# Peripheral Mechanisms of Touch and Pain

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# Declaration

I, Jane Elizabeth Sexton confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

# Abstract

This thesis uses transgenic approaches to alter expression of candidate mechanosensors in physiological and pathological conditions to determine their contribution to the sensations of touch and pain.

Transient receptor potential (TRP) ion channels have highly conserved roles in sensory function and a great deal of evidence implicates them in the process of mechanotransduction. Their propensity to form heteromeric complexes as well as the functional redundancy they exhibit makes them difficult to study. We used animals with global deletion of multiple canonical TRP (TRPC) channels to minimise the effects of these features and found that TRPC1, TRPC3, TRPC5 and TRPC6 have a modality specific and combinatorial role in innocuous mechanosensation.

Next, we looked at the role of TRPC channels and TRPA1 and TRPV1 in mechanical hypersensitivity in the monosodium-iodoacetate (MIA) model of Osteoarthritis (OA). The results show TRPC3, TRPC6 and TRPV1 do not contribute to mechanical hypersensitivity in OA. However, TRPA1 appears to be necessary for the full manifestation of OA induced mechanical hypersensitivity.

We also investigated the role of Annexin A6 in mechanosensation and OA. The Annexin A6 protein binds to NMB-1, a blocker of mechanically activated currents. We found that it negatively regulates mechanosensation and that by overexpressing the protein using a gene therapy approach we are able to partially attenuate mechanical hypersensitivity in the MIA model of OA.

Finally, we investigated the role of ASIC4 in pain and mechanosensation through generation and behavioural characterisation of a global knockout mouse. The function of ASIC4 is largely unknown but *in vitro* it regulates expression and function of other ASIC subunits, some of which have roles in noxious and mechanosensory processes. We found a role for ASIC4 in formalin induced pain and in visceral inflammation which is the first report of a somatosensory function for this poorly understood protein.

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wellcome trust

## Publications

**Sexton, JE,** Desmonds, T, Quick, K, Taylor, R, Abramowitz, J, Forge, A, Kros, CJ, Birnbaumer, L and Wood, JN. (2015). The contribution of TRPC1, TRPC3, TRPC5 and TRPC6 to touch and hearing. Neuroscience letters 610:36-42.

**Sexton, JE**, Vernon, J and Wood, JN (2014) TRPs and Pain. In: Nilius, B. and Flockerzi, V. (eds.) Mammalian Transient Receptor Potential (TRP) Cation Channels, Volume II. Handbook of Experimental Pharmacology Volume 223. Springer International Publishing. pp 873-897

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# Abbreviations

**5-HT** Serotonin

**AAV** Adenoassociated virus

**ABR** Auditory brainstem response

**AITC** Allyl isothiocyanate

**AMPAR** α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor

**Anxa6** AnnexinA6

**ASIC** Acid sensing ion channel

**ATP** Adenosine triphosphate

**BDNF** Brain derived neurotrophic factor

**cAMP** Cyclic adenosine monophosphate

**CCI** Chronic constriction injury

**CFA** Complete Freund's adjuvant

**CGRP** Calcitonin gene related peptide

**CHO** Chinese hamster ovary

**CNS** Central nervous system

**COX** Cyclo-oxygenase

**Cre** Cyclization recombinase protein

**Ct** Cycle threshold

**DAG** Diacylglycerol

**DKO** Double knockout mouse

**dNTPs** Deoxyribonucleotide triphosphate

**DRG** Dorsal root ganglia

**GDNF** Glial cell derived neurotrophic factor

**GFP** Green fluorescent protein

**GPCR** G protein coupled receptor

**HEK** Human embryonic kidney cells

**IA** Intermediately adapting current

**IB4** Isolectin B4

**IHC** Immunohistochemistry

**IL-1** Interleukin-1

**KO** Knockout

**LTMR** Low threshold mechanoreceptor

**MA** Mechanically activated current

**MIA** Monosodium iodoacetate

**mRNA** Messenger RNA

**Neo** Neomycin resistance cassette

**NGF** Nerve growth factor

**NMDAR** N-methyl D-aspartate receptor

**NSAIDs** Non-steroidal anti-inflammatory drugs

**OA** Osteoarthritis

**OHC** Outer hair cells

**PAG** Periaqueductal grey

**PEPD** Paroxysmal extreme pain disorder

**PCR** Polymerase chain reaction

**PKA/C** Protein kinase A/C

**PLC** Phospholipase C

**PWT** Paw withdrawal threshold

**QKO** Quadruple knockout mouse

**qPCR** Quantitative real time PCR

**RA** Rapidly adapting current

**RVM** Rostroventral medulla

**SA** Slowly adapting current

**SAC** Stretch activated channel

**SKO** Single knockout mouse

**SNI** Spared nerve injury

**SNL** Spinal nerve ligation

**SNT** Sciatic nerve transection

**TG** Trigeminal ganglia

**TKO** Triple knockout mouse

**TNFα** Tumour necrosis factorα

**TRP** Channel Transient Receptor potential channel

**TTX** Tetrodotoxin

**VGCC** Voltage-gated calcium channel

**VGSC** Voltage gated sodium channel

**WB** Western Blot

**WDR** Wide dynamic range

**WT** Wildtype

## Acknowledgements

I would like to thank my supervisor, Professor John Wood, for giving me the opportunity to do a PhD in his lab where I have learnt so much under his guidance.

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# 1 Introduction

## 1.1 Introduction to Neurobiology

The nervous system can be divided into its central and peripheral components. The central nervous system consists of the brain and spinal cord while the peripheral nervous system encompasses all neurons outside of this which include both sensory and motor components. The motor component consists of efferent neurons which can be classified as somatic (involved in voluntary functions) or autonomic (involved in automatic functions); the latter can then be subdivided into sympathetic and parasympathetic nervous systems which are responsible for 'fight or flight' functioning and maintenance of normal 'at rest' functions, respectively. The sensory component of the peripheral nervous system (PNS) consists of afferent neurons which convey information to the CNS.

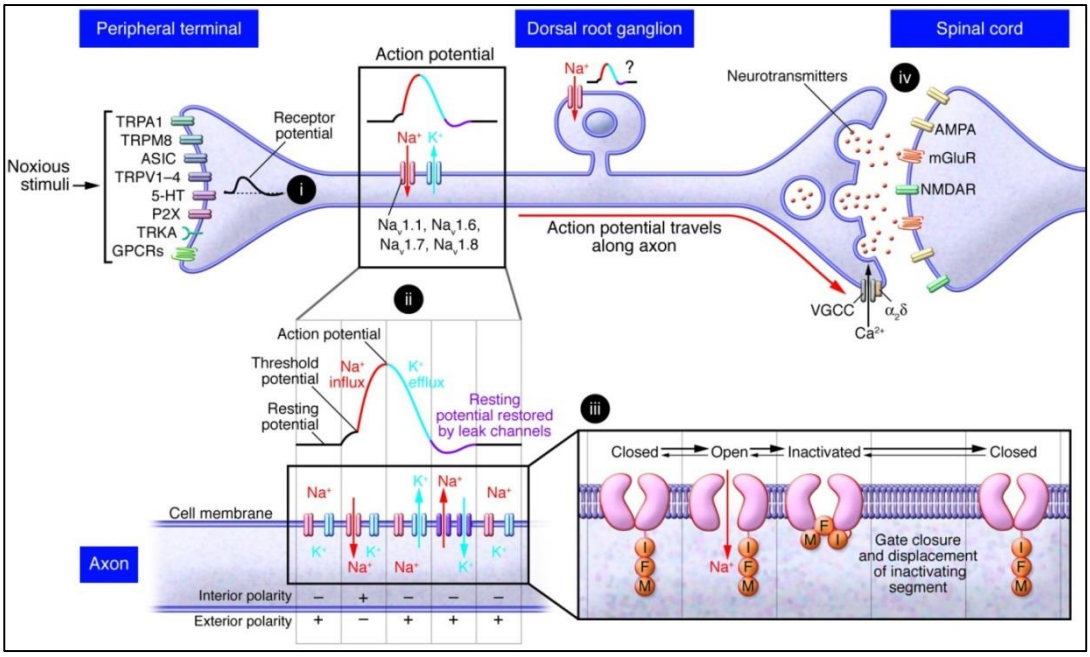



Figure 1.1 Nociceptors are a subtype of primary afferent neuron, all of which have their cell bodies in dorsal and trigeminal root ganglia. They express transduction channels at their peripheral terminals which confer sensitivity to thermal, chemical and mechanical stimuli. Voltage gated sodium channels and potassium channels are involved in propagation of the action potential to the dorsal horn of the spinal cord and voltage gated calcium channel allow release of neurotransmitter across the synapse. (Adapted from Raouf et al. (2010)).

The primary afferent neurons of the PNS all have their cell bodies in dorsal or trigeminal root ganglia; they are pseudo-unipolar neurons which project from these cell bodies to the periphery and to the dorsal horn of the spinal cord (Figure 1.1). These neurons have been classified according to a number of different features including conduction velocity, myelination state, diameter and presence or absence of specific molecular markers. So called Aβ fibres have a large diameter and are thickly myelinated hence their rapid conduction velocity of around 10-30m/s. Aδ fibres are lightly myelinated while C fibres have no myelination and have a much smaller diameter leading to conduction velocities of 1.2-10m/s and <1.5m/s, respectively.

The Aδ and C fibres described are considered 'nociceptors' which are defined by the International Association for the Study of Pain (IASP) as "a central or peripheral neuron of the somatosensory nervous system that is capable of encoding noxious stimuli". Their differences in conduction velocity therefore give rise to separate nociceptive responses involving a 'first pain' (mediated by Aδ fibres) and a 'second', slower pain (mediated by the slower conducting C fibres). These subpopulations of sensory neurons respond to combinations of thermal, mechanical and chemical stimuli though their sensitivity profiles can be altered in pathological pain states. Aβ fibres, however, fall under the functional category of 'non-nociceptors' which are largely responsible for conveying light touch information. This category also includes Aα fibres which are involved in conveying proprioceptive information and can be extended to some Aδ and C fibres which respond to low threshold mechanical inputs.

These subsets of sensory neurons also have defining patterns of projection to the spinal cord and terminals in the skin and muscle. While C fibres terminate largely in lamina I and II of the spinal cord, Aδ fibres mostly terminate in inner lamina II and in lamina III and Aβ fibres in deeper laminae III-V (though there is a degree of overlap)(Lallemend and Ernfors, 2012). Nociceptive neurons largely exhibit free endings in the periphery whereas non nociceptive neurons are more commonly associated with various mechanosensory end organs.

As well as being distinguished by the qualities described above, primary afferent neurons can be classified by the molecular markers they express. Large diameter fibres which convey proprioceptive information are sensitive to neurotrophin-3 as they express the TrkC receptor. Fibres which convey low threshold mechanical inputs express TrkB and

Ret, sometimes with TrkC, so are sensitive to BDNF and GDNF. C fibres, on the other hand, can be subdivided into peptidergic and non peptidergic groups; peptidergic C fibres express the TrkA receptor and respond to nerve growth factor (NGF), while non-peptidergic nociceptors bind isolectin B4 (IB4) and, during development, lose sensitivity to NGF and instead begin to express the Ret receptor.

More recently, single cell RNA sequencing technology has provided a detailed molecular profile of sensory neurons leading to differentiation of eleven functionally distinct subtypes (Usoskin et al., 2015). This includes four main families of neurons (1) those expressing neurofilament (NF), traditionally associated with myelinated neurons (2) a class of peptidergic (PEP) neurons expressing Substance P, TrkA and calcitonin gene related peptide (CGRP), (3) non peptidergic (NP) and (4) unmyelinated neurons defined by the presence of tyrosine hydroxylase (TH) which express high levels of Piezo2 (Figure 1.2). As well as correlating transcript expression with known functions, the markers of these cell subtypes allow prediction of roles in other sensory functions. This work builds on the existing knowledge of molecular expression patterns and cell size as markers of cell subtypes and highlights functionally relevant gene expression patterns providing a fundamental foundation for an increased understanding of the contribution of different neuronal populations to touch, pain and itch sensation. It is important to note that this classification was performed in tissue taken from naïve animals and therefore the transcriptional profile of these neuronal subsets may be altered in pathological conditions.

In order to convey sensory information from the periphery, these neurons express channels which selectively transduce sensory stimuli (Figure 1.1). These include TRP channels, acid sensing ion channels (ASICs), ATP receptors and G protein coupled receptors (GPCRs) among others and can be activated by thermal, chemical or mechanical stimuli. Once the primary afferent neuron has been depolarised, voltage gated sodium channels are involved in transmission of the action potential along the length of the fibre (Figure 1.1). $Na_v1.1$-1.3 and $Na_v1.6$-1.9 are expressed in the nervous system. Voltage gated calcium channels are also involved and play a crucial role in the release of neurotransmitter vesicles across the synaptic cleft. The roles played by these channels are vital for normal functioning and alterations in channel function can hugely influence sensitivity to sensory inputs.

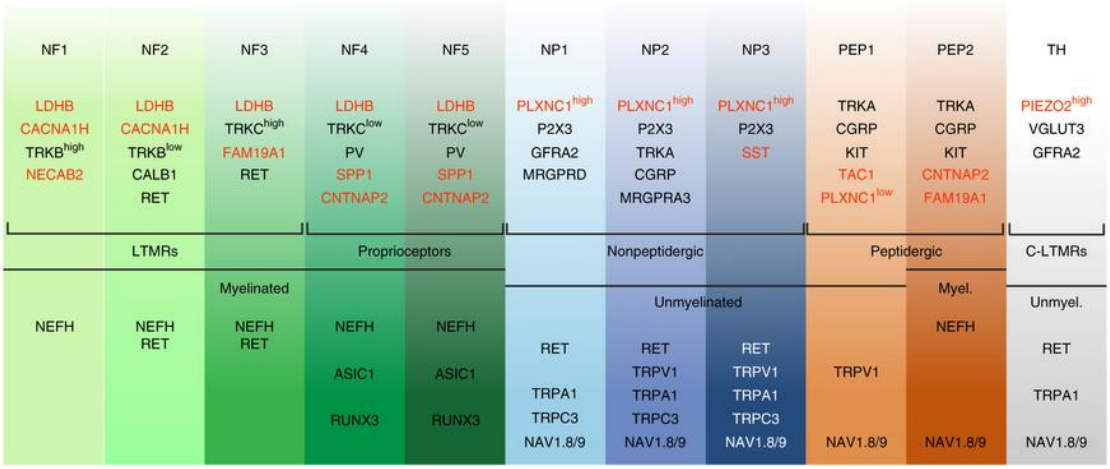



Figure 1.2 Single cell RNA sequencing has identified eleven distinct subtypes of sensory neurons. Gene products on top are suggested markers for identification of subtypes (red, new markers; black, selected previously used markers). Gene products at bottom are examples of a distribution of gene products commonly studied in the field. Proposed relation of sensory subtypes to modality-specific functions or previous functional classification is indicated below brackets. Myel., myelinated; Unmyel., unmyelinated (Adapted from Usoskin et al. (2015)).

## 1.2 Mechanosensation

Mechanosensation, the sensitivity to a mechanical stimulus, is a sensory modality which is vital for normal functioning. It is a feature which is highly conserved across species from basic organisms exhibiting sensitivity to osmotic pressure and more complex species requiring cutaneous sensitivity to touch and noxious mechanical stimuli for normal life. The importance of mechanosensitivity in humans is vast, ranging from the ability to manipulate objects, requiring sensitivity to tactile input, to avoiding aversive or noxious stimuli. Moreover, dysfunction of the mechanisms involved in mechanosensitivity can lead to touch hypersensitivity which is seen in many instances of inflammatory and neuropathic, chronic pain conditions.

### 1.2.1 *Primary afferent neurons and mechanosensory end organs*

Primary afferent neurons are involved in conveying information regarding mechanical stimuli which are transduced by mechanosensory end organs at their peripheral terminals. Different classes of end organs are innervated by different combinations of primary afferent neuron and work to encode specific qualities of mechanosensory input.

Mechanosensory end organs involved in cutaneous touch are all involved in detection of different qualities of a mechanical stimulus. Meissner's corpuscles, found in the glabrous skin, are responsive to the deformation and motion of skin and are innervated by rapidly adapting type I (RAII) Aβ low threshold mechanoreceptors (LTMRs). Pacinian corpuscles, located deeper in the dermis, are innervated by RA type II (RAII) Aβ LTMRs and are sensitive to vibrations. Slowly adapting type I (SAI) Aβ LTMRs are involved in detection of indentation depth via Merkel endings in glabrous skin. These endings consist of groups of cells which are all linked to the same Aβ fibre and are specialised for their function through their high spatial resolution. Importantly it has recently been shown that Merkel cells themselves, and not just the fibres which innervate them, are sites of mechanotransduction (Poole et al., 2014). Skin stretch is perceived via the activity of slowly adapting type II (SAII) Aβ LTMRs which associate with Ruffini endings. Free nerve endings in the epidermis from Aδ and C fibres are responsible for the detection of noxious stimuli however the molecularly distinct C-LTMRs (Li et al., 2011)are also implicated in the sensation of pleasant touch, showing intense firing in response to brush strokes at a velocity considered to be pleasant (Loken et al., 2009) as well as noxious mechanosensation and mechanical hyperalgesia (Seal et al., 2009).

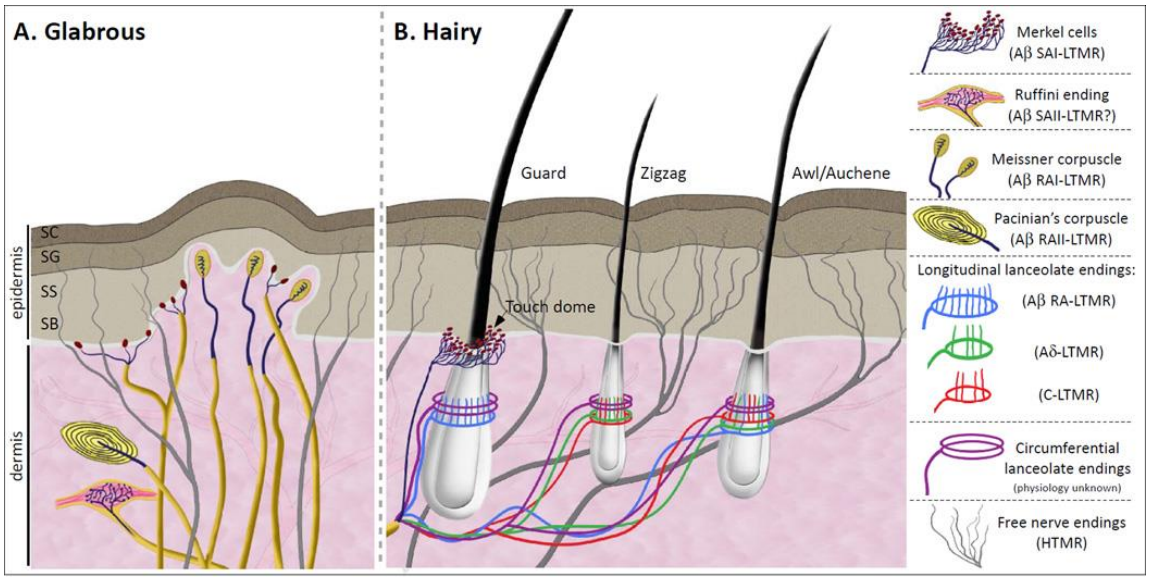



Figure 1.3 Mechanosensory end organs are differentially innervated and respond to distinct components of mechanical stimuli (Taken from Abraira and Ginty (2013)).

These end organs are largely associated with mechanosensitivity in glabrous skin. The hair follicles found in hairy skin form a separate, complex class of mechanosensory end organ. Li et al. (2011) performed a comprehensive investigation into the peripheral and central LTMRs innervating the hairy skin of mice. The most abundant hair type (~76% trunk hairs) found in mice are the fine Zigzag hairs while the shorter Auchene hairs make up ~23% of the hairs on the trunk. Guard hairs are the least abundant and longest hair type, making up a mere ~1% of the hairs on the trunk.

C-LTMRs and Aδ-LTMRs associate with Zigzag and Auchene hairs, often forming interdigitated (or interlocking) innervation patterns in a single hair follicle. Aβ-LTMRs are separated into SAI Aβ LTMRs, which innervate Merkel cells which are in turn linked to Guard hairs and RAI Aβ LTMRs which associate with Guard and Auchene hairs (Figure 1.3). This complex pattern of sensory innervation of hair follicles led Li et al. (2011) to suggest that each of the three hair types are distinct mechanosensory end organs which form peripheral LTMR units consisting of a central Guard hair surrounded by ~20 Auchene hairs and ~80 zigzag hairs. This, they argue, means individual qualities of a mechanical stimulus involve different combinations of hair follicles which in turn will lead to unique combinations of activity in LTMRs. This is furthered by their finding that

columns of LTMR terminals occur in the dorsal horn and that these columns are linked to innervation of the same or adjacent LTMR units in the periphery (Figure 1.4). This complex and integrated suggestion for the expression patterns of LTMRs provides a detailed insight into the complexities surrounding cutaneous mechanosensory innervation.

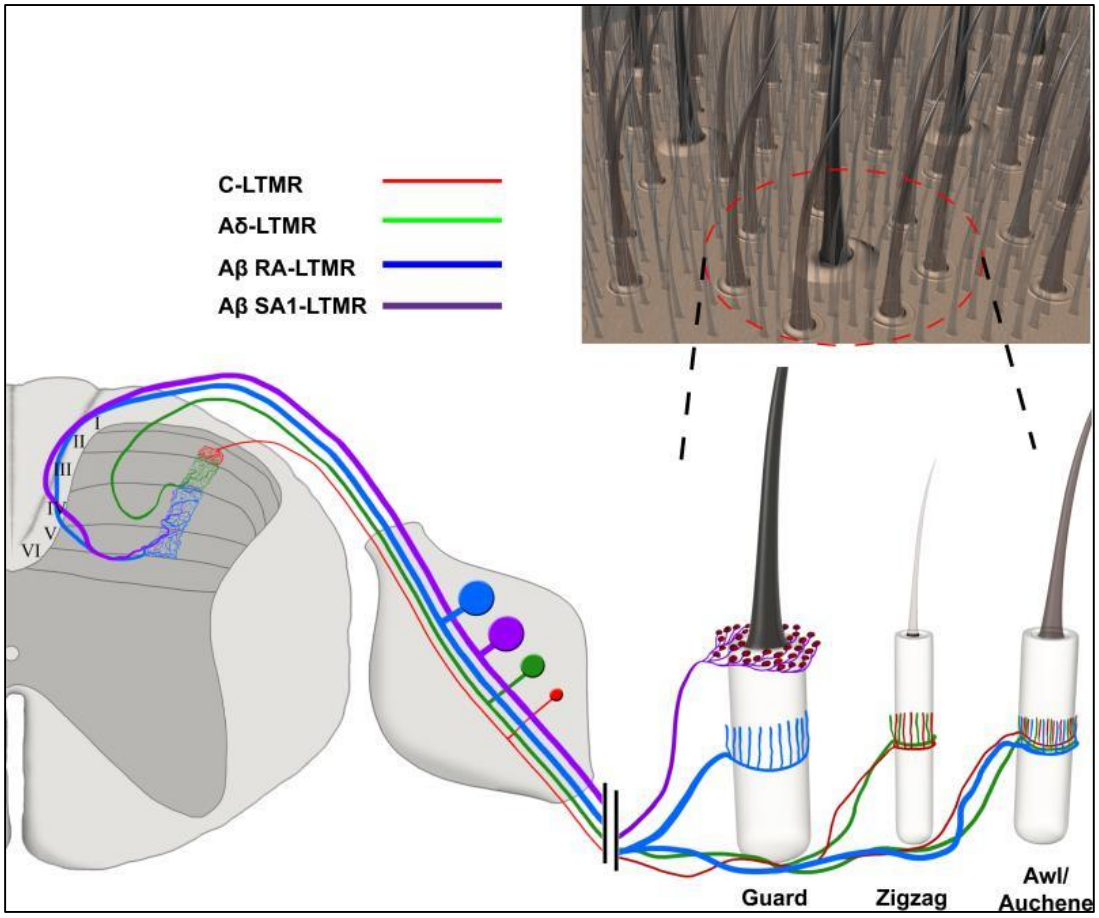



Figure 1.4 LTMRs are arranged in unique combinations around each of the three types of hair follicles. Peripheral LTMR units project to the dorsal horn where they form columns. Each column is made up of projections from the same or adjacent peripheral LTMR units (Taken from Li et al. (2011)).

### 1.2.2 *The electrophysiological properties of mechanosensitive fibres*

Mechanosensory neurons exhibit characteristic electrophysiological properties. The first whole cell patch clamp recordings of rat DRG neurons in culture showed inward cationic currents in response to mechanical stimuli (McCarter et al., 1999). It was subsequently determined, through the use of mechano-clamp electrophysiology, that these mechanically induced currents could be classified into three groups, rapidly adapting (RA), intermediately adapting (IA) and slowly adapting (SA), which could be detected in the soma and neurites of sensory neurons *in vitro* (Drew et al., 2002, Hu and Lewin, 2006) (Figure 1.5) and whose defining decay kinetics are distinct. RA decline in <20ms whereas SA currents are sustained for around 100ms.

Fibres responding to low threshold stimuli exhibit rapidly adapting currents while slowly and intermediately adapting currents, as well as some rapidly adapting currents, are associated with neurons expressing nociceptive markers (Drew et al., 2004). Indeed, NMB-1, a blocker of SA and IA currents selectively inhibits behavioural responses to high threshold, noxious, mechanical stimuli (Drew et al., 2007).

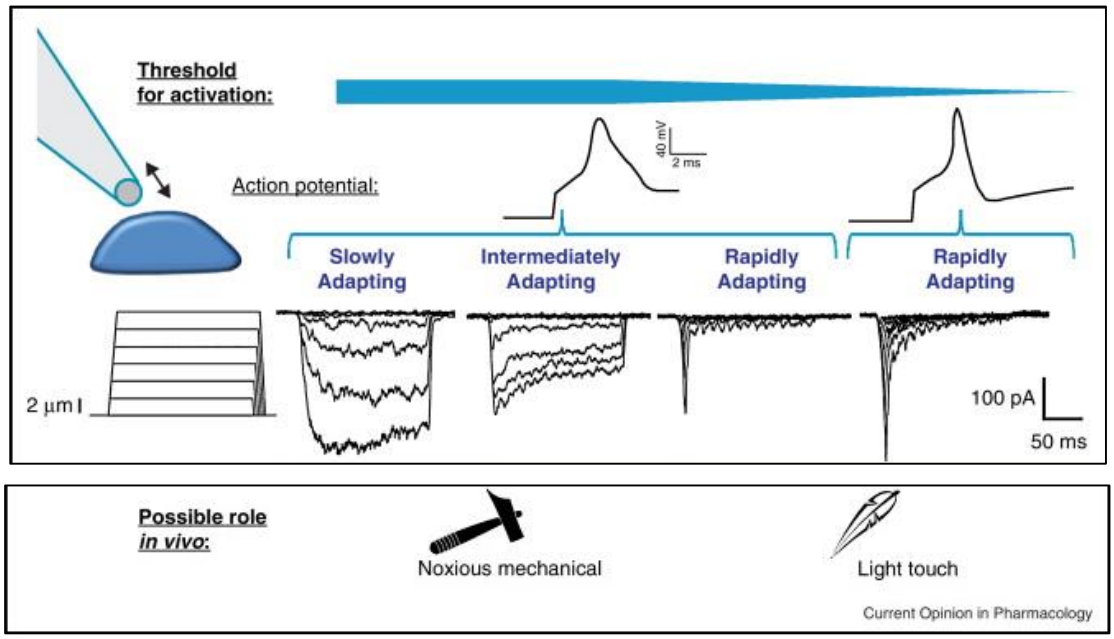



Figure 1.5 Electrophysiological properties of mechanosensory fibres. Slowly and intermediately adapting currents are associated with fibres which have a high threshold and respond to noxious mechanical inputs. Responses to light touch are more commonly associated with rapidly adapting currents, though there is some overlap in these current response profiles (Modified from Wood and Eijkelkamp (2012)).

### 1.2.3 *Potential mechanisms of mechanotransduction*

There is much debate surrounding the mechanisms by which mechanotransduction channels may be activated. Direct activation may occur whereby ion channels respond to distortion of, or changes in tension of, the membrane. Alternatively, the activation may be mediated by tethering molecules linking accessory proteins to the membrane or may involve an indirect mechanism involving second messenger pathways (Figure 1.6).

There is much support for a direct gating mechanism in bacterial mechanosensitive channels, MscL and MscS (Kung, 2005) and the stretch activated channel (SAC) in Xenopus oocytes which has a response latency of <5ms (Hamill and McBride, 1992). Indeed, for a channel to be directly gated by mechanical stimuli the latency from stimulation to activation would be short. For example, it has been shown that currents induced in cultured sensory neurons are activated 0.4-0.8ms after application of a mechanical stimulus (Hu and Lewin, 2006). This latency is likely insufficient for a second messenger mediated response and thus supports the idea of a directly activated channel.

In the hair cells of the inner ear, it is widely supported that mechanotransduction is mediated by a tethering mechanism. Although it has been shown that tip links are necessary for mechanotransduction (Assad et al., 1991) it is unclear if they are attached to the channel and mediate opening in this way or if displacement or motion of the surrounding membrane is involved (Kung, 2005). NMB-1, a blocker of SA and IA currents, is able to reduce the uptake of FM1-43 dye into the hair cells of the cochlear. Since this dye is known to be taken up through mechanosensitive channels, it is argued that the mechanisms involved in cutaneous and hair cell mechanosensation have common features (Drew et al., 2007). As such, the potential for a tethering mechanism in cutaneous touch has also been investigated and it was found that treatment of cultured sensory neurons with proteases that ablate an extracellular protein tether led to abolition of RA mechanosensory currents (Hu et al., 2010).

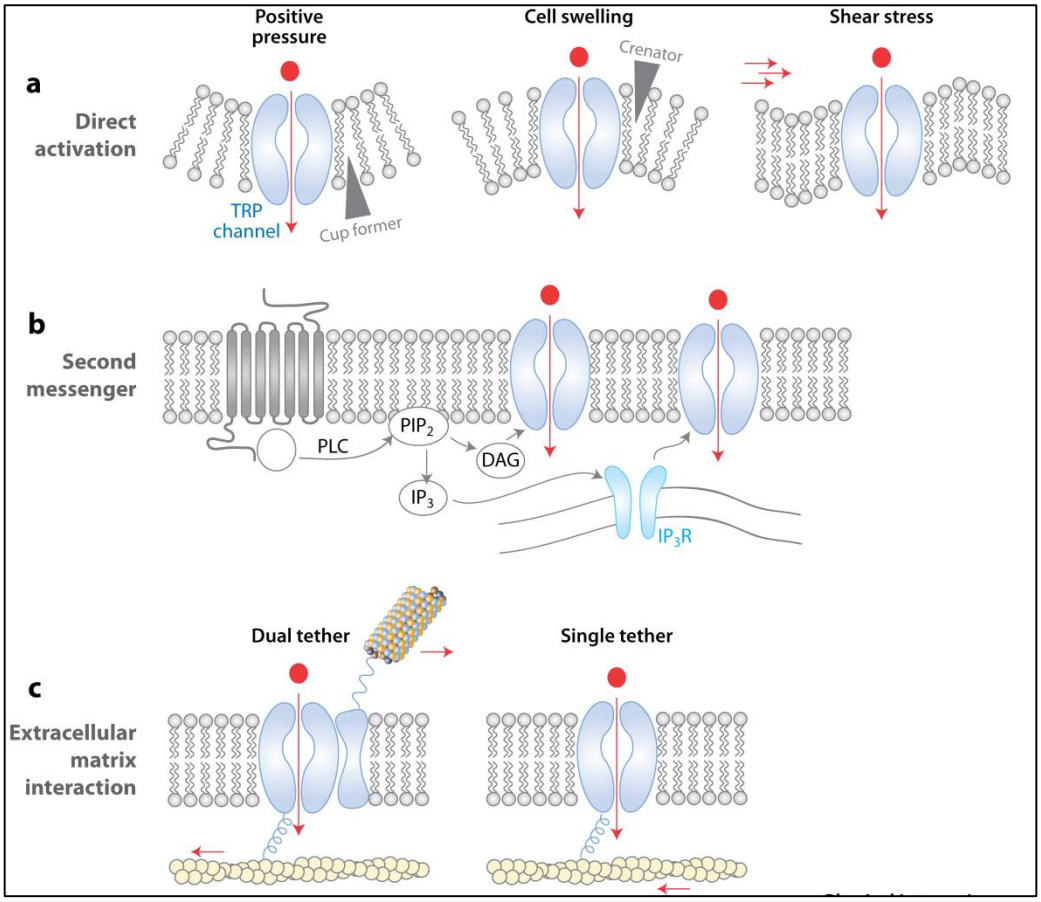



Figure 1.6 Potential mechanisms of mechanotransduction include direct activation of the channel by deformation of the cell membrane, second messenger pathways, interaction of the channel with a tethering protein (Modified from Eijkelkamp et al. (2013b)).

### 1.2.4 *Mechanotransduction in the inner ear*

Hair cells in the Organ of corti are responsible for mechanotransduction that allows the detection of sound (Figure 1.7). They are present on the basilar membrane with one row of inner hair cells and three rows of outer hair cells arranged in a gradient whereby responses to high frequency stimuli occur at the basal end of the basilar membrane and to low frequency stimuli at the apical end. Oscillations in air pressure created at the tympanic membrane are converted to oscillations in fluid pressure in the cochlea that cause vibration of the basilar membrane. The outer hair cells are connected to the tectorial membrane which remains static when the basilar membrane vibrates. The resulting shearing movement deflects hair cell bundles on the inner hair cells as they are not connected to the tectorial membrane. Each hair cell projects a bundle of stereocilia which are arranged in rows of increasing height with the tallest row being made up of kinocilium. These stereocilia contain tip links which connect ion channels and which are displaced, upon movement of the bundle, towards the tallest stereocilia in the bundle. The tension put on the proteinaceous tip links causes ion channels to open and the auditory input is transduced into an electrical impulse that can be propagated along the auditory nerve.

Hair cells are also a key component of the vestibular system which is made up of three semi-circular canals and two otolith organs which, along with the cochlea, make up the inner ear. Hair cell mechanotransduction in the vestibular system is similar to that in the auditory system whereby deflection of projecting stereociliary bundles by fluid movement leads to opening of mechanosensitive ion channels.

Although the process of inner ear mechanotransduction is fairly well understood (Phillips et al., 2008, Schwander et al., 2010), the identity of the channel responsible has remained a mystery. However it is considered that, once elucidated, a mechanotransduction channel in the cochlea may also be involved in cutaneous mechanotransduction.

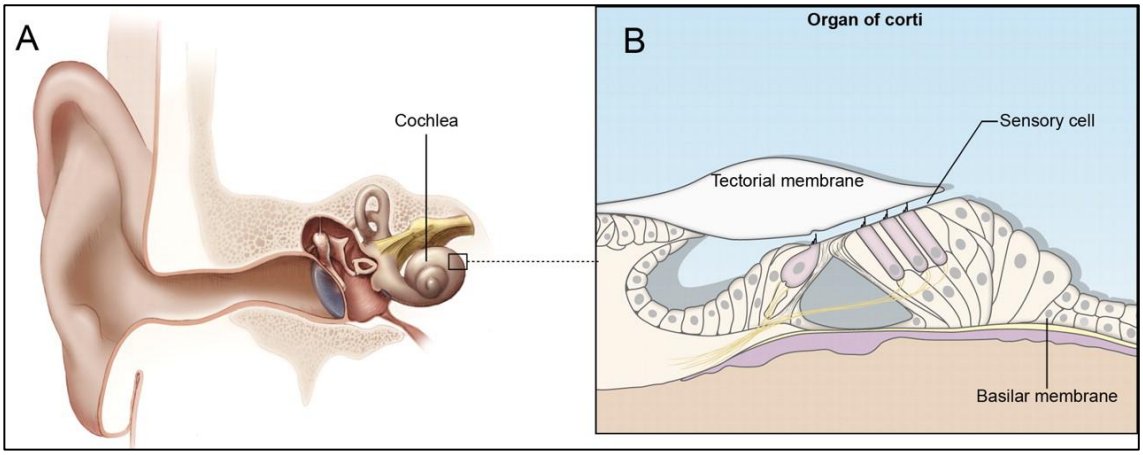



Figure 1.7 The outer, middle and inner ear make up the auditory sense organ. The cochlea is located in the inner ear. The Organ of corti contains the basilar membrane which is caused to vibrate by fluid pressure oscillations leading to displacement of stereociliary bundle located on the hair cells. (Adapted from Schwander et al. (2010)).

### *1.2.5 Mechanotransduction candidates*

The search for mechanotransduction channels has led to the emergence of a number of favourable candidate groups.

The family of Piezo proteins 1 and 2, previously known as Fam38a and Fam38b, are critical in mechanotransduction. Mechanically activated currents in a mouse neuroblastoma cell line were found to be dependent on Piezo1 expression; the protein was also able to confer mechanical sensitivity to a number of other cells lines (Coste et al., 2010). Similarly, expression of Piezo2 in cultured cells robustly induced a mechanically activated RA current (Figure 1.8) while knockdown of Piezo2 in cultured DRG neurons reduced these kinetically distinct currents (Coste et al., 2010). Piezo2 has been shown to contribute to the pore of mechanically sensitive channels (Coste, 2012).

Piezo2 is expressed in sensory neurons and is the major transducer of touch stimuli. Knockdown of Piezo2 results in increased threshold to touch stimuli and reduces allodynia induced by cAMP and in neuropathic pain conditions (Eijkelkamp et al., 2013a). In addition to expression in sensory neurons, Piezo2 is expressed in Merkel cells. Use of an inducible Cre recombinase driven by the Advillin promoter deleted Piezo2 from both of these cell types and caused a dramatic reduction of behavioural responses to a variety of innocuous mechanical stimuli (Ranade et al., 2014). Interestingly, knocking out Piezo2 from Merkel cells alone produced only a mild light touch impairment though cultured Merkel cells lost their mechanosensitivity (Maksimovic et al., 2014, Woo et al., 2014). Piezo2 deletion from sensory neurons caused a significant reduction in the proportion of cells expressing RA currents and an increase in mechanically insensitive Aβ fibres (Ranade et al., 2014). Conditional Piezo2 knockout mice also showed a loss of velocity sensitivity in the RA fibres which innervate hair suggesting Piezo2 mediates mechanosensitivity in hairy as well as glabrous skin.

Identification of Piezo2 has produced remarkable progress in the understanding of mechanotransduction. However, there are likely to be other proteins involved in responses to mechanical stimuli; for example the threshold for Piezo2 activation can be modulated by other proteins (Poole et al., 2014) while mechanisms responsible for production of the SA mechanically activated current are incompletely understood. Candidate proteins for mechanotransduction complexes have included the TRP family of ion channels as well as ASICs, both of which are involved in mechanosensory processing

in other systems, such as cardiovascular and renal mechanosensation, and have mechanosensory roles in other species.

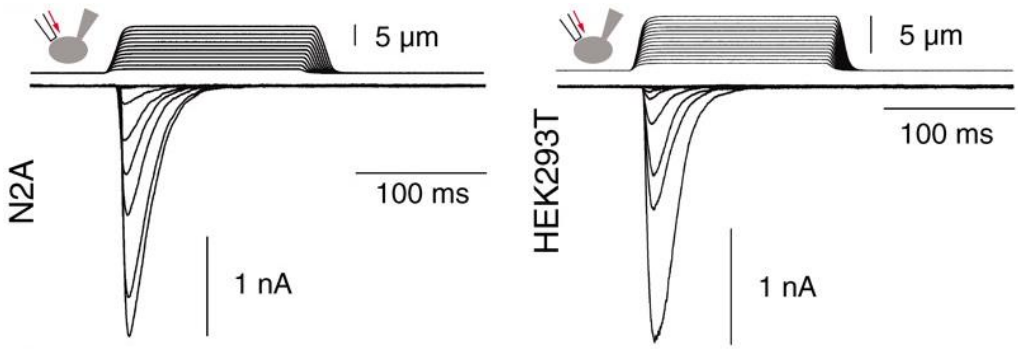



Figure 1.8 Example traces of mechanically activated, RA currents produced by Piezo2 when transfected into Neuro2A and HEK293T cells (Modified from Coste et al. (2010)).

## 1.3 Pain and nociception

The International Association for the Study of Pain defines pain as 'an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage'. Pain is a complex phenomenon which is usually the result of tissue damage caused by a noxious stimulus and as such has a protective function as well as being important for allowing healing of damaged tissue. Nociception is defined as 'the neural process of encoding noxious stimuli' and while it leads to activation of neurons, it does not necessarily result in pain sensation.

Noxious stimuli can be mechanical, thermal or chemical, and they activate nociceptors, a type of sensory afferent neuron whose cell bodies lie in the dorsal and trigeminal root ganglia. As discussed in section 1.1, the two main categories of nociceptor are commonly known as Aδ and C fibres which are thinly myelinated and unmyelinated, respectively. As a result of their myelination state and their larger diameter, Aδ fibres have a greater conduction velocity than C fibres and are responsible for the so-called first pain, a pinprick sensation which precedes the burning sensation, and the 'second pain' mediated by small-diameter unmyelinated C fibres.

Although pain usually arises from a peripheral stimulus it can occur without noxious input or outlast the initial insult, becoming chronic at which point it has no physiological or protective function. Indeed, human microneurography studies have demonstrated that the relationship between noxious stimulation and pain perception is not linear and that in pathological conditions this relationship can be altered (Torebjork et al., 1984, Torebjork et al., 1992). A large-scale survey estimated that approximately 19 % of people in Europe suffer from chronic pain (Breivik et al., 2006) making it a huge clinical problem; the comorbidities associated with chronic pain are numerous and debilitating, including depression, anxiety and insomnia. Chronic pain is a problem in a huge number of prevalent conditions including arthritis, fibromyalgia, irritable bowel syndrome, diabetic or herpetic neuropathy and cancer or chemotherapeutic drug induced pain. The processes responsible for pain in these inflammatory and neuropathic conditions have been shown to be mechanistically different (for example (Abrahamsen et al., 2008, Minett et al., 2014b, Lolignier et al., 2015a)) while the mechanisms for some are incompletely understood. Moreover the mechanisms underlying the transition from acute to chronic pain are poorly understood and reasons why persistent acute pain does not always become chronic is also unclear (Reichling and Levine, 2009).

Current analgesic therapies include opioids and nonsteroidal anti-inflammatory drugs (NSAIDs) which are often successful as acute pain therapies. However, a number of issues are associated with these drug classes. For example, the NNT (number needed to treat) values assigned to analgesics identify the number of people who need to take a drug for one person to achieve 50% pain relief; for morphine this value is three (McQuay et al., 1999) indicating the limited efficacy of one of the most commonly used analgesics. In addition, long term analgesic use in chronic pain conditions is frequently associated with undesirable side effects. Long term treatment with NSAIDs are associated with adverse gastrointestinal (Wolfe et al., 1999), cardiovascular and renal (Bleumink et al., 2003) issues while use of opioids can induce side effects including nausea, pruritus and opioid induced hyperalgesia as well as causing tolerance and dependence (Moore and McQuay, 2005) while overdosing can cause respiratory depression and ultimately death meaning that they are not favourable for long term use. The use of selective serotonin and noradrenaline reuptake inhibitor and tricyclic antidepressant drugs have also proved clinically useful, though they too produce undesirable side effects.

The search for new, improved analgesics has not been without success. The development of the anticonvulsant drug, gabapentin was a huge development in the treatment of neuropathic pain; it is believed to act through binding of α2-δ subunits of voltage gated calcium channels (Field et al., 2006). Monoclonal antibodies designed as analgesics show improved target specificity, for example binding to NGF (Lane et al., 2010). However many other blockers of key pain mediators are less effective than preclinical studies predicted indicating other mechanisms are likely to contribute to the analgesia produced by knockout studies. A recent development which may go some way to explaining this discrepancy is the finding that loss of function of VGSC, Nav1.7 produces a concurrent upregulation of the endogenous opioid precursor, preproenkephalin (Minett et al., In Press). Loss of function mutations in Nav1.7 produce robust pain insensitivity in humans and mice (discussed further in section 1.6) but pharmacological blockers of Nav1.7 are surprisingly ineffective analgesics, for example Protoxin II which alters action potential firing without having any analgesic effect on acute nociception (Schmalhofer et al., 2008). Minett et al. (In Press) suggest that in addition to its role in electrical signalling, Nav1.7 may have other functions and as such, the fact that total loss of function of Nav1.7 in mice leads to an increase in endogenous opioid signalling is something pharmacological inhibitors are unable to recapitulate. Newly developed analgesics demonstrate the benefit

of identifying novel analgesic targets to act where existing therapeutics have shown limited success. Similarly, the development of new, more effective analgesics clearly requires a more complete understanding of the mechanisms underlying pain and nociception in order to prove effective in the clinic.

### *1.3.1 Inflammatory and neuropathic pain*

In states of chronic inflammatory and neuropathic pain, sensitivity to sensory inputs is altered as a result of peripheral and central sensitisation. This manifests as enhanced sensitivity to noxious stimuli (hyperalgesia), pain that occurs in response to a normally non noxious stimulus (allodynia) and in the case of neuropathic insults, spontaneous pain (Figure 1.9) (Sandkuhler, 2009).

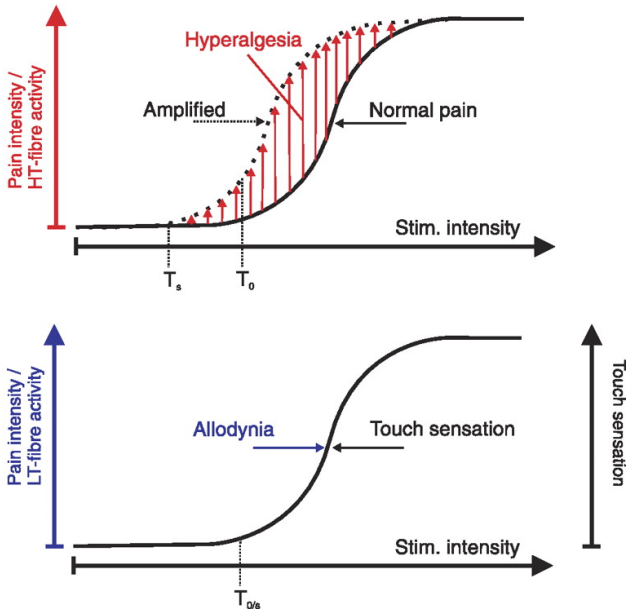



Figure 1.9 In pathological pain states, there is a shift in pain thresholds. Hyperalgesia is increased pain sensitivity which can occur from a decrease in threshold or increased response to a suprathreshold stimulus. Allodynia is pain which occurs in response to a non-noxious stimulus normally by a stimulus which is incapable of activating nociceptors (Modified from Sandkuhler (2009)).

Inflammation induces thermal and mechanical hyperalgesia that can occur at the site of inflammation (primary hyperalgesia) or at distal sites (secondary hyperalgesia). Characteristic features of neuropathic pain include tactile allodynia, spontaneous pain, sensations such as pins and needles, deficits in proprioceptive feedback as well as numbness all at sites innervated by the injured or dysfunctional nerve. Animal models aim to recapitulate these symptoms. Rodent models of inflammation include subcutaneous injection of Freund's Adjuvant and carrageenan which produce thermal and mechanical hyperalgesia while neuropathic pain is commonly modelled in rodents

through surgical lesions (Figure 1.10), or using chemically induced disease models. For example, the chemotherapeutic agent oxaliplatin causes neurotoxicity leading to mechanical and cold allodynia, while streptozocin is used to induce diabetic neuropathy. These rodent models have provided indispensable insight into the differential mechanisms underlying different types of neuropathic and inflammatory pain, some of which are examined below.

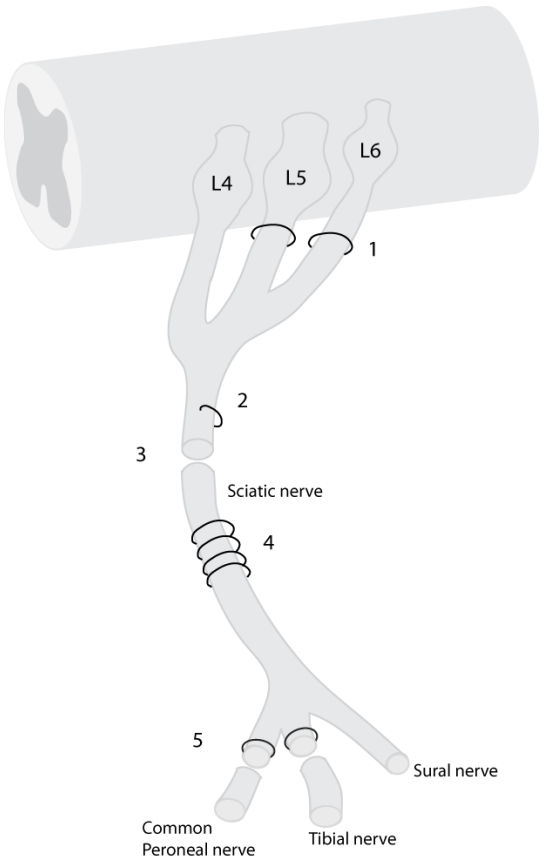



Figure 1.10 Surgical models of neuropathic pain. 1. **SNL**; *spinal nerve ligation*; tight ligation of L5 and L5 spinal nerves. 2. **PSNL**; *partial sciatic nerve ligation*; tight ligation of one third – one half of the sciatic nerve. 3. **SNT**; *sciatic nerve transection*; complete transection of sciatic nerve. 4. **CCI**; *chronic constriction injury*; 3-4 loose ligatures around sciatic nerve. 5. **SNI**; *spared nerve injury*; tibial and common peroneal branches of the sciatic nerve are ligated and transected distal to its trifurcation. (Adapted from Sexton et al. (2014)).

Inflammation is associated with pain, redness, heat and swelling. These symptoms are the result of tissue damage which causes accumulation of pro-inflammatory mediators and endogenous factors which are released by neurons and surrounding non neuronal cells into the affected area. Pro inflammatory mediators include peptides such as substance P, neurotransmitters, eicosanoids, cytokines and neurotrophins and form what is known as an 'inflammatory soup'. These pro-algesic agents mediate their effects through direct binding to cell surface receptors or through activation of second messenger cascades leading to an alteration in the sensitivity profile of cells in the affected area (Figure 1.11). For example, the neurotrophin NGF binds the TrkA receptor activating signalling pathways which induce modifications of proteins at the peripheral terminals of nociceptors causing them to become sensitised, for example through phosphorylation of TRPV1 (Chuang et al., 2001). NGF can also be retrogradely transported to the cell soma where it causes upregulation of transcription of pro-nociceptive factors, such as TRPV1 and substance P. NGF also activates non neuronal cells such as mast cells which release further pro-inflammatory cytokines such as prostaglandins, bradykinin, ATP and 5-HT which adds to the inflammatory milieu and potentiates the inflammatory response. The analgesic action of non-steroidal anti-inflammatory drugs (NSAIDs) is achieved by inhibiting cyclooxygenase enzymes (COX-1 and COX2) to prevent synthesis of prostaglandins though a ceiling effect is often problematic as many other pro-algesic agents remain.

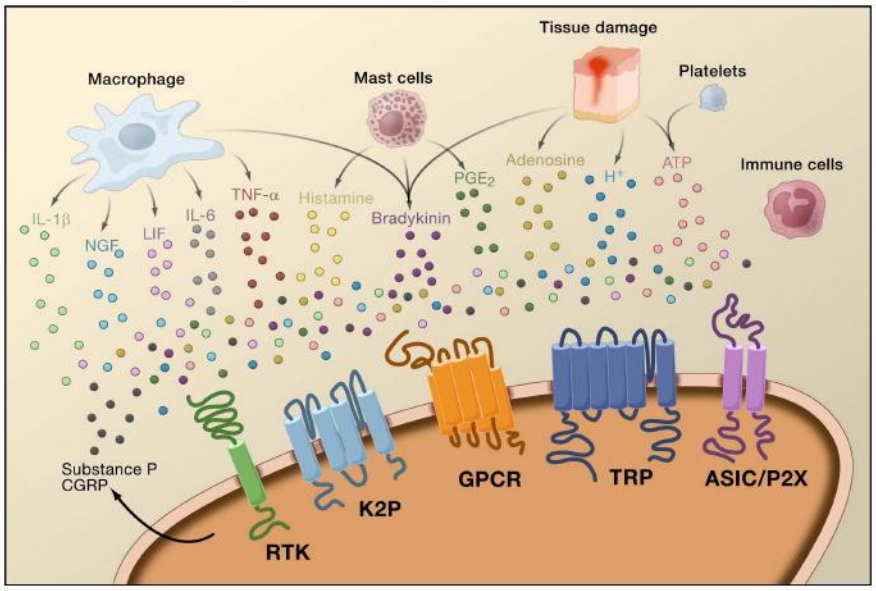



Figure 1.11 Pro-inflammatory mediators are released in the periphery by neuronal and non-neuronal cells following tissue damage. These pro-algesic agents act on cell surface receptors and lead to neuronal sensitisation (Taken from Basbaum et al. (2009).

In neuropathic pain conditions, damage to peripheral neurons leads to a loss of trophic support to the damaged neuron, which is unable to transport neurotrophins to the cell soma where they would normally have neuroprotective functions. Impaired neuronal trafficking can also lead to accumulation of ion channels in the damaged neuron that may cause ectopic firing activity. Degeneration of damaged neurons leads to release of compounds such as neurotrophins, neurotransmitters and inflammatory mediators into the surrounding area which sensitises intact neurons (Li et al., 2000). Altered gene expression is also found in both neuronal and non-neuronal cells in the peripheral and central nervous systems. Indeed, sensitisation of peripheral neurons can occur through changes in both transduction and transmission processes. For example, upregulation of expression of transduction channels such as TRP channels and ASICs are observed, while Piezo2, a primary mechanotransduction channel is sensitised in a model of neuropathic pain (Eijkelkamp et al., 2013a). Similarly, voltage gated ion channels which contribute to action potential transmission show changes in expression levels as well as aberrant firing activity (Figure 1.6).

Under these pathological conditions, the response profile of peripheral neurons changes. Small diameter, unmyelinated C fibres become sensitised and some subsets of these neurons which are normally sensitive to just thermal stimuli can become polymodal. Microneurographic recordings in humans have demonstrated the role for a subset of C fibres, known as mechanically insensitive afferents (MIAs), in the maintenance of chronic pain (Orstavik et al., 2003, Orstavik et al., 2006). These MIAs have been shown to develop mechanical sensitivity in conditions such as erythromelalgia and diabetic neuropathy and therefore contribute to altered sensitivity in chronic pain conditions. Furthermore, a population of C-LTMRs (which appear to be implicated in normal noxious mechanical responses too) have been found to be responsible for the maintenance of mechanical hypersensitivity in inflammatory and neuropathic pain states (Seal et al., 2009).

Although many of the molecular and downstream intracellular mechanisms underlying peripheral sensitisation in mechanical hyperalgesia and mechanical allodynia are thought to share some features, distinct differences have also been observed (Lolignier et al., 2015b). For example, cAMP is implicated in nerve injury induced mechanical allodynia and inflammatory hyperalgesia. Similarly, PKC activity is implicated in peripheral sensitisation in both inflammatory and neuropathic pain, as well as the additional 'primed state' where nociceptors exhibit normal, basal nociceptive thresholds but show increased sensitivity to agents such as inflammatory mediators (Aley et al., 2000, Parada et al., 2003). However, PKA, which is produced downstream of cAMP does not appear to be necessary for the development of allodynia although evidence suggests it contributes to mechanical hyperalgesia (Hucho and Levine, 2007).

Central sensitisation is typically characterised by increased responses to suprathreshold stimuli in both areas of tissue damage and surrounding areas, increased spontaneous activity and reduced thresholds for activation of dorsal horn neurons of the spinal cord and expansion of receptive fields (Latremoliere and Woolf, 2009). This contributes to hypersensitivity in a number of ways through mechanisms involving neuronal sensitisation and neuronal disinhibition as well as changes mediated by non-neuronal cells. The barrage of peripheral neuron input into the spinal cord which occurs after injury is considered to be a trigger for further central changes which potentiate hypersensitivity (Han et al., 2000). The increase in neuronal activity leads to an increase in neurotransmitter release at the central terminals of primary afferents. Under normal

conditions, depolarisation of second order neurons in the spinal cord occurs through activation of postsynaptic glutamate receptors such as the AMPA receptor. However, in the presence of increased neurotransmitter, sufficient levels of depolarisation can be reached to activate the NMDA receptor which is normally blocked by the presence of an $Mg^{2+}$ ion. NMDA receptor activity leads to potentiation of the activity in second order neurons when depolarised by subsequent afferent input as well as through increased intracellular $Ca^{2+}$ levels which activate a number of signalling cascades further potentiating activity of spinal projection neurons. Altered activity and signalling in inhibitory interneurons in the spinal cord can also contribute to the maintenance of persistent pain. For example, activity of TRPV1 in a set of GABAergic interneurons can cause long term depression (LTD) of these interneurons which leads to central sensitisation through disinhibition of ascending spinothalamic tract projections (Kim et al., 2012b). This ultimately leads to neuropathic pain induced mechanical allodynia which is independent of peripherally expressed TRPV1 function. A loss of GABAergic transmission has also been reported after nerve injury (Moore et al., 2002). Facilitation of $GABA_A$ activity in spinal cord interneurons can attenuate inflammatory and neuropathic hypersensitivity (Knabl et al., 2008) while prostaglandins have been shown to reduce the inhibitory activity of glycinergic interneurons, the net effect of which is disinhibition of spinal projection neurons (Ahmadi et al., 2002, Harvey et al., 2004).

As well as changes in neuronal properties, other non-neuronal cells contribute to peripheral and central sensitisation. For example, microglia in the CNS proliferate after nerve damage and surround the central terminals of damaged primary afferent neurons and display changes in surface receptor expression and expression of inflammatory mediators. Indeed, activation of microglial ATP receptors has been shown to be critical for the development and maintenance of neuropathic pain. Activation of the microglial ATP receptor, P2X4 leads to release of factors such as BDNF which contribute to a depolarising shift such that the activity of GABAergic neurons produces depolarising, rather than hyperpolarising responses in second order neurons (Tsuda et al., 2003, Coull et al., 2005, Trang et al., 2009, Beggs et al., 2012). Altogether, these mechanisms potentiate the activity of ascending signalling pathways and contribute to the maintenance of persistent pain.

### 1.3.2 *Central pain pathways*

Peripheral afferent neurons terminate in the dorsal horn of the spinal cord where they synapse onto second order spinal neurons. Peripheral nociceptive neurons primarily project to the superficial laminae (I and II) of the dorsal horn (Todd et al., 2002) though the terminals of muscle and visceral afferents show more sparse terminations through superficial and deeper laminae (Sugiura et al., 1989) while deep dorsal horn neurons mainly receive input from non-nociceptive afferents. Spinal neurons include nociceptive specific neurons which are activated by noxious stimulus intensities and wide dynamic range (WDR) neurons which respond to both noxious and non-noxious inputs. These spinal neurons form ascending pathways such as the spinothalamic tract which project to brainstem nuclei, thalamocortical structures and onto to the cortex where pain perceptions are integrated (Figure 1.12). Projection neurons play a critical role in pathological pain states. For example, a subset of neurons which project from lamina I and express the NK1 receptor, which binds substance P, develop in early postnatal stages (Man et al., 2012) and are critical for the presence of thermal and mechanical hypersensitivity in persistent pain states through activation of descending pathways (Nichols et al., 1999, Suzuki et al., 2002). Indeed, descending pathways which arise from structures such as the periaqueductal grey (PAG), the rostral anterior cingulate cortex (rACC) and the rostroventral medulla (RVM) project to the spinal cord can both facilitate and inhibit spinal nociceptive processing (Figure 1.12). Descending serotonergic pathways which synapse onto spinal 5-$HT_3$, and some other 5-HT, receptors are thought to primarily promote pain (Svensson et al., 2006, Geranton et al., 2008) though other 5-HT mechanisms in the spinal cord contribute to pain inhibition (Ossipov et al., 2010, Song et al., 2011). Descending noradrenergic pathways are primarily inhibitory and act on α2 adrenoreceptors in the dorsal horn (Ossipov et al., 2010) while dopaminergic pathways can be both pro and anti-nociceptive and can contribute to the transition to chronic pain through activation of $D_1/D_5$ receptors (Kim et al., 2015). The balance of descending spinal pathways is therefore critical for normal pain processing while the plasticity of these pathways is hugely influential on the processing of pathological pain.

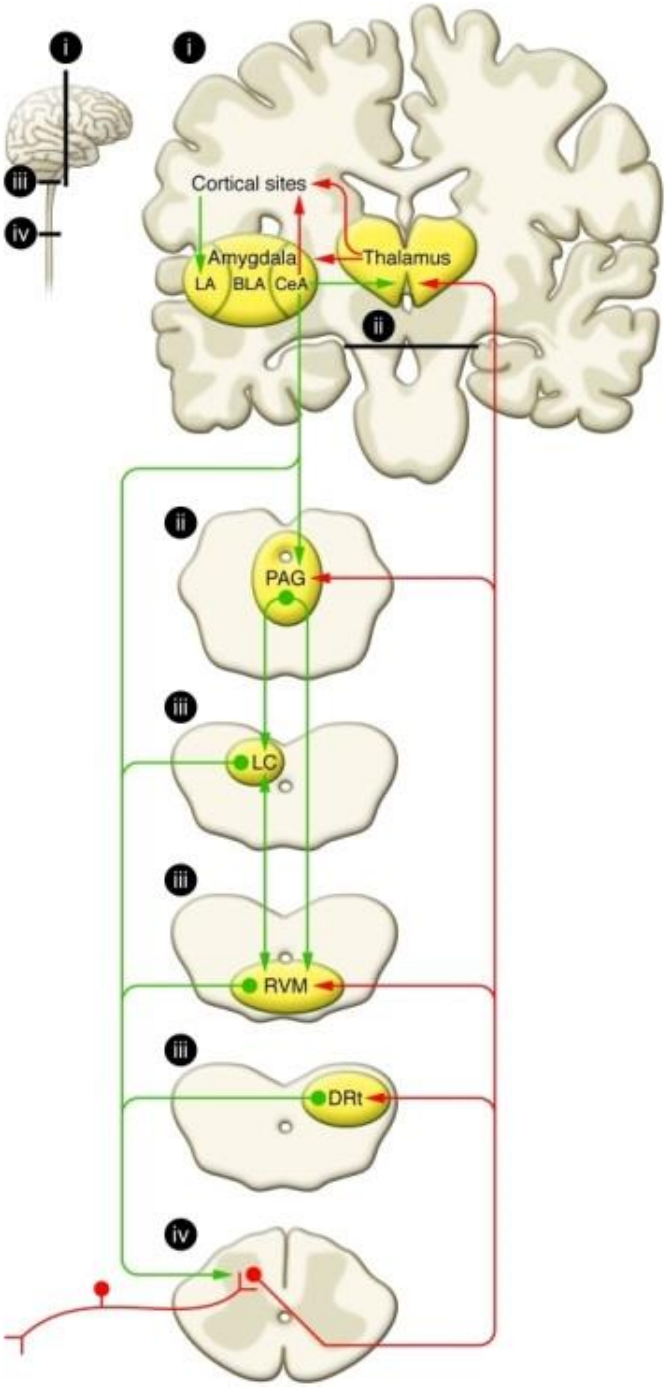



Figure 1.12 Central pain circuitry. Nociceptive inputs to the dorsal horn of the spinal cord synapse onto second order spinal neurons which project to corticothalamic structures as well as nuclei such as the RVM and PAG. Descending pathways also project to, and arise from, these nuclei and can both facilitate and inhibit pain signalling. (Taken from Ossipov et al. (2010)).

## 1.4 Transient Receptor Potential (TRP) channels

The transient receptor potential family of ion channels is a structurally homologous superfamily of cation channels which are highly conserved across many species. TRP channels can be subdivided into families TRPA, C, M, ML, N, P and V according to their amino acid sequences and are implicated in a variety of sensory functions. The first TRP channel was identified in Drosophila where a mutant form of the channel responded transiently to a light stimulus resulting in a decreased $Ca^{2+}$ influx (Hardie and Minke, 1992). Subsequently identified TRP channels have also been associated with roles in many sensory functions.

TRP channels consist of six transmembrane domains which form tetramers around a nonselective cation pore (Figure 1.13). TRPs are all non-selective cation channels, though some exhibit selectivity for $Ca^{2+}$ over $Na^{+}$. Each family of channels have distinguishing patterns of domains expressed at the intracellular C and N terminals and display great diversity in expression, activation and function. TRP channels are believed to show a huge amount of functional redundancy which may be explained by their role in processes vital for survival.

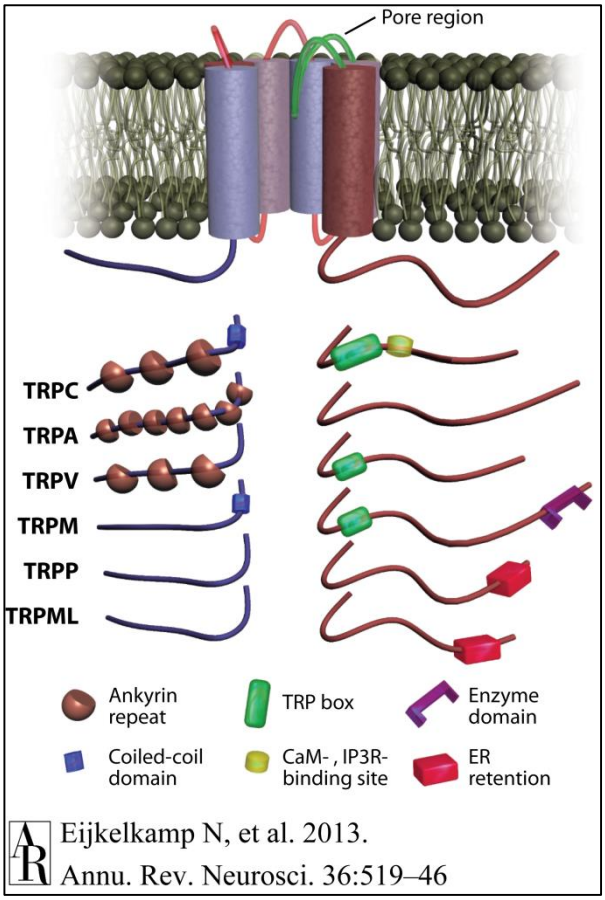



Figure 1.13 TRP channels consist of six transmembrane domains, however other defining features at the N and C termini vary between subfamilies (Adapted from Eijkelkamp et al. (2013))

### 1.4.1 *TRP channel expression in sensory neurons*

Many TRP channels are expressed in DRG neurons and are significant contributors to the functional properties of different neuronal populations. TRPV1 serves a marker for a subset of small diameter, unmyelinated fibres which express the TrkA receptor and is first detected at E12.5 (Hjerling-Leffler et al., 2007). TRPA1 and TRPM8 expression occurs later as these classes of neurons derive from the TRPV1 positive population. TRPA1 is expressed in a portion of TRPV1 positive neurons while TRPM8 is only found in capsaicin insensitive neurons which are neither peptidergic nor IB4 positive. Interestingly, it is more highly expressed in the trigeminal ganglia than the DRG (McKemy et al., 2002). TRPV3 is coexpressed with TRPV1 though TRPV2 overlaps less with TRPV1, predominantly being expressed in medium and large diameter neurons (Caterina et al., 1999, Smith et al., 2002). TRPV4 is expressed in small diameter fibres and is found at high levels in the trigeminal ganglia (Liedtke et al., 2000, Facer et al., 2007) while TRPP2 transcript is the most highly expressed of the TRP channels at all levels of sensory ganglia (Vandewauw et al., 2013). All of the functional TRPC channels are found in primary sensory neurons (Table 1.1) and are some of the most highly expressed in the mammalian nervous system (Elg et al., 2007). TRPC1 is expressed in myelinated and unmyelinated, IB4 negative fibres, in particular large diameter neurons. TRPC3 is one of the most abundantly expressed TRPC channels in the cell bodies of primary afferent neurons and is found in most TRPV1 negative cells (Elg et al., 2007, Quick et al., 2012). In addition, TRPC5 has been found to be localised to small and medium diameter sensory neurons in the periphery (Zimmermann et al., 2011) while TRPC6 is expressed in most DRG neurons (Quick et al., 2012).

| **TRP channel** | **Sensory neuron expression** | **References** |
|---|---|---|
| TRPA1 | Peptidergic small- and medium-diameter Neurons | (Story et al., 2003, Vandewauw et al., 2013) |
| TRPC1 | Predominantly large diameter neurons | (Staaf et al., 2009a) |
| TRPC3 | Predominantly small diameter neurons | (Quick et al., 2012) |
| TRPC4 | Nociceptors and non-nociceptors | (Buniel et al., 2003) |
| TRPC5 | Small diameter TG and DRG neurons | (Gomis et al., 2008) |
| TRPC6 | Most DRG neurons | (Quick et al., 2012) |
| TRPC7 | | (Elg et al., 2007) |
| TRPM2 | Rostrocaudal distribution in sensory neurons | (Vandewauw et al., 2013) |
| TRPM3 | | |
| TRPM4 | | |
| TRPM6 | | |
| TRPM7 | | |
| TRPM8 | Small diameter non-peptidergic neurons; higher expression in TG than DRG | (McKemy et al., 2002, Peier et al., 2002a) |
| TRPML1 | Rostrocaudal distribution in sensory neurons | (Vandewauw et al., 2013) |
| TRPP2 | Rostrocaudal distribution in sensory neurons | (Vandewauw et al., 2013) |
| TRPV1 | Peptidergic small and medium-diameter neurons | (Caterina et al., 1997) |
| TRPV2 | Medium and small diameter neurons | (Caterina et al., 1999) |
| TRPV3 | Peptidergic small and medium-diameter neurons | (Smith et al., 2002) |
| TRPV4 | TG and small and medium diameter neurons | (Liedtke et al., 2000, Facer et al., 2007) |

Table 1.1 TRP channel expression in sensory neurons.

### 1.4.2 *TRP channel structure and heteromer formation*

Recently, the atomic structure of TRPV1 was examined with cryo-electron microscopy. Liao et al. (2013) and Cao et al. (2013) showed that the structure of TRPV1 is comparable to that of voltage gated ion channels. The channel is arranged as a tetrameric structure around a pore which is formed by transmembrane segments S5 and S6 and a pore loop while the voltage sensing residues are replaced by aromatic residues which are proposed to impart stability onto the ion channel (Liao et al., 2013). They found that the TRP domain is positioned to allow interaction with the S4-S5 transmembrane domains and a helix located near to the S1 transmembrane domains and suggest this allows modulation of conformation of the ion channel pore (Liao et al., 2013). In contrast to voltage gated ion channels, TRPV1 is believed to exhibit a dual gating system; it is proposed that a pore helix mediates this complex mechanism by allowing coupling between these gates (Cao et al., 2013). This development in the understanding of TRPV1 channel structure will likely aid understanding of the structure and mechanisms of activation of other TRP channel families.

Despite the atomic structure of TRP channels only recently being confirmed, it has been known for some time that these channels can function in homomeric and heteromeric complexes. Indeed, the tetrameric structure of TRP channels allows different combinations of proteins to form around a pore and their structural homology means that there is promiscuity with TRP channels forming heteromers within subfamilies as well as between subfamilies. For example, TRPC1 is believed to heteromultimerise with all other TRPC channels (Storch et al., 2012) as well as channels from other subfamilies, such as TRPP2 (Kobori et al., 2009) and TRPV4 (Ma et al., 2011). Indeed, homomers overexpressed *in vitro* fail to exhibit currents suggesting that alone, TRPC1 cannot form functional channels (Storch et al., 2012). Other heteromeric complexes also show different functional properties than homomeric forms of their constituent proteins. For example, TRPC4 and TRPC5 when in complex with TRPC1 exhibit a different current-voltage relationship than when expressed as homomeric channels (Clapham, 2003). TRPC5 in HEK293 cells is activated by temperatures below 37$^{o}$C whereas expression of TRPC1 and TRPC5 heteromers did not induce the same current changes in response to cold (Zimmermann et al., 2011). Additionally, when in complex with TRPC1, TRPV4 exhibits altered electrophysiological properties and $Ca^{2+}$ permeability than when expressed as a homomeric channel (Ma et al., 2011). This structural promiscuity conveys

huge functional diversity to TRP channels but also makes dissecting out their individual contributions to sensory function particularly complex.

### 1.4.3 *TRP channels in Mechanosensation*

TRP channels have been widely linked to a role in mechanosensation. In *C.elegans*, the TRP family isoform *osm-9* is required for normal mechanosensory responses to nose touch (Colbert et al., 1997). The *NOMPC* (no mechanoreceptor potential C) gene in Drosophila encodes a channel which is expressed in mechanosensitive bristles. Flies expressing the *NOMPC* mutation have reduced sensitivity to touch shown by a decrease in mechanosensitive currents in these mechanosensory end organs (Kernan et al., 1994, Walker et al., 2000). Furthermore, interfering with the normal function of a vertebrate orthologue of the *NOMPC* channel was subsequently found to induce deafness and vestibular deficits in zebrafish which occurred as a result of altered mechanotransduction in hair cells (Sidi et al., 2003). Additionally, TRPY, the yeast TRP channel, is sensitive to osmotic pressure (Christensen and Corey, 2007).

TRPA1 has also been implicated in mechanosensation. *Drosophila* larvae lacking expression of the TRPA1 homologue, *painless*, are insensitive to noxious mechanical stimuli (Tracey et al., 2003) while Kwan et al. (2006) found TRPA1 null mice exhibit an attenuated response to high-force innocuous and noxious mechanical stimuli. Slowly and intermediately adapting currents are associated with noxious mechanosensation and are absent from small diameter, non peptidergic DRG fibres taken from TRPA1 null mice (Vilceanu and Stucky, 2010, Brierley et al., 2011). Inflammatory and neuropathic models have indicated a complex role for TRPA1 in mechanical hypersensitivity. For example, mechanical hyperalgesia induced by mustard oil, CFA and SNL has been shown to be TRPA1 dependent (Petrus et al., 2007, Eid et al., 2008, Perin-Martins et al., 2013). However, contention over the role of TRPA1 in mechanosensation arose from other findings which suggest no alteration in the mechanical thresholds of TRPA1 knockout mice (Bautista et al., 2006) and that nerve injury induced mechanical allodynia was not resolved by antisense oligonucleotides against TRPA1 (Obata et al., 2005). Another reason that TRPA1 was a favourable candidate for primary mechanotransduction was the finding that it is expressed in mechanosensory hair cells in the inner ear and is blocked by a number of blockers of mechanically induced currents including gadolinium, amiloride and ruthenium red (Nagata et al., 2005). Subsequently however, this idea was challenged by the finding that TRPA1 null mice have unimpaired hearing (Kwan et al., 2006). Differently designed gene knockouts, experimental approaches and use of single or mixed sex mice have been proposed as possible explanations for the discrepancies in

these TRPA1 studies. Importantly, heterologously expressed TRPA1 is not sufficient to confer mechanical sensitivity (Vilceanu and Stucky, 2010) leading to suggestions that TRPA1 may have an indirect role in mechanosensation. For example, one group propose it may regulate mechanical hyperalgesia rather than acute mechanical sensitivity (Petrus et al., 2007) or may only confer mechanical sensitivity to a certain subset of fibres (Brierley et al., 2011). Similarly, it is possible that TRPA1 alone is not sufficient to produce mechanically activated currents in non-neuronal cell lines, as has been reported for other TRP proteins believed to contribute to mechanosensation (Quick et al., 2012). Although TRPA1 may not be a primary mechanotransducer it is interesting to note that human Familial Episodic Pain Syndrome, generated by a point mutation in TRPA1 leads to the onset of cold evoked mechanical pain as a result of increased basal channel activity (Kremeyer et al., 2010).

TRPV1 has been linked to roles in mechanical hypersensitivity in inflammatory and neuropathic pain conditions (Kanai et al., 2005). TRPV4 does not respond to stretching of the membrane *in vitro* and thus may not be gated by mechanical stimulation (Strotmann et al., 2000) however it does appear to be involved in mediating sensitivity to high threshold stimuli in mice (Suzuki et al., 2003). It is expressed in the DRG and in terminals of mechanosensory neurons (Suzuki et al., 2003) and also seems to mediate mechanical hypersensitivity in inflammatory and neuropathic pain states (Alessandri-Haber et al., 2008). Furthermore, TRPV4 is frequently coexpressed with TRPC1 and TRPC6 in DRG and it has been proposed that they may act in concert to mediate these changes in hypersensitivity (Alessandri-Haber et al., 2009).

Many members of the TRPC subfamily are implicated in mechanosensation in both cutaneous and other systems. TRPC1 null animals show a decrease in sensitivity to innocuous mechanical stimuli and sensory neurons in culture show a reduction of SA Aβ fibre firing in response to mechanical stimuli (Garrison et al., 2012). TRPC1, along with a number of other TRP channels, is expressed in Merkel cells where they have a role in mechanically induced $Ca^{2+}$ influx (Haeberle et al., 2008). When both TRPC3 and TRPC6 are deleted together, mice display attenuated sensitivity to light touch as well as a reduction in mechanically activated, RA currents in cultured DRG (Quick et al., 2012).Vascular smooth muscle tone is believed to rely on mechanical sensitivity of TRPC6 while it also has mechanosensory roles in sensing glomerular pressure in the kidney where members of the TRPP family are also implicated in mechanosensory

function (Dietrich et al., 2005, Huber et al., 2006, Spassova et al., 2006, Christensen and Corey, 2007). The role of TRPC channels in mechanosensation is discussed further in section 4.2.1.

In spite of the substantial evidence that TRP channels play a role in mechanosensation, their function as a pore forming, directly mechanosensitive ion channel has only recently been shown. In *C.elegans*, TRP-4 (a member of the NOMPC/TRPN subfamily) exhibits a rapid onset, mechanically activated current. A point mutation in the pore encoding region of the channel is able to alter its ion selectivity providing convincing evidence that the channel itself forms a pore subunit (Kang et al., 2010).

### 1.4.4 *TRP channels and pain*

As well as a role in mechanosensation TRP channels have critical roles in physiological and pathological pain sensation.

One of the most widely studied TRP channels, TRPV1 was identified as a capsaicin sensitive ion channel which also responds to noxious thermal stimuli (temperatures >43 °C) and low pH (Caterina et al., 1997, Caterina et al., 2000). However, its role in sensing noxious heat was called into question when it was shown that while TRPV1 KO mice lose sensitivity to capsaicin and low pH, they exhibit limited alteration in acute noxious heat sensitivity (Caterina et al., 2000, Davis et al., 2000, Woodbury et al., 2004). TRPV2 is also activated by temperatures above 52ºC *in vitro* (Caterina et al., 1999), although 82% of heat-sensitive neurons in TRPV1 null mice do not express TRPV2 suggesting that the retained sensitivity to noxious heat is also independent of TRPV2 (Woodbury et al., 2004). *In vitro*, TRPV3 and TRPV4 produced currents at temperatures of 33–39 °C and 25–34 °C, respectively though neither single nor double TRPV3/TRPV4 KO mice showed total loss of noxious thermal sensitivity (Liedtke et al., 2000, Peier et al., 2002b, Smith et al., 2002, Xu et al., 2002, Huang et al., 2011). Diphtheria toxin mediated ablation of TRPV1-positive sensory neurons does, however, abolish responses to noxious heat and thermal hyperalgesia showing this subset of neurons is critical for heat sensing (Mishra et al., 2011).

Although the role for TRPV channels in acute noxious thermal sensitivity *in vivo* is not conclusive, they play crucial roles in pathological conditions. TRPV1 null animals do not display inflammation induced thermal hyperalgesia (Caterina et al., 2000, Davis et al., 2000). Also, elevated TRPV1 expression is seen in sensory neurons of the DRG and in lamina I and II of the spinal cord following tissue damage and inflammation (Amaya et al., 2003, Zhou et al., 2003, Luo et al., 2004). TRPV1 is also sensitised by pro-inflammatory mediators; this leads to induction and maintenance of thermal hyperalgesia, via protein kinase A (PKA) and protein kinase C (PKC) dependent mechanisms. For example, PKA enhances gating and increases TRPV1 translocation to the membrane (Zhang et al., 2008) while PKC phosphorylates TRPV1 to lower its activation threshold and potentiate channel activity (Premkumar and Ahern, 2000, Vellani et al., 2001). Other inflammatory mediators act to increase TRPV1 activity allowing it to contribute to nociceptor sensitisation in pathological conditions. These include prostaglandins, for example PGE2 and PGI2 which reduce the thermal threshold of TRPV1 to ~35 °C

(Moriyama et al., 2005), chemokines (Zhang et al., 2005a, Zhang et al., 2005b), 5- HT (Loyd et al., 2011), phosphoinositides (Zhuang et al., 2004) and bradykinin (Ferreira et al., 2004). TRPV1 expression is downregulated in damaged neurons following nerve injury and upregulated in surrounding, undamaged neurons (Hudson et al., 2001, Fukuoka et al., 2002). TRPV1 expression is also reduced in human diabetic and motor neuropathies (Lauria et al., 2006, Facer et al., 2007). NGF acts on TRPV1 in a number of ways including upregulation of its transcription, increased TRPV1 phosphorylation and increase translation and transport of TRPV1 to the periphery (Ji et al., 2002) which contributes to and maintains thermal hyperalgesia in both inflammatory and neuropathic conditions.

In addition to TRPV1, other TRPV channels play a role in inflammatory and neuropathic pain conditions. TRPV4 is sensitised by ATP (Phelps et al., 2010) which contributes to the inflammatory response. Although loss of TRPV3 function reportedly does not affect thermal hyperalgesia *in vivo* (Moqrich et al., 2005), other studies have found that TRPV3 activation in keratinocytes leads to release of prostaglandins and causes increased sensitivity to acute noxious heat and thermal hyperalgesia in a TRPV1- independent manner (Huang et al., 2008) and that TRPV3 expression is seen in intact nerves after injury (Facer et al., 2007).

Cold hyperalgesia is a symptom of diseases such as rheumatoid arthritis (Jahanshahi et al., 1989), and cold allodynia is a common feature of many neuropathic pain states including those caused by traumatic nerve injury and post-herpetic neuralgia (Jorum et al., 2003). Under physiological conditions, the threshold for noxious cold, as distinct from innocuous cool, is considered to occur at temperatures <15 °C. Two TRP channels, TRPM8 and TRPA1 both contribute to transduction of cool and cold stimuli.

TRPM8 is sensitive to menthol (a cooling compound) and cold, with a threshold of ~25°C, and is activated by temperatures encompassing the innocuous cool and noxious cold range(McKemy et al., 2002, Peier et al., 2002a) Though TRPM8 has an established role for the channel in detection of innocuous cooling its role in detection of noxious stimuli is more controversial as two groups found loss of TRPM8 function *in vivo* did not alter cold sensitivity (Bautista et al., 2007, Dhaka et al., 2007) while two others report decreased cold sensitivity in the absence of TRPM8 (Colburn et al., 2007, Gentry et al., 2010). Because TRPV1 is expressed in neurons expressing TRPM8 during development

but TRPV1 is subsequently downregulated, toxin mediated ablation of TRPV1 containing sensory neurons also caused ablation of TRPM8 and caused abolition of responses to acute noxious cold (Mishra et al., 2011). In neuropathic pain models, TRPM8 is critical for cold allodynia evoked by cooling compounds such as acetone (Colburn et al., 2007, Caspani et al., 2009) though nerve injury induced changes in TRPM8 expression are controversial (Proudfoot et al., 2006, Caspani et al., 2009) and menthol induced cold hypersensitivity is mediated by TRPA1, not TRPM8 (Gentry et al., 2010).

TRPA1 was first suggested as a noxious cold sensor as it has an activation threshold of 17$^{o}$C when expressed heterologously which is close to the cold temperature considered to be painful and it is co-expressed with TRPV1 in nociceptive neurons (Story et al., 2003). Contention over the role of TRPA1 in acute cold sensation has arisen from behavioural studies in TRPA1 KO mice. Some groups, including Kwan et al. (2006), observed a decrease in cold sensitivity while others, such as Bautista et al. (2006), did not observe any changes in cold sensitivity at the behavioural or electrophysiological level. A number of explanations have attempted to resolve this contention, for example that different techniques of cold assessment (Gentry et al., 2010) and experimental setup (Kwan et al. found a greater deficit in the response of female mice while Bautista et al. only used males) are responsible for the conflict. The design of the gene knockout has also been suggested as a potential source of variability. TRPA1 'null' mice used by both groups are thought to express a truncated form of the protein, it has been suggested that this truncated form may exert an effect on other implicated channels, thus affecting the phenotype of the null mice (Foulkes and Wood, 2007). It has also been suggested that rather than being directly activated by cold, TRPA1 is activated by a rise in intracellular $Ca^{2+}$ which is induced by cold (Zurborg et al., 2007) though it was subsequently shown that TRPA1 could be activated by cold in the absence of both intra- and extracellular $Ca^{2+}$ (Karashima et al., 2009).

In pathological conditions, genetic and pharmacological disruption of TRPA1 function reduces cold hypersensitivity (Stucky et al., 2009, del Camino et al., 2010, Gentry et al., 2010). This includes rodent models of neuropathic and inflammatory pain; for example TRPA1 activation is responsible for the pain behaviour produced in the first phase of the Formalin test by neuronal excitation (Macpherson et al., 2007). As well as this, rodent models of neuropathic pain have shown that damaged neurons display a reduction in TRPA1 expression while expression is increased in intact neurons (Obata et al., 2005,

Katsura et al., 2006, Caspani et al., 2009). TRPA1 does not confer cold sensitivity to HEK cells, despite producing allyl isothiocyanate (AITC) induced currents, however in both HEK cells and cultured sensory neurons, agonist and inflammatory agent evoked TRPA1 currents are markedly potentiated in the presence of even mild cooling (del Camino et al., 2010). Together, this data provides convincing evidence that TRPA1 is necessary for cold hypersensitivity in pathological conditions though its role as an acute noxious cold sensor requires more investigation.

Importantly, it has been shown that a small population of cold sensitive neurons does not respond to menthol or AITC suggesting TRPM8 and TRPA1 independent mechanisms of cold sensitivity (Babes et al., 2004). This may also provide some explanation for the discordance between results from single channel animal KO studies.

## 1.5 Acid sensing ion channels

Acid sensing ion channels (ASICs) are a family of voltage-independent, ligand gated ion channels which are constituents of the amiloride-sensitive degenerin/epithelial sodium (DEG/ENaC) channel family. They are highly conserved across evolution, are widely expressed in the nervous system, form homomeric and heteromeric complexes and, much like TRP channels, have diverse roles in sensory functions.

Encoded by ACCN (amiloride sensitive cation channel) genes 1-4, there are six 'major' ASICs; ASIC1a, ASIC1b, ASIC2a, ASIC2b, ASIC3 and ASIC4. ASIC1a and b are alternative splice products of ACCN2; ASIC2a and b are alternative splice products of ACCN1. Another protein, ASIC5, or BASIC, is amiloride sensitive but only shares low sequence homology with other ASICs (Schaefer et al., 2000, Lin et al., 2015b). ASICs show high sequence homology and form trimeric complexes; each constituent protein consists of two transmembrane domains, cytosolic N and C termini and large, multidomain extracellular regions. The trimeric structure of ASICs (Figure 1.14) was confirmed when the crystal structure of the chicken ASIC1 protein was resolved (Jasti et al., 2007). The transmembrane domains of ASIC subunits are crucial; their interactions with surrounding lipids and each other provides stability to the channel while they also define the interior of the pore and TM2 contributes to its ion selectivity (Jasti et al., 2007, Gonzales et al., 2009). The expression pattern, biophysical properties and functional roles of ASICs vary greatly and are examined in brief below.

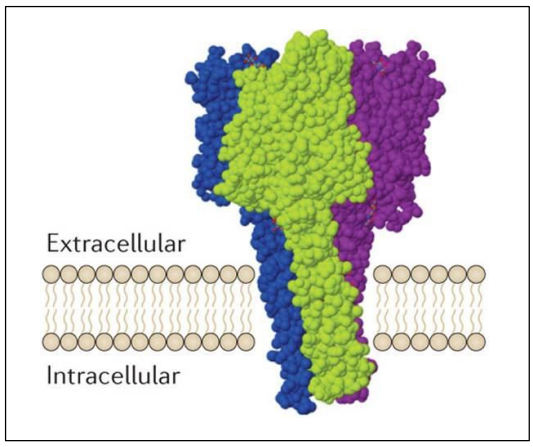



Figure 1.14 The trimeric structure of ASICs was confirmed through resolution of the crystal structure of chicken ASIC1 (Modified from Wemmie et al. (2013)).

### 1.5.1 *ASIC expression*

ASICs are expressed in neuronal and non-neuronal tissue including in the cell bodies and peripheral terminals of primary sensory neurons and mechanosensory end organs. ASIC1a is localised to a subset of TRPV1 positive neurons, whereas ASIC1b has specific expression and ASIC3 preferential expression in the peripheral nervous system. ASIC1, 2 and 3 are all found in large and small diameter DRG neurons (Table 1.2). ASIC1, 2 and 3 are also expressed in cell bodies of the trigeminal ganglia (Rahman et al., 2011).

In hairy skin, ASIC2a and ASIC3 are expressed in lanceolate endings surrounding the guard hair follicles (Price et al., 2000, Price et al., 2001) while in glabrous skin, ASIC2 and ASIC3 are present in Meissner corpuscles. Both of these mechanosensory end organs are associated with RA mechanoreceptors. In addition, ASIC3 appears to be present in Merkel cell complexes and free nerve endings associated with the SA mechanically activated current (Price et al., 2001). Expression of ASIC3 in rodents is primarily restricted to sensory ganglia and while some expression has been reported in the spinal cord (Baron et al., 2008), in humans ASIC3 is highly expressed in both spinal cord and brain (Delaunay et al., 2012). ASIC4 was originally cloned from a DRG cDNA library and was found in large diameter fibres (Akopian et al., 2000, Usoskin et al., 2015), though reports of ASIC4 expression in sensory ganglia are contested (Lin et al., 2015a) (see section 7.2.1).

In the central nervous system, ASIC1a, ASIC2 and ASIC4 are all broadly expressed in regions including the spinal cord, cerebellum, amygdala and hippocampus (Table 1.2) in both homomeric and heteromeric forms. ASIC4 expression in cerebellum is increased in adult compared to newborn rats while its expression in spinal cord is downregulated after birth (Akopian et al., 2000); the highest expression of ASIC4 however is observed in the pituitary gland (Lin et al., 2015a).

| ASIC | Tissue expression | Reference |
|---|---|---|
| ASIC1a | DRG; TG; nodose ganglia | Waldmann et al. (1997b) (rat); Rahman et al. (2011); Walder et al. (2010); Page et al. (2004); Alvarez de la Rosa et al. (2002) (rat) |
| | Spinal cord | Wu et al. (2004) (rat); Baron et al. (2008) (mouse) |
| | Brain | Waldmann et al. (1997b) (human; rat); Alvarez de la Rosa et al. (2003) (mouse) |
| ASIC1b | DRG; TG; nodose ganglia | Page et al. (2004) (mouse); Page et al. (2005) (mouse); Rahman et al. (2011) ; Alvarez de la Rosa et al. (2002) (rat) |
| ASIC2a | DRG; TG; nodose ganglia | Page et al. (2005) (mouse); Price et al. (2000) (mouse); Cano14 (human); Alvarez de la Rosa et al. (2002) (rat) |
| | Mechanosensory end organs | (Cabo et al., 2015) (human) |
| | Spinal cord | (Wu et al., 2004)(rat); (Baron et al., 2008) (mouse) |
| | Brain | (Price et al., 2000) (mouse); (Roza et al., 2004) (mouse) |
| ASIC2b | DRG; TG; nodose ganglia | (Page et al., 2005) (mouse); (Alvarez de la Rosa et al., 2002) (rat) |
| | Spinal cord | (Wu et al., 2004)(rat); (Baron et al., 2008) (mouse) |
| | Brain | (Roza et al., 2004) (mouse) |
| ASIC3 | DRG; TG; nodose ganglia | (Page et al., 2005) (mouse); (Price et al., 2001) (mouse); (Chen et al., 2002) (mouse); (Alvarez de la Rosa et al., 2002)(rat); (Sluka et al., 2003) (mouse); (Waldmann et al., 1997a)(rat) |
| | Mechanosensory end organs | (Price et al., 2001) (mouse); |
| | Spinal cord | (Baron et al., 2008) (mouse); (Delaunay et al., 2012) (human) |
| | Brain | (Delaunay et al., 2012) (human) |
| ASIC4 | Brain (regions include: amygdala, striatum, hippocampus, cerebellum) | (Lin et al., 2015a) (mouse); (Grunder et al., 2000) (rat); (Akopian et al., 2000) (rat) |
| | Pituitary gland | (Lin et al., 2015a) (mouse) |
| | Spinal cord | (Lin et al., 2015a)(mouse); (Grunder et al., 2000) (rat); (Akopian et al., 2000) (rat) |
| | DRG | (Akopian et al., 2000) (rat; low levels); (Alvarez de la Rosa et al., 2002)(rat); |
| | Vestibular system | (Grunder et al., 2000) (rat) |

Table 1.2 ASIC subunit expression patterns.

### 1.5.2 *Biophysical properties of ASICs*

An excess of protons ($H^+$) is found in conditions such as inflammation, ischaemia and at the synaptic cleft following release of neurotransmitter. Proton gated currents in the nervous system are heterogeneous and mediated by TRPV1 (Bevan and Yeats, 1991) and ASICs which form homomeric and heteromeric complexes. The description of proton gated currents in sensory neurons by Krishtal and Pidoplichko (1981) was followed in 1997 (Waldmann et al.) by the cloning of the first ASIC, shown to produce a proton gated current which was selective for $Na^+$ ions. All ASICs, excluding ASIC2b and ASIC4 (Lingueglia et al., 1997), are gated by protons; it has recently been suggested that it is likely ASICs bind more than one proton during activation and that many ASIC residues are involved in binding protons (Grunder and Pusch, 2015). The large extracellular domain of ASICs contains a negatively charged cavity containing carboxyl-carboxylate groups which allow proton binding in low pH conditions (Jasti et al., 2007). Proton binding is thought to cause movement of the disulphide rich region, also in the extracellular loop, leading to rotation of the transmembrane domains to open the activation gate (Jasti et al., 2007, Gonzales et al., 2009). Sustained exposure to low pH can cause ASICs to desensitise through movement of the transmembrane domains that form a desensitisation gate (Gonzales et al., 2009). Desensitisation occurs over a period of hundreds of milliseconds to seconds, depending on the subunit involved (Table 1.3). ASIC3, however, does not fully desensitise (Waldmann et al., 1997a) and ASIC1a homomers exhibit the phenomenon of tachyphylaxis (Chen and Grunder, 2007, Li et al., 2012), though this sustained desensitisation after repeated activation requires strong acidification so may not play a major role in physiological activation (Grunder and Pusch, 2015).

ASIC subunits show different cation selectivity with most subunits preferentially conducting $Na^+$ and only ASIC1a showing $Ca^{2+}$ permeability. Heterologous expression systems have been used to identify the characteristic currents produced by ASIC homomeric and heteromeric complexes, which has led to a better understanding of the contributions of different ASIC subunits *in vivo* (Figure 1.15). In particular, the functional properties of many heteromeric complexes have been linked to physiological roles in the nervous system that cannot be accounted for by homomeric complexes alone.

ASIC1a is activated by a rapid drop in pH (<1ms) to below 6.9 and produces a transient, rapidly desensitising inward current which is slow to recover (Waldmann et al., 1997b).

Similar to ASIC1a, ASIC1b produces an amiloride sensitive, rapidly activating and desensitising current at pH5.9 (Chen et al., 1998, Sutherland et al., 2001); it is permeable to $Na^+$ and $K^+$ but its human orthologue is additionally permeable to $Ca^{2+}$ (Hoagland et al., 2010). ASIC2a is activated at a much lower pH than ASIC1a or ASIC3 and has slower inactivation kinetics (Lingueglia et al., 1997). When expressed alone, ASIC2b is insensitive to protons though it can form heteromeric complexes with ASIC2a where it produces a non-selective slowly inactivating current, not seen with ASIC2a expression alone (Lingueglia et al., 1997). ASIC2b also forms functional heteromeric complexes with ASIC3. As well as producing a $Na^+$ selective transient current, ASIC3 exhibits a sustained current which is not inhibited by amiloride (Waldmann et al., 1997a). When expressed as a homomer, this current is $Na^+$ selective, though when coexpressed with ASIC2b, the sustained current appears to be non-selective which is consistent with the sustained current seen in sensory neurons. This sustained current is likely to be key to acid sensitivity in the periphery where pH decreases slowly and over a sustained period in inflammation and ischaemia (Grunder and Pusch, 2015). Importantly, this current is also able to sensitise neurons to other stimuli (Deval et al., 2003) while the transient currents of ASIC1 and ASIC3 are sufficient to depolarise sensory neurons (Mamet et al., 2002, Deval and Lingueglia, 2015).

ASIC1a also forms functional heteromeric complexes with ASIC2a and 2b (Bassilana et al., 1997, Sherwood et al., 2011, Bartoi et al., 2014). Proton gated currents in hippocampal neurons are consistent with the properties of ASIC1a/2a heteromers (Askwith et al., 2004) as well as ASIC1a/2b complexes which also appear to mediate neuronal cell death in CNS neurons (Sherwood et al., 2011). The ratio of ASIC1a/2a subunits in heteromeric complexes is believed to regulate their function in CNS neurons (Jones et al., 2004, Li et al., 2010); ASIC1a/2a complexes can form in a stoichiometry of 2:1 or 1:2 and do so in cells also expressing functional homomeric channels (Bartoi et al., 2014). It has been suggested that the ASIC1a/2a complex is less proton sensitive than ASIC1a homomers in order to prevent sustained desensitisation in the presence of continued acidification (Baron et al., 2008). Clearly, the heteromerisation of ASIC subunits confers functional diversity and allows fine tuning of their biophysical properties; as such, understanding their subunit interactions has, and continues, to elucidate more about the physiological roles of ASICs.

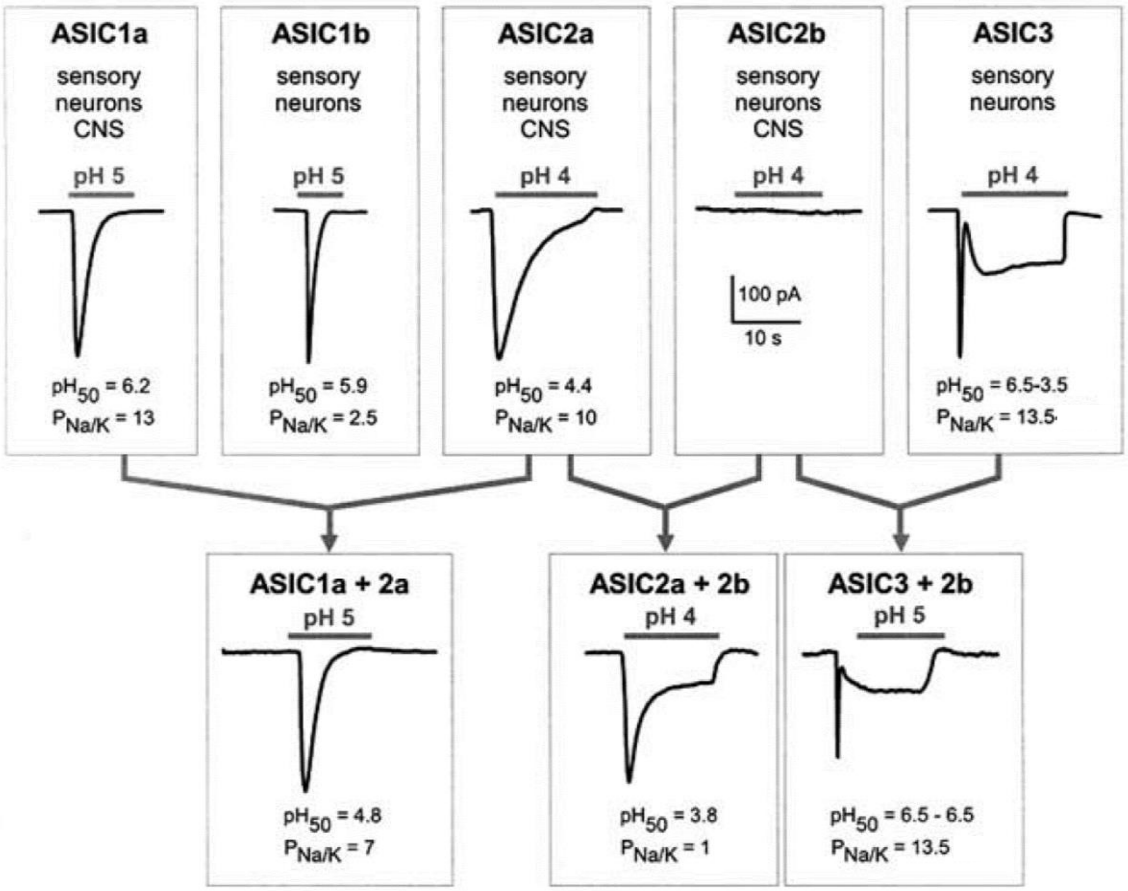



Figure 1.15 Currents produced by ASIC subunits expressed in COS cells vary by subunit as does ion selectivity. Expression of multiple subunits likely leads to formation of heteromeric channels with different characteristics to their component homomers. Taken from Voilley (2004)).

| ASIC | Ion permeability | Activation pH | $\tau_{activation}$ (ms at pH 6.0) | $\tau_{desensitisation}$ (s) | $\tau_{recovery}$ (s, at pH 7.4) | References |
|---|---|---|---|---|---|---|
| ASIC1a | $Na^+$>Ca2$^+$>$K^+$ | 6.4-6.6 | 6-13 | 1.2-3.5 | 13 | (Waldmann et al., 1997b); (Sutherland et al., 2001); (Bartoi et al., 2014); (Yermolaieva et al., 2004) |
| ASIC1b | $Na^+$, $K^+$ ($Ca^{2+}$ human orthologue) | 5.9 | | 1.7 | 5.9 | (Chen et al., 1998); (Sutherland et al., 2001); (Hoagland et al., 2010) |
| ASIC2a | $Na^+$ | 4.5-5.5 | | 3.15 | | (Lingueglia et al., 1997); (Bartoi et al., 2014) |
| ASIC2b | - | - | - | - | - | (Lingueglia et al., 1997) |
| ASIC3 | $Na^+$>$K^+$ | 6.5-6.7 (transient); ≤4 (sustained) | <5 | 0.3 | 0.4-0.6 | (Waldmann et al., 1997a); (Sutherland et al., 2001) |
| ASIC4 | - | - | - | - | - | (Akopian et al., 2000) |
| ASIC1a/2a | $Na^+$, $K^+$ | 4.8-5.4 | | 0.6-0.9 | 0.4-0.6 | (Bassilana et al., 1997); (Bartoi et al., 2014) |
| ASIC1a/2b | $Na^+$, $K^+$, $Ca^{2+}$ | 6.4 | | 2.6 | | (Sherwood et al., 2011) |
| ASIC3/2b | Non selective | 6.5 | | | | (Lingueglia et al., 1997) |

Table 1.3 Biophysical properties of ASICs.

### 1.5.3 *ASICs in mechanosensation*

The mec-4 and mec-10 members of the family of DEG/ENaC channels contribute to a complex that transduces touch in *C.elegans* (Goodman et al., 2002, Chalfie, 2009). Mutations in these proteins alter ion selectivity of the channel complex showing that these channels are pore forming subunits (O'Hagan et al., 2005). These orthologues of mammalian ASICs are vital for production of normal mechanically activated currents and behavioural responses to touch. Stomatin-like protein3 is needed for normal touch sensation and associates with and blocks the currents produced by ASIC2a, ASIC2b and ASIC3 (Wetzel et al., 2007). These findings, along with ASIC structure, expression in mechanosensory structures, and early knockout data showing impaired mechanosensory function *in vivo* indicated that ASICs may be potentially interesting candidate mechanotransducers.

An ASIC3 knockout mouse showed increased sensitivity to light touch but decreased responses to noxious pinch stimuli (Price et al., 2001) while impairing the function of ASIC2 in the firing of RA mechanosensitive fibres produced conflicting data (Price et al., 2000, Roza et al., 2004). In light of the heteromerisation of ASICs, some groups have investigated their function in combination. For example, a model which inactivated all native neuronal ASIC currents through expression of a dominant negative ASIC3 subunit found heightened sensitivity to cutaneous mechanical stimuli (Mogil et al., 2005). Similarly, an ASIC1a, ASIC2 and ASIC3 triple KO mouse showed an increased sensitivity to stimulation with von Frey fibres (Kang et al., 2012). Altogether these data indicated that ASICs may negatively regulate mechanosensation.

Electrophysiological properties of mechanosensitive fibres in ASIC null mice have also highlighted ASIC mediated changes in mechanosensitivity but which conflict with some of the behavioural findings. Though ASIC1a, ASIC2, ASIC3 triple KOs display increased spike frequency in response to mechanical stimulation (Kang et al., 2012) in the absence of just ASIC2, RA and SA MA currents were decreased (Price et al., 2000) while the absence of ASIC1a produced no changes and lack of ASIC3 caused decreased A mechanoreceptor firing but an increase in RA currents (Chen and Wong, 2013). Furthermore, Drew et al. (2004) found that cultured DRGs from ASIC2 and ASIC3 null mice did not show any difference in mechanically induced currents compared to wild types. In agreement with this, it was found that ASIC1a, ASIC2 and ASIC3 single KO

mice show no changes in acute mechanosensation (Roza et al., 2004, Staniland and McMahon, 2009).

It has been suggested that while ASICs may not be involved in cutaneous mechanosensation, their presence in deep tissues may be more important. For example, ASIC2 appears to play a role in mechanosensitivity associated with the baroreflex (Lu et al., 2009) and it may also have a role in the viscera (Roza et al., 2004, Page et al., 2005). Loss of ASIC1a produces an increase in visceral mechanosensitivity while loss of ASIC3 decreases mechanical sensitivity in a subset of visceral afferents (Page et al., 2004, Roza et al., 2004, Jones et al., 2005, Page et al., 2005).

### 1.5.4 *ASICs in pain*

Protons are a feature of inflammation and countless animal and human studies have shown that administration of acidic stimuli leads to pain (Wemmie et al., 2013); understanding the contribution of ASICs is therefore particularly important to develop a better understanding of mechanisms involved in pain and inflammation.

In DRG neurons, acid evoked currents can be produced *in vitro* in capsaicin sensitive and insensitive neurons and can lead to neuron depolarisation (Bevan and Yeats, 1991, Steen et al., 1992, Leffler et al., 2006). Indeed, proton sensitivity of DRG neurons involves activity of TRPV1 (Bevan and Yeats, 1991, Tominaga et al., 1998) but amiloride, a blocker of ASICs, reduces pain in inflammatory conditions (Waldmann et al., 1997b) while NSAIDs such as ibuprofen and aspirin inhibit proton induced currents independently of TRPV1 (Voilley et al., 2001) suggesting proton induced ASIC currents also contribute to nociceptive function in sensory neurons. Proton-gated currents are still present in rat DRG neurons in the presence of TRPV1 agonist, capsazepine and these currents are inhibited by administration of morphine (Cai et al., 2014). In humans, cutaneous acid infusion produced pain shown to be dependent on both ASICs and TRPV1 (Jones et al., 2004) while another study found that amiloride but not capsazepine block currents evoked by mildly acidic pH (Ugawa et al., 2002) suggesting the contribution of these channels to acid sensation may be pH dependent. Importantly, TRPV1 requires pH <6 for activation but cutaneous nociceptors can be activated by a drop to just pH6.9. Moreover, in muscle tissue, a drop in pH from 7.4 to 7.0 can be sufficient to induce persistent neuronal activity, suggesting TRPV1 is unlikely to be the sole mediator of acid evoked currents in nociceptive neurons (Issberner et al., 1996, Ugawa et al., 2002). Though TRPV1 plays an important role in acid evoked nociception, there is a great deal of evidence that ASICs are critical as well.

Inflammatory mediators such as NGF, BDNF and 5-HT promote upregulation of ASIC transcription. It has also been shown that NGF-mediated-increase in ASIC3 levels leads to an increase in the proportion of cells expressing ASICs, increased ASIC3 current amplitude, and ultimately to increased neuronal excitability (Mamet et al., 2002). In addition to changes in sensory neurons, expression of ASIC1a and ASIC2a is increased in the spinal cord after peripheral inflammation (Wu et al., 2004). Dorsal horn neurons produce acid evoked currents which are independent of capsaicin and appear to be

mediated by ASIC1a with ASIC2a and ASIC2b contributing to some of the current types produced, likely through heteromer formation (Baron et al., 2008).

Animal models have been used to investigate the effect of disrupting ASIC function on cutaneous and deep tissue acute and inflammatory pain *in vivo*. Since ASIC3 produces a sustained current which is thought to be critical for the pain resulting from tissue acidosis (Waldmann et al., 1997a), it has been particularly well studied in animal models with sometimes conflicting results. While some groups report loss of ASIC3 function leads to a decrease in acid sensitivity and a decrease in heat sensitivity (Price et al., 2001) others have found hypersensitivity to acidic, thermal and mechanical stimuli (Chen et al., 2002). Yen et al. (2009) and Deval et al. (2008) found ASIC3 siRNA mediated knockdown and ASIC3 KO attenuated inflammatory thermal and mechanical hypersensitivity. Another investigation of single KO of ASIC1, ASIC2 and ASIC3 showed unimpaired acute and inflammatory sensitivity in all groups (Staniland and McMahon, 2009). Disrupting the function of all acid evoked, neuronal ASIC currents through expression of a dominant negative ASIC3 subunit produced hypersensitivity to a number of inflammatory visceral and cutaneous insults as well as acute, noxious mechanical but not thermal stimuli (Mogil et al., 2005). Two models of neuropathic pain produced mechanical and thermal hypersensitivity which was reversed by treatment with the ASIC1a blocker, Psalmotoxin1 (PcTx1) through activation of endogenous enkephalins (Mazzuca et al., 2007). On the other hand, ASIC3 KO mice showed an increase in sensitivity to dynamic mechanical stimuli in neuropathic conditions (Borzan et al., 2010). Clearly, the role for ASICs in cutaneous mechanosensation is complex, though the use of specific toxins and synthetic peptides which bind to ASICs have gone some way to elucidating more about the way these channels may be activated and thus contribute to nociception.

It has been suggested that through their large, extracellular domain, ASICs may have ligands other than protons which lead to pain sensation or that may increase their sensitivity to protons. For example, a small synthetic molecule, 2-guanidine-4-methylquinazoline (GMQ), binds to ASIC3 outside of its $H^+$ binding site (Figure 1.16) and when injected *in vivo*, causes pain behaviour which is not observed in ASIC3 knockout mice (Yu et al., 2010). Agmatine, an endogenous polyamide, also produced this pain behaviour acting through the same ligand binding site. Its activity was potentiated by mild acidosis and coadministration of Agmatine with other inflammatory mediators augments pain behaviour produced by inflammatory mediators alone (Li et al., 2010).

Importantly, these molecules produce sustained activation of ASIC3, as opposed to the transient currents produced by low pH, which may indicate a mechanism for the long term effects of ASICs in painful conditions (Yu et al., 2010, Wemmie et al., 2013). Similarly, hind paw injection of a snake venom, MitTx (*Micrurus tener tener*) produces pain behaviour in WT but not ASIC1a KO mice and to a lesser degree in ASIC3 KO mice while *in vitro*, this venom selectively produces persistent activation of ASIC1a, ASIC1b and ASIC3 and enhances the proton efficacy of ASIC2a (Bohlen et al., 2011, Wemmie et al., 2013). It is possible, therefore, that endogenous ligands of ASICs potentiate their sensitivity to protons allowing their activation at physiologically relevant pH; for example, through binding of polyamines such as agmatine, which accumulate in inflammatory conditions (Yu et al., 2010). Also, Deval et al. (2008) argue that in the presence of arachidonic acid, ASIC3 can be activated at physiological pH, 7.4. Indeed, arachidonic acid and hyperosmolarity are both features of inflammatory conditions and synergistically reduce the pH dependence of ASIC3 in rat DRG and can activate the channel at physiologically relevant levels (Deval et al., 2008).

Deep tissue pain in structures such as muscles, joints and viscera is a huge clinical problem (Sikandar and Dickenson, 2012). ASICs are expressed in peripheral sensory neurons which innervate skeletal muscle (Gautam and Benson, 2013); interestingly, evidence suggests that ASIC1 and ASIC3 may contribute differentially to deep tissue pain. In a model of chronic muscle pain, the development of secondary mechanical hyperalgesia is ASIC3, but not ASIC1, dependent (Sluka et al., 2003, Sluka et al., 2007). Similarly, joint and muscle inflammation produces secondary mechanical hyperalgesia of the paw which is ASIC3 dependent, while primary mechanical hyperalgesia at the site of inflammation was shown to be mediated by ASIC1 (Ikeuchi et al., 2008, Walder et al., 2010). ASIC3 mRNA is upregulated in fibres innervating muscle (Walder et al., 2010) though ASIC3 was shown to be necessary for the development rather than the maintenance of mechanical hyperalgesia (Karczewski et al., 2010, Chen et al., 2014). In a human study of patients with inflammatory bowel conditions, ASIC expression was upregulated in the intestine compared to patients with uninflamed viscera (Yiangou et al., 2001), while animal models showed the importance of ASIC1 and ASIC3 in mediating mechanical hypersensitivity in inflammatory conditions (Jones et al., 2005, Staniland and McMahon, 2009). Acidosis in cancer tumour microenvironment is believed to contribute to pain though the role of ASICs in this mechanism is still incompletely understood (Sluka

et al., 2009, Damaghi et al., 2013). For example, ASIC1a and ASIC1b mRNA was upregulated in a rodent model of cancer induced bone pain (Nagae et al., 2007) while conversely, ASIC2 upregulation was shown to attenuate migration of glioma cells (Vila-Carriles et al., 2006, Damaghi et al., 2013). ASIC3 is also implicated in pain associated with cardiac ischaemia (Sutherland et al., 2001). Clearly there is an important role for ASICs in pain sensitivity in normal as well as pathological states though a better understanding of the nociceptive signalling mechanisms of these channels in the nervous system would be beneficial to the understanding of pain processing and potentially to the search for more effective analgesic targets.

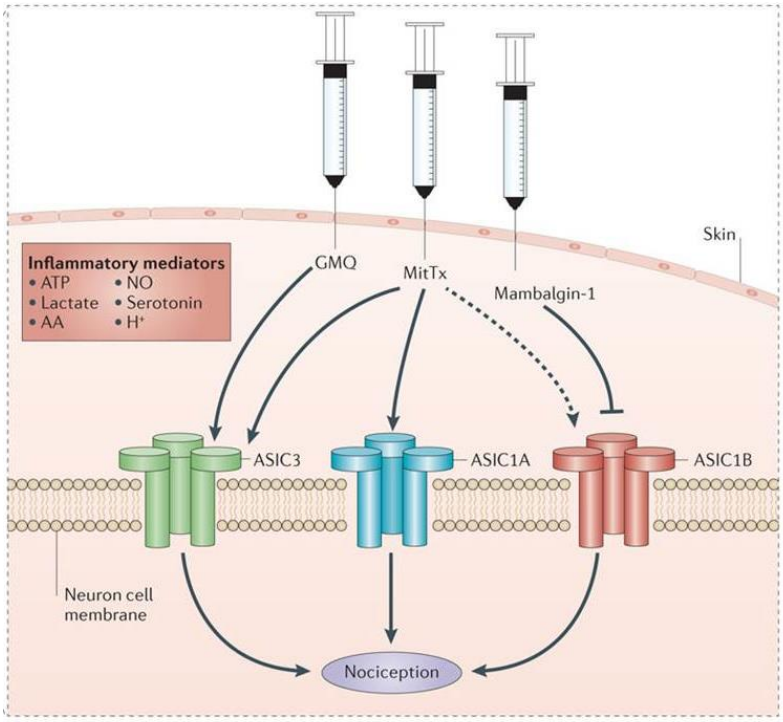



Figure 1.16 Synthetic (GMQ) and naturally occurring (MitTx; Mambalgin-1) agonists and antagonists of ASICs demonstrate proton independent mechanisms of ASIC activation and have helped elucidate more about the roles of ASICs in pain. Pro-algesic agents such as Arachidonic Acid (AA) have also been identified as modulators of ASICs in inflammatory conditions. (Modified from Wemmie et al. (2013)).

## 1.6 Annexin A6

In this thesis, we identify a novel role for Annexin A6 in regulation of mechanosensation. Annexins are a superfamily of $Ca^{2+}$-dependent, phospholipid-binding proteins which are involved in diverse functional processes. They are highly evolutionarily conserved; vertebrate annexins are denoted by an A in their name (for example, Annexin A6) while classes B-E refer to invertebrate, unicellular and plant orthologues.

The annexin 'core' consists of four homologous domains made up of approximately 70 amino acids which pack around a hydrophilic hole and form a convex surface which binds $Ca^{2+}$. Resolution of the crystal structure of vertebrate annexins showed that these domains are arranged into a bent disc shape. The homologous repeat domains are critical in allowing annexins to bind to $Ca^{2+}$ and phospholipids while the N terminal domains of these proteins, which are much more diverse, are believed to be important for conferring function to individual annexins as well as mediating stability, and protein interactions (Moss et al., 1992, Turnay et al., 2005, Lizarbe et al., 2013).

Annexin A6 is unique among vertebrate annexins in having eight, rather than four, homologous domains. The resolution of the ANXA6 crystal structure showed that these domains form two core lobes connected by a 40 amino acid loop (Benz et al., 1996, Avila-Sakar et al., 1998). The two lobes are perpendicular to one another but upon membrane binding they reorientate themselves such that they are coplanar with the membrane (Benz et al., 1996, Kawasaki et al., 1996). Two such conformations of membrane bound ANXA6 have been reported; in one, both lobes have their convex surface facing the membrane while in the other, the lobes are closer together and only one has the convex side facing the membrane while the other lobe is flipped such that the convex surface faces away from the membrane (Avila-Sakar et al., 2000). It has been suggested that the flexibility of ANXA6 and its orientation to the membrane is regulated by phosphorylation of specific residues and may be critical for its role in membrane fusion and vesicle aggregation (Benz et al., 1996, Freye-Minks et al., 2003).

*Anxa6*, previously known as p68, calelectrin and protein III (Moss et al., 1992) is a compound gene which appears to derive from Annexin A5 and Annexin A10 being spliced together, early in evolution. Expression of ANXA6 is restricted to specific tissues and cell types. In both rat and mouse, ANXA6 is expressed in large and small diameter sensory neurons and at low levels in Schwann cells but not in other types of glial cells in

the DRG (Naciff et al., 1996a, Naciff et al., 1996b). It is highly expressed at the plasma membrane of these neurons and in intracellular regions consistent with association of ANXA6 with the endoplasmic reticulum (Naciff et al., 1996a, Naciff et al., 1996b). In the spinal cord, ANXA6 is distributed rostrocaudally, primarily in the cell bodies, axons and dendrites of motor neurons. In cultured neurons taken from DRG and spinal cord, ANXA6 has been shown to suppress $K^+$ conductance as well as decreasing $Ca^{2+}$ current in sensory neurons (Naciff et al., 1996a) though a clear understanding of the function of ANXA6 in nervous tissue has yet to be resolved.

## 1.7 Voltage gated sodium channels

The family of mammalian voltage-gated sodium channels consist of nine alpha subunits, Nav1.1-1.9, encoded by the genes SCN1A-5A and SCN8A-11A. Beta subunits of voltage gated sodium channels associate with the alpha subunits acting to anchor and localise the channels (Figure 1.17). Voltage gated sodium channels allow flux of $Na^+$ into the cell, producing the depolarising upstroke of the action potential. Neuronally expressed sodium channels in peripheral sensory neurons have crucial roles in pain and mechanosensation.

Nav1.3, 1.7, 1.8 and 1.9 are preferentially expressed in the peripheral nervous system. Nav1.3 is more highly expressed during embryonic development than in adult nervous system, and Nav1.3 null animals have normal pain sensitivity (Nassar et al., 2006) though in pathological conditions it is upregulated in DRG (Black et al., 2004) and spinal cord (Waxman et al., 1994).

Nav1.7 is expressed in sensory and sympathetic neurons as well as the thalamus and olfactory bulb. In humans, a nonsense mutation mapped to SCN9A, the gene encoding Nav1.7, produced total insensitivity to pain and anosmia while leaving other sensory modalities unaffected (Cox et al., 2006), subsequently, other loss of function mutations have also been mapped to Nav1.7 which cause pain insensitivity. Paroxysmal extreme pain disorder (PEPD) (Fertleman et al., 2006) and primary erythermalgia (Yang et al., 2004, Drenth et al., 2005) are conditions characterised by severe, ongoing pain which occurs in the absence of a stimulus as a result of gain of function mutations in SCN9A. Mice lacking functional expression of Nav1.7 in sensory neurons show decreased thermal and mechanical sensitivity and significantly reduced inflammatory hypersensitivity (Nassar et al., 2004) while impairing the function of Nav1.7 in sensory and sympathetic neurons leads to loss of mechanical allodynia produced by specific models of neuropathic pain (Minett et al., 2012). Interestingly, Nav1.7 KO mice develop comparable mechanical and cold hypersensitivity to WT in some models of neuropathic pain as well as in models of cancer induced bone pain (Minett et al., 2014b).

Nav1.8 is selectively expressed in a population of small diameter fibres in dorsal root and trigeminal ganglia (Akopian et al., 1996). It produces a TTX resistant, slowly inactivating current and is crucial in the upstroke of the action potential and repetitive firing of neurons in which it is expressed. Its role in some thermal and mechanical sensitivity in acute and inflammatory conditions (Akopian et al., 1999) has been shown through the use of KO

mice which show normal behavioural responses to all other stimuli. Ablation of the Nav1.8 positive population of sensory neurons leads to almost total insensitivity to cold and noxious mechanical pain (Abrahamsen et al., 2008).

Nav1.9 is responsible for production of the persistent current in peripheral sensory neurons (Ostman et al., 2008) and the hyperexcitibility of those neurons in inflammatory conditions. Loss of Nav1.9 leads to a loss of mechanical and thermal hypersensitivity in inflammatory conditions (Lolignier et al., 2011). Nav1.9 is also critical for normal cold sensitivity in physiological and pathological conditions including as a result of oxaliplatin induced neuropathic pain (Lolignier et al., 2015a) which is a problem in up to 90% of people treated with this chemotherapeutic agent (Kiernan, 2007).

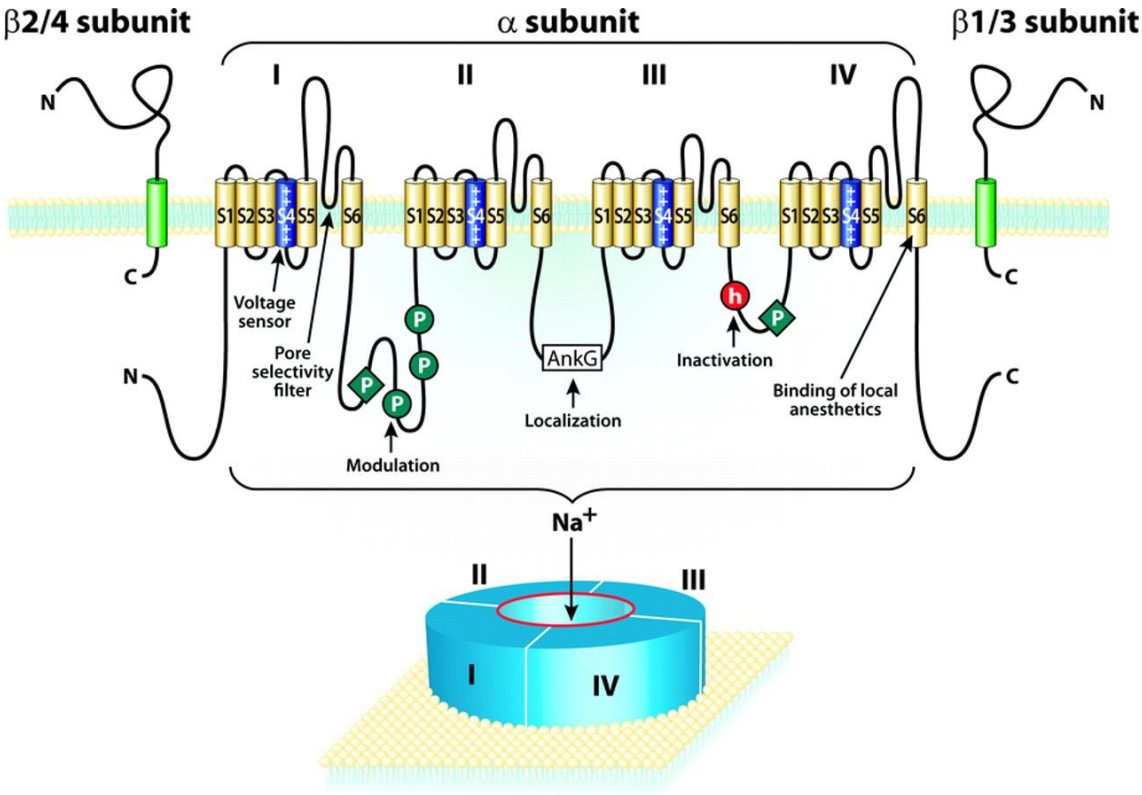



Figure 1.17 The primary structure of voltage gated sodium channels. There are 9 α-subunits in the family of VGSCs; all consist of four homologous domains (DI-DIV) each of which has six transmembrane segments (S1–S6). The S4 segment of each domain contains positive charge while the linker that connects S5 and S6 forms the external pore and selectivity filter. The β-subunits associate with the α-subunits acting to anchor and localise the channels. (Taken from (Benarroch, 2007).

## 1.8 Targeted gene deletion strategies in transgenic mice

### *1.8.1 Cre/loxP system*

The Cre/lox recombination system allows selective modification of gene expression. In order to create a gene knockout, the gene of interest is flanked by 2 *loxP* sites (red triangles; Figure 1.18), derived from Bacteriophage P1 (Sternberg et al., 1981, Sauer and Henderson, 1988), which are 34 base pairs in length: **ATAACTTCGTATAGCATACATTATACGAAGTTAT**. The central 8bp sequence, (5'-**GCATACAT**-3') where recombination occurs, is flanked by 13bp inverted repeat sequences. The recombination occurs in the presence of a Cre enzyme which is under the control of a promoter of interest. The promoter can be ubiquitous, tissue specific or under the control of an inducible system which allows selectivity in the spatial or temporal modification of gene expression. In cells where the Cre recombinase is expressed, the floxed sequence is excised and removed while one loxP site remains in the targeting allele; in tissues not expressing Cre, gene expression is unaffected.

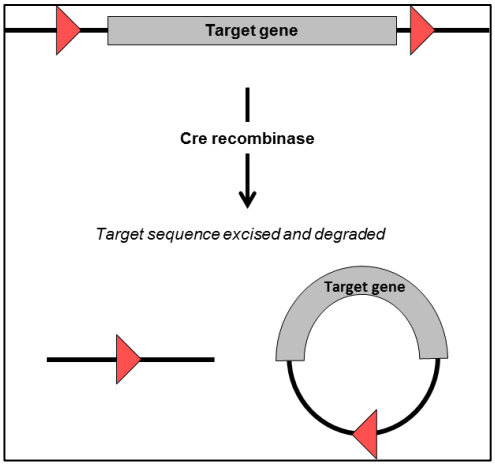



Figure 1.18 The Cre-loxP system allows selective modification of gene expression (red arrows, loxP sites).

### *1.8.2 Flp/FRT system*

The Flp /FRT system works in a similar way to the Cre/loxP system. The flippase recombination target (FRT) sites flank the gene of interest and the flippase (Flp) recombinase enzyme, from the *Saccharomyces cerevisiae* yeast (Dymecki, 1996), acts to excise the gene of interest at these FRT sites: **GAAGTTCCTATTCTCTAGAAAGTATAGGAACTTC**.

## 2 Aims of this project

The aim of this thesis is to use transgenic techniques to investigate the contribution of candidate mechanosensors to touch and pain in physiological and pathological conditions.

- First, mice with knockout of multiple TRPC channels are used to investigate their role in mechanosensation with a particular focus on TRPC1, TRPC3, TRPC5 and TRPC6. TRPC3 and TRPC6 are expressed in a subpopulation of sensory neurons which are known to be responsible for sensitivity to mechanical stimuli (Abrahamsen et al., 2008) and have been shown to have a combined role in innocuous, but not noxious, mechanosensation (Quick et al., 2012). TRP channel are believed to show functional redundancy and their known protein interactions are vast. As such, we hypothesised that additional TRPC channels may work in combination with TRPC3 and TRPC6 to mediate innocuous mechanosensation. TRPC1 and TRPC5 are expressed in DRG, have demonstrable roles in mechanosensation (Gomis et al., 2008, Garrison et al., 2012) and are known to interact with TRPC3 and TRPC6. Using a novel multiple knockout approach, we aimed to minimise the effects of functional redundancy by studying TRPC1, TRPC3, TRPC5 and TRPC6 quadruple KO mice and elucidate a combinatorial role for these TRPC channels in innocuous mechanosensation where monogenic studies may have failed.
- Next, we use a multiple knockout approach to investigate whether TRP channels which have roles in mechanosensation contribute to mechanical hypersensitivity in Osteoarthritis. Mechanical hypersensitivity is a key clinical feature of Osteoarthritis and is poorly understood. Although TRPA1 and TRPV1 have been implicated in Osteoarthritis induced pain in pharmacological studies and inhibitors of these channels have been tested in preclinical and clinical trials, there is some contention over whether these channels do, in fact, play any role in OA pain. Although TRPC3 and TRPC6 have established roles in mechanosensation, there is no evidence arguing for or against a role in OA induced mechanical hypersensitivity. As all of these channels are viable candidates for such a role, we hypothesised that their contribution to mechanical hypersensitivity may be elucidated most effectively using multiple knockout mice. This novel transgenic approach aimed to minimise the influence of characteristics such as functional redundancy and heteromerisation and provide insight into the roles of TRP

channels in Osteoarthritis pain where pharmacological approaches have produced complex and sometimes conflicting findings.

- Using a combination of gene knockout and gene therapy approaches, the role of Annexin A6 in mechanosensation is reported. Mechanically activated currents can be classified according to their adaptation profiles and the recent discovery of Piezo2 as a transducer of rapidly adapting currents was a huge step towards a better understanding of this sensory modality. In spite of this, little is known about the protein(s) responsible for the slowly adapting, mechanically activated current which is produced by high threshold mechanical stimulation and is associated with noxious mechanosensation. NMB-1 is a peptide which selectively blocks the slowly adapting current. We identified Annexin A6 as an NMB-1 binding partner and found it is also present in a subpopulation of sensory neurons which are known to be responsible for sensitivity to mechanical stimuli (Abrahamsen et al., 2008). After identifying Annexin A6 as a strong mechanotransduction candidate, we performed behavioural assessment of global knockout mice and found they were hypersensitive to noxious mechanical stimuli. Subsequently, we hypothesised that Annexin A6 may in fact be a negative regulator of noxious mechanosensation and used electrophysiological and behavioural assessment to determine the effect of Annexin A6 overexpression on mechanosensation.
- Finally, an ASIC4 knockout mouse is used to determine whether this poorly understood protein has a role in pain or mechanosensation. ASICs have been widely studied as potential contributors to mechanosensation and pain though the resulting evidence has provided conflicting insight into the roles these channels may be playing. The function of ASIC4 is poorly understood, however *in vitro* studies suggested that it may be involved in the regulation of other ASICs. We hypothesised that if ASIC4 also regulates other ASICs in vivo, it may be indirectly involved in regulating mechanosensation or sensitivity to other noxious stimuli. As such, we characterised the expression of ASIC4 in nervous tissue and performed behavioural assessment of the sensory function of a novel, global ASIC4 knockout mouse.

# 3 Materials and Methods

## 3.1 Molecular Biology

### *3.1.1 DNA extraction for genotyping*

Ear tissue samples were taken from mice and digested in 30µl lysis buffer for 60 minutes at 55ºC then for 5 minutes at 95ºC to inactivate Proteinase K. Samples were vortexed, spun down and stored at -20ºC.

| Component | Volume (ml) |
|---|---|
| **10X GB** | 3.60 |
| **25% TritonX-100** | 0.72 |
| **β-Mercaptoethanol** | 0.36 |
| **$H_2O$** | 31.32 |
| **Total** | 36.00 |

Table 3.1 Ear Lysis Buffer reagents

| Component | Volume (ml) |
|---|---|
| **1.5M Tris pH8.8** | 4.47 |
| **1.0M Ammonium Sulphate** | 1.66 |
| **1.0M Magnesium Chloride** | 0.67 |
| **$H_2O$** | 3.20 |
| **Total** | 10.00 |

Table 3.2 10X GB buffer reagents

*1µl 19.7mg/ml Proteinase K (Roche) added per 499µl Lysis Buffer.*

Samples were then used to check gene expression with appropriate primers for each gene of interest.

### *3.1.2 Polymerase chain reaction for genotyping*

The following components were added to 0.2ml tubes and set up for PCR. A typical reaction consisted of:

11µl Dreamtaq PCR Mastermix (Thermo Scientific)

11µl $H_2O$

1µl each primer (10µM)

1ng template DNA

(Dreamtaq contains all components necessary for PCR, dNTPs, Taq, buffer)

Samples were then subject to PCR in conditions appropriate for Dreamtaq and specific for each primer set. A typical programme is given below:

Initial denaturation, 95$^{o}$C for 2 minutes;

The next 3 steps were cycled 30 times:

Denaturation, 95$^{o}$C for 30 seconds;

Annealing, 55-65$^{o}$C for 30 seconds (determined from Tm of primers-5$^{o}$C);

Extension, 72$^{o}$C for 30 seconds;

Final extension, 72$^{o}$C for 5 minutes

### *3.1.3 Gel electrophoresis*

Samples were run on a 1% agarose gel to separate bands by size. Gels were prepared by dissolving 0.5g agarose (Sigma) per 50ml 1X TAE buffer (40mM Tris-acetate, 1mM EDTA). Ethidium bromide (EtBr) was added (0.5µg/ml) and the gel was set. 5µl of an appropriate molecular weight marker was run alongside samples in each gel. Samples were run for 45 minutes at 100 volts to separate bands which were visualised using a Biodoc System.

### *3.1.4 RNA extraction*

Animals were culled according to Home Office protocols and DRGs were dissected onto ice and stored at 4$^{o}$C in RNA*later*. DRGs were immersed in 0.8ml Trizol and homogenised for 3 x 10seconds using a MINILYS benchtop homogeniser (Peqlab).

RNA extraction was then performed using an RNEasy Minelute Cleanup Kit (QIAgen). Samples were passed through a Qiashredder mini column and spun at 13,000rpm; the supernatant was removed and, with 2/10$^{th}$ of the volume of chloroform, was added to a phase lock gel tube. The sample was spun at 13,000rpm for 15 minutes at 4$^{o}$C. The resulting colourless, aqueous layer was added to an equal volume of 70% ethanol (in DEPC $H_2O$) and spun for 15 seconds in an RNeasy MinElute spin column. Following application of a wash buffer to the column membrane, 80µl DNase I in an RNase free RDD buffer was added and incubated at RT for 15 minutes to digest genomic DNA. Wash buffer was applied to the column membrane, followed by 80% ethanol. The column was spun for 15 seconds at 13,000rpm both times and placed in a new 2ml collection tube for each step. Once the membrane was dried by spinning, 12µl nuclease free water was applied and the column spun for 2 minutes at 13,000rpm to elute the RNA. RNA concentration (ng/µl) was checked using a Nanodrop spectrophotometer (Thermo Scientific) and RNA was stored at -80$^{o}$C.

### *3.1.5 cDNA synthesis*

The following components were incubated at 25$^{o}$C for 5 minutes and then at 42$^{o}$C for 30 minutes followed by a reverse transcriptase heat inactivation step at 85$^{o}$C for 5 minutes. All reagents were obtained from the iScript cDNA synthesis kit (BioRad). cDNA was stored at -20$^{o}$C.

| **Component** | **Quantity** |
|---|---|
| **RNA** | 1 µg |
| **Random Primers** | 2µl |
| **Reverse Transcriptase** | 1µl |
| **5x iScript Select Reaction Mix** | 4µl |
| **Nuclease Free $H_2O$** | Volume adjusted as necessary |

Table 3.3 cDNA synthesis reagents

### *3.1.6 Agarose gel extraction and DNA sequence analysis*

gDNA and cDNA samples which required sequence confirmation were amplified using protocols listed above. Next, samples were run on a 1% agarose gel and excised using a scalpel before being excised with a Qiagen gel extraction kit. Briefly, the gel fragment

was put in a 1.5ml Eppendorf, weighed and dissolved in 3x gel volume (1mg gel~1µl) of solubilisation buffer at 50ºC for 10mins. 1 gel volume of isopropanol was added to the sample which was then applied to a spin column and centrifuged. 750µl wash buffer was passed through the membrane and spun before another 1min spin to remove residual buffer. DNA was eluted in 50µl elution buffer and centrifuged for 1min and the concentration measured using a Nanodrop spectrophotometer (Thermo Scientific). Samples were sent to Source Bioscience (Cambridge) for Sanger sequencing. Results were analysed using SnapGene viewer software and sequence alignments performed using NCBI nucleotide BLAST.

### *3.1.7 Reverse transcription polymerase chain reaction (RT-PCR)*

RT-PCR was used to confirm gene knockout in RNA which was isolated from DRG samples and reverse transcribed into cDNA as described above. Primers were designed to target exon-exon junctions of cDNA sequences. A typical PCR protocol was used, as outlined above. The resulting products were run on an agarose gel alongside a housekeeping gene control, GAPDH to ensure the cDNA sample and PCR reaction worked.

### *3.1.8 Quantitative RT-PCR*

In order to detect levels of gene expression in different tissues, RNA was isolated from tissue samples and reverse transcribed into cDNA as described above. Reactions were performed in triplicate on a 96-well reaction plate in 20µl volume using Bio-Rad SYBR Green Supermix. Reactions were run using a Biorad Real-Time PCR Detection System. A typical programme was:-

Denaturation at 95ºC for 3 min

The next 3 steps were cycled 40 times:

Denaturation at 95 ºC for 30 seconds

Annealing at 60 ºC for 30 seconds

Extension at 72 ºC for 30 seconds

Melt curve to check for one specific product

Results were normalised to GAPDH expression levels and analysed using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

## 3.2 Immunohistochemistry

### *3.2.1 Tissue collection*

Animals were administered sodium pentobarbitone via intraperitoneal (i.p.) injection (150mg/kg) to induce terminal anaesthesia. They were then transcardially perfused with 0.1 M phosphate buffered saline (PBS) with 0.01µl/1000 ml heparin followed by 4% PFA in PBS for 5-10 minutes. Tissues were dissected and post-fixed for 2 h in 4% PFA and transferred to 30% w/v sucrose in 0.1 M PBS at 4°C overnight. Tissues were then mounted in OCT medium and stored at - 80 °C.

### *3.2.2 Sectioning*

Tissues were sectioned using a Cryostat and mounted onto Superfrost Plus slides (Thermo Scientific). DRG were sectioned at 10µm thickness and spinal cord at 30µm thickness. Slides were dried for 2 hours at RT then stored at -20$^{o}$C.

### *3.2.3 Immunostaining*

Tissue was removed from -80$^{o}$C and dried at 4$^{o}$C for 30 mins then at RT for 120 mins. Slides were washed with 1xPBS containing 0.3% Triton X-100 (PBST) for 3 x 5 mins then with PBST containing 10% goat serum (blocking buffer) for 60 mins at RT. Primary antibodies (Table 3.4) were applied to the slides, diluted in blocking buffer, and left at 4$^{o}$C overnight. Slides were then washed for 3 x 10 mins in PBST before secondary antibodies, diluted in blocking buffer (Table 3.4), were applied for 2 hours at RT. Secondary antibodies were removed by washing in PBST for 3 x 10 mins. The slides were dried at RT in the dark and then mounted with Vectashield mounting medium and a coverslip before being sealed and stored in the dark.

### *3.2.4 Analysis*

After drying, sections were imaged using a Leica SP5 confocal microscope or Leica DMRB microscope with Hamamatsu ORCA-R2 digital camera and HCImage software. Images were processed using EMBL ImageJ software using global adjustments in brightness and contrast.

## 3.3 Western blotting

### *3.3.1 Tissue preparation*

Animals were culled according to Home Office protocols and tissues were dissected onto dry ice and homogenized for 90 seconds with Precellys ceramic beads (Peqlab) in ~300µl modified RIPA buffer. Cell lysates were incubated for 1hr shaking on ice. Following a final homogenisation step, samples were centrifuged for 8 minutes at 14,000 RPM at 4$^{o}$C.

### *3.3.2 Protein quantification*

Samples were prepared using the bicinchoninic acid (BCA) kit (Thermo Scientific) according to manufacturer's instructions and compared to 5 standards of bovine serum albumin (BSA) from 0µg/µl to 2µg/µl. Briefly, protein samples were made up to dilution factors of 1, 2, 5 and 10 using 1ml solution A and 20µl solution B. Samples and standards were incubated at 37$^{o}$C for 30 minutes. Standards were analysed using a Nanodrop spectrophotometer (Thermo Scientific) to produce a standard curve which allowed quantification of sample concentrations.

### *3.3.3 SDS-PAGE*

3X loading buffer (NEB; 187.5mM Tris-HCl, 6% w/v SDS, 30% glycerol) with 1ml DTT (1.25M) was added to the protein samples and denatured at 95$^{o}$C for 5 mins. 20-60µg protein was loaded onto a Mini-PROTEAN TGX Stain-Free precast gels (4-12% Gradient, Bio-Rad) alongside a Spectra Multicolor High Range Protein ladder (Thermo Scientific) in 1X running buffer (10X stock; 30.3g Tris Base, 144g glycine, 10g SDS at pH 8.3) and run for 15 minutes at 120V and then 45 minutes at 100V.

### *3.3.4 Wet transfer*

Immobilin-P membranes (Millipore) were activated with Methanol. Proteins were transferred onto the membrane in 1X ice cold transfer buffer (10X Stock Transfer Buffer, 30g Tris base, 144g glycine in 1L $dH_2O$) for 1 hour at 100V.

### *3.3.5 Blocking and antibody incubation*

Membranes were incubated in blocking solution (1x PBST; 10X PBS stock (Gibco) pH7.5, 0.1% Tween 20 (Sigma); 5% Marvel) for 2 hours at RT and then at 4$^{o}$C overnight in primary antibody (Table 3.4) and blocking solution. For peptide control samples, primary antibody was pre-incubated with peptide antigen at RT for 30 minutes with shaking.

Membranes were washed 4 times in 1X PBST before incubation with horse radish peroxidase (HRP) conjugated secondary antibodies (Table 3.4) in blocking buffer (1x PBST with 2.5% Marvel) for 90 minutes at room temperature with shaking. Membranes were washed 4 times in 1X PBST. Protein was incubated with Pierce ECL chemiluminescent substrate (Thermo Scientific) for 1 minute before visualisation on X-ray films (GE healthcare) using an X-ray film developer.

## 3.4 Antibodies

| Experiment | Primary Antibody | Dilution | Company | Secondary Antibody | Dilution | Company |
|---|---|---|---|---|---|---|
| **WB** | anti-ASIC1 | 1:600 | Alomone | ECL anti-Guinea pig HRP conjugated IgG (from goat) | 1:16000 | GE Life sciences |
| **WB** | anti-ASIC4 | 1:200 | Alomone | ECL anti-Rabbit HRP conjugated IgG (from donkey) | 1:16000 | GE Life sciences |
| **WB** | anti-βactin | 1:1000 | Santa Cruz | ECL anti-Mouse HRP conjugated IgG (from sheep) | 1:16000 | Abcam |
| **WB** | anti-GFP | 1:200 | Abcam | ECL anti-Rabbit HRP conjugated IgG (from donkey) | 1:2000 | Abcam |
| **WB** | anti-Anxa6 | 1:400 | Abcam | ECL anti-Rabbit HRP conjugated IgG (from donkey) | 1:2000 | Abcam |
| **IHC** | anti-Peripherin | 1:200 | Sigma | Alexa Fluor 594 conjugated goat anti-mouse IgG | 1:1000 | Life technologies |
| **IHC** | anti-GFP | 1:2000 | Abcam | Alexa Fluor 488 conjugated goat anti-rabbit IgG | 1:1000 | Abcam |
| **IHC** | anti-IB4 | 1:500 | Sigma | Alexa Fluor 568 Streptavidin conjugates | 1:1000 | Life technologies |

Table 3.4 Antibodies used for Western Blot (WB) and Immunohistochemistry (IHC)

## 3.5 Behavioural testing

Animals were acclimatised for an appropriate amount of time for each test. Tests determined to be painful were performed on different groups of animals where possible. Mice were used at 6-12 weeks old and 5-8 weeks old for hearing tests.

### *3.5.1 Von Frey*

von Frey testing is a behavioural paradigm designed to investigate sensitivity to an innocuous mechanical stimulus. A set of 20 nylon hairs of weights ranging from 0.008g – 300g were used to apply pressure to the plantar surface of the paw. A hair of 0.4g was always used first. von Frey testing was performed using the up-down method (Chaplan et al., 1994) to determine the 50% paw withdrawal threshold. This involved applying a von Frey filament to the paw; if a positive response was obtained, a filament of lesser force was applied next but if no response was seen a filament of greater strength was applied. This was continued until 6 responses were obtained with a positive response consisting of lifting, flinching or licking of the paw during or immediately after stimulus application. In order to complete the test, animals were kept in a mesh bottomed enclosure in which they were acclimatised for at least 60 minutes prior to testing.

The same technique was used when testing mechanical threshold on the hairy skin of the abdomen. Since glabrous and hairy skin receive different sensory innervation, both types of skin were investigated in order to determine whether deletion of TRP channels may have an effect on mechanical threshold in either or both types of skin.

### *3.5.2 Cotton Swab test*

The cotton swab test is a different measure of innocuous sensitivity than the up-down von Frey technique. It is a dynamic stimulus; a cotton swab puffed out to 15mm diameter (3x normal size) was moved across the glabrous skin of the paw in a ~1 second sweep as described by Garrison et al. (2012). The cotton swab stimulus was applied 5 times to each animal with a break of ≥60 seconds and the number of responses was recorded to produce a score out of 5.

### *3.5.3 Randall Selitto*

The Randall Selitto test is used to assess the noxious mechanical pain threshold and was used on the tail and paw of animals. The test designed by Randall and Selitto (1957, as cited in Minett (2011)) and modified by Takesue et al. (1969) involves application of a

blunt probe to a surface (paw or tail) with increasing pressure until the animal exhibits a painful response. Such signs include struggling, withdrawal of tail or paw or vocalisation. The test was repeated 3 times for each animal.

When testing the tail, animals were restrained in a clear plastic tube with an acclimatisation period of approximately 5 minutes while for testing the paw, animals were restrained through holding the scruff of the neck in such a way that a response could still be observed.

### *3.5.4 Hargreaves*

The Hargreaves test, originally designed to assess thermal nociception in inflammatory pain states (Hargreaves et al., 1988), was used to assess spinal reflex responses to noxious thermal stimulation. Animals were acclimatised in an enclosure with a glass base for a minimum of 60 minutes. A radiant heat light source was applied to the plantar surface of the hind paw with a cut off time of 30 seconds. The stimulus was stopped when a painful response was observed (licking, lifting or shaking the paw) and the latency to paw withdrawal (in seconds) was recorded. Three recordings were made from each animal and an average taken.

### *3.5.5 Hot Plate*

The hot plate test is designed to test responses to noxious thermal stimuli but unlike the Hargreaves test, it is believed to involve supraspinal mechanisms (Chapman et al., 1985). Prior to testing, mice were acclimatised on the unheated plate. Once heated to the desired temperature, mice were placed on the plate and the time until the first distinctive pain behaviour (licking or shaking of hind paw) was recorded, at which point mice were removed from the plate. Temperatures used were 50$^{o}$C and 55$^{o}$C (±0.2$^{o}$C) according to the initial description of the technique by Macdonald et al. (1946) and amended by Eddy and Leimbach (1953) with testing for each temperature performed in separate sessions.

### *3.5.6 Thermal Place Preference*

Cold sensitivity tests provide insight into sensitivity of animals to noxious cold temperatures. It is suggested that temperatures <15$^{o}$C can be considered to be at a noxious level of cold. For this project, cold sensitivity was assessed using a place preference set up. Two adjacent plates (a test plate and second plate) were held at 20$^{o}$C while animals were allowed to explore for 120 seconds. The temperature of the test plate was then

dropped to 4ºC and animals explored for a further 120 seconds. In both trials, animals were placed onto the test plate (in order that they were exposed to the cold temperature) and the time spent on the test plate was recorded. The test plate and second plate were switched in order that environmental effects did not bias the time spent on the test plate.

### *3.5.7 Rotarod*

The accelerating rotarod test was used as a measure of motor coordination (Jones and Roberts 1967). Mice were placed onto a rotarod machine turning at 6 rpm. This speed was maintained for 30 seconds to allow animals to acclimatise. Time spent on the rotarod was recorded from the end of this period at which point the speed was increased from 6rpm to 40 rpm on a 3 minute ramp with a cut-off point of 5 minutes from the start of acceleration. The timer was stopped when animals fell from the rotarod or made 3 full rotations within a 10 second period. This was repeated 3 times per animal.

### *3.5.8 Swim Test*

The forced swim test was, in this case, used as a measure of vestibular function. Although this test can be used to investigate many different faculties, applying simple parameters allow it to be used as a means of studying vestibular function while unaffected by proprioceptive inputs seen in other tests performed on a solid surface. The test was initially designed by Porsolt et al. (1978) and since has frequently been used to monitor vestibular deficits in rodents (Gray et al., 1988, Kaiser et al., 2001, Burne et al., 2006). A protocol similar to that performed by Can et al. (2012) was employed.

Animals were placed in a 15cm deep water bath which was maintained at 23-25ºC and allowed to swim for 1 minute. They were then removed and dried in heated chamber. The test was recorded and analysed in slow motion using Realtime player software and the number of times the head of the animal was submerged under water was recorded.

### *3.5.9 Trunk Curl Test*

The trunk curl test acts as a crude measure of vestibular function. Each animal is held in the air by the tail 5 cm away from a horizontal surface for 5 seconds. A wildtype mouse should demonstrate a reaching movement towards the horizontal surface, whereas animals with vestibular deficits are inclined to curl towards the abdomen (Hardisty-Hughes et al., 2010). A scoring system was used to quantify this; if the mouse reached its

forelimbs towards the surface, it received a score of 1. If the mouse curled towards the abdomen, it received a score of 0. A partial curl received a score of 0.5.

### *3.5.10 Auditory testing*

#### *3.5.10.1 Click Box*

The click box test was performed to investigate hearing function. Animals were put in individual enclosures and allowed to acclimatise for 60 minutes. The device was held 20cm above each animal and an 89dbSPL click with a frequency of 19.5 kHz was emitted. The Preyer reflex response is the response of a wild type mouse to such a sound and involves the ears moving flat against the back (Jero et al., 2001). The test was recorded and analysed in slow motion using Realtime player software. If the response was observed, a score of 1 was given; for no response a score of 0 was given and for a partial response, a score of 0.5 was given. All animals were tested with 3 consecutive clicks, separated by a period of at least 5 seconds.

#### *3.5.10.2 Auditory Brainstem Response recordings*

*Performed by Dr. Ruth Taylor*

Mice were anaesthetised and an active electrode placed between the ears at the vertex with a reference electrode at the mastoid and a ground electrode at the tail. Tone pip stimuli of 8, 12, 24, 32 and 40 kHz were applied at varying sound pressure levels to allow determination of the threshold (dB) required to elicit a response. (Protocol as previously reported (Quick et al., 2012) and, this time, provided and implemented by Dr Ruth Taylor, UCL Ear Institute). C57BL/6 mice have been shown to develop early onset hearing loss (Henry and Chole, 1980), so mice were used between the ages of 4 and 8 weeks of age.

#### *3.5.10.3 Mechano-electrical Transducer Current recordings*

*Performed by Terri Desmonds*

Mechano-electrical transducer (MET) currents were recorded from outer hair cells in cultured cochlea from early postnatal animals (~P2) using whole-cell patch clamp. A fluid jet was used to generate a 45 Hz sinusoidal force with a driver voltage (DV) amplitude of 40V. The holding potential of -84mV was stepped in 20 mV increments from -164 mV to +96 mV. (Protocol as previously reported (Quick et al., 2012) and, this time, provided and implemented by Terri Desmonds, University of Sussex).

### *3.5.11 Inflammatory pain models*

#### *3.5.11.1 Formalin Test*

The Formalin test was first modified for mice in order to provide a model of continuous, moderate pain which allowed non-invasive assessment of pain behaviour (Hunskaar et al., 1985). Animals were acclimatised to a Perspex box for at least 1 hour prior to testing when a 20µl intraplantar injection of 5% Formalin was given in the hind paw. Animals were observed for the next 60 minutes and the time spent licking, shaking or biting the affected paw was measured in five minute blocks.

The test involves an early acute phase, which is believed to involve direct activation of C fibres via TRPA1 (Tjolsen et al., 1992, McNamara et al., 2007) and occurs around 5-10 minutes after injection. The second, inflammatory phase begins around 20 minutes after injection and believed to result from the action of prostaglandins and other inflammatory mediators as well as spinally mediated changes (Dubuisson and Dennis, 1977, Hunskaar and Hole, 1987, Tjolsen et al., 1992).

#### *3.5.11.2 CFA*

Complete Freund's Adjuvant (CFA) injection is used as a model of lasting inflammatory hypersensitivity to both thermal and mechanical stimuli and is a well-established model for use in mice (Larson et al., 1986). It causes sustained thermal and mechanical hyperalgesia which are linked to TNFα release in the periphery (Cunha et al., 1992) as well as spinal changes such as increased density of postsynaptic NMDA receptors (Yang et al., 2009).

Animals received a single, intraplantar injection of 20µl of 100% CFA (Sigma) and were tested for mechanical and thermal hypersensitivity using the von Frey up-down method and the Hargreaves test, respectively for up to 14 days.

### *3.5.12 Model of chronic muscle pain*

Mice received an injection of 20µl of pH 4 acidic saline into the gastrocnemius muscle on days 0 and mechanical hypersensitivity was analysed by von Frey stimulation on the ipsilateral and contralateral paw using the up-down method and using a static weight bearing machine to assess weight distribution over the hind paws. This model has been shown to induce chronic mechanical hypersensitivity in the absence of tissue damage (Sluka et al., 2001), unlike comparable administration of inflammatory stimuli.

### *3.5.13 Model of visceral pain and referred hypersensitivity*

0.1% w/v capsaicin was dissolved in 10% ethanol, 10% Tween80 and 80% saline and 50µl was administered into the colon using a synthetic cannula, 4cm long and 0.61mm diameter according to the protocol designed by (Laird et al., 2001). Animals were then filmed for 20 minutes after administration of capsaicin to allow analysis of visceral pain. Pain behaviours were analysed using Realtime player and counted when behaviours including pressing the abdomen onto the floor of the holding box, licking or biting the abdomen and stretching or retracting the abdomen were observed. Subsequently 10 applications of a 0.16g von Frey filament to the abdomen and the number of responses were counted. The hair was applied for 1–2 s to the abdomen with an inter-stimulus interval of 10 s. Responses included licking or biting the abdomen at the site of stimulation, arching the abdomen away from the site of stimulation or jumping.

### *3.5.14 Induction of Arthritis*

The monosodium iodoacetate model of Osteoarthritis was first described by Kalbhen (1987) (as cited in (Combe et al., 2004)) and has been widely used in rats to produce symptoms allied to OA in humans. These include cartilage degradation, synovial inflammation, osteoclast formation and ultimately bone remodelling. This model, though beginning to be used in mice (Harvey and Dickenson, 2009), has been less thoroughly investigated than in rats. We therefore began by doing a dose-response study and proceeded using a dose of 0.5mg Sodium Iodoacetate (Sigma) dissolved in 5µl sterile 0.9% Saline (Sigma) injected intra-articularly into the knee joint of each animal. Pain behaviour was assessed by stimulation with von Frey filaments using the up-down method and using a static weight bearing machine to assess weight distribution over the hind paws.

## 3.6 Gene therapy

### *3.6.1.1 AAV production and purification*

*Performed by Dr Julie Tordo*

The human Annexin A6 gene was cloned into two different serotypes of adeno associated virus (AAV), 6 and X. The gene was inserted downstream of an IRES-eGFP construct to allow subsequent observation of transduced neurons.

To produce recombinant AAV (rAAV) vectors, cells were transiently co-transfected with two plasmids: the plasmid containing the transgene flanked by the inverted terminal repeat (ITRs) of AAV2, and a helper plasmid that provides the excised AAV-specific genes rep and cap in *trans* and the adenovirus helper functions necessary for virion formation. To produce AAV6 or AAVx recombinant vectors, the specific capsid gene (cap) of each virus was inserted into the helper plasmid.

To produce recombinant AAV virions, $5x10^8$ HEK293T cells were seeded per cell factory (CF10). 14 to 18 hours later, the cells were double transfected with the ITR-containing plasmid pAAV-CMV-AnxA6-IRES-GFP, and either of the helper plasmids to produce AAV6 or AAVx-CMV-AnxA6-IRES-GFP vectors. The cells were harvested after 72 hours of incubation at 37°C by centrifugating the media and cells; The supernatant was removed and kept for further treatment, and the cell pellets were resuspended in lysis buffer (150mM NaCl, 50mM Tris pH8.5). The cell pellets were then lysed by 4 cycles of freeze and thaw to release the virus, where each cycle consists of 30 minutes at -80°C followed by 30 minutes at 37°C. After the last thaw the lysate was treated with benzonase and incubated at 37° for 30 minutes. The lysate was then spun at 4000 rpm for 30 minutes for clarification and the resulting supernatant was filtered. The cell culture supernatant was also harvested and precipitated by adding 31.3 g ammonium sulfate salt per 100 ml of collected media supernatant. After shaking to dissolve the salts, the supernatant was incubated for 30 minutes on ice then spun for 30 minutes at 8300 g. The resulting pellet was resuspended in lysis buffer, treated with benzonase, clarified by centrifugation and filtered.

The recombinant AAV virus preparations were purified by FPLC using the ÄKTApurifier chromatography system (GE Healthcare) and an AVB sepharose affinity column (buffer A: PBS, pH 8; buffer B: 0.5M glycine, pH2.7). The collected fractions were then dialysed

against PBS overnight. For mice injections, the virus samples had to be concentrated as only limited volumes of vectors can be injected in vivo. We then performed real time quantitative PCR and SDS polyacrylamide gel electrophoresis (SDS-PAGE) assays to assess the viral genomes titers and capsids titers, respectively, of the concentrated vectors. The vectors were then titer-matched before injection.

#### *3.6.1.2 Real-time Quantitative PCR analysis*

*Performed by Dr Julie Tordo*

The number of genome copies of AAV in the viral stock was determined by quantitative PCR (qPCR) using primers targeted to the GFP transgene; GFP forward (5' GAC GGC AAC ATC CTG GGGCAC AAG 3') and GFP reverse (5' CGG TCA CGA ACT C 3').

qPCRs were performed in a master mix solution containing SYBR Green PCR master mix with MgCl2 (Applied Biosystems), forward and reverse GFP primers, Reference Dye, and dH2O. Samples were added into each well of a 384-well plate and each condition was assayed in duplicate. The PCR conditions consist of an initial denaturation step at 94°C for 5 min followed by 40 cycles of denaturation at 94°C for 10 sec, annealing at 60°C for 20 sec, and extension at 72°C for 40 sec.

#### *3.6.1.3 SDS-PAGE*

*Performed by Dr Julie Tordo*

Protein samples were electrophoresed on a polyacrylamide gel in the presence of SDS, and stained using Sypro Ruby Stain.

12% polyacrylamide gels were loaded with various dilutions of viral samples, along with bovine serum albumin at quantities ranging from 500ng to 62.5ng to realise a standard curve. Each sample was mixed with 6x loading buffer and heated for 5 minutes at 95°C prior to loading. The gels were run at 110V in 1x Tris-glycine buffer until the loading dye exited the bottom of the gel. The gel was then immersed and agitated in fixing solution (50% ethanol, 15% (v/v) acetic acid in ultrapure water) twice for 10 minutes before immersion and agitation in ultrapure water for 5 minutes. An overnight stain of the gel was then performed with Sypro Ruby Stain (Pierce Biotechnology) following manufacturer's instructions.

#### *3.6.1.4 Administration of viral vectors*

A viral titre of $5x10^{12}$VP/ml was administered intrathecally to mice at the sixth lumbar level, following an intravenous injection of 25% Mannitol. Pre-treatment with mannitol has been shown to increase viral transduction into primary sensory neurons (Vulchanova et al., 2010).

## 3.7 Patch clamp electrophysiology

*Performed by Dr. Stéphane Lolignier*

### 3.7.1.1 *Culture and transfection of dorsal root ganglia*

Immediately after CO2 euthanasia, DRG were removed from 6-12 week old mice and digested for 35 min at 37°C in $Ca^{2+}$- and $Mg^{2+}$-free Hanks' balanced salt solution (HBSS) containing 5 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 10 mM glucose, 5 mg/ml collagenase type XI and 10 mg/ml dispase. After digestion, DRG were gently triturated in Dulbecco's modified Eagle's medium (DMEM) containing 10% qualified foetal bovine serum (FBS) using fire-polished glass Pasteur pipettes. When required, neurons were electroporated using the Neon Transfection System (Life Technologies) according to the supplier's recommendations, in 10 µl tips and with 0.6 µg plasmid per reaction, applying 2 x 20 ms pulses of 1100 V. Cells were plated on poly-L-lysine and laminin coated dishes, in DMEM containing 10% FBS and 125 ng/ml 7S nerve growth factor (NGF). Neurons were kept at 37°C in 5% CO2 and used for patch clamp studies the two following days, or at 48 ± 4 h for electroporated neurons.

### 3.7.1.2 *Patch clamp recordings*

Small (< 30 pF) or large (> 30 pF) DRG neurons whose soma were not in contact with those of other neurons, and who were displaying clear fluorescent signal when transfection was performed, were selected for electrophysiological recordings. Patch pipettes were pulled from borosilicate glass capillaries using a PC-10 puller (Narishige Group) and had a resistance of 1.5-3.5MΩ. The pipette solution contained (in mM): 125 CsCl, 4.8 CaCl2, 1 MgCl2, 4 MgATP, 0.4 Na2GTP, 10 ethylene glycol tetraacetic acid (EGTA) and 10 HEPES (pH: 7.4 with CsOH; osmolarity: 310 mOsm with sucrose). The bath solution contained (in mM): 132 NaCl, 3 KCl, 2.5 CaCl2, 1 MgCl2, 10 glucose, 10 HEPES (pH: 7.4 with NaOH; osmolarity: 310 mOsm with sucrose). Voltages were not corrected for junction potentials and recordings were performed at room temperature.

Voltage and current clamp recordings were performed using a MultiClamp 200B amplifier and an Axon DigiData 1440A digitizer (Molecular Devices). Data were recorded and stored using Clampex 10 (Molecular Devices). Current recordings were low-pass filtered at 5 kHz and sampled at 20 kHz. Capacity transients were cancelled; however, series resistances were not compensated. Holding command was set at -70 mV

before and during mechanical stimulation. Off-line analysis and fits were performed using Clampfit 10 (Axon Instruments, Molecular Devices Inc.).

#### *3.7.1.3 Mechanical stimulation and analysis*

Mechanical stimulation of cell bodies was achieved using a heat-polished glass pipette (tip diameter of approximately 2 µm) controlled by a piezo-electric crystal drive (Burleigh LSS-3000) and placed above the centre of the cell soma at an angle of 70° to the surface of the dish. The probe was positioned so that a 10 µm movement did not visibly contact the cell but that an 11 µm movement, considered as a 1 µm stimulus, produced an observable membrane deflection. The probe was moved at a speed of 1 µm/ms. 250 ms mechanical steps were applied every 10 s in 1 µm increments.

Currents were characterized as a function of the signal given by the highest stimulation intensity applied before the seal was lost. Cells were considered as non-responding when no current > 20 pA was observed with a 12 µm stimulus. Responding cells were then classified regarding to the adaptation kinetics of their currents. Rapidly adapting currents had a decay kinetic that was best described by a bi-exponential fit. Intermediately adapting currents had a decay kinetic that was best described by a mono-exponential fit. Slowly adapting currents showed minimal decay (< 20%) during the 250ms stimulation.

## 3.8 Statistical analysis

Data were analysed using Graphpad Prism 6. Student's unpaired t tests were used to compare groups or one or two-way ANOVA with post-hoc tests, where appropriate. If data could not be assumed to be normally distributed or did not satisfy the Shapiro-Wilk normality test, non-parametric analysis was used; Mann Whitney or Kruskal Wallis with post hoc tests; are stated for each experiment where appropriate. Data are presented as Mean±SEM. *$p$<0.05; **$p$<0.01; ***$p$<0.001; ****$p$<0.0001

# 4 The contribution of TRPC1, TRPC3, TRPC5 and TRPC6 to touch and hearing

## 4.1 Summary

Transient receptor potential channels have diverse roles in mechanosensation. Recently, TRPC3 and TRPC6 have been shown to be necessary for normal touch and hearing as animals lacking functional expression of both channels show reduced innocuous mechanosensitivity and altered hearing and vestibular function. However, the fact that single knockout mice are apparently normal suggests that TRPC3 and TRPC6 together, contribute to mechanosensation. We have extended these studies to analyse deficits by additionally knocking out TRPC1 and TRPC5 in triple and quadruple knockout mice. TRPC1 and TRPC5 have both been shown to have roles in mechanosensation and stretch activation, respectively, and are coexpressed in sensory neurons with TRPC3 and TRPC6. We examined both touch and hearing in behavioural and electrophysiological assays, and provide evidence that triple and quadruple knockout mice have larger deficits than double knockout mice. Mechano-electrical transducer currents of cochlear outer hair cells were however normal. This suggests that TRPC1, TRPC3, TRPC5 and TRPC6 channels contribute to cutaneous and auditory mechanosensation in a combinatorial manner, but have no direct role in cochlear mechanotransduction.

## 4.2 Introduction

### *4.2.1 TRPC channels*

The classical or canonical family of TRP channels, known as TRPCs, were the first family of TRP channels to be identified (Wes et al., 1995). The seven members of the mammalian TRPC family can be subdivided into groups according to amino acid sequence homology; TRPC1; TRPC3/6/7; TRPC4/5 and TRPC2. The latter acts as a pseudogene in humans but has a role in pheromone sensing in rodents (Montell, 2005). Characteristics of the family include 3 to 4 ankyrin repeats located near to the N terminus and a TRP domain located close to the C terminus. Of particular interest to this study are canonical TRP channels 1, 3, 5 and 6 due to their known roles in mechanosensory function, their known interactions and their coexpression in DRG neurons.

Canonical TRP channels exhibit low levels of expression early in development but their increase in expression levels aligns, temporally, with the gain of sensory functions (Elg et al., 2007). TRPC3 is the most highly expressed of the TRPC channels in tissues such

as the brain, skeletal muscle and heart and is also expressed in the kidney, pancreas and lung (Kunert-Keil et al., 2006). In addition, TRPC1, TRPC5 and TRPC6 are expressed in the brain, with TRPC6 found at particularly high levels in the Purkinje cells of the cerebellum (Kunert-Keil et al., 2006) and TRPC5 in growth cones of the hippocampus (Table 4.1) (Clapham, 2003). In the DRG, TRPC1, TRPC3 and TRPC6 are the most abundantly expressed TRPC subunits and their expression has been observed in most sensory neurons in adult mice (Elg et al., 2007, Quick et al., 2012). In addition, TRPC5 has been found to be localised to small and medium diameter sensory neurons (Table 4.1) (Zimmermann et al., 2011). A single cell RNA sequencing study also determined a non-peptidergic subset of neurons which express all four TRPC subunits (Usoskin et al., 2015) meaning there is substantial potential for interaction between different combinations of these TRPC subunits. Additionally, all TRPC channels are found within the inner ear (Takumida and Anniko, 2009). As this study investigates the roles of TRPC channels using global KO mice, the expression of these channels must be taken into account.

The function of TRPC channels in sensory systems has been widely investigated and many TRPC channels have been implicated in roles in mechanosensation. An understanding of their mechanisms of activation is an integral part of this (Table 4.1).

TRPC1 is believed to be a store operated channel which is activated as a result of depletion of intracellular $Ca^{2+}$ (Wes et al., 1995, Zitt et al., 1996). Much evidence suggests TRPC3 functions as a receptor activated channel (McKay et al., 2000, Venkatachalam et al., 2001) linked to the presence of the phosphotidylinositol bisphosphate ($PIP_2$) metabolite, diacylglycerol (DAG) (Trebak et al., 2003a) though other studies have indicated that it functions as a store operated channel (Vazquez et al., 2001). Subsequently it was proposed that the activation mechanism of TRPC3 may be dependent on its expression levels; low levels of expression allow it to function as a store operated channel while at higher levels of expression it is coupled to receptor activation (Vazquez et al., 2003). Functionally distinct splice variants of TRPC3 have also been identified suggesting potentially even greater diversity in the mechanisms of activation of this channel (Kim et al., 2012a). TRPC5 is also considered to be activated by receptor coupling (Okada et al., 1998) and is sensitive to intracellular $Ca^{2+}$ levels (Blair et al., 2009). Like TRPC3, TRPC6 has been shown to be activated by DAG in a Protein Kinase C independent manner and independently of intracellular $Ca^{2+}$ stores and $PIP_2$ metabolite inositol trisphosphate ($IP_3$) (Hofmann et al., 1999, Trebak et al., 2003b). For example, it

is believed to contribute to mechanosensory function in the cardiovascular system indirectly though activation of the Angiotensin II (AT1) receptor (Saleh et al., 2006). TRPC3 shows significantly higher levels of basal activity than TRPC6 owing to different levels of N terminal glycosylation (Dietrich et al., 2003) however that both can be activated by DAG which is also involved in activity of stretch activated channels further highlights their potential relevance to mechanosensory function. It has been proposed that the process of DAG production from the breakdown of $PIP_2$ can lead to membrane stress which is comparable to stretch and that this is potentially a mechanism by which DAG sensitive TRPC channels could be sensitive to mechanical force (Hardie and Franze, 2012).

More recently, it has been suggested that TRPC3 and TRPC6 may also be subject to more direct regulation by $PIP_2$ independent of DAG (Lemonnier et al., 2008, Imai et al., 2012). Interestingly, Hardie and Franze showed that exposure to light evoked PLC-mediated contractions of the lipid membrane in photoreceptor cells of Drosophila. When mechanosensitive channels were expressed in these cells, they were activated by these contractions suggesting lipid mediated mechanical gating of TRP channels in sensory function (Hardie and Franze, 2012, Rohacs, 2013). The complexities of the mechanisms of channel activation for TRPC channels are somewhat difficult to define. This is complicated further by the fact that many *in vitro* investigations may not accurately mimic behaviour of the native forms of the channels. Crucially, the ability of TRPC channels to form functional heteromers which, evidence suggests, do not necessarily behave in the same way as homomeric channels (Clapham, 2003) adds to the difficulty of dissecting out the mechanisms of action and function of each TRPC channel subunit. This has been shown to be true in the case of TRPC3 and TRPC6 in mechanosensation.

| TRPC Channel | Mechanism of Activation (proposed) | Expression | Selectivity $P_{Na}$ : $P_{Ca}$ | Evidence of heteromerisation |
|---|---|---|---|---|
| TRPC1 | Store operated channel | Heart, brain, kidney, DRG | Non selective | TRPC3 (Liu et al., 2005)<br>TRPC5 (Strubing et al., 2001)<br>TRPC6<br>TRPV4 (Ma et al., 2011)<br>TRPP2 (Kobori et al., 2009) |
| TRPC2 | Store operated channel, DAG | Vomeronasal organ, testis | 2.7 | TRPC6 |
| TRPC3 | Store operated channel, DAG | Brain, kidney, lung, pancreas, DRG | 1.6 | TRPC1 (Liu et al., 2005, Yuan et al., 2007)<br>TRPC6 (Hofmann et al., 2002)<br>TRPC7 (Hofmann et al., 2002) |
| TRPC4 | Store operated channel | Brain, endothelia, adrenal gland, retina, DRG | 7 | TRPC1 (Shin et al., 2011)<br>TRPC3 (Shin et al., 2011)<br>TRPC5 (Shin et al., 2011)<br>TRPC6 (Shin et al., 2011) |
| TRPC5 | Store operated channel, DAG | Brain, kidney, liver, TG, DRG | 9.5 | TRPC1 (Strubing et al., 2001) |
| TRPC6 | DAG | Brain, lung, heart, DRG | 5 | TRPC3 (Hofmann et al., 2002)<br>TRPC4 (Yuan et al., 2007)<br>TRPC7 (Hofmann et al., 2002) |
| TRPC7 | Store operated channel, DAG | Eye, heart, lung | 1.9-5 | TRPC1 (Shin et al., 2011)<br>TRPC3 (Shin et al., 2011)<br>TRPC5 (Shin et al., 2011)<br>TRPC6 (Shin et al., 2011) |

Table 4.1 Properties of canonical TRP channels. TRP-protein interactions are also mapped in the TRIP database (Shin et al., 2011, Shin et al., 2012).

TRPC3 and TRPC6, which are known to heteromerise (Hofmann et al., 2002), came to light as potential cutaneous mechanosensors as a result of their expression in a subset of sensory neurons which are necessary for normal mechanosensation (Abrahamsen et al., 2008). Knockout mice were created with global deletion of either TRPC3 or TRPC6 or both genes. It was shown that mice with global deletion of both TRPC3 and TRPC6 (double knockouts; DKO) have attenuated innocuous mechanosensitivity which is reflected by a decrease in RA mechanically activated currents in DRG neurons taken from DKO mice (Quick et al., 2012). In the past, other potential mechanotransducers have lost favour as they lacked a role in transduction of auditory stimuli in the cochlea. In contrast to this, the previous data also suggested that DKO animals had a high frequency hearing deficit. Interestingly, when expressed heterologously, TRPC3 with or without TRPC6 is able to confer mechanosensitivity to ND-C cells but not HEK cells. ND-C cells are a sensory neuron derived cell line which display a slight endogenous mechanosensitivity (Rugiero and Wood, 2009). The context dependent ability of TRPC3 and TRPC6 to confer mechanosensitivity to cell lines suggests that these channels are, alone, insufficient to form a primary mechanotransduction complex. For example, Piezo2 produces a robust RA mechanically activated current in both Neuro2A and HEK293 cells (Coste et al., 2010). This, combined with the knowledge that TRPC channels interact with each other to produce functional heteromeric complexes (Table 4.1) led us to investigate the effect of knocking out additional TRPC channels, TRPC1 and TRPC5. Both of these channels are expressed in sensory neurons and are able to form heteromers with other TRPC channels. Moreover both *in vivo* and *in vitro* evidence suggests they are capable of contributing to mechanosensory function, for example TRPC1 confers sensitivity to osmotically induced membrane stretch in cultured DRG neurons (Staaf et al., 2009a) while TRPC5 is also sensitive to this in HEK293 cells (Gomis et al., 2008). TRPC5, in addition to TRPC6, is blocked by a tarantula toxin known to inhibit mechanosensitive channels (Spassova et al., 2006). TRPC1 KO mice show impaired responses to low threshold mechanical stimuli and a reduction in of firing of SA-Aβ and RA-Aδ fibres (Garrison et al., 2012).

As well as taking into consideration the likelihood of TRPC heteromers influencing function, using mice with multiple TRPC channel knockout provides a way of minimising the effects of the likely compensatory upregulation of other TRPC channels. The importance of considering these factors has been demonstrated by Quick et al. (2012) in

behavioural phenotype analysis and by Dietrich et al. (2005) who showed that TRPC3 is upregulated in the smooth muscle cells of TRPC6 KO mice where both channels are involved in regulation of vascular smooth muscle tone. Finally, it is thought that TRPC1 is not able to function as a homomeric channel (Storch et al., 2012), and is believed to require other channels to allow its translocation into the membrane (Hofmann et al., 2002) suggesting that the interaction between these TRPC subunits has potentially vast functional consequences.

The data presented below use a novel multiple knockout approach to investigate the effect of TRPC1, TRPC3 and TRPC6 triple KO (TKO) and TRPC1, TRPC3, TRPC5 and TRPC6 quadruple KO (QKO) on mechanosensory function *in vivo.* Behavioural paradigms have been chosen to investigate the sensory function of these animals while electrophysiological recordings investigated mechanosensory function in the inner ear.

## 4.3 Results

### *4.3.1 Gene knockout and breeding strategy of triple and quadruple knockout mice*

Triple and quadruple KO mice were generated on a mixed C57BL/6J:129SvEv background by combinatorial breeding of single KO alleles (TRPC1 (Dietrich et al., 2007), TRPC3 (Hartmann et al., 2008), TRPC5 (Phelan et al., 2013), TRPC6 (Dietrich et al., 2005)) at the Comparative Medicine Branch of the NIEHS in North Carolina by Dr. J Abramowitz and Prof. L. Birnbaumer.

Both TRPC1 and TRPC6 gene knockouts were created by interruption of an exon with a neomycin cassette (PGK-neo). In TRPC1, the cassette was placed in exon 8 downstream of an *Eco*RI site which is destroyed, leading to creation of a stop codon in the exon; while in the TRPC6 gene, exon 7 was replaced with the cassette. In both cases, the interrupted regions normally encode 86 amino acid long sequences which form transmembrane domains 4 and 5 and contribute to the pore domain of the channels (Figure 4.1).

TRPC3 and TRPC5 gene knockouts were produced by global Cre recombinase mediated excision of floxed exons. Exon 7 of TRPC3 was flanked by *loxP* sites; the floxed mouse was crossed with a mouse expressing a Cre recombinase under the control of the Mox2 promoter (which is ubiquitously expressed, early in development), potentially producing a truncated protein. In TRPC5, a mouse with floxed exon 5 was excised by crossing with a mouse expressing Cre recombinase under the control of the Sox-2 promoter (also expressed early in development). If a truncated protein is expressed, it would be lacking expression of the $5^{th}$ and $6^{th}$ transmembrane domains (Figure 4.1).

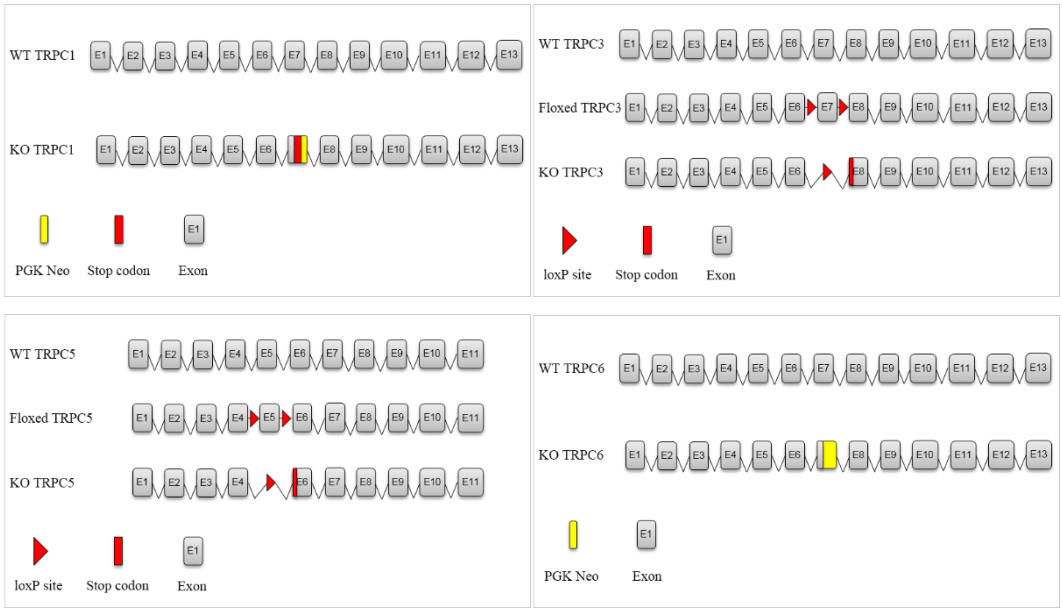



Figure 4.1 Construct designs for TRPC single KO mice which were bred together to produce multiple KOs.

TRPC3 and TRPC6 double KO mice (also produced from single KOs) were crossed with C57BL/6J mice to generate heterozygous offspring. Heterozygotes were then bred together to produce WT and DKO mice which acted as controls for QKO mice. Though not littermate controls, these animals were age and sex matched as far as possible and produced on the same background. TKO mice were compared to TRPC1, 3, 6 heterozygous mice which were produced by breeding TKO mice with C57BL/6J mice. Such controls were not an option for QKO mice as the TRPC5 gene is on the X chromosome so male mice can only be WT or KO for TRPC5.

TRPC gene knockouts were confirmed through genotyping using PCR of genomic DNA isolated from ear samples of each mouse (Figure 4.2). Primers were used to identify WT, heterozygous or KO gene expression; primer sequences and band sizes are listed in the table below (Table 4.2).

| Gene | Primer | Sequence (5'-3') | WT band size | KO band size |
|---|---|---|---|---|
| **TRPC1** | F | GGGATGATTTGGTCAGACATTAAG | 368 bp | 250 bp |
| **TRPC1** | R | GTGTACCTAACATCAACCATGGTAC | | |
| **TRPC1** | R1 | TGGATGTGGAATGTGTGCGAGGC | | |
| **TRPC3** | F2 | GAATCCACCTGCTTACAACCATGTG | 800 bp | 300 bp |
| **TRPC3** | R | GGTGGAGGTAACACACAGCTAAGCC | | |
| **TRPC5** | R | GGATGTTGGCTCTGTGAAACAATGACTC | 2.3 Kb | 450 bp |
| **TRPC5** | F2 | AGCTAACAGTATCCCTAAGTGATCC | | |
| **TRPC6** | 3 | CAGATCATCTCTGAAGGTCTTTATGC | 234 bp | N/A |
| **TRPC6** | 4 | TGTGAATGCTTCATTCTGTTTTGCGCC | | |
| **TRPC6** | 1 | ACGAGACTAGTGAGACGTGCTACTTCC | N/A | 339 bp |
| **TRPC6** | 2 | GGGTTTAATGTCTGTATCACTAAAGCCTCC | | |

Table 4.2 Genotyping primers for TRPC channels

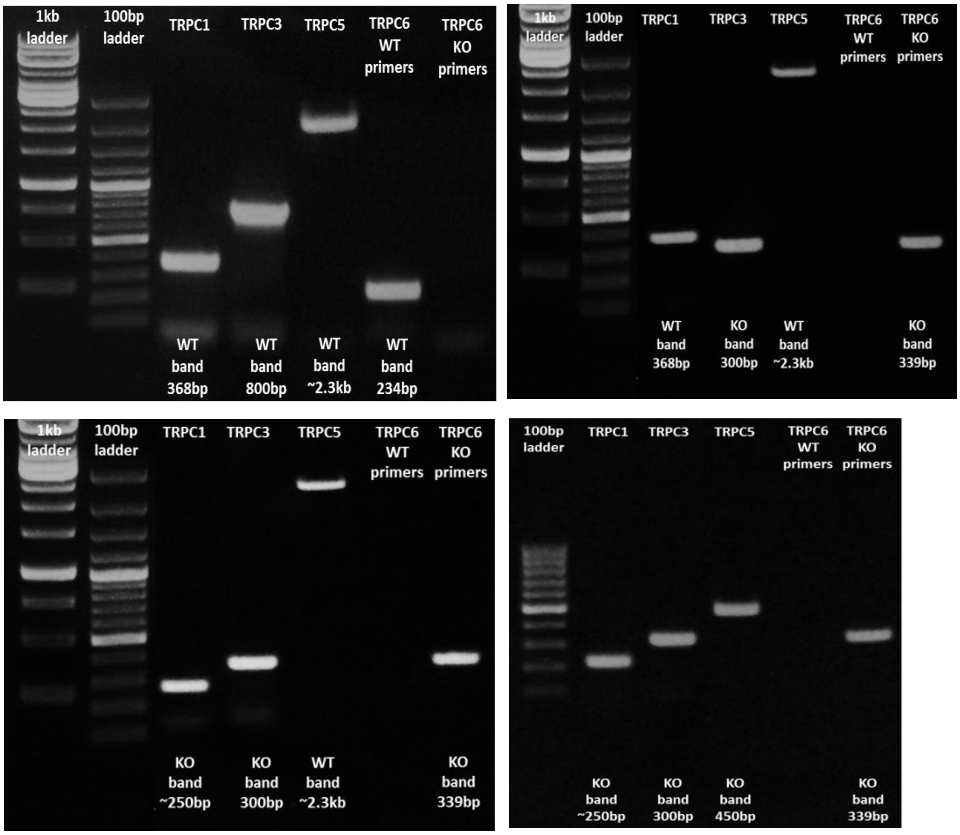



Figure 4.2 PCR genotyping was performed to identify WT, DKO, TKO and QKO mice.

### *4.3.2 Mice with global deletion of multiple TRPC channels develop normally*

Mice with TRPC1, 3, 6 and TRPC1, 3, 5, 6 deletions develop normally to adulthood, are born in average litter sizes and exhibit largely normal health. At 6 weeks old, there was no significant difference in the mass of the animals (Figure 4.3); WT=20.30g±1.77g; DKO=21.47g±1.38g; QKO =24.36g±1.58g; TKO=22.58g±1.22g; Het=21.64g±1.08g ($p$>0.05).

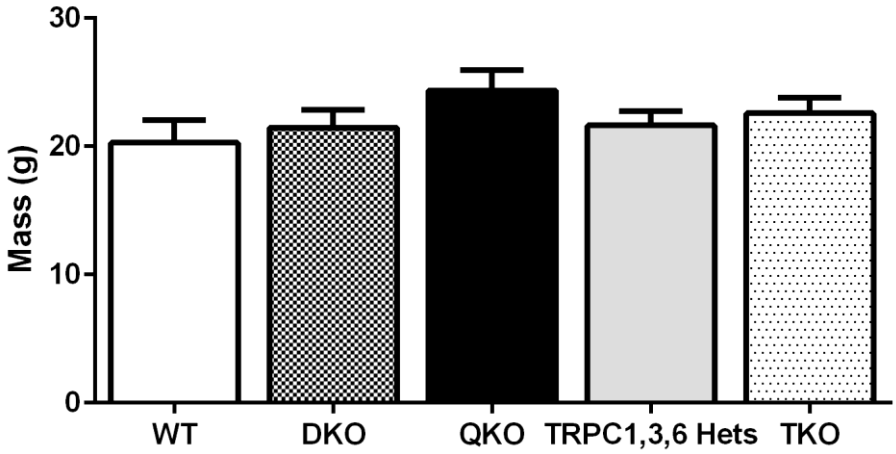



Figure 4.3 Mass of animals at 6 weeks old Was not significantly different between groups (WT n=6; DKO n=9; QKO n=5; TKO n=6; TRPC Het n=9 Data are shown as Mean±SEM with one-way ANOVA).

### *4.3.3 Mice with global deletion of multiple TRPC channels have deficits in light touch sensitivity*

Previous work identified a combinatorial role for TRPC3 and TRPC6 in innocuous mechanosensation (Quick et al., 2012) shown by an increase in the paw withdrawal threshold (PWT) and a decrease in response frequency to stimulation with a cotton swab. We repeated these tests in QKO and TKO mice to determine whether these deficits were augmented by additional loss of TRPC1 and TRPC5.

In the von Frey test QKO mice show an increase in PWT when compared to WT controls (effect of genotype, ANOVA $p$=0.004) (WT= 0.36g, QKO =0.69g; $p$=0.003). This is not, however, significantly different than the PWT of DKO mice (0.69g; $p$>0.05). TKO mice (0.55g) show a similar PWT to heterozygous controls (0.53g, $p$>0.05) though a Student's t test showed a significant increase compared to WT mice ($p$=0.045) though no significant difference was observed when compared to DKO or QKO groups ($p$>0.05) (Figure 4.4a).

Sensory innervation of hairy and glabrous skin is different. von Frey testing was performed on the hairy skin of the abdomen to allow comparison of this to the glabrous skin of the paw as phenotypic differences between these two skin types have been observed (Minett et al., 2014a). Though there was a trend towards KO groups showing less sensitivity to mechanical stimuli than control groups, this was not significant ($p$>0.05) (Figure 4.4b).

Different mechanosensory end organs with different neuronal innervation are associated with responses to different stimulus modalities. As well as assessing innocuous mechanosensitivity using von Frey fibres, we used a dynamic cotton bud stimulus to investigate the roles of TRPC channels in mechanosensation. In the cotton swab test, the number of responses out of a total of five stimuli was counted. Data show there is a stepwise decrease in the response frequency from WT to DKO to QKO mice (scores 2.83±0.31, 1.80±0.20 and 0.80±0.25, respectively) (Figure 4.4c). QKO mice showed a significant decrease in the percentage responses when compared to WT controls (genotype effect, Kruskal Wallis test $p$=0.004; QKO v. WT $p$=0.0006). TKO mice responded fewer times to cotton swab stimulation than heterozygous controls (Figure 4.4c) (scores 1.13±0.40 and 2.60±0.27, respectively; $p$=0.01) and if compared to WT mice, their decrease in sensitivity is also significant ($p$=0.02; not shown on graph for clarity).

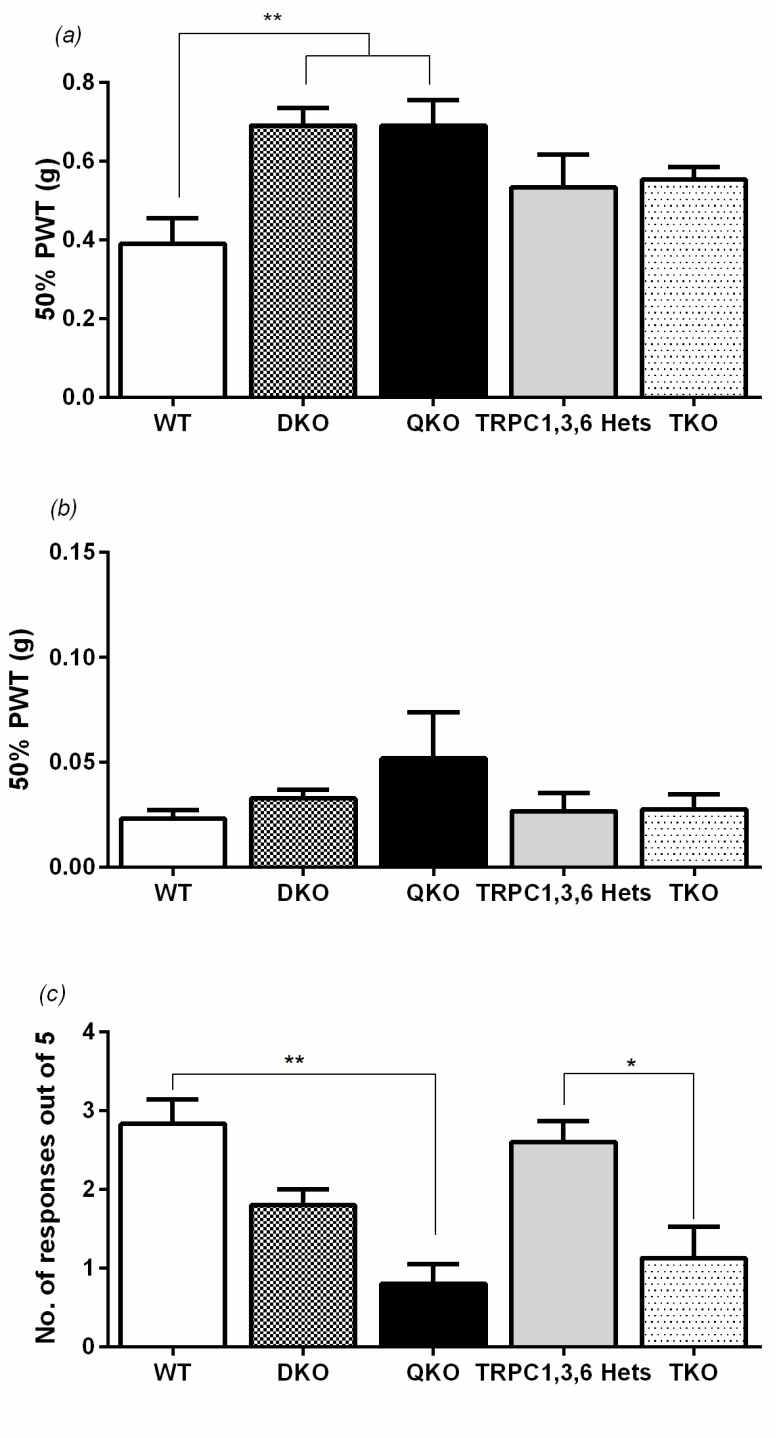



Figure 4.4 Innocuous mechanical sensitivity is impaired in multiple KO mice, demonstrated by an increase in PWT on the glabrous skin of the paw (a) though no change is observed on the hairy skin of the abdomen (b) and a decrease in the number of responses to a cotton swab stimulus (c) (von Frey paw: WT n=9; DKO n=10; QKO n=10; TKO n=8; TRPC Het n=9; von Frey abdomen: n=6 per group; cotton swab: WT n=6; DKO n=10; QKO n=10; TKO n=8; TRPC Het n=10 Data are shown as Mean±SEM with one-way ANOVA and Tukey post-hoc test (a,b) and Kruskal Wallis with Dunn's post hoc test (b) *$p$<0.05; **$p$<0.01; ***$p$<0.001; ****$p$<0.0001).

### *4.3.4 Mice with global deletion of multiple TRPC channels show normal responses to noxious mechanical stimuli*

Sensitivity to noxious mechanical stimuli is assessed using Randall Selitto equipment applied to the paw or the tail. As some groups have reported that a phenotype is only produced using one of these tests, both paw and tail responses have been assessed (Minett et al., 2014a).

The force at which QKO animals responded to noxious mechanical stimulation on the tail (173.5g±4.72g) was comparable to WT (165.42g±3.90g) and DKO (159.25g±4.64g) animals ($p$>0.05) (Figure 4.5a). Heterozygous and TKO mice also showed no significant difference in responses with the force required to elicit withdrawal as 170.42g±4.93g and162.08g±4.93g, respectively ($p$>0.05) (Figure 4.5a).

Similarly, the force required to elicit a response when stimulating the paw was consistent between groups; WT 98.26g±5.17g, DKO 101.71g±4.63g, QKO 100.13g±3.87g and TKO 94.46g±3.81g compared to heterozygotes 105.18g±6.35g ($p$>0.05) (Figure 4.5b).

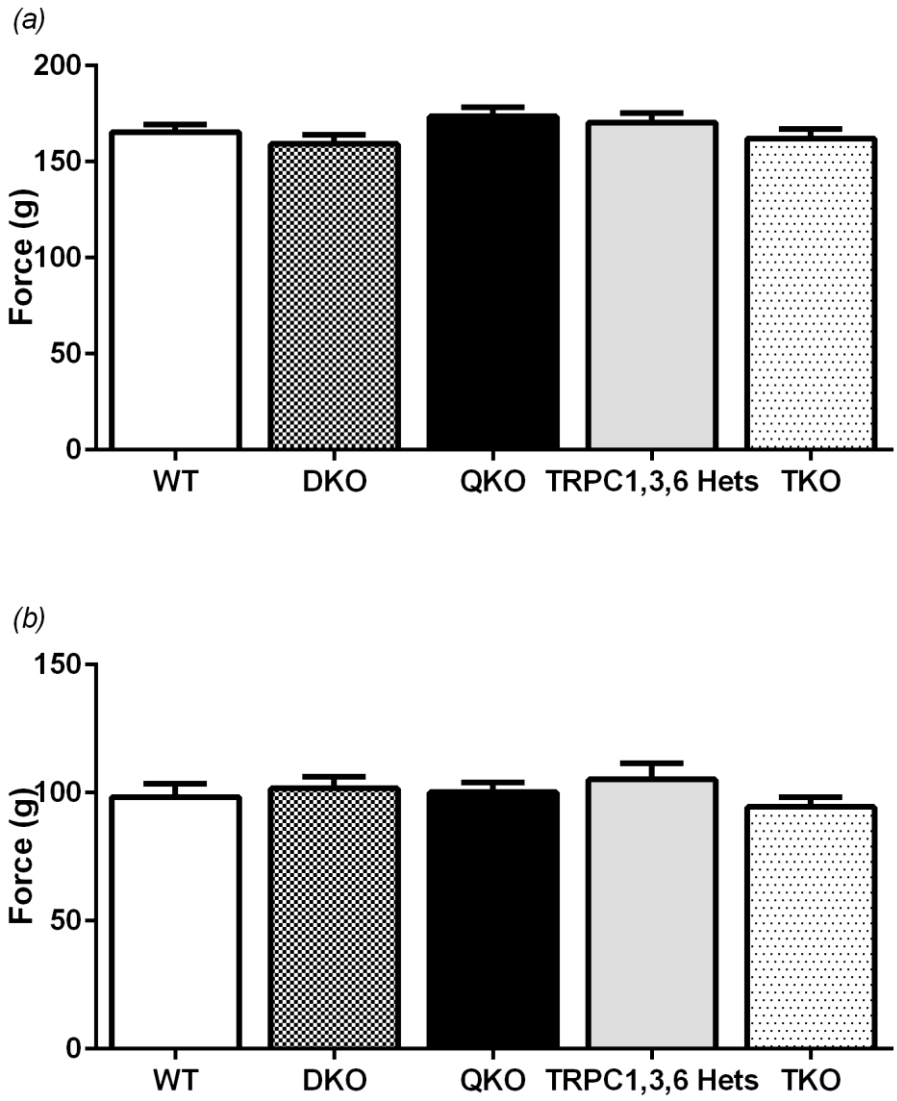



Figure 4.5 Noxious mechanosensation is unimpaired in all groups. Sensitivity was tested using Randall-Selitto apparatus applied to the tail (a) and to the paw (c) (R-S tail: WT n=6; DKO n=10; QKO n=10; TKO n=6; TRPC Het n=6; R-S paw: WT n=6; DKO n=10; QKO n=10; TKO n=9; TRPC Het n=7 Data are shown as Mean±SEM with one-way ANOVA).

### *4.3.5 Mice with global deletion of multiple TRPC channels show normal responses to thermal stimuli*

Exposure to a radiant heat source in the Hargreaves test indicated no difference in heat sensitivity between genotypes. The latency to a nocifensive response was 18.12s±1.62s for WT, 17.42s±1.09s for DKO, 16.81s±0.61s for QKO and 18.53s±1.81s and 15.66s±0.89s for TKO and heterozygous controls, respectively (Figure 4.6a). There was no significant difference between test groups and their controls. While the Hargreaves test is believed to be primarily a test of spinal reflex responses, it is suggested that the hot plate test involves some supraspinal mechanisms (Chapman et al., 1985, Minett, 2011); since these mice have global deletion of TRPC channels comparing these assessment types is important. However, consistent with the Hargreaves test results, responses in the hot plate test were not significantly different between groups. At 50$^{o}$C the latency to response times were WT=33.37s±3.48s; DKO=39.78s±1.97s; QKO=33.08s±1.86s; TKO=36.05s±2.65; Het=40.02s±2.01s and at 55$^{o}$C, WT=19.78s±2.00s; DKO=16.97s±0.52s; QKO=12.77s±0.72s; TKO=14.80s±1.11s and Het =15.18s±1.37s (Figure 4.6b).

A place preference set up was used to assess cold sensitivity; a baseline recording was taken with both plates set at 20$^{o}$C for 2 minutes before one plate was dropped to 4$^{o}$C for another 2 minutes. QKO mice spent 8.61%±6.17% of the time on the 4$^{o}$C test plate compared to 47.22%±10.49% time spent on the same plate when held at 20$^{o}$C (Figure 4.6c). TKO mice spent 15.42%±10.72% of the time on the noxious cold (4$^{o}$C) test plate compared to 61.67% time spent on the test plate at 20$^{o}$C (Figure 4.6c). The time spent on the test plate when held at a noxious cold temperature (4$^{o}$C) was not significantly different between groups (WT=9.028%±5.35%; DKO=15.28%±6.17%; Het 15.83s±8.56s).

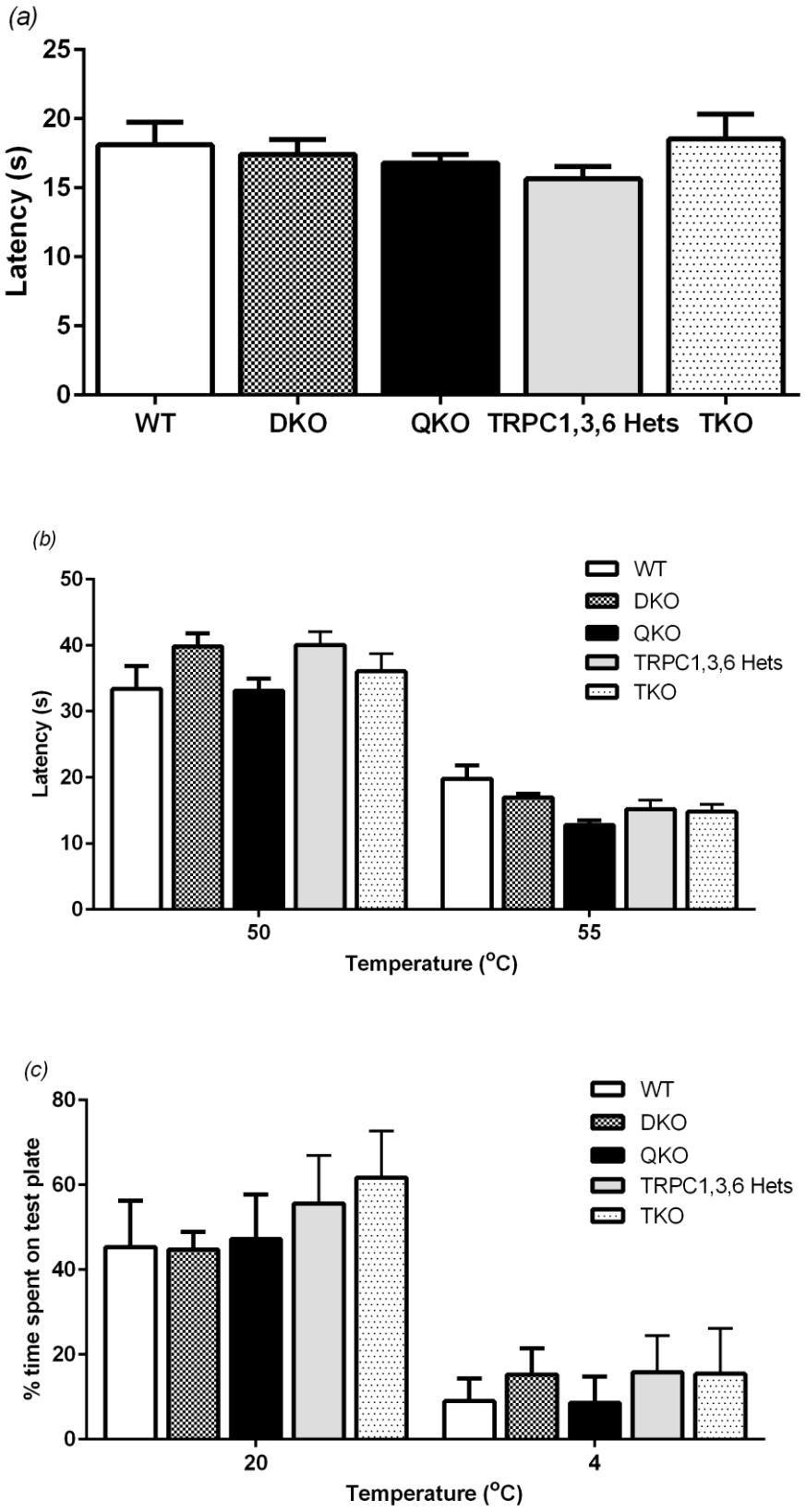



Figure 4.6 Thermosensation is unimpaired in all groups shown by comparable responses to a latent heat stimulus (a), a hot plate test (b) and a noxious cold place preference test (c) (All tests, n=6 per group Data are shown as Mean±SEM with two-way ANOVA (a, b, c).

### *4.3.6 Mice with global deletion of multiple TRPC channels show some deficits in vestibular function*

In the forced swim test, an increase in the number of head submerges was seen in QKO animals compared to WT animals (scores of 3.17 and 0.50, respectively; $p$=0.11) though this change was less pronounced than that seen in DKO mice (genotype effect Kruskal Wallis test, p=0.0005; WT v. DKO $p$=0.002; DKO v. QKO $p$>0.05) (Figure 4.7a). TKO mice also showed an average of 4.33 head submerges compared to the 0.83 seen in heterozygous controls ($p$=0.04) suggesting impaired vestibular function in TRPC multiple KO mice.

In the trunk curl test, scores were attributed to each mouse according to a basic scoring system which assesses vestibular function. WT mice scored 0.75±0.11; DKO, 0.33±0.11; QKO, 0.33±0.11 (genotype effect Kruskal Wallis test, p=0.003; WT v DKO and v QKO $p$>0.05) while TKO scored 0.10±0.10 compared to the heterozygotes score 0.67±0.11 ($p$=0.01) (Figure 4.7b).

These findings suggest some deficit in the normal vestibular function of all the groups of knockout animals. However, the time taken to fall from an accelerating rotarod was similar in all groups with latencies of 181.6s±10.70s, WT; 181.60s±21.99s, DKO; 202.80s±26.13s; 204.70s±24.77s, QKO; 202.80s±26.13s, TKO and 202.4s±29.07s, heterozygotes (Figure 4.7c). Although the rotarod test can be used to investigate vestibular function, other studies have found that the test does not always correlate with the alterations in vestibular deficits presented by other relevant tests (Minasyan et al., 2009, Quick et al., 2012).

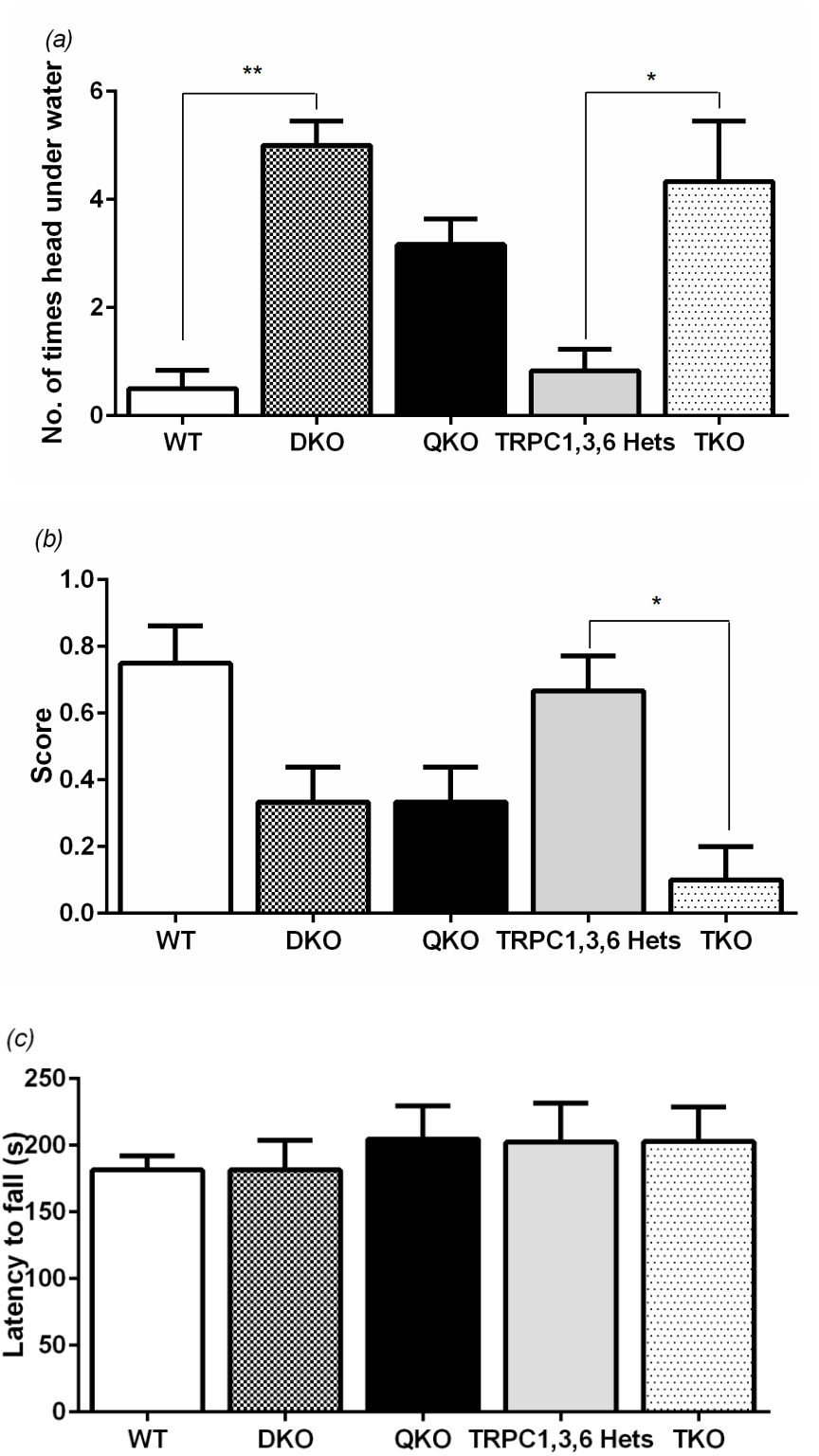



Figure 4.7 Vestibular function is impaired in TRPC KO mice. The number of head submerges in a swim test (a). Trunk curl test score (b). Latency to fall from an accelerating rotarod is unimpaired in KO mice (c). (Swim test n=6 per group; Trunk curl: WT, DKO, QKO, TRPC Het n=6 per group; TKO n=5; Rotarod WT n=6; DKO n=7; QKO n=6; TKO n=6; TRPC Het n=9. Data are shown as Mean±SEM with Kruskal Wallis with Dunn's post hoc test (a,b) and one-way ANOVA (c) *$p$<0.05; **$p$<0.01; ***$p$<0.001; ****$p$<0.0001).

### *4.3.7 Mice with global deletion of multiple TRPC channels have some hearing deficits*

*Auditory brainstem response (ABR) recordings were performed by Dr Ruth Taylor (UCL Ear Institute)*

A calibrated click box was used for a crude assessment of auditory function; the normal Preyer reflex response it provokes was assessed in multiple KO animals. QKO animals responded with an average score of 0.31±0.12 compared to 0.75±0.06 in WT and 0.53±0.14 in DKO (Figure 4.8a). The mean TKO response score was 0.47±0.11 compared with 0.86±0.04 in heterozygous controls (Figure 4.8b). The difference between WT and QKO animals was found to be significant (genotype effect Kruskal Wallis test, $p$=0.03; $p$=0.04) and when compared to the TKO score of 0.47, there is a clear downward trend with deletion of each additional gene. The TKO score was also significantly reduced compared to the heterozygote score of 0.86 ($p$=0.02). Nonetheless, all genotypes maintained some responses showing hearing at this frequency was not completely abolished.

Recordings from the brainstem were taken in response to stimuli at a range of frequencies. TKO mice were compared to WT and heterozygous controls and showed an elevated threshold in response to frequencies of 24 kHz (TKO 46dB±3.23dB; Het 30.00dB±6.89dB) and 32 kHz (TKO 68.50dB±3.25dB; WT 54.17dB±5.39dB) though these were not significant. Also at the highest frequency of 40kHZ, responses of all groups were seen at similar thresholds (~70DbSPL) (Figure 4.8d). These values can be considered unexpectedly high for WT animals.

Separately tested QKOs were compared to DKO and WT mice and showed a much higher threshold for a response at all frequencies tested over 24kHz. (genotype effect ANOVA, p=0.0002; 24kHz, QKO v. WT $p$=0.004; QKO v. DKO $p$=0.002; 32kHz, QKO v. WT $p$<0.0001; QKO v. DKO $p$<0.0001; 40kHz, QKO v. WT $p$<0.0001; QKO v. DKO $p$=0.0006). A higher response threshold was also seen in QKO animals upon stimulation with a low frequency stimulus of 8kHz (WT, 25.83dB±4.17dB; DKO, 32.22dB±2.90dB; QKO, 50.00dB±5.70dB; QKO v. WT $p$=0.004; QKO v. DKO $p$=0.03) (Figure 4.8c). Altogether, these data substantiate the finding that loss of function of multiple TRPC channels leads to impaired hearing function and suggests that deletion of each additional TRP channel is able to augment this sensory deficit.

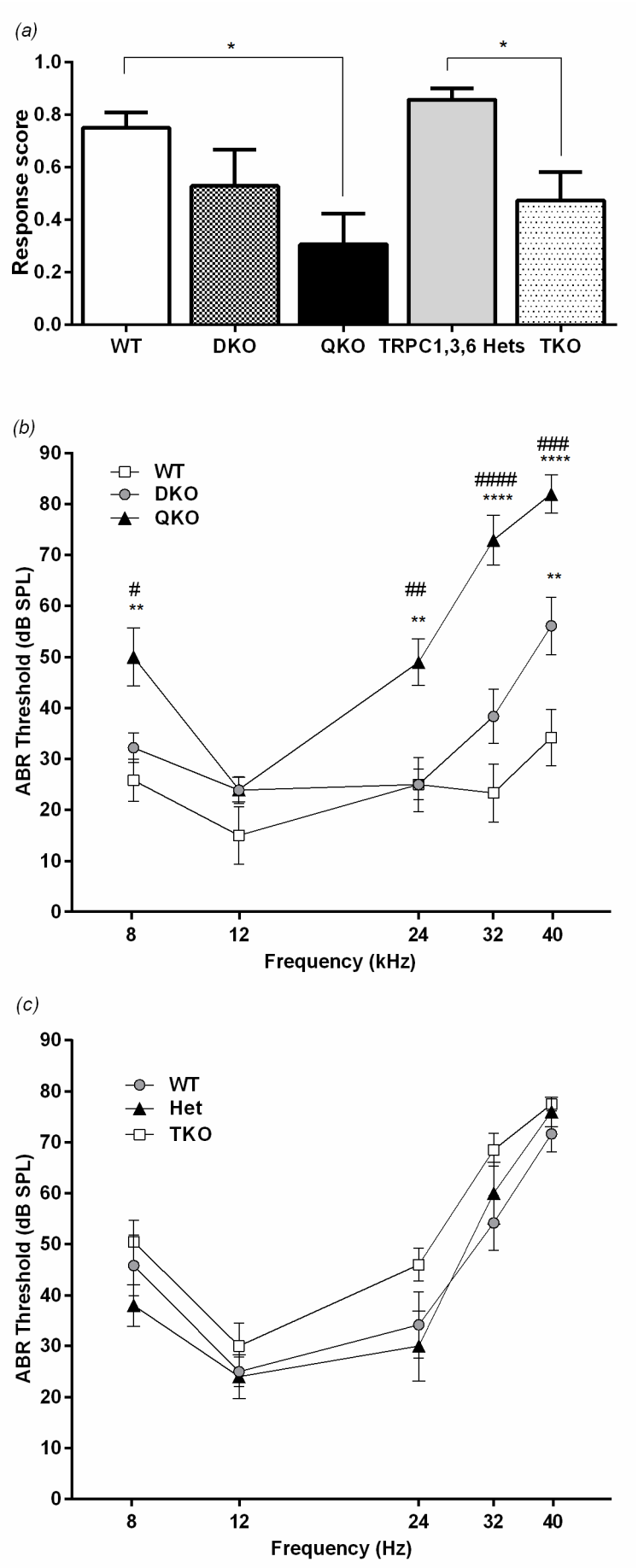

(a)
Response score
1.0
0.8
0.6
0.4
0.2
0.0
*
*
WT
DKO
QKO
TRPC1,3,6 Hets
TKO
(b)
ABR Threshold (dB SPL)
90
80
70
60
50
40
30
20
10
0
WT
DKO
QKO
#
**
##
**
####
****
###
****
**
8
12
24
32
40
Frequency (kHz)
(c)
ABR Threshold (dB SPL)
WT
Het
TKO
Frequency (Hz)

Figure 4.8 Impaired hearing in QKO and TKO mice (a) compared to controls shown by a decrease in Preyer reflex response score and higher threshold responses to auditory pip-tone stimuli in Auditory Brainstem Response recordings in QKO mice compared to DKO and WT (b) and in TKO mice compared to TRPC Het and WT mice (c). (a) WT, DKO, QKO, TKO n=6 per group; TRPC Het n=5 (b) WT n=6; DKO n=9; QKO n=5; (c) WT n=6 TKO n=10; TRPC Het n=5. Data are shown as Mean±SEM with Kruskal Wallis with Dunn's post hoc test (a) and two-way ANOVA and Tukey post-hoc test (b,c) *$p$<0.05; **$p$<0.01; ***$p$<0.001; ****$p$<0.0001 (in b,c *denotes significance compared to WT; # denotes significance compared to DKO)).

### *4.3.8 Mice with global deletion of multiple TRPC channels show normal mechano-electrical transduction currents in the outer hair cells of the cochlea*

*This work was performed by Terri Desmonds (University of Sussex)*

Mechano-electrical transducer (MET) currents recorded from outer hair cells of the cochlea are evoked by sinusoidal force stimuli. These currents are of normal amplitude in cells taken from the apical end of the cochlea which responds to low frequency sounds and at the basal end which is responsive to high frequency sounds in all genotypes. Previously, reduced amplitude currents were observed in basal coil hair cells, though these traces show that multiple KO animals exhibit the same features as WT mice (Figure 4.9). These features include current reversal near to 0mV and small numbers of channels open at rest which increase with each depolarisation as a result of reduced $Ca^{2+}$-dependent adaptation. Whereas attenuation of the current amplitude may indicate impaired cochlear mechanotransduction, these currents demonstrate that this is not the case in multiple KO animals and that, therefore, TRPC channels are not direct mechanotransducers in the cochlea.

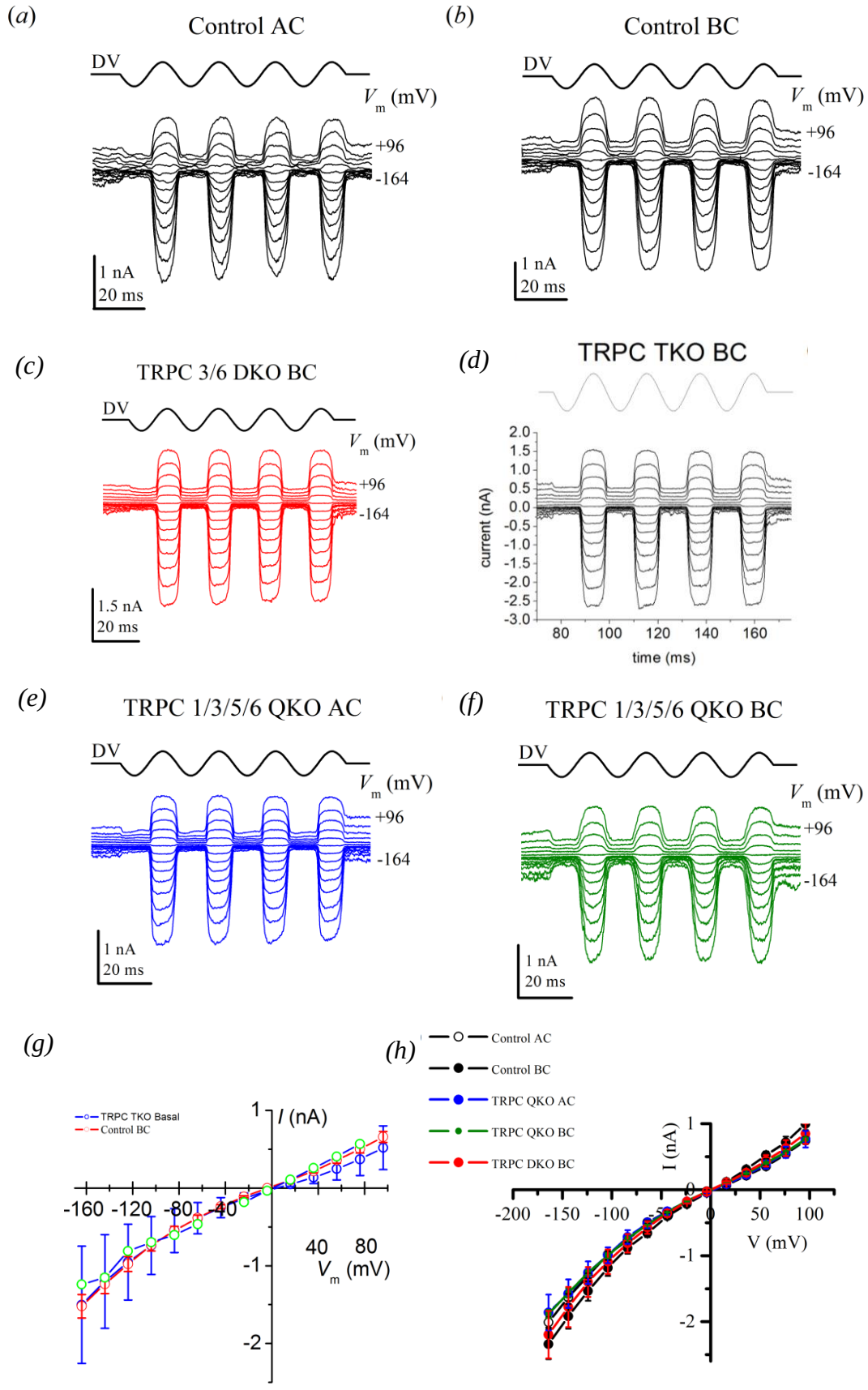

(a)
Control AC
DV
$V_m$ (mV)
+96
-164
1 nA
20 ms
(b)
Control BC
DV
$V_m$ (mV)
+96
-164
1 nA
20 ms
(c)
TRPC 3/6 DKO BC
DV
$V_m$ (mV)
+96
-164
1.5 nA
20 ms
(d)
TRPC TKO BC
current (nA)
time (ms)
(e)
TRPC 1/3/5/6 QKO AC
DV
$V_m$ (mV)
+96
-164
1 nA
20 ms
(f)
TRPC 1/3/5/6 QKO BC
DV
$V_m$ (mV)
+96
-164
1 nA
20 ms
(g)
TRPC TKO Basal
Control BC
I (nA)
$V_m$ (mV)
(h)
Control AC
Control BC
TRPC QKO AC
TRPC QKO BC
TRPC DKO BC
I (nA)
V (mV)

Figure 4.9 Representative MET current recording from the apical and basal coil (AC and BC, respectively) of control (a, b) and QKO mice (e,f) and from the basal coil of DKO (c) and TKO (d) mice . Current-voltage curves averaged from (g) 16 mid-basal coil control OHCs, 4 mid-basal coil TRPC1/TRPC3/TRPC6 TKO OHCs (h) 5 mid-apical WT OHCs, 4 mid-basal WT OHCs, 3 mid-apical QKO OHCs; 2 mid-basal QKO OHCs and 3 mid-basal DKO OHCs.

## 4.4 Discussion

The aim of this work was to characterise the sensory function of mice with global deletion of multiple members of the canonical subfamily of TRP channels. Previous work showed that global deletion of both TRPC3 and TRPC6 attenuates sensitivity to innocuous mechanical stimuli and impairs hearing and vestibular function (Quick et al., 2012). No such phenotype was observed in mice with global deletion of only one of these genes, therefore it seems that TRPC channels may have a combinatorial role in mediating specific sensory functions. As TRP channels are known to heteromultimerise and are believed to show functional redundancy, the development of these multiple knockout animals provided a novel approach to investigating the combined roles of the TRPC channels where monogenic studies may have been unsatisfactory. We pursued this line of investigation by characterising the sensory function of TRPC1, TRPC3 and TRPC6 triple KO and TRPC1, TRPC3, TRPC5 and TRPC6 quadruple KO mice and observed that additional KO of TRPC1 and TRPC5 in addition to TRPC3 and TRPC6 augments specific sensory deficits.

### *4.4.1 TRPC channels are required for normal sensitivity to innocuous mechanical stimuli*

Sensitivity to innocuous mechanical stimuli is impaired in TKO and QKO mice. QKO mice show an increase in paw withdrawal threshold compared to WT controls in the von Frey test. Interestingly, this hyposensitive phenotype is not augmented compared to DKO mice. The threshold of TKO animals is comparable to heterozygous controls however, although it is not significant, it does appear to be higher than the WT threshold. The cotton swab test provides a different means of assessing sensitivity to mechanical stimuli as it an unequivocally light touch stimulus which is dynamic and thus has different qualities to stimulation with punctate von Frey fibres. In this test, there was a decrease in touch sensitivity which appears to be augmented in a stepwise manner by deletion of each additional channel, suggesting a combinatorial role for these channels in light touch sensitivity.

Based on the existing knowledge of a role for TRPC1 in light touch mechanosensation and TRPC5 in stretch activation in other physiological systems we hypothesised that additional deletion of these TRPC channels may augment the phenotype seen in DKO mice. As such, the fact that we did not see such a change in the von Frey test is perhaps

surprising, however in the cotton swab test, we observed a stepwise decrease in the response score with each additional gene knockout. Garrison et al. (2012) identified a similar issue in mice lacking TRPC1. The responses of these mice to suprathreshold mechanical stimuli were unchanged when compared to controls but the percentage responses to sub threshold stimuli, including a cotton swab and low threshold von Frey fibres, were attenuated. Different subsets of sensory neurons are responsible for specific sub-modalities, for example a specific subset of C-LTMR fibres are believed to be responsible for responses to pleasant touch (Loken et al., 2009), while activation of different mechanosensory end organs by different stimulus qualities leads to activation of different combinations of primary afferent neurons. As such, it is possible that the different nature of these assays may involve TRPC channels expressed in different subsets of sensory neurons. Indeed, single cell RNA sequencing identified one subset of neurons which expresses all of the four TRPC subunits and many others that expressed two or three (Usoskin et al., 2015); it may be that by knocking out all four, we are interfering with multiple, distinct mechanosensory mechanisms. Importantly, TRPC1 KO mice showed unimpaired firing in SA-Aβ mechanoreceptors (Garrison et al., 2012) whereas we previously identified a loss of RA currents in TRPC3 and TRPC6 DKO mice (Quick et al., 2012) which substantiates this hypothesis. As such, it could be that the different stimulus qualities of the cotton swab test compare to the von Frey fibres means that there is more of a role for large diameter fibres in responses to this particular stimulus type. Also, TRPC1 but not TRPC6 is believed to contribute to mechanically activated $Ca^{2+}$ influx in Merkel cells (Haeberle et al., 2008) so it is possible that differential TRPC channel expression in mechanosensory end organs means multiple TRPC KO mice have altered mechanotransduction in multiple neuronal subtypes and also mechanosensory end organs. As such, one potential interpretation of this data is that by knocking out TRPC1, TRPC3, TRPC5 and TRPC6 we are affecting mechanosensory processing in multiple ways and that the behavioural assessment we have performed is indicative of an augmented deficit in a specific mechanosensory sub-modality. In order to determine whether this is the case, electrophysiological characterisation of TKO and QKO neurons would need to be performed. Both mechanoclamp stimulation of cultured DRG and recording from the *ex-vivo* skin-saphenous nerve preparation would allow a better indication of the way in which multiple TRPC channel KOs have altered the properties of mechanosensitive fibres.

In spite of the expression of TRPC3 and TRPC6 in $Na_v1.8$ expressing nociceptive neurons (Abrahamsen et al., 2008), sensitivity to noxious mechanical stimuli were normal in our multiple KO mice. TRPC5 is also expressed in some small diameter neurons (Zimmermann et al., 2011) but is also found in some large diameter neurons where TRPC1 is also expressed. Garrison et al. (2012) showed that there was no alteration in the behaviour of TRPC1 knockout animals in response to noxious mechanical stimuli, nor was there any change in the firing properties of C and Aδ nociceptors. Alessandri-Haber et al. (2009) found antisense oligonucleotides knockdown of TRPC1 and TRPC6 did not alter baseline responses to Randall-Selitto testing. Together, these are consistent with the conclusion that these channels do not play a significant role in noxious mechanical sensitivity and substantiate a modality specific role for TRPC channels in mediating sensitivity to innocuous, and not noxious, mechanical stimuli.

Sensitivity to acute noxious heat stimuli is unaltered in all groups which is consistent with previous findings about members of the TRPC subfamily of channels (Garrison et al., 2012, Quick et al., 2012). Interestingly, TRPC5 has been linked to a role in cold sensing; when expressed in HEK293 cells, currents were induced by temperatures 25-37ºC. However these currents were not inducible in TRPC1/5 heteromers and TRPC5 null mice did not display any changes in behavioural responses to cold (Zimmermann et al., 2011). In the current study, QKO animals (the only group which lack TRPC5 expression) exhibited normal responses to cold. In light of the findings mentioned above, it may be interesting to look at responses of these animals to cool temperatures within the 25-37ºC range which induced currents in the heterologously expressed channels. Alternatively, it may also be indicative of TRPC5 functioning cooperatively with other TRP channels which are linked to a role in cold sensitivity. Further investigation into the roles of these channels, for example through electrophysiological characterisation of KO sensory neurons, may elucidate more about the sensory functions of these channels.

### *4.4.2 TRPC channels indirectly contribute to auditory and vestibular function*

It has been suggested that cutaneous mechanotransduction complexes and complexes of the inner ear are likely to have a common identity as loading of FM1-43, a styryl dye which is taken up by mechanosensitive channels in the cochlea, is inhibited by NMB-1, the peptide which blocks mechanically activated SA currents in sensory neurons (Gale et al., 2001, Drew et al., 2007). Previous work indicated a role for TRPC3 and TRPC6 in hearing and vestibular function (Quick et al., 2012). These new data support this

suggestion and also implicate TRPC1 and TRPC5 in normal hearing function as ABR thresholds are higher in QKO mice than control mice.

In order to determine whether the observed hearing deficits in TRPC multiple KO mice are the result of altered mechanotransduction in the cochlea, mechano-electrical transducer (MET) currents were recorded from cultured OHCs. As the amplitude of the currents produced by mechanotransduction complexes in the hair cells were comparable in all KO groups to WT, it is unlikely that TRPC1, TRPC3, TRPC5 or TRPC6 are directly involved in auditory mechanotransduction. Instead, it is likely that the loss of TRPC channel function affects the auditory process downstream of the MET channel, though it is possible that function is impaired elsewhere in the cochlea. Indeed, since vestibular function is also impaired, it is likely that mechanosensory processing in the inner ear is altered by loss of function of these TRPC subunits. However, in order to identify the way in which TRPC channel deletion alters this mechanosensory processing, more in depth behavioural and electrophysiological phenotyping would need to be performed.

### *4.4.3 A role for TRPC channels in mechanosensation*

The role of TRPC channels in sensory function is not clear cut. The characterisation of mice with multiple TRPC channels knocked out is beneficial as these channels are known to heteromultimerise and are believed to show functional redundancy. As a result, animals with a single null gene may have a masked phenotype if other channels are able to compensate for the loss of function of the affected channel. Through using mice with loss of function of multiple TRPC channels, this is likely to have been avoided to some extent. However, the fact that mice used in the current study have a deletion of these channels from birth means other changes may have occurred outside of the TRPC family to compensate for these mechanisms. Moreover, the fact that these channels are deleted globally suggests that sensory phenotypes may be affected by changes outside of sensory neurons; TRPC channels are expressed in keratinocytes (Tu et al., 2005), for example, which are believed to contribute to mechanosensation (Lumpkin et al., 2010). Though current understanding of the role of keratinocytes in mechanosensation is limited, there is evidence that these cells respond to mechanical stress (Tsutsumi et al., 2009). TRPV channels are expressed in keratinocytes and have been shown to mediate responses to stretch stimuli and noxious thermal stimuli in these cells (Huang et al., 2008, Mihara et al., 2011). It has been suggested that sensory transduction through keratinocytes may lead to neuronal activation through release of mediators such as prostaglandins and ATP

(Huang et al., 2008, Mihara et al., 2011). In addition, Merkel cells, another form of epidermal cell, have recently been shown to have a direct role in mechanosensation where they are sites of mechanotransduction (Maksimovic et al., 2014). It is therefore possible that the role we observe for TRPC channels in mechanosensation could be partly due to their expression in keratinocytes. Indeed, teasing out the contribution of epidermal cells and neurons is difficult, however a conditional KO approach, as was used to determine the role of Piezo2 in Merkel cells, may elucidate further whether epidermally expressed TRPC channels contribute to innocuous mechanosensation.

Unimpaired responses to other stimuli such as noxious heat in the Hargreaves test suggest that reflex responses are unimpaired. This is despite the known 'moonwalker' phenotype produced in TRPC3 knockout mice (Becker et al., 2009) though normal performance in the rotarod test also suggests these animals have unimpaired motor coordination.

Indeed, a conditional deletion of these channels may be a more reliable way of investigating the role of these channels in primary afferent neurons while an inducible knockout model may avoid compensation of loss of function by other mechanisms. There are, therefore, many ways in which this work could be expanded to tease apart the roles for these channels in sensory function.

Although this study does not provide evidence that TRPC channels are directly gated by mechanical force they may instead be involved more indirectly in mechanosensitivity. The mammalian transient receptor potential channel-interacting protein (TRIP) database identifies a total of 276 protein interaction partners for TRPC1, TRPC3, TRPC5 and TRPC6 (Shin et al., 2011, Shin et al., 2012). Of these, a number have been linked to roles in mechanosensation. For example, Orai1 interacts with TRPC1, TRPC3 and TRPC6 and mediates stretch sensitivity in cardiomyocytes (Volkers et al., 2012). Phospholipases are also activated by stretch in a number of sensory systems and, as discussed earlier are linked to activation of a number of TRPC channels. Moreover, evidence already exists to suggest that TRPC channel activation occurs downstream of other mechanosensitive proteins. For example, Mederos y Schnitzler et al. (2008) found that ligand independent activation of $G_q$ protein coupled receptors by membrane stretch activate $PIP_2$ and subsequently cause release of DAG which is able to induce cation currents through TRPC6 leading to membrane depolarisation in HEK cells. TRPC channel contribution to mechanosensitivity in the kidney has also been linked to interactions with other proteins

(Huber et al., 2006). The idea that TRPC channel interactions are critical for their role in mechanosensation is supported by previous work which showed that their mechanosensory function is context dependent (Quick et al., 2012). When expressed in neuronally derived cell lines, TRPC3 and TRPC6 produce mechanically activated currents though they are unable to confer mechanosensitivity to non-neuronal cell lines.

This new data suggests that the role of TRPC channels in the inner ear are indirect as transducer currents in the cochlea are unimpaired. They are, however, critical for cutaneous touch sensation and appear to function combinatorially in their contribution to specific elements of innocuous mechanosensation. The huge and diverse range of TRPC channel interactions indicate that it is likely that they act as part of a bigger mechanosensory complex to contribute to or coordinate cutaneous mechanosensation and serves to highlight the potentially complex roles these channels may be playing in mechanosensation but also provides an interesting route to identifying other constituents of mechanotransduction complexes.

### *4.4.4 Future work*

The work presented shows TRPC channels have a modality specific role in innocuous mechanosensation. DRG neurons taken from TRPC3 and TRPC6 showed a selective loss of RA mechanically activated currents and a corresponding increase in the proportion of non-responding neurons (Quick et al., 2012). In order to better understand the contributions of TRPC1 and TRPC5 to mechanosensory function, it is necessary to determine the changes in electrophysiological properties of sensory neurons. This would include patch clamp recording of multiple KO DRG neurons with mechanical stimulation to determine whether additional KO of TRPC1 and TRPC5 alters any other mechanically activated current type and if any other changes in neuronal properties are observed. TRPC3 and TRPC6 only confer mechanosensitivity to a neuronally derived cell line, while Piezo2, a bone fide primary mechanotransducer is able to produce currents in HEK cells. First, it would be interesting to transfect TRPCs 1, 3, 5 and 6 into HEK cells and investigate whether, together, these channels are able to confer mechanosensitivity to this cell line. Indeed, pursuing the idea that TRPC channel interactions may lead to information about the identity of mechanotransduction complex means that it may be useful to investigate the effect of coexpressing TRPC interacting proteins with TRPC channels in non-mechanosensitive cell lines. This could be prioritised by beginning with proteins with mechanosensory roles already identified.

Evidence which implicates TRPC channels in neuronal sensitisation in pathological conditions is increasing (Sisignano et al., 2014). For example TRPC channels 1 and 6 have a role in mediating mechanical hyperalgesia in inflammatory conditions despite not contributing to the nociceptive threshold in uninjured neurons (Alessandri-Haber et al., 2009) while TRPC3 and TRPC5 are downregulated in an SNI model of neuropathic pain (Staaf et al., 2009b). TRPC3 knockdown attenuates nociceptor sensitisation induced by inflammatory mediators (Alkhani et al., 2014). This suggests that characterising inflammatory and neuropathic pain induced mechanical hypersensitivity in TKO and QKO may elucidate more roles for these channels in mechanosensory function. Indeed, TRPC3 and TRPC6 are expressed in a subset of small diameter fibres which express RA mechanically activated currents; a subset of neurons with these qualities are integral to the development of mechanical hypersensitivity (Seal et al., 2009). Moreover, low threshold mechanotransduction complexes have been implicated in neuropathy induced mechanical allodynia (Eijkelkamp et al., 2013a). As such, there is potential that the combinatorial mechanosensory function of TRPC channels may extend to pathological pain conditions.

There are, therefore a number of ways in which this work could be developed to better understand the contribution of TRPC channels to mechanosensory function.

(See Appendix A (Sexton et al., 2015) for related article).

# 5 The role of TRP channels in mechanical hypersensitivity in Osteoarthritis

## 5.1 Summary

Osteoarthritis is a debilitating condition which is increasingly prevalent with old age and is predicted to affect 130 million people worldwide by 2050. Pain is the predominant clinical symptom however the relationship between joint damage and pain is unclear. Existing analgesic therapies often provide incomplete pain relief and, with chronic administration, are associated with adverse effects. As such a better understanding of the mechanisms responsible for pain in OA is required for the development of more effective analgesics. Transient receptor potential ion channels have established roles in pain and mechanosensation in physiological and pathological conditions. We hypothesised that putative mechanosensory TRP channels, TRPC3, TRPC6, TRPA1 and TRPV1 may contribute to mechanical hypersensitivity in OA. Pharmacological studies have produced conflicting data regarding the roles of TRPA1 and TRPV1 in OA pain so by using transgenic approaches we hoped to confirm whether these channels do indeed contribute to OA induced mechanical hypersensitivity. However we find that only TRPA1 has any role in mechanical hypersensitivity in OA suggesting that pharmacological agents targeting TRPA1 which are currently involved in clinical trials may be successful in conferring some analgesia to patients in OA. Importantly however, we found that TRPA1 does not play a role in the referred hypersensitivity seen in the paw. A role for TRPC3 and TRPC6 in OA pain has not been investigated, however these channels are expressed in a subpopulation of sensory neurons required for mechanical and inflammatory pain and have demonstrable roles in innocuous mechanosensation. Using unique multiple KO mice, we hoped to determine whether these channels play a role in mechanical hypersensitivity in OA however we found that both single and double TRPC3 and TRPC6 knockout mice display normal hypersensitivity after injection of MIA. Mechanisms responsible for pain in OA are poorly understood; this multiple knockout approach therefore provides a novel insight into the role of key pain mediators in OA.

## 5.2 Introduction

### *5.2.1 Osteoarthritis*

Osteoarthritis (OA) is a chronic, degenerative joint disease which the World Health Organisation estimate affects 9.6% men and 18% women over the age of 60, worldwide (WHO, 2013).

OA has a severe impact on quality of life; a recent survey by ArthritisCare (2010) found the majority of respondents reported experiencing 'severe levels of pain on a regular basis'. Indeed, musculoskeletal conditions, of which OA is one of the most common, are the largest single cause of work loss in Europe (EUMUSC) and in the UK alone, an estimated 9.3 million working days were lost through work related musculoskeletal disorders in 2009/10 (HSE, 2010). It is estimated that OA alone leads to 164,000 hip and knee replacements in the UK per year and costs the NHS at least £1 billion per year (ArthritisResearchUK, 2011).

Prevalence of OA increases with age. The UN estimates that the ageing population means that by 2050, people over the age of 60 will account for 20% of the world's population of whom 15%, or an estimated 130 million people, will have symptomatic OA (WHO, 2013).

Many complex pathological changes occur in OA (Wieland et al., 2005, Suri and Walsh, 2012) beginning with progressive loss of articular cartilage, most commonly in joints such as the knees and hips. Normally, this cartilage is responsible for uptake and release of synovial fluid during load bearing movements. Loss of cartilage in OA leads to production of inflammatory mediators and changes in the subchondral bone such as sclerosis and the development of osteophytes. OA also entails inflammation and thickening of the synovium, leading to production of excess synovial fluid whilst the capsule thickens and contracts. Changes in the composition of the cartilage, such as a loss of proteoglycans, can lead to vascular and neuronal invasion of the cartilage. Increased osteoclast activity can result in replacement of the subchondral bone marrow with fibrovascular tissue such as macrophages, which also produce inflammatory mediators. In late stages of the disease these changes can, together, lead to severe bone malformation and remodelling (Figure 5.1).

The predominant clinical feature of OA is pain though presenting symptoms also include stiffness, crepitus, swelling and limitation of movement (ArthritisCare, 2010, Davis et al., 2010). Pain in OA has a strong mechanical component and can be triggered by specific activities such as climbing the stairs; in early stages of the disease, this pain can be relieved by rest. As the disease progresses pain becomes more constant, with reports of spontaneous as well as evoked pain, and neuropathic-like symptoms can develop (Hochman et al., 2011). These include sensations of pins and needles and burning and deficits in vibration sensation and proprioception.

Despite its high incidence and well characterised pathology, the relationship between joint damage and pain is not completely understood. Cartilage degradation is a characteristic feature of OA but cartilage is an aneural tissue while other structures such as the synovium and subchondral bone are much more highly innervated (Saito and Koshino, 2000, Hunter et al., 2013) but their damage produces widely varying levels of pain (Hunter et al., 2008). Similarly, a metastudy suggests that pain is not a useful indicator of the degree of joint damage in OA of the knee (Bedson and Croft, 2008). Moreover, although total joint replacement is often the last resort for patients suffering, some patients still report pain after surgery (Wylde et al., 2011).

Currently, OA treatments are symptomatic; first line therapies include recommending lifestyle changes, such as weight loss given the risk factor of obesity, and a number of pharmacological interventions (NICE-Clinical-Guidelines-177, 2014). These include administration of NSAIDs such as aspirin, and other cyclooxygenase inhibitors, as well as opioids and antidepressants such as SNRIs. These drugs only provide pain relief in a subset of patients; a meta-analysis estimated the effect size for NSAIDs in treating OA of the knee as ‘small to moderate’ (Bjordal et al., 2004). Undesirable side effects are also problematic, particularly as these drugs are often administered chronically to OA patients (Wolfe et al., 1999). New classes of drugs have attempted to target different mechanisms underlying pain in OA. Tanezumab is a humanised monoclonal antibody which targets nerve growth factor (NGF) and produced significant analgesia in phase II and phase III clinical trials of patients with OA of the knee (Lane et al., 2010, Wood, 2010, Brown et al., 2012). Adverse effects in these patients including an increased need for total joint replacement means the safety of this class of drugs is yet to be resolved. Pain relief in OA therefore represents a huge unmet clinical need.

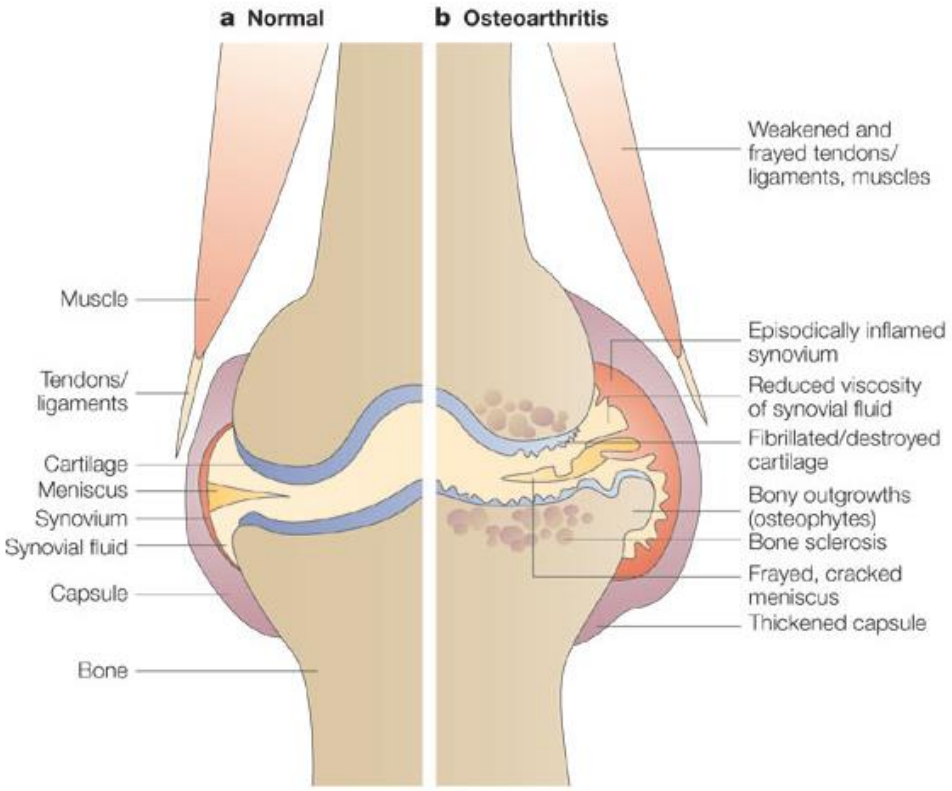



Figure 5.1 Changes present in the osteoarthritic joint. (a) In a healthy joint, cartilage is healthy, aneural and avascular while in an osteoarthritic joint (b) cartilage degradation can ultimately lead to bone remodelling while synovial inflammation and thickening of the capsule also occur. Taken from (Wieland et al., 2005).

### *5.2.2 Monosodium Iodoacetate model of Osteoarthritis*

Animal models of OA aim to mimic disease pathology. In order to produce results which can be translated to the human condition, it is vital that the models accurately reflect the histological and pain phenotype observed in OA.

The monosodium iodoacetate (MIA) model is well established in rats and is beginning to be used in transgenic mice (Harvey and Dickenson, 2009). It is a rapid onset, chemical model involving a single intraarticular injection of sodium iodoacetate, which acts through inhibition of glycolysis to cause chondrocyte degradation. The pathological changes associated with MIA injection have been well characterised. In the first days after MIA injection, there is a loss of proteoglycans (Fernihough et al., 2004), while cartilage degradation is visible by day 14 (Fernihough et al., 2004, Mapp et al., 2013). Persistent synovial inflammation, which is sensitive to NSAIDs (Fernihough et al., 2004), is also reported for as long as 49 days after MIA injection (Mapp et al., 2013) though other studies report that this inflammation subsides after day 7 (Guzman et al., 2003). In later stages of the MIA model, degradation of the subchondral bone, an increase in osteoclasts and ultimately, bone remodelling occur (Guzman et al., 2003, Fernihough et al., 2004, Mapp et al., 2013). Dose-dependent changes consistent with neuropathic pain have also been reported (Thakur et al., 2012) and gabapentin, which is often used as a first line therapy for neuropathic pain, has been shown to reverse MIA induced tactile allodynia but not mechanical hyperalgesia (Fernihough et al., 2004).

The MIA model produces robust pain behaviours which transpire from an early stage. Hypersensitivity is restricted to the affected limb with no contralateral changes occurring; thermal sensitivity is also unaltered (Harvey and Dickenson, 2009). Both primary and secondary mechanical hypersensitivity are present at an early stage in the MIA model (Fernihough et al., 2004). Assessing pain behaviour in the MIA model in rodents predominantly relies on the use of tactile stimulation of the paw on the affected limb and assessment of weight distribution of affected and unaffected limbs (Fernihough et al., 2004, Neugebauer et al., 2007, Harvey and Dickenson, 2009). In rats, many more pain outcomes have been used including grip strength and vocalisation in response to knee bend, though using these to produce reliable and reproducible data in mice has proved difficult. The use of von Frey hairs applied to the plantar surface of the hind paw of rodents is an established and reliable means of assessing mechanical hypersensitivity, although it is important to note that measurement of hind paw mechanical sensitivity in a

model of knee OA relates to a measure of secondary hyperalgesia (given that the primary site of injury is not being directly assessed). Indeed this contributes to the translational value of OA models that produces secondary changes, as referred pain is commonly reported in OA patients as a result of central sensitisation (Bajaj et al., 2001). Using an incapacitance meter to assess weight distribution arguably also provides clinically relevant means of assessing hypersensitivity as patients with OA often exhibit compensatory behaviours to limit joint loading and have altered joint biomechanics which can affect their gait (Malfait et al., 2013). High doses of MIA produce weight bearing asymmetry in rats (Fernihough et al., 2004) though at lower doses reports vary in the significance of these changes (Thakur et al., 2012, Mapp et al., 2013, Yu et al., 2013, Rahman et al., 2015). Used in combination, these behavioural assessments of pain outcomes provide a more rounded insight into the development and maintenance of mechanical hypersensitivity in OA.

The MIA model produces histological changes which are consistent with some of the changes seen in human OA, it also induces rapid onset pain behaviour and therefore provides a useful means of assessing the mechanisms responsible for OA pain (Fernihough et al., 2005).Although the MIA model has been best characterised in rats, applying the model to mice provides the opportunity to investigate the molecular mechanisms contributing to OA induced pain using transgenic approaches.

Although the role for transduction channels in OA has been investigated using pharmacological inhibitors which have produced complex and sometimes conflicting findings (McGaraughty et al., 2010, Okun et al., 2012), there is little literature using transgenic alteration of gene expression to study the role of putative mechanosensory TRP channels in mechanical hypersensitivity in OA. We use a multiple KO approach to reduce the influence of functional redundancy which TRP channels are believed to exhibit and to investigate the potential combinatorial role of TRP channels in mechanical hypersensitivity in OA.

## 5.3 Results

### 5.3.1 *Dose-response study for induction of pain behaviour with MIA*

*This work was performed together with Dr Stéphane Lolignier and Dr Ana Paula Luiz*

Although the MIA model is widely used, other studies report a wide range of doses. A brief literature search indicates in rats, doses from 0.1mg to 4.8mg per knee are used while in mice, there are similar levels of variability. For the current study, three doses of MIA were tested and the pain behaviour they produced was monitored over 14 days (effect of dose ANOVA, $p$=0.005). 0.2mg MIA produced a significant reduction in 50% paw withdrawal threshold at day 7 ($p$=0.02) but by day 14 this hypersensitivity was beginning to resolve ($p$=0.21). The 0.5mg and 0.75g doses both produced significant hypersensitivity that was maintained for 2 weeks (Day 7, p=0.001 and 0.03, respectively; Day 14, p=0.001 and 0.02, respectively) (Figure 5.2a). Subsequent experiments were performed using 0.5mg MIA in 5µl 0.9% saline in order to use the lowest dose that produces sustained hypersensitivity and reproducible pain behaviours.

In line with other reports, we observed development of weight bearing asymmetry, but unlike observations in rat studies, this phenotype present at day 7 was largely resolved by day 14 (Figure 5.2b). No alteration in thermal sensitivity was observed in the Hargreaves test following administration of 0.2mg MIA (Figure 5.2d), and motor coordination was not significantly altered at any time point after administration of 0.5mg MIA (Figure 5.2c), consistent with reports of MIA use in C57BL/6 mice (Harvey and Dickenson, 2009). After confirming no changes in thermal sensitivity arose from the study and that motor coordination was unimpaired, subsequent experiments were analysed using the von Frey and weight bearing tests enabling us to focus specifically on changes in mechanical sensitivity in OA.

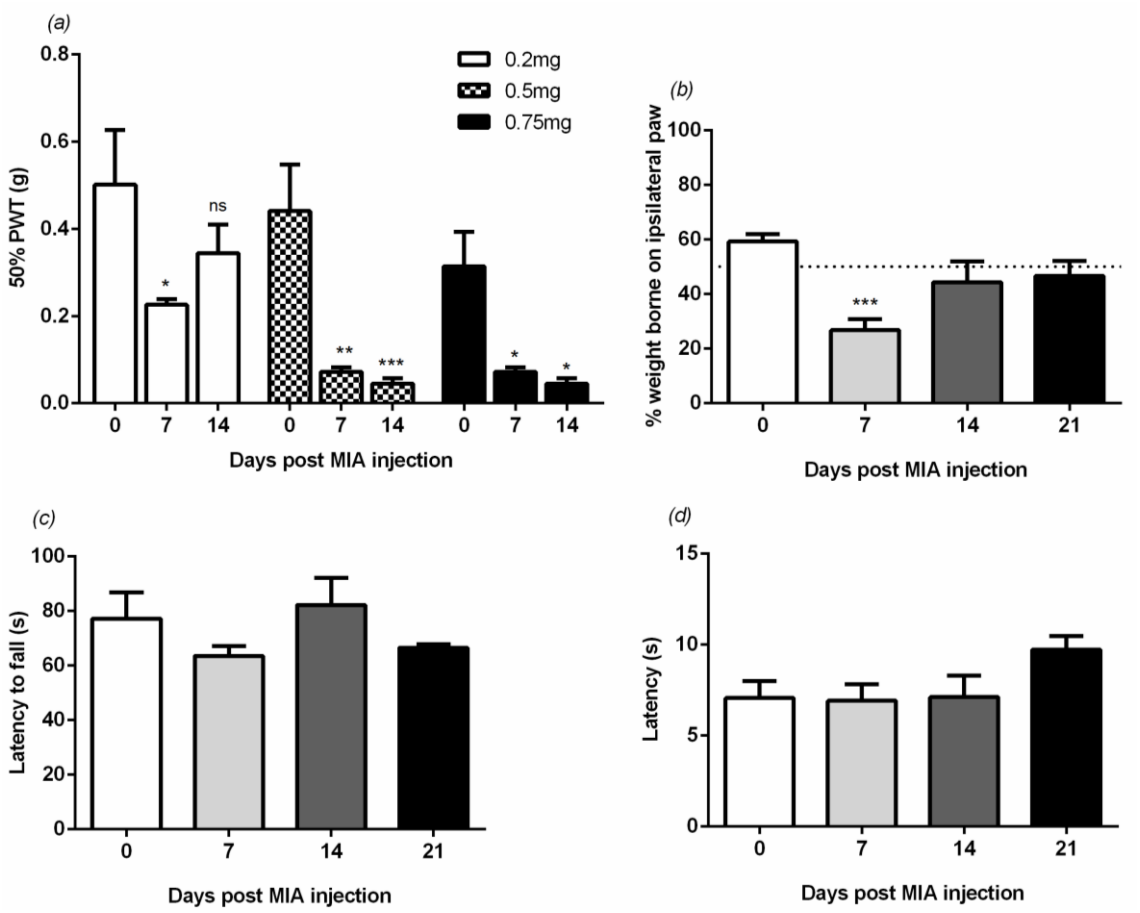



Figure 5.2 Dose-dependent pain behaviour following intra-articular injection of MIA. Three doses of MIA were tested using von Frey assessment over 14 days. Both 0.5mg and 0.75 mg produced lasting hypersensitivity so were used for subsequent experiments. (b) A weight bearing asymmetry was present at day 7 but resolved by day 14. No changes in motor coordination (d) or thermal sensitivity in the Hargreaves test (c) were observed following administration of 0.5mg and 0.2mg MIA, respectively. (Dose response trial, n=5 per group. Data shown as Mean±SEM with one way ANOVA with Tukey post-hoc test. Weight bearing test, n=6; Hargreaves test n=3; Rotarod test n=9. Data shown as Mean±SEM with one-way ANOVA with Dunnett's post-hoc test).

### 5.3.2 *TRPC3 and TRPC6 knockout animals develop mechanical hypersensitivity following injection of MIA*

*This work was performed together with Louisa Townson*

TRPC3 and TRPC6 are, together, required for normal touch sensitivity and contribute to RA mechanically activated currents in a subset of sensory neurons. As a result of this important role in mechanosensation, we hypothesised that they may have contribute to mechanical hypersensitivity in OA. In order to investigate this, TRPC3 single KO, TRPC6 single KO and TRPC3, TRPC6 DKO animals were compared to WT mice following administration of 0.5mg MIA. A decrease in 50% withdrawal threshold at day 7 was significant in all groups compared to baseline (MIA treatment effect, ANOVA $p<0.0001$; WT 0.43g±0.08g to 0.18g±0.06g ($p=0.002$); TRPC3 SKO 0.34g±0.07g to 0.13g±0.03g ($p=0.006$); TRPC6 SKO 0.33g±0.04g to 0.15g±0.05g ($p=0.04$); TRPC3, TRPC6 DKO 0.52g±0.06g to 0.20g±0.05g ($p=0.001$)) (Figure 5.3a). There was no significant difference in withdrawal threshold between groups at any time point ($p>0.05$), though WTs showed a threshold of 0.12g and TRPC3 and TRPC6 double KO mice have a threshold of 0.24g.

Similarly, although a weight bearing asymmetry was visible in all groups by day 7 (MIA treatment effect, ANOVA $p<0.0001$), it was beginning to resolve by day 14 in all groups (Figure 5.3b). That WT mice show resolution of this phenotype suggests that the changes are independent of the loss of function of TRPC3 and TRPC6. There was no difference between genotype groups at any time point. The weight bearing and von Frey data together suggest that TRPC3 and TRPC6 do not contribute to the development or maintenance of mechanical hypersensitivity in OA.

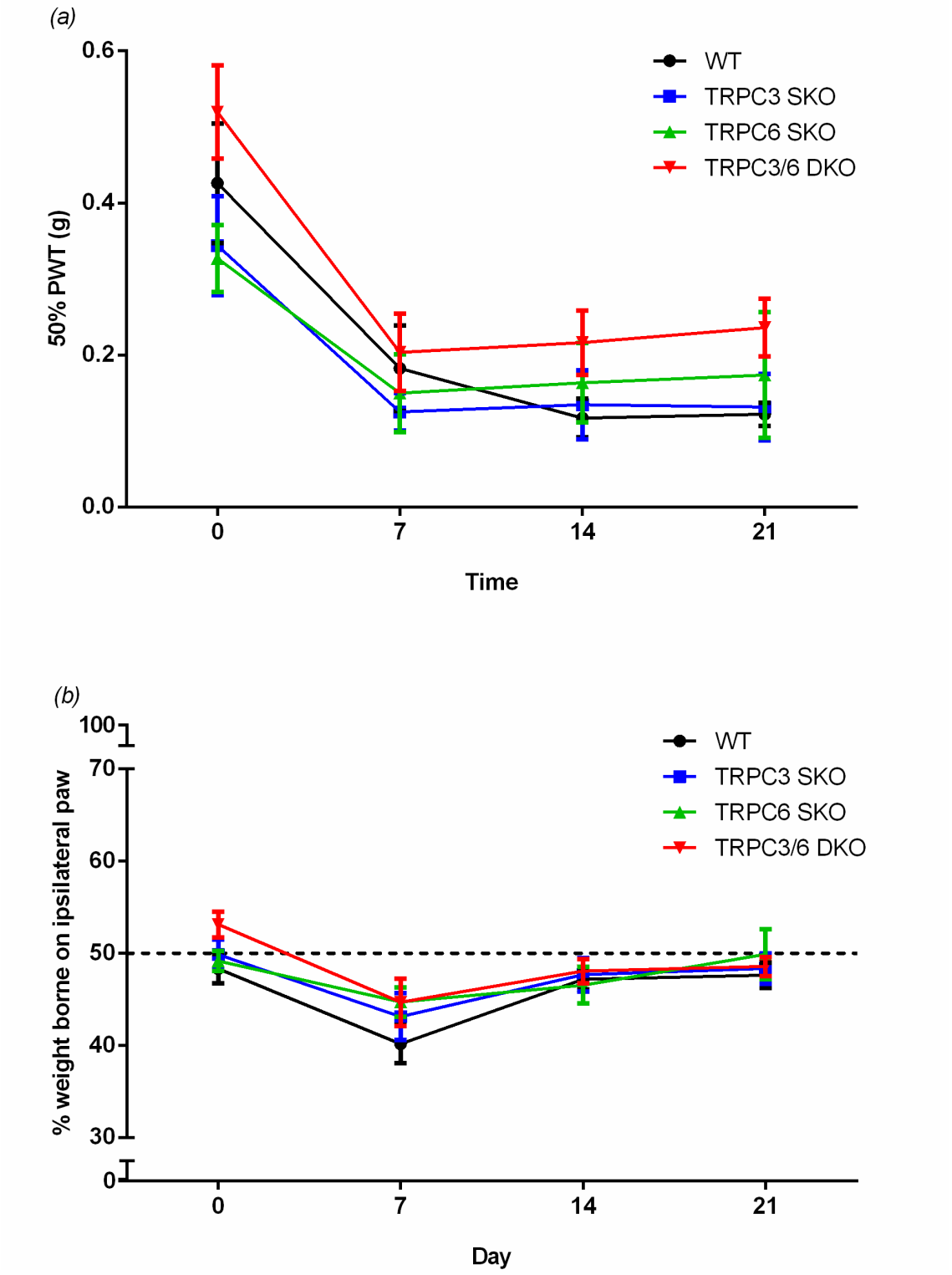



Figure 5.3 TRPC3 and TRPC6 do not contribute to mechanical hypersensitivity in OA. (a) von Frey withdrawal thresholds were significantly decreased compared to baseline in all groups. (b) a weight bearing asymmetry was present in all groups at day 7 but resolved by day 14. (n=10 per group; Data shown as Mean±SEM with two way ANOVA with Tukey post-hoc test).

### *5.3.3 TRPA1-TRPC3-TRPC6 triple knockout animals show partial attenuation in mechanical hypersensitivity following injection of MIA*

*This work was performed together with Dr. Stéphane Lolignier*

TRPA1, TRPC3 and TRPC6 have all been implicated in mechanosensory roles and are co-expressed in some subsets of sensory neurons, allowing the potential for interaction, though TRPA1 is also found in an additional peptidergic subset (Usoskin et al., 2015). Although these channels are unlikely to be primary mechanotransducers, they all have established roles in mechanosensation. As a result of the functional redundancy which TRP channels are believed to exhibit, we considered that although inhibiting the function of TRPC3 and TRPC6 did not alter mechanical hypersensitivity produced by arthritis induction, additionally inhibiting the function of TRPA1 may. As such, we tested TRPA1-TRPC3-TRPC6 triple KO mice and compared them to TRPC3-TRPC6 double KO mice to determine whether these channels, together, have any role in mechanical hypersensitivity in Osteoarthritis.

Both groups showed a significant decrease in withdrawal threshold from baseline to day 7 (MIA treatment effect, ANOVA $p<0.0001$; TRPC3-TRPC6 double KO mice, $p=0.001$; TRPA1-TRPC3-TRPC6 triple KO mice, $p=0.001$ statistics not shown on graph for clarity) which was maintained for the duration of the experiment (Figure 5.4a). TRPC3-TRPC6 DKO mice showed a slight weight bearing asymmetry at day 7 though this was not significant and returned to baseline distribution from day 14 for the duration of the experiment (Figure 5.4b). TRPA1-TRPC3-TRPC6 triple KO mice did not develop a significant weight bearing asymmetry at day 7 compared to baseline ($p=0.82$). There was no significant difference between genotype groups at any time point.

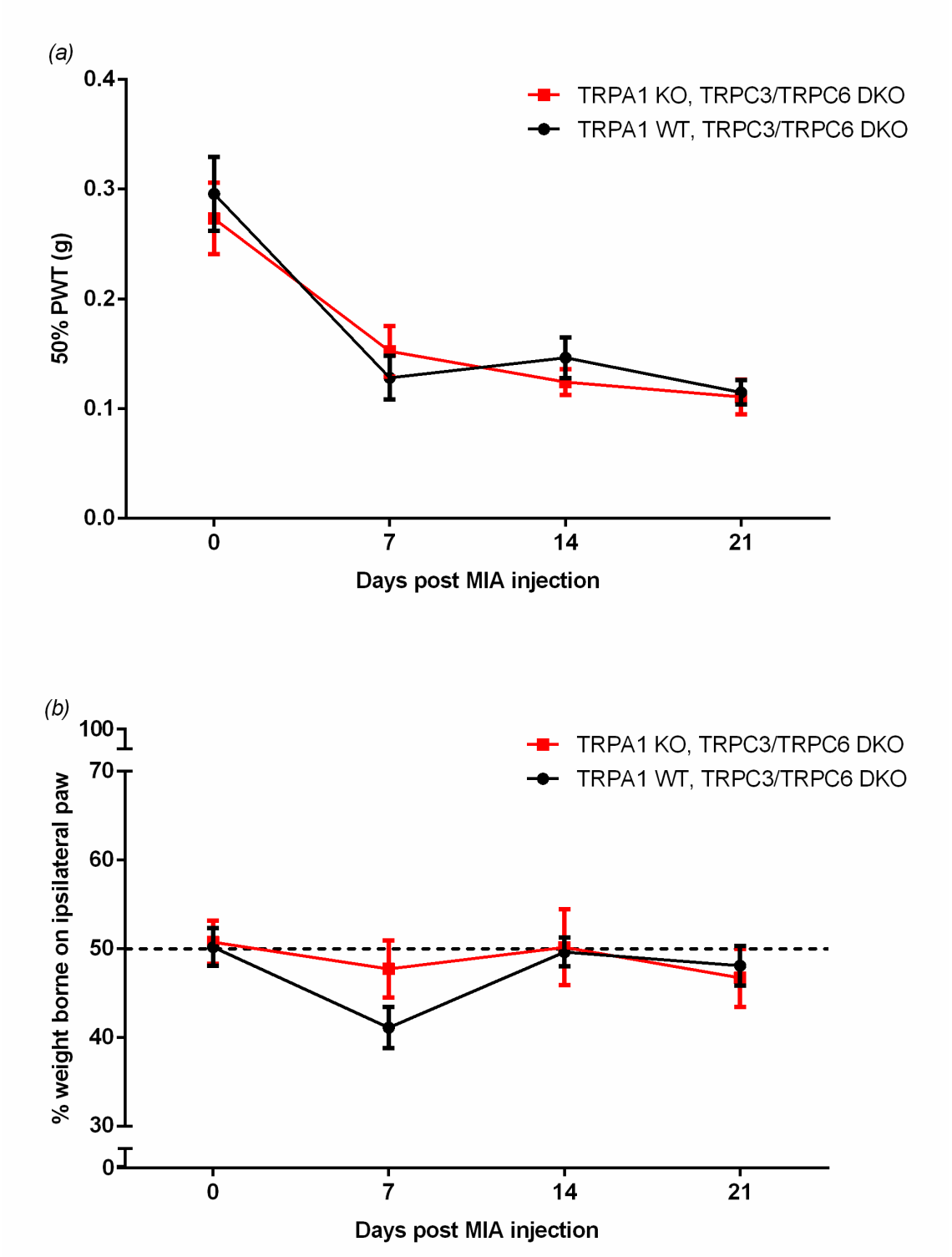



Figure 5.4 TRPA1 is required for the full manifestation of mechanical hypersensitivity in OA. (a) von Frey withdrawal thresholds were significantly decreased compared to baseline in all groups. (b) No significant weight bearing asymmetry was observed after MIA injection. (n=10 per group; Data shown as Mean±SEM with two way ANOVA with Tukey post-hoc test)

### 5.3.4 *TRPA1- TRPV1 double knockout animals develop mechanical hypersensitivity following injection of MIA*

*This work was performed together with Dr. Stéphane Lolignier*

TRPA1 and TRPV1 have both separately been linked to a role in mechanical hypersensitivity in pathological conditions and recent evidence shows that as well as being co-localised in sensory neurons, they are able to form functional heteromeric complexes (Salas et al., 2009). In order to pursue this, TRPA1 single KO, TRPV1 single KO and TRPA1, TRPV1 double KO mice were compared to WT animals in order to see the contribution of TRPA1 and TRPV1 alone and in combination in OA induced mechanical hypersensitivity.

Assessment of mechanical hypersensitivity using von Frey hairs did not identify any difference between genotype groups at any time point, though all groups showed a significant reduction in withdrawal threshold at day 7 compared to baseline which was maintained for the duration of the experiment (WT 0.45g±0.03g to 0.28g±0.04g ($p$=0.002); TRPA1 KO 0.34g±0.07g to 0.13g±0.03g ($p$=0.006); TRPV1 SKO 0.33g±0.04g to 0.15g±0.05g ($p$=0.04); TRPA1, TRPV1 DKO 0.52g±0.06g to 0.20g±0.05g ($p$=0.001)) (Figure 5.5a).

A weight bearing asymmetry was produced in WT, TRPV1 SKO and TRPA1, TRPV1 DKO animals at day 7 but this was resolved back to baseline levels by day 14. Interestingly, TRPA1 KO mice did not develop a weight bearing asymmetry at any time point and this was significant, at day 7, compared to WT mice ($p$=0.01) (Figure 5.5b).

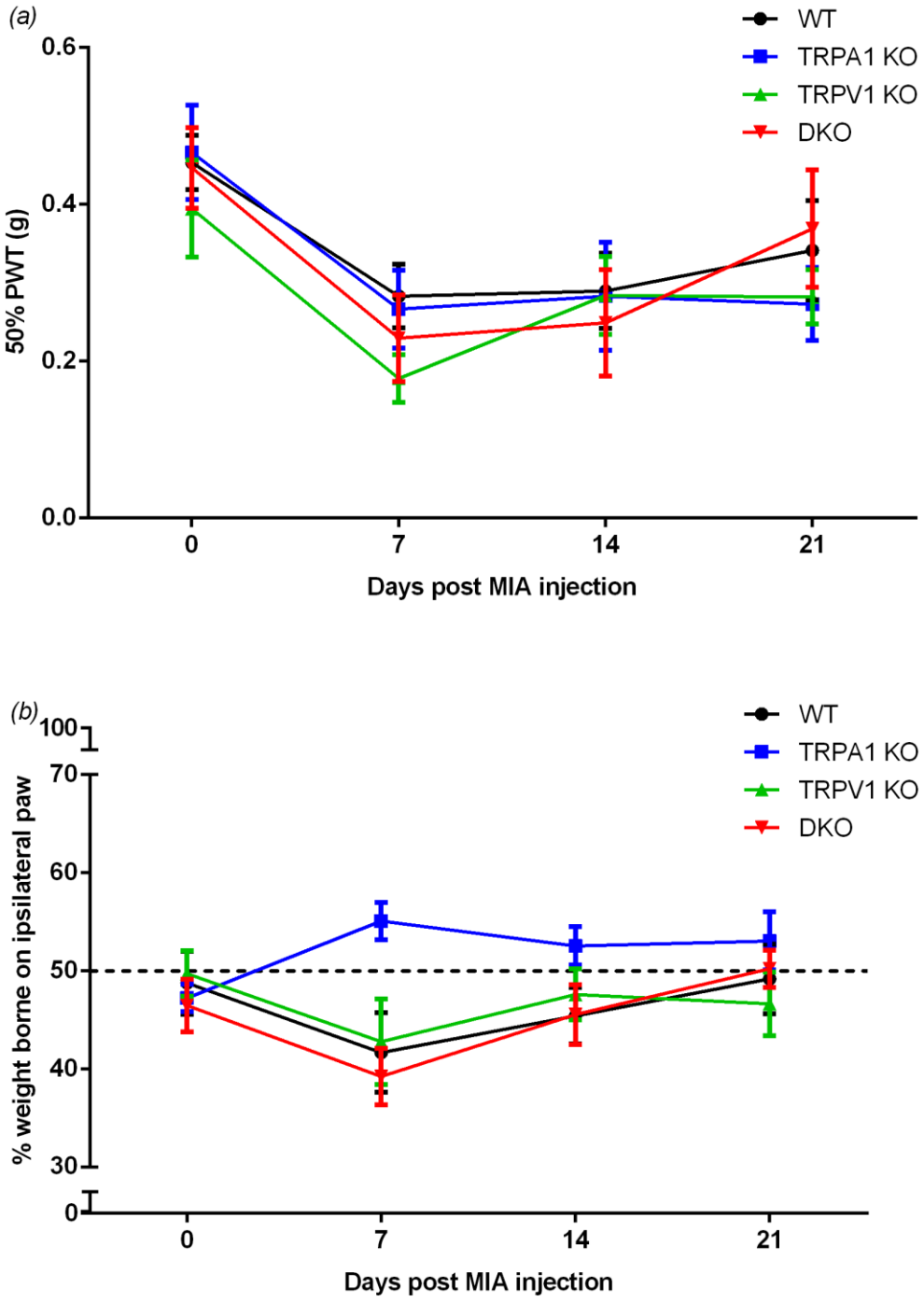



Figure 5.5 TRPA1 but not TRPV1 contributes to mechanical hypersensitivity in OA. (a) von Frey withdrawal thresholds were significantly decreased compared to baseline in all groups. (b) A weight bearing asymmetry did not develop in TRPA1 SKO mice but was present in all other groups. (WT, n=7; TRPA1 SKO, n=8; TRPV1 SKO, n=8, DKO, n=7; Data shown as Mean±SEM with two way ANOVA with Tukey post-hoc test).

## 5.4 Discussion

The aim of the work in this chapter is to explore the role of TRP channels in OA. The MIA model of OA is a commonly used rodent model, partly due to its rapid onset and production of pain and joint pathology comparable to that seen in the human condition. We used this model to investigate whether putative mechanosensors in the family of TRP channels contribute to mechanical hypersensitivity in OA.

### 5.4.1 *MIA produces a weight bearing asymmetry and referred tactile hypersensitivity*

The MIA model is well characterised in rats in that it produces a unilateral, mechanical hypersensitivity which elicits a biphasic, dose-dependent weight bearing asymmetry. Its use in mice is less widely reported and the dose used in both of these groups is highly variable. We found that injection of 0.5mg MIA produced a lasting tactile hypersensitivity in the von Frey test as well as a phasic weight bearing asymmetry. Testing the distribution of weight on the hind paw is considered to be a useful measure as OA patients report pain on load bearing. In our experiments, weight bearing asymmetry was only visible 7 days after injection of MIA and was back to an equal distribution by day 14.

Inflammation has been shown to be present in the early stages following injection of MIA though the length of time it is present has been debated with some groups suggesting it is attenuated after 7 days. Weight bearing asymmetry can be resolved by treatment with NSAIDs suggesting that early stage inflammation contributes to the phenotype (Bove et al., 2003). In rats, this measure produces a biphasic response where an initial asymmetry is resolved and then reappears around 7 days after administration of MIA. It is possible that a similar pattern is visible in mice, though assessments at time points between injection and 7 days would be needed to confirm this. After 7 days, the asymmetry remains in rats which is not what we observed in the current tests in mice. It is possible that the phenotype in the current work is resolved partially after day 7 as the dose is only sufficient to produce a short period of inflammation up to 7 days but becomes milder or absent by day 14. However it is also possible that if the inflammation is milder after 7 days that the static weight bearing measurement is not sensitive enough to detect such a mild phenotype in mice.

In line with other reports in C57BL/6 mice, thermal sensitivity was unaltered by MIA injection at all time points and motor coordination was also unimpaired (Harvey and

Dickenson, 2009). As such, in subsequent experiments only von Frey and weight bearing assessments were performed.

### 5.4.2 *TRPA1, but not TRPC3, TRPC6 or TRPV1, contributes to mechanical hypersensitivity in OA*

TRPC3 and TRPC6 are together necessary for normal touch sensation (Quick et al., 2012). Though they are not primary mechanotransducers alone, they contribute to the RA currents displayed by a subset of small diameter neurons. TRPC6 has a demonstrable role in inflammatory mechanical hyperalgesia (Alessandri-Haber et al., 2009). Also, TRPC3 and TRPC6 DKO mice show attenuated pain behaviours in the second phase of the formalin test and a partial attenuation of mechanical hypersensitivity evoked by carrageenan (Quick, 2011); altogether existing evidence makes them favourable candidates for a role in mechanical hypersensitivity in pathological conditions.

The current data show that mice lacking functional expression of TRPC3, TRPC6 and both channels develop mechanical hypersensitivity to the same extent as WT controls. This suggests that the mechanosensory function of these channels does not contribute to the development or maintenance of mechanical hypersensitivity in OA. Although existing evidence implicates TRPC3 and TRPC6 in mechanical hyperalgesia in some models of inflammatory pain (see above), there is also evidence to argue against a role for these channels in pathological conditions. For example, DKO mice show normal allodynia following SNL surgery and develop hyperalgesia following CFA injection (Quick, 2011). Notably, the Nav1.8 positive population of sensory neurons, where TRPC3 in particular is expressed, are not needed for neuropathic pain (Abrahamsen et al., 2008).

MIA has been shown to have a dose-dependent neuropathic component as it leads to upregulation of ATF-3, a marker of peripheral neuron injury, and produces a pain phenotype which is sensitive to gabapentin (Thakur et al., 2012). It is unlikely that the dose we are using has a neuropathic element as it is quite low, though this would need to be confirmed using histological or pharmacological analysis. Although other groups have reported a role for TRP channels in OA induced pain at specific doses of MIA, if this experiment was repeated at a higher dose it is unlikely our findings would change since TRPC3 and TRPC6 are unlikely to contribute to mechanical allodynia. It is therefore likely that, TRPC3 and TRPC6 play a selective role in mechanical hypersensitivity in other inflammatory conditions that may be unimportant in OA pain. Also, it is possible

that other mechanically activated TRP channels compensate for the loss of function of TRPC3 and TRPC6 allowing the hypersensitivity to remain in spite of their loss of function.

TRPC3 is expressed throughout the DRG while TRPA1 and TRPC6 show slightly more restricted expression however Usoskin et al. (2015) identified four subsets of neurons expressing all three channels so there is potential interaction between TRPC3 and TRPC6 with TRPA1 in the DRG. All of these channels contribute to mechanosensation in some way though they may contribute differentially. As such, knocking out TRPC3 and TRPC6 is unlikely to completely impair pathways involving TRPA1 and it is possible that compensatory activity through TRPA1 pathways allows mechanical hypersensitivity to remain in the absence of functional TRPC3 and TRPC6. We used mice with deletion of TRPA1, TRPC3 and TRPC6 to reduce the effect of such functional redundancy and used TRPC3 and TRPC6 DKO mice as controls knowing that they alone were not refractory to mechanical hypersensitivity. The current data show that TKO mice do not develop a weight bearing asymmetry at any time point whereas DKO mice show a slight, non-significant, decrease in the weight borne on the affected limb from 50.20%±2.12% down to 41.11%±2.33%.

Though TRPA1 KO mice do not develop a weight bearing asymmetry, suggesting resistance to the development of some pain, they do display hypersensitivity in the von Frey test. The application of von Frey hairs to the paw, a site away from the affected joint is considered to be representative of a secondary hyperalgesia or referred pain, which is also a feature of the human condition. The von Frey test demonstrates that neither TRPA1, TRPC3 nor TRPC6 have a role in the referred hypersensitivity produced by OA. In a separate experiment, TRPA1 single KO mice were compared to WT, TRPV1 KO and TRPA1-TRPV1 DKO mice. TRPA1 KO mice did not develop a weight bearing asymmetry at day 7 while all other groups did. All groups developed a referred hypersensitivity, demonstrated by a sustained reduction in the withdrawal threshold of the paw. This latter experiment is important as it demonstrates that loss of function of TRPA1 alone is sufficient to prevent full manifestation of OA induced mechanical hypersensitivity.

Existing data both supports and contests a role for TRPA1 in OA induced pain based on studies where pharmacological inhibitors, A-967079 and HC030031 are administered to

block TRPA1 (McGaraughty et al., 2010, Okun et al., 2012). The former compound, A-967079 attenuates mechanical allodynia as well as reducing the responses of spinal neurons to noxious mechanical stimuli in rats (McGaraughty et al., 2010). However HC030031 does not resolve weight bearing asymmetry produced by high dose MIA (Okun et al., 2012). Okun et al. (2012) suggested that the differential findings may be explained by the fact that A-967079 had no effect on spontaneous firing of spinal neurons and that this activity contributes to the ongoing pain they see in the weight bearing test. They concluded therefore that TRPA1 blockade is insufficient to provide relief from ongoing pain in advanced OA. Such a conclusion is compatible with our findings that a relatively low dose of MIA can produce a weight bearing asymmetry which is attenuated by loss of TRPA1, yet the referred pain to the paw, which may be the result of changes in the CNS, is independent of TRPA1 activity. Indeed, spinal pain pathways have been shown to play a critical role in OA and joint pain (Harvey and Dickenson, 2009, Rahman et al., 2009, Carr et al., 2014) and systemic administration of a TRPA1 antagonist was unable to reverse the heightened activity of spinal cord neurons in the MIA model of OA (McGaraughty et al., 2010). Therefore, it seems that TRPA1 only contributes to specific components of OA induced mechanical hypersensitivity. Our conclusions in this area would be strengthened by looking at the effect of higher doses of MIA in TRPA1 KO mice as we may find loss of TRPA1 function with higher MIA doses does not attenuate the weight bearing asymmetry, as was reported by Okun et al. (2012).

Our data suggest that TRPV1 does not have a role in OA induced mechanical hypersensitivity as TRPV1 KO mice develop a weight bearing asymmetry and decreased paw withdrawal threshold comparable to WT controls (Figure 5.5). TRPV1 is upregulated in DRG neurons innervating the knee following administration of MIA (Fernihough et al., 2005) as well as in the synovium of the joint following injection of MIA (Kelly et al., 2015) and has therefore been identified as a potentially interesting analgesic target for the treatment of OA pain. However, existing preclinical studies which focus on the use of pharmacological antagonists provide conflicting evidence regarding the role of TRPV1 in mechanical hypersensitivity. Okun et al. (2012) report that TRPV1 blockade can attenuate weight bearing asymmetry following administration of 3mg, but not 4.8mg, MIA in rats and thus suggest that blocking the function of TRPV1 may only be clinically beneficial in mild to moderate OA. Our data does not support a role for TRPV1 in OA mechanical hypersensitivity, although it is important to note that the dose we used is not

particularly high and therefore is unlikely to model the advanced OA described by Okun et al. On the other hand, it has been shown that spinal TRPV1 does not contribute to mechanical hypersensitivity in OA (Kelly et al., 2015) and TRPV1 antagonists have proved unsuccessful in clinical trials as a result of the hyperthermia they produce through binding to centrally expressed TRPV1 (Gavva et al., 2008). As such, it has been suggested that a peripherally targeted antagonist may be more successful. Our data does not provide evidence to support this, however another group have reported that analgesia through blockade of TRPV1 can be more effective when performed in combination with inhibition of the endocannabinoid pathway, components of which can lead to TRPV1 sensitisation (Malek et al., 2015). It is therefore possible that the reason we do not observe analgesia resulting from loss of TRPV1 function is that loss of TRPV1 alone is insufficient to produce analgesia as unimpaired nociceptive pathways remain.

TRPA1 and TRPV1 expression in sensory neurons overlaps in a portion of peptidergic, small diameter, unmyelinated fibres. It is well established that there is functional interaction between these channels and it has recently been demonstrated that TRPA1 and TRPV1 form functional heteromeric complexes (Salas et al., 2009, Staruschenko et al., 2010, Fischer et al., 2014). As such, we used TRPA1 and TRPV1 double KO mice to investigate whether these channels show any combinatorial role in mediating mechanical hypersensitivity in OA. DKO mice developed hypersensitivity to the same extent as WT mice in both the weight bearing and von Frey tests. This result was somewhat surprising as we have shown that loss of TRPA1 prevents the development of a weight bearing asymmetry in both single KOs in this experiment and in TRPA1 KO mice. It is not clear why TRPA1 and TRPV1 DKO mice showed an asymmetry when TRPA1 KO mice did not however we do consistently find that loss of TRPA1 does not alter the hypersensitivity shown in the von Frey test. The von Frey test produces a nocifensive, reflex response at a site away from the injured joint which is representative of secondary hypersensitivity resulting from complex central processing. As such, it seems unlikely that centrally expressed TRPA1 contributes to pain in OA though the conflicting results in the weight bearing test results remain particularly difficult to interpret. Aforementioned studies looking at the effect of TRPA1 and TRPV1 blockade on MIA induced weight bearing produce conflicting data (McGaraughty et al., 2010, Okun et al., 2012) which suggests a more thorough characterisation of the relationship between MIA dose, behavioural

outcome and changes in neuronal activity, in mice is required for us to better understand these findings.

### 5.4.3 *Mechanisms underlying mechanical hyperalgesia in OA*

Altogether, the current data is valuable as it indicates that mechanical hypersensitivity in OA is likely to have mechanisms distinct from other pathological pain conditions as loss of function of important mechanosensory TRP channels does not alter mechanical hypersensitivity in OA. Even in the human condition, the lack of correlation between joint destruction and pain illustrates the complex nature of the underlying pain mechanisms. Animal models of OA are varied in the histological and pain behaviour phenotypes they produce; their use in rats and mice of different backgrounds also contributes to the variability in model outcomes as strain-dependent differences can be highly influential on development of pain and behavioural outcomes in noxious testing (Mogil, 1999, Wilson and Mogil, 2001). Even work performed using the same model in the same animals has produced differing reports of cartilage degradation, duration of inflammation and pain phenotype. In the case of the MIA model of OA, the influence of the dose used to induce OA has a demonstrably influential effect on the success of blocking the function of transduction channels which further highlights the complex mechanisms responsible for mechanical hypersensitivity in the different stages of OA. As a result, there are clear limitations on the use of the data in this study alone and a great deal of work using other models of OA and, likely, different doses of MIA will be needed to make more firm conclusions from our findings. Nonetheless, these data are unique in the multiple KO approach used to characterise the roles of mechanosensory channels in mechanical hypersensitivity in OA as many previous studies have focused on the use of pharmacological inhibitors which do not take into account the influence of functional redundancy and TRP channel subunit interaction.

In conclusion, we find that TRPA1 plays a selective role in mechanical hypersensitivity in OA suggesting that targeting TRPA1 may alleviate pain in some stages of the disease. However we do not find evidence to support such a role for TRPV1, TRPC3 or TRPC6. Clearly, an all rounded approach is necessary to determining the contribution of putative mechanosensors to mechanical hypersensitivity in OA. This includes determining whether the dose used causes neuropathy, the time course of inflammation and a better characterisation of the changes in neuronal activity which are produced by this dose of

MIA. As well as this, more may be gained from examining the roles of these channels in other models of OA which mimic different features of the human condition.

### 5.4.4 *Future work*

To complement the findings in this study, surgical models such as destabilisation of the medial meniscus of the knee which causes the joint to become unstable through transection of the medial meniscotibial ligament may be useful. In this model, altered joint biomechanics lead to progressive degradation of cartilage, thickening of the subchondral bone and formation of osteophytes over a longer time course than the MIA chemical model (Glasson et al., 2007, Loeser et al., 2013). Used in combination, these models encompass pathological changes seen in OA, produce pain behaviour in animals and provide a suitable means of testing potential analgesics. It would be interesting to corroborate the data produced in TRPA1 KO mice by testing these mice using the DMM model. The current approach to determining the role of putative mechanosensory in OA induced mechanical hypersensitivity can also be continued through the use of other KO mice.

The varying doses of MIA widely reported in the literature have been shown to produce different pathological and behavioural phenotypes. A full characterisation of these doses in our transgenic mice would be beneficial as it may allow a better understanding of the contribution of, in particular TRPA1, to mechanical hypersensitivity in OA, and therefore allow a better dissection of the mechanisms which underlie the different behavioural phenotypes observed. It is likely that the doses used in the present study models only mild OA. Since some studies have demonstrated a differential role for transduction channels in mild and advanced OA, this work would be strengthened by using a higher dose of MIA to model a more advanced form of the disease. In addition, such work would likely strengthen the clinical relevance of this work as higher doses of MIA reportedly produce thermal hyperalgesia (Okun et al., 2012) which is a symptom reported in quantitative sensory testing studies on humans with OA (Suokas et al., 2012).

Finally, many studies in rats have used spinal electrophysiology to characterise the effects of pharmacological inhibitors on OA induced pain. Using this approach in our transgenic mice may add to the wealth of data available as many of the transduction channels expressed peripherally are also expressed in the in the spinal cord, as well as on the central terminals of primary afferents.

# 6 Investigating the role of Annexin A6 in mechanosensation

## 6.1 Summary

Mechanically activated currents in sensory neurons can be classified according to their adaptation profiles. Rapidly adapting currents are associated with low threshold mechanical stimulation while slowly and intermediately adapting currents are produced by high threshold mechanical stimuli and are blocked by the conotoxin, NMB-1 (Drew et al., 2007). This peptide also attenuates behavioural responses to noxious mechanical stimuli in mice. Annexin A6 was identified as a binding partner for NMB-1 thereby highlighting it as a candidate transducer of the slowly and intermediately adapting mechanically activated current. Our hypothesis that Annexin A6 may be a noxious mechanotransducer was strengthened by the fact it is present in the Nav1.8 positive subset of sensory neurons (Raouf et al. unpublished data) which are necessary for noxious mechanosensation. In the present study we demonstrate that, in fact, Annexin A6 is a negative regulator of mechanosensation and is able to attenuate the mechanically activated currents of primary mechanotransducer Piezo2. Consistent with this, loss of ANXA6 function leads to mechanical hypersensitivity in mice and overexpression of ANXA6 is able to partially attenuate mechanical hypersensitivity in Osteoarthritis. Together these data demonstrate an exciting and novel mechanism of mechanosensory regulation.

## 6.2 Introduction

### *6.2.1 Annexin A6 binds to NMB-1, a potent blocker of mechanically activated currents*

NMB-1 (noxious mechanosensation blocker 1) is a conotoxin peptide which inhibits the persistent, non-adapting component of intermediately-adapting (IA) and slowly-adapting (SA) mechanically activated currents produced by sensory neurons (Figure 6.1) (Drew et al., 2007). In addition to this NMB-1 binds to small diameter, peripherin positive neurons (Drew et al., 2007), which is consistent with the neuronal subtypes which express SA and IA currents. NMB-1 also inhibits mechanotransduction currents in the cochlea and blocks behavioural responses to noxious mechanical stimuli.

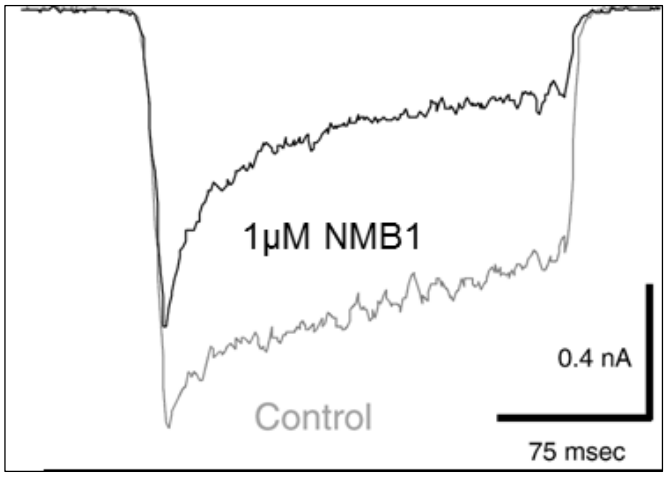



Figure 6.1 Mechanically activated currents are inhibited by NMB-1 peptide in cultured sensory neurons. (Adapted from Drew et al. (2007)).

In order to identify the peptides which NMB-1 binds to, and therefore identify proteins which are likely to contribute to mechanotransduction, a co-immunoprecipitation assay followed by mass spectrometry analysis was performed and identified ANXA6 as a binding partner of NMB-1 (Raouf et. al, unpublished). Strengthening the candidacy of ANXA6 as a mechanotransducer is its downregulation in the population of Nav1.8 expressing neurons (unpublished) which have been shown to be critical for mechanical and inflammatory pain (Abrahamsen et al., 2008). In order to investigate the potential role of ANXA6 in mechanosensation, we looked at the effects of gene knock out on pain behaviour and overexpression on mechanically activated currents *in vitro*.

### *6.2.2 Annexin A6 knockout mouse*

*Anxa6* global knockout mice were kindly provided by Prof. S. Moss. The *Anxa6* gene was disrupted through insertion of a 1.1kb sequence containing a neomycin cassette into exon 3 (Figure 6.2); it has been previously reported that these animals are healthy, fertile and develop normally (Hawkins et al., 1999).

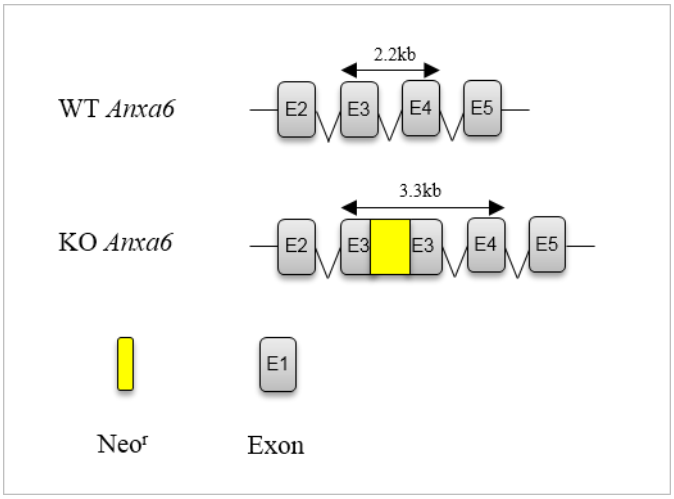



Figure 6.2 Targeting construct for *Anxa6* gene knockout. Exon 3 is disrupted by insertion of a 1.1kb sequence which renders the gene non-functional.

### *6.2.3 Gene therapy*

Gene therapy is an increasingly popular method of treating monogenic diseases which are not successfully targeted by existing pharmacological tools. Administration of vectors allows selective gene knockdown through the use of short hairpin RNA sequences and delivery of functional genes for overexpression or to confer function when the endogenous gene is defective. The success of current analgesics and the development of newer therapies is marred by problematic side effects and lack of specific blockers for homologous ion channels such as voltage gated sodium channels. Gene therapy offers many potential advantages which may tackle these issues and allow a more focused, long term means of targeting nociceptive pathways (Glorioso and Fink, 2009).

Viruses are commonly used as vectors for gene therapy. Herpes simplex virus (HSV) (e.g. (Majima et al., 2015)), lentivirus (LV) (e.g. (Meng et al., 2015)) and adeno-associated virus (AAV) (e.g. (Samad et al., 2013)) have all been successfully used to transduce gene expression in nervous tissue in rodents for the purposes of producing analgesia without any reported adverse effects. Adenoassociated virus (AAV) is a small, non-enveloped virus with an approximately 4.7kb single stranded DNA genome. There are twelve human serotypes of AAV, all of which consist of Rep and Cap genes, necessary for the AAV life cycle, transcription regulation and capsid formation, and which are flanked by 145bp inverted terminal repeats. Their ability to transduce dividing and non-dividing cells, lack of toxicity and persistent expression for long periods of time make them well suited to a role in gene therapy (Daya and Berns, 2008, Ojala et al., 2015). AAV1, AAV5 and AAV6 have been identified as being particularly successful at transducing DRG neurons and have distinct cell type transduction specificity; AAV5 preferentially transduces large diameter fibres while AAV6 favours small diameter neurons (Mason et al., 2010, Towne et al., 2010). Early phase clinical trials also indicate that the use of gene therapy for analgesia (Fink et al., 2011) and the use of AAVs in human CNS (Kaplitt et al., 2007, Eberling et al., 2008, LeWitt et al., 2011) is safe, despite concerns of a problematic immune response. We selected AAV6 and AAVx, a novel vector provided by Prof. M. Linden which effectively transduces neurons (AAVx is an arbitrary name as the viral serotype is currently under patent processing), to induce sensory neuron overexpression of ANXA6 via the clinically relevant (Samad et al., 2013), intrathecal route of administration.

## 6.3 Results

### *6.3.1 Global deletion of Annexin A6 selectively impairs noxious mechanosensation*

Using von Frey and Randall-Selitto testing, we investigated the role of ANXA6 in mechanosensation *in vivo*. We found that *Anxa6* knockout mice had a 50% withdrawal threshold of 0.62g±0.10g compared to 0.64g±0.06g in WTs when tested on the paw suggesting that impairing the function of ANXA6 does not alter innocuous mechanosensation ($p$=0.84) (a). However, in response to noxious mechanical pressure, *Anxa6* knockout mice showed a 17.5% decrease in the force required to elicit a response. WT mice responded at an average of 188.75g±5.00g compared to 155.83g±7.73g in KO ($p$=0.003) (b). These data suggest that ANXA6 has a negative regulatory role in noxious mechanosensation and that its role is modality specific which is consistent with the findings of Drew et al. (2007) who found NMB-1 selectively attenuated SA and IA currents, which are associated with high threshold mechanical stimuli, and behavioural responses to noxious mechanical stimuli.

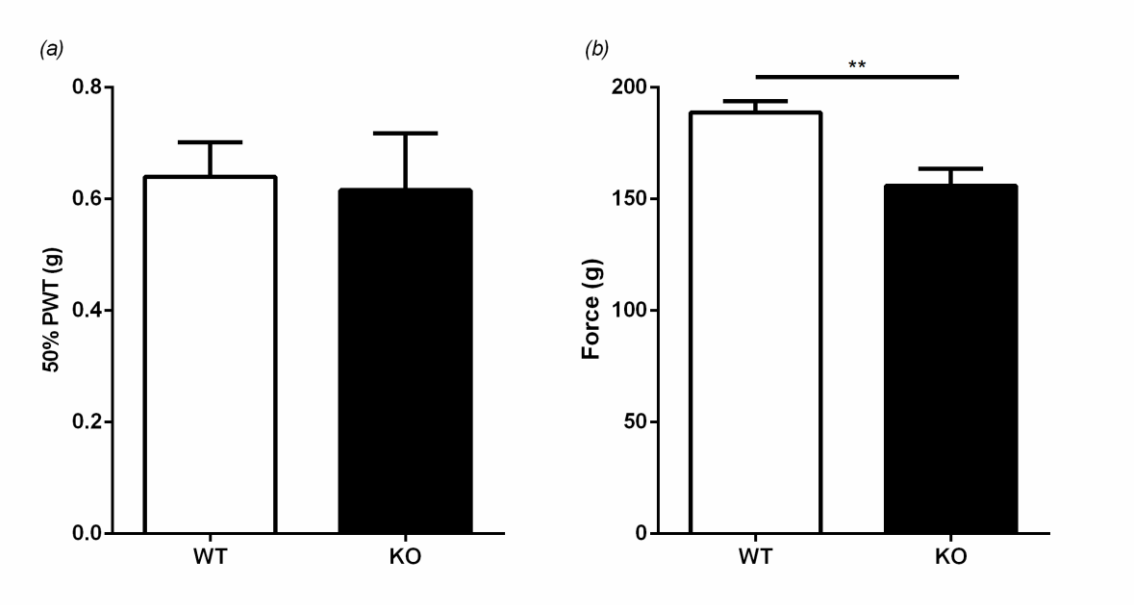



Figure 6.3 *Anxa6* KO mice show normal responses to von Frey stimulation (a) but show a decrease in the force required to elicit a response in the Randall-Selitto test of noxious mechanical sensitivity (b). (WT, n=9; KO, n=8) Data are shown as Mean±SEM with Student's t test. *p<0.05; **p<0.01; ***p<0.001, ****$p$<0.0001.

### *6.3.2 Electrophysiological properties of Annexin A6*

*This work was performed by Dr. Stéphane Lolignier (see Appendix B for methods)*

#### *6.3.2.1 Overexpression of Annexin A6 alters mechanosensitivity of sensory neurons*

Cultured DRG neurons produce RA, IA and SA currents following mechanical stimulation of the cell soma. Overexpression of ANXA6 in cultured sensory neurons did not alter the proportion of cells expressing each current type (Figure 6.4a), or significantly alter the displacement threshold required to produce a 40pA current (Figure 6.4b). However it significantly reduced the amplitude of the IA currents produced from displacements of 7-9µm (treatment effect, ANOVA $p<0.0001$; EV v ANXA6 $p=0.02$ at 7µm displacement, $p=0.0001$ at 8µm displacement and $p<0.0001$ at 9µm displacement) (Figure 6.4d). Though there is a substantial reduction in the Imax of IA currents, this reduction was not significant (ANOVA $p>0.05$). At the displacement thresholds shown, there was no difference in the amplitude of SA currents produced (Figure 6.4e), however the numbers of cells expressing SA currents tested is substantially lower than cells expressing RA and IA currents (Figure 6.4 c, d, e, f). Both SA and IA currents are associated with high threshold mechanical stimulation so increasing the number of SA current recordings is vital to fully characterise the effect of ANXA6 on high threshold mechanically activated currents.

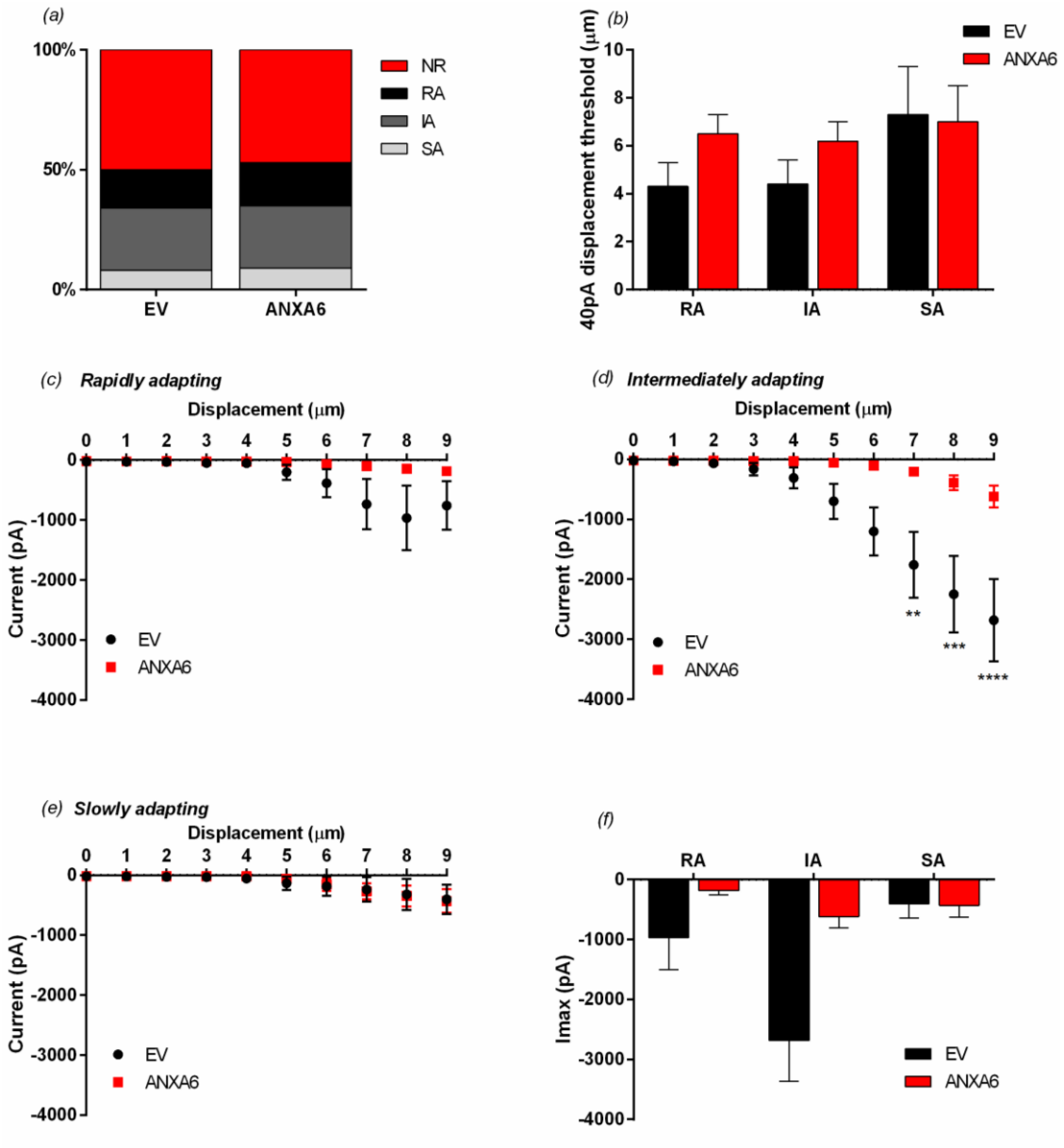



Figure 6.4 ANXA6 electrophysiology (a) Sensory neurons overexpressing ANXA6 show normal proportions of RA, IA and SA currents. (b) Mechanical displacement required to elicit a 40pA current. (c-e) I/displacement kinetics of RA, IA and SA currents in cells overexpressing ANXA6 (RA current; EV n=5, ANXA6 n=6; IA current; EV n=10; ANXA6 n=9; SA current; EV n=3; ANXA6 n=3). (f) Peak current recorded throughout displacement. Data are shown as Mean±SEM. Total n numbers; EV, n=38; ANXA6, n=34. *p<0.05; **p<0.01; ***p<0.001, ****$p$<0.0001.

#### *6.3.2.2 Overexpression of Annexin A6 reduces mechanosensitivity of Piezo2*

Piezo2 produces a robust, RA mechanically activated current when expressed heterologously. We overexpressed Piezo2 and ANXA6, separately and together in ND-C cells, a neuronally derived cell line with a slight, endogenous mechanosensitivity which is seen when empty expression vectors were used as controls. Expression of Piezo2 produces a peak current of 2036.92pA±360.4pA which is reduced to 786.9pA±130.7pA when ANXA6 is coexpressed (treatment effect ANOVA, $p<0.0001$; Piezo2 + EV v. Piezo2 + ANXA6 $p=0.003$) (Figure 6.5a) suggesting ANXA6 is able to negatively regulate Piezo2 mechanosensitivity. Similarly, the threshold required to evoke a 40pA current response was slightly, but not significantly higher in cells co-expressing ANXA6 and Piezo2 compared to Piezo2 alone (4.92±0.67µm and 3.31±0.56µm, respectively; $p>0.05$) (Figure 6.5b). Stimulus response curves show that ANXA6 was able to significantly reduce the magnitude of Piezo2 current produced by high threshold mechanical stimulation (treatment effect ANOVA $p=0.006$; Piezo2 + EV v. Piezo2 + ANXA6 $p<0.0003$ at displacements greater than 7µm) (Figure 6.5c).

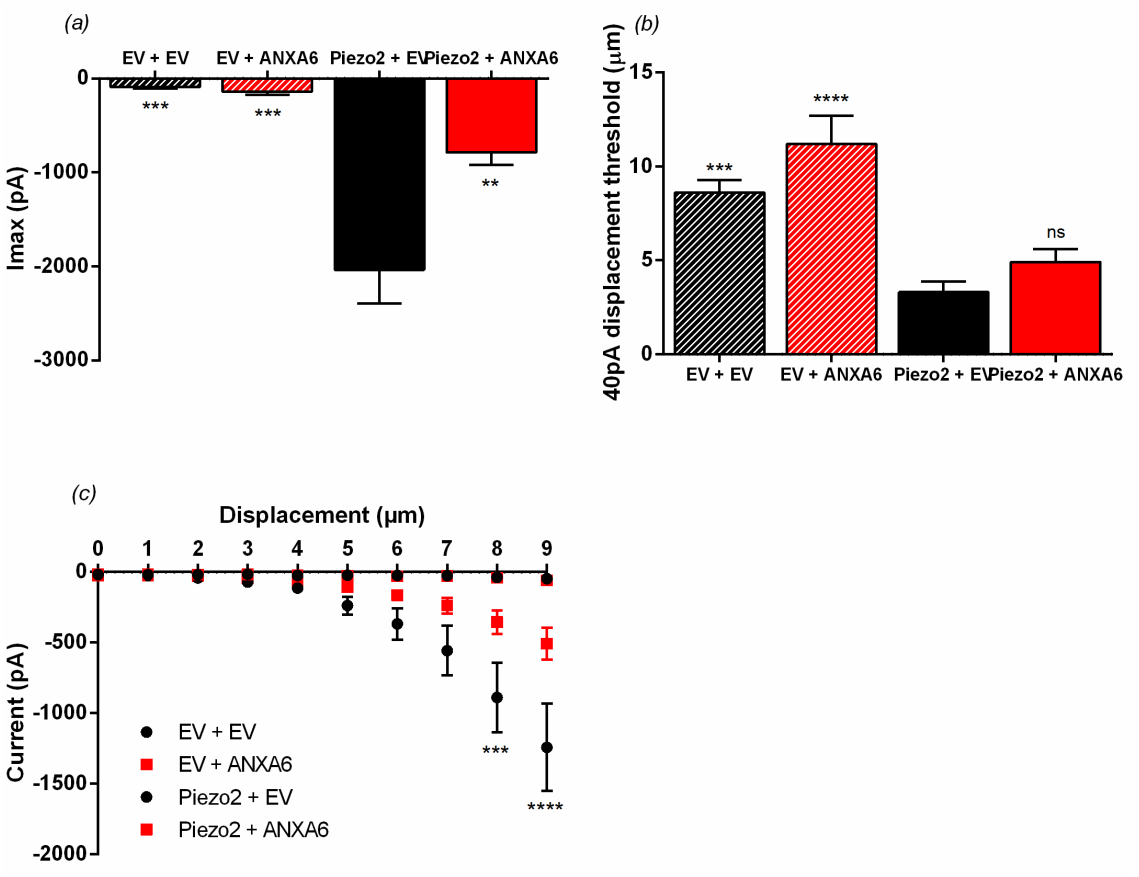



Figure 6.5 ANXA6 coexpressed with Piezo2 in ND-C cells. (a) Peak current recorded throughout displacement. (b) Mechanical displacement required to elicit a 40pA current. (c) Current displacement kinetics of Piezo2 in the presence and absence of ANXA6. Data are shown as Mean±SEM. *p<0.05; **p<0.01; ***p<0.001 ****$p$<0.0001 all groups are compared to Piezo2+EV treated cells in (a) and (b); in (c) only Piezo2+EV vs. Piezo2+ANXA6 are shown.

### *6.3.3 Virally mediated overexpression of Annexin A6*

#### *6.3.3.1 Viral vector construct design*

*The viral vector was provided by Prof. M. Linden and this work was performed by Dr Julie Tordo*

As knocking out *Anxa6* produced hypersensitivity to noxious mechanical stimuli, we hypothesised that overexpression of the protein may produce hypoalgesia and therefore be a useful target for gene therapy and used viral vectors to test this hypothesis.

Human *ANXA6* was cloned into the AAV, downstream of an IRES-eGFP construct and under the control of a CMV promoter (Figure 6.6). Recombinant virus was produced and a viral titer of $5x10^{12}$VP/ml was administered by a single intrathecal injection.

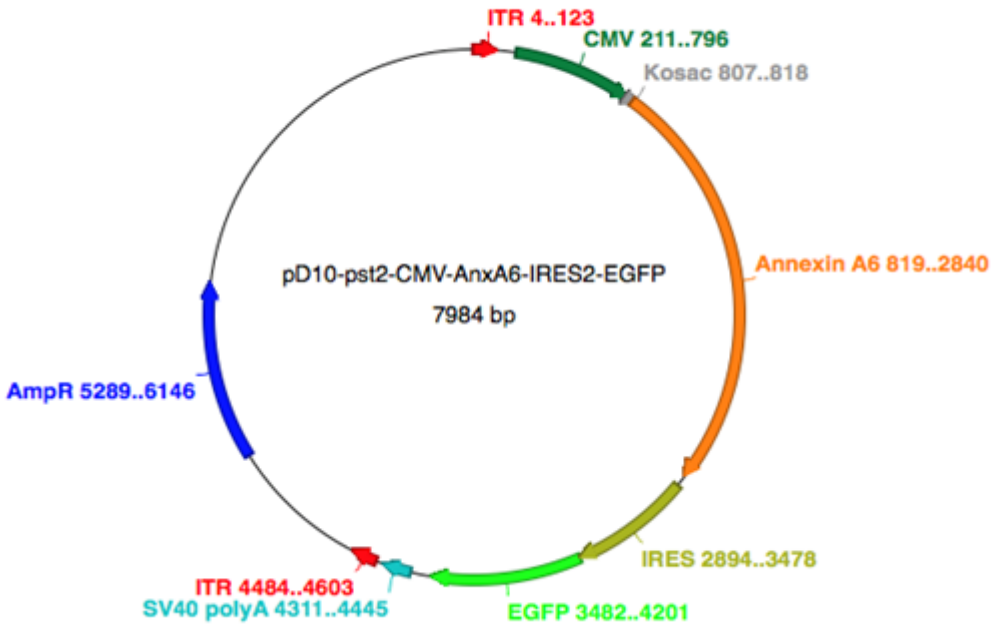



Figure 6.6 Construct design for production of virus particles containing AnnexinA6 transgene.

### *6.3.3.2 Viral overexpression of Annexin A6 leads to reductions in mechanical sensitivity*

*The Hargreaves test was performed by Sonia Santana-Varela and the weight bearing test was performed by Dr. Stéphane Lolignier.*

The human *ANXA6* gene was cloned into two viral vectors; AAVx and AAV6. Each serotype was administered intrathecally into separate groups of mice with a third group treated with an empty AAVx vector (EV-AAVx). Behavioural analysis was used to determine whether ANXA6 overexpression in these vectors was able to reduce mechanical sensitivity in physiological and pathological conditions (Figure 6.7).

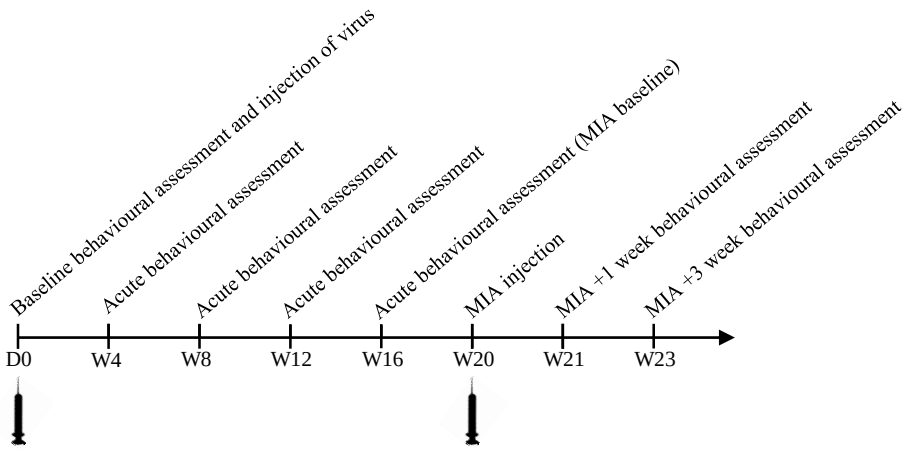



Figure 6.7 Time course of AAV study. 20 weeks after viral vector injection, Osteoarthritis was induced by a single injection of MIA. Behavioural assessment was performed throughout.

Innocuous and noxious mechanical and noxious thermal sensitivity were monitored regularly over a number of weeks. After 20 weeks, 0.5mg MIA was administered into the left knee of mice in all groups and behavioural analysis of mechanical sensitivity was continued using von Frey and weight bearing asymmetry analysis while Randall-Selitto (noxious mechanical) and Hargreaves (noxious thermal) measures were discontinued (Figure 6.7).

We observed that overexpression of ANXA6 did not significantly alter sensitivity to noxious or innocuous mechanical stimuli in naïve mice at any time point ($p$>0.05) (Figure 6.8a,c). However, following a slight, non-significant, increase in noxious mechanical threshold, we administered a unilateral injection of MIA in order to see if ANXA6 expression could instead attenuate mechanical hypersensitivity. A baseline weight bearing measurement was taken at week 16 as well as an additional von Frey threshold assessment prior to this MIA injection at week 20.

Thermal sensitivity was not significantly altered from baseline at any time point and was not significantly different between treatment groups after AAV administration (Figure 6.8a).

One week after injection of MIA, all treatment groups showed a significant decrease in 50% threshold compared to the threshold recorded at 16 weeks (MIA treatment effect, ANOVA $p$<0.0001; EV-AAVx $p$=0.02; *ANXA6*-AAVx $p$=0.04; *ANXA6*-AAV6 $p$<0.001) (Figure 6.8c). Three weeks after MIA injection, control (EV-AAVx treated) mice showed a further drop in 50% threshold from 0.42g±0.04g (one week post MIA) to 0.28g±0.03g (three weeks post MIA) while the 50% threshold of *ANXA6*-AAVx treated mice dropped only slightly from 0.45g±0.04g (one week post MIA) to 0.41g±0.06g (three weeks post MIA). Results from *ANXA6*-AAV6 treated mice are less clear with significant hypersensitivity present one week after MIA injection but a less severe hypersensitivity three weeks after MIA injection; withdrawal thresholds dropped from 0.57g±0.04g to 0.30g±0.06g after one week and rose to 0.40g±0.04g after three weeks. A two-way ANOVA with Tukey post hoc comparison showed no significant effect between vector-treatment groups at any time point.

An attenuation of mechanical hypersensitivity in *ANXA6*-AAVx treated mice was observed in the weight bearing test (Figure 6.8d). While control (EV-AAVx treated) mice developed an asymmetry which was significant compared to baseline one week after MIA injection (MIA treatment effect, ANOVA $p$=0.0005), *ANXA6*-AAVx treated mice did not develop a significant weight bearing asymmetry at any time point (MIA treatment effect, ANOVA $p$>0.05). Indeed, in *ANXA6*-AAVx treated mice weight on the ipsilateral paw at baseline was 50.59%±1.77, one week after MIA injection was 49.21%±1.95 and three weeks after MIA injection was 51.38%±1.76. Whereas EV-AAVx treated mice showed weight on the ipsilateral paw dropped from 50.56%±1.31 to 43.98%±1.58 ($p$=0.007) one

week after MIA and 46.77%±1.48 ($p$=0.18) three weeks after MIA injection. *ANXA6*-AAV6 treated mice showed a significant weight bearing asymmetry following MIA injection thus this vector produced no ANXA6 mediated analgesia.

As in the von Frey test, a two-way ANOVA with Tukey post hoc comparison showed no significant effect between treatment groups at any time point in the weight bearing test. However, since the EV-AAVx treated mice developed a weight bearing asymmetry which was significant compared to baseline and *ANXA6*-AAVx treated mice did not, it seems this treatment produces a partial analgesic effect. Altogether, these data show through both von Frey and weight bearing tests that ANXA6 overexpression in the AAVx vector is able to partially attenuate MIA induced mechanical hypersensitivity.

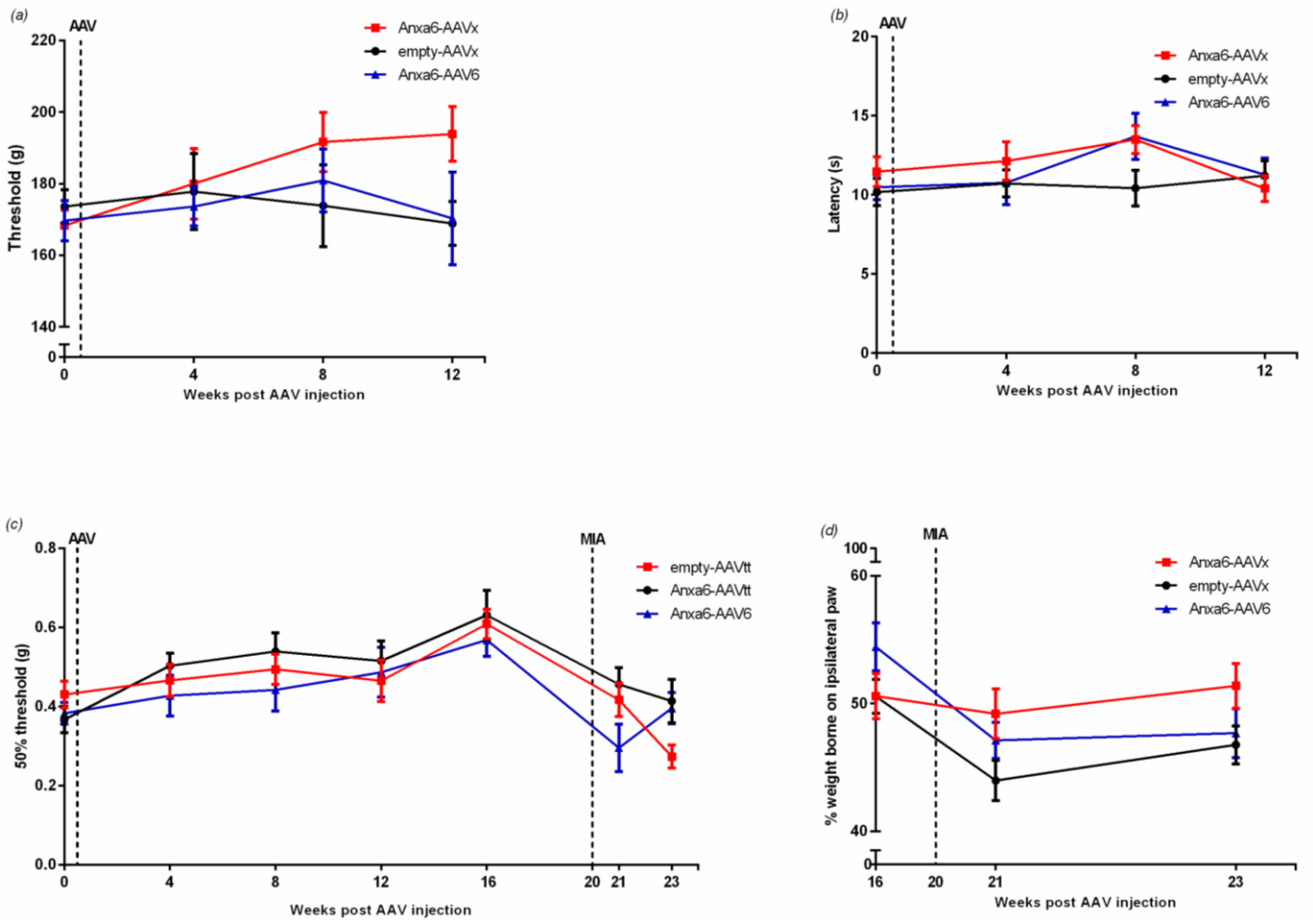


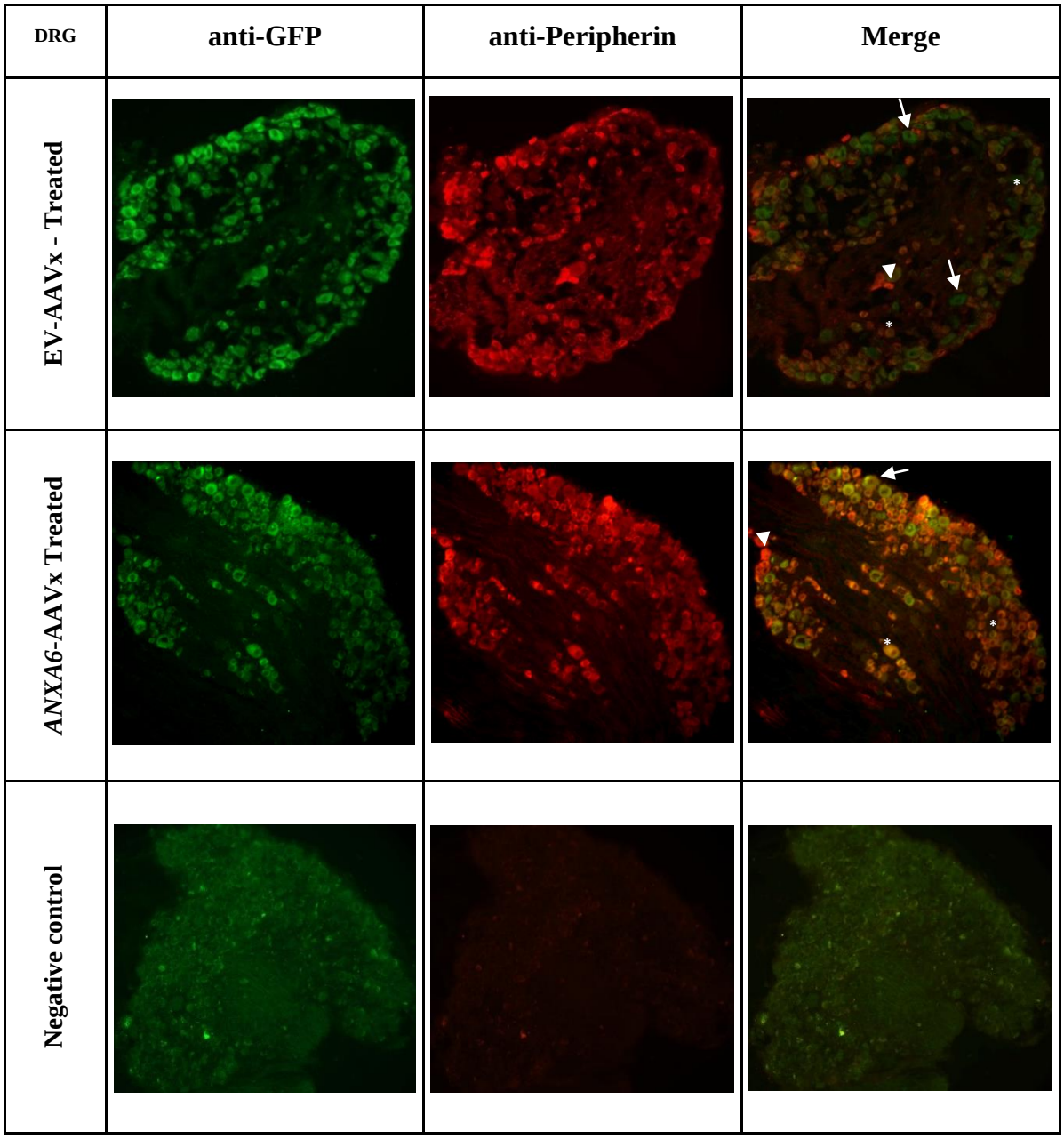

Figure 6.8 Effects of ANXA6 overexpression on mechanical and thermal sensitivity. *ANXA6*-AAVx treatment partially attenuates noxious mechanosensation (a) but has no effect on thermal sensitivity (b). ANXA6 overexpression has no effect on acute innocuous mechanosensation but partially attenuates MIA induced mechanical hypersensitivity (c,d) (n=12 for all groups) Data are shown as Mean±SEM.

#### *6.3.3.3 Viral overexpression of Annexin A6 leads to increased protein in DRG and spinal cord*

*This work was performed by Dr. Stéphane Lolignier and Anna Biller*

Since the most prominent analgesic phenotype was observed after treatment with *ANXA6*-AAVx, *ANXA6* expression was assessed in tissue taken from these mice to observe. In DRG neurons, an anti-GFP antibody shows staining in the cell bodies of sensory neurons (Figure 6.9). Co-staining with an anti-Peripherin antibody showed expression of GFP in both small and large diameter sensory neurons in both EV and *ANXA6*-AAVx treated neurons. A no-primary-antibody treated sample of *ANXA6*-AAVx tissue was used as a negative control; no peripherin staining is observed as expected however low levels of GFP expression are visible, demonstrating effective amplification of the signal with the anti-GFP antibody. The percentage numbers of DRG neurons transduced with the *ANXA6*-AAVx vector is shown in Figure 6.9 and is estimated to be 87.98%±3.76%. In line with the behavioural results observed, these expression studies strongly suggest that the viral vectors had effectively transduced sensory neurons following intrathecal administration. In order to better study the expression pattern of GFP it would be valuable to perform staining of untreated DRG neurons as both EV-AAVx and *ANXA6*-AAVx treated mice show GFP expression.

In the spinal cord of untreated mice, IB4 binding is seen in the superficial dorsal horn (Figure 6.10) and in mice treated with *ANXA6*-AAVx, widespread, diffuse GFP staining is visible throughout the grey matter. Tissue from the spinal cord of untreated mice showed no GFP expression, as expected



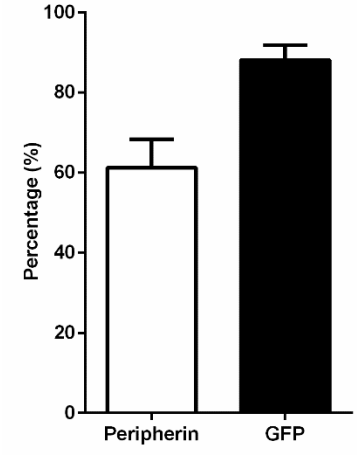




Figure 6.9 Immunohistochemistry following treatment with AAVx shows GFP expression (green; arrows) in DRG in *ANXA6*-AAVx and EV-AAVx treated mice. All sections were co-stained with an antibody to Peripherin (red; arrowhead) which labelled small fibres in the DRG. A sample of *ANXA6*-AAVx treated tissue was tested without primary antibody as a negative control. Quantification of *ANXA6*-AAVx treated cells estimates 87.98%±3.76% neurons are transduced by the viral vector (n=4); 61.15%±7.08% neurons are positive for peripherin. Double labelled neurons marked with asterisk (*).

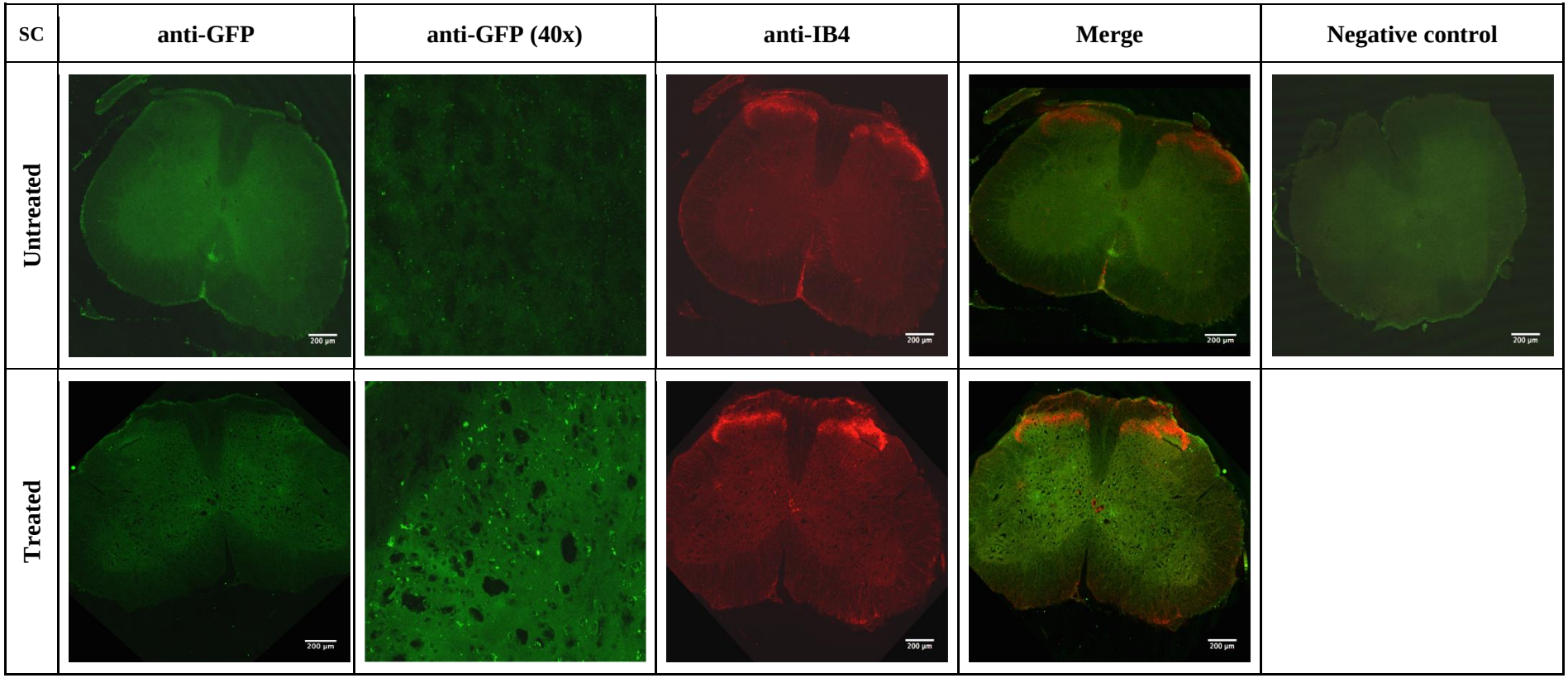



Figure 6.10 Immunohistochemistry following treatment with AAVx showing GFP expression (green) in the lumbar spinal cord. Panels show GFP expression in *ANXA6*-AAVx treated mice while untreated mice show no GFP expression. Higher magnification of GFP staining is shown in the second panel. All sections were co-stained with an antibody to IB4 (red) which labelled inner lamina II of spinal cord sections. No primary antibody control images for spinal cord sections were used as a negative control.

Immunohistochemical analysis demonstrated that sensory neurons were effectively transduced by the AAVx viral vector. Next, we used Western blot analysis to determine whether ANXA6 protein expression in the DRG of *ANXA6*-AAVx treated mice had successfully increased above endogenous levels.

Western blots show expression of ANXA6 protein in the DRG of EV-AAVx treated and *ANXA6*-AAVx treated mice. ANXA6 has a molecular weight of 76kDa and β-actin 42kDa (Figure 6.11a). A β-actin loading control was used to quantify the bands to determine whether the levels of ANXA6 protein were increased beyond endogenous levels in ANXA6-AAVx treated mice. Though quantification indicates that levels of ANXA6 are higher in the DRG of *ANXA6*-AAVtt treated mice (ratio of ANXA6:β-actin 2.01±.58) compared to EV-AAVx treated mice (ratio of ANXA6:β-actin 1.67±0.49), this increase was not significant ($p$=0.26) Figure 6.11c). Our behavioural data indicated a notable latency from administration of *ANXA6* in the viral vector to the development of a hyposensitivity to noxious mechanical stimuli. It is possible that the time required for translation or trafficking of ANXA6 protein contributed to this delay though the current data suggest that by the end of the experiment, ANXA6 protein had successfully been overexpressed, in the cell bodies of sensory neurons, following viral administration. ANXA6 expression was also observed in the spinal cord, though quantification shows no significant difference in *ANXA6*-AAVx and EV-AAVx treated groups (Figure 6.11c,d).

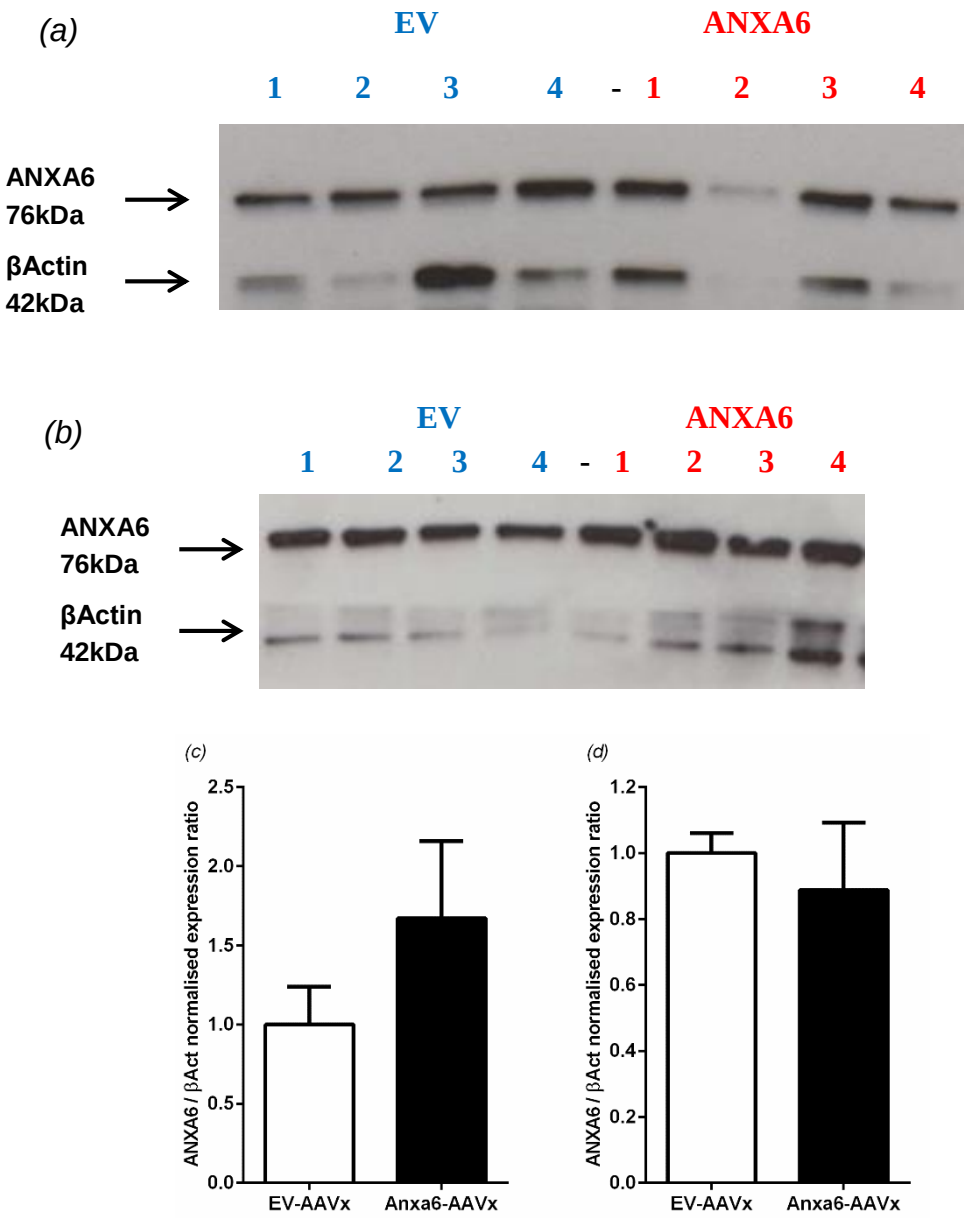



Figure 6.11 Western Blots show expression of ANXA6 protein and βactin in nervous tissue in EV-AAVx and *ANXA6*-AAVx treated mice. Expression levels from 4 mice in each treatment group from DRG tissue (a) and spinal cord tissue (b) which were quantified using βactin for normalisation in DRG (c) and spinal cord (d).

## 6.4 Discussion

Mechanical hyperalgesia and allodynia are symptoms frequently presented by patients with chronic pain conditions. We identified ANXA6 as a potential contributor to mechanotransduction as it binds to NMB-1, a peptide blocker of SA currents. ANXA6 is known to bind phospholipid membranes, regulate membrane fluidity and is also involved in second messenger pathways; we used *Anxa6* KO mice and virus-mediated ANXA6 overexpression to investigate its role in acute mechanosensation and OA induced mechanical hypersensitivity.

### *6.4.1 Annexin A6 negatively regulates mechanosensation*

Our data clearly demonstrate that Annexin A6 has a selective role in noxious mechanosensation. *Anxa6* KO mice showed increased sensitivity to noxious mechanical stimuli while leaving responses to innocuous mechanical stimuli unaltered. Mechanical stimulation of cultured DRG neurons overexpressing ANXA6 at high stimulus thresholds reduced the amplitude of the IA current, which is associated with noxious mechanosensation (Drew et al., 2007). Interestingly, overexpressing ANXA6 with Piezo2 reduced the peak current produced by mechanical stimulation compared to expression of Piezo2 alone. Taken together, these data provide strong evidence that ANXA6 negatively regulates mechanosensation.

The current data provides the first evidence of a role for ANXA6 in cutaneous mechanosensation. Interestingly, ANXA6 has been linked to roles in mechanosensory functions in other physiological systems. For example, in mice overexpressing ANXA6 in the heart, there is a decrease in the contractility of cardiomyocytes, presumed to be the result of reduced free intracellular $Ca^{2+}$ and reduced $Ca^{2+}$ release upon depolarisation (Gunteski-Hamblin et al., 1996). Similarly, in skeletal muscle, ANXA6 regulates the gating properties of the sarcoplasmic reticulum $Ca^{2+}$ release channel, potentially linking it to a role in excitation-contraction coupling responsible for muscle contraction (Diaz-Munoz et al., 1990).

Current understanding of the functions of ANXA6 provide insight into the potential mechanisms by which it could regulate mechanotransduction. In conditions of low $Ca^{2+}$ annexins are soluble monomeric proteins though when intracellular $Ca^{2+}$ levels rise, annexins can bind to phospholipid membranes (Naciff et al., 1996a). It is proposed that to bind the membrane, annexins form trimers and aggregate into a hexagonal array

surrounding a target protein (Concha et al., 1993, Voges et al., 1994, Naciff et al., 1996a). This is known to affect membrane properties, for example altering $Ca^{2+}$ mediated membrane fluidity (Newman et al., 1989, Junker and Creutz, 1993, Sobota et al., 1993, Megli et al., 1998) and potentially interfering with protein-protein or protein-phospholipid interactions as well as affecting $Ca^{2+}$ dependent second messenger pathways (Kaetzel et al., 1994). If ANXA6 aggregates are able to influence membrane fluidity, increasing membrane rigidity in the same way as Annexin A5 for example (Megli et al., 1998), it is possible that this could increase the force required to elicit a mechanically activated response. Alternatively, if ANXA6 aggregates interact with membrane bound proteins they may be able to regulate the properties of mechanotransduction channels. For example, in sensory and spinal cord neurons, ANXA6 negatively regulates $Ca^{2+}$ channel conductance (Naciff et al., 1996a). If ANXA6functions to bind to and regulate $Ca^{2+}$ permeable ion channels in the cell membrane this may be true for regulation of mechanotransduction complexes. We have shown *in vitro*, that overexpressing ANXA6 alters the current produced by the primary mechanotransducer, Piezo2 which is a $Ca^{2+}$ permeable channel. As such, it is possible that the effect of ANXA6 on mechanosensation is conferred through regulation of conductance of mechanotransduction channels. As well as being able to target membrane proteins, it has been shown that annexins, including ANXA6, are able to regulate membrane permeability. ANXA6 has been shown to be able to have both protective and disruptive effects on the lipid bilayer under stressful conditions, including in the presence of arachidonic acid, a key inflammatory mediator (Creutz et al., 2012). Other features of ANXA6 include reports of it acting as a $Ca^{2+}$ channel (Pollard et al., 1992), though reconciling this with the reported negative regulation of $Ca^{2+}$ conductance by ANXA6 is somewhat difficult.

### *6.4.2 Annexin A6 is a useful gene therapy target for treating pain in OA*

Our data show that ANXA6 negatively regulates mechanosensation; as a result we asked whether overexpressing ANXA6 would reduce sensitivity to noxious mechanical stimuli in naïve animals and if it would attenuate the mechanical hypersensitivity associated with OA. Gene therapy has been shown to be successful in producing analgesia in experimental models of pain including cancer induced bone pain (Meng et al., 2015), HIV induced pain (Kanao et al., 2015), inflammatory pain (Xu et al., 2003b), surgical models of neuropathic pain (Storek et al., 2008, Samad et al., 2013, Fan et al., 2014), diabetic neuropathy (Tan et al., 2015) and rheumatoid arthritis (Braz et al., 2001) through

intraganglionic, intrathecal and cutaneous routes. In these models, the targeting strategies have included knockdown of genes encoding ion channels, for example Nav1.3 (Samad et al., 2013) and overexpression of proteins such as the mu opioid receptor (Xu et al., 2003b). We used a novel AAV serotype which has a high tropism for neurons, AAVx, and AAV6 to overexpress human ANXA6 *in vivo*.

ANXA6 overexpression reduced noxious mechanosensation, though this hyposensitivity was only seen 12 weeks after administration of the viral vectors. Throughout this 12 week period, innocuous mechanosensation remained unaltered which is consistent with our *Anxa6* KO data, indicating that ANXA6 does not contribute to light touch sensitivity. We also performed a noxious thermal sensitivity test to ensure the hyposensitivity we observed was the result of modality specific changes and found no significant effect on thermal thresholds compared to baseline or between treatment groups. AAVx appeared to be more effective at producing analgesia than AAV6. It has been reported that transduction of sensory neurons by AAV6 peaks at a period of 4 weeks and levels of AAV6 expression subsequently decline (Mason et al., 2010). It is therefore possible that this feature of AAV6 makes it incompatible with our experiment which appears to require a substantially longer period to produce sufficient ANXA6 expression to affect pain sensitivity.

After observing the development of analgesia in response to noxious mechanical stimulation, we injected MIA into the left knee of virus-injected mice. Mice treated with *ANXA6* in the AAVx vector did not develop a significant weight bearing asymmetry at any time point (whereas control mice did develop a significant asymmetry) and showed an attenuation in hypersensitivity of the paw compared to mice treated with the control viral vector. The weight bearing measure of mechanical hypersensitivity is considered to be a clinically relevant assessment of pain as OA patients report pain on load bearing while the von Frey test represents a referred pain to sites distal to the affected joint. Existing analgesic therapies for OA are often only partially effective, associated with side effects and not appropriate for long term use (see section 5.1). In section 5.3, we also demonstrate that the molecular mechanisms underlying mechanical hypersensitivity in OA may be distinct from other pathological conditions; similarly, the correlation between joint damage and pain is weak which together illustrate the complexities of understanding mechanisms responsible for pain in OA and the difficulties in achieving effective analgesia. Gene therapy offers a target specific alternative to existing pharmacological

interventions and has already been shown to be a viable treatment method for analgesia in the clinic (Fink et al., 2011) while in preclinical models, gene therapy for pain relief has already been shown to be useful in rheumatoid arthritis (Braz et al., 2001). The current data indicate that overexpressing ANXA6 is sufficient to produce partial attenuation of the mechanical hypersensitivity induced by OA. However different doses of MIA have been shown to produce different pathological and behavioural phenotypes so using a higher dose of MIA would allow us to investigate whether this effect is maintained in a model of more advanced OA.

Immunohistochemistry of DRG neurons shows that 23 weeks after injection of viruses, a large proportion of neurons were positive for virally expressed GFP, however it would be interesting to inject an empty AAVx vector and dissect tissue at different time points throughout the 12 week experiment in order to determine at what stage our vectors effectively transduce DRG neurons. Validating this data through quantitative PCR would provide further information as to the efficiency of virus transduction over the time course of the experiment while looking at levels of protein expression throughout would also be informative. In the current experiment, after 23 weeks, levels of ANXA6 in DRG appear to be higher in *ANXA6*-AAVx treated mice than the levels of endogenous ANXA6 seen in mice treated with empty vector only. Other groups report a maximum of 1.5 fold increase in expression of protein levels compared to endogenous protein expression which is similar to the increase we observed (1.6 fold increase) however the values were highly variable meaning our data is not significant. This conclusion may be strengthened by quantifying tissue from a higher number of animals.

Interestingly, other groups have reported efficient transduction of viruses to DRG within a few weeks (Meng et al., 2015, Tan et al., 2015) whereas we only observed a behavioural phenotype around 12 weeks after virus injection. It is important to note that many of these groups were using short RNA sequences to inhibit transcription of specific genes, whereas other groups have reported that – despite efficient transduction of sensory neurons by AAV vectors and increased mRNA expression after as little as 1-3 weeks – increases in protein levels were much more gradual, including when viral vectors were administered directly into DRG (Xu et al., 2003a, Mason et al., 2010, Fan et al., 2014). It is also possible that trafficking of the newly translated protein to the peripheral terminals of primary sensory neurons increased the latency to an observable phenotype.

Another factor which may affect the data we have observed is our initial focus on the non-diseased state. Many other reports of gene therapy in animal models administer viral vectors concurrent to induction of the pain model. Our data taken from after the injection of MIA produce exciting results which indicate that overexpressing ANXA6 using the AAVx viral vector can reduce mechanical hypersensitivity in OA. This is substantiated by our use of two different methods of behavioural assessment, the benefits of each have been discussed (see section 5.1). It is possible that if we were to induce OA at an earlier time after virus administration we would see similar results to other groups as it may be that at the earlier stages of the experiment, any change in acute mechanical threshold is subtle and a phenotype is difficult to observe. It is therefore important that the time course of the effects of this gene therapy approach is addressed if it is to be a clinically useful gene therapy tool.

### *6.4.3 Annexin A6 in mechanosensation*

This chapter has demonstrated a novel role for ANXA6 in noxious mechanosensation. ANXA6 negatively regulates behavioural responses to noxious mechanical stimuli and attenuates IA mechanically activated currents as well as reducing current produced by the primary mechanotransducer, Piezo2. When expressed in a viral vector and administered intrathecally, ANXA6 is able to attenuate the mechanical hypersensitivity induced by OA. Altogether, the current data highlight an exciting potential analgesic therapy for OA as well as identifying a novel regulatory mechanism of mechanosensation. We have observed that overexpression of ANXA6 is most effective using the AAVx serotype; a full characterisation of the time course of this effect is necessary before this can be considered as a useful therapy at which point, it may confer analgesia to mechanical hyperalgesia and allodynia in other pathological pain conditions.

### *6.4.4 Future work*

Our data indicates an exciting novel function for ANXA6 in negative regulation of mechanotransduction and cutaneous mechanosensation. In order to completely understand the contribution of ANXA6 to mechanosensation and to fully explore its potential use in gene therapy a number of additional experiments are required.

First, as our current data does not elucidate a mechanism for ANXA6 regulation of mechanotransduction, it would be interesting to perform further studies to determine the mechanism by which ANXA6 has its effects. For example, measuring membrane fluidity

may indicate whether the effect of ANXA6 on mechanotransduction currents is through alteration of membrane properties or if it is more likely through another mechanism such as direct regulation of mechanotransducers. The latter could be addressed by performing a co-immunoprecipitation with Piezo2 to assess whether ANXA6 directly binds Piezo2

As previously discussed, looking at the time course of the transduction efficiency of *ANXA6*-AAVx through immunohistochemistry and quantitative PCR may be useful to see whether it is possible to produce an earlier onset phenotype in the disease model as well as allowing optimising the concentration of virus administered to identify the most effective dose.

Also, despite the many benefits of the MIA model of OA, including rapid onset, minimal cost and production of robust pain behaviour, there are some criticisms over the clinical relevance of the model. The fact that the model produces arthritis pathology so rapidly is not in line with the slow, degenerative progression of human OA. The DMM surgical model of OA (see section 5.4.4) produces different features of OA than the MIA model. It has been reported that this easy to induce-and-reproduce model produces a pathology similar to that seen in spontaneous models of mouse OA (Glasson et al., 2007). The altered joint biomechanics produced by severing the medial meniscotibial ligament do not produce such an inflammatory response as MIA and onset of symptoms such as cartilage damage occur much later after induction than is seen in the MIA model (Glasson et al., 2007, Loeser et al., 2013). Moreover, the slowly progressing nature of this model makes it more comparable to the human condition so administering our viral vector containing *ANXA6* to mice with this model of OA may substantiate our claims of the clinical usefulness of this therapy. Finally, administering a higher concentration of MIA (up to 1mg can be used in mice (Ogbonna et al., 2013)) would strengthen a role for ANXA6 in producing OA in analgesia as a result of the dose-dependent effects of MIA (Thakur et al., 2012) which have been suggested to model different stages of the human condition (Okun et al., 2012). Together, administering *ANXA6*-AAVx to animals with OA induced by DMM surgery and high dose MIA in combination with the current data would allow us to further demonstrate the clinical usefulness of our gene therapy approach.

# 7 Does ASIC4 have a role in mechanosensation or pain?

## 7.1 Summary

The family of acid sensing ion channels (ASIC) have established roles in sensory function, including in pain and mechanosensation. Unlike other subunits of this family ASIC4 is poorly characterised and its function *in vivo* is incompletely understood. Also unlike other subunits, ASIC4 is proton insensitive and its expression is limited to specific regions of the nervous system and the pituitary gland. *In vitro,* it has been shown that ASIC4 can regulate the expression of other ASIC subunits, namely ASIC1 and ASIC3. We considered that if this function was the same *in vivo,* ASIC4 may indirectly contribute to pain and mechanosensory function. In order to investigate whether ASIC4 has any role in pain or mechanosensation either directly or indirectly through regulation of expression of ASIC1 or ASIC3 we produced and characterised a global ASIC4 KO mouse. We find that ASIC4 is expressed in the central nervous system though this does not appear to lead to altered ASIC1 protein expression. We also find that loss of ASIC4 function leads to altered Formalin induced pain behaviour and decreased sensitivity in a model of visceral pain. This is the first report of a role of ASIC4 in these modalities and suggests that pursuing an understanding of the role of ASIC4 *in vivo* will contribute to a better understanding of the roles of ASICs in pain and mechanosensation.

## 7.2 Introduction

### *7.2.1 ASIC4*

Unlike other members of the ASIC family, ASIC4 has remained in relative obscurity. Early studies were performed using rat ASIC4 which shares 94% amino acid homology with human ASIC4; murine ASIC4 also shares 94% homology to the human orthologue. However while it was cloned from rat DRG and brain in 2000, the function of the proton-insensitive ASIC4 remained unknown until 2008 when it was found to be a regulator of the expression of other ASIC proteins (Donier et al., 2008). ASIC4 reduces total protein levels of ASIC1a in the cell and reduces the current densities of ASIC1a and ASIC3 when the channels are co-expressed. Importantly, the gating properties of these channels are not affected by ASIC4.

Though ASIC4 appears to regulate functional expression of ASIC1a and ASIC3, a yeast-two hybrid study looking at N-terminal interactions of ASIC subtypes did not identify these proteins as binding to ASIC4, suggesting that they may not interact directly (Donier

et al., 2008). Through its stretch of amino acids 2-21, ASIC4 is ubiquitinated to a much higher level than the other ASIC subtypes (Donier et al., 2008). When this amino acid sequence is removed, the ubiquitination levels of ASIC4 drop to a level similar to ASIC1a and ASIC3, suggesting this region may be important for regulating ASIC4 function. Interestingly however, removal of the 2-21 amino acid region of ASIC4 does not affect ASIC4 mediated downregulation of ASIC1a and ASIC3 (Donier et al., 2008).

The high levels of ASIC4 ubiquitination are interesting. Though ubiquitination of a protein is often a signal for its degradation, it can also mediate subcellular location of the protein and alter protein interactions (Donier et al., 2008). In the case of ASIC4, deletion of the ubiquitin binding region of amino acids 2-21 causes an increase in the total pool of ASIC4 and also increases its cell surface expression (Donier et al., 2008).

During acquisition of data in the present study, Lin et al. (2015a) published characterisation of another ASIC4 KO mouse and identified a role for ASIC4 in fear and anxiety, but not acute nociception. Importantly, they speculated that ASIC4 is unlikely to regulate ASIC3 *in vivo* because they found no ASIC4 expression in adult mouse DRG, where ASIC3 is almost selectively expressed. Indeed, reports of ASIC4 expression vary with some studies reporting low levels of expression in DRG (Akopian et al., 2000, Usoskin et al., 2015); it is important to clarify this in order to determine the role of ASIC4 i*n vivo*.

As ASICs have been implicated in mechanosensation and pain and ASIC4 is a potential modulator of other ASICs, we generated a targeting construct to produce a mouse with a floxed ASIC4 gene. We crossed this mouse with a Cre recombinase line where Cre is ubiquitously expressed from a cytomegalovirus (CMV) promoter, leading to global excision of the floxed ASIC4 sequence. In order to determine whether the *in vitro* findings of Donier et al. (2008) translate *in vivo*, we began by investigating ASIC4 transcript expression in different tissues. We also performed behavioural characterisation of these global KO animals in order to determine if ASIC4 regulation of other ASICs has any influence over their role in pain sensitivity.

### *7.2.2 ASIC4 targeting construct design*

*The ASIC4 targeting construct was created by Dr. Emmanuelle Donier.*

Homologous recombination of the targeting vector into ES cells produced a targeted gene with exons 2, 3 and 4 flanked by *loxP* sites. A neomycin cassette was inserted between exons 4 and 5 to confer selectivity and was flanked by FRT sites to allow removal of the cassette without affecting the gene of interest. The ASIC4 KO allele retains a single loxP site, and does not express exons 2, 3 and 4 which, if translated, is predicted to lead to production of a truncated protein similar to that shown in Figure 7.1, producing a protein of 218 amino acids compared to the full-length 539 amino acid WT protein.

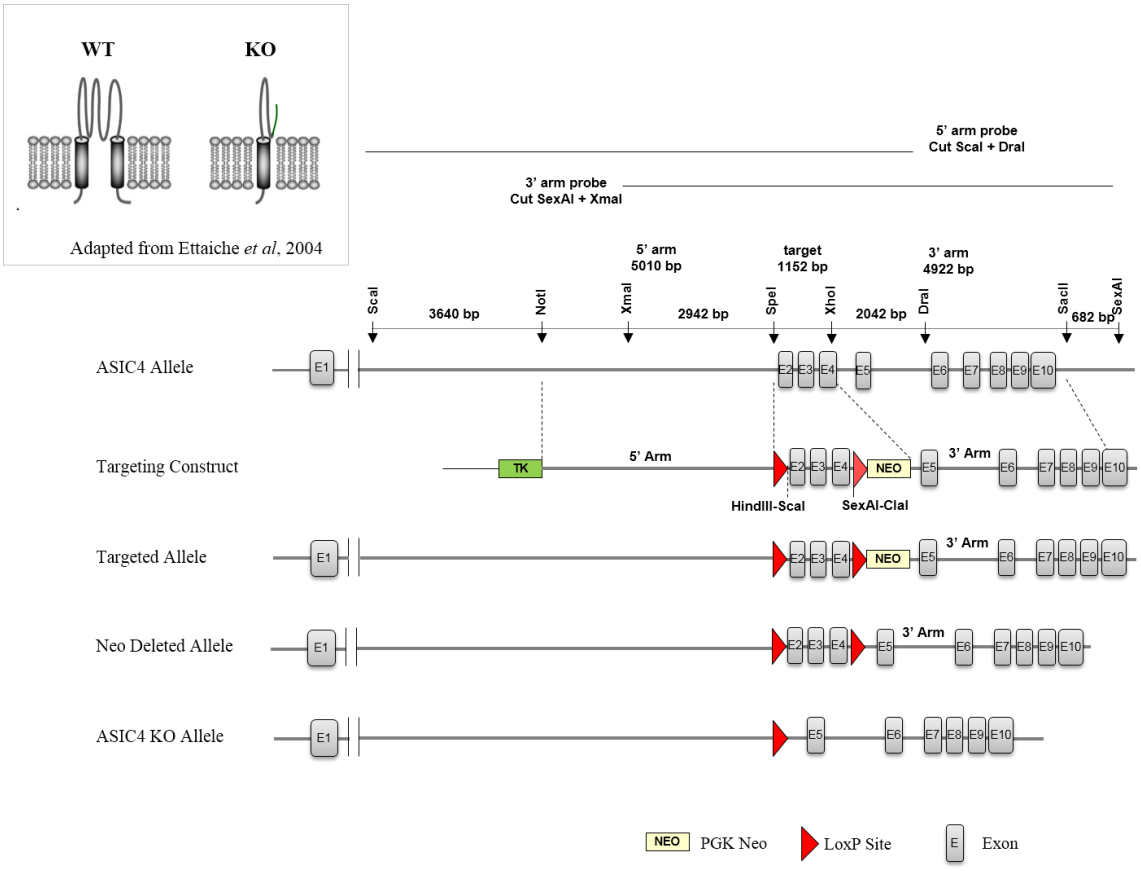



Figure 7.1 ASIC4 knockout construct design. Exons 2-4 are flanked by loxP sites allowing excision by Cre recombinase (Figure by Dr. E Donier).

## 7.3 Results

### *7.3.1 Gene knockout and breeding strategy*

The ASIC4 protein is encoded by the *Accn4* gene; animals with a heterozygous floxed *Accn4* gene ($ASIC4^{fl/+}$) were bred together to produce $ASIC4^{+/+}$, $ASIC4^{+/fl}$ and $ASIC4^{fl/fl}$ pups. Homozygous floxed mice were then crossed with animals expressing a single copy of a Cre recombinase under the control of the ubiquitously expressed CMV promoter (B6.C-Tg(CMV-cre)1Cgn/J; Jackson Laboratories). Offspring which inherited a single floxed allele and Cre recombinase were thus heterozygous knockouts for ASIC4 (ASIC4 Het; $Cre^{+}$). These animals were crossed with a mouse ubiquitously expressing Flp recombinase, which removed the neomycin cassette present between exons 4 and 5 and flanked by FRT sites. Offspring which were ASIC4 Het; $Neo^{-}$ were crossed to produce ASIC4 WT, ASIC4 Het and ASIC4 KO (all $Neo^{-}$). The FLP recombinase was also bred out of these mice.

Genomic DNA, isolated from the ear, was used to confirm that the *Accn4* gene was flanked by *loxP* sites (Figure 7.2a) and subsequently that the floxed exons had been excised (Figure 7.3a). Primers were targeted to the genomic DNA sequence of the targeting construct (Table 7.1; Table 7.2).

The first set of primers were used to identify WT and floxed animals (Table 7.1); PCR products were excised from the gel and the purified DNA was Sanger sequenced. Figure 7.2b and c show the gDNA sequence obtained from this process confirming the insertion of *loxP* sites into the genomic DNA and the corresponding WT sequence.

The next set of primers were used to identify WT and KO animals (Table 7.2) and bands were also excised and purified for sequencing (Figure 7.3b,c). Sequencing results confirmed the deletion of a 1012bp sequence from the WT genome leading to the production of a KO gene.

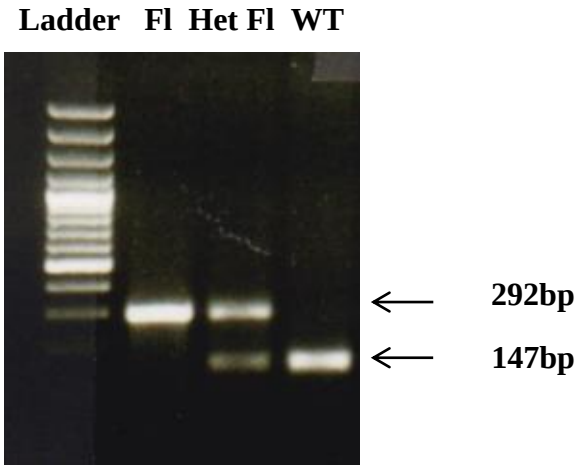
| Forward Primer | Reverse Primer | WT band | Fl band |
|---|---|---|---|
| 5'-GAGCAGGATTGATAGGATAGC | 5'-ATTTGCTACACTGTGTAGCTACAAG | 147bp | 292bp |

Table 7.1 Primers to identify floxed and WT ASIC4 gDNA

| Forward Primer | Reverse Primer | WT band | KO band |
|---|---|---|---|
| 5'-GAGCAGGATTGATAGGATAGC | 5'-GAACAGTTCTGAGATCTACAGTC | 1430bp | 418bp |

Table 7.2 Primers to identify WT and KO ASIC4 gDNA

(a)




(b)

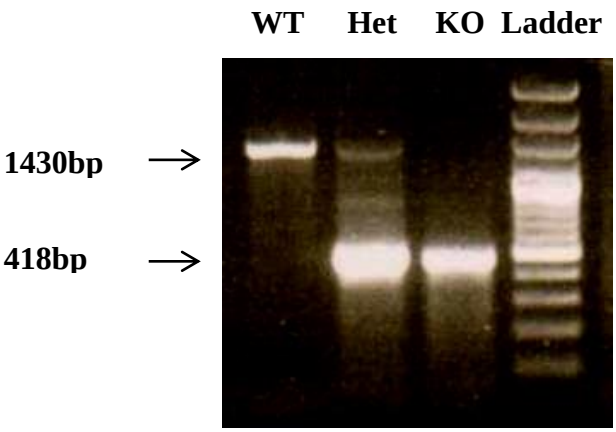
GAGCAGGATTGATAGGATAGCAAGAGTTCTGAGCCTGGTGTGGTGGTGAATCTCTGTACTCC
CAGCTCTTGGGTGGTTAAAGCAGGAGGATTGGGAATTCAAGGCCGGCTACACAGGGAGTCC
TTGTAGCTACACAGTGTAGCAAAT

(c)

GAGCAGGATTGATAGGATAGCAAGAGTTCTGAGCCTGGTGTGGTGGTGAATCTCTGTACTCC
CAGCTCTTGGGTGGTTAAAGCAGGAGGATTGGACTAGTGAATTCCGATCATATTCAATAACC
CTTAATATAACTTCGTATAATGTATGCTATACGAAGTTATTAGGTCTGAAGAGGAGTTTACG
TCCAGCCAAGCTAGCTTGGCTGCAGCAGCTGCATATCGAGAAGCTTCAGTACTGAATTCAAG
GCCGGCTACACAGGGAGTCCTTGTAGCTACACAGTGTAGCAAAT

Figure 7.2 Genotyping of WT and floxed ASIC4 mice. (a) WT, heterozygous floxed and homozygous floxed bands produced by PCR. Sequence of gDNA extracted from PCR bands showing (b) WT and (c) floxed ASIC4 genes. Primer complement sequences are in green, *loxP* sites are in blue and residual plasmid regions in red. Sequence regions confirmed by Sanger sequencing are underlined.

(a)




(b)

GAGCAGGATTGATAGGATAGCAAGAGTTCTGAGCCTGGTGTGGTGGTGAATCTCTGTACTCC
CANCTCTTGGGTGGTTAAAGCAGGAGGATTGGGAATTCAAGGCCGGCTACACAGGGAGTTCT
TGTAGCTACACAGTGTAGCAAATCTTGGGCAGGGGAATTTCTGGGTTACTGCAAGGCCCTGG
GGAGGAGCAGATCCAATCTCAACTACACAGTAAAAACCTGCCAAAAAAATATTAAGAGTTCT
GTGTCTGCTGTGTTCTTTCTCCCTCCAGGTCTATACTCGCTATGGGAAGTGTTACACCTTTAAT
GCAGATCCTCAGAGTTCACTGCCCAGTAGGGCAGGCGGCATGGGTAGTGGCCTGGAGATCAT
GCTAGACATCCAGCAGGAGGAATACCTACCCTTATGGAGGGAGACAAGTATGCAGGCGGGA
AGGGGATAAGGGCCAGCTCATGGATGGGGCCTGGGCTCGNNTNTACCCCTGAAGGGGGGTN
TGAAGCCTCTCATCTCTGCTGNTCAACGAGACGCCNTTTCNAGGNTCATTCGAGGCAGGGAT
CCGGGTGCAGATCCACAGCCAGGAGGAGCCGCCCTACATCCACCAGCTGGGGTTcGGCGTGT
CCCCAGGCTTCCAGACTTTTGTGTCCTGCCAGGAACAGCGGGTGAGCATCCCCAGCTAGGCC
TAGGCTGGGCACAGGGCAACTCACTGCTTAAAGGGATGCTGGCCACATCTGCCTGGCAGATA
CTCAGTCTGCCAGCCCCATGGCCCTGGCTGGGGCTATTGACACTGGAGGACTGGCCCTGATG
GACACGCTTGGCCTTGACCTTGTCATGCCCCATCTCTGCAGCTAACTTATCTGCCCCAGCCTT
GGGGCAACTGCCGGGCGGAAAGCGAGCTCAGGGAGCCTGAGCTTCAGGGCTACTCAGCCTA
TAGTGTGTCTGCCTGCCGACTGCGCTGTGAGAAGGAGGCCGTGCTTCAGCGCTGCCACTGCC
GGATGGTGCACATGCCAGGTGGGCACCTCCAGCCAGTCTTTGGTGCCACTCTTCTGAGCTCCC
GAACTTCCAGGAGGCAGAGATGCATGCGTACCACATGTACACGCATCTACACGCATGAGCGT
GTGCTCATGTGTATGTCTGGATACTTACACATGGGTTCGTATACTTGGGAGCGCATAGATGTG
CATGCTTGCGTGCATGCTAATATATATTCGTGGGTATGTGTTTTGGGGCTGTGTGCATGACCC
GAAGACTGTAACATGATTGTGTAAGCAAATGTGCAATTACCTGCATATGTGTATGTATGCATT
TTTACTGTTTTTATGCGTGTGTGTGTGTATATACATTGAACTGTTATGCATATGTGTAAACATG
CATACATGTAGATGTCTACACAGGTGTGTGGTGAGGGAGAAGGGAAAGGACTGTAGATCTCA
GAACTGTT

(c)

GAGCAGGATTGATAGGATAGCAAGAGTTCTGAGCCTGGTGTGGTGGTGAATCTCTGTACTCC
CAGCTCTTGGGTGGTTAAAGCAGGAGGATTGGACTAGTGAATTCCGATCATATTCAATAACC
CTTAATATAACTTCGTATAATGTATGCTATACGAAGTTATTAGGTCTGAAGAGGAGTTTACGT
CCAGCCAAGCTAGCTTGGCTGCAGGATCCGAGCTCTAGCGAAGTTCCTATTCCGAAGTTCCTA
TTCTCTAGAAAGTATAGGAACTTCGCTAGCGTCGAGGATTGTGTAAGCAAATGTGCAATTACC
TGCATATGTGTATGTATGCATTTTTACTGTTTTTATGCGTGTGTGTGTGTATATACATTGAACT
GTTATGCATATGTGTAAACATGCATACATGTAGATGTCTACACAGGTGTGTGGTGAGGGAGA
AGGGAAAGGACTGTAGATCTCAGAACTGTTC

Figure 7.3 Genotyping of WT and KO ASIC4 mice. (a) WT, heterozygous and KO bands produced by PCR. Sequence of gDNA extracted from PCR bands showing (b) WT and (c) KO ASIC4 genes. Primer complement sequences are in green, *loxP* sites are in blue, FRT sites in purple and residual plasmid regions in red. Sequence regions confirmed by Sanger sequencing are underlined.

### *7.3.2 ASIC4 is expressed in mouse brain and spinal cord*

In order to determine the expression pattern of ASIC4 in the nervous system, both qualitative and quantitative RT-PCR were performed using tissue from adult mice at 8 weeks of age.

RT-PCR was performed on cDNA from wild-type mouse tissues using primers specific to ASIC4, designed to target exons 2 – 4 and to overlap exon-exon junctions (Table 7.3). A band of 147bp was produced (Figure 7.4a) which was consistent with the mRNA sequence length produced on NCBI Primer BLAST. Bands were produced in cortex, cerebellum and spinal cord while liver was used as a negative tissue control and no band was seen in the negative template control or in any tissue taken from the ASIC4 KO. Importantly, only a very faint band was seen in WT DRG though this was reproduced when repeated. All samples, excluding the no template negative control produced bands of 200bp when targeted with primers to GAPDH.

These data were confirmed using quantitative RT-PCR and results were normalised to GAPDH before being displayed as a ratio of ASIC4 expression in the cortex (Figure 7.4b). The qPCR suggests ASIC4 expression in adult DRG is negligible as levels were comparative to the liver negative control tissue. This is in line with findings from other groups, who report little or no ASIC4 expression in adult DRG and indicates that overlap of ASIC4 and ASIC3 is likely to be minimal and therefore that ASIC4 influence on ASIC3 function is unlikely to be of major physiological relevance. It is still possible that, as (Lin et al., 2015a) report, that ASIC4 is expressed in DRG early in development.

| Gene | Forward Primer | Reverse Primer | WT band | KO band |
|---|---|---|---|---|
| **ASIC4** | 5'-GGAGGGAGACAAACGAGACG | 5'-AAGTTAGCCGCTGTTCCTGGC | 147bp | No band |
| **GAPDH** | 5'-TGCGACTTCAACAGCAACTC | 5'-CTTGCTCAGTGTCCTTGCTG | 200bp | 200bp |

Table 7.3 ASIC4 cDNA primers

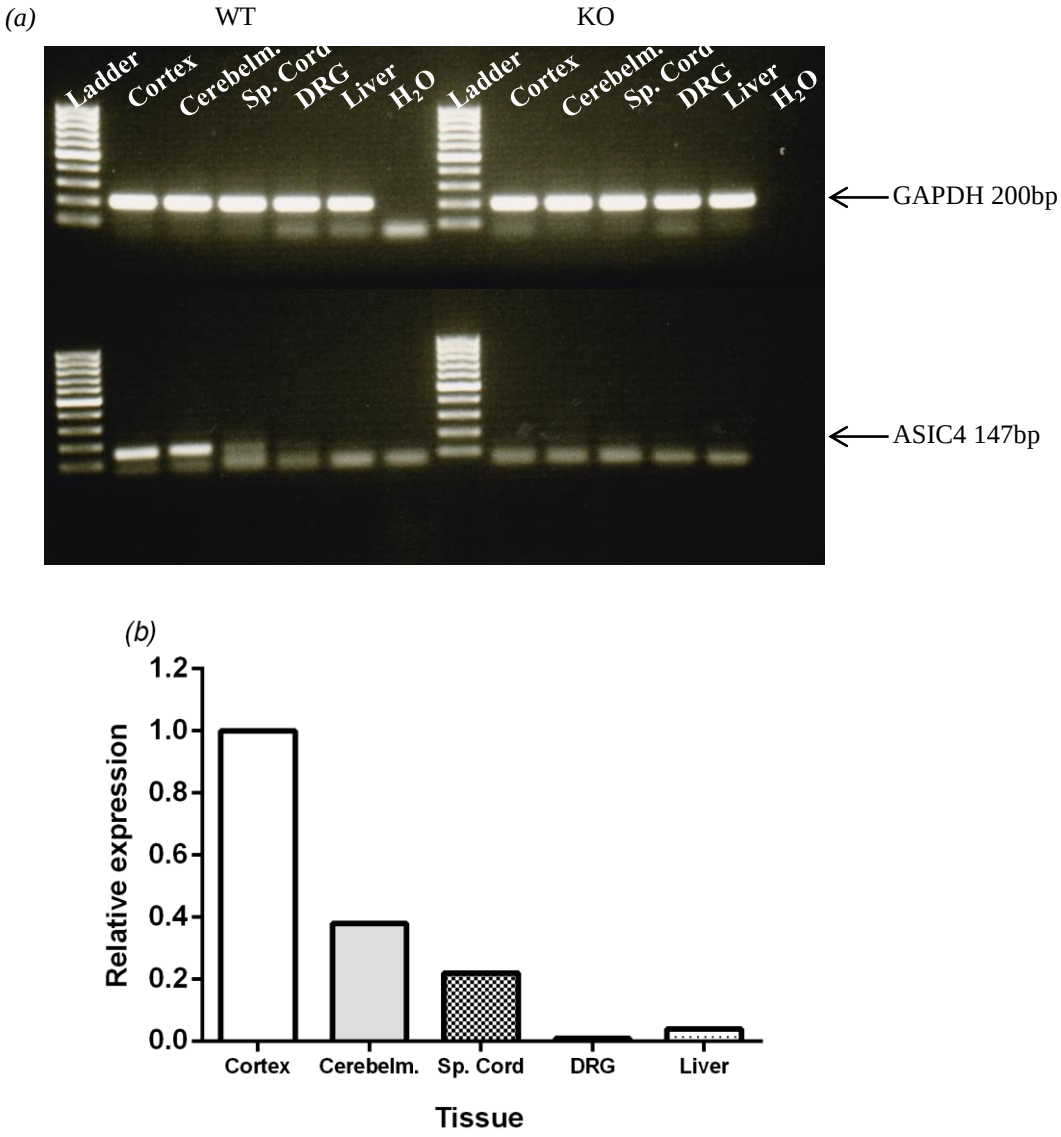



Figure 7.4 ASIC4 transcript expression in cortex, cerebellum, spinal cord, DRG and liver. (a) RT-PCR bands show GAPDH expression in all tissue from WT and KO mice; ASIC4 expression was only observed in cortex, cerebellum and spinal cord of WT mice but not in any tissue taken from KO mice. (b) qRT-PCR expression shown relative to GAPDH expression using the $2^{-\Delta\Delta Ct}$ method confirms expression of ASIC4 in cortex, cerebellum and spinal cord with only negligible expression in DRG and the negative tissue control, liver.

### *7.3.3 ASIC protein expression*

*This work was performed by Queensta Millet.*

A Western blot was performed to quantify the expression of ASIC1 in the brain, spinal cord and DRG of ASIC4 KO mice, compared to WT controls, as well as to investigate alteration of ASIC4 protein expression in these tissues. *In vitro* data suggests that ASIC4 alters levels of total protein of other ASIC subunits so a Western blot was necessary to investigate if these changes occur *in vivo* following ASIC4 deletion.

β-actin was used as a loading control for each lane (43kDa) and a peptide control antigen was used in each tissue to confirm the specificity of the ASIC band.

An antibody targeted to ASIC4 (Alomone) showed staining at around 65kDa; the estimated mass of the mouse protein on the Uniprot database is 59.22kDa suggesting the presence of post-translational modifications which increase the size of the protein (Figure 7.5a). Due to the fact that the ASIC4 antibody epitope is tagged to the N-terminus and the region encoding this was not deleted in the KO, a truncated protein may be produced in KO tissue. A peptide mass search estimated the size of the ASIC4 band would be 24.63kDa in KO tissue, however, as the blot shows, the band in KO tissue was the same size as in WT tissue. Moreover, the strong band present in DRG is not in line with the results obtained from RT-PCR which suggest that ASIC4 DRG expression is negligible. Although the use of a control peptide antigen led to loss of the bands, it is likely that the ASIC4 antibody is not specific to this subunit and that the bands are produced through non-specific binding to other ASIC subunits as the epitope tag of this antibody is targeted to the N terminus which is homologous in many ASIC subunits.

An anti-ASIC1 antibody which recognises both ASIC1a and ASIC1b splice variants produced bands in both ASIC4 WT and ASIC4 KO tissue from DRG, spinal cord and brain at 65-70kDa (Figure 7.5b). In the spinal cord and brain a doublet band was produced however it is unlikely that this is representative of the two splice variants as ASIC1b expression is only reported in the DRG though this could be confirmed through RT-PCR. Instead it is possible that one in spinal cord and brain there are some tissue-specific post-translational modifications to the ASIC1a subunit as this antibody has been shown to be specific to ASIC1 (Wemmie et al., 2003). Quantification of band intensity in WT and KO tissue relative to β actin controls showed that no significant change in ASIC1 expression levels occur following loss of ASIC4 (Figure 7.5c).

No ASIC3 antibody was used as the negligible expression of ASIC4 in DRG where ASIC3 is almost exclusively expressed renders it unlikely that the two subunits interact *in-vivo*.

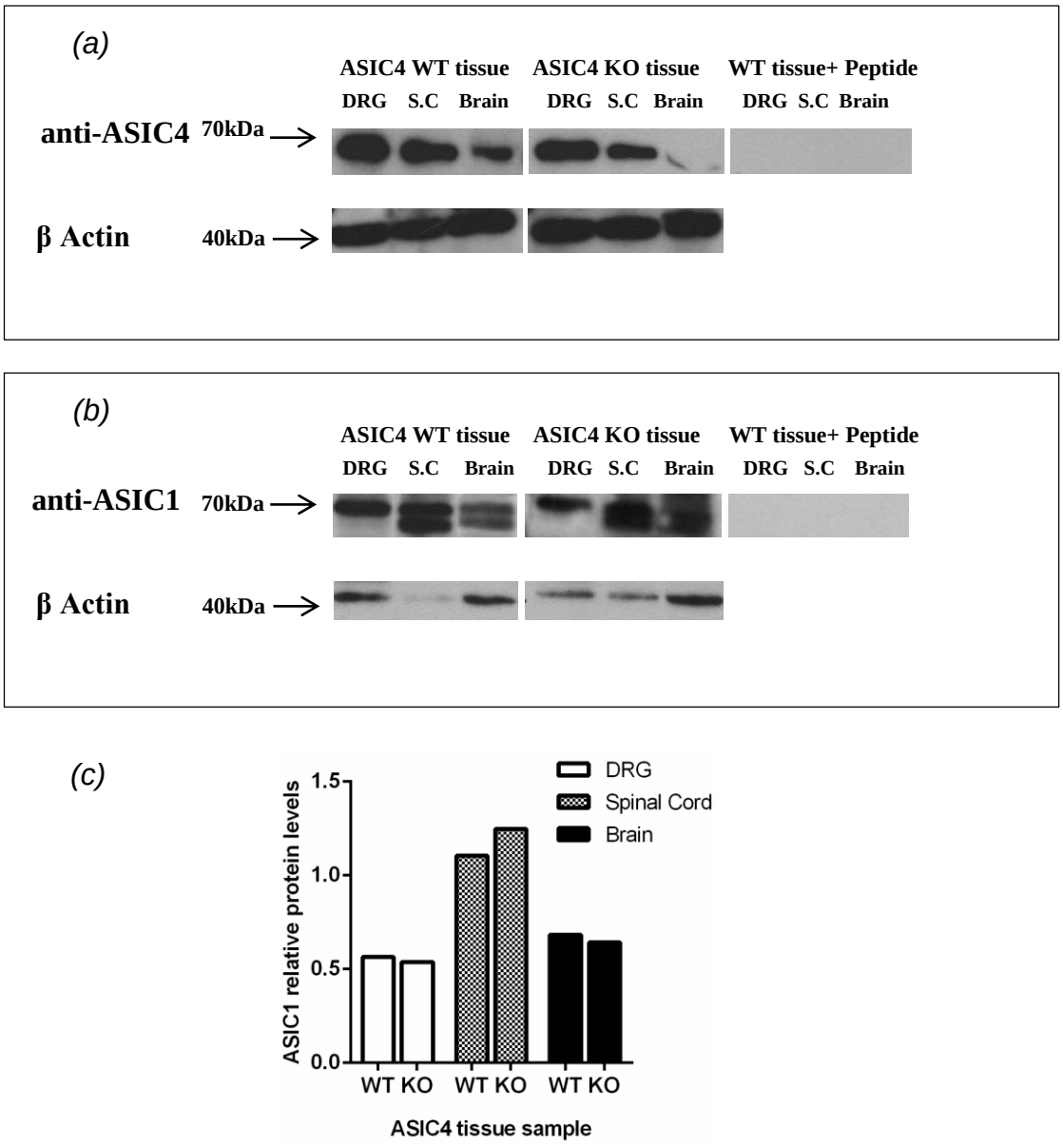



Figure 7.5 Quantification of ASIC4 protein expression (a) ASIC4 protein is present in DRG, spinal cord and brain at 70kDa in WT and KO tissue but is lost in the presence of the peptide control. (b) ASIC1 protein expression in ASIC4 WT and ASIC4 KO tissue was detected in DRG, spinal cord and brain. A loading control, β actin antibody shows expression in all tissues at 43kDa. (c) ASIC1 protein levels relative to βactin in ASIC4 WT and KO tissue from DRG, spinal cord and brain; ASIC1 expression levels are not altered in ASIC4 KO.

### *7.3.4 Behavioural characterisation of ASIC4 knockout mice*

ASICs have been repeatedly shown to play a role in mechanosensory and nociceptive function which has often been demonstrated at the behavioural level (See sections 1.5.3, 1.5.4). We hypothesised that inhibiting the function of ASIC4 may produce an altered pain phenotype as a result of differential expression of other ASIC subunits and used a thorough behavioural characterisation to achieve this.

#### *7.3.4.1 ASIC4 knockout mice develop normally and have normal motor coordination*

ASIC4 heterozygous (Het) mice were produced as described above and bred together to produce WT, KO and heterozygous animals. Out of 92 pups born, 28.3% were WT, 42.3% were Het and 29.3% were KO; close to Mendelian ratios. This normal frequency of KO pups suggests loss of ASIC4 function is not developmentally lethal. Litters were also of expected sizes and mass of the animals was not different between groups (Figure 7.6a).

ASIC4 KO mice show a comparable amount of time to fall from an accelerating rotarod (59.79± 8.53s) compared to ASIC4 WT (63.04± 5.39s) and ASIC4 Het (49.17±5.87) suggesting that these animals have unimpaired motor coordination ($p$>0.05) (Figure 7.6b). As a result, it is reasonable to assume that responses to subsequent sensory tests are not altered as a result of motor impairment.

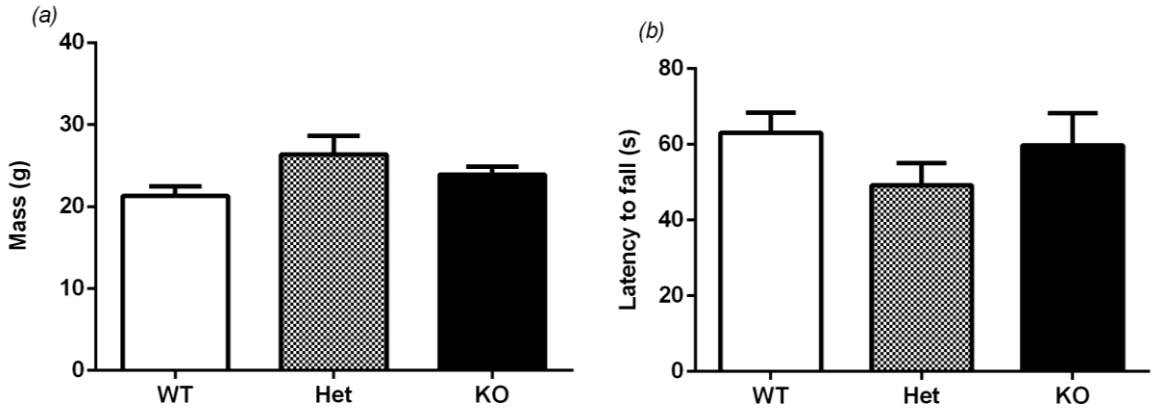



Figure 7.6 ASIC4 KO mice show unimpaired motor coordination compared to WT and Het controls demonstrated by a comparable latency to fall from an accelerating rotarod. (Mass: WT, n=2; Het, n=3; KO, n=3; Rotarod, n=8 per group. Data are shown as Mean±SEM with one-way ANOVA with Tukey post-hoc test).

#### *7.3.4.2 ASIC4 knockout mice show unimpaired responses to mechanical stimuli*

von Frey fibres were used to test the responses of animals to innocuous mechanical stimuli on the glabrous skin of the paw and the hairy skin of the abdomen (as hairy and glabrous skin are known to have differential innervation patterns). On the paw, the 50% withdrawal threshold for ASIC4 WT was 0.63±0.10g, ASIC4 Het were 0.54±0.07g and ASIC4 KO were 0.52±0.06g with no significant differences between groups ($p$>0.05 between all groups) (Figure 7.7a).

The noxious application of a blunt probe to the tail, in the Randall-Selitto test of sensitivity to noxious mechanical stimuli, led to similar responses in all groups. ASIC4 WT, ASIC4 Het and ASIC4 KO, responded at forces 136.3±11.50g, 133.8±8.69g and 140.4±9.89g, respectively ($p$>0.05) (Figure 7.7b). When the same probe was applied to the hind paw ASIC4 WT, ASIC4 Het and ASIC4 KO responded at forces 63.75±4.02g, 84.79±6.82g and 72.50±12.28g, respectively but no significance was found in the different responses of these groups ($p$>0.05) (Figure 7.7c).

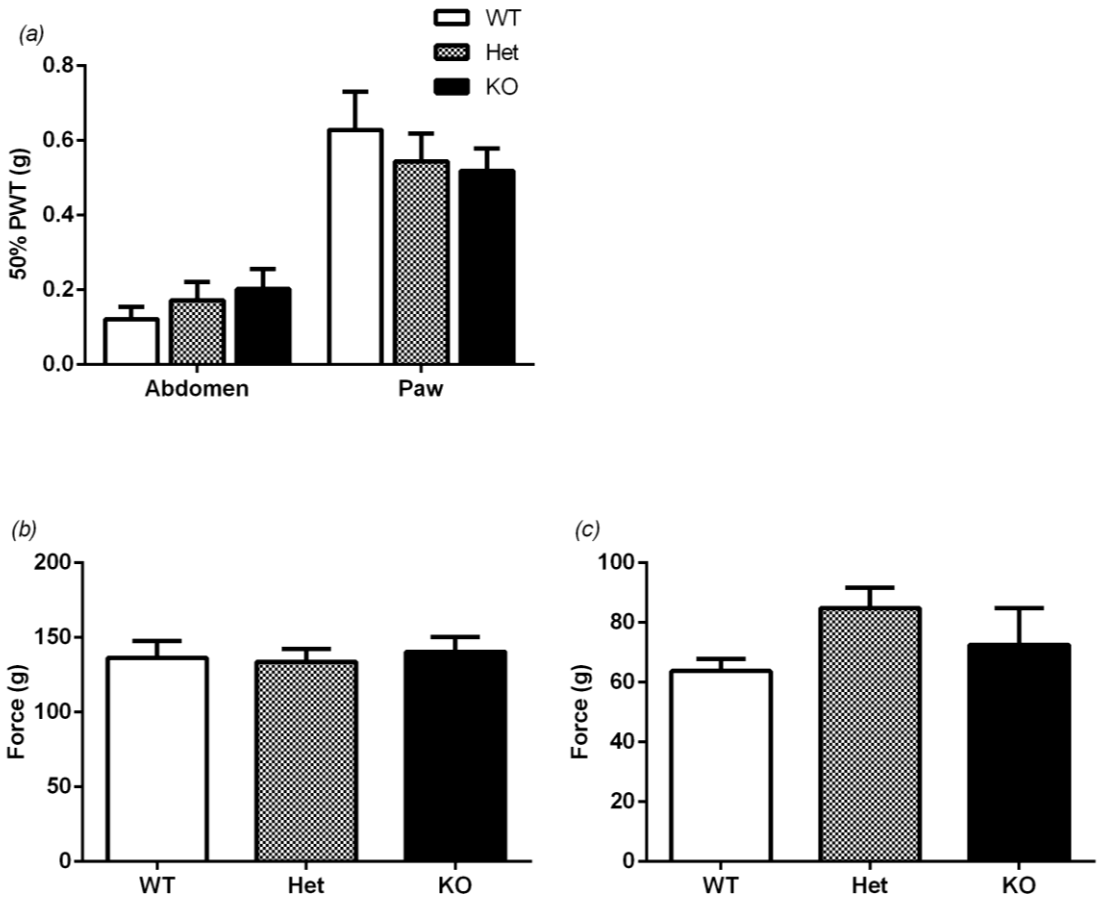



Figure 7.7 Mechanical sensitivity in ASIC4 KO mice is unaltered. (a) responses to von Frey hairs on hairy and glabrous skin (b) noxious mechanical sensitivity of the tail and (c) noxious mechanical sensitivity on the paw are comparable between all groups. (All tests, n=8 per group. Data are shown as Mean±SEM with one-way ANOVA with Tukey post-hoc test).

#### *7.3.4.3 ASIC4 knockout mice have unimpaired responses to thermal stimuli*

The latency to respond to a thermal stimulus in the Hargreaves test were similar in all groups; ASIC4 WT (14.63±1.69s), ASIC4 Het (14.95±2.00s), ASIC4 KO (13.91±2.29s) ($p$>0.05) (Figure 7.8a). In the hot plate test, the latency to respond to temperatures of 50$^{o}$C and 55$^{o}$C were measured and support results from the Hargreaves test which indicate that ASIC4 does not play a role in noxious thermosensation. At 50$^{o}$C, ASIC4 WT responded after 38.71±4.85s, ASIC4 Het after 35.43±3.48s and ASIC4 KO after 33.63±3.24s ($p$>0.05 between all groups). At 55$^{o}$C ASIC4 WT responded after 15.43±1.81s, ASIC4 Het after 14.34±0.95s and ASIC4 KO after 16.36±1.22s ($p$>0.05 between all groups) (Figure 7.8b).

Sensitivity to a noxious cold plate was tested in a place preference set up. ASIC4 WT animals spent 8.85±5.93% time on a 4$^{o}$C test plate in a 2 minute testing period and spent the remainder of the time on a baseline temperature plate (Figure 7.8c). ASIC4 Het spent 8.23±2.56% time and ASIC4 KO spent 17.81±10.2% of the time on the test plate, suggesting no difference between ASIC4 WT and ASIC4 Het ($p$>0.05).

In the acetone test, ASIC4 KO animals show a slight increase in pain behaviour compared to ASIC4 WT and ASIC4 Het groups, though these data are not significant ($p$>0.05) (Figure 7.8d).

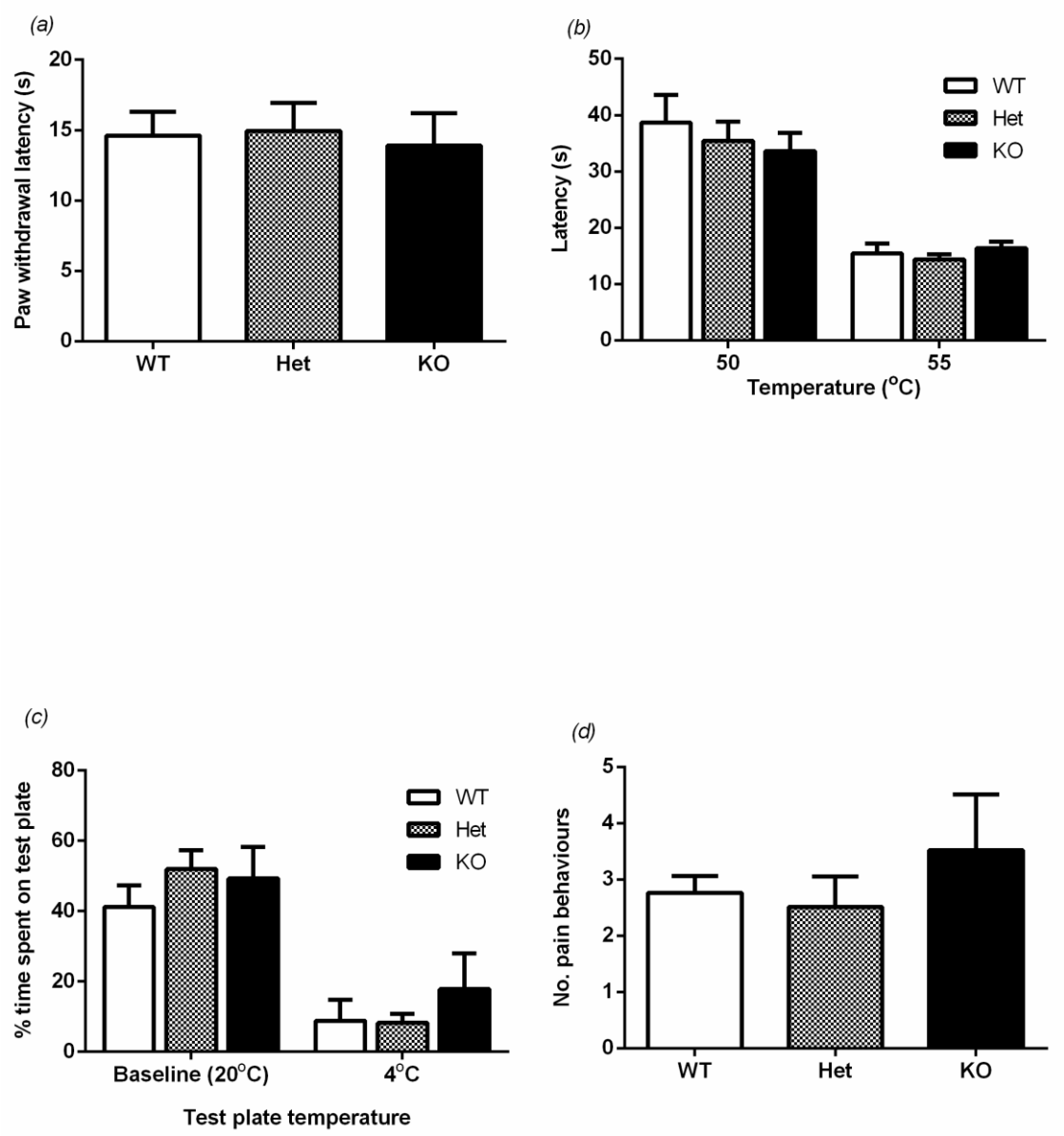
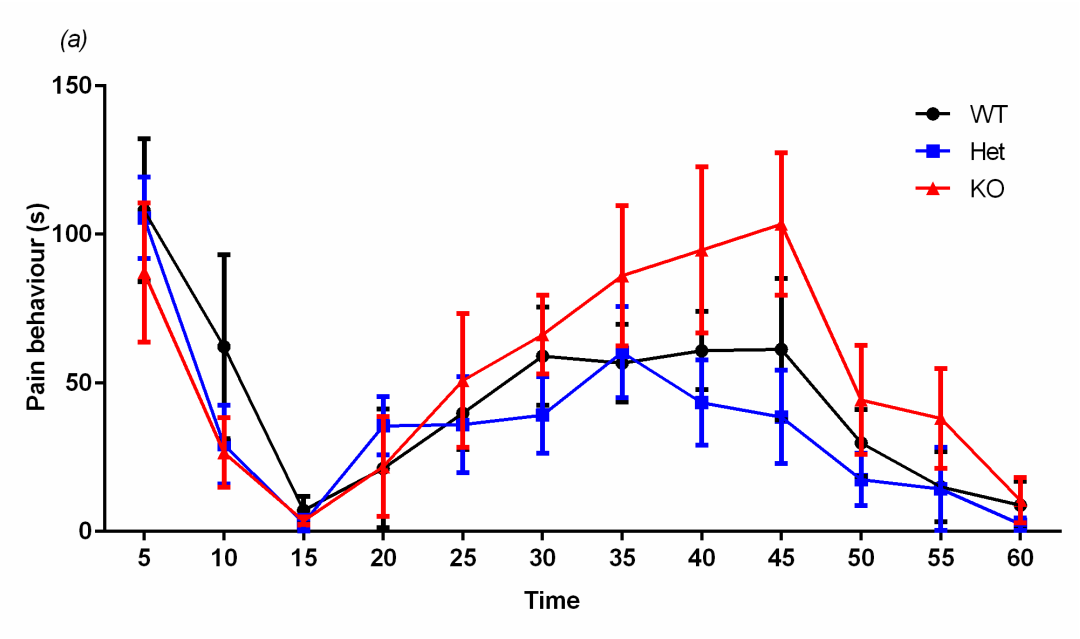



Figure 7.8 ASIC4 has no role in thermosensation. Responses to (a) latent heat in the Hargreaves test, (b) noxious heat in the hot plate test, (c) noxious cold stimuli in the thermal place preference test and (d) cooling following Acetone application show that no change in thermal sensitivity occurs as a result of loss of ASIC4 function. (All tests, n=8 per group. Data are shown as Mean±SEM with one-way ANOVA with Tukey post-hoc test for (a, d) and two-way ANOVA with Tukey post hoc tests for (b,c)).

#### *7.3.4.4 ASIC4 knockout mice show altered responses to certain inflammatory stimuli*

##### 7.3.4.4.1 Formalin Test

The formalin test has two, well defined phases; the former is an acute phase linked to direct activation of nociceptive fibres while the second phase involves a more complex inflammatory response. ASIC4 KO mice show comparable pain behaviour to ASIC4 WT and ASIC4 WT animals in the first, acute phase. However, the ASIC4 knockouts show a significant increase in pain behaviour overall in the second phase of the formalin test. This was significant compared to both ASIC4 WT and ASIC4 Het (ANOVA $p$=0.03; WT v KO $p$=0.047 and Het v KO $p$=0.004) though not significant at any individual time point (Figure 7.9).



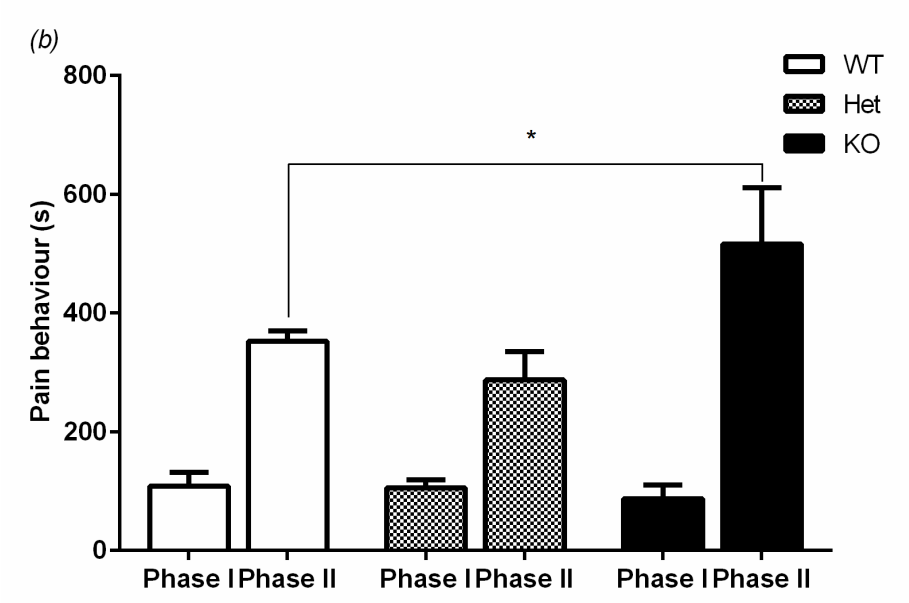




Figure 7.9 Loss of ASIC4 function has no effect on pain behaviour in phase I of the Formalin test though a partial but not significant increase in Phase II pain behaviour is observed. (n=6 per group. Data are shown as Mean±SEM with two-way ANOVA with Bonferroni post-hoc test *$p$<0.05; **$p$<0.01; ***$p$<0.001; ****$p$<0.0001).

#### 7.3.4.4.2 Complete Freund's Adjuvant

As ASIC4 KO mice show increases sensitivity in the inflammatory phase of the Formalin test, we tested sensitivity to another inflammatory agent, CFA. Following CFA injection, WT and Heterozygous mice developed both thermal and mechanical hypersensitivity. The 50% withdrawal threshold dropped from 0.47±0.06g to 0.21±0.17g by 24 hours while the response latency to the thermal stimulus dropped from 12.79±1.25s to 4.20±0.55s. At all time points tested following CFA injection, KO mice show comparable mechanical and thermal hypersensitivity to WT and Het groups ($p$>0.05 between all groups at all time points) (Figure 7.10).

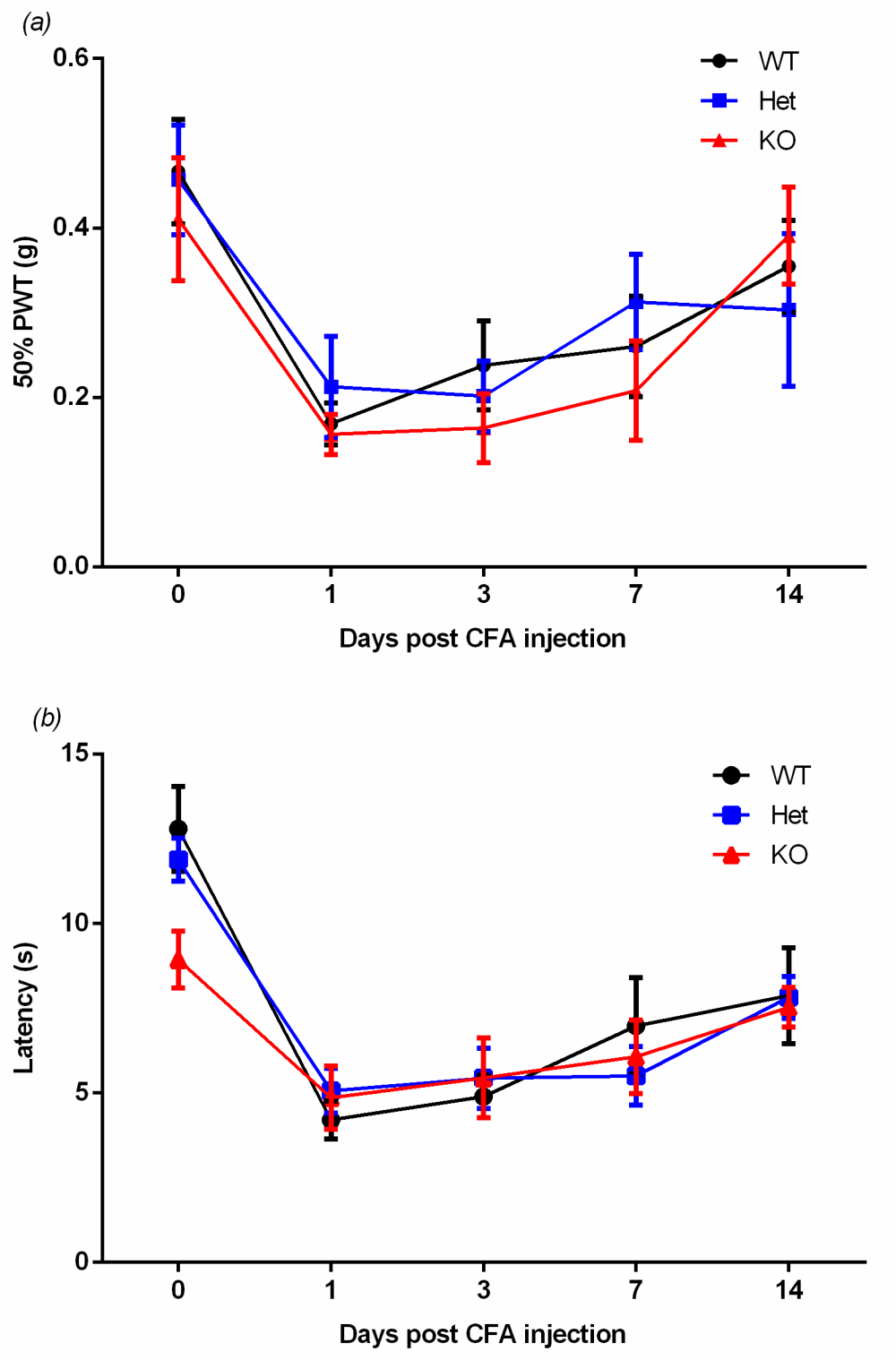



Figure 7.10 Mechanical (a) and thermal (b) hypersensitivity induced by administration of CFA was present in ASIC4 KO mice and not significantly different from any other group at any time point. (n=6 per group. Data are shown as Mean±SEM with two-way ANOVA with Tukey post-hoc test).

#### *7.3.4.5 ASIC4 knockout mice develop acute hypersensitivity in a model of acid induced muscle pain*

ASIC subunits have been implicated in a role in mediating deep tissue pain including musculoskeletal pain. If ASIC4 regulates the expression of these channels, it is possible that loss of function of ASIC4 will affect the normal development of mechanical hypersensitivity in a model of muscle pain. We used the established approach of administration of acidic saline into the gastrocnemius muscle and assessed mechanical hypersensitivity using von Frey testing and a weight bearing asymmetry measure.

Control animals develop ipsilateral mechanical hypersensitivity by 4 hours after the first injection of acidic saline into the gastrocnemius muscle, which was still present at 24 hours but by 3 days was beginning to resolve. KO animals developed hypersensitivity to the same extent and for the same duration as WT controls (Figure 7.11a). Although a slight decrease in withdrawal threshold developed by day 1 on the contralateral paw, this was not significant compared to baseline or between groups.

The mechanical hypersensitivity shown by the change in 50% withdrawal threshold was not reflected by any change in weight bearing on the ipsilateral paw, which was not significantly different from baseline at any time point in control or KO groups ($p>0.05$ between all groups at all time points) (Figure 7.11b).

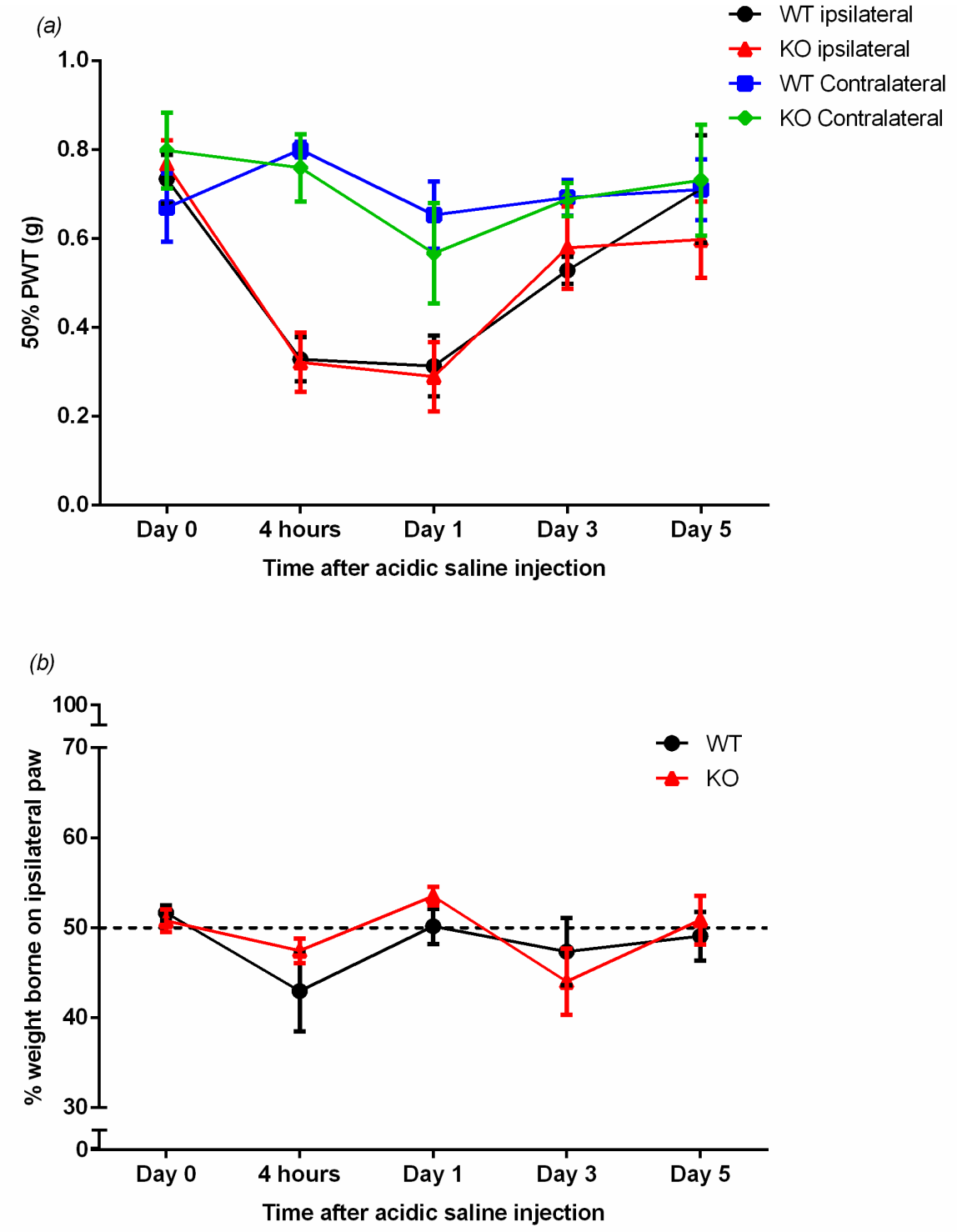



Figure 7.11 ASIC4 does not contribute to mechanical hypersensitivity in a model of muscle pain. (a) ASIC4 KO mice developed hypersensitivity to the same extent as controls which was restricted to the ipsilateral side. (b) No significant weight bearing asymmetry was seen in either groups at any time point. (n=6 per group. Data are shown as Mean±SEM with two-way ANOVA with Tukey post-hoc test).

#### *7.3.4.6 ASIC4 knockout mice show reduced hypersensitivity in a model of visceral pain*

ASIC subunits, ASIC1 and ASIC3, have been shown to have a role in pain and mechanosensation in the viscera; we used an established visceral pain model to determine whether ASIC4 has any role, direct or indirect in these functions.

In the 20 minutes immediately following colonic administration of capsaicin visceral hypersensitivity was monitored through observing behaviours indicating pain. These include writhing, licking the abdomen and pressing the abdomen to the floor. WT mice showed an average of 38±5.81 pain behaviours compared to only 24.8±1.28 shown by KO mice (Figure 7.12a). This decrease was significant ($p$=0.04) demonstrating increased visceral nociception in ASIC4 KO mice.

A 12.5% decrease was observed in the number of responses to 10 stimulations of the abdomen with a 0.16g von Frey filament in KO compared to WT mice, though this was not significant ($p$>0.05) (Figure 7.12b).

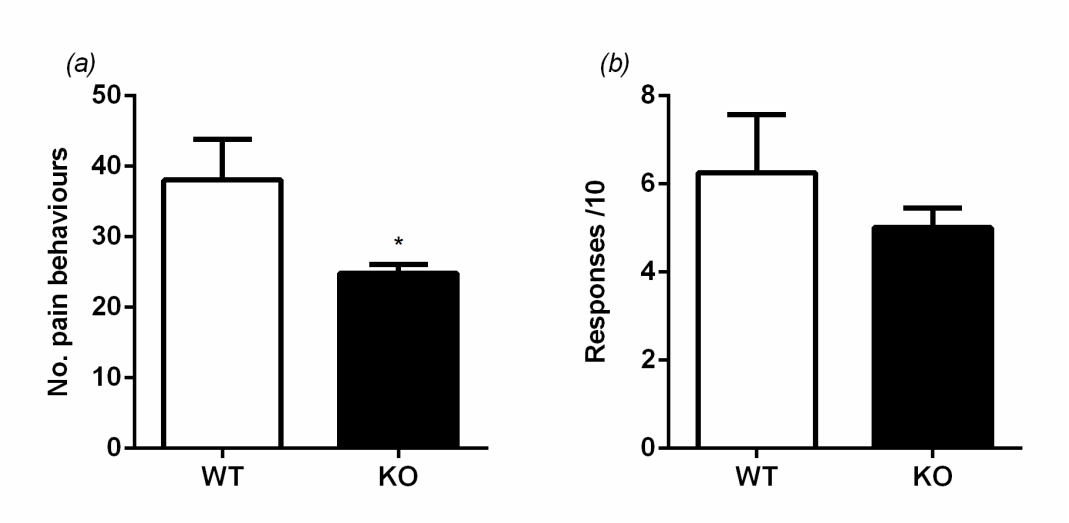



Figure 7.12 Loss of ASIC4 function reduces pain behaviours following colonic administration of capsaicin (a) though no significant change in visceral mechanical hypersensitivity is observed (b). (n=5 per group. Data are shown as Mean±SEM with Student's unpaired t test (a) and Mann-Whitney test (b) *$p$<0.05; **$p$<0.01; ***$p$<0.001; ****$p$<0.0001).

## 7.4 Discussion

ASICs have been widely studied for many years and have been shown to have roles in sensory functions including mechanosensation and pain. ASIC4, by comparison, is less well understood. The work of Donier et al. (2008) highlighted a potential modulatory role for this protein *in vitro*; the creation of an ASIC4 knockout mouse has allowed us to investigate whether this role is reflected *in vivo* and to better understand the contribution of this protein to sensory function.

### *7.4.1 ASIC4 is expressed in the central nervous system*

In Chinese hamster ovary (CHO) cells, ASIC4 down regulates current density and total protein levels of ASIC1a and ASIC3 however its role *in vivo* has not been fully explored. If such a role is reflected in the nervous system, the loss of ASIC4 function may lead to increased levels of ASIC1 and ASIC3 in the cell which may, in turn, have pro-nociceptive effects. We began this study by confirming that a global knockout mouse had been successfully been produced through sequencing of genomic DNA products. We found expression of ASIC4 transcript in adult wild-type mouse cortex, cerebellum and spinal cord though we saw negligible expression in DRG. Other studies have produced conflicting results regarding ASIC4 expression in DRG. The first reports of ASIC4 by Akopian et al. (2000) found low levels of protein and Grunder et al. (2000) found no ASIC4 transcript in DRG; where both studies were performed in rat. Alvarez de la Rosa et al. (2002) reported ASIC4 protein expression in adult rat DRG, whereas an RT-PCR performed by Du et al. (2014) showed no expression in mouse DRG. A database produced by single cell RNA sequencing identified low levels of ASIC4 transcript in mouse DRG (Usoskin et al., 2015) and though Lin et al. (2015a) report expression in DRG during development, they did not observe it in adult tissue. It is possible that if ASIC4 is only expressed at low levels in DRG, as reported by Akopian et al. (2000) using *in situ* hybridisation, other techniques are not sensitive enough to identify its expression. For example, Lin et al. used a tdTomato reporter protein with an inducible Cre under the control of the ASIC4 promoter however a number of studies have demonstrated that the efficiency of this system can be dose and tissue dependent, including in peripheral neurons and DRG (Hayashi and McMahon, 2002, Leone et al., 2003, Zhao et al., 2006).If the induction of Cre activity was not fully penetrant in the case of the ASIC4 CreERT2, it is possible that in the small number of cells expressing ASIC4 in DRG there was no recombinase activity or it was insufficient to produce visible expression of tdTomato.

Similarly, it is possible that the RT PCR used in the present study was not sensitive enough to detect low levels of ASIC4 in the DRG which were able to be detected through the single cell RNA sequencing performed by Usoskin et al. (2015). Alternatively, it is possible that there is a different expression pattern of ASIC4 in rat and mouse nervous tissue. This is the case with ASIC3 in rodents and humans for example, where rodent expression is predominantly seen in DRG and human expression is higher in the brain and spinal cord (Delaunay et al., 2012). Determining the expression pattern of ASIC4 is important because if it is not coexpressed with ASIC1 and ASIC3 then it is unlikely to be modulating their function; as ASIC3 is almost exclusively expressed in the periphery, findings in this chapter suggest that it is unlikely to be modulated by ASIC4 *in vivo* due to the likely negligible expression of ASIC4 in DRG.

We performed a Western blot to determine whether loss of ASIC4 function alters levels of total ASIC1 protein. No significant difference was observed between levels of ASIC1 protein expression in ASIC4 WT and ASIC4 KO tissue, which suggests that the *in vitro* role of ASIC4 is not reflected *in vivo*. However, it may be the case that the total ASIC1 protein level is not altered in the cell; rather, there may only be a change in the levels of ASIC1 expression at the membrane if its ubiquitination state is altered as this could regulate the normal functional activity and trafficking of the protein. Performing the same Western blot using a membrane preparation could confirm this, but overall the current data do not allow an accurate conclusion to be drawn as to whether ASIC4 alters ASIC1 protein expression *in vivo*. Importantly, Donier et al. (2008) reported the function of ASIC4 based on its expression in CHO cells. Though this is a mammalian cell line it is not neuronal; it is possible that the function of ASIC4 is context dependent and that this was not represented in this exogenous expression study. As such, it is likely that further features of ASIC4 function will be elucidated by *in vivo* studies.

### *7.4.2 ASIC4 knockout mice show altered responses to inflammatory stimuli*

The major aim of this study was to determine the role of ASIC4 in sensory function *in vivo* with a particular focus on mechanosensation and nociception, both of which involve the function of other ASIC subunits. For example, ASIC1 and ASIC3 have roles in cutaneous inflammatory pain, visceral pain and mechanosensation as well as chronic muscle pain. If ASIC4 does contribute to these processes it could do so through modulation of other ASIC subunits as happens *in vitro*, or as a direct result of ASIC4 activity.

ASIC4 global knockout mice were viable, developmentally normal and showed unaltered motor coordination in the rotarod test despite expression of ASIC4 in the cerebellum (Lin et al., 2015a). Although ASICs have been implicated in mechanosensation, our mechanical sensitivity tests did not identify any acute changes in mechanosensitivity in ASIC4 KO animals. Similarly, thermal sensitivity was unimpaired in KO mice. In a model of cutaneous inflammatory pain, produced by administration of CFA, mechanical and thermal hypersensitivity developed to the same extent and for the same duration as in WT mice. Staniland and McMahon (2009) found no role for ASIC1 in thermal or mechanical inflammatory hyperalgesia which was substantiated by the ineffectiveness of ASIC1 block with PcTx1, even though it has been shown that CFA induced thermal hyperalgesia can be prevented by administration of APETx2, an ASIC3 blocker (Deval et al., 2008). Our findings here indicate that both thermal and mechanical inflammatory hyperalgesia develop independently of functional ASIC4.

ASIC4 KO mice showed a slight increase in the duration of pain behaviours in the second phase of the formalin test (Figure 7.9). While the first phase of the test is associated with direct activation of afferent neurons, the second phase is believed to represent an inflammatory response and increased activity in neurons in the dorsal horn of the spinal cord (Abram and Yaksh, 1994, Ji et al., 1999). If indeed peripheral expression of ASIC1 and/or ASIC3 is altered by loss of ASIC4 function, the partially augmented pain behaviour we observed in the second phase of the formalin test may be due to this negative regulation of ASIC expression by ASIC4 and consequent heightened sensitivity of peripheral neuron to inflammatory mediators. Moreover, at the spinal cord level, upregulation of ASIC1a could also account for the hypersensitivity observed in the second phase of the formalin test. Duan et al. (2007) and Baron et al. (2008) showed that ASIC1a is a major contributor to ASIC currents in the dorsal horn and levels of ASIC1a transcript are increased in spinal cord following inflammation (Wu et al., 2004). ASIC1a antagonists such as PcTx1 have been shown to reduce inflammatory hyperalgesia (Dube et al., 2005, Duan et al., 2007) and Mazzuca et al. (2007) showed that intrathecal block of ASIC1a produces analgesia through activation of endogenous enkephalin pathways. These data indicate ASIC1 has a pro-nociceptive role in inflammatory pain, which supports the possibility that ASIC4 KO mice have an upregulation of ASIC1 that underlies the increased pain behaviour we observed. In contrast to this, Staniland and McMahon (2009) report that ASIC1a KO mice show increased pain behaviour in the

formalin test, as do mice where all ASIC currents are abolished (Mogil et al., 2005) which would suggest that the phenotype observed in the ASIC4 KO is not dependent on changes which occur in ASIC1 expression.

A model of visceral inflammation involving colonic administration of capsaicin produces spontaneous pain behaviours and hypersensitivity to von Frey hairs applied to the abdomen. Previously, it has been shown that ASIC1 KO mice show increased activity in visceral mechanoreceptors (Page et al., 2004). The current data shows ASIC4 KO leads to reduction in both pain behaviours and mechanical hypersensitivity. This is consistent with a role for ASIC4 in upregulation of ASIC1 protein levels as the opposite phenotype was observed to data published using ASIC1 KO mice.

The increased sensitivity of ASIC4 knockout mice in the formalin test along with the decreased sensitivity in the model of visceral inflammation and the normal phenotype in the CFA model of cutaneous inflammation are difficult to reconcile given that all are based on types of inflammatory pain. In the formalin test ASIC4 KO mice show an increase in pain behaviour whereas following administration of CFA no hypersensitivity was observed. It is possible that no hypersensitivity was observed in the latter test as a result of limitations of the behavioural assessment approaches used or a ceiling effect of the dose of CFA; using a milder inflammatory stimulus may produce a similar outcome of increased hypersensitivity in the formalin test. Alternatively, it is also possible that these inflammatory models are regulated by different mechanisms which do not both involve ASICs, as other groups have observed similar discrepancies in ASIC contribution in different models of inflammatory pain (Mogil et al., 2005, Staniland and McMahon, 2009). It is important to mention that structures such as the viscera and skeletal muscles are differentially innervated than cutaneous structures and it has been shown that the relative contribution of ASICs to nociceptive signalling depends on the neurons they are expressed in (e.g. (Page et al., 2004)). This highlights the importance of investigating the effect of ASIC4 KO in multiple models of pain. Nonetheless, it is a recurring issue in studies using transgenic approaches to determine ASIC function that ASICs have been shown to be both pro- and anti-nociceptive, thus complicating conclusive findings on ASIC function in normal and pathophysiological pain.

Intramuscular injection of acidic saline is a widely used model of muscle pain, where repeated injections of saline produce chronic hypersensitivity (Sluka et al., 2001). ASICs

are expressed in afferents innervating skeletal muscle, in particular ASIC3, and have been shown to play a role in mediating the development of hypersensitivity and, critical to this study, the development of spinal sensitisation following acid injection which induces a localised increase in protons. We used a single injection of acidic saline to model acute muscle pain which produced significant mechanical hyperalgesia on the ipsilateral paw of WT mice. Unlike some reports from Sluka and colleagues (Sluka et al., 2001), the current data show that no contralateral hypersensitivity developed in WT mice. Similarly, ASIC4 KO mice developed ipsilateral but not contralateral hypersensitivity to the same extent as WT controls and neither group showed a significant weight bearing asymmetry suggesting ASIC4 has no direct role in development or maintenance of secondary muscle hyperalgesia. Sluka et al. (2003) observed that although ASIC3 was necessary for mechanical hypersensitivity, ASIC1a was not. Mogil et al. (2005) reported that inhibiting ASIC currents led to hypersensitivity to intramuscular acidic saline which contradicts the findings of Sluka et al, though this was attributed to a strain difference in the mice. Sluka et al. used mice with a C57BL/6 background which is consistent with our ASIC4 KO mice. As such, though the current data rule out a direct role for ASIC4 in acid-evoked muscle pain, it is still possible that ASIC4 regulates ASIC1, and since ASIC1 is not involved in muscle pain no phenotype was observed in this model.

While these data are insufficient to conclude whether ASIC4 regulates other ASICs *in vivo*, they suggest that such a function is possible with regard to ASIC1 and unlikely for ASIC3 due to the negligible DRG expression of ASIC4. This is consistent with the findings of Lin et al. (2015a) who performed assessment of their own, different ASIC4 KO mouse. Moreover, they highlight that ASIC4 function influences hypersensitivity in some cutaneous and visceral inflammatory pain conditions.

### *7.4.3 Understanding the function of ASIC4*

A distinctive feature of ASIC4 is its insensitivity to protons. ASIC2b is also insensitive to protons. However, by heteromerising with other, pH sensitive channels ASIC2b is able to modulate their gating properties, thereby having an indirect effect on proton sensitivity (Lingueglia et al., 1997). However, if ASIC4 is mutated in ways which cause constitutive activation of ASIC2b it remains insensitive to protons (Grunder et al., 2000). Also, ASIC2b is able to contribute to proton sensitive heteromeric complexes though it is unlikely that ASIC4 does the same as a pull-down assay did not identify other ASIC subunits as binding partners for ASIC4 (Donier et al., 2008). It has been suggested that

the proton sensitivity of ASICs may not determine their primary function as the rapidity and degree of pH drop required to activate them is rarely seen in physiological conditions. Examples of non-proton ligands have been described for a number of ASIC subunits (see section 1.5.4) which alter their proton sensitivity and can cause direct activation of ASICs. Specific residues in ASIC3 have been shown to be the site of a ligand binding domain which is distinct from the proton binding site (Yu et al., 2010). Understanding the contribution of other ligands to ASIC function is crucial for both understanding the contribution of their proton sensitivity in physiological conditions and potentially elucidating other functions such as in mechanosensation where lipid activators may be influential. It is possible that ASIC4 is also bound by non-proton ligands; it is particularly highly expressed in the pituitary gland where many peptides are secreted. A snail ENaC orthologue of ASICs is only activated by a neuropeptide and when first cloning ASIC4 and discovering it was proton insensitive, Grunder et al. (2000) suggested this may be true for ASIC4. The presence of a large extracellular domain in all ASIC subunits has also fostered the idea of ligands other than protons as it seems unlikely such a structure exists merely to sense protons (Wemmie et al., 2006). It is possible that through its extracellular domain, ASIC4 binds other non-proton ligands or that it interacts with extracellular matrix, as is the case with ASIC orthologues in *C.Elegans* (Gu et al., 1996), in a way which influences its function.

ASIC4 was determined as a member of the acid sensing ion channel family because of its amino acid sequence, however it bears some crucial differences to other ASICs in its structure. This includes more N-linked glycosylation sites in the large extracellular domain of the protein. N-linked glycosylation of ASIC1a has been shown to regulate efficiency of trafficking to the cell membrane which in turn affects current density (Jing et al., 2011) while mutating these residues in ASIC2a reduces proton affinity and current amplitude, demonstrating the influence of N-linked glycosylation on channel function (Saugstad et al., 2004). Two N-glycans present in ASIC1b but not ASIC1a are necessary for its expression and function; ASIC4 is the only other ASIC subunit to contain these two N-linked glycosylation sites (Delaunay et al., 2012). It has been proposed that these may contribute to protein folding and stabilisation and this same group also report that all eight glycosylation sites in ASIC4 are likely to bind glycans suggesting a drastically different extracellular domain is present in ASIC4 compared to other ASIC subunits. In addition to this, a pull down assay used to identify proteins which interact with ASIC4

found that the protein interacted with substantially fewer proteins than other ASIC subunits (Donier et al., 2008). Its N-terminus has five lysine residues which have the potential for ubiquitination and this has already been shown to alter its expression and function *in vitro* as well as providing a possible mechanism by which presence of ASIC4 reduces expression of other ASIC subunits. As such, pursuing a better understanding of the structurally unique features of ASIC4 are likely to provide critical insight into its function.

The current data suggest that ASIC4 is unlikely to have a major role in acute nociception though it appears to be important in inflammatory pain. Thus far, it does not provide us with a mechanism by which inhibiting ASIC4 function alters noxious sensation. Speculating that its functions *in vitro* translate to ASIC4 function *in vivo*, it is reasonable to assume that modulating the functional expression of other ASICs is one way in which these changes could be occurring. However, it remains possible that ASIC4 itself is directly activated by non-proton ligands which have shown to be able to activate other ASICs. As a result of its expression in the pituitary gland, Lin et al. investigated the effect of loss of ASIC4 function on body weight and composition and activity in the cage but found no changes in KO mice (Lin et al., 2015a). However, peptide release from the hypothalamic-pituitary axis has been linked to pain sensation. For example, corticotrophin-releasing factor (CRF) attenuates bone cancer induced mechanical allodynia (Fan et al., 2015). If ASIC4 is sensitive to neuropeptides produced by the pituitary gland it is possible that its function in the spinal cord is also mediated by their presence. Obtaining a complete understanding of ASIC4 function in pain will require investigations into its role in areas where it most highly expressed, including the pituitary gland and in specific cell subtypes in the cortex, subcortical structures and cerebellum and importantly these supraspinal structures that are involved in central nociceptive processing and top-down modulation of spinal cord excitability.

### *7.4.4 Future work*

The work produced in this study has begun to demonstrate roles for ASIC4 function in the nervous system. Corroborated by the findings of Lin et al. (2015a), it suggests that ASIC4 function is not involved in acute nociception though it influences cutaneous and visceral inflammatory pain. At the behavioural level, it appears that ASIC4 contributes to spinally mediated hypersensitivity in the inflammatory phase of the formalin model. In order to determine the mechanism underlying this phenotype, it would be interesting to

perform spinal cord recordings to see if there is hyperactivity of spinal neurons after inflammatory insults.

In order to determine if there is a role for ASIC4 in mediating ASIC1a expression in the cell membrane, more work needs to be done. Performing a Western blot focussing on protein expression in the cell membrane may provide more insight than the whole cell protein lysate study performed so far.

It may also be interesting to determine whether impairing the function of ASIC4 alters expression of other genes in areas where it is highly expressed through use of a microarray or RNA sequencing. If the primary function of ASIC4 is modulatory of other ASICs, expression arrays would be useful; however, as its modulation of ASIC1 *in vitro* was post-translational it may be more helpful to investigate further the functional links to its protein binding partners identified by (Donier et al. (2008)).

# 8 Summary

The work in this thesis used transgenic techniques to explore the mechanisms responsible for pain and mechanosensation in physiological and pathological conditions which remain incompletely understood.

Characteristic features of TRP channels include their high structural homology and their propensity to form heteromeric complexes which suggests potential functional redundancy. Using a novel, multiple knockout approach we examined both touch and hearing in behavioural and electrophysiological assays, and provide evidence that TRPC1, TRPC3, TRPC5 and TRPC6 multiple knockout mice have deficits in innocuous mechanosensation as well as in hearing and vestibular function. In some cases, these deficits were larger than the deficits found in double KO mice which have previously been shown to be critical for normal touch sensation, suggesting a combinatorial role for these channels in light touch and hearing. Importantly, mechano-electrical transducer currents of cochlear outer hair cells were normal. This suggests that TRPC1, TRPC3, TRPC5 and TRPC6 channels contribute to cutaneous and auditory mechanosensation in a combinatorial manner, but have no direct role in cochlear mechanotransduction.

Pain in Osteoarthritis represents a huge, unmet clinical need as existing analgesics are often ineffective or produce undesirable side effects with chronic use. For reasons stated above, we used multiple knockout approaches to examine the combined roles of putative mechanosensory TRP channels in mechanical hypersensitivity in OA. Using the MIA model of OA we, surprisingly, found no role for TRPC3, TRPC6 or TRPV1 in mechanical hypersensitivity. However our data suggest that TRPA1 is necessary for the complete manifestation of mechanical hypersensitivity in OA. Until now, the MIA model has been widely used in rats where pharmacological compounds have been tested for their analgesic efficacy. This multiple knockout approach therefore provides a novel insight into the role of key pain mediators in OA as well as taking into account the potential influence of heteromerisation or functional redundancy which other monogenic studies may have been unable to do.

We have firmly established a role for Annexin A6 in negative regulation of mechanosensation. This was demonstrated using knockout animals which show increased behavioural responses to noxious mechanical stimuli. Annexin A6 reduces IA current responses in sensory neurons and attenuates the current produced by the primary

mechanotransducer, Piezo2 in heterologous expression systems. Critically, we demonstrate that virally mediated overexpression of Annexin A6 is a viable gene therapy approach for reducing mechanical hypersensitivity in OA. These findings demonstrate a previously unidentified regulatory mechanism of cutaneous mechanosensation and provide an exciting insight into a potential, clinically relevant approach to treating pain in OA.

Finally, we identified a role for ASIC4 in formalin induced pain and potentially in visceral pain and mechanical hypersensitivity. This is the first report of such a role for this poorly understood protein and suggests that further investigation may elucidate more about the contribution of acid sensing ion channels to nociceptive function.

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# 10 Appendices

## 10.1 Appendix A: Publication

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Research paper

# The contribution of TRPC1, TRPC3, TRPC5 and TRPC6 to touch and hearing

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## HIGHLIGHTS

- The contribution of multiple TRPC channels on mechanosensory function is demonstrated.
- We observe a critical role for TRPC channels in touch sensation.
- TRPC channels contribute to cutaneous and auditory mechanosensation in a combinatorial manner, but have no direct role in cochlear mechanotransduction.

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## ABSTRACT

Transient receptor potential channels have diverse roles in mechanosensation. Evidence is accumulating that members of the canonical subfamily of TRP channels (TRPC) are involved in touch and hearing. Characteristic features of TRP channels include their high structural homology and their propensity to form heteromeric complexes which suggests potential functional redundancy. We previously showed that TRPC3 and TRPC6 double knockout animals have deficits in light touch and hearing whilst single knockouts were apparently normal. We have extended these studies to analyse deficits in global quadruple TRPC1, 3, 5 and 6 null mutant mice. We examined both touch and hearing in behavioural and electrophysiological assays, and provide evidence that the quadruple knockout mice have larger deficits than the TRPC3 TRPC6 double knockouts. Mechano-electrical transducer currents of cochlear outer hair cells were however normal. This suggests that TRPC1, TRPC3, TRPC5 and TRPC6 channels contribute to cutaneous and auditory mechanosensation in a combinatorial manner, but have no direct role in cochlear mechanotransduction.



### 1. Introduction

The mechanisms underlying mechanotransduction in mammals are incompletely understood. Piezo2 has been shown to be essential for light touch sensitivity, in mechanical allodynia in neuropathic conditions and produces a mechanically activated, rapidly adapting current [5,8,24,33]. Transient receptor potential (TRP) channels are a superfamily of structurally homologous cation channels which have diverse roles in sensory functions. We have previously discussed the extensive evidence implicating TRP channels in mechanosensory roles in many different species, including TRPA1 which has an important role in cutaneous mammalian mechanosensation [2,18,21,32].

We also reported previously, a combinatorial role for TRPC3 and TRPC6 in mediating normal touch and hearing [23]. The canonical subfamily of TRP (TRPC) channels have known roles in mechanosensory function in mammalian systems including the cardiovascular system [7] and the kidneys [16] and there is an increasing pool of evidence implicating members of the TRPC subfamily in cutaneous mechanosensory functions. In the DRG, TRPC1, TRPC3 and TRPC6 are the most abundantly expressed TRPC subunits

*Abbreviations:* DKO, double knockout mice; QuadKO, quadruple knockout mice.

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and their expression has been observed in most sensory neurons in adult mice [10,23]. In addition, TRPC5 has been found to be localised to small and medium diameter sensory neurons [34]. A single cell RNA sequencing study also determined a non-peptidergic subset of neurons which express all four TRPC subunits [30] meaning there is substantial potential for interaction between different combinations of these TRPC subunits. TRPC1 and TRPC6 are coexpressed with TRPV4 in dorsal root ganglia (DRG) and it has been proposed that they may act in concert to mediate mechanical hypersensitivity in neuropathic and inflammatory pain states [1]. TRPC1 null animals show a decrease in sensitivity to innocuous mechanical stimuli and show a reduction in down hair Aδ and slowly adapting Aβ fibre firing in response to innocuous mechanical stimulation [11]. TRPC1 and TRPC5 confer sensitivity to osmotically induced membrane stretch in cultured DRG neurons and HEK293 cells, respectively [13,28]. TRPC6 is also activated by membrane stretch while both TRPC5 and TRPC6 activity is blocked by a tarantula toxin known to inhibit mechanosensitive channels [27]. In addition, TRPC channels are ubiquitously expressed in the inner ear in structures including the organ of Corti and the spiral and vestibular ganglia [29] suggesting that, in addition to TRPC3 and TRPC6, there is potential for other TRPC subunits to play a mechanosensory role in hearing.

In the current study we extended our analysis of TRPC channels and their role in mechanosensation. TRP channels are known to function in heteromeric complexes and are believed to show functional redundancy. In order to minimise the effects of compensation mechanisms which these qualities confer, we progressed from investigating sensory function in TRPC3 and TRPC6 double knockout animals (both knockout, or DKO, animals) to looking at animals with global knockouts of TRPC1, TRPC3, TRPC5 and TRPC6 channels (quadruple knockout, or QuadKO, animals). We previously provided evidence that TRPC3 channels contribute to mechanotransduction in some cell lines, but not others, consistent with some role for TRPC channels in mechanotransduction [23]. Here we provide further evidence of a combinatorial role for TRP channels in mechanosensation.

## 2. Results

### 2.1. TRPC1, TRPC3, TRPC5 and TRPC6 knockout animals have selective deficits to light touch stimuli but normal responses to thermal stimuli

We found that QuadKO animals showed deficits in light touch sensitivity compared to WT animals, shown by an increase from 0.39 g to 0.69 g in the 50% withdrawal threshold to von Frey hairs (WT v. DKO $p = 0.003$; WT v. Quad KO $p = 0.003$; DKO v Quad KO $p = 0.99$; Fig. 1a) and a 41% decrease in the percentage response to a dynamic cotton swab application to the paw (WT v. DKO $p = 0.20$; WT v. Quad KO $p = 0.0006$; DKO v Quad KO $p = 0.07$; Fig. 1b). Interestingly, QuadKO animals did not show any difference in 50% withdrawal threshold compared to DKO animals but showed a decrease in the response to cotton swab stimulation compared to DKO, though this was not significant ($p = 0.07$).

Responses to high force mechanical stimuli, on the tail, were unimpaired in all groups (WT v. DKO $p = 0.54$; WT v. Quad KO $p = 0.95$; DKO v Quad KO $p = 0.27$; Fig. 1c).

Unimpaired responses to noxious heat stimuli in knockout animals (WT v. DKO $p = 0.97$; WT v. Quad KO $p = 0.85$; DKO v Quad KO $p = 0.95$; Fig. 1d, e) suggest that these TRPC channels are unlikely to be involved in transduction of noxious heat. We used a place preference paradigm to study QuadKO sensitivity to noxious cold temperatures. A baseline recording showed all groups spent ~50% of the test session on the test plate; when the temperature was lowered to 4 °C, this dropped to ~5% of the test session indicating all groups were aversive to the noxious cold temperature (WT v. DKO $p = 0.83$; WT v. Quad KO $p = 0.99$; DKO v Quad KO $p = 0.81$; Fig. 1f).

### 2.2. TRPC1, TRPC3, TRPC5 and TRPC6 knockout animals have impaired auditory and vestibular function

Using the trunk curl test [14], we found that QuadKO animals show some vestibular deficits which are comparable to deficits in DKO animals (WT v. DKO $p = 0.03$; WT v. Quad KO $p = 0.03$; DKO v Quad KO $p = 0.99$; Fig. 2a) but that TRPC multiple KO animals show latencies to fall from an accelerating rotarod that are comparable to those observed for WT and DKO mice (WT v. DKO $p = 0.96$; WT v. Quad KO $p = 0.72$; DKO v Quad KO $p = 0.87$; Fig. 2b) suggesting unimpaired motor coordination. As we reported previously, the role for the rotarod test in assessing vestibular function has been disputed as other studies have found that it does not always correlate with vestibular deficits presented by other relevant tests [19,23]. Also, the trunk curl test is a rudimentary measure of vestibular function therefore more in depth tests would likely provide more information about the nature of these deficits [14].

Auditory brainstem response recordings (ABRs) were used to assess the auditory function of these animals where auditory pip tone stimuli are used to determine the threshold in decibels which is required to elicit a response at different frequencies. We found at frequencies of 8, 24, 32 and 40 kHz that QuadKO animals had a significantly higher response threshold than both WT and DKO animals (8 kHz, WT v. DKO $p = 0.57$; WT v. Quad KO $p = 0.004$; DKO v Quad KO $p = 0.03$; 12 kHz, WT v. DKO $p = 0.34$; WT v. Quad KO $p = 0.43$; DKO v Quad KO $p = 0.99$; 24 kHz, WT v. DKO $p = 0.99$; WT v. Quad KO $p = 0.004$; DKO v Quad KO $p = 0.002$; 32 kHz, WT v. DKO $p = 0.05$; WT v. Quad KO $p = 0.0001$; DKO v Quad KO $p = 0.0001$; 40 kHz, WT v. DKO $p = 0.002$; WT v. Quad KO $p = 0.0001$; DKO v Quad KO $p = 0.0006$; Fig. 2c).

### 2.3. TRPC1, TRPC3, TRPC5 and TRPC6 knockout animals have unimpaired mechano-electrical transducer currents in the hair cells of the inner ear

Mechano-electrical transducer (MET) currents evoked by sinusoidal force stimuli in both the basal (associated with responses to high frequency stimuli) and apical (associated with responses to low frequency stimuli) coil of the cochlea, show normal amplitudes in outer hair cells (OHCs) of QuadKO animals, of comparable size to currents recorded from OHCs of matching WT control mice [12,17] (Fig. 3a–d). MET currents of the QuadKO OHCs were similar in all respects to those of the WT control OHCs: currents reversed near zero mV, a fraction of the MET channels were open at rest and this fraction increased for depolarized membrane potentials due to a reduction in $Ca^{2+}$-dependent adaptation [4].These observations suggest that the process of mechanotransduction in the cochlea is unaltered in knockout animals. Earlier data [23], suggested that MET currents in basal-coil OHCs of TRPC3/TRPC6 DKO OHCs were on average substantially smaller than those of WT controls. Further experiments using the same methods for MET current recording showed that it is possible to record large MET currents from TRPC3/TRPC6 DKO OHCs in the basal coil (Fig. 3e). The current–voltage curves (Fig. 3f) were similar between the five groups of OHCs being compared. For example, MET current size of OHCs at −104 mV (mean ± SEM) was: WT control apical coil: −983 ± 47 pA, $n = 5$; WT control basal coil: −1185 ± 121 pA, $n = 4$; QuadKO apical coil: −993 ± 120 pA, $n = 3$; QuadKO basal coil: −997 ± 37 pA, $n = 2$; DKO basal coil: −1091 ± 187 pA, $n = 3$. There were no significant differences ($p > 0.05$; ANOVA with Tukey post-test) between any of these groups. This negates our earlier finding of on-average smaller currents in basal-coil DKO OHCs. The pre-

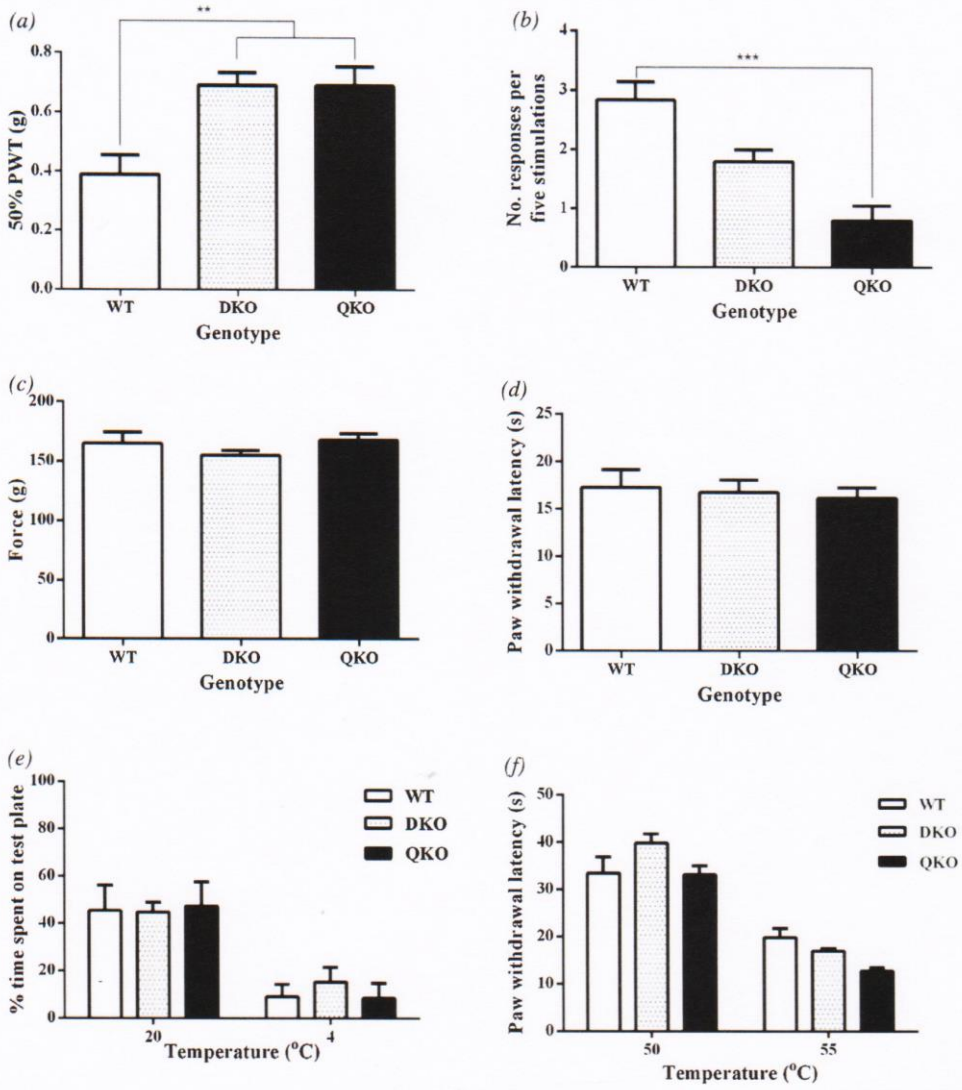


**Fig. 1.** Modality specific sensory deficits in multiple KO animals. (a) DKO (0.69 g ± 0.04 g) ($n = 10$) and QuadKO (0.69 g ± 0.06 g) ($n = 10$) show an increase in 50% withdrawal threshold compared to WT (0.39 g ± 0.06 g) ($n = 10$) but no difference is seen between the two test groups. (b) QuadKO (0.8 ± 0.25) ($n = 10$) show a stepwise decrease in the percentage responses to a dynamic cotton swab stimulus compared to WT (2.83 ± 0.31) ($n = 6$) and DKO (1.8 ± 0.2) ($n = 10$). (c) No difference was observed in sensitivity to noxious mechanical force between groups (WT $n = 6$, DKO $n = 10$, QuadKO $n = 10$). (d) No difference was observed in sensitivity to noxious heat between groups ($n = 6$ all groups). (e) No difference was observed in sensitivity to a hot plate between groups ($n = 6$ all groups). (f) No difference was observed between groups of the time spent on a noxious cold plate (4 C) following an acclimatisation session (20 C) ($n = 6$ all groups). Data are shown as Mean ± SEM with one-way ANOVA with Tukey post test (a,c,d); Kruskal Wallis Test with Dunn's post test (b) and two-way ANOVA with Tukey post test (e,f) $^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; $^{****}p < 0.0001$.

viously observed diminished inward currents in basal-coil DKO OHCs may be explained by sub-optimal organotypic cultures. The present results do not support a role for TRPC channels in primary mechanotransduction in the inner ear.

## 3. Discussion

TRPC1, TRPC3, TRPC5 and TRPC6 are all expressed in sensory ganglia [10,31] and TRPC3 and TRPC6 have been shown to be expressed in cochlear hair cells [23]. We previously reported that TRPC3 and TRPC6 DKO mice show selective deficits in sensitivity to innocuous mechanical stimuli and in hearing and vestibular function. TRPC3 and TRPC6 single KO animals, on the other hand, showed unimpaired responses to all sensory stimuli. Therefore it seems that TRPC channels may have a combinatorial role in mediating specific sensory functions. As TRP channels are known to heteromultimerise and are believed to show functional redundancy, the development of TRPC1, TRPC3, TRPC5 and TRPC6 QuadKO animals, generated on a mixed C57BL/6J:129SvEv background at the Comparative Medicine Branch of the NIEHS in North Carolina, by combinatorial breeding of single KO alleles (TRPC1:[6], TRPC3:[15], TRPC5:[22], TRPC6: [7]), has provided us with a novel way of investigating the combined roles of the TRPC channels where monogenic studies may have been unsatisfactory. Using this

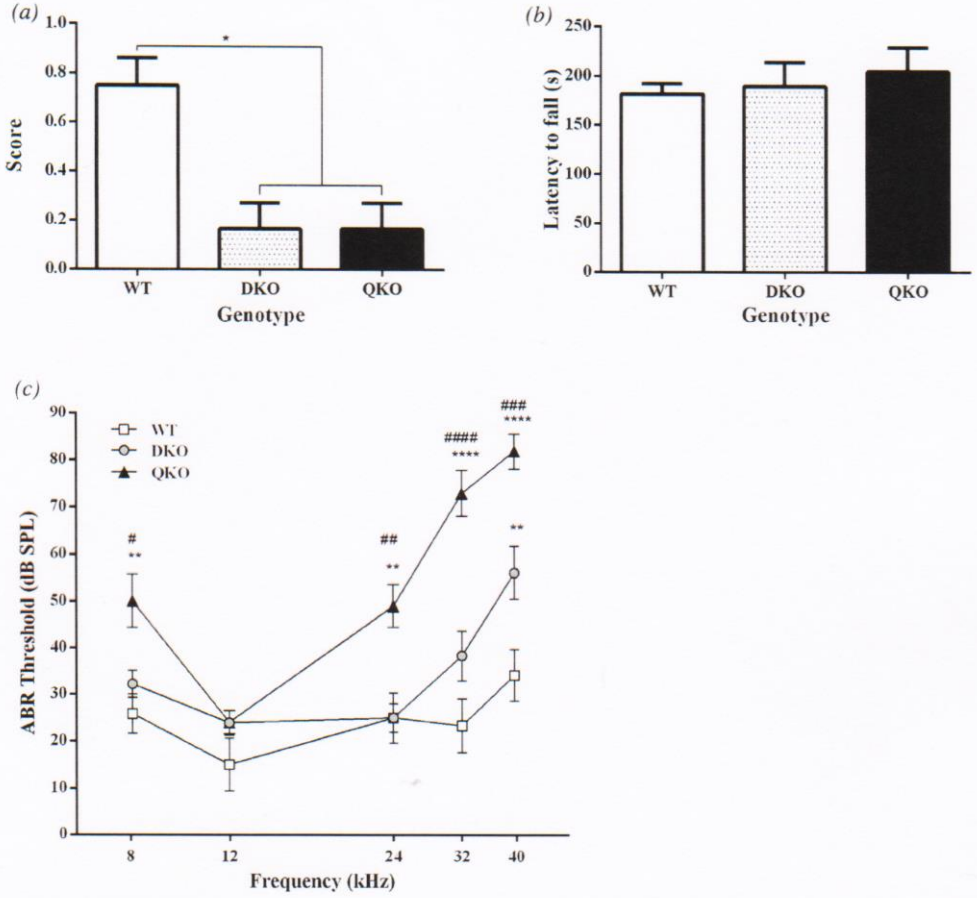


**Fig. 2.** Impaired vestibular function seen in (a) trunk curl response ($n = 6$ all groups) but not in (b) rotarod test ($n = 6$ all groups) (c) Higher threshold responses to auditory piptone stimuli in Auditory Brainstem Response recordings in QuadKO ($n = 5$) compared to DKO ($n = 9$) and WT ($n = 5$) groups. Data are shown as Mean ± SEM with Kruskal–Wallis test with Dunn's post test (a), one-way ANOVA with Tukey post test (b) and two-way ANOVA with Tukey post test (c); $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; $^{****}p < 0.0001$. (*denotes significance compared to WT; # denotes significance compared to DKO).

approach, we have been able to show that knocking out TRPC1 and TRPC5 in addition to TRPC3 and TRPC6 augments specific sensory deficits.

Sensitivity to light touch sensitivity is impaired in QuadKO mice. We found, however, that the impairment was only augmented compared to DKO animals in the cotton swab test while the von Frey withdrawal threshold remained comparable. The cotton swab stimulus is an unequivocally light touch stimulus which is dynamic and thus has different qualities to stimulation with punctate von Frey fibres. Garrison, et al. [11] have previously found that in TRPC1 knockout animals the withdrawal threshold was unaltered but that the responses to subthreshold cotton swab stimuli were impaired. They suggest that this is indicative of a role for TRPC1 involvement in subthreshold mechanical responses which may also be reflected in our multiple KO animals.

Responses to noxious mechanical stimuli were normal in these animals; this is consistent with other data showing TRPC channels do not appear to play a role in mediating noxious mechanosensation [1,11,23]. This also highlights a modality specific role for TRPC channels in mediating sensitivity to innocuous, and not noxious, mechanical stimuli. Similarly responses to noxious heat and noxious cold stimuli were unimpaired in QuadKO animals. Although it has been suggested that cold-evoked currents can be produced following heterologous expression of TRPC5, Zimmermann, et al. [34] found behavioural responses in TRPC5 null mice were unaltered. This may be indicative of TRPC5 functioning cooperatively with other TRP channels which are linked to a role in cold sensitivity.

Cochlear hair cells are arranged in a frequency gradient along the basilar membrane in the organ of Corti. They project stereocilia which are deflected by shearing movements between the tectorial and basilar membranes in the organ of Corti in the inner ear, leading to opening of mechanosensitive channels. A similar mechanism of mechanotransduction is found in the vestibular system. Previously, we reported that TRPC3 and TRPC6 were, together, important for normal hearing and vestibular function. These new data support this suggestion and also implicates TRPC1 and TRPC5 in normal hearing function as ABR thresholds were higher in QuadKO animals than DKO animals. In order to determine whether the observed hearing deficits are the result of altered mechanotransduction in the cochlea, mechano-electrical transduction (MET) currents were recorded from cultured OHCs. Since the recordings taken from QuadKO animals were normal, similar both to matching WT controls and previous recordings from OHCs of CD-1 mice [12,17], we are led to conclude that the loss of TRPC channel function affects the auditory process downstream of the MET channel, though it is possible that function is impaired elsewhere in the cochlea, and

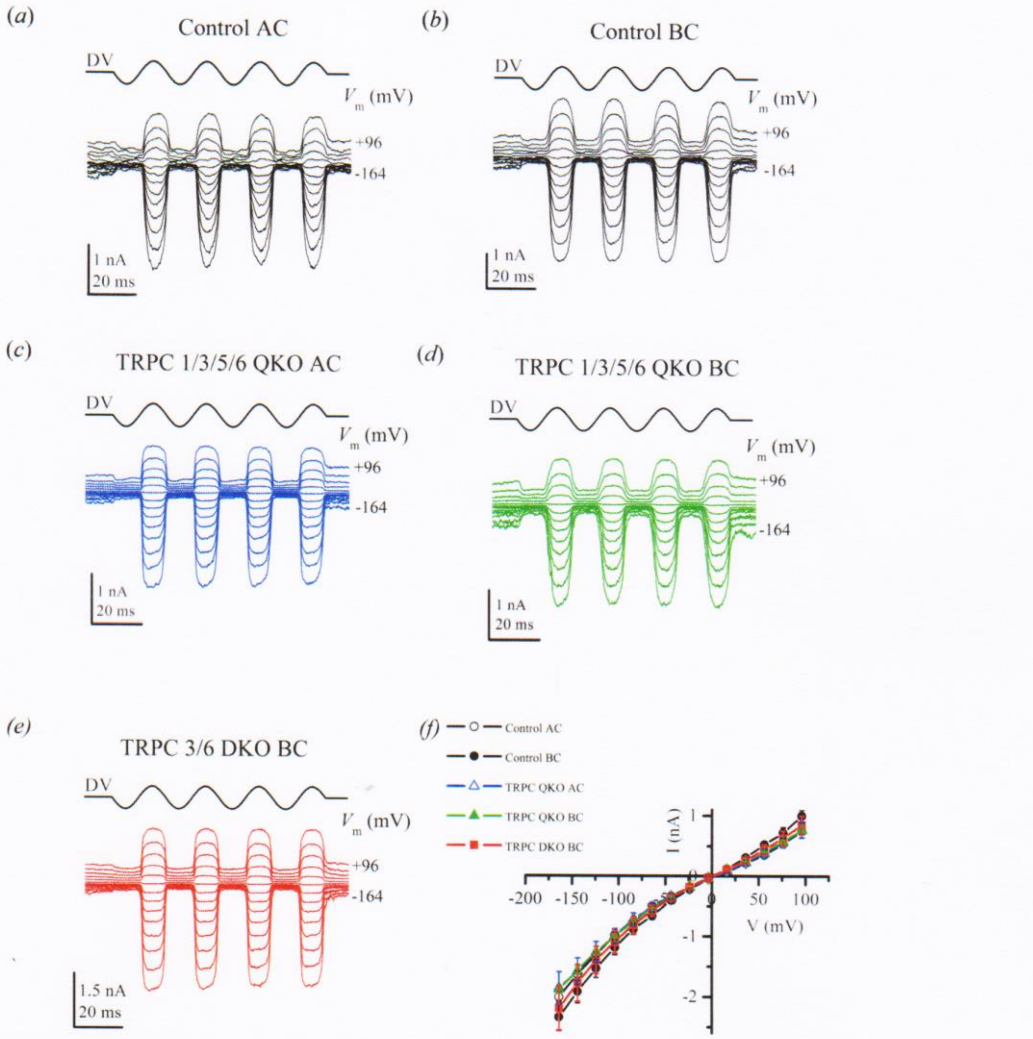


**Fig. 3.** Unimpaired mechanotransduction in OHCs of QuadKO and DKO animals. (a–d) MET currents in response to 45 Hz sinusoidal force stimuli from a fluid jet. Holding potential was −84 mV and the membrane potential was stepped between −164 mV and +96 mV in 20 mV increments. Driver voltage (DV) amplitude was 40 V. Positive DV moves the hair bundles in the excitatory direction towards the kinocilium. (a) WT control OHC, postnatal day 2 + 1 (P2 + 1), mid-apical coil. (b) WT control OHC, P2 + 2 mid-basal coil. (c) QuadKO OHC, P2 + 1, mid-apical coil. (d) QuadKO OHC, P2 + 2, mid-basal coil. (e) DKO OHC, P2 + 1, mid-basal coil. (f) Current-voltage curves averaged from 5 mid-apical WT OHCs (black open circles), 4 mid-basal WT OHCs (black closed circles), 3 mid-apical QuadKO OHCs (blue open triangles); 2 mid-basal QuadKO OHCs (green closed triangles) and 3 mid-basal DKO OHCs (red squares). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

that therefore, TRPC channels do not form part of a mechanotransduction complex in the inner ear.

Our earlier electrophysiological work suggests that the role of TRPC channels in mechanosensation is context dependent [23]. TRP channels are notoriously difficult to study in exogenous expression systems because of their function as heteromeric complexes and their interaction with other TRP proteins. Altogether, our data lead us to conclude that the function of TRPC channels involves combined activity of multiple TRPC proteins, something which has been elucidated as a result of the multiple knockout approach. The current work shows that by impairing the function of a further 2 members of the TRPC subfamily we can augment some of the sensory deficits we reported in DKO animals, reinforcing the concept that TRPC channels play a supporting role in in mediating or coordinating mechanosensation. This supports the view that this interaction within the TRPC subfamily is functionally relevant in mechanosensation as interfering with a single TRPC channel leaves behavioural responses unaltered [23] while QuadKO animals show augmented deficits compared to DKO in specific sensory modalities.

The current study substantiates our earlier conclusions that TRPC channels are critical for cutaneous touch sensation. We can now be confident their role in the auditory system is likely to be indirect, as TRPC channels are clearly not primary mechanotransducers. The expression of mechanosensitive currents in neuronal but not non-neuronal cell lines transfected with TRPC3 [23] is intriguing, and suggests that TRPCs may interact with other proteins to form a mechanotransduction complex. TRPC channels are known to interact with a huge list of other proteins and signalling molecules, many of which have already been implicated in mechanosensory roles, including Orai1 which mediates stretch

sensitivity in cardiomyocytes and phospholipases which are activated by stretch in a number of sensory systems [3,9,25,26]. This serves to highlight the potentially complex roles these channels may be playing in mechanosensation but also provides an interesting route to identifying other constituents of mechanotransduction complexes.

## 4. Methods

Mice were obtained from the Comparative medicine Branch at the NIEHS, Research Triangle Park, North Carolina, USA. TRPC1, TRPC3, TRPC5 and TRPC6 QuadKO animals were generated on a mixed C57BL/6J:129SvEv background by combinatorial breeding of single KO alleles, TRPC1 [6], TRPC3 [15], TRPC5 [22], TRPC6 [7]. Quad KO mice exhibited generally good health and TRPC3/6 DKO mice were crossed with C57BL/6 mice to generate WT control animals (as previously reported [23]). Both were used for comparison to a TRPC1/3/5/6 QuadKO test group, unless otherwise stated, and mice were aged and sex matched. Behavioural tests, ABRs, MET current recordings from OHCs in organotypic cultures made at postnatal day 2 (P2) and maintained in vitro for 1–2 days and statistical analyses were performed as previously reported [20,23].

## Conflict of interest

The authors declare no conflict of interest.

## Ethics

All behavioural tests were approved by the United Kingdom Home Office Animals (Scientific Procedures) Act 1986.

## Contributors

JNW designed experiments. JES and KQ performed animal behaviour and analysis. RT and AF performed ABRs and analysis. TD performed MET recordings and TD and CJK performed analysis. JA and LB generated KO mice. All authors contributed to manuscript preparation.

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