid. A synthetic agonist, BTB09089, has also been described for the receptor. The activation of distinct intracellular signalling pathways has been described upon engagement of the receptor by these agonists, suggesting an inherent signalling bias (ability of agonist of the same receptor to coordinate distinct intracellular signalling cascades). However, studies of GPR65 thus far have used a variety of cellular backgrounds and receptor species, focussed on different signalling outputs and employed different methods to study intracellular activity. To assess signalling bias at GPR65, the signalling signatures of the reported agonists were compared in a uniform cellular background. The ability of the agonists to coordinate accumulation of cAMP, mobilise Ca^{2+} from intracellular stores, activate the mitogen-activated protein kinases ERK1/2 and recruit β -arrestins was assessed. It was found that the signalling signatures of protons and BTB at GPR65 were closely aligned, while engagement of the receptor with psychosine triggered distinct cascades. The residues important for activation of the receptor by the agonists were assessed by generating a number of mutations in the receptor, the importance of extracellular histidine residues in activation of GPR65 was apparent for all agonists. Following activation all three agonists of GPR65 evoked receptor internalisation, which was shown to be dependent on clathrin and dynamin. The results described here offer comprehensive pharmacological profiling of GPR65 confirming the coordination of distinct signalling outputs upon its activation by protons or lipids. Given the synthetic agonist BTB, which was shown to be selective for GPR65 over other PS-GPCRs, mimics signalling responses elicited by protons at GPR65 this compound represents a useful tool to probe the role of GPR65 as a proton-sensor in primary cells and *in vivo*, separating the consequences of GPR65 activation from other proton-sensitive receptors.

5.2. Introduction

GPCRs represent the largest family of transmembrane receptors and their activation by a diverse range of ligands means that they are involved in disparate physiological processes and diseases alike. In recent years GPCR biology has become yet more complex with concepts such as biased agonism, receptor oligomerisation, compartmentalised signalling and non-canonical interactions with proteins other than traditional heterotrimeric G-proteins increasing signalling diversity (Schneditz et al., 2019; Smith et al., 2018; Wang et al., 2018).

Considering that more than a third of approved pharmaceuticals exert their actions on GPCRs (Santos et al., 2017), there is rightly considerable interest in understanding the pharmacology of this important family of receptors. However, a large proportion of GPCRs remain poorly characterised. Among the least characterised GPCRs are the PS-GPCRs, a family of six receptors reported to be activated by extracellular protons. The ability of protons to activate GPR68 was described first, GPR4, GPR65 and GPR132 were also identified as PS-GPCRs shortly thereafter owing to a high degree of homology between the receptors (Ludwig et al., 2003; Murakami et al., 2004; Wang et al., 2004). More recently, GPR31 and GPR151 have also been reported to be activated by protons (Mashiko et al., 2019). Despite PS-GPCRs first being identified over 15 years ago, characterisation of proton-induced signalling and the relevance of this to health and disease remains poorly understood. This may in part be due to technical difficulties associated with using protons as agonists in traditional pharmacological assays, which are often sensitive to perturbations in pH. In addition, the large number of other receptors known to be activated or modulated by protons (Pattison et al., 2019) adds further complexity to assigning physiological roles to individual proton-sensors, especially given their widespread expression.

Many of the PS-GPCRs are also reported to be activated by lipids, including GPR65 which has been shown to be activated by psychosine, a glycosphingolipid implicated in the pathology of globoid cell leukodystrophy, whereby loss of a lysosomal enzymes sees psychosine concentration reach cytotoxic levels ensuing cell death and neurogenic inflammation (Hawkins-Salsbury et al., 2013). While protons cause GPR65-mediated accumulation of cAMP and give rise to Ca^{2+} flux (Lin et al., 2017; Wang et al., 2004), psychosine inhibits adenylyl cyclase and mobilises intracellular Ca^{2+} (Im et al., 2001). The nature of these interactions of protons and lipids with PS-GPCRs is debated, such that some groups report lipids as agonists in their own right whilst others claim that they act as antagonists (Im, 2005). The contrasting reports may be an artefact of the different approaches and cellular backgrounds used to interrogate PS-GPCR pharmacology and make a strong case for more systematic profiling of agonists reported to activate PS-GPCRs.

GPR65 represents an important candidate PS-GPCR for further study of its signalling following activation by protons and lipids because of the relevance of endogenous activation by protons or psychosine, which could potentially facilitate diverse physiological responses as a result of distinct intracellular signalling events. Additionally a synthetic agonist of GPR65 is also available, BTB09089 (BTB) which has been shown to increase cAMP accumulation via GPR65 and used to assess how GPR65 can modulate the production of cytokines by immune

cells (Onozawa et al., 2011). The aim of this Chapter was thus to profile the pharmacological consequences of GPR65 activation by reported agonists, exploring multiple pathways known to be orchestrated by PS-GPCRs in a uniform system to permit comparisons to drawn between agonists. By furthering basic pharmacological understanding of the relevance of GPR65 activation by endogenous protons versus lipids, it is hoped studies into the physiological relevance of this might be better informed. For this reason, the mouse homolog of GPR65 (MmGPR65) was studied so that results may be better interpreted alongside downstream *in vivo* studies.

5.3. Methods

See Materials and Methods chapter for detailed descriptions of RNA extraction and cDNA synthesis (Section 2.9), molecular cloning (Section 2.10), site-directed mutagenesis (Section 2.10.1), generation and culture of cell lines (Section 2.10), immunostaining (Section 2.13.1), Agonists, activators and inhibitors (Section 2.1), BRET assays (Sections 2.16 and 2.17), quantification of cAMP accumulation (Section 2.18), Ca²⁺ mobilisation (Section 2.19) and ERK activation (Section 2.20).

5.4. Results

Given most reports of GPR65 signalling have focussed on modulation of adenylyl cyclase to alter levels of cAMP, the ability of the agonist panel (protons, psychosine and BTB) to influence cAMP levels through activation of mouse GPR65 (MmGPR65) was first assessed. To eliminate any potential influences of pH in the detection of cAMP, a 'stimulate, lyse and detect' approach was taken for these experiments. To do this, MmGPR65 cells were first exposed to stimulatory solutions buffered to various pH or containing agonists for 15 minutes and then transferred to ice, stimulatory solutions removed and cells lysed with a neutral pH lysis buffer. Consistent with previous reports, increasing the concentration of protons, resulted in increased accumulation of cAMP until the pH surpassed 6.6, when activity then decreased (Figure 5.1A-B) (Wang et al., 2004). Similarly, BTB was also found to cause a concentration-dependent increase in cAMP accumulation, with an EC₅₀ of 5.2 µM (Figure 5.1A). Given psychosine is reported to decrease forskolin-induced cAMP accumulation, cells exposed to psychosine were co-stimulated with 1 µM forskolin and a concentration-dependent decrease in the amount of cAMP produced by cells was observed (EC₅₀ 8.8 µM, Figure 5.1A), which corroborates previous data (Im, 2005).

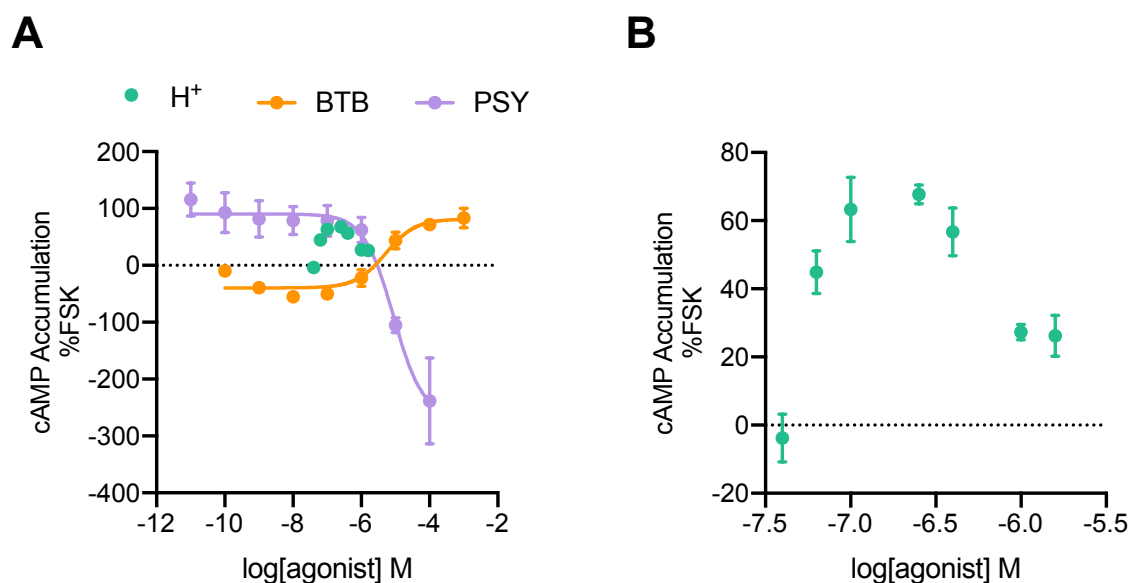


Figure 5.1. Modulation of cAMP levels by GPR65 agonists. (A) CHO cells stably expressing MmGPR65 were stimulated with various concentrations of protons (H⁺) or BTB09089 (BTB) for 15 minutes before cells were lysed and cAMP levels quantified. Given psychosine (PSY) has been reported to inhibit adenylyl cyclase, cells were co-stimulated with 1 μ M forskolin and psychosine prepared at various concentrations to study psychosine-induced inhibition of cAMP production. (B) Expanded concentration-response data for proton-induced cAMP accumulation. Data were normalised to the cAMP accumulation induced by 100 μ M forskolin (H⁺ and BTB) or 1 μ M forskolin (PSY) and are presented as means $\pm$ SEM from 5 independent experiments performed in duplicate.

Activation of GPCRs can lead to changes in intracellular Ca²⁺ concentration through the mobilisation of Ca²⁺ from intracellular stores, or engagement of Ca²⁺ permeable ion channels at the plasma membrane (Dhyani et al., 2020). The ability of protons, BTB and psychosine to coordinate the release of Ca²⁺ from intracellular stores was assessed using the Ca²⁺-sensitive dye Fluo4, omission of Ca²⁺ from the assay buffer allowed any responses to be attributed to release of Ca²⁺ intracellularly, most likely through engagement of G α_q subunits. Only psychosine and protons, were found to be able to cause the mobilisation of Ca²⁺ (Figure 5.2A-C). Psychosine was only able to coordinate Ca²⁺ flux when the concentration used to stimulated cells exceeded 1 μ M (Figure 5.2D) and stimulation of cells with 10 μ M psychosine raised intracellular Ca²⁺ levels to comparable levels to those achieved by stimulation with the positive control ionomycin. Proton-driven increases in Ca²⁺ levels intracellularly also appeared to be dependent on proton concentration, with the EC₅₀ estimated to be approximately pH 6.2 (Figure 5.2E). To confirm that the observed increases in Ca²⁺ flux were due to engagement of GPR65, experiments were repeated using FlpIN parental cells, which do not express endogenous PS-GPCRs (Figure 4.1B). 10 μ M psychosine did not induce a Ca²⁺ flux in FlpIN cells (FlpIN AUC, 0.33 $\pm$ 1.62, MmGPR65 AUC, 85.37 $\pm$ 6.87, $p < 0.0001$, two-way ANOVA with Bonferroni post hoc, mean $\pm$ SEM, $n = 3 - 5$, Figure 5.2F), but a small increase in the intracellular concentration of Ca²⁺ was achieved when cells were stimulated with assay buffer prepared at pH 6, although this was significantly smaller than the increase induced in

MmGPR65 expressing cells (FlpIN AUC, 7.58 ± 2.20 , MmGPR65 AUC, 25.36 ± 0.17 , $p < 0.01$, two-way ANOVA with Bonferroni post hoc, mean $\pm$ SEM, $n = 3 - 5$, Figure 5.2F). Due to the proton-induced Ca^{2+} response in FlpIN CHO cells at pH 6 being smaller in magnitude than that observed for MmGPR65 cells, it would suggest that GPR65 mediates a proton-induced Ca^{2+} flux. However, the experimental set up used was likely affected by more extreme pH, given that no response was seen when FlpIN cells were stimulated with pH 7 (FlpIN AUC, 0.483 ± 0.43 , MmGPR65 AUC, 2.19 ± 2.29 , mean $\pm$ SEM, $n = 3 - 5$, Figure 5.2F). Possible explanations for this include endogenous expression of other proton-sensitive receptors capable of driving Ca^{2+} flux, although this could not be any of the other PS-GPCRs, ASIC1a nor TRPV1 which are not expressed by CHO cells (Figure 4.1A) or a direct effect of high concentrations of protons on the dye Fluo4 used to infer intracellular Ca^{2+} concentration. These findings reinforce the idea that proton-induced signalling needs careful consideration and that lipids and protons drive distinct responses upon activation of the same receptor.

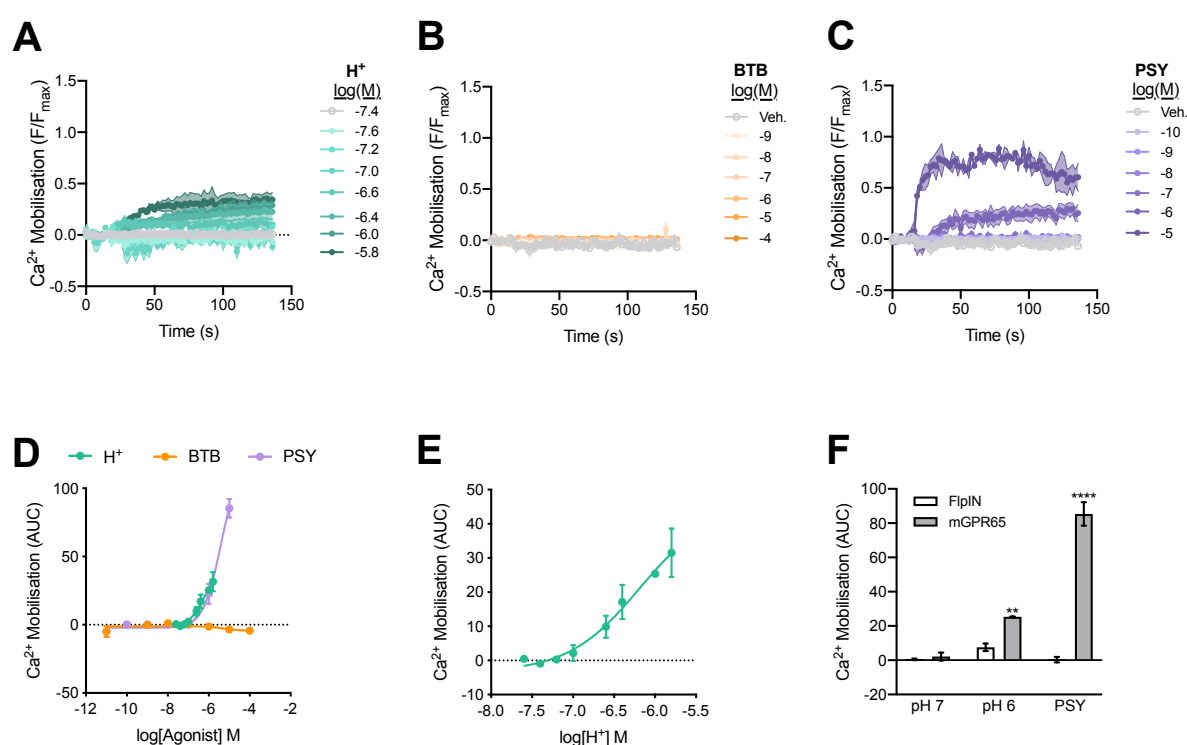


Figure 5.2. Mobilisation of Ca^{2+} following GPR65 stimulation with various agonists. MmGPR65 cells were loaded with the Ca^{2+} -sensitive dye Fluo4 and stimulated with various concentrations of (A) protons (H^+), (B) BTB or (C) psychosine (PSY) to assess the ability of agonists to evoke an intracellular Ca^{2+} flux. (D) The area under the curve (AUC) obtained from kinetic data was used to explore concentration dependence of the agonist-induced Ca^{2+} mobilisation. (E) Expanded view of proton dependence of Ca^{2+} mobilisation. (F) The specificity of agonist-induced Ca^{2+} mobilisation was assessed by comparing the responses achieved by stimulating MmGPR65 cells to responses observed in parental FlpIN cells. Data were normalised to the maximum change in Fluo4 intensity evoked by stimulation of cells with 10 μM ionomycin and are presented as mean $\pm$ SEM from ≥ 3 independent experiments performed in duplicate. ** $p < 0.01$, *** $p < 0.001$, two-way ANOVA followed by Bonferroni post-hoc; * denote differences between the response of annotated agonist for MmGPR65 cells and FlpIN cells.

Another facet of GPCR signalling is the activation of ERK, which may be achieved through several routes and is crucial for many GPCRs to fulfil their physiological roles (Jain et al., 2018). A similar approach to that used to assess cAMP levels was used to assess the activation of ERK: cells were firstly stimulated with agonists, then lysed in a neutral buffer and the level of phosphorylated ERK1/2 measured as a readout of ERK activation. Initial experiments exploring the time course suggested that ERK activation peaked 5-minutes post-stimulation and so 5 minutes was used as the time point for assessing concentration-dependence of the agonists. Both protons and BTB were found to coordinate the activation of ERK1/2, with respective EC_{50} estimates of pH 6.2 and 27.7 μ M (Figure 5.3A-B). However, the efficacy of BTB for ERK activation was higher than for protons, with a maximum response of 62% of that achieved when cells were stimulated with 10 μ M phorbol 12,13-dibutyrate (PDBu, a PKC activator which causes ERK phosphorylation), compared to 47% achieved by proton stimulation. Stimulation of MmGPR65 cells with 100 μ M psychosine caused a small increase in ERK activation (11% of the PDBu-induced response), but was the only concentration shown to lead to ERK phosphorylation. To assess the specificity of agonist-induced ERK activation, the ability of pH 6 and 1 mM BTB to coordinate ERK activation for FlpIN cells not expressing MmGPR65 was investigated, the responses achieved for the parental cell line were minimal and were found to be statistically distinct from those achieved with MmGPR65 cells (pERK activation as % of PDBu-induced response – pH 6: FlpIN, 5.60 ± 0.55 , MmGPR65, 41.02 ± 4.15 , $p < 0.0001$; 100 μ M BTB: FlpIN, 3.44 ± 0.17 , MmGPR65, 62.27, $p < 0.0001$; two-way ANOVA with Bonferroni post hoc, mean $\pm$ SEM, $n = 4$, Figure 5.3C), indicated the responses measured were driven by MmGPR65.

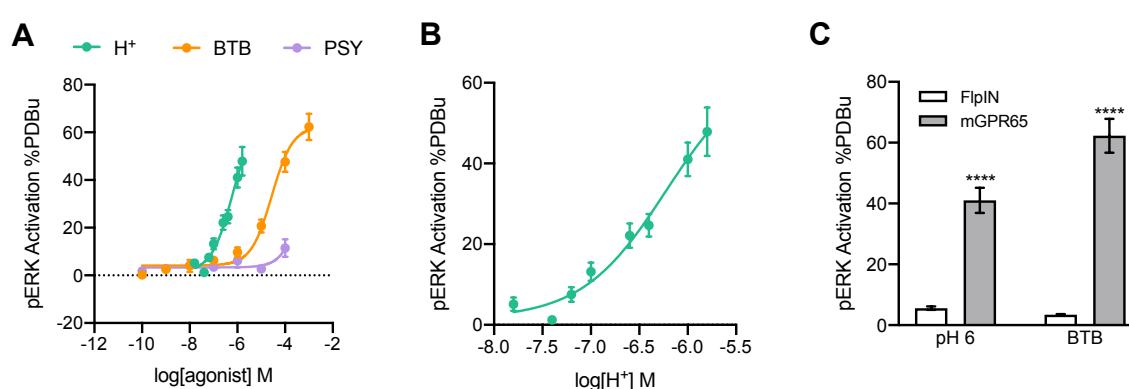


Figure 5.3. GPR65-mediated activation of ERK kinases. (A) MmGPR65 cells were stimulated with various concentrations of protons (H⁺), BTB or psychosine (PSY) for 5 minutes to assess activation of ERK kinases. **(B)** Expanded view of the relationship between concentration of protons used to stimulate MmGPR65 cells and ERK activation. **(C)** The specificity of proton- and BTB-induced ERK activation was assessed by comparing ERK activation for MmGPR65 cells to the parental FlpIN CHO cell line following stimulation with pH 6 or 1 mM BTB. Data were normalised to responses seen following stimulation of cells with 10 μ M PDBu and are presented as mean $\pm$ SEM from 4 independent experiments performed in duplicate. **** $p < 0.0001$, two-way ANOVA followed by Bonferroni post-hoc; * denote differences between the agonist response achieved for MmGPR65 cells and FlpIN cells.

β -arrestins mediate desensitisation of many GPCRs, but can also coordinate signalling events in their own right (Gurevich and Gurevich, 2019). The ability of GPR65 agonists to induce recruitment of β -arrestins to active receptors was assessed using a BRET-based assay. Briefly, Flp-In parental CHO cells expressing luciferase tagged GPR65 (MmGPR65-RLuc8) and yellow fluorescent protein (YFP) tagged β -arrestin 1/2 (β -arrestin1/2-YFP) or pcDNA 3.1 were challenged with various concentrations of protons, BTB or psychosine. 15 minutes after initial stimulation, the BRET signal (YFP emission/RLuc8 emission) between the receptor and β -arrestins was measured and corrected by subtracting the BRET signal obtained between cells expressing MmGPR65-RLuc8 and pcDNA 3.1 stimulated in the same way. Given BRET can only occur when the YFP (appended to the β -arrestins) is in sufficient proximity to the luciferase, such that luciferase emission can excite YFP molecules, the greater the BRET signal the stronger the recruitment of arrestins. Following 15 minutes of exposure to protons or BTB, increased BRET was detected between MmGPR65-RLuc8 and β -arrestin 1-YFP, suggesting agonist-induced recruitment (Figure 5.4A-B). No change in BRET was observed upon psychosine treatment, indicating that psychosine does not coordinate recruitment of β -arrestin 1 to MmGPR65. Similar results were obtained when exploring recruitment of β -arrestin 2, with protons and BTB able to evoke recruitment of β -arrestin 2, whereas psychosine did not (Figure 5.4C-D). EC_{50} values for the recruitment of β -arrestins to MmGPR65 induced by protons and BTB cannot be estimated reliably due to uncertainty when the maximal response is achieved.

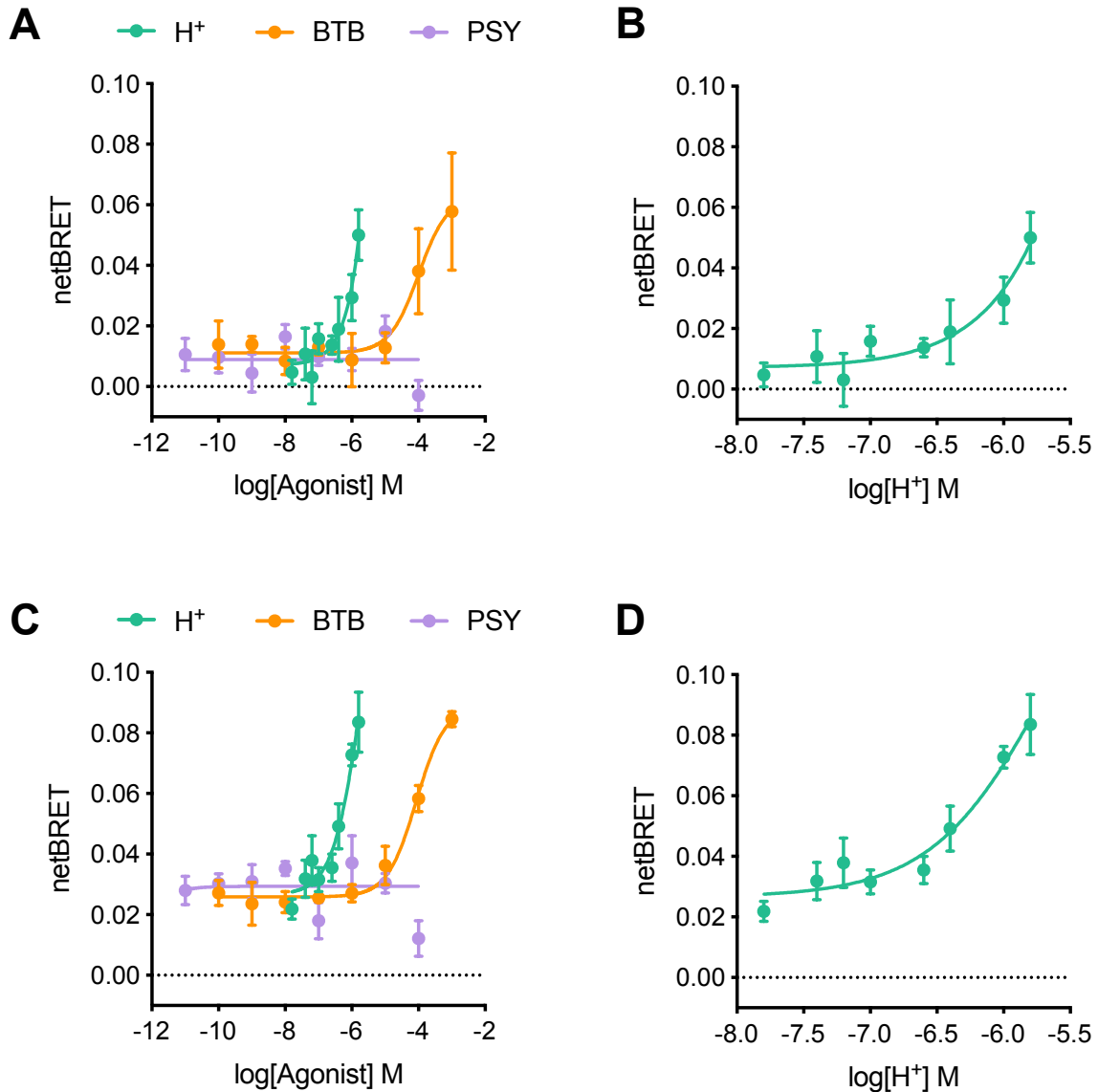


Figure 5.4. β -arrestin recruitment to GPR65 following receptor activation with protons, BTB or psychosine. To assess the ability of GPR65 agonists to recruit β -arrestins FlpIN CHO cells were transiently transfected with luciferase tagged GPR65 (MmGPR65-RLuc8) and YFP-tagged β -arrestins, the proximity of (A) β -arrestin 1 and (C) β -arrestin 2 to GPR65 was assessed 15 minutes after stimulation of cells with protons (H⁺), BTB or psychosine (PSY) through assessing BRET between the luciferase tagged receptor and YFP-tagged arrestins. Dose-response data for proton-induced recruitment of (B) β -arrestin 1 and (D) β -arrestin 2 were plotted separately to exemplify the proton-dependence of arrestin recruitment. BRET signals from experiments performed in the absence of YFP-tagged arrestins were subtracted from those achieved in the presence of β -arrestin 1 or 2 to negate any effect of agonists altering luciferase activity. Data are mean $\pm$ SEM from ≥ 4 independent experiments performed in duplicate.

Having observed differences in the ability of protons, BTB and psychosine to activate intracellular signalling events through GPR65, the molecular determinants of receptor-agonist interactions were further explored. Histidine residues present in extracellular portions of GPR65 have been shown to be critical in conferring proton-sensitivity to the receptor, more specifically, mutating histidines 10 and 243 of human GPR65 to phenylalanine has been shown to ablate proton-induced cAMP accumulation, while mutation of histidine 14 reduced

the efficacy of protons to coordinate cAMP generation (Wang et al., 2004). To assess whether homologous residues (H13, H16 and H245) of MmGPR65 were required for sensitivity of the receptor to protons, BTB and psychosine, each histidine was mutated to phenylalanine independently (Figure 5.5A). Fluorescently tagged variants of the histidine mutants still localised to the plasma membrane when visualised with confocal microscopy, meaning that the mutant receptors would still be exposed to agonists and that any change in agonist sensitivity would most likely be due to altered sensitivity of the receptor, rather than improper expression (Figure 5.5B). The ability of each agonist to alter intracellular levels of cAMP was compared to responses seen with the wild-type receptor. The H13F and H16F mutants were shown to prevent proton-induced cAMP accumulation, while stimulation of the H245F mutant shifted the pH range at which MmGPR65 could raise intracellular cAMP to higher proton concentrations (Figure 5.5C). The H13F and H16F mutations also prevented BTB-induced cAMP accumulation at MmGPR65, with some activity retained for the highest concentrations against H245F (Figure 5.5D). Similarly, loss of extracellular histidines dampened the ability of psychosine to inhibit forskolin-induced cAMP accumulation, although some activity was observed for H245F when stimulated with 100 μ M (Figure 5.5E). When considered with the results already described in this Chapter, data obtained with these mutant receptors suggest that while the GPR65 agonists being investigated differ in their abilities to coordinate certain signalling phenomena, the residues of the receptor important for activation are conserved across agonists.

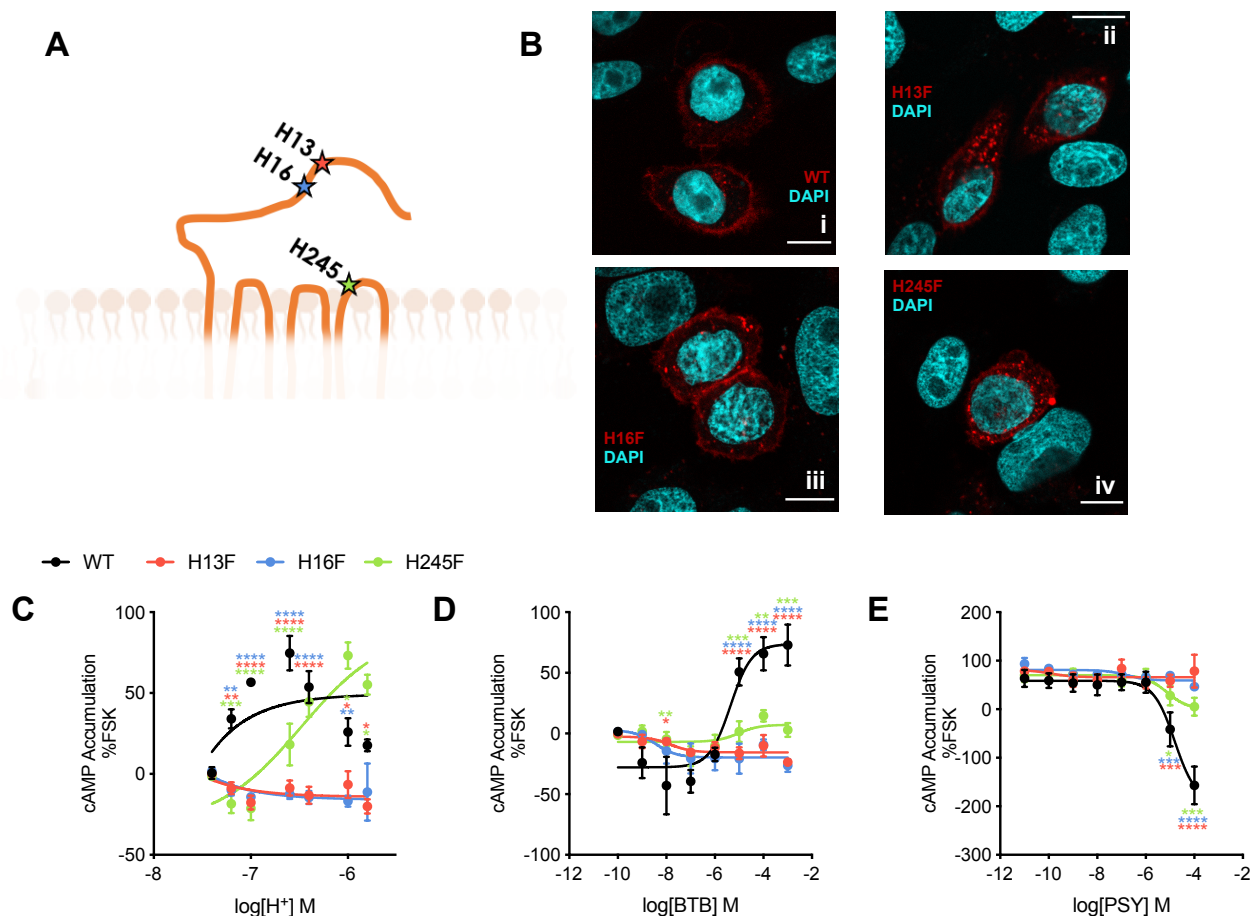


Figure 5.5. Mutation of histidine residues in extracellular portions of MmGPR65 reduces receptor sensitivity to agonists in cAMP assays. (A) Schematic diagram showing the location of histidine residues mutated to phenylalanine in the extracellular N-terminus and third extracellular loop of GPR65. (B) CHO cells transfected with i) wild-type MmGPR65 or histidine mutants ii) H13F iii) H16F or iv) H245F tagged with mCherry (red) at the C-terminus were expressed at the plasma membrane, nucleus stained with DAPI (blue), scale bar = 10 μm. The ability of (C) protons (H⁺) (D) BTB and (E) psychosine (PSY) to modulate cAMP levels was assessed for the wild-type and histidine mutants. Data were normalised to the cAMP accumulation induced by 100 μM forskolin (H⁺ and BTB) or 1 μM forskolin (PSY) for each receptor and are presented as means ± SEM from 4-5 independent experiments performed in duplicate. * p < 0.05 ** p < 0.01, *** p < 0.001, **** p < 0.0001 two-way ANOVA followed by Bonferroni post-hoc; * are colour coded to signify differences between individual histidine mutants and the wild-type receptor for each concentration of agonist tested.

Following activation many GPCRs are internalised, to assess whether this is the case for GPR65, BRET assays to quantify the proximity of MmGPR65-RLuc8 and fluorescent fusion proteins resident at the plasma membrane (RIT-Venus), or early endosomes (Rab5a-Venus) were performed. Following stimulation with protons, BRET between MmGPR65 and the plasma membrane marker decreased in a time- and concentration-dependent manner, with an estimated EC₅₀ of pH 6.7 (Figure 5.6A,D). BTB and psychosine were also found to induce receptor internalisation, with half the maximal response being achieved upon stimulation with 2.67 μM and 3.17 nM respectively (Figure 5.6B-C, E-F). Concomitant experiments quantifying the proximity of MmGPR65-RLuc8 to the early endosomal marker Rab5a-Venus, showed an

increase in BRET signal upon stimulation of cells with protons, BTB and psychosine (Figure 5.6G-I). A concentration-dependent increase in BRET was observed for each agonist (EC_{50} s: protons: pH 7.1, BTB: 25 nM, PSY: undetermined; Figure 5.6J-L). Taken together these results suggest that shortly after activation of GPR65 by protons, BTB or psychosine, the receptor is internalised to early endosomes.

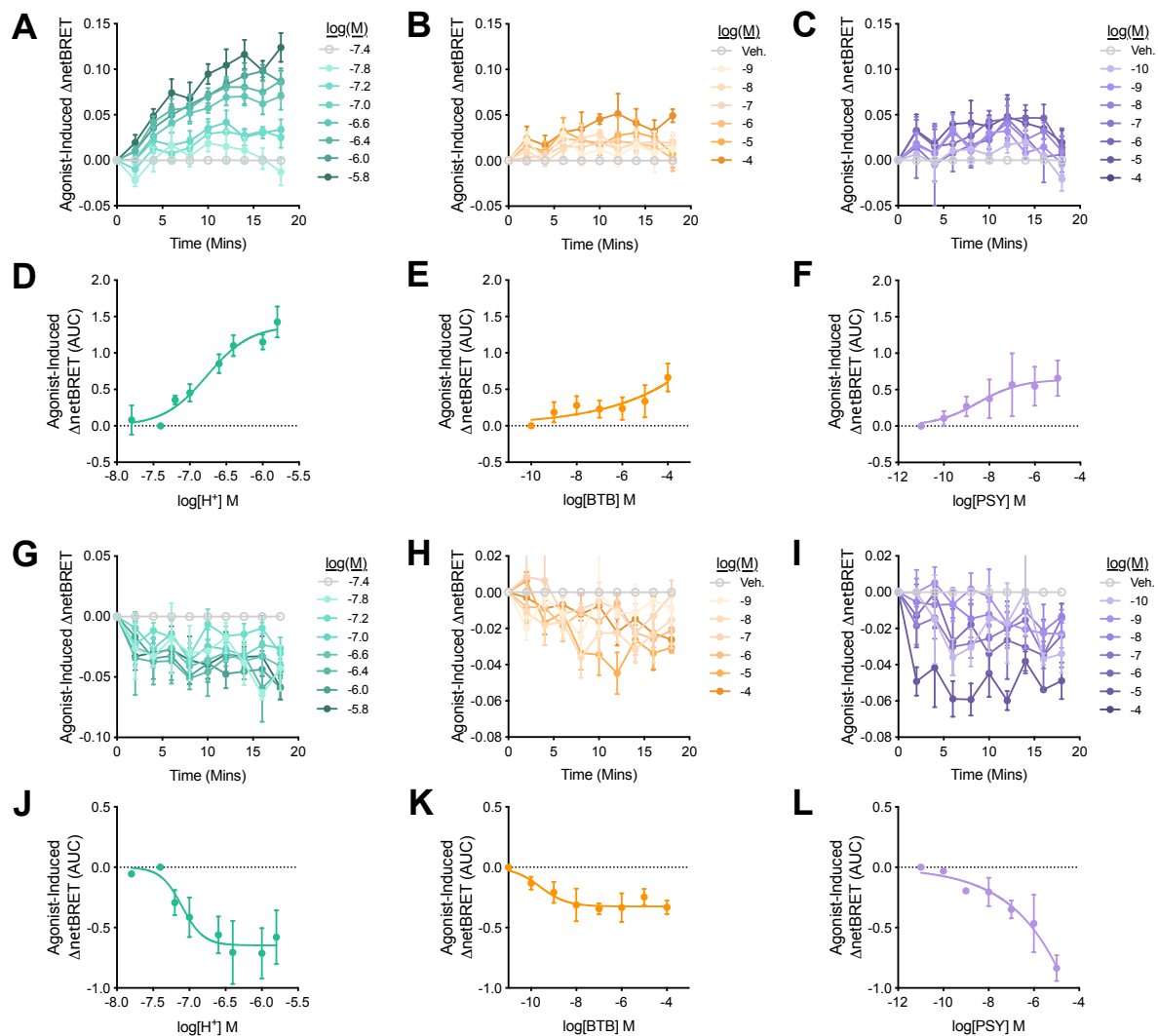


Figure 5.6. GPR65 internalises to early endosomes following activation. BRET between MmGPR65-RLuc8 and RIT-Venus (a protein resident at the plasma membrane) was monitored over time following stimulation of cells with (A) protons (H^+), (B) BTB or (C) psychosine (PSY), the decrease in BRET indicates GPR65 moves away from the plasma membrane following activation. (G-I) In separate experiments proximity of MmGPR65-RLuc8 to Rab5a-Venus, a marker of early endosomes was assessed. (D-F, J-L) The area under the curve (AUC) was quantified for kinetic data to explore the dependence of receptor internalisation on the concentration of agonist used to stimulate GPR65. BRET signals from experiments performed in the absence of Venus-tagged acceptors were subtracted from those achieved in the presence of RIT- or Rab5a-Venus. Data are mean $\pm$ SEM from 5 independent experiments performed in duplicate.

Having established that protons, BTB and psychosine all induce internalisation of MmGPR65 to early endosomes, the molecular mechanisms behind intracellular trafficking of the receptor were explored. GPCRs may be internalised by several routes (Marchese et al., 2003), and those responsible for MmGPR65 internalisation were examined by inhibition of endocytic machinery via genetic or pharmacological approaches, and studying the effect of this on ability of pH 6, 100 μ M BTB or 1 μ M psychosine to cause internalisation to early endosomes. First, the dependence on dynamin, a GTPase responsible for cleaving budded receptors from the plasma membrane, was explored through assessing BRET between MmGPR65-RLuc8 and the early endosomal marker Rab5a-Venus when wild-type or a dominant negative mutant dynamin (K44E), deficient in GTPase activity, were overexpressed. Compared to control cells expressing pcDNA3.1, DynaminK44E prevented internalisation of MmGPR65 in response to stimulation with all agonists (pH 6 AUC: pcDNA3.1, 1.31 ± 0.14 , Dyn-K44E, -0.32 ± 0.16 , $F = 35.69$, $p < 0.0001$; 100 μ M BTB AUC: pcDNA3.1, 0.49 ± 0.07 , Dyn-K44E, -0.27 ± 0.08 , $F = 46.42$, $p < 0.01$; 1 μ M PSY AUC: pcDNA3.1, 0.68 ± 0.14 , Dyn-K44E, -0.45 ± 0.12 , $F = 26.10$, $p < 0.001$, one-way ANOVA with Bonferroni post-hoc, mean $\pm$ SEM, $n = 5$, Figure 5.7A-F). Overexpression of wild-type dynamin did not affect agonist-induced internalisation (pH 6: 1.04 ± 0.13 , 100 μ M BTB: 0.60 ± 0.06 , 1 μ M PSY: 0.58 ± 0.10 , mean $\pm$ SEM, $n = 5$). Next, the role of clathrin, a protein responsible for budding of GPCRs due to be internalised from the plasma membrane, in GPR65 internalisation was assessed using the clathrin inhibitor PitStop2 (PS2). Pre-treatment of cells expressing MmGPR65-RLuc8 and Rab5a-Venus with 30 μ M PS2 reduced the ability of GPR65 agonists to coordinate internalisation of the receptor to early endosomes relative to cells pre-treated with a vehicle control (pH 6 AUC: DMSO, 1.48 ± 0.14 , PS2, 0.57 ± 0.09 , $p < 0.001$; 100 μ M BTB AUC: DMSO, 0.66 ± 0.06 , PS2, -0.29 ± 0.05 , $p < 0.0001$; 1 μ M PSY AUC: DMSO, 0.78 ± 0.08 , PS2, -0.07 ± 0.05 , $p < 0.0001$, unpaired t-test, mean $\pm$ SEM, $n = 5$, Figure 5.7G-L). Taken together these results indicate that internalisation of GPR65 following activation by protons, BTB or psychosine is dependent on both dynamin and clathrin. The observation that psychosine induces GPR65 internalisation in a clathrin-dependent manner is inconsistent with the inability of psychosine to coordinate recruitment of β -arrestins to the GPR65, given β -arrestins often serve a scaffold between GPCRs due to be internalised and clathrin molecules, further exploration of the mechanism of psychosine-induced internalisation is thus required.

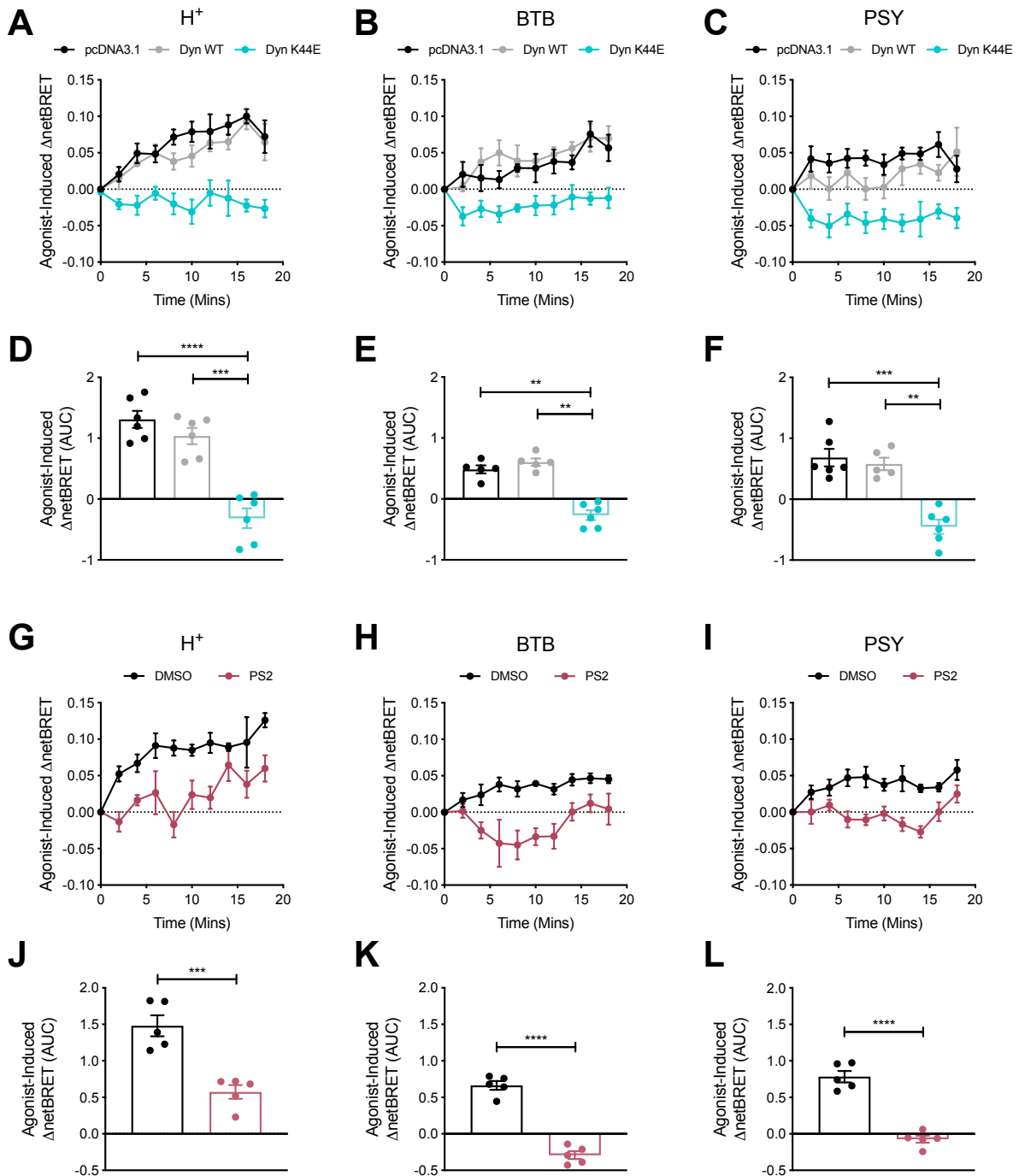


Figure 5.7. Assessing the molecular mechanisms behind agonist-induced internalisation of GPR65. BRET assays assessing internalisation of MmGPR65 in response to agonist stimulation were repeated following (A-C) overexpression of wild-type (Dyn WT) or a dominant negative form of Dynamin (Dyn K44E) or (G-I) pre-treatment with the clathrin inhibitor PitStop2 (PS2) or DMSO as a vehicle control. (D-F, J-L) The area under the curve (AUC) was quantified for kinetic data and used to assess the significance of inhibiting endocytic machinery, BRET signals from experiments performed in the absence of Rab5a-Venus were subtracted from those achieved in the presence of Rab5a-Venus to negate any non-specific effect of agonists altering luciferase activity. Data are mean $\pm$ SEM from 5 independent experiments performed in duplicate. ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$: (D-F) one-way ANOVA followed by Bonferroni post hoc analysis, (J-L) unpaired t-test.

Results thus far have identified differences in the ability of GPR65 agonists to engage intracellular signalling cascades, but similar trafficking patterns upon receptor activation. The

ability of different agonists of the same receptor to induce distinct signalling cascades, often referred to as functional selectivity or biased agonism, is of significant interest in general pharmacology, as such, methods to quantify the 'bias' of agonists for certain cascades over others have been described (Kenakin and Christopoulos, 2013). However, these methods rely on a similar range of agonist concentrations being assessed for pathways of interest. Consequently, it is not possible to extend these methods for quantifying bias at PS-GPCRs given the far narrower range of proton concentrations able to be assessed. As a rudimentary analysis of signalling bias, the potencies of PS-GPCRs agonists were compared instead of efficacy-based parameters, to assess how similar reported agonists of PS-GPCRs are in their ability to coordinate signalling events. The maximal signalling responses recorded in assays discussed in this Chapter (accumulation/inhibition of cAMP, Ca^{2+} mobilisation, ERK activation and β -arrestin recruitment) were thus normalised to those achieved with protons, i.e. protons were set as the reference ligand to assess how BTB- and psychosine-induced signalling compares. As previously described, this revealed that the signalling responses coordinated by GPR65 upon stimulation with protons or BTB are very similar, the only major difference is the inability for BTB to drive Ca^{2+} mobilisation, which protons were found to induce (H^+ , 1.00, BTB, -0.03 ± 0.03 , mean $\pm$ SEM, $n = 3 - 5$, Figure 5.8A). Psychosine, on the other hand elicits a very different signalling signature at GPR65, most evident when considering its effect on intracellular cAMP levels, which are reduced by psychosine, whereas protons lead to cAMP accumulation (H^+ , 1.00, PSY, -1.27 ± 0.95 , $p < 0.0001$, two-way ANOVA with Bonferroni post hoc, mean $\pm$ SEM, $n = 3 - 5$, Figure 5.8A), as well as the difference in potency to cause Ca^{2+} mobilisation (H^+ , 1.00, PSY, 2.71 ± 0.22 , $p < 0.05$, two-way ANOVA with Bonferroni post hoc, mean $\pm$ SEM, $n = 3 - 5$, Figure 5.8A) and inability of psychosine to coordinate recruitment of β -arrestin 1 (H^+ , 1.00, PSY, -0.06 ± 0.10 , $p < 0.05$, two-way ANOVA with Bonferroni post hoc, mean $\pm$ SEM, $n = 3 - 5$, Figure 5.8A). It also appears that psychosine fails to induce recruitment of β -arrestin 2 to GPR65 or activate ERK, however, the differences in these responses were not deemed statistically different to those achieved with protons, this is likely an artefact of the method used to assess significance, which is heavily affected by the extreme difference in the ability of protons and psychosine to modulate cAMP levels.

The fact that BTB mimics most of the signalling responses of protons at GPR65 highlights a potential use of this agonist as a surrogate for studying the physiological role of proton-induced GPR65 signalling, which is far more complicated to do for protons due to their potential to influence many different receptor and process, which might confound observations. Indeed, one of the main issues plaguing studies of the relevance of proton-induced signalling events is the vast number of receptors that are activated (Pattison et al., 2019) or modulated (Kapolka et al., 2021) by the extracellular concentration of protons. To further assess the utility of BTB

to initiate similar intracellular signalling cascades as protons at MmGPR65, the selectivity of BTB for GPR65 over other PS-GPCRs was assessed. Given most of the reported PS-GPCRs have been shown to modulate cAMP levels (Figure 4.2A-B), the cAMP assay was used to assess possible interactions of BTB with PS-GPCRs. It was found that BTB only altered the intracellular cAMP concentration of cells expressing MmGPR65 relative to the parental FlpIN CHO cells, an observation first deemed significant following stimulation with 1 μ M BTB (FlpIN, 4.47 ± 4.22 , MmGPR65, 50.90 ± 14.03 , $p < 0.05$, two-way ANOVA with Bonferroni post hoc, mean $\pm$ SEM, $n = 4$, Figure 5.8B), suggesting that BTB is selective for GPR65, at least among PS-GPCRs.

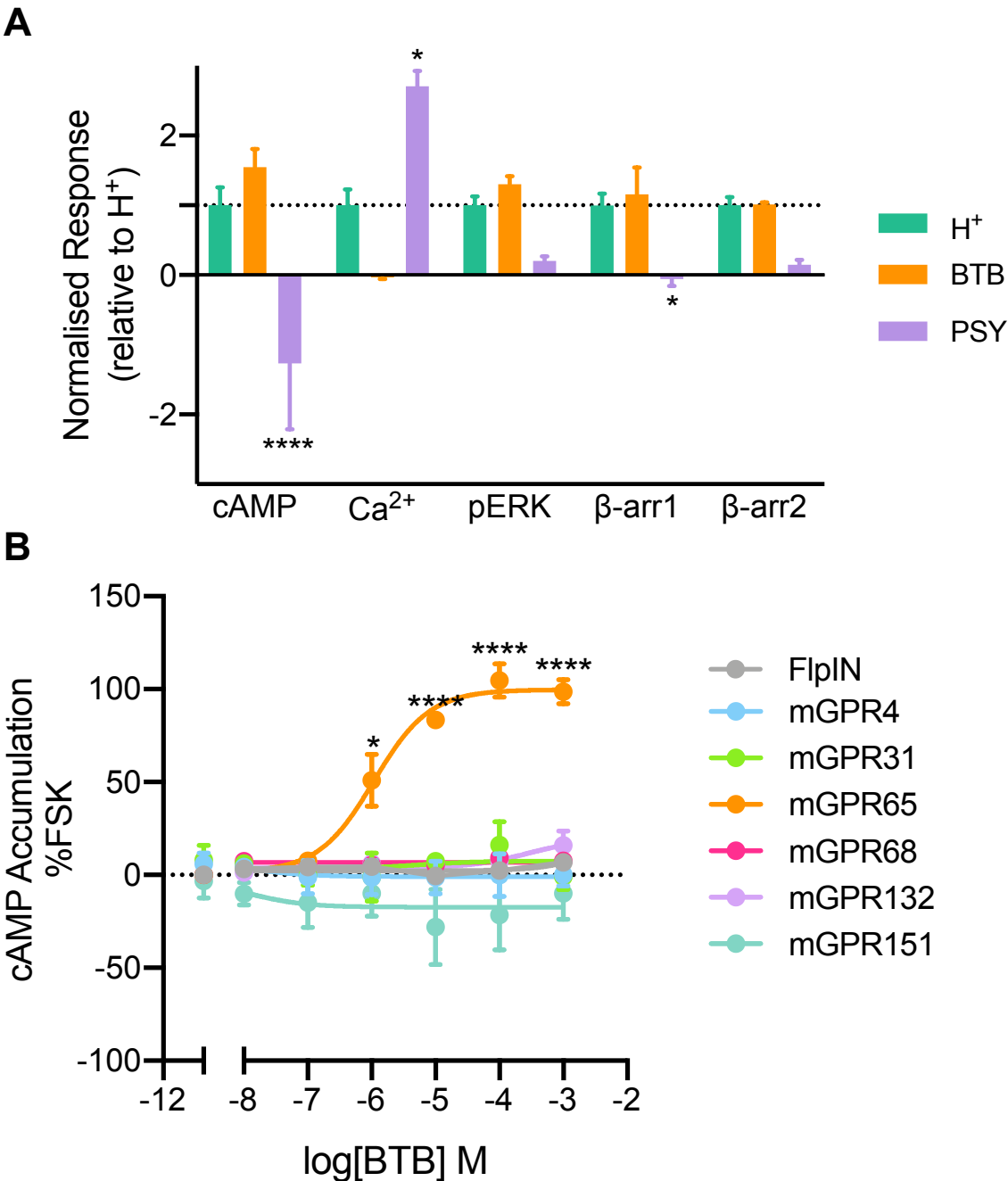


Figure 5.8. BTB and psychosine are specific for GPR65 and induce distinct intracellular signalling cascades. (A) To quantify signalling bias of GPR65 agonists, peak responses of assays were normalised to protons as the reference ligand (peak H⁺ responses were elicited by pH 5.8 for all pathways except cAMP for which proton-induced accumulation peaked at pH 6.6). The responses elicited by 1 mM BTB closely resembled those of protons, while 100 µM psychosine differed in its ability to activate intracellular cascades considerably. (B) Given BTB mimics proton-induced activation of GPR65 the ability of BTB to activate other PS-GPCRs was explored by assessing cAMP generation. Data are presented as mean ± SEM from 4 independent experiments performed in duplicate. * p < 0.05, **** p < 0.0001, two-way ANOVA followed by Bonferroni's post hoc analysis: (A) * denote differences between the annotated agonist to elicit a particular response in comparison to protons. (B) * denote differences between the response of the indicated cell line in relation to parental FlpIN CHO cells.

5.5. Discussion

Experiments discussed in this Chapter have systematically characterised GPR65 signalling in a consistent manner and uniform cellular background, extending previous reports of protons and other compounds capable of interacting with GPR65. Studies were further extended to investigate the potential of GPR65 agonists to induce internalisation of the receptor. Key findings include the observation of a distinct signalling signature elicited upon GPR65 activation with the lipid agonist psychosine compared to that achieved with protons. This highlights the importance of further consideration of PS-GPCRs as sensors of both extracellular proton concentration and lipids given the distinctness of signalling elicited by each agonist. Expanding studies of the synthetic agonist, BTB, have revealed that BTB is selective for GPR65 over other members of the PS-GPCR family and elicits signalling responses largely akin to those induced by protons. Such findings highlight the potential use of BTB as a more specific surrogate agonist for GPR65 in elucidating the role of GPR65 as a proton-sensor in physiological processes and diseases. Additionally, histidine residues in extracellular portions of GPR65 were shown to be crucial for all GPR65 agonists to evoke signalling responses. Protons, BTB and psychosine were also alike in their ability to induce internalisation of GPR65 to early endosomes upon receptor activation, a process shown to be mediated by clathrin and dynamin.

The only difference between proton-induced signalling cascades at GPR65 and the responses coordinated by BTB at the receptor in this study, is the inability of BTB to coordinate mobilisation of intracellular Ca²⁺. GPCR-mediated increases in intracellular Ca²⁺ concentration are predominantly achieved through the engagement of Gα_q subunits. Of the pathways explored in this study a Ca²⁺ response is the most likely to be coordinated upon activation of Gα_q, this suggests the difference between the abilities of protons and BTB to evoke such a response may lie in their ability to cause GPR65 engagement of Gα_q subunits. The ability of GPCRs to discriminate between the Gα's has been shown to be largely dictated by differences in the C-termini of G proteins (Okashah et al., 2019), additionally the ligand-induced structural

changes of GPCRs have been shown to be similar for receptors coupling to the same Ga: for example the extent of displacement of the sixth transmembrane domain is much greater for both Class A and Class B GPCRs that couple to Gas subunits than those which engage Gai (Glukhova et al., 2018). This suggests that the conformational change imposed on GPR65 by protons and BTB may explain their differing ability to interact and catalyse activation with Gαq subunits and evoke release of Ca²⁺ from internal stores. At the time of writing, structures of a single GPCR in complex with distinct agonists and Gα subunits are not available, although this would be a massive achievement for the field and provide rich insight to the structural basis of biased signalling. It would thus be extremely worthwhile to seek to solve the structure of GPR65 in its inactive form and upon activation with BTB and/or psychosine which would certainly provide valuable understanding on how the receptor coordinate downstream signalling. Resolving structures of GPCRs, like most membrane proteins, is extremely challenging though due to issues stabilising the proteins in the absence of the plasma membrane. This may prove even more ambitious for PS-GPCRs such as GPR65, given the ability of protons to activate the receptor by changing its conformation and the reliance on reagents that may have large impacts on local proton concentrations to purify membrane proteins for structural evaluation. An alternative means of first verifying if BTB and protons differ in their ability to engage Gαq, might be to employ BRET assays for monitoring engagement of specific Gα's (Masuho et al., 2015).

The lack of a GPR65 antagonist has made it impossible to directly attribute observed responses in signalling assays to GPR65. This has partly been addressed by using a CHO cell background, which was shown to be devoid of endogenous expression of known proton-sensitive receptors (Figure 4.1A-B). Additionally, the specificity of proton-, psychosine- or BTB-induced signalling being driven by GPR65 was assessed, where appropriate, by stimulation of the parental FlpIN CHO cell line with concentrations shown to induce substantial responses for the pathway in question for MmGPR65 cells. Inhibitors of certain effectors of GPCR signalling may also be employed in assays to assess the specificity of GPR65 signalling in assay readouts, particular those evoked by protons. Such tools may also help better understand the mechanisms by which intracellular cascades are evoked, for example ERK activation, which may be achieved a number of ways (Jain et al., 2018). Until an antagonist has been validated for GPR65, specificity of signalling events will always have to be attributed to GPR65 by these indirect means. Alternatively, an inhibitory pepducin could be developed for GPR65 for use in basic *in vitro* pharmacological studies; pepducins are short peptides that are lipidated allowing them to interact with GPCRs at their intracellular face, which can be utilised as a means of disrupting the ability of the receptors to signal, thus antagonising them

(Zhang et al., 2015). As pepducins can be designed using the primary sequence of receptors alone, this might represent the most productive way of achieving GPR65 antagonism in the short term (McKeown et al., 2014). Although a traditional pharmacological antagonist would be more useful for *in vivo* studies, or even eventually for clinical trials, due to easier administration and should be the focus of future research.

The search for traditional pharmacological antagonists relies on a good understanding of molecular interaction between receptors and known pharmacophores. The histidine mutants generated here may thus prove useful in such efforts: the importance of histidine residues in driving observed signalling phenomena is evident and suggests that occlusion of these residues might permit blockade of GPR65 activation by the agonists profiled here. Of course it remains to be assessed whether these histidine mutants also disrupt the ability of GPR65 agonists to engage other signalling pathways, as only cAMP modulation was studied here. As well as studying the effect of the histidine mutations in functional assays, the pharmacological workflow described in this chapter would benefit from being extended to assess the binding of psychosine and BTB to GPR65 as well, which may be achieved using fluorescently tagged versions of these agonists or traditional radioligand binding approaches (Stoddart et al., 2016). Given BTB can interact with GPR65, the molecular scaffolds of this compound could be used as starting points for iterative development of GPR65 antagonists, which have affinity but no efficacy at GPR65. Of course the same approach of modifying the structure of BTB as a means to develop a GPR65 antagonist, could also be used to refine the tuning of synthetic agonists of the receptor to improve efficacy. The hunt for novel pharmacological modulators of GPR65 would be considerably easier if structural insight on how the receptor is activated were available, however as discussed above obtaining structural data for GPR65 and other PS-GPCRs will likely even more difficult than is usual for GPCRs activated exclusively by complex ligands. Alternatively, a homology might be able to be made from GPCRs that have had their structures solved by looking for regions of homology across sequences, success has previously been achieved in identifying compounds capable of binding the melanin-concentrating hormone receptor 1 (MCH-R1, GPR24) using *in silico* docking performed on a homology model of the receptor (Cavasotto et al., 2008). If the structure of GPR65 can be modelled or solved, it would permit *in silico* drug discovery approaches to be applied to the receptor which would likely represent the best hope of identifying novel compounds which might modulate GPR65 activity and prove useful for future pharmacological profiling of this PS-GPCR. The power of *in silico* drug design approaches is evident from the recent unveiling of a platform capable of screening over 11 billion compounds against receptor structures (Sadybekov et al., 2021).

The experiments described in this Chapter highlight the complexities faced when studying proton-induced signalling. Although the majority of methods described here were indirectly shown to be specifically driven by GPR65 due to the lack of proton-induced signalling when assays were extended to parental FlpIN CHO cells, it was not always the case that protons did not seem to drive responses solely through GPR65. For example, during assessment of Ca^{2+} mobilisation with Fluo4, stimulation of FlpIN cells with pH 6 triggered a small increase in the signal, although this was smaller than for when MmGPR65 cells were stimulated (Figure 5.2F) it calls in to question the specificity of this and thus accuracy of the proton profiling. This may be addressed by studying other readouts of the same signalling pathway: in this case Ca^{2+} mobilisation from intracellular stores relies on the generation of inositol trisphosphate (IP_3) from PIP_2 at the plasma membrane, the generation of IP_3 or its metabolites, most commonly IP_1 has successfully been used as a readout of PS-GPCR engagement of $\text{G}\alpha_q$ signalling (Wang et al., 2004) and as such a similar approaches may provide more clarification here.

As well as consideration of the methodology for studying proton-induced signalling the interpretation of such data needs to be tailored to the underlying research aims, for example issues absolute or relative quantification of proton-induced signalling were considered in the discussion of Chapter 4. Additionally, in this Chapter an attempt to quantify signalling bias at GPR65 was made, although this could not be achieved as is commonly done for GPCRs capable of being activated by multiple traditional agonists. This is due to the intrinsic biochemical differences between protons and conventional compound agonists, which mean they cannot be compared across the same concentration range. Thus, comparisons of bias, may need to be weighted on potency, as described here, rather than efficacy, although potency is far less relevant as a pharmacological parameter when it comes to studying receptors *in vivo* or possibly in clinical trials on human patients, so this likely requires further thought.

Another example of where restrictions on the choice of experimental approach to study proton-induced signalling may limit pharmacological understanding of PS-GPCRs is the reliance on end-point assays taken at certain timepoints following receptor stimulation. It is well documented that GPCR signalling outputs heavily depend on the time points at which they are taken (Klein Herenbrink et al., 2016), hence *in vitro* pharmacological assays are more routinely performed using biosensors, which afford high temporal resolution of signalling outputs following receptor activation of living cells. Such techniques however usually rely on resonance energy transfer between two fluorophores (FRET) or a bioluminescent enzyme and fluorophore (BRET) as the readout of pathway activation (Kankanamge et al., 2019) which is well placed for studies of GPCRs activated by canonical peptides or synthetic compounds, but

may not be appropriate when using protons as an agonist due to the possibility that protons might directly alter the conformation of biosensors and confound results. It is far easier to limit confounding effects of protons having a direct effect on signalling readouts for end-point assays, i.e. by terminating reactions, lysing cells in a neutral buffer and quantifying the production or activation of signalling intermediaries. Extending studies to look at other timepoints would thus be an interesting avenue to explore, albeit a time-consuming process if it must be done using end-point methodologies and varying the stimulation length. The recent advent of single-fluorophore biosensors to study intracellular signalling might hold more promise for capturing the temporal aspects of proton-induced signalling as such sensors are generally less reliant on large conformational changes (Mehta et al., 2018), although protons may affect the maximal fluorescence limiting the dynamic range of such approaches. It would certainly be worth an assessment of the practicalities of using such tools to assess proton-induced signalling, but even if they were just employed to further study BTB- and psychosine-driven signalling that would be useful.

The idea of studying the temporal profiles of GPR65 signalling is all the more interesting given it has been shown that agonists of GPR65 can induce internalisation of the receptor. In recent years the internalisation of GPCRs has been shown to influence the signalling responses they can engender, which can be crucial in their ability to drive physiological processes and disease (Jimenez-Vargas et al., 2018; Yarwood et al., 2017). It is thus of interest to further study the relevance of the subcellular localisation of GPR65 in relation to its signalling outputs, this is all the more intriguing for GPR65 given the progressive acidification of the endomembrane network (Lafourcade et al., 2008), which would mean that the local concentration of protons may change with the intracellular location of the receptor. The role of receptor internalisation may first be studied by inclusion of the genetic and pharmacological tools shown here to disrupt GPR65 internalisation in signalling assays. It would also be useful to further characterise the intracellular trafficking of GPR65 following internalisation to early endosomes. Further spatial resolution of signalling events can be studied using biosensors targeted to specific subcellular locales (Halls and Canals, 2018), as discussed above these may not be applicable to studying proton-induced signalling, but would certainly be useful to further profile BTB and psychosine as GPR65 agonists. To summarise, this Chapter has shown that lipids and protons exert functional selectivity over GPR65 driven signalling, by comparing the abilities of reported agonists to engage various GPCR-driven signalling nodes in the same cellular background. A number of further lines of enquiry have been discussed mainly relating to addressing the relevance of the observed receptor internalisation to signalling responses.

6. A BTB09089-GPR65 signalling axis in inflammatory joint pain.

6.1. Authorship statement

Rebecca H. Rickman assisted with the acquisition and analysis of animal behavioural studies discussed in this chapter.

6.2. Chapter Summary

Inflammation is often accompanied by localised acidosis. The increased concentration of extracellular protons at the site of inflammation both activates and sensitises sensory neurones through various proton-sensitive receptors, which has been shown to contribute to the inflammatory pain, as well as acid-evoked nociception. Tenable links between the PS-GPCR, GPR65, and inflammatory pain have been made. However, the links between GPR65 and inflammatory pain are mostly based on less hypersensitivity of animals undergoing models of inflammation following GPR65 expression knockdown or knockout. Having established that the signalling signature of BTB at GPR65 is very similar to that engendered by engagement of the receptor by protons, BTB was used here to selectively activate GPR65 and assess its ability to drive inflammatory pain. The synovial fluid of rheumatoid arthritis patients is known to be acidic compared to that of healthy controls, and thus BTB was injected to the knees of mice to selectively interrogate the role of GPR65 in the pain associated with joint inflammation. Injection of BTB to the knee joint of mice resulted in robust inflammation that peaked within 24-hours and coincided with behavioural responses consistent with heightened nociception. Electrophysiology recordings of knee-innervating sensory neurones found a greater excitability of those cells which innervated the BTB-injected knee. Interestingly, stimulation of acutely cultured sensory neurones with BTB *in vitro* did not affect excitability upon electrophysiological characterisation, suggesting the involvement of other cell types resident in the knee joint. A role of fibroblast-like synoviocyte (FLS) cells in contributing to the development and pain associated with rheumatoid arthritis has been previously demonstrated. FLS were found to express GPR65 and following stimulation with BTB secreted pro-inflammatory cytokines which sensitised sensory neurones. The findings reported here reaffirm the importance of non-neuronal cells in the development of inflammatory joint pain and highlight a novel role of GPR65 as a mediator of cell-cell communication in the joint responsible for the manifestation of pain.

6.3. Introduction

Chronic inflammatory conditions such as rheumatoid arthritis and inflammatory bowel disease are associated with a localised increase in the concentration of extracellular protons (Andersson et al., 1999; Cummings and Nordby, 1966; Sasaki et al., 1997). Localised acidosis is known to both activate and sensitise sensory neurones (Steen et al., 1992). Thus far, the contributions of protons to nociception have mostly been attributed to ASIC mediated signalling (Ugawa et al., 2002), with ASIC3 having a particularly well-established role in both acid-induced and inflammatory pain in the periphery (Deval et al., 2008; Sluka et al., 2003). The involvement of PS-GPCRs in nociception is less clear, however their expression in nociceptors (Huang et al., 2007) and a number of genetic associations between PS-GPCRs and conditions associated with pain (Chen et al., 2009; de Vallière et al., 2015; Reinhold et al., 2015) make a strong case for the roles of PS-GPCRs in nociception, particularly peripheral sensitisation to be explored.

Among the PS-GPCRs, GPR65 expression is the most heavily upregulated by commonly used models of inflammatory pain (Chen et al., 2009), indeed work previously reported in this thesis has also confirmed that this is the case with the intraplantar carrageenan model (Figure 3.4A). Genome-wide association studies have also highlighted correlations between single nucleotide polymorphisms in *GPR65* and the inflammatory conditions chronic obstructive pulmonary disease, ankylosing spondylitis and ulcerative colitis (Hardin et al., 2014; Tcymbarevich et al., 2019a; Yuan et al., 2019). siRNA-mediated silencing of *Gpr65* has remedied the mechanical hypersensitivity observed in murine models of bone cancer pain when delivered intrathecally, suggesting GPR65 may contribute to nociception on the central and peripheral levels (Hang et al., 2012). However, knockdown of *Gpr65* following intraplantar injection of siRNA also reduced mechanical hypersensitivity following carrageenan- or CFA-induced inflammation or acid injection, strengthening the notion that GPR65 activation may drive peripheral sensitisation and enhanced nociception (Dai et al., 2017). There is also evidence to suggest that GPR65 can sensitise the nociceptive channel TRPV1 (Lin et al., 2017). Further to this, studies of knockout mice have shown that GPR65, TRPV1 and ASIC3 all contribute to the chronic stages of experimental rheumatoid arthritis, with GPR65^{-/-} mice also exhibiting less physical inflammation and mechanical hypersensitivity during acute stages of the model (Hsieh et al., 2017). As well as less pain being reported following induction of models of inflammatory pain when GPR65 is silenced or ablated, direct activation of GPR65 via intraplantar injection of the synthetic agonist BTB has been shown to result in mechanical hypersensitivity. RNA-interference mediated knockdown of GPR65 and inhibition of protein

kinase A were both shown to reduce the BTB-induced mechanical hypersensitivity (Dai et al., 2017; Dai et al., 2020) suggesting GPR65 mediated engagement of $G\alpha_s$ and the resulting activation of protein kinase A underlie the observed behaviour.

Effective therapeutic options for managing inflammatory pain are lacking and as such chronic pain represents a significant burden across the world. Many of the problems associated with currently approved analgesics stem from a lack of specificity: morphine and other opioid-based analgesics, remain the mainstay for treating pain despite a number of issues including unwanted side effects, such as respiratory depression and constipation. This may be due to the widespread expression of opioid receptors in the peripheral nervous system and their blanket mechanism of toning down neuronal activity (Figure 6.1A) (Stein, 2016). In contrast GPR65 expression among sensory neurones innervating the colon reveals a relatively restricted expression among peptidergic cells (Figure 6.1B), with high co-expression with the nociceptive markers such as TRPV1 (Figure 6.1C) and P2X3 (Figure 6.1D). This perhaps furthers the idea that GPR65 might contribute to nociceptive networks, but also provokes great promise for antagonism of the receptor as a novel way to achieve analgesia if it is shown to influence nociception, due to the discrete expression it enjoys among sensory neurones suggesting side-effects would be less of an issue, that said GPR65 is expressed by other cell types such as immune cells and fibroblasts resident in the knee joint (Chakrabarti et al., 2020a; Tcymbarevich et al., 2019b).

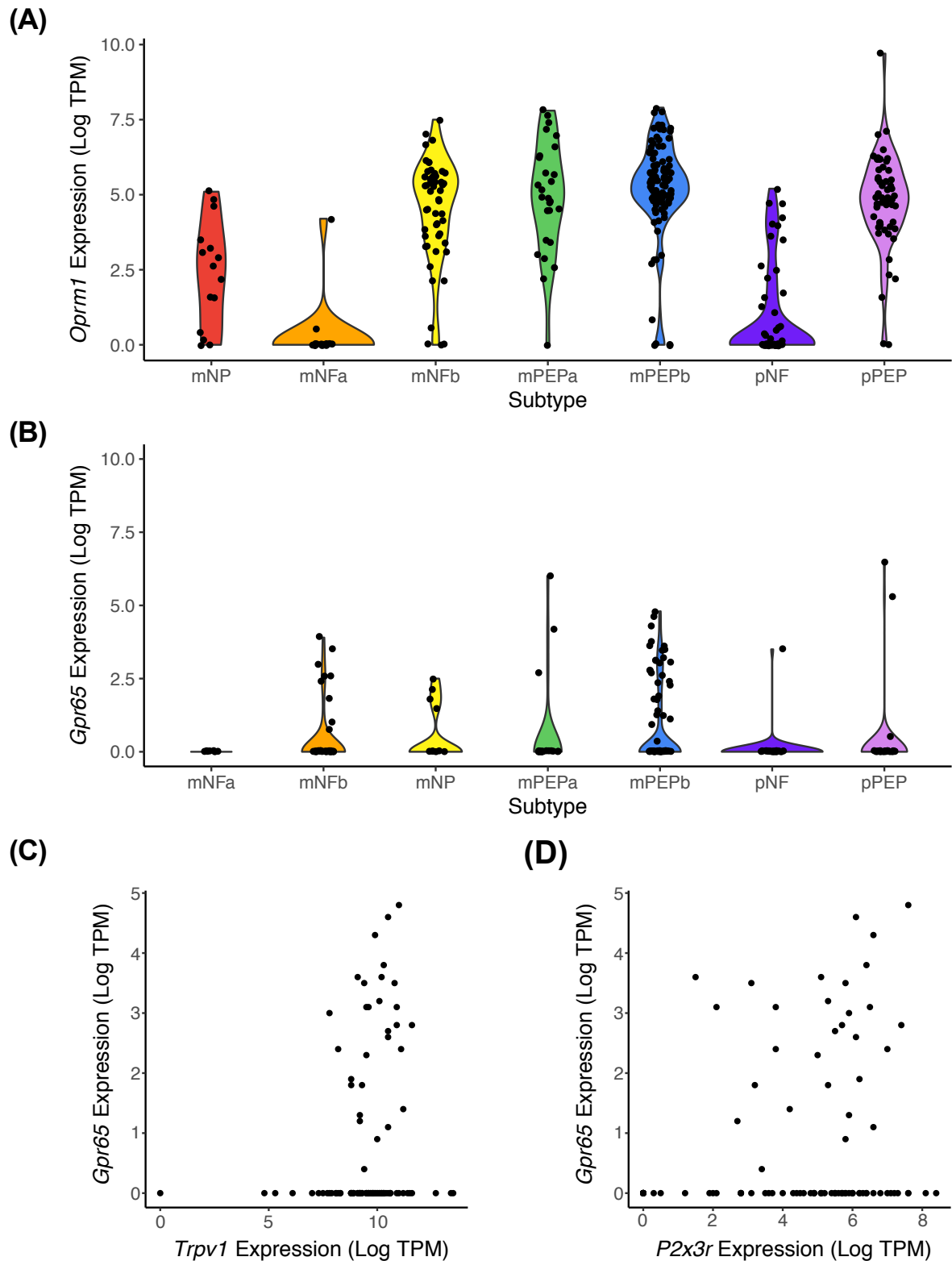


Figure 6.1. Single-cell RNA-seq of colonic sensory neurons in mouse. (A) The mu opioid receptor (*Oprm1*) is highly expressed across all classes of colonic sensory neurone, perhaps explaining its efficacy at reducing the pain of inflammatory bowel diseases, but also causing constipation. (B) By comparison expression of *Gpr65* is more restricted to the nociceptive peptidergic population (mPEPb), demonstrating strong co-expression with (C) *Trpv1* and (D) P2X3 (*P2x3r*) in this population. Each point represents data from a single colonic sensory neuron, expression values are presented as Transcripts-Per-Million (Log [TPM]), data previously published by Hockley et al. (2018).

Working on the basis that BTB has already been shown to induce mechanical hypersensitivity, the role of GPR65 in inflammatory pain was further characterised using BTB as a surrogate for selective activation of GPR65. Given reports of arthritic synovial fluid having a higher concentration of protons than that of healthy patients (Cummings and Nordby, 1966) and expression of GPR65 by multiple cell-types in the knee joint (Zeisel et al., 2018; Riemann et al., 2015) focus was set on the knee joint. BTB was injected into the knee joint before behavioural analysis of mice covering both spontaneous and evoked aspects of pain, the effects of BTB at the cellular level were also assessed through electrophysiology, qPCR and cytokine release.

6.4. Methods

See Materials and Methods chapter for detailed descriptions of mouse intra-articular injections (Section 2.3), assessment of mouse behaviour (Section 2.5), DRG dissociation and culture (Section 2.6), culture of FLS cells (Section 2.7), whole-cell patch clamp electrophysiology (Section 2.21), qPCR (Section 2.12 and 2.12.1), immunocytochemistry (Section 2.13.1) and assessment of inflammatory mediator production (Section 2.15).

6.5. Results

Given the many intricacies of pain previously discussed, the importance of a comprehensive behavioural battery of tests to assess the wellbeing of animals following administration of BTB was paramount. To this end, preliminary studies were performed with a better characterised model of inflammatory joint pain, unilateral injection of the immunostimulant Complete Freund's adjuvant (CFA) into the knee joint, which has previously been shown to reduce ethological behaviours, consistent with the spontaneous pain as well as induce sensory neurone hyperexcitability after 24-hours (Chakrabarti et al., 2018). A similar timescale was followed for these experiments (Fig 6.2A), 24-hours post-injection of CFA, significant swelling of the afflicted knee was observed (pre-CFA, 3.14 ± 0.44 mm, post-CFA, 4.69 ± 0.47 mm, $F = 29.18$, $p < 0.0001$, ANOVA with Bonferroni post-hoc, mean $\pm$ SD, $N = 6$, Figure 6.2B). No such swelling was observed for the un-injected contralateral knee (pre-CFA, 3.01 ± 0.27 mm, post-CFA, 3.11 ± 0.18 mm, mean $\pm$ SD, $N = 6$, Figure 6.2B). A common report of patients experiencing from chronic inflammatory pain is spurts of spontaneous pain, which have a negative impact on an individual's motivation to perform routine tasks (Walsh and McWilliams, 2014), this apathetic pain type behaviour can be assessed in mice by observing ethological behaviours, such as digging. Naturally inquisitive, mice will dig when placed in a novel

environment with deep burrowing substrate and decreases in this behaviour are thus akin to the lack of motivation caused by pain (Wodarski et al., 2016). The act of digging may also be painful during joint inflammation, so such an assay captures both central and peripheral aspects of nociception. Following induction of inflammation of the knee joint with CFA, mice spent less time digging (pre-CFA, 30.14 ± 21.08 s, post-CFA, 8.34 ± 10.62 s, $p < 0.05$, paired t-test, mean $\pm$ SD, $N = 6$, Figure 6.2C) which also presented as a decrease in the number of burrows produced during testing (pre-CFA, 3.33 ± 0.81 , post-CFA, 1.00 ± 0.63 , $p < 0.001$, paired t-test, mean $\pm$ SD, $N = 6$, Figure 6.2D). To ensure the decreases in digging behaviour could be attributed to apathy and/or the act being painful following inflammation rather than any incapacitation caused by the inflammation, digging assessments were followed by the rotarod test, which forces mice to walk and assesses motor coordination. No deficit in the ability of mice to stay on the rotarod was seen following CFA-induced inflammation (pre-CFA, 305.3 ± 75.29 s, post-CFA, 332.5 ± 44.35 s, mean $\pm$ SD, $N = 6$, Figure 6.2E), indicating that the decreased digging behaviour was likely driven by enhanced nociception resulting from the inflammation. As well as experiencing spontaneous pain, individuals with chronic inflammation experience hyperalgesia, whereby painful stimuli are experienced as more painful, this is best assessed in lab animals using evoked tests. To assess mechanical sensitivity of the knee joint, pressure was applied medially, the force at which mice demonstrated pain (limb withdrawal) was recorded with a force transducer and occurred after much lower stimulation following CFA-induced inflammation (pre-CFA, 319.1 ± 50.26 g, post-CFA, 149.1 ± 58.80 g, $F = 17.21$, $p < 0.001$, ANOVA with Bonferroni post-hoc, mean $\pm$ SD, $N = 6$, Figure 6.2F). Together these behavioural tests allowed an assessment of both spontaneous pain and mechanical hypersensitivity.

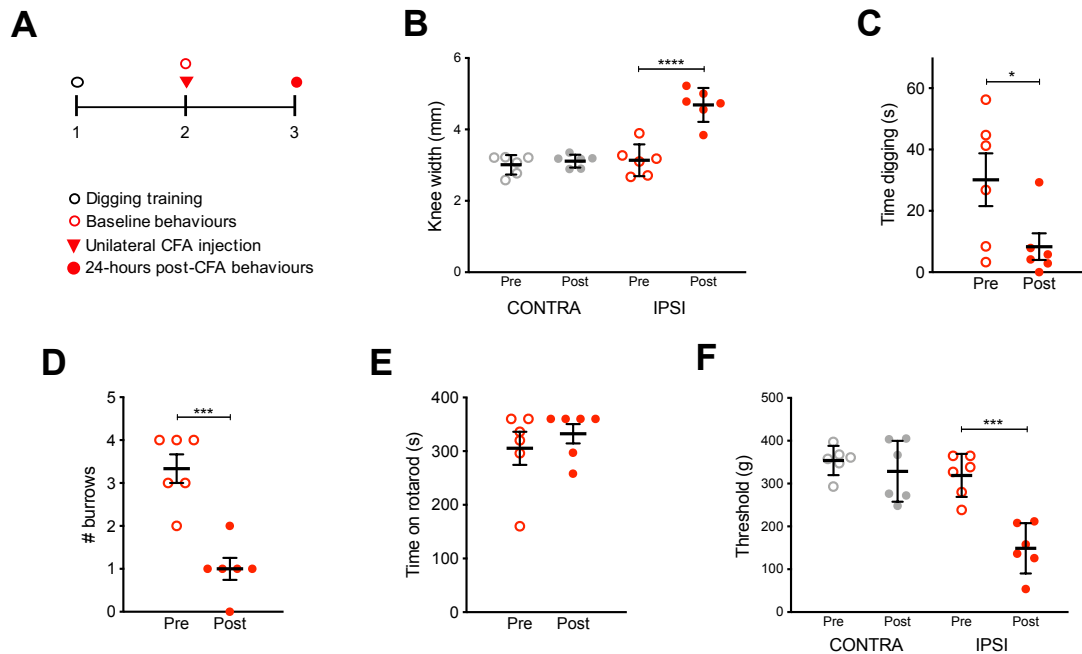


Figure 6.2. Unilateral injection of CFA into the knee joint of mice results in swelling and pain-like behavioural defects. (A) Overview of experimental timeline. (B) Swelling of the knee joint was quantified for both limbs with digital callipers before and 24-hours post-induction of inflammation with CFA. The digging behaviour of mice was also analysed before and after induction of inflammation, experimental readouts included (C) time spent digging and (D) number of burrows produced. (E) The time mice spent on an accelerating rotarod was recorded to assess motor coordination. (F) Mechanical hypersensitivity of the knee joint was also assessed. Data are mean $\pm$ SD. * $p < 0.05$, *** $p < 0.001$, **** $p < 0.0001$; (B-F) one-way ANOVA followed by Bonferroni post-hoc, * denote time difference (C,D) paired t-test. N = 6 animals.

Having validated a behavioural battery for profiling joint pain, this was used to study mice which received unilateral injections of 100 μ M BTB or DMSO as control (0.1% (v/v), the same as BTB). As this was the first instance of BTB being injected into the knee joint, behavioural characterisation was carried out for 7-days post-injection as per the timeline (Figure 6.3A). Injection of BTB resulted in robust swelling of the knee joint, which peaked at 24-hours (Ipsi: pre, 2.96 ± 0.20 mm, 24-hours, 3.60 ± 0.20 mm, $p < 0.001$, two-way ANOVA with Bonferroni post-hoc, Figure 6.3B), persisted at a similar level 48-hours after injection (Ipsi: 48-hours, 3.61 ± 0.19 mm, $p < 0.001$, two-way ANOVA with Bonferroni post-hoc, Figure 6.3B), before beginning to subside and resolving 5 days post-injection. A decrease in the time mice spent digging (% baseline: 24-hours, DMSO, $93.79 \pm 52.05\%$, BTB, $33.54 \pm 33.44\%$, $p < 0.05$; 48-hours, DMSO, $86.07 \pm 49.60\%$, BTB, $37.83 \pm 35.79\%$, $p < 0.05$, two-way ANOVA with Bonferroni post-hoc, mean $\pm$ SD, N = 8, Figure 6.3C) and number of burrows produced (24-hours: DMSO, 3.38 ± 0.52 , BTB, 1.88 ± 1.13 , $p < 0.05$; 48-hours: DMSO, 3.88 ± 0.99 , BTB, 2.13 ± 0.84 , $p < 0.01$, two-way ANOVA with Bonferroni post-hoc, Figure 6.3D) was also observed during early timepoints, correlating with the observed physical inflammation. Mice injected with BTB demonstrated similar abilities to negotiate the rotarod as those that received DMSO injections (Figure 6.3E), suggesting the observed decrease in digging behaviour was likely caused by inflammation-associated nociception, rather than any gross locomotion

impairment. A lower threshold for mechanical pressure was reported for the BTB-injected knee 24-hours post injection (Ipsilateral 24-hours: contra., 282.06 ± 77.32 g, ipsi., 170.59 ± 57.35 g, $p < 0.01$, two-way ANOVA with Bonferroni post-hoc, mean $\pm$ SD, $N = 8$, Figure 6.3F), but was not dissimilar to that of the contralateral side by 48-hours. Taken together, these results demonstrate that administration of BTB stimulates an inflammatory response, associated with an increase in pain-like behaviours and thus implicate GPR65 activation as sufficient to drive inflammation and ensue enhanced nociception.

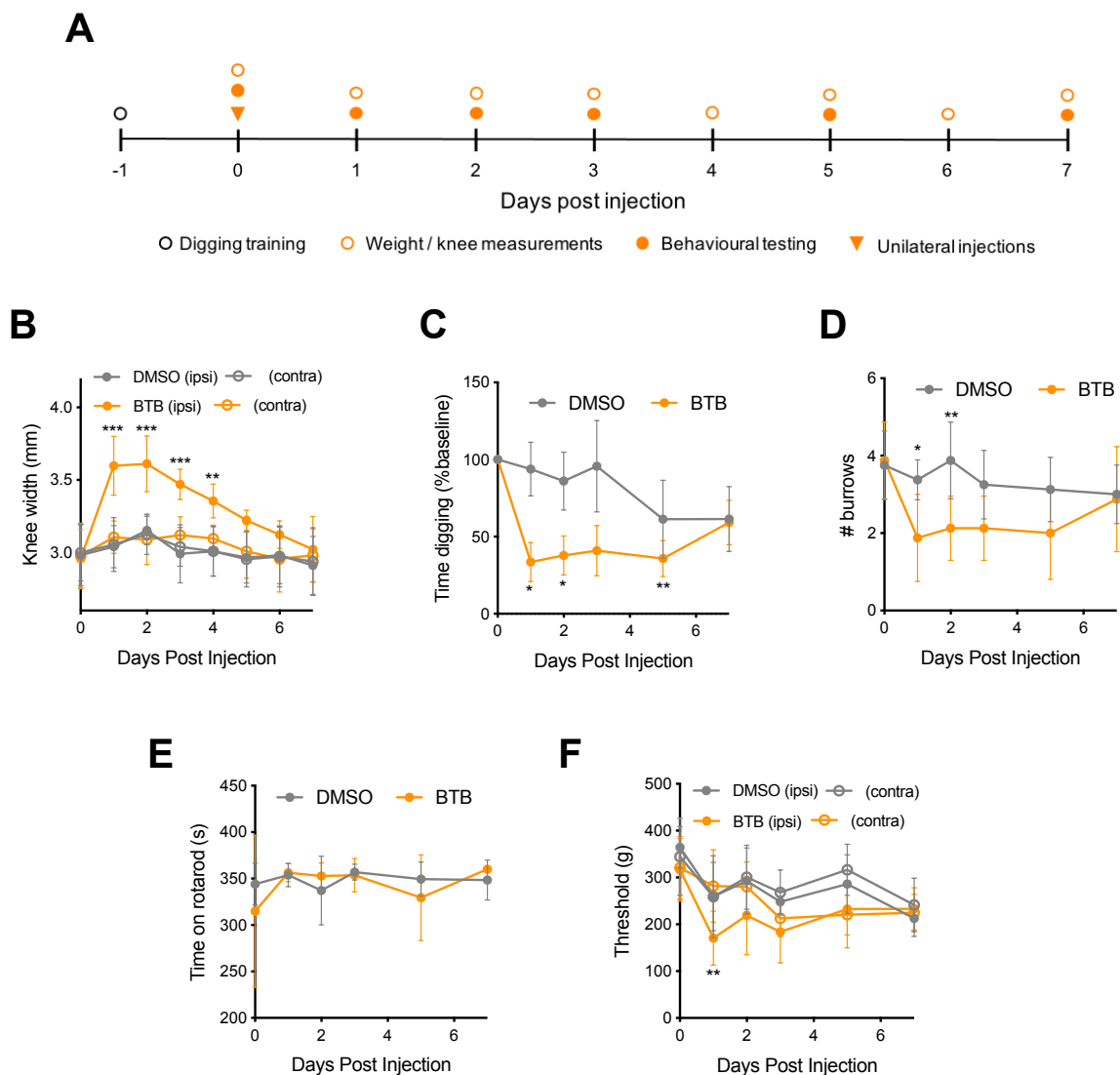


Figure 6.3. Unilateral injection of BTB into the knee joint of mice evokes swelling and pain-like behavioural deficits. (A) Timeline overview of experiments to assess mouse behaviour (B) Knee joint diameters were measured to monitor physical inflammation. (C) The time mice spent digging and (D) number of burrows produced were measured to assess spontaneous pain. (E) Motor coordination was assessed using a rotarod. (F) A digital force transducer was used to assess mechanical hypersensitivity of each knee joint. Data are mean $\pm$ SD. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, two-way ANOVA followed by Bonferroni post-hoc; (B,F) * denote difference between contra. and ipsi. for indicated time point; (C,D) * denote difference between DMSO and BTB treatment for indicated time point. $N = 8$ animals per group.

Inflammatory pain is usually a consequence of the sensitisation and thus higher excitability of sensory neurones. Therefore, to better understand the molecular basis of the observed pain-like behaviours following injection of BTB into the knee joint, electrophysiology was used to study the effect of BTB on sensory neurones. Using the timepoint shown to correspond with the greatest pain behaviours, 24-hours, naïve DRG were incubated with either DMSO or 100 μ M BTB overnight, to model exposure of sensory neurones in the knee joint to BTB in an *in vitro* setting (Figure 6.4A). Neurones incubated with BTB or DMSO showed no difference in their resting membrane potential compared to those cultured in standard medium (Control, -44.22 ± 0.94 mV, DMSO, -46.38 ± 1.04 mV, BTB, -44.75 ± 0.94 mV, mean $\pm$ SEM, $n = 23 - 24$, $N = 4$, Figure 6.4B). The rheobase (current needed to evoke action potential discharge) of neurones from each group was also comparable (Control, 345.2 ± 51.13 pA, DMSO, 263.3 ± 52.33 pA, BTB, 316.3 ± 66.08 pA, mean $\pm$ SEM, $n = 23 - 24$, $N = 4$, Figure 6.4C), and similarly overnight incubation with DMSO or BTB had no effect on the number of action potentials evoked by stimulation of cells with a suprathreshold ($2 \times$ rheobase) current injection for 500 ms (Control, 1.8 ± 0.70 , DMSO, 3.19 ± 0.49 , BTB, 2.92 ± 0.58 , mean $\pm$ SEM, $n = 23 - 24$, $N = 4$, Figure 6.4D). The neurones recorded from did not differ in any other intrinsic or active parameter measured for any condition, as summarised in Table 6.1.

	Control Medium ($n = 23$)	DMSO ($n = 24$)	BTB ($n = 24$)
Resting membrane potential (mV)	-44.22 ± 0.94	-46.38 ± 1.04	-44.75 ± 0.94
Capacitance (pF)	41.22 ± 3.38	36.63 ± 4.60	40.89 ± 5.13
Rheobase (pA)	345.2 ± 51.13	263.3 ± 52.33	316.3 ± 66.08
Action potential amplitude (mV)	76.91 ± 5.36	83.07 ± 3.39	78.78 ± 3.87
Half peak duration (ms)	2.11 ± 0.28	2.64 ± 0.27	2.35 ± 0.26
Afterhyperpolarization amplitude (mV)	15.89 ± 0.93	14.68 ± 0.96	15.92 ± 0.83
Afterhyperpolarization duration (ms)	16.84 ± 2.66	16.68 ± 1.80	15.09 ± 2.3
# Action potentials at suprathreshold	1.8 ± 0.70	3.19 ± 0.49	2.92 ± 0.58

Table 6.1 Intrinsic and active properties of dorsal root ganglion neurones cultured overnight in either control medium, or medium containing with 100 μ M BTB or 0.1% (v/v) DMSO. Data are mean $\pm$ SEM. $n = 23$ cells (Control Medium), 24 cells (DMSO), 40 cells (BTB) from $N = 4$ independent cultures.

To examine the effect of overnight incubation of sensory neurones with DMSO or BTB on the currents mediated by voltage-gated ion channels, current-voltage curves were produced. Macroscopic voltage-gated inward currents did not differ in any parameter measured for any treatment group (V_{half} : control, -38.52 ± 3.48 mV, DMSO, -40.78 ± 1.76 mV, BTB, -39.12 ± 0.93 mV; $I_{max} / I_{max \text{ control}}$: control, -1.00 , DMSO, -1.06 ± 0.16 , BTB, -1.09 ± 0.20 , mean $\pm$ SEM,

n = 23 – 24, N = 4, Figures 6.4E-F). Similarly, no differences were observed in the magnitude of voltage-gated outward currents normalised to that of the control condition (Control: 1.00, DMSO, 1.056 ± 0.30 , BTB, 0.79 ± 0.41 , mean $\pm$ SEM, n = 23 – 24, N = 4, Figure 6.4G-H). Hyperexcitability of sensory neurones usually underpins pain-like behaviours, however these results suggest that the direct action of BTB on sensory neurones fails to produce any change at the cellular level. However, there was no selection over which DRG subpopulations were recorded from during these experiments, so the possible dilution of any BTB-induced changes in electrophysiological properties, brought about by patching more cells that do not express *Gpr65* than do may also explain these results.

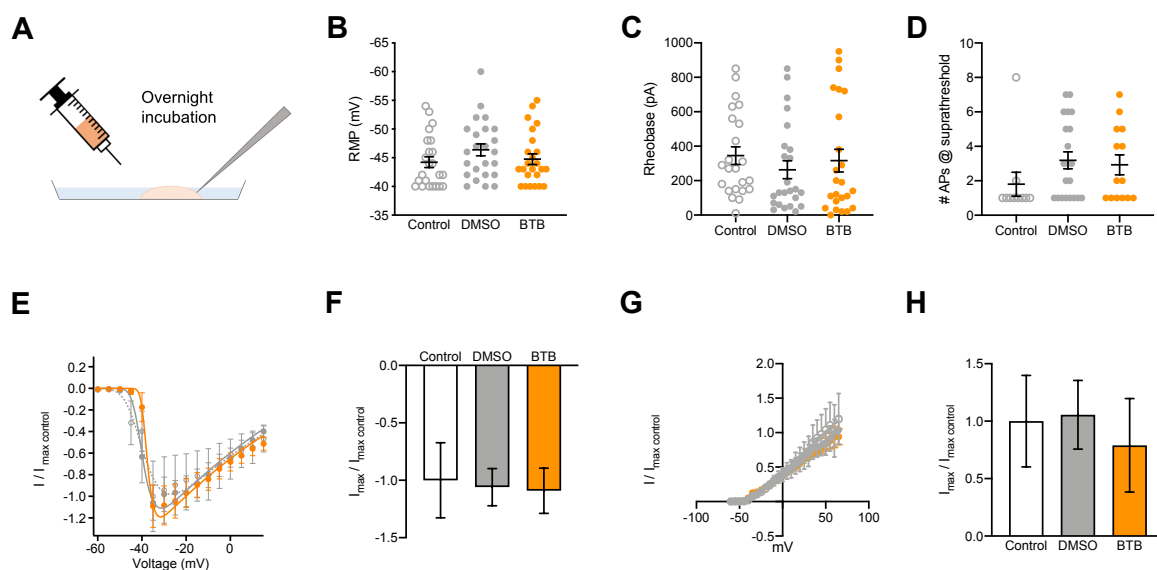


Figure 6.4. Overnight stimulation of naïve dissociated DRG sensory neurones with BTB does not affect neuronal excitability or voltage-gated currents (A) Graphical summary of experimental set up. Electrophysiology was used to assess the (B) resting membrane potential, (C) rheobase and (D) hyperexcitability of sensory neurones following overnight stimulations. Voltage-clamp recordings were taken to assess whether overnight stimulation affected macroscopic voltage-gated (E-F) outward and inward (G-H) currents. Data are mean $\pm$ SEM. n = 23 cells (Control Medium), 24 cells (DMSO), 40 cells (BTB) from N = 4 independent cultures.

As mentioned, the lack of an effect of BTB on naïve cultures of DRG sensory neurones might be explained by the well document heterogeneity of DRG neurones and relatively low expression of GPR65 (Zeisel et al., 2018). Thus to better understand the pain driven by BTB-injection, electrophysiological characterisation of DRG specifically innervating the knee joint was performed (Figure 6.5A). Given the most prominent pain behaviours were observed 24-hours post injection these studies focussed on that time point. 5-7 days before BTB injection, the knees were injected with the retrograde tracer FB to allow identification of knee-innervating neurones following acute dissociation and culture. Pre- and post-BTB behavioural states were assessed for this cohort to confirm a similar pain phenotype to that previously described, results were akin to those obtained in earlier studies: BTB-induced swelling of the injected

knee (pre-BTB, 2.76 ± 0.14 mm, post-BTB, 3.74 ± 0.31 mm, $F = 32.79$, $p < 0.0001$, ANOVA with Bonferroni post-hoc, mean $\pm$ SD, $N = 5$, Figure 6.5B) which was concomitant with reduced digging behaviour (time digging: pre-BTB, 46.06 ± 10.45 s, post-BTB, 14.05 ± 4.93 s, $p < 0.01$, paired t-test, mean $\pm$ SD, $N = 5$, Figure 6.5C; number of burrows: pre-BTB, 5.60 ± 1.14 , post-BTB, 2.40 ± 0.55 , $p < 0.01$, paired t-test, Figure 6.5D) and a lower threshold for mechanical stimuli (ipsi.: pre-BTB, 368.9 ± 48.29 g, post-BTB, 114.3 ± 34.77 g, $F = 26.45$, $p < 0.0001$, ANOVA with Bonferroni post-hoc, mean $\pm$ SD, $N = 5$, Figure 6.5E). Additionally the weight-bearing of this cohort of animals was assessed, while mice placed less weight through the hind paw of their injected limb (Ipsilateral: pre-BTB, $47.00 \pm 2.14\%$, post-BTB, $44.94 \pm 2.77\%$, mean $\pm$ SD, $N = 5$, Figure 6.5F) this was not significant. A difference assessment of dynamic-weight bearing did highlight though that mice spent more time immobile following injection of BTB (pre-BTB, $27.72 \pm 10.23\%$, post-BTB, $40.31 \pm 4.59\%$, $p < 0.05$, paired t-test, mean $\pm$ SD, $N = 5$, Figure 6.5G), which aligns with the observed diminished digging and supports the notion of inflammation-induced apathy, which manifests in less motivation to explore the open field arena. During studies performed in the open arena it was also noticed that mice displayed several overt visual signs of pain including orbital tightening, bulging of the nose and a hunched posture (Figure 6.5H). Thus, it might be worthwhile scoring visual signs of pain and distress of animals in future studies as an additional non-evoked assessment of animal wellbeing, which has been well documented as the grimace scale (Langford et al., 2010), but equipment was not available to make a full assessment of this.

While the resting membrane potential of neurones isolated from the injected, ipsilateral, side did not differ from the contralateral side (contra., -46.15 ± 0.91 mV, ipsi., -48.76 ± 1.19 mV, mean $\pm$ SEM, $n = 33 - 34$, $N = 5$, Figure 6.5I), ipsilateral neurones exhibited higher excitability, evident from the lower rheobase (contra., 562.7 ± 47.62 pA, ipsi., 320.9 ± 44.00 mV, $p < 0.001$, Mann Whitney test, mean $\pm$ SEM, $n = 33 - 34$, $N = 5$, Figure 6.5J). However, ipsilateral neurones had a similar propensity to fire multiple action potentials when stimulated with a suprathreshold (contra., 1.38 ± 0.26 , ipsi., 2.00 ± 0.48 , mean $\pm$ SEM, $n = 33 - 34$, $N = 5$, Figure 6.5K). As detailed in Table 6.2 the neurones recorded from did not differ any other active or intrinsic property.

	Contra. (n = 33)	Ipsi. (n = 34)
Resting membrane potential (mV)	-46.15 ± 0.91	-48.76 ± 1.19
Capacitance (pF)	54.53 ± 5.23	42.83 ± 5.50
Rheobase (pA)	$562.7 \pm 47.62^{***}$	$320.9 \pm 44.00^{***}$
Action potential amplitude (mV)	81.29 ± 4.16	81.13 ± 4.53

Half peak duration (ms)	2.41 ± 0.50	2.23 ± 0.23
Afterhyperpolarization amplitude (mV)	17.51 ± 0.62	17.43 ± 0.62
Afterhyperpolarization duration (ms)	10.40 ± 1.29	10.84 ± 1.85
# Action potentials at suprathreshold	1.38 ± 0.26	2.00 ± 0.48

Table 6.2 Intrinsic and active properties of knee innervating sensory neurones from the BTB-injected side (Ipsi.) and contralateral (Contra.) side. Data are mean ± SEM. *** $p < 0.001$, Mann Whitney test. $n = 33$ cells (Contra.), 34 cells (Ipsi.) from $N = 5$ animals.

To explore the observed differences further, voltage-sensitive currents were interrogated in voltage-clamp mode. A decrease in the half-maximal activation potential of macroscopic inward currents was observed for ipsilateral neurones, compared to those of the un-injected side (contra., -34.91 ± 2.94 mV, ipsi., -46.54 ± 2.66 mV, $p < 0.05$, unpaired t-test, mean ± SEM, $n = 33 - 34$, $N = 5$, Figure 6.5L), however the peak inward currents (contra., -1.00 , ipsi., -1.28 ± 0.27 , mean ± SEM, $n = 33 - 34$, $N = 5$, Figure 6.5M) elicited in neurones from each side did not differ. Similarly, the normalised magnitude of voltage-gated outward currents was unaffected by BTB-induced inflammation (contra., 1.00 , ipsi., 1.28 ± 0.30 , mean ± SEM, $n = 33 - 34$, $N = 5$, Figure 6.5N-O).

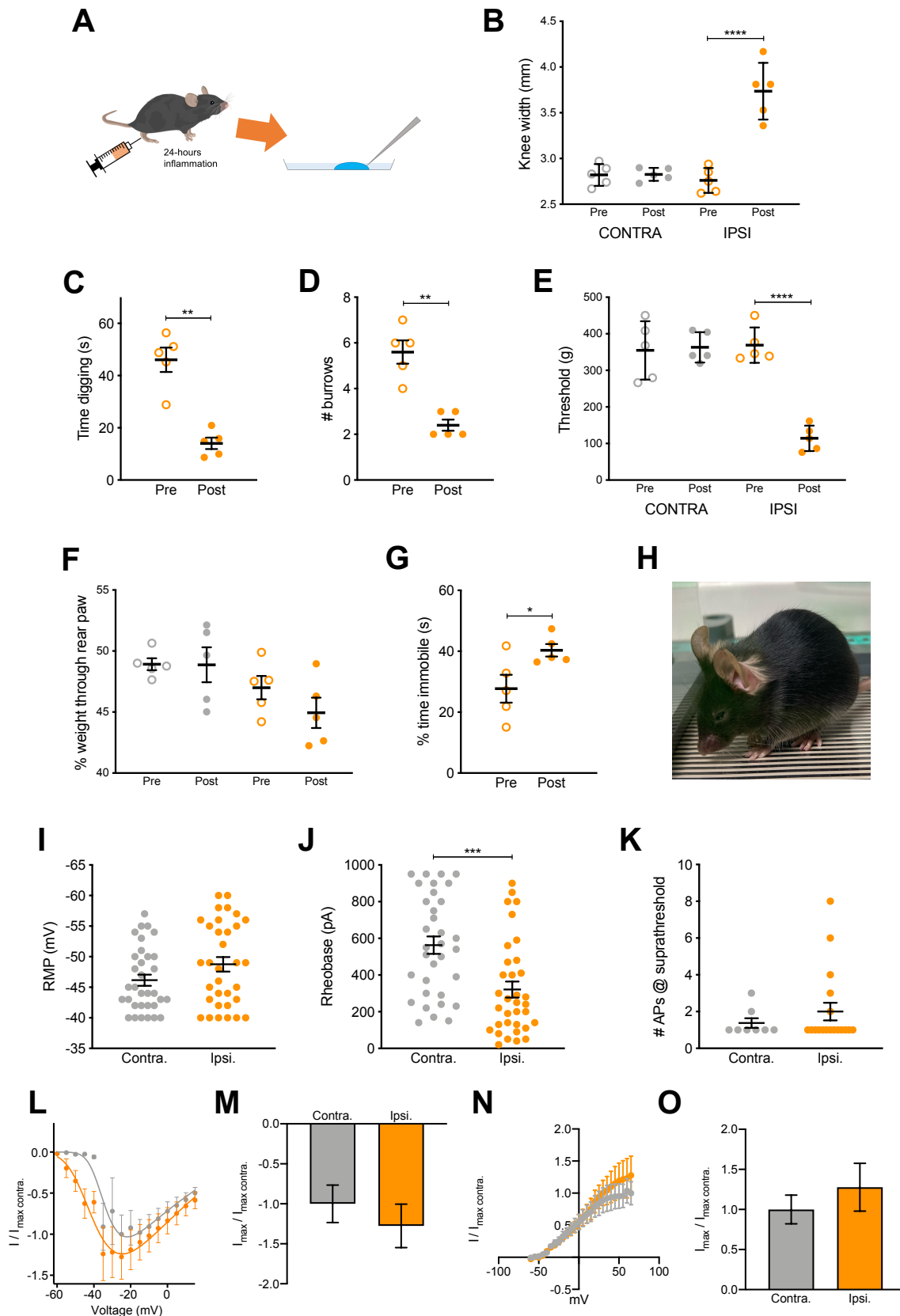


Figure 6.5. Intra-articular injection of BTB increases the excitability of sensory neurones innervating the knee joint 24-hours post-injection. (A) Graphical overview of experimental design. (B) Knees injected with BTB showed swelling 24-hours post injection which corresponded with animals spending (C) less time digging, (D) less burrows being dug and (E) higher sensitivity to mechanical pressure. (F) The dynamic weight bearing of mice was also assessed, during which animals spent (G) more time immobile and displayed overt signs of discomfort (H) following BTB-injection. Electrophysiology was performed on sensory neurones innervating the injected (ipsi.) and non-injected (contra.) knee joints: the (I) resting membrane potential, (J) rheobase and (K) ability to fire multiple action potentials was assessed in current clamp. Voltage clamp recordings were also made to study voltage-gated ion channels that dictate (L-M) inward and (N-O) outward currents. Data are presented as mean $\pm$ SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, (B, E) one-way ANOVA followed by Bonferroni post-hoc, (C, D, G) paired t-test, (J) Mann Whitney test. $n = 33$ cells (Contra.), 34 cells (Ipsi.) from $N = 5$ animals.

Results thus far have shown that intra-articular injection of BTB results in prominent inflammation which is associated with pain-like behaviour. Profiling of the sensory neurones which project to the site of inflammation revealed an increase in excitability, which may offer some explanation for the observed pain, however, stimulation of naïve sensory neurones with BTB *in vitro* failed to recapitulate these changes in neuronal excitability. The lack of an effect of BTB on dissociated naïve neurones might be explained by the differences in dissociated neurones compared to those intact *in vivo*, as the coordination of BTB-driven excitability may arise at the nerve terminal, i.e. nerve terminals may have particularly high GPR65 expression compared to that at the cell body. It may also be the case that the specific peptidergic population expressing *Gpr65* were underrepresented or diluted by recordings made from cells of other populations, as no selection over the populations recorded from was applied. An alternative explanation may lie in the relatively restricted expression of GPR65, which might be further diluted when studying the larger population of naïve, unlabelled lumbar DRG. Previous work has shown that fibroblast-like synoviocytes (FLS), which reside in the knee joint, may become activated during inflammation triggering the release of additional soluble mediators that can influence sensory neurone excitability, these cells have also been shown to express PS-GPCRs (Chakrabarti et al., 2020a). Therefore, the possibility that FLS cells might also contribute to BTB-driven inflammation and pain in the knee joint was explored.

Cells which grew from dissected mouse patellae were characterised after three passages, due to previous reports that by this stage FLS dominated cultures over other immune cells. Indeed, expression of the FLS markers *Cdh-11* and *Cd-248* was detected by immunostaining after passage 3 (Figure 6.6A-B), thus confirming cells were FLS in phenotype. qPCR was employed to further characterise the expression profile of FLS and in agreement with immunostaining high expression of the FLS markers *Cdh-11* and *Cd-248* was observed. The endothelial marker *Cd-31* could not be detected and only one of the three biological samples contained transcripts of the macrophage marker *Cd-68* at a very low level, indicating cultures were highly enriched for FLS. Expression of the six PS-GPCRs was also assessed, FLS were found to express, ordered from highest expressed to lowest, *Gpr65*, *Gpr4*, *Gpr31* and *Gpr132*, whereas *Gpr68* and *Gpr151* transcripts could not be detected (Figure 6.6C). Given that GPR65 expression was highest among the PS-GPCRs by FLS, it was hypothesised that BTB could activate FLS via GPR65 to induce the secretion of factors which may sensitise sensory neurones driving the pain observed. To address this, P3 FLS were stimulated with either 100 μ M BTB or an equivalent concentration of DMSO overnight and the following day media was collected. Cultures of naïve, unlabelled sensory neurones were prepared and incubated overnight with the conditioned media taken from stimulated FLS (Figure 6.6D). Electrophysiology experiments performed on neurones incubated with conditioned media

revealed no change in the resting membrane potential between those incubated with the media from FLS stimulated with BTB or DMSO compared to control media from unstimulated cells (Control, -51.07 ± 1.43 mV, DMSO, -46.78 ± 1.36 mV, BTB, -46.12 ± 1.42 mV, mean $\pm$ SEM, $n = 15 - 19$, $N = 4$, Figure 6.6E). However, as observed in neurones innervating BTB injected knees, the rheobase of cells cultured in conditioned media from FLS stimulated with BTB was lower than that of neurones cultured in control of DMSO conditioned media (Control, 464.7 ± 57.82 pA, DMSO, 412.6 ± 59.13 pA, BTB, 213.7 ± 47.35 pA, $F = 5.844$, $p < 0.01$, Kruskal-Wallis test with Dunn's post-hoc, mean $\pm$ SEM, $n = 15 - 19$, $N = 4$, Figure 6.6F). The number of action potentials discharged following stimulation of cells with a suprathreshold did not differ between conditions (Control, 1.00 ± 0.00 , DMSO, 1.00 ± 0.00 mV, BTB, 2.88 ± 1.06 , mean $\pm$ SEM, $n = 15 - 19$, $N = 4$, Figure 6.6G).

	Control (n = 15)	DMSO (n = 19)	BTB (n = 19)
Resting membrane potential (mV)	-51.07 ± 1.43	-46.78 ± 1.36	-46.12 ± 1.42
Capacitance (pF)	52.53 ± 7.91	41.04 ± 6.17	46.79 ± 7.15
Rheobase (pA)	464.7 ± 57.82	412.6 ± 59.13	$213.7 \pm 47.35^{**}, ^\wedge$
Action potential amplitude (mV)	95.11 ± 4.54	82.29 ± 5.08	88.15 ± 5.27
Half peak duration (ms)	1.48 ± 0.22	1.84 ± 0.41	2.06 ± 0.32
Afterhyperpolarization amplitude (mV)	15.77 ± 0.45	16.93 ± 1.00	14.52 ± 1.44
Afterhyperpolarization duration (ms)	10.57 ± 2.07	6.71 ± 1.13	7.38 ± 1.75
# Action potentials at suprathreshold	1.00 ± 0.00	1.00 ± 0.00	2.88 ± 1.06

Table 6.3 Intrinsic and active properties of dorsal root ganglion neurones cultured overnight in either control FLS medium, or the media of FLS stimulated with 100 μ M BTB or 0.1% (v/v) DMSO. Data are mean $\pm$ SEM. * $p < 0.05$, ** $p < 0.01$, Kruskal-Wallis test with Dunn's post hoc, * denote differences between indicated condition and control condition; $^\wedge$ denote differences between indicated condition and DMSO condition $n = 15$ cells (Control), 19 cells (DMSO) and 19 cells (BTB) from $N = 4$ animals.

Next voltage-sensitive macroscopic currents were studied in voltage-clamp. Inward currents showed similar properties to those recorded from knee-innervating neurones isolated from mice injected with BTB, with a decrease in the half-maximal activation potential of macroscopic inward currents observed for neurones cultured with media from BTB-stimulated FLS, compared to control conditioned FLS media or that collected from FLS treated with DMSO (Control, -41.48 ± 1.55 mV, DMSO, -38.17 ± 2.52 mV, BTB, -51.96 ± 3.09 mV, $F = 8.025$, $p < 0.05$, ANOVA with Bonferroni post-hoc, mean $\pm$ SEM, $n = 15 - 19$, $N = 4$, Figure 6.6H). The peak inward currents (Control, -1.00 , DMSO, -1.13 ± 0.60 , BTB, -1.25 ± 0.40 , Figure 6.6I) elicited by neurones from each side did not differ however. Similarly, the

normalised magnitude of voltage-gated outward currents was unaffected (Control, 1.00, DMSO, 0.84 ± 0.23 , BTB, 1.53 ± 0.89 , mean $\pm$ SEM, $n = 15 - 19$, $N = 4$, Figure 6.6J-K).

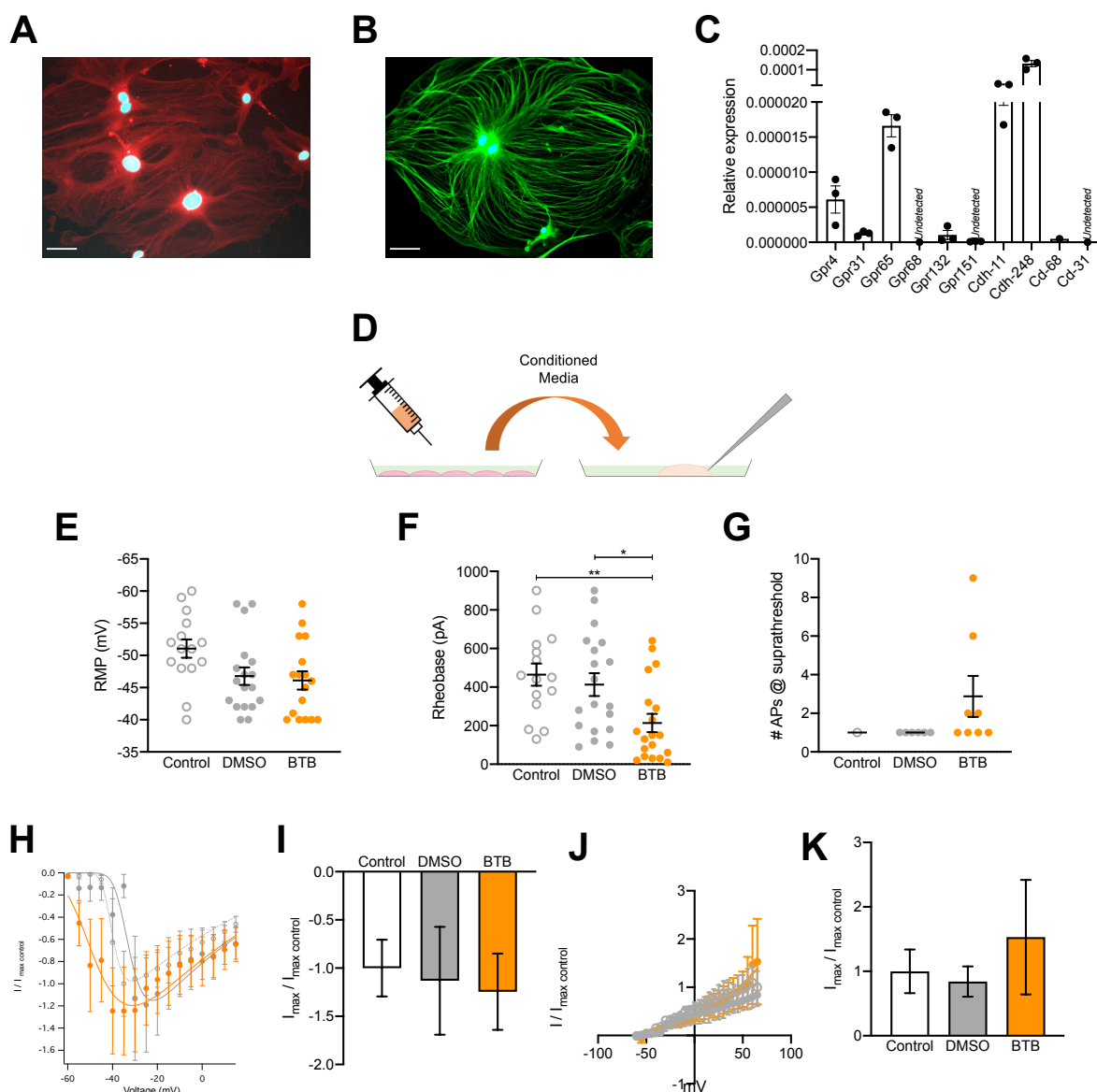


Figure 6.6. Following stimulation with BTB the conditioned media of FLS increases the excitability of sensory neurones. Fibroblast-like synoviocytes express (A) CDH-11 and (B) CD248, (C) the expression profile of FLS was further assessed by qPCR ($n = 3$), expression is relative to the housekeeping gene *18srRNA*. (D) Graphical representation of experimental design. (E) The resting membrane potential, (F) rheobase and (G) ability of DRG to fire multiple action potentials when stimulated with 2x rheobase (suprathreshold) was assessed. Macroscopic voltage-gated (H-I) inward and (J-K) outward currents were also assessed. * $p < 0.05$, ** $p < 0.01$, Kruskal-Wallis test with Dunn's post-hoc, * denote differences between indicated condition and control condition. $n = 15$ cells (Control), 19 cells (DMSO) and 19 cells (BTB) from $N = 4$ animals.

To further explore how FLS stimulated with BTB can sensitise sensory neurones, an antibody array which detects inflammatory mediators was employed to analyse the conditioned media samples (Figure 6.7A). Compared to the media of unstimulated control cells and DMSO-stimulated cells, the media of FLS exposed to BTB contained higher levels of several interleukins (IL): IL-7 (1.83 ± 0.47 fold change from control, $p < 0.05$, two-way ANOVA with

Bonferroni post-hoc, mean $\pm$ SEM, $n = 3$, Figure 6.7B), IL-10 (1.77 ± 0.06 fold change from control, $p < 0.05$, two-way ANOVA with Bonferroni post-hoc, Figure 6.7B) and the active heterodimer IL-12 p40/p70 (2.33 ± 0.51 fold change from control, $p < 0.0001$, two-way ANOVA with Bonferroni post-hoc, mean $\pm$ SEM, $n = 3$, Figure 6.7B). A higher level of the CCL chemokine MIP- γ (CCL9, 5.17 ± 0.52 fold change from control, $p < 0.0001$, two-way ANOVA with Bonferroni post-hoc, mean $\pm$ SEM, $n = 3$, Figure 6.7B) was also reported. The CXC chemokine MIG (CXCL9) was also detected at a higher level (1.93 ± 0.05 fold change from control, $p < 0.01$, two-way ANOVA with Bonferroni post-hoc, mean $\pm$ SEM, $n = 3$, Figure 6.7B), as was GCSF (1.92 ± 0.51 fold change from control, $p < 0.01$, two-way ANOVA with Bonferroni post-hoc, mean $\pm$ SEM, $n = 3$, Figure 6.7B). Interestingly the media of DMSO treated FLS had lower levels of MIP- γ (CCL9, 0.20 ± 0.02 fold change from control, $p < 0.05$, two-way ANOVA with Bonferroni post-hoc, mean $\pm$ SEM, $n = 3$, Figure 6.7B) and Eotaxin (CCL11, 0.33 ± 0.33 fold change from control, $p < 0.001$, two-way ANOVA with Bonferroni post-hoc, mean $\pm$ SEM, $n = 3$, Figure 6.7B) compared to that of control FLS. It is thus apparent that FLS cells secrete inflammatory mediators recognised by both immune cells and sensory neurones, which may contribute to and drive both the inflammation and pain observed following injection of BTB to the knee joint.

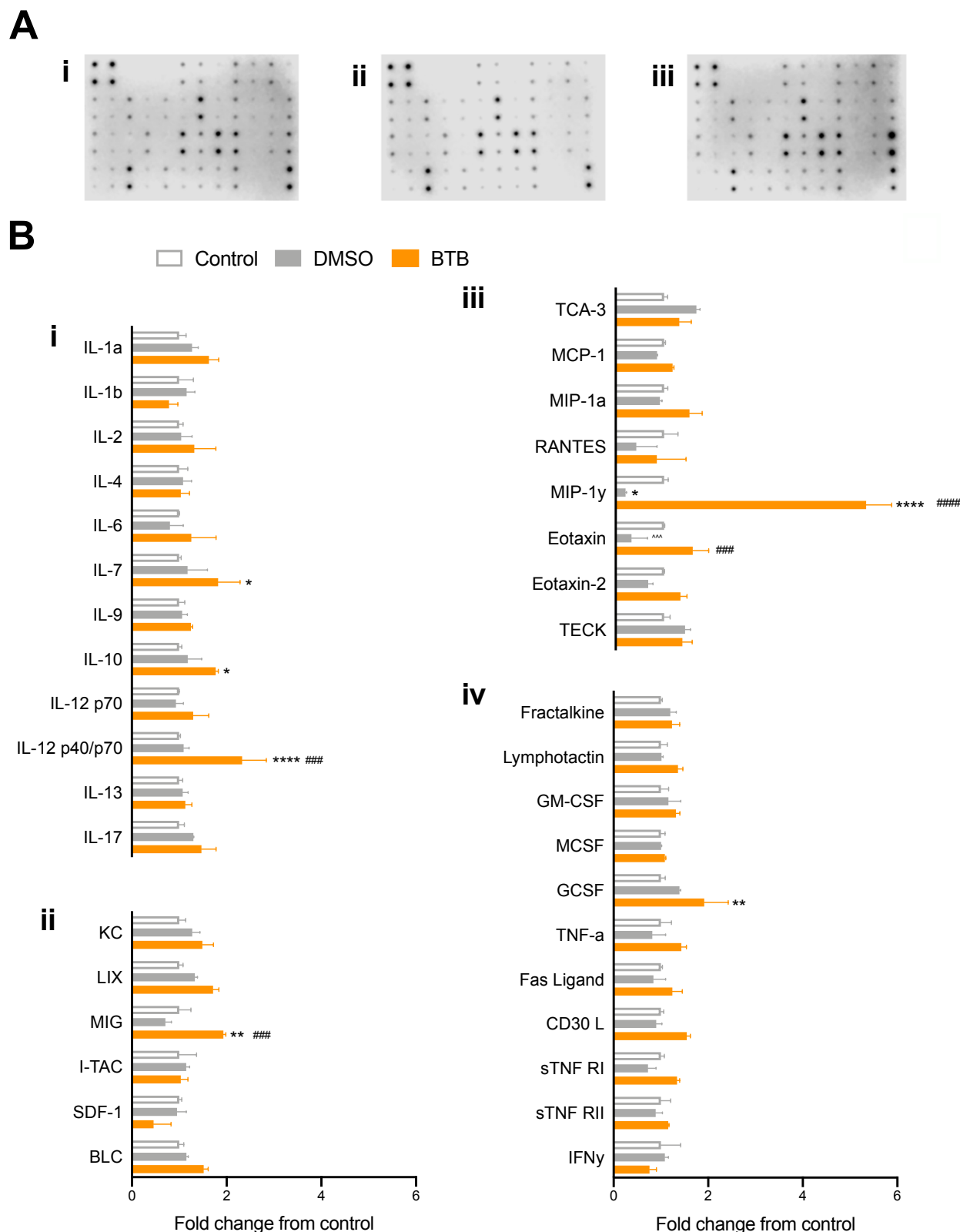


Figure 6.7. Stimulation of FLS with BTB causes secretion of inflammatory mediators (A) Images of array membranes used to assess the presence of inflammatory mediators in conditioned media from (i) unstimulated control FLS, (ii) DMSO-stimulated FLS and (iii) BTB-stimulated FLS. **(B)** Fold change in detection of inflammatory mediators on arrays, normalised levels observed in media from unstimulated control FLS. Data are mean $\pm$ SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, one-way ANOVA followed by Bonferroni post-hoc; ^ denote differences between control and BTB conditions, # denote differences between DMSO and BTB conditions. n = 3 independent stimulations per condition pooled and assayed in duplicate.

6.6. Discussion

Given reports of acidosis in the arthritic joint and interest in PS-GPCRs as mediators of inflammatory pain, this Chapter sought to assess the contribution of GPR65 to joint inflammation and associated pain using the selective GPR65 agonist BTB. Following injection of BTB into the mouse knee joint an inflammatory response is initiated, which is evident from joint swelling that persists a few days. Mice also demonstrate behaviours consistent with pain, including mechanical hypersensitivity of the afflicted joint 24-hours after inflammation induction and longer lasting defects in digging behaviour reminiscent of spontaneous pain and inflammation-induced apathy. Electrophysiology recordings made from sensory neurones innervating the inflamed knee showed higher excitability and a leftward-shift in the activation of voltage-gated inward currents, hyperexcitability that could not be reproduced by incubating sensory neurones with BTB alone. However, *in vivo*, sensory neurones do not innervate empty space, but rather a variety of cell types with which they directly and, through the release of mediators, indirectly interact. One key cell type in synovial joints are synoviocytes that line the capsule, often proliferating and releasing inflammatory mediators during different forms of arthritis. Here, it was observed that FLS express GPR65 and when incubated with BTB secreted inflammatory mediators. Moreover, overnight culture of DRG sensory neurones in the conditioned media of BTB stimulated FLS resulted in increased excitability and a leftward-shift in the activation of voltage-gated inward currents, i.e., effects similar to those seen in knee-innervating neurones isolated from mice that had received BTB knee injections. It is thus postulated that the inflammation and associated pain resulting from injection of BTB into the knee joint at least partially arises from activation of FLS resident in the joint, which are triggered to release mediators that can stimulate immune cells and sensory neurones in the vicinity to drive the resulting inflammation and pain.

The lack of a validated antagonist for GPR65 makes attributing the inflammation and pain evoked by BTB specifically to its action at GPR65 very difficult and is a considerable limitation of the work presented here: although BTB appears to be selective among the PS-GPCRs (Figure 5.8B), it cannot be ruled out that this compound might be able to interact with other receptors. Until an antagonist is developed, genetic silencing or ablation of GPR65 represents the best way of ascribing contributions of GPR65 to normal physiology and diseases. Indeed, it would be well worth repeating some of the experiments discussed in this chapter with cells isolated from established GPR65 knockout animals or RNA-mediated approaches to knockdown expression of certain genes (Han, 2018; Radu et al., 2006), however that was beyond the scope of work presented for this Thesis. More advanced methods of silencing

gene expression in particular cell types, such as the Cre-lox system (Kim et al., 2018) or the use of viruses which can selectively silence gene expression using cell-specific promoters (Sliva and Schnierle, 2010) might be of more use for teasing out the contributions of GPR65 in different cell types to inflammatory pain. As well as permitting mechanistic studies on primary tissue, such approaches have the benefit of being able to study the effect of loss of GPR65 function in specific cell types *in vivo*. This might also prove useful in narrowing down roles of different fibroblast populations in the inflammation observed following stimulation of GPR65 (Croft et al., 2019).

Once effects have been irrefutably tied to GPR65 activation it would also be of interest to elucidate the exact signalling events responsible for driving inflammatory pain given BTB can engage a number of downstream cascades at GPR65 (Figure 5.8A), fortunately there exist many pharmacological tools for inhibiting the effectors downstream of GPCR activation. As well as using inhibitors *in vitro* and *in vivo*, reporter gene assays could be performed with FLS to gauge further signalling modalities that can influence gene transcription following BTB-induced activation of GPR65, e.g. a detection of a reporter gene under the control of cAMP response element (CRE) would highlight the G_{α_s} -cAMP-PKA pathway as driving gene expression, but similarly engagement of a serum response element (SRE) would rather implicate ERK activation. Results could then be validated using qPCR to look at how inhibition of different pathways affects the ability of BTB to coordinate transcription of those genes encoding mediators known to be secreted by FLS following stimulation with BTB.

Here, the role of GPR65 in pain and inflammation has only been studied using the selective synthetic agonist BTB. This was to isolate engagement of GPR65 selectively given protons activate numerous receptors, however, the role of GPR65 as a proton-sensor in inflammatory pain should be explored as this is more relevant to human disease. Again, given the lack of GPR65 antagonists, this would most likely involve the use of knockout animals, which could be subjected to various models of inflammatory pain, although this requires careful consideration as models of inflammatory pain do not always correspond to localised acidosis (Wright et al., 2018). An alternative to this might be to use the acid-induced hyperalgesia model, although this does appear to be largely dependent upon ASIC3 (Sluka et al., 2001). The ability of the endogenous GPR65 agonist, psychosine to coordinate inflammation could also be explored as a means of assessing the role of endogenous activation of GPR65 in inflammation. Considering the lowered pH of the synovial fluid in different forms of arthritis, another approach could be to assess whether synovial fluid samples from arthritic patients can activate GPR65, this could first be done using MmGPR65-CHO cells (Figure 4.1B) to avoid any convolution from the other proton-sensitive receptors; this would have the added

benefit of translational value from using samples from human patients to stimulate the receptor. However, again this would require careful consideration before testing on primary cells as the diseased synovial fluid will already contain many mediators capable of activating a multitude of cell types (Chakrabarti et al., 2020b).

BTB stimulation of FLS triggered the release of inflammatory mediators which likely drove both the inflammation (through activation and recruitment of immune cells) and enhanced nociception (via direct or indirect actions on sensory neurones) observed *in vivo*. Indeed one of the cytokines detected at higher levels in media from BTB-treated FLS, MIG (CXCL9) has a well-established role in promoting immune cell recruitment (Van Raemdonck et al., 2015). A body of evidence implicating IL-7, which was also detected at higher levels, in arthritic conditions also exists including a genetic association between an *IL-7* SNP and osteoarthritis among a Chinese Han population (Zhang et al., 2016), reports of increased production of IL-7 by chondrocytes during osteoarthritis, which had the effect of increasing matrix metalloproteinase-13 production and accelerating joint degradation (Long et al., 2008). Finally inhibition of the IL-7 receptor with a monoclonal antibody has been shown to ameliorate arthritis severity in a proteoglycan-induced arthritis model in mice, attributed to reduced immune cell recruitment (Hillen et al., 2015). The active form of IL-12 was also found to be upregulated, production of IL-12 has also been reported to be increased in patients of rheumatoid arthritis and correlates with disease severity (Kim et al., 2000) and exacerbated collagen-induced arthritis in mice (Germann et al., 1995). Neutralisation of IL-12 with an antibody has been shown to remediate histological signs of arthritis in a murine model, in part due to reduced infiltration of cells to the inflamed joint (Joosten et al., 2000). A common mechanism of recruiting immune cells to the site of inflammation, which drives disease progression, is thus evident for those mediators upregulated. In the *in vitro* model used in this study, the opportunity for the mediators present in the conditioned media to activate immune cells is limited. It would thus seem that the mediators secreted by FLS in response to BTB might also have an effect upon neurones, either direct activation and or sensitisation or initiation of neurogenic inflammation, which is also worth further exploration considering the evidence discussed above regarding CXCL9, IL-7 and IL-12.

A potentially confounding result from the antibody array was the detection of a higher level of IL-10 in the media of FLS stimulated with BTB. IL-10 is an anti-inflammatory cytokine and has been shown to reduce secretion of IL-1 β by synovial cells cultured from rheumatoid arthritis patients (Chomarat et al., 1995), which likely ameliorates inflammation. Moreover, lentiviral constructs encoding IL-10 under the control of promoters specific for genes upregulated in rheumatoid arthritis have also been shown to slow the progression of experimental arthritis

(Vermeij et al., 2015). The increased secretion of IL-10 may have occurred secondary to BTB-induced secretion of pro-inflammatory mediators and serve as a form of negative feedback to reduce levels of pro-inflammatory mediators after they have been elevated. Indeed, higher levels of IL-10 have been reported among rheumatoid arthritis patients (Cush et al., 1995), thus highlighting the importance of relativity between pro- and anti-inflammatory mediators in driving disease states.

In summary, work described here has added to the evidence that the PS-GPCR GRP65 is a regulator of inflammatory pain. The findings presented here suggest that GPR65 coordinates inflammatory joint pain through non-neuronal FLS cells, which are shown to release inflammatory mediators in response to the GPR65 agonist BTB. These results provoke further questions regarding the molecular mechanisms between engagement of GPR65 on FLS and the resulting painlike behaviours and moreover highlight the importance of further study of PS-GPCRs.

7. Conclusions and Future Directions

This thesis has explored PS-GPCR biology from basic pharmacological characterisation to investigating the roles of these receptors in inflammatory pain. Using both *in vitro* and *in vivo* techniques, the studies discussed have culminated in the discovery of a novel role of the PS-GPCR GPR65 in driving inflammatory joint pain but have also sparked many additional questions. The following paragraphs return to the key questions posed in the introduction, summarising relevant findings and suggesting how these might be built upon to further explore the role of PS-GPCRs in inflammatory pain.

1) How does the time course of inflammation correspond with sensory neurone activity and pain-like behaviours? Are PS-GPCRs involved in commonly used models of inflammatory pain?

In Chapter 3, the carrageenan model of inflammatory pain was used to correlate behavioural readouts of inflammatory pain with profiling of sensory neurones, the principal drivers of inflammatory pain. The peak of physical inflammation was shown to correlate with changes in evoked and spontaneous pain-like behaviour, as well as sensory neurone hyperexcitability, which was possibly due to greater total conductance of inward currents and contribute to neuronal depolarisation. 24-hours post-injection of carrageenan, inflammation had almost completely subsided and neuronal excitability had normalised, however, mice were still found to exhibit thermal hyperalgesia. Analysis of the expression of nociceptive channels by sensory neurones at this time point revealed higher levels of TRPV1, an ion channel activated by noxious heat, which may explain the persistent heightened sensitivity to thermal stimuli. Expression of the PS-GPCRs was also found to be elevated, particularly in the case of GPR65, suggesting an involvement of the receptor in the later stages of the inflammation. This Chapter highlights the need for careful consideration of experimental endpoints when assessing inflammatory pain in rodents, as well as the types of assessment and timepoints at which these are taken. Although this work suggests inflammation driven changes in sensory neurone excitability and expression might drive the observed pain-like behaviour, further study is necessary to fill in mechanistic detail, such as the identification of mediators that drive the changes in cell conductance. Given the overall focus of this thesis is on PS-GPCRs, it would be of particular interest to elucidate the cause of the reported upregulation of GPR65 expression by sensory neurones and functional relevance of this, which may be achieved using GPR65^{-/-} mice.

2) Does PS-GPCR expression and function differ in the naked mole-rat, a species that does not perceive acid as noxious?

Chapter 4 focussed on differences in the expression and function of PS-GPCRs cloned from NMRs and mice, results demonstrated that the majority of NMR PS-GPCRs were unable to coordinate the accumulation of cAMP in response to proton stimulation. While expression levels of PS-GPCRs appeared broadly consistent between mice and NMRs, only the NMR PS-GPCR GPR151 that drove an inhibitory signalling response could be activated by protons. These findings further support a role of PS-GPCRs in acid-induced nociception, a consequence of inflammatory pain, given previous reports of NMR's experiencing no pain in response to acid administration. Whether this is the case for native primary cells with endogenous expression of PS-GPCRs remains to be determined, the lipid-sensitivity of NMR PS-GPCRs should also be explored. Nonetheless, these findings suggest that NMRs might represent a valuable tool for studying PS-GPCR function, especially if NMR receptors are shown to be activated by lipids, which would allow delineation of the consequences of lipid- and proton-induced activation of PS-GPCRs.

3) How do the signalling and trafficking signatures elicited by reported agonists of the PS-GPCR GPR65 compare to one and other?

Independent reports of GPR65 activation by protons, the sphingolipid psychosine and a synthetic agonist BTB09089 have all been reported using disparate techniques to study signalling phenomena and a variety of cellular backgrounds. In Chapter 5, the pharmacological consequences of GPR65 activation with these agonists was studied systematically using a uniform cell background. Engagement of GPR65 by protons and psychosine resulted in distinct intracellular cascades, given that both of these agonists may activate the receptor endogenously GPR65 thus represents an opportunity to study the consequences of biased agonism in a completely endogenous system. Further study on the physiological and pathophysiological events which would permit activation of GPR65 by both protons and psychosine would be a good starting point for addressing this. This work also showed that the synthetic agonist BTB could elicit a similar signalling response to that induced by protons at GPR65, the selectivity of BTB for GPR65 over other acid sensitive receptors thus makes it a valuable tool for probing the roles of GPR65 in health and disease by using BTB as a surrogate agonist for protons.

Additionally, all of the reported agonists of GPR65 assessed were shown to drive internalisation of the receptor to early endosomes via clathrin- and dynamin-dependent mechanisms following activation. The significance of this in orchestrating signalling outputs

represents an interesting line of further enquiry, given the signalling outputs of GPCRs have been shown to be heavily dictated by their location within the cell (Halls et al., 2016). Furthermore, endosomal signalling of several GPCRs implicated in chronic pain has been shown to be responsible for the sustained signalling necessary to manifest in pain (Jensen et al., 2017; Jimenez-Vargas et al., 2018; Yarwood et al., 2017). Given the central hypothesis of this work surrounds a role of PS-GPCRs in inflammation, compartmentalised signalling of PS-GPCRs is well worth further investigation. This might first be addressed by taking the validated approaches which prevented receptor internalisation in trafficking assays forward to signalling assays, additional temporal resolution of signalling responses may be gained through the use of biosensors targeted to specific subcellular locales (Halls and Canals, 2018). The endomembrane system is progressively acidified (Breton and Brown, 2013), thus PS-GPCR signalling from within the endomembrane system represents an extra layer of intrigue given the shift in local concentrations of protons resulting from trafficking has the potential to impact upon the signalling response, this is well deserving of further exploration.

4) Can a surrogate GPR65 agonist alter nociception and sensory neurone activity in rodents?

Knowing BTB can selectively activate GPR65 to elicit a similar intracellular signalling signature as protons, work described in Chapter 6 used BTB to explore the role of GPR65 in driving inflammatory joint pain. Intraarticular injection of BTB to mice resulted in swelling of the knee joint and a change in behaviours consistent with inflammatory pain. Using primary cultures of sensory neurones and fibroblast-like synoviocytes (FLS) to probe the molecular mechanisms behind the observed pain revealed that BTB could stimulate FLS cells resident in the joint environment, triggering a release of inflammatory mediators capable of sensitising neurones. These findings heavily implicate GPR65 as a mediator of cell-cell communication in the joint which can drive inflammation and the associated pain. Future work should address the roles of the cytokines released in response to BTB-activation of FLS in driving both inflammation and nociception, which might be achieved by studying the effects of the individual mediators on cultures of immune cells and sensory neurones independently and will enable better understanding of the interplay between inflammation and the manifestation of pain that can later be confirmed by *in vivo* work or using co-culture techniques. The relevance of proton-engagement of GPR65 in inflammatory diseases should also be addressed, as this would hold far greater translational value, however, this requires careful thought due to the widespread expression of other proton-sensitive receptors, which will make it hard to pinpoint any contributions directly to GPR65. This may first be assessed by assessing the ability of OA and RA synovial fluid to activate cells expressing only GPR65.

Ultimately work presented in this thesis has demonstrated an involvement of PS-GPCRs in inflammation: the expression of this family is increased following induction of a commonly used model of inflammatory pain, PS-GPCR homologs of the NMR have been shown to be insensitive to protons and finally activation of GPR65 with a synthetic agonist that recapitulates many of the signalling events induced by protons at the receptor can drive inflammation of the knee joint and an concomitant increase in nociceptive behaviour. Reflecting on the hypothesis proposed at the start of this thesis, work presented suggest that PS-GPCRs, particularly GPR65, do contribute to inflammation and nociception, although signalling from non-neuronal cells present at the site of inflammation seem to have a greater contribution than their action directly on DRG, suggesting this family of receptors are critical integrators of neuroimmune responses to tissue acidosis in inflammation. It is hoped that the work discussed in this thesis might inspire wider interest in this long-neglected family to further explore their roles in health and disease. A major hurdle to categorically attributing roles of any of the PS-GPCRs in biological phenomena is the current lack of antagonists. While this work has hopefully built a stronger case for prioritising the development of PS-GPCR antagonists, particularly GPR65, revisiting experiments described here when such a tool is available would unequivocally allow roles of PS-GPCRs in coordinating pain and inflammation to be proved. Antagonists of the PS-GPCRs would also serve as a first means of assessing the potential of therapeutic targeting of these receptors, which based on work presented here may be a novel way of achieving analgesia and reducing inflammation. Conveniently, if the link between GPR65 and inflammatory pain is further verified and an antagonist can be developed that shows promise in alleviating pain, recent developments in pH-sensitive drug delivery using nanoparticles would permit highly targeted inhibition of GPR65 in inflamed acidified locales and increase therapeutic benefit of targeting the receptor (Ramírez-García et al., 2019).

8. References

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