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Sex- and injury-dependent nociceptive C-fibre temporal relay

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Declaration

The work presented here was carried out in the Centre for Discovery Brain Sciences, School of Biomedical Sciences at the University of Edinburgh. I declare this thesis was written by me and the experiments and analysis were conducted by me, except for where highlighted in the text. No part of this thesis has been submitted for any other degree or professional qualification.

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Abstract

Chronic pain is a debilitating condition, affecting significantly more females than males. It poses a significant clinical burden due to the currently limited effective treatments, reflecting limited understating of the mechanism underlying chronic pain states. Chronic pain symptoms include increased sensitivity to noxious heat stimuli (heat hypersensitivity), which is known to involve changes in the function of the pain-sensing C-fibres. It can also cause pain triggered by innocuous stimuli (allodynia), which is thought to involve central plasticity driven by the ongoing C-fibre activity. Given that majority of the studies investigating chronic pain mechanisms have been conducted primarily in males, it is therefore important to study the function of the pain-sensing C-fibres in both sexes. C-fibres display a unique phenomenon termed activity-dependent slowing (ADS) whereby repetitive stimulation results in a progressive slowing of action potential conduction velocity, which manifests as a progressive increase in response latency in both humans and animals. It has been shown to also alter the temporal relay of pain signals to the spinal cord, where pain processing occurs. In addition, C-fibre ADS has been demonstrated to be altered in neuropathic pain states and following tissue inflammation in a sex-dependent manner.

Given the accumulating evidence for sex difference in acute and chronic pain sensitivity along with the recent findings of sex differences in the temporal relay of C-fibre mediated pain signals to the spinal cord in inflammatory pain states, this thesis aimed to study C-fibre ADS in both sexes in normal physiology and under pathological conditions. In addition, it aimed to determine the impact of C-fibre ADS on spinal heat pain processing in both sexes.

C-fibre ADS was investigated, using compound action potential recordings in dorsal roots *ex vivo,* in normal physiology and in the incision-induced model of postoperative pain in juvenile rats of both sexes, with findings of incision-induced enhancement of ADS, which was more pronounced in females. This is proposed to contribute to the less pronounced reduction in peak heat hypersensitivity observed

following incision in females. Given that the voltage-gated sodium (Na_V) channels are implicated in C-fibre ADS, the impact of Na_V channels on ADS in normal physiology and in the pathology of postoperative pain was explored in both sexes. Pharmacological manipulation of the tetrodotoxin-sensitive Na_V channels suggests sex- and incision-dependent changes in Na_V functional expression in different C-fibre subtypes.

It has been previously demonstrated that blood plasma levels of the glycolytic metabolite methylglyoxal (MG) differentiate between diabetic neuropathy patients with or without pain symptoms, with one proposed mechanism of the MG-induced pain involving post-translational modifications of the Na_V channels, which are also implicated in C-fibre ADS. Chronic but not acute application of MG was found to alter C-fibre ADS in a sex-dependent manner in juvenile rats, with females showing enhancement of ADS and males showing a reduction in ADS. These ADS findings can potentially contribute to the observed MG-induced heat hypersensitivity in males only.

ADS has been suggested to provide a 'memory' of previous levels of activity, which could in turn influence responses to subsequent high-frequency stimuli, such that ADS induced by prolonged low-level firing, similar to spontaneous C-fibre firing observed in pain states, could impact subsequent responses to high frequency inputs by altering their ADS profile. This thesis has shown that prolonged low-frequency C-fibre stimulation could alter ADS levels in juvenile rats and adult mice in a sex-dependent manner. Given the fact that sex differences in thermal pain sensitivity have been shown to be strain- and species-dependent, the findings in this thesis of a more pronounced ADS in juvenile female Sprague-Dawley (SD) rats and in adult male *C57BL/6* mice suggest that these may contribute to the previously observed higher heat thresholds in female SD rats and in male *C57BL/6* mice.

Patch clamp recording from noxious heat sensitive superficial dorsal horn neurons in adult mouse spinal cord slices, in both sexes, was used to assess the impact of ADS and dynamic memory on noxious heat processing on an individual spinal

neuron level. In line with the observed more pronounced C-fibre ADS in the male *C57BL/6* mice, there was more ADS in monosynaptic C-fibre inputs to the heat sensitive spinal neurons, which, however, was associated with an overall less pronounced evoked activity in those neurons in males. This finding likely reflects the initial less pronounced evoked activity of the heat sensitive spinal neurons in males, independent of C-fibre ADS. Using the length-dependency of ADS, it was demonstrated that pronounced ADS in monosynaptic C-fibre inputs is associated with less pronounced reduction in the activity of the heat sensitive spinal neurons in both sexes, suggesting C-fibre ADS as a potential mechanism involved in maintaining the activity of the heat sensitive spinal neurons following repetitive simulation. Dynamic memory significantly altered monosynaptic C-fibre input to noxious heat sensitive superficial dorsal horn neurons in both sexes, increasing the number of C-fibre synaptic input failures, which was reflected in the pronounced reduction of the activity of the heat sensitive spinal neurons in both sexes, suggesting that dynamic memory may act as an intrinsic self-inhibitory mechanism to limit the activity of the heat sensitive spinal neurons.

This thesis proposes a role for C-fibre ADS in modulating noxious heat sensitivity in a sex-dependent manner in normal physiology and in different pain pathologies. In addition, this work highlights the potential influence of ADS on spinal circuits involved in noxious heat processing.

Lay Summary

Chronic pain, defined as pain that lasts for more than three months, is a debilitating condition, which affects more females than males. It poses a huge clinical burden, with current treatment strategies having limited effects. For more efficient treatment options, it is important to understand how pain develops in both sexes. The symptoms of chronic pain arise due to changes in the function of nerves that detect and convey pain signals, termed C-fibres, to the spinal cord, where pain gets processed before being sent to the brain. Because the majority of research so far has been conducted primarily in males, it is important to study the function of these pain-sensing C-fibres in both sexes because there could be differences in how pain signals are conveyed and processed between the sexes. The pain-sensing C-fibres have a unique characteristic feature that allows them to slow down pain signals, which effectively could put a 'brake' on pain. This is termed activity-dependent slowing (ADS) and has been shown to be altered in pain states following damage to nerves and following tissue inflammation in a sex-dependent manner. Given the increasing amount of evidence for sex difference in acute and chronic pain along with the recent findings of sex differences in the C-fibre 'pain brake', this thesis aimed to study C-fibre 'pain bake' in both sexes in normal conditions and in different pain states. In addition, it aimed to determine the effect of the C-fibre 'pain brake' on the processing of heat pain signals in the spinal cord in both sexes.

C-fibre ‘pain brake’ was explored in normal conditions and in conditions of pain following surgery in rats of both sexes. It was found that surgery increased C-fibre ‘pain brake’, with a bigger increase seen in females. Thus, this increased ‘pain brake’ is proposed to be involved in the reduced peak sensitivity to heat pain in females compared to males. The C-fibre ‘pain brake’ is thought to be regulated by a group of special molecules located on the C-fibres, called voltage-gated sodium (NaV) channels. Therefore, the role of NaV channels on C-fibre ‘pain brake’ was studied in normal condition and after surgery in both sexes. Findings showed that the function of the NaV channels is altered and these alterations depend on sex and presence of surgery.

Previous studies have identified a product of glucose breakdown, called methylglyoxal (MG), which is present at higher levels in the blood of patients with diabetic pain. It has been shown that MG has an effect on the NaV channels that are also involved in the C-fibre ‘pain brake’ and this is thought to affect the speed at which pain signals are conveyed. This thesis revealed that MG alters C-fibre ‘pain brake’ differently in males and females. Females had increased C-fibre ‘pain brake’, while males showed a reduced ‘pain brake’. These findings are proposed to contribute to the demonstrated increased sensitivity to heat following MG in males but not females.

C-fibre ADS, or the C-fibre ‘pain brake’, has been also though to provide a ‘memory’ of previous activity, which, as this thesis demonstrated, is altered in juvenile rats not adult mice. Given the fact that sex differences in sensitivity to heat pain depend on strain and species, the findings of a more pronounced ‘pain brake’ in juvenile female Sprague-Dawley (SD) rats and a less pronounced ‘pain brake’ in adult female *C57BL/6* mice suggest that these may contribute to the previously observed higher heat tolerance in female SD rats and lower heat tolerance in female *C57BL/6* mice.

The effect of C-fibre ‘pain brake’ and ‘memory’ on the processing of heat pain signals in the spinal cord was also studied. Increased C-fibre ‘pain brake’ was shown to result in less blockade of the respective pain-processing spinal cord neurons in

both sexes. In contrast, C-fibre 'memory' was demonstrated to block pain signals to neurons in the spinal cord responsible for processing of painful heat signals.

This thesis proposes a role for C-fibre 'pain brake' in regulating painful heat sensitivity in a sex-dependent manner in normal and in different pain conditions. In addition, this work highlights the potential sex-dependent influence of the C-fibre 'pain brake' on the processing of painful heat signals in the spinal cord.

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List of Abbreviation

5-HT	5-hydroxytryptamine
ADS	activity-dependent slowing
AGE	advanced glycation end products
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid
AMPAR	α -amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptor
AP	action potential
ASICs	acid-sensing ion channels
ATP	adenosine triphosphate
AUC	area under the curve
BDNF	brain-derived neurotrophic factor
Calb	calbindin
CaMKII	Ca2+/calmodulin-dependent kinase II
CAP	compound action potential
Ca_V	voltage-gated calcium channels
CCI	chronic constriction injury
CCK	cholecystokinin
CFA	complete Freund's adjuvant

CGRP	calcitonin gene related peptide
CGRP1-R	calcitonin gene related peptide 1 receptor
CIPN	chemotherapy-induced painful neuropathy
CM	mechano-sensitive
CMi	mechano-insensitive
CMiHi	mechano and heat insensitive c-fibres
CNS	central nervous system
CR	calretinin
CREB	cyclic-amp response element binding protein
DN	diabetic neuropathy
DRG	dorsal root ganglia
DTX	dendrotoxin
eEPSP	evoked excitatory post-synaptic potential
ENaC	epithelial sodium channel
EphBR	ephrinb receptor
EPSC	excitatory post-synaptic current
ERK	extracellular signal-regulated kinase
eVF	electronic von Frey apparatus
eIPSCs	evoked inhibitory postsynaptic current

GABA	δ-aminobutyric acid
$GABA_AR$	δ-gamma aminobutyric acid A receptor
GAD	glutamic acid decarboxylase
GLO	glyoxalase
Glu	glutamate
GlyR	glycine receptor
GPR	G-protein coupled receptors
GPR	G-protein coupled receptors
GRP	gastrin releasing peptide
HCN	hyperpolarisation-activated cyclic nucleotide-gated cation channels
HTMR	high-threshold mechanoreceptors
i.p	intraperitoneal
IA	intermediate adapting
IASP	international association for the study of pain
IB4	isolectin-B4
IEGs	Immediate early genes
Ih	hyperpolarisation-activated current
IL-1β	interleukin-1β
IL-6	interleukin-6

ir-DIC	infrared-differential interference contrast
IVC	individually ventilated cages
JSTX	synthetic Joro spider toxin
KAR	kainate receptor
KCC2	potassium-chloride co-transporter
KO	knockout
K_V	voltage-gated potassium channels
LTMR	low-threshold mechanoreceptors
LTP	long term potentiation
MAPK	mitogen-activated protein kinase
mEPSCs	miniature excitatory post-synaptic currents
MG	methylglyoxal
MG-Hs	methylglyoxal-derived cyclic hydroimidazones
mGluRs	metabotropic glutamate receptors
MHC-II	major histocompatibility complex class II
mIPSC	miniature inhibitory postsynaptic current
MRGPRB4	mas related g protein-coupled receptor member 4
MRGPRD	mas related g protein-coupled receptor family member d
MRI	magnetic resonance imaging

NACWO	named animal care and welfare officer
NaV	voltage-gated sodium channel
NCX2	Na/Ca exchanger
NGF	nerve growth factor
NIH	National Institute of Health
NK1-R	neurokinin-1 receptor
NK1-R+	neurokinin-1 receptor positive
NLRP3	NACHT, LRR and PYD domains-containing protein 3
NMDA	N-methyl-D-aspartate
NMDARs	N-methyl-D-aspartate receptors
NMDG	N-methyl-D-glucamine
NPY	neuropeptide Y
NVS	named veterinary surgeon
PDN	painful diabetic neuropathy
PI3K	phosphoinositide 3-kinases
PKA	protein kinase A
PKC	protein kinase C
PNS	peripheral nervous system
PSD	post-surgical day

PSNI	peripheral saphenous nerve ligation injury
PSNL	partial sciatic nerve ligation
PTM	posttranslational modifications
RA	rapidly adapting
RAGE	receptor for advanced glycation end products
RNA	ribonucleic acid
RNAi	interference ribonucleic acid
ROS	reactive oxygen species
SA	slowly adapting
SD	sprague–dawley
SNL	spinal nerve ligation
SNT	spinal nerve transection
SOM	somatostatin
SP	substance P
STD	short-term depression
STP	short-term plasticity
STZ	streptozotocin
TG	trigeminal ganglia
TH	tyrosine hydroxylase

TLR4	Toll-like receptor
TNF	tumour necrosis factor
TNFR1	tumour necrosis factor receptor 1
TNF-α	tumor necrosis factor-α
TPRA1	transient receptor potential channel subfamily A, member 1
TrkB	tyrosine receptor kinase B
TRP	the transient receptor potential
TRPM8	TRP subtype melastatin 8
TRPV1	transient receptor potential (TRP) vanilloid 1
TTX	tetrodotoxin
TTX-R	tetrodotoxin-resistant
TTX-S	tetrodotoxin-sensitive
VGLUT2	vesicular glutamate receptor 2
VGLUT3	vesicular glutamate transporter 3
WDR	wide dynamic range
WT	wild type
ZDF	zucker diabetic fatty

Chapter 1

General Introduction

1.1 Chapter Summary

- The somatosensory system provides information about the external and the internal body environment that is essential for normal health and survival.
- The nociceptive system is part of the somatosensory system and allows the detection and processing of noxious stimuli. Different primary afferent fibre subtypes detect, transduce and transmit specific types of sensory information to the dorsal horn of the spinal cord, where the information gets processed before being sent to higher brain centres, which allow the experience of the sensory information.
- C-fibre primary afferents detect and convey primarily noxious stimuli, particularly noxious thermal stimuli. These nociceptive fibres display a special feature, called C-fibre activity-dependent slowing, that has been proposed to be involved in the processing of nociceptive information.
- Acute pain is an adaptive response essential for survival. Chronic pain, however, is maladaptive and considered a disease that significantly affects quality of life and poses a huge clinical burden upon society.
- Chronic pain affects significantly more females than males but due to historic reasons sex has not been considered as a factor in research, partially pain research, until recently. The requirement for inclusion of both males and females in research studies has begun to identify a number of sex differences in important biological mechanism, which, however, are still not fully explored.
- Varied pathological states and mechanisms can give rise to chronic pain. Given the crucial role of C-fibres in detection and transmission painful stimuli, these have been extensively studied in patients and in animals. However, the activity-dependent slowing phenomenon of the C-fibre remains poorly understood in the context of sex-differences in chronic pain.

1.2 Somatosensory Nervous System and Nociception

The somatosensory system is part of the sensory nervous system, a complex network of sensory neurons and neural pathways that respond to external and internal changes at the surface or inside the body (Willis, 2007). It is comprised of interoceptive (proprioceptive and autonomic) and exteroceptive (cutaneous) receptors and their associated afferent nerve fibres. These relay information about the body to the spinal cord, where the information gets processed by dorsal horn neurons before being sent to the brain for further processing (**Figure 1.1**) (Koch et al., 2018). The interoceptive arm of the somatosensory system is composed of proprioceptive and visceral receptors and their associated afferents, which provide proprioceptive information about muscle tension and body position, essential for the sense of self and self-agency (Windhorst, 2007). The exteroceptive arm comprises specialized skin receptors and their afferent sensory neurons, which relay information about the body's interaction with the immediate environment, primarily conveying information about tactile, thermal, pruritic and noxious stimuli to the central nervous system (CNS) (Abraira and Ginty, 2013, Lallemend and Ernfors, 2012, Bautista et al., 2014). The ability of the interoceptive and exteroceptive receptors and their associated afferent nerve fibres to be activated by different stimuli has given their corresponding names, for example proprioceptors, mechanoreceptors, thermoreceptors, chemoreceptors, and nociceptors.

Nociceptors are specialised sensory receptors and their associated peripheral afferent fibres, which detect and become activated by intense thermal, mechanical and chemical stimuli. This neuronal process of encoding and processing noxious stimuli is referred to as nociception (Basbaum and Jessell, 2000). Given the existing ambiguity around the terms 'pain' and 'nociception' in pain research and literature (Fabrega and Tyma, 1976, Bourke, 2012), it is important to note the distinct definitions of these terms, which are not synonymous (Weisman et al., 2019, Basbaum and Jessell, 2000) because each can occur without the other. Nociception may not always result in pain, and it is well recognised now that pain can occur with

and without actual nociceptors activation. This is reflected by the International Association for the Study of Pain (IASP) very definition of pain, which was recently revised as '*an unpleasant sensory and emotional experience associated with, or resembling that associated with, actual or potential tissue damage*' (Raja et al., 2020).

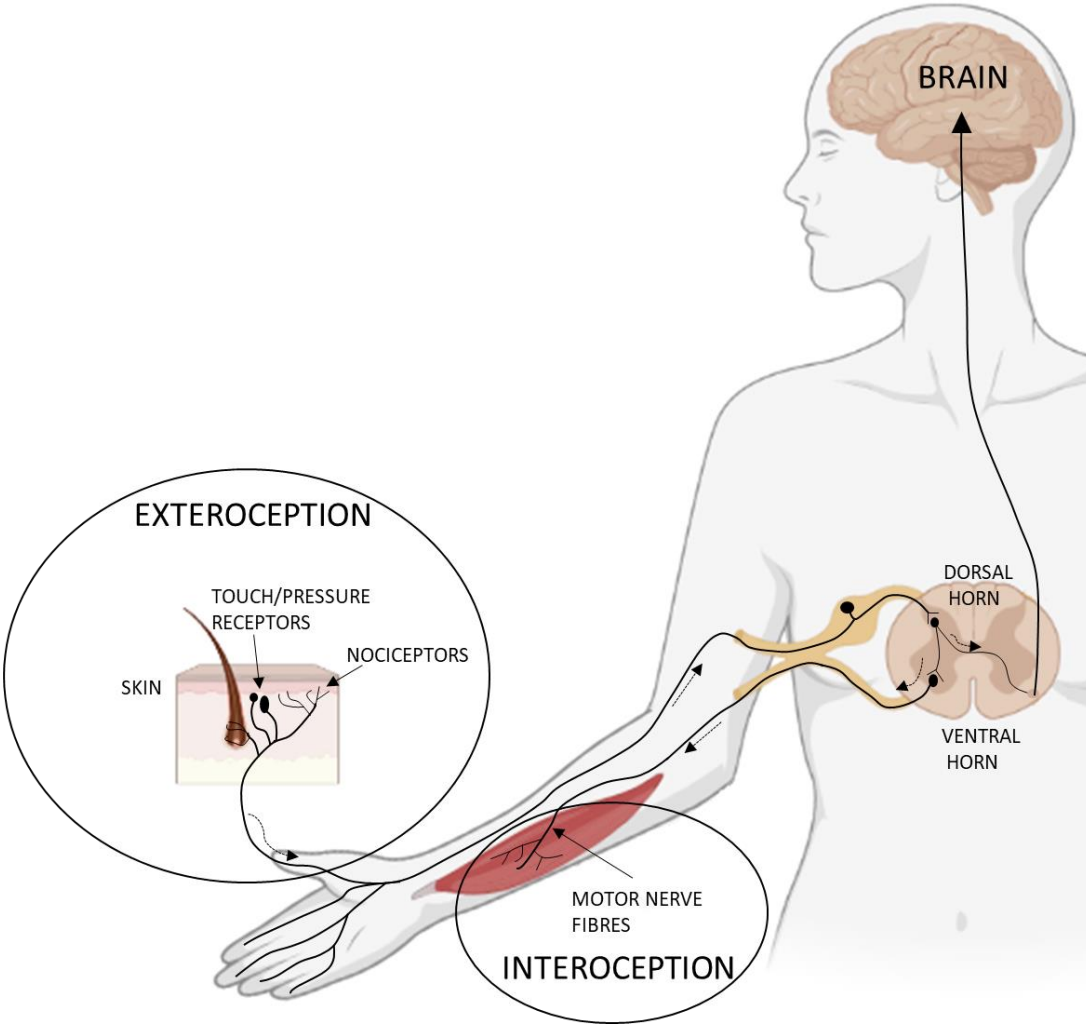


Figure 1.1 Somatosensory system

The exteroceptive and interoceptive arms of the somatosensory system. The interoceptive arm is composed of proprioceptive and visceral receptors and provide information about muscle tension, body position etc. The exteroceptive arm comprises specialised skin receptors and their afferent sensory neurons, which conveying information about tactile, thermal, pruritic and noxious stimuli. Created using Biorender.

1.3 Acute and chronic pain

Pain is an essential biological process that acts as an adaptive sensation to warn and protect the body from tissue injury and could also aid in repair after tissue damage (Koch et al., 2018, Basbaum and Jessell, 2000, Scholz and Woolf, 2002, Woolf and Ma, 2007). Thus, it is important for the survival of organisms in a potentially harmful environment and for their wellbeing (Nagasako et al., 2003). This is dramatically illustrated by rare cases where individuals suffer from congenital abnormalities that make them incapable of detecting noxious stimuli, which could be life threatening (Bennett and Woods, 2014, Nagasako et al., 2003, Drissi et al., 2020, Axelrod and Hilz, 2003). However, pain can also become maladaptive, reflecting a pathological function of the nervous system (Scholz and Woolf, 2002).

Pain can be classified in many forms. An important classification is the one that distinguishes between acute and chronic pain. Acute pain is normally triggered by noxious stimuli and is often considered an adaptive sensation because it can elicit a flexion-withdrawal response (removing tissue from the noxious stimuli), with the associated unpleasant sensation being instrumental in learning to avoid potentially harmful environments (Latremoliere and Woolf, 2009, Scholz and Woolf, 2002). Chronic pain has been defined as pain that persists beyond normal healing time (Bonica, 1953), thus not having the acute warning function of physiological nociception (Treede, 2016). Usually pain that lasts or recurs for more than 3 to 6 months is regarded as chronic (Treede et al., 2015). Chronic pain can be broadly subdivided into inflammatory and neuropathic pain. Inflammatory pain usually arises following injury or inflammation as a result of mechanical, thermal and/or chemical stimulation of the nociceptors. Neuropathic pain refers to pain caused by an insult to the nervous system or by changes in the nervous system involving neural plasticity. It must be noted that this is a relatively simple classification because in many cases chronic pain is not strictly inflammatory or neuropathic as neuropathy may involve inflammatory components and neuropathic components may contribute to inflammatory pain states. Pain research now recognises different pain pathologies, including pain after surgery, cancer-induced pain, diabetes-

induced pain, and pain during degenerative diseases (Treede et al., 2015). All these different types of pain have been suggested to have distinct and specific underlying mechanisms. However, they all exhibit similar characteristic symptoms such as spontaneous pain, hyperalgesia (exaggerated responses to noxious stimuli) and allodynia (normally innocuous stimuli trigger painful sensations) (**Figure 1.2**). Soon after injury, these symptoms could have an adaptive function, resulting in behaviour that will protect the site of injury and aid healing by preventing further harm and or infection. However, if these symptoms persist beyond the normal healing time, their adaptive properties are lost and they become maladaptive, impacting patients' quality of life (Basbaum et al., 2009, Latremoliere and Woolf, 2009, Scholz and Woolf, 2002).

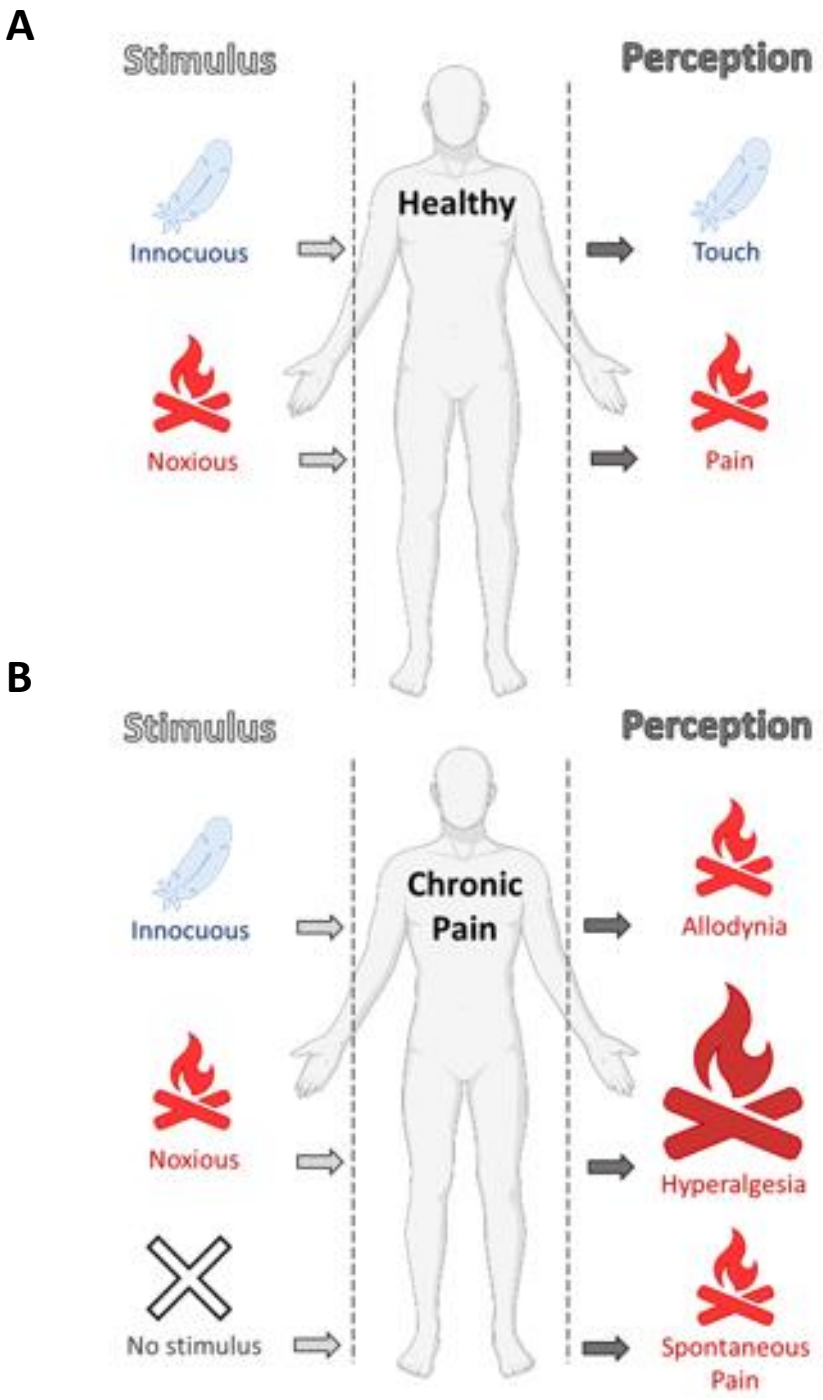


Figure 1.2 Perception of sensory stimuli in normal physiology and in pain pathology

In normal physiology, innocuous stimuli are felt and perceived as non-painful, while noxious stimuli result in the sensation of pain (**A**). Chronic pain pathologies present with the characteristic symptoms of allodynia (perceived pain in response to a non-noxious stimulus), hyperalgesia (enhanced pain response) and spontaneous (non-evoked) pain (**B**). Adapted by Wilson (2021) from Torsney and Fleetwood-Walker (2012)

Chronic pain affects an estimated 20% of the people worldwide (Breivik et al., 2006, Goldberg and McGee, 2011, Simon, 2012) and has been reported to last on average approximately 7 years (Institute of Medicine Committee on Advancing Pain Research and Education, 2011). European studies have found that chronic pain accounts for 15%-20% of physician visits (Mäntyselkä et al., 2001, Koleva et al., 2005), making it one of the most frequent causes for seeking medical care (Mäntyselkä et al., 2001). Whilst not immediately life threatening, chronic pain remains a leading cause of human suffering and disability (Goldberg and McGee, 2011), significantly affecting quality of life and increasing morbidity rates (Mäntyselkä et al., 2001, Smith et al., 2001, Breivik et al., 2006). Thus, chronic pain is increasingly perceived as a disease in itself rather than a symptom of another underling condition (Treede et al., 2015, Niv and Devor, 2004, Siddall and Cousins, 2004). As such, it also brings considerable costs to society, with European estimates at €441 billion annually (EuroStat, 2017) and U.S estimates at $630 billion annually (Institute of Medicine Committee on Advancing Pain Research and Education, 2011, Gaskin and Richard, 2012). Current treatment options do not provide adequate relief to the majority of patients (Breivik et al., 2006), with many currently used analgesics having undesirable side effects (Scholz and Woolf, 2002, Woolf, 2010) and/or having the potential for abuse (Stannard and Johnson, 2003, Breivik et al., 2006). This makes chronic pain one of the largest medical health problems worldwide (Breivik et al., 2006). Interestingly, accumulating evidence suggests that the susceptibility to and the severity of chronic pain might not be evenly distributed across the population due to a number of contributing factors, including sex, with many studies demonstrating sex differences in chronic pain (Mogil, 2016, Mogil, 2018, Sorge and Strath, 2018).

1.4 Sex differences in pain research

Over the past 30 years, sex has been accounted for as a biological variable by the National Institute of Health (NIH) in the US to address the fact that disease and

treatment outcomes can present differently in men and women (Bale and Epperson, 2017, Arnegard et al., 2020). The first major policy required the inclusion of women in all NIH-funded clinical investigations in 1990. However, it was not until 2014 that a more general policy included sex as a factor in designing studies in animals and human subjects, with a requirement now to justify the exclusion of any sex in grant applications (Clayton and Collins, 2014). Clearly, the implementation of these policies was a direct response to the unequal representation of male and female subjects in research. Despite both sexes being nearly equally represented in more recent studies with human subjects, Beery and Zucker (2011) have shown that at least 80% of the animal studies published in 2009 exclusively used male subjects only, with the most prominent male bias being in neuroscience. Consequently, important biological mechanisms still remain underexplored and poorly understood, with inadequate female representation potentially leading to either overgeneralisation of male findings or missed mechanistic insights. Thus, inclusion of sex as a biological variable is essential for rigorous and transparent research investigations that will have major implications for future healthcare.

Research in the pain field has been a particularly important area of investigation for sex differences (Hardy and Du Bois, 1940, Berkley, 1992, Greenspan et al., 2007, Melchior et al., 2016, Mogil, 2012, Mogil, 2016, Mogil, 2018, Mogil, 2020). Studies have shown that women are disproportionately affected by chronic pain, with a greater female prevalence of conditions such as migraines and headaches, back and musculoskeletal pain, arthritis, chronic fatigue syndrome and fibromyalgia (Gerdle et al., 2008, Fillingim et al., 2009, Mogil, 2012, Steingrímsdóttir et al., 2017). When it comes to preclinical studies, historically, the used of female animals has been avoided due to perceived challenges of oestrus cycle phases on nociception and analgesic responsiveness, but recent evidence suggests that the behavioural variability associated with the different oestrus cycle stages is similar to that occurring intrinsically in males (Beery, 2018). Now there is accumulating evidence suggesting quantitative (suggestive of different levels of pain-related variable) and

qualitative (suggestive of differential mechanisms) sex differences in both acute and chronic pain sensitivity (Mogil, 2020).

Over the last 15 years preclinical studies have explicitly investigated sex differences in basal nociceptive sensitivity in rodents and have shown that the pain thresholds in experimental models of noxious heat (Chesler et al., 2002, Terner et al., 2003b, Terner et al., 2003a, Mogil et al., 2000, Kest et al., 1999), chemical (Barrett et al., 2003, Gaumond et al., 2002) and mechanical (Barrett et al., 2002) nociception are different between males and females. Sex differences have also been reported in different injury-induced models of chronic pain in rodents (Sorge and Totsch, 2017, Fillingim, 2002, Mogil and Bailey, 2010).

The majority of the preclinical evidence of quantitative sex differences in acute and chronic pain (**Figure 1.3A**) (Fillingim and Maixner, 1995, Fillingim et al., 2009, Popescu et al., 2010), in line with the clinical evidence (Fillingim et al., 2003, Barnabe et al., 2012, Tang et al., 2012, Mogil, 2012), supports the findings of greater pain sensitivity in females compared to males (Mogil, 2020). In contrast to clinical studies, preclinical studies in rodents have reported males as more sensitive to analgesics than females (**Figure 1.3B**) (Mogil, 2020). Interestingly, meta-analysis has reported that majority of the preclinical studies published up to 2019 showed experimentally induced effects in one sex only (**Figure 1.3C**), suggesting there are sex differences in pain processing mechanisms. However, it is also possible that this conclusion is confounded by other factors such as pain severity, environmental conditions, social expectations, heath care access, sample diversity, quantitative methodology, and statistical power (Wiesenfeld-Hallin, 2005, Steingrímsdóttir et al., 2017, Fillingim et al., 2009, Hashmi and Davis, 2014).

Such disparities have largely been attributed to genetic, hormonal and neuroimmune differences between the sexes (Gregus et al., 2021, Mogil, 2020). Given the fact that biological sex includes complex interactions among genetics, anatomical development and hormones, it has been suggested that multiple biological mechanisms are involved in the differential regulation of pain in males

and females, including the peripheral and central nervous systems as well as the immune system (Mogil, 2020).

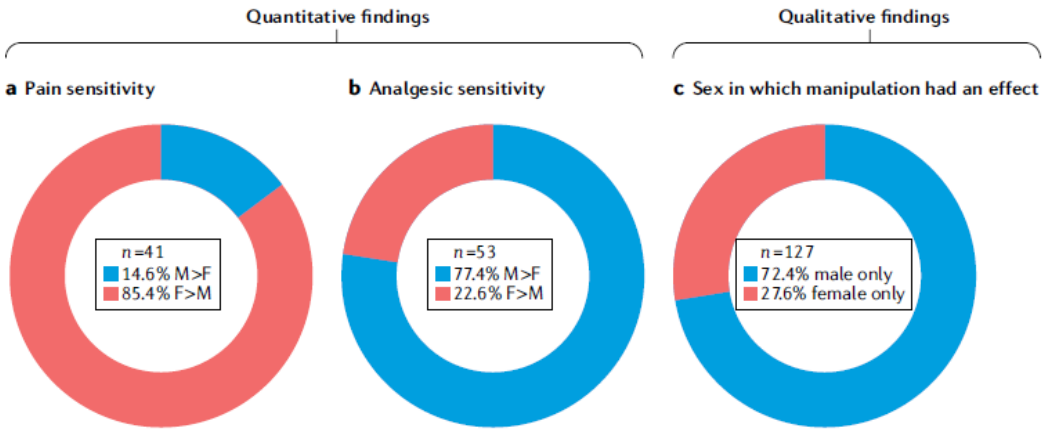


Figure 1.3 Quantitative and qualitative sex differences in pain research

The include data shown from a PubMed database search on 1st August 2019 including the search terms 'sex differences AND pain AND (mouse OR rat)'. There were 41 findings of quantitative sex differences in pain sensitivity with 85.4% reporting female rodents as more sensitive (**A**). Sensitivity to analgesics was reported to be greater in males in 77.4% of the 53 findings (**B**). There were 127 findings of qualitative sex differences, with 72.4% showing statistically significant effects of experimental manipulation in male rodents only. F denotes females, M denotes males. (Mogil, 2020).

1.4.1 Influence of gonadal hormones

Sex differences can be the result of organisational (hormone action during critical periods of gestation) and/or activational (contemporaneous circulating hormone action on particular behaviour) effects of gonadal hormones.

1.4.1.1 Effects of testosterone

Investigations on the organisational effects of testosterone have demonstrated that neonatal exposure to testosterone is necessary for lower baseline nociceptive sensitivity and increased morphine analgesia in adult males (Borzan and Fuchs, 2006). The antinociceptive organisational effects of testosterone have also been demonstrated in chronic pain models, in particular the lumbar radiculopathy model of low back pain where it has been shown that neonatal testosterone treatment of

females decreases tactile and thermal hypersensitivity (LaCroix-Fralish et al., 2005b). Sex differences in opiate analgesia appeared to be due to the organisational rather than the acute activational effects of testosterone, with neonatally-castrated males showing reduction in morphine analgesia similar to control females, while neonatally-androgenised females displaying increased morphine analgesia in adulthood similarly to males (Cicero et al., 2002, Krzanowska et al., 2002). Studies in human subjects have further supported the organisational antinociceptive effects of testosterone, where digital ratio (the ratio of the length of the second finger to the fourth finger (2D:4D)) was used as an estimate of prenatal androgen levels. A low ratio has been shown to correspond to prenatally high testosterone and low oestrogen levels, and a high ration was indicative of prenatally low testosterone and high oestrogen levels. Interestingly, further support for the organisational antinociceptive effects of testosterone comes from studies revealing an association between a low digital ratio and less postoperative pain in men, but more postoperative pain in women (Kasielska-Trojan et al., 2017).

The activational effects of testosterone still remain unclear. Some studies have reported activational antinociceptive effects of testosterone, reducing inflammation-induced sensitivity to mechanical but not thermal stimuli in adult males (Borzan and Fuchs, 2006). However, testosterone has also been shown to have pronociceptive activational effects, with its removal reducing lipopolysaccharide-induced allodynia and its replacement in gonadectomised male and female mice reinstating the lipopolysaccharide-induced allodynia (Sorge et al., 2011).

1.4.1.2 Effects of oestrogen and progesterone

The effects of oestrogen seem to be more complex. There are many reports of effects of gonadectomy, oestradiol or hormone replacement therapy in both directions in pain modulation. Overall, gonadectomy has been shown to increase acute pain sensitivity (Kuba and Quinones-Jenab, 2005), while oestradiol and progesterone given to ovariectomised animals have been reported to generally

decrease pain sensitivity (Kuba and Quinones-Jenab, 2005, Craft, 2007). Studies of the activational effects of oestrogen on rodent chronic pain models have also shown that its systemic administration is able to decrease nociceptive behaviours in gonadectomised rats of both sexes following intraplantar formalin injections (Gaumond et al., 2005, Kuba et al., 2006, Mannino et al., 2007) as well as in nerve injured intact mice (Vacca et al., 2016). In contrast, some chronic pain states, such as complete Freund's adjuvant (CFA)-induced arthritis, have been shown to be exacerbated by oestrogen, with females in proestrus (high ovarian hormones) displaying the greatest thermal hyperalgesia and the greatest mechanical hyperalgesia (Bradshaw et al., 2000, Cook and Nickerson, 2005). The pronociceptive effects of oestrogen have been further supported by radiculopathy studies where ovariectomy six weeks prior to surgery was able to abolish the lumber radiculopathy-induced enhanced tactile and thermal hypersensitivity in adult and pubertal female rats (LaCroix-Fralish et al., 2005b)

Progesterone, on the other hand, has been shown to serve mainly a protective function, mediating pregnancy-related analgesia (Rosen et al., 2017) and attenuating hyperalgesia caused by CFA- or carrageenan-induced monoarthritis (Ren et al., 2000, Tall and Crisp, 2004), excitotoxic spinal cord injury (Gorman et al., 2001) and peripheral diabetic neuropathy (Leonelli et al., 2007).

1.4.2 Influence of genetics

Genetic studies and studies in different rodent strains have revealed interactions between the influences of sex and genotype on baseline pain sensitivity and injury-induced pain sensitivity and processing (Mogil, 2003, Mogil et al., 2000).

Studies in different rodent strains have revealed that genetics could play a role in sex differences in baseline nociception and analgesia. Studies have reported strain-dependent sex differences in nociceptive behaviour (Mogil et al., 2000, Kest et al., 1999, Mogil et al., 1999, Smith, 2019). For example, female *AKR/J*, *C3H/HeJ* and *C57BL/6J* mice have been demonstrated to be more sensitive to noxious heat stimuli than male mice of the same strains, but the males of these three strains

required lower morphine doses to achieve half-maximal nociception (Kest et al., 1999). Moreover, further studies have reported substrain-dependent sex differences in nociceptive behaviour, with reports of the *C57BL/6J* and *C57BL/6N* mouse substrains showing sex differences in thermal nociception, where males exhibited more pronounced baseline sensitivity to thermal stimuli than females (Bryant et al., 2019). There is also evidence that swim-induced analgesia is more pronounced in female Wistar but not Lewis rats (Vendruscolo et al., 2004). In contrast, stress-induced analgesia in *C57BL/6* and Swiss Webster mice is greater in males than in females (Mogil and Belknap, 1997). These studies indicate that the quantitative sex differences in stress-induced analgesia are strain/species-dependent, suggesting that sex differences in pain sensitivity and analgesia could also depend on stain/species.

Recent gene expression profiling studies have also shown sex differences in the expression of basal nociception-related genes.

Investigations of the transcriptome and translatome (actively translated messenger RNAs) of mouse dorsal root ganglia (DRG) have revealed 80 genes with sex differential expression in whole DRG transcriptome and 66 genes that were differentially translated in a sex-dependent manner in nociceptor-enriched translatomes (Tavares-Ferreira et al., 2020). Interestingly, the females were found to have higher expression of proteins in the prostaglandin family, with pharmacological studies supporting the prostaglandins-induced sex differences in acute pain behaviours. Additional RNA sequencing studies have identified female-selective differential expression of genes in sensory neurons that have been associated with inflammatory, synaptic transmission and extracellular matrix reorganisation processes that could enhance neuro-inflammation severity (Mecklenburg et al., 2020). Two main biological processes were related to male-selective differential expression of genes - reactive oxygen species and protein metabolism, which have been proposed to likely play a protective role in neuropathic pain states (Mecklenburg et al., 2020). These quantitative findings

further support the presence of neural sex differences in nociceptive pathways that are influenced by genetics.

Sex differences in injury-induced rodent pain models could also be influenced by genetics. For example, it has been reported that female Sprague–Dawley (SD) and Long–Evans but not Holtzman rats develop more severe mechanical allodynia following lumbar radiculopathy (LaCroix-Fralish et al., 2005a) or spinal nerve transection (SNT) (DeLeo and Rutkowski, 2000). Furthermore, the different mouse substrains *C57BL/6J* and *C57BL/6N* have been reported to display differences in nociceptive behaviours following intraplantar formalin injection but not in the CFA or chronic constriction injury (CCI) models (Bryant et al., 2019). These findings further suggest that species/strain could also be a factor contributing to sex differences in injury-induced pain sensitivity.

Gene expression profiling studies have also reported sex differences in the expression of chronic pain-related genes. RNA sequencing studies in the rat CCI model have revealed that only 16% of the injury-induced genes were shared between the sexes, with some of those being regulated in opposite directions in males and females (Stephens et al., 2019). Similar analysis has been conducted in human tissue. The human tibial nerve transcriptome of patients with or without arthritis or type II diabetes showed that 149 genes are expressed in a sex-dependent manner (Ray et al., 2019), with macrophage related genes upregulated primality in men with the pain-related conditions (consistent with neuroimmune differences in rodents as discussed in section 1.4.3). A similar study in DRG from patients with neuropathic pain revealed that there are different transcriptome signatures in males and females, with macrophage related genes upregulated primarily in men. Interesting the study was able to predict patients' sex and pain status with 95% accuracy from the RNA profiling (North et al., 2019).

Thus, the studies in different rodent species and strains together with the transcriptome and translatome analyses provide comprehensive evidence that

genetics could influence baseline and injury-induced pain processing in a sex-dependent manner.

1.4.3 Sex differences in neuro-immune interactions

There is accumulating evidence suggesting a role for sex-specific immune responses that may explain some of the disparities in incidences of pain conditions and other neurological disorders. It has been recognised that glial cells of the immune system are important players in the development and maintenance of chronic pain (Ji et al., 2016, Grace et al., 2014) as well as in its inhibition (Averitt et al., 2019) and resolution (Chen et al., 2018b).

Mouse studies have reported that the absence of Toll-like receptor 4 (TLR4) reduces mechanical hypersensitivity following nerve injury in males but not females (Sorge et al., 2012, Tanga et al., 2005). Additional genetic and pharmacological experiments confirmed the importance of spinal cord TLR4 signalling in mediating neuropathic and inflammatory pain hypersensitivity in males only (Bastos et al., 2013). Thus, it has been suggested that spinal cord microglia, where TLR4 is expressed, are important in pain processing in males (Beggs and Salter, 2010, Chen et al., 2018b), but are not actually required for pain hypersensitivity in females (Sorge et al., 2015). Females, however, have been suggested to use T-cells of the adaptive immune system instead to produce chronic pain hypersensitivity (Sorge et al., 2015). Additional pharmacological studies have shown that microglial-inhibiting drugs (including minocycline, fluorocitrate and propentofylline) or inhibitors of proteins known to be expressed primarily on microglia (including TLR4, p38 mitogen-activated protein kinase, P2X4 and caspase 6) have effects in males but not females (Chen et al., 2018a, Berta et al., 2016, Taves et al., 2016, Woller et al., 2016, Mapplebeck et al., 2018, Rosen et al., 2017, Luo et al., 2018, Paige et al., 2018). However, other studies have shown that nerve injury ‘activates’ microglia in the spinal cord equally in both sexes (Sorge et al., 2015, Mapplebeck et al., 2018), with hypothesis of the sex difference being in the apparently male-specific upregulation of P2X4 receptor expression in activated microglia (Sorge et al., 2015).

Other immune cells and associated molecules have been shown to also display sex differences of relevance to chronic pain, such as major histocompatibility complex class II (MHC-II) (Hartlehnert et al., 2017), TLR9 (Luo et al., 2019), NACHT, LRR and PYD domains-containing protein 3 (NLRP3) (Cowie et al., 2019b), and tumour necrosis factor receptor 1 (TNFR1) (Del Rivero et al., 2019).

It must be noted that additional sex-specific mechanistic drivers could exist but have not been yet explored because female subjects are still significantly underrepresented in preclinical pain research. It has been shown that 79% of the non-human pain studies in animals between 1996 and 2005 have been conducted in males only (Mogil, 2012) and although these numbers have improved since then (Mogil, 2020), the exclusion of female subjects in preclinical pain research and thus unexplored pain mechanisms still remain.

1.4.4 Sex differences within the nervous system

In addition to the sex differences in immune responses, there is evidence for sex differences within the peripheral and the central nervous system. In the peripheral nervous system (PNS), studies from this lab have demonstrated that the relay of pain signals in nociceptive C-fibres differs between the sexes (Dickie et al., 2017). Further studies in the periphery have demonstrated sex differences in the contribution of PKCε and PKCδ signalling in the development of diabetes-induced mechanical hyperalgesia (Joseph and Levine, 2003). In addition, transcriptomic studies in the human tibial nerve have shown sex differences in neuronal gene expression in basal and chronic pain states (Ray et al., 2019). Interestingly, studies in the PNS, showing more hyperpolarised resting membrane potential in DRG neurons and higher mechanical C-fibres thresholds in females, have suggested sex-differences in CNS mechanisms, which can overcome the opposing differences in the peripheral mechanisms and explain the lower mechanical nociceptive thresholds observed in females (Hendrich et al., 2012). There are also reports of sex differences in CNS plasticity, with studies showing a reduction in axonal initial segment in prefrontal cortex neurons in males only, linking it with their greater

cortical dysfunction (Shiers et al., 2018). Further studies in the CNS have demonstrated sex differences in the anti-allodynic effects of angiotensin II and oxytocin in the PSNL-induced neuropathic pain model, with potent anti-allodynia achieved only in males (Chow et al., 2018). Furthermore, sex differences have been demonstrated in the mechanisms involved in the transition from acute to chronic pain, with spinal atypical PKCδ being essential for this transition in males but not in females (Baptista-de-Souza et al., 2020). Interestingly, functional MRI studies in rats have showed that sex can influence pain modulatory networks in healthy and chronic pain states, with sex differences in descending inhibitory control that can influence chronic pain vulnerability (Da Silva et al., 2021).

1.5 Cutaneous primary afferent neurons

The exteroceptive somatosensory system comprises the cutaneous primary afferent neurons, which convey information about the external environment and the body's interaction with it. Distinct cutaneous primary afferent fibre subtypes detect specific innocuous, noxious or pruritoceptive stimuli (Abraira and Ginty, 2013, Lallemend and Ernfors, 2012, LaMotte et al., 2014) and transduce them into inward 'generator' currents. If these 'generator' currents are sufficiently large, they drive action potentials along the axon, thus transmitting the information from the periphery to specific regions of the dorsal horn of the spinal cord.

The cell bodies (somas) of the cutaneous primary afferents that innervate the periphery are found within the dorsal root ganglia (DRG), while those innervating the face are located within the trigeminal ganglia (TG). A single process emanates from the cell body in the DRG or the TG and bifurcates to send a peripheral axon to innervate the skin and a central axon to the dorsal horn of the spinal cord or the trigeminal subnucleus caudalis, respectively, where it forms synapses with second order neurons (Basbaum et al., 2009, Smith and Lewin, 2009). This unique morphology of the primary afferent fibres makes them pseudo-unipolar and allows the propagation of electrical signals between the periphery and the spinal cord (brain stem) to follow a direct axonal pathway, reducing the risk of conduction

failures (Amir and Devor, 2003). Unlike prototypical neurons, the pseudo-unipolar nature of the primary afferents makes their peripheral and central branches biochemically equivalent. This means that the primary afferents could send and receive information from either end. Although both the peripheral and central terminals can be regulated by various endogenous molecules, only the peripheral terminals are able to respond to stimuli from the external environment.

The primary afferent neurons can be broadly divided into three main subgroups based on their anatomical and biophysical properties, such as their cell body size, the presence of myelin sheaths and the diameter of their axons, the latter two directly correlating to the speed of their transmission (Basbaum and Jessell, 2000, Lallemend and Ernfors, 2012, Abraira and Ginty, 2013). These are the large diameter, thickly myelinated, fast conducting Aβ-fibres, the medium diameter, thinly myelinated, medium conducting Aδ-fibres, and the small diameter, unmyelinated, slow conducting C-fibres (**Table 1.1**). These can be further subdivided into non-nociceptors and nociceptors, which are able to detect and transduce innocuous and noxious stimuli, respectively. The sensory specificity of the primary afferents is determined by the expression of ion channels (transducers) with different thresholds to mechanical, thermal and chemical stimuli, such that the high threshold transducers of the nociceptors differentiate them from the sensory neurons responding to innocuous stimuli, which express low threshold transducers (Woolf and Ma, 2007).

Table 1.1 Primary afferent fibres characteristics

Information about myelination was taken from Le Pichon and Chesler (2014). Axon diameters were taken from Millan (1999) and conduction velocities values of juvenile rat recordings were taken from Nakatsuka et al. (2000). The primary afferents laminar targets in the spinal cord were taken from Todd (2010).

Primary afferent fibre	Myelination	Axon diameter (µm)	Conduction velocity (m/s)	Spinal cord laminar target
Aβ	Heavily myelinated	>10	5.5-8.8	IIi-V
Aδ	Lightly myelinated	2-6	1.6-5.5	I (D-hair in Iii/III)
C	Unmyelinated	0.4-1.2	0.5-0.9	I/II

Most of the Aβ-fibres detect and transduce low threshold, innocuous stimuli like light touch and are thus classified as non-nociceptive. The majority of the Aδ-fibres and the C-fibres are classified as nociceptors because they are activated primarily by high threshold stimuli with intensities in the noxious range (Basbaum and Jessell, 2000, Lallemend and Ernfors, 2012, Abraira and Ginty, 2013). This classification assigns each fibre type to different cutaneous sensory modalities, but it must be noted that this is an oversimplification. There are reports of Aβ-fibres that respond to noxious stimuli (as discussed in section 1.5.1) as well as low threshold Aδ-fibres and C-fibres that transmit innocuous dynamic and affective touch stimuli, respectively (as discussed in section 1.5.2 and 1.5.3). Initial fast-onset pain is thought to be conducted by the faster Aδ-fibre nociceptors, while the more diffused 'slow' pain is associated with the slow conducting C-fibre nociceptors (Djouhri and Lawson, 2004, Woolf and Ma, 2007, Magerl et al., 2001, Ochoa and Torebjörk, 1989).

The majority of nociceptors are known to be polymodal because they have been demonstrated to respond to a variety of different types of noxious stimuli (mechanical, thermal or chemical) (Basbaum et al., 2009). Distinct classes of nociceptors have been established based on single unit recordings from nerve fibres in peripheral nerves (microneurography) and skin-nerve preparations in mammals (Zimmermann et al., 2009) as well as from microneurography studies in humans (Namer and Handwerker, 2009, Schmelz, 2009). This allowed the differentiation of the nociceptive fibres based on their sensitivity and threshold to noxious mechanical (M), heat (H) and chemical (C) stimuli (Raja et al., 1988, Mizumura and Kumazawa, 1996). The Aδ-fibres have been shown to be predominantly sensitive to heat and mechanical stimuli (A-MH, A-H, A-M) (Lewin and Moshourab, 2004, Cain et al., 2001). The most common type of C-fibre nociceptor responds to thermal, mechanical and chemical stimuli (C-MH, C-MC, C-MHC) (Van Hees and Gybels, 1981, Raja et al., 1988). This fibre class is functionally heterogeneous because of the differential repertoires of transducer receptors expressed in C-fibres. The use of electrical rather than mechanical stimulation allowed the identification of a special type of C-fibre nociceptor that is normally insensitive to mechanical and heat stimuli (C-MiHi), called silent. They have been shown to develop sensitivity to noxious mechanical or heat stimuli as a result of sensitisation induced by inflammatory mediators (Schmidt et al., 1995, Meyer et al., 1991). Thus, it has been proposed that silent nociceptors may contribute to inflammation-induced mechanical hyperalgesia (Gold and Gebhart, 2010). While silent nociceptors have been shown to be rare in rodent skin, around 25% of all C-fibre nociceptors in human skin have been reported as silent (Schmidt et al., 1995). Around 10% of the C-fibre nociceptor have been shown to respond only to noxious heat stimuli (C-H) and have been implicated in heat sensation (Dubin and Patapoutian, 2010).

1.5.1 Aβ fibres

Aβ-fibres are large diameter and thickly myelinated neurons (Basbaum et al., 2009) and as a result display the fastest conduction velocity (5.5-8.8 m/s for juvenile rats)

of all three primary afferent subtypes (Nakatsuka et al., 2000). The peripheral terminals of the Aβ-fibres innervate specialised non-neuronal structures in the skin, which confer high sensitivity to tactile spatial stimuli, fine dynamic touch, stretch, vibration and hair movement, called Merkel cells, Meissner's corpuscles, Ruffini endings, Pacinian corpuscles, and Lanceolate endings, respectively (Abraira and Ginty, 2013, Smith and Lewin, 2009, Handler and Ginty, 2021). Each of these structures displays a unique morphology that likely contributes to the tuning specializations of their associated nerve fibres (Abraira and Ginty, 2013). Because of their sensitivity to weak, innocuous stimuli, most Aβ-fibres have low mechanical thresholds and are known as low-threshold mechanoreceptors (LTMR).

However, there is evidence of myelinated peripheral fibres that respond to noxious mechanical stimuli with conduction velocities within the Aβ fibre ranges (Burgess and Perl, 1967, McIlwrath et al., 2007, Woodbury and Koerber, 2003, Djouhri and Lawson, 2004). Under normal conditions these 'myelinated nociceptors' have been also shown to respond to innocuous mechanical stimuli and some of them to noxious heat (Treede et al., 1998). This suggests that they are likely to serve both LTMR and nociceptive functions. Myelinated nociceptors have been reported to be present in both glabrous and hairy skin, but their anatomical morphologies are not known yet (Abraira and Ginty, 2013, Lawson et al., 2019).

The central branches of the Aβ-fibres terminate predominantly in laminae IIi-V in the dorsal horn of the spinal cord (**Figure 1.4**) with projections from there to the dorsal column nuclei in the brainstem (Todd, 2010).

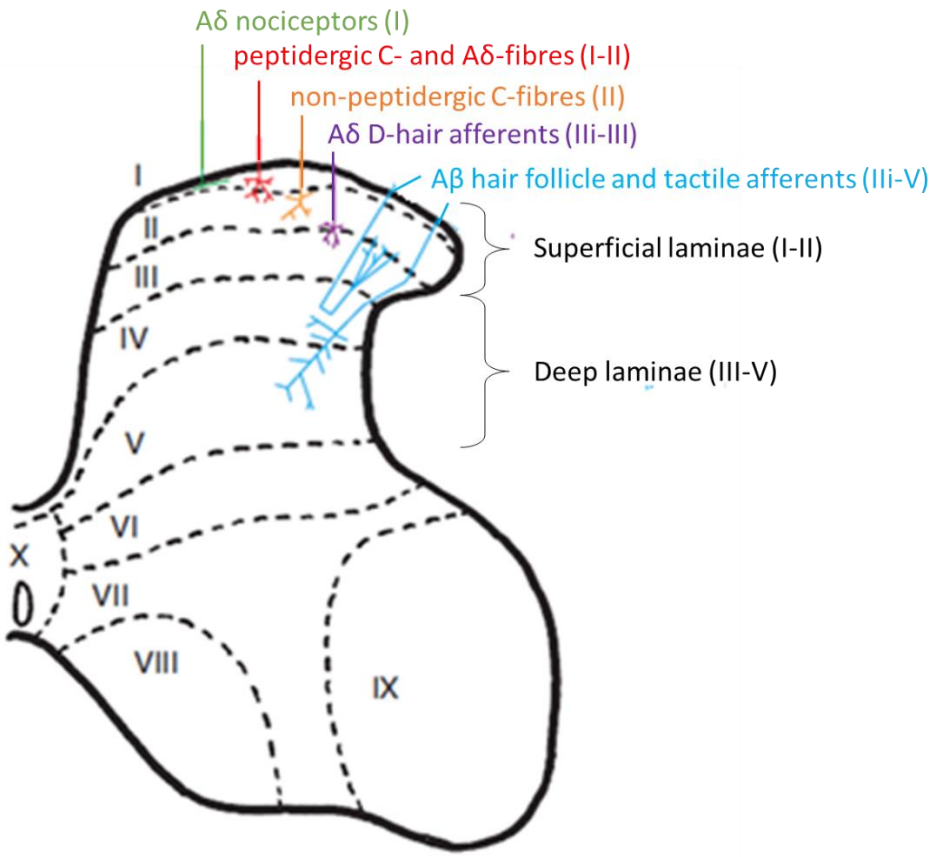


Figure 1.4 Laminar organisation of the spinal dorsal horn and laminar targets of the primary afferent inputs.

Diagram based on a transverse section form the lumbar (L4) region of the spinal cord. Adapted from Todd (2010).

1.5.2 Aδ fibres

The peripheral afferent Aδ-fibres have medium diameter cell bodies and axons, which are lightly myelinated (Basbaum et al., 2009) and as a result they conduct with intermediate conduction velocities in the range of 1.6-5.5m/s for juvenile rats (Nakatsuka et al., 2000).

The peripheral branch of the Aδ-fibres innervates the skin, where it loses its myelin sheath, and terminates as unmyelinated free nerve endings (Smith and Lewin, 2009). These make high density clusters, the anatomical substrate for their

receptive field (Dubin and Patapoutian, 2010). Majority of the Aδ free nerve endings that innervate the skin are high-threshold mechanoreceptors (HTMR). Given their faster conduction velocity, Aδ-HTMR are associated with the initial highly localised 'fast pain' rather than the more diffused 'slow' pain associated with C-fibre nociceptors (Basbaum et al., 2009). Unlike LTMR, the HTMR are often polymodal, with Aδ-HTMR being further subdivided based on their sensitivity to heat as A-MH, A-H, A-M(Lewin and Moshourab, 2004, Cain et al., 2001). However, sensitivity to noxious cold could be also observed (Cain et al., 2001, Koltzenburg et al., 1997). The thermal threshold of Aδ-fibres has been demonstrated to decrease following prolonged exposure to noxious stimuli (Eilers and Schumacher, 2005, Lewin and Moshourab, 2004) or following injury, which likely supports their involvement in inflammatory pain (Basbaum et al., 2009).

Subset of the Aδ-fibres have been shown to be LTMR that transmit innocuous stimuli resulting from the slow movement of hair from hairy skin (Lewin and Moshourab, 2004, Basbaum et al., 2009). They have been described as Aδ hair follicle afferents or D-hair afferents (Lewin and Moshourab, 2004). The Aδ-LTMR are the most sensitive mechanoreceptor in skin as they display some of the lowest mechanical thresholds and highest dynamic sensitivity (Koltzenburg et al., 1997).

The central branches of the nociceptive Aδ-fibres project primarily to the lamina I region of the dorsal horn of the spinal cord, while the non-nociceptive D-hair afferents terminate in lamina IIii/III (**Figure 1.4**) (Todd, 2010).

1.5.3 C-fibres

The C-fibre afferents are the most abundant afferent fibre type, comprising more than half of all somatosensory neurons (Le Pichon and Chesler, 2014). They have a small diameter and axon. They are unmyelinated and as a result they display the slowest conduction velocity of the primary afferents (0.5-0.9m/s) (Table 1.1) (Nakatsuka et al., 2000).

The peripheral C-fibre branch terminates in the skin, organs and/or bone as free nerve endings (Le Pichon and Chesler, 2014), which are very broadly distributed (Smith and Lewin, 2009), thus precluding exact identification of the stimulus localisation. Similarly to the Aδ-fibre nociceptors, the majority of C-fibres are classified as high threshold polymodal nociceptors and as such respond to differing combinations of intense thermal, mechanical or chemical stimuli (Le Pichon and Chesler, 2014, Basbaum et al., 2009). Given their slow conduction velocity and the broad distribution of their peripheral terminals, the C-fibre nociceptors have been associated with a more diffuse 'slow' pain (Basbaum et al., 2009).

Not all C-fibres are classified as nociceptors. Previous research has identified subgroup of unmyelinated low threshold mechanoreceptors with conduction velocities within the C-fibre range (C-LTMR) that mediate pleasant touch (Zotterman, 1939, Douglas and Ritchie, 1957, Iggo, 1960, Iggo and Kornhuber, 1977). These C-LTMR have been shown to be present only in hairy skin, with greater proportion of C-LTMR than myelinated fibres innervating the skin (Li et al., 2011). Further studies in human hairy skin have suggested that the C-LTMR could be involved in mediating 'emotional touch' (Löken et al., 2009, McGlone et al., 2007, Olausson et al., 2008).

C-fibres can also be classified on their neurochemical basis as peptidergic and non-peptidergic. The peptidergic C-fibres express the peptides substance P (SP) and calcitonin gene related peptide (CGRP), while the non-peptidergic neurons lack neuropeptide storage vesicles and can be identified by their reactivity for isolectin-B4 (IB4) (Averill et al., 1995, Basbaum et al., 2009, Woolf and Ma, 2007). The peptidergic fibres innervate mostly the viscera and the deeper skin layers, the non-peptidergic fibres - the more superficial skin layers (Basbaum et al., 2009). Functional evidence in mice points to differences between the two C-fibre subpopulations. The non-peptidergic MAS Related G Protein-coupled Receptor (GPR) Family Member D (MRGPRD) expressing C-fibres have been associated with noxious mechanical sensitivity, while the peptidergic transient receptor potential

(TRP) vanilloid 1 (TRPV1) expressing C-fibres have been implicated in noxious heat sensitivity (Cavanaugh et al., 2009). It must be noted, however, that there are reports of non-peptidergic C-fibres that are not labelled by IB4 (Abraira and Ginty, 2013) as well as findings of overlap between these markers (McCoy et al., 2013). Thus, the functional significance of these markers, especially given striking species differences (Woodbury et al., 2004, Price and Flores, 2007), should be interpreted with caution (Dubin and Patapoutian, 2010). More recent transcriptomic studies offer an improved understanding of the different DRG neurons subclasses and their molecular profiles (Usoskin et al., 2015, Li et al., 2016), which will help further understanding of nociceptors subtypes.

The C-LTMRs have been shown to express tyrosine hydroxylase (TH) , vesicular glutamate transporter 3 (VGLUT3) or MRGPR Family Member B4 ($MRGPRB4^{+}$) (Koch et al., 2018), and have been shown to innervate the hairy skin only (Liu et al., 2007, Vrontou et al., 2013). The VGLUT3-expressing C-LTMR have been found only in mouse and have been suggested to be implicated in mechanical allodynia in inflammatory and neuropathic pain (Seal et al., 2009), but this still remains controversial (Lou et al., 2013).

In addition, *in vivo* electrophysiological recordings have been used to functionally classify C-fibres of different sensory modalities utilising the C-fibre activity dependent slowing (ADS) phenomenon as a tool (Schmelz et al., 2000, Gee et al., 1996, Raymond et al., 1990, Thalhammer et al., 1994, Obreja et al., 2010, Serra et al., 1999, Weidner et al., 1999). C-fibre ADS is the progressive reduction in C-fibres conduction velocity following repetitive stimulation at high frequencies (see section 1.7.2 for more detail) (Gee et al., 1996, Thalhammer et al., 1994, Weidner et al., 1999, Serra et al., 1999). It has been used to differentiate the mechano-sensitive (CM) from the mechano-insensitive (CMi), or silent, C-fibre nociceptors (George et al., 2007, Hilliges et al., 2002, Jørum et al., 2007, Schmelz et al., 2000). Thus, the CM are characterised by their minimal amount of ADS, while the CMi are distinguished

by their pronounced degree of ADS (Weidner et al., 1999, Weidner et al., 2000, Weidner et al., 2002, Bostock et al., 2003, Obreja et al., 2010)

The C-fibre central branches project to the superficial layers of the dorsal horn of the spinal cord, an essential region for the first stage of noxious and thermal stimuli processing (Le Pichon and Chesler, 2014), with the peptidergic C-fibres shown to project to lamina I/IIo and majority of the non-peptidergic - to lamina II (**Figure 1.4**) (Todd, 2010).

1.6 Transduction of sensory information

The terminal ends of the primary afferent fibres express various ligand-gated ion channels and G-protein coupled receptors (GPR) that are involved in the detection and transduction of sensory information into electrical current and the subsequent generation of action potentials (Basbaum et al., 2009, Woolf and Ma, 2007, Smith and Lewin, 2009). Transduction of the sensory stimulus involves the stimulus activating a specific receptor (transducer receptor) expressed on the peripheral terminals of the afferent fibres. This drives the influx of Na^+ and Ca^{2+} ions though ion channels, leading to depolarisation (generator potential) of the terminal end of the afferent fibres. If the depolarisation is of large enough magnitude and duration, this triggers the generation of action potential, which propagates along the afferent fibre towards the CNS.

1.6.1 Transduction of thermal stimuli

The transduction of nociceptive thermal stimuli involves members of the transient receptor potential (TRP) channel family (Wang and Woolf, 2005). The best characterised member of the TRP channel family is the transducer receptor TRP subtype vanilloid 1 (TRPV1), which is activated by temperatures in the noxious range (>43^{o}C), and also by the agonist capsaicin, the active component of chillies, which gives them their burning sensation (Caterina et al., 1997).

Early microneurography studies in humans have demonstrated that in normal conditions the activity in a subset of heat-responsive fibres (C-MH, A-MH type I, A-

MH type II) correlates to the degree of pain perceived (Raja et al., 1988, Van Hees and Gybels, 1981). The A-MH Type II fibres in the hairy skin, which activate rapidly, adapt during prolong heat stimulation and are sensitive to capsaicin (Ringkamp et al., 2001). They have been proposed to mediate 'first' fast onset pain in humans (Treede et al., 1995). The slowly developing 'second' pain has been proposed to be associated with the activation of C-fibres and A-MH type I–fibres (Raja et al., 1988, Van Hees and Gybels, 1981).

Detailed studies in mice have supported the involvement of TRPV1 in the detection and transduction of noxious heat stimuli. It has been reported that in the absence of TRPV1, mice show deficiencies in their responses to acute noxious heat and failed to develop complete Freund's adjuvant (CFA)-induced hypersensitivity to heat, but their heat sensitivity following nerve injury remains unaltered (Caterina et al., 2000). Similar studies in TRPV1 knockout mice have also revealed no development of heat hypersensitivity following carrageenan hindpaw injection, but the responses to acute noxious thermal stimuli were reported as normal (Davis et al., 2000). This partial reduction of noxious heat sensitivity in behavioural assays in mice following TRPV1 deletion and the lack of effect of the TRPV1 deletion on the heat responsiveness of the tested C-fibres (Caterina et al., 2000, Zimmermann et al., 2005, Woodbury et al., 2004) suggest that heat sensors additional to TRPV1 probably also contribute to the acute heat-induced pain in mice. Further studies have functionally characterised C-MH neurons in mice and revealed that they are not immunoreactive for TRPV1, but all the heat sensitive C-fibres (C-H) (~10% of the C-fibre population) had TRPV1 immunoreactivity and no functionally identified C-H fibres were detected in the TRPV1-deficient mice (Lawson et al., 2008). The differences regarding the contribution of TRPV1 to fibre subtype functionality (especially C-MH fibres) may depend on species, stimulation techniques, tissue preparation, and sensitivity of the assay. However, these studies suggest that acute heat responses, at least in mice, are mediated by mechanisms in addition to TRPV1 activation. In contrast, in the context of tissue injury, polymodal C-MH fibres certainly seem important in TRPV1-mediated thermal hyperalgesia in mice,

indicating that inflammation upregulates the contribution of TRPV1 to heat-evoked nocifensive behaviours.

Other members of the TRP family have also been shown to be important for detection of various temperatures. These include the TRP subtype ankyryn 1 (TRPA1) that detects temperatures below 17^{o}C as well as noxious mechanical stimuli and irritants (Huang et al., 2006, Nassini et al., 2011, Petrus et al., 2007). It has also been associated with capsaicin–induced cold allodynia (Caterina et al., 1997, Nassini et al., 2011, Vay et al., 2012). TRP subtype vanilloid 2 (TRPV2) has been shown to detect temperatures above 52^{o}C, TRP vanilloid subtype 3 (TRPV3) – 33-39^{o}C, TRP vanilloid subtype 4 (TRPV4) – 37-34^{o}C and TRP subtype melastatin 8 (TRPM8) - 8-28^{o}C (Wu et al., 2010). TRPV3 has been shown to undergo sensitisation to repeated heat stimuli, but it has not been reported to be directly involved insensitisation of C-fibre heat responses (Zimmermann et al., 2005).

1.6.2 Transduction of mechanical stimuli

In the mammalian somatosensory system mechanosensitive neurons mediate the sense of touch and pain. Despite the increasing efforts to identify candidate transducer molecules underlying mechanotransduction, the channels responsible for the detection and transduction of mechanical stimuli are still currently not fully understood (Wood and Eijkelkamp, 2012).

Genetic studies in flies and worms have identified mechanically gated ion channels, which are members of the epithelial sodium channel (ENaC) family (Arnadóttir and Chalfie, 2010). Although the mammalian homologues of these channels have been extensively explored, there is no conclusive evidence for members of the ENaC family acting as mechanotransducers in sensory neurons (Chatzigeorgiou et al., 2010).

Three members of the acid-sensing ion channels (ASICs), that are highly expressed in sensory neurons, have been linked to mechnotransduction (Deval et al., 2010, Wu et al., 2012). While ASIC1 is in most somatosensory neurons, ASIC2 and ASIC3

are co-expressed in the medium- and large-diameter DRG neurons (Alvarez de la Rosa et al., 2002). ASIC2 and ASIC3 are also found in the peripheral terminals of cutaneous mechanoreceptors, which is where transduction occurs (Price et al., 2000, Price et al., 2001). Despite the abnormal sensory transduction in mice lacking ASIC1a, ASIC1, ASIC2, or ASIC3 (Price et al., 2000, Price et al., 2001, Page et al., 2005), the phenotypes in knockout studies were subtle or controversial, which makes the role of ASICs in sensory mechnotransduction still illusive (Wu et al., 2012, Drew et al., 2004).

Members of the TRP channel family have also been proposed as potential mammalian mechanotransducers (Wood and Eijkelkamp, 2012). These channels have been localised to variety of mechanoreceptors in different species and their disruption has been associated with alterations in detecting mechanical stimuli (Brierley, 2010). For example, studies in mice with a TRPV4 deletion have demonstrated altered responses to noxious mechanical pressure (Tabuchi et al., 2005). Moreover, TRPA1, which is expressed in DRG and TG neurons as well as in hair cells, has been found to be involved in noxious but not innocuous mechanosensation (Kwan et al., 2006). Deletion of TRPA1 in mice has been associated with silencing of a set of small peptidergic sensory neurons and with behavioural deficits in noxious mechanosensation (Vilceanu and Stucky, 2010, Andersson et al., 2009). TRPA1 selective blocker has been shown to inhibit mechanosensory currents in sensory neurons and to inhibit injury-induced mechanical hypersensitivity in various pain models (Eid et al., 2008). Interestingly, human studies have found that a gain of function TRPA1 mutation causes episodic pain syndrome (Kremeyer et al., 2010). All these data are consistent with a role for TRPA1 as a mechanically-gated ion channel, but this has not yet been demonstrated using heterologous expression of TRPA1 (Wood and Eijkelkamp, 2012). Other TRP channels have been also linked to mechnotransduction, including TRPC1 and TRPC6, which have been implicated in inflammation-induced mechanical hyperalgesia. While TRPC1 has been liked with mechanical hyperalgesia only, TRPC6 has been

shown to play a role in both mechanical and thermal hyperalgesia (Alessandri-Haber et al., 2009).

More recently, the Piezo channel family, which comprises the cation channels Piezo1 and Piezo2, shown to detect mechanical stimuli, has been implicated in mechnotransduction processes, including touch sensation (Coste et al., 2010). While Piezo1 channels have been localised primarily to non-neuronal tissues such as skin, lungs bladder, the Piezo2 channels have been found in neuronal tissues, including DRG and the terminals of specific primary afferents (Coste et al., 2010).

Some Piezo1 channels have been shown to be present in small diameter DRG neurons in mice and thus have been suggested to be potentially implicated in detecting and transducing noxious mechanical stimuli (Wang et al., 2019). Clinical studies have found that patients with autosomal recessive inheritance of Piezo2 display deficits in discriminative touch and inflammation-induced tactile allodynia (Szczot et al., 2018). Preclinical studies in Piezo2 knockout rodents have also demonstrated deficits in gentle touch responses, with responses to noxious mechanical stimuli remaining unaffected (Maksimovic et al., 2014, Ranade et al., 2014, Woo et al., 2014). However, recent studies in mice point to Piezo2 involvement in mechanical allodynia pain (Murthy et al., 2018, Szczot et al., 2018).

1.6.3 Transduction of chemical stimuli

Primary afferents are also able to detect environmental irritants and endogenous molecules produced by physiological stress via a process called chemo-nociception (Basbaum et al., 2009). The TRP channel family has also been implicated in this process since some of the members of this family also function as receptors for plant-derived irritants such as capsaicin (TRPV1), menthol (TRPM8), as well as the pungent ingredients in mustard and garlic (TRPA1) (Bandell et al., 2004, Caterina et al., 1997, Jordt et al., 2004, McKemy et al., 2002, Peier et al., 2002). TRPA1 has been shown to respond to structurally diverse environmental irritants, including allyl isothiocyanate (from wasabi) or allicin (from garlic) as well as acrolein, present for example in smoke from burning vegetation (Hinman et al., 2006, Macpherson et al.,

2007). Thus, mice lacking TRPA1 have been shown to exhibit reduced sensitivity to such environmental agents (Caceres et al., 2009). Given that TRPA1 has been shown to be also activated by formalin, it is thought to mediate nociceptive responses in the formalin test (McNamara et al., 2007).

Some of the chemicals and other pro-algesic agents, produced endogenously following tissue damage or oxidative stress, such as protons (H^+), ATP, serotonin or lipid, could also alter neuronal excitability by directly acting on the peripheral nerve terminals (Woolf and Salter, 2000). These express several proton-sensitive channels, but the members of the TRP channel family and ASICs have been suggested as the main contributors to proton sensitivity (White et al., 2010). The receptor for adenosine triphosphate (ATP), P2X2, also plays a major role in chemo-nociception (Fabbretti, 2013).

1.7 Conduction of sensory information

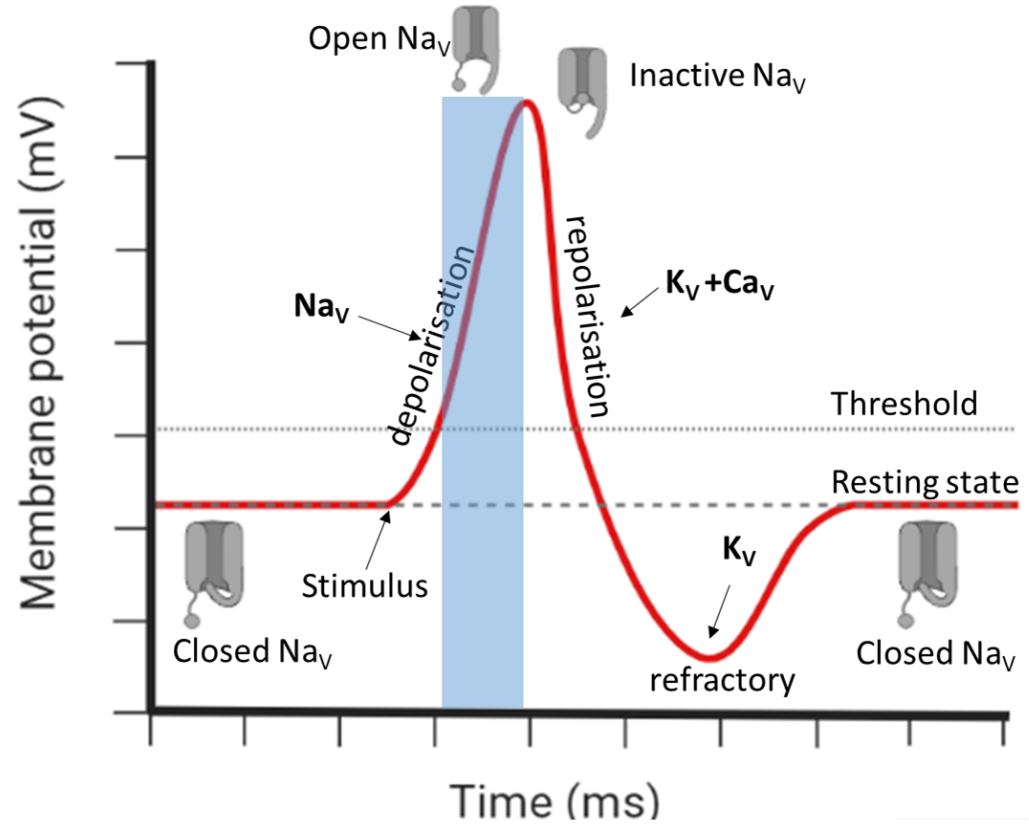
The primary afferent fibres express a variety of voltage-gated channels, including Na_V, Ca_V, K_V, which are involved in the transduction of the receptor potential into an action potential, the basic currency of communication in the nervous system. Since the action potentials are all-or-none events, information is encoded by the number of action potentials and the time intervals between them (Adrian and Zotterman, 1926a, Adrian and Zotterman, 1926b, Adrian, 1926). For nociceptors, the intensity of the noxious stimulus is encoded by the frequency of the action potentials: the more noxious the stimulus, the more action potentials with closer intervals between successive action potentials (Torebjörk et al., 1984, Yarnitsky and Ochoa, 1990).

There are 9 Na_V, 10 Ca_V, and 40 K_V genes in mammals, the majority of which have multiple splice variants with different functional characteristics (Schulz et al., 2008). The complement of these channels and those contributing to frequency modulation, for example hyperpolarisation-activated cyclic nucleotide-gated cation

channels (HCN), A-type $K_V4.3$ and $K_V3.4$ channels) underlie cell excitability and firing behaviour (threshold for action potential generation, action potential amplitude and duration, maximum firing frequency) (Hille, 1978).

1.7.1 Voltage-gated sodium channels

Na_V are of particular interest because they are essential for the transmission of sensory information from the periphery to the CNS (Gold and Gebhart, 2010, Liu and Wood, 2011). They contribute to the generator potential in the peripheral terminals and the initiation and propagation of action potentials along the axon as they amplify the generator potential, by allowing the rapid influx of sodium into the neurons, and drive the depolarising upstroke of the action potential (**Figure 1.5**) (von Hehn et al., 2012, Catterall et al., 2005). They are also involved in neurotransmitter release from central terminals (Bennett et al., 2019).


Open Na_V
Inactive Na_V
Membrane potential (mV)
Na_V
depolarisation
repolarisation
K_V+Ca_V
Threshold
Resting state
K_V
Stimulus
Closed Na_V
refractory
Closed Na_V
Time (ms)

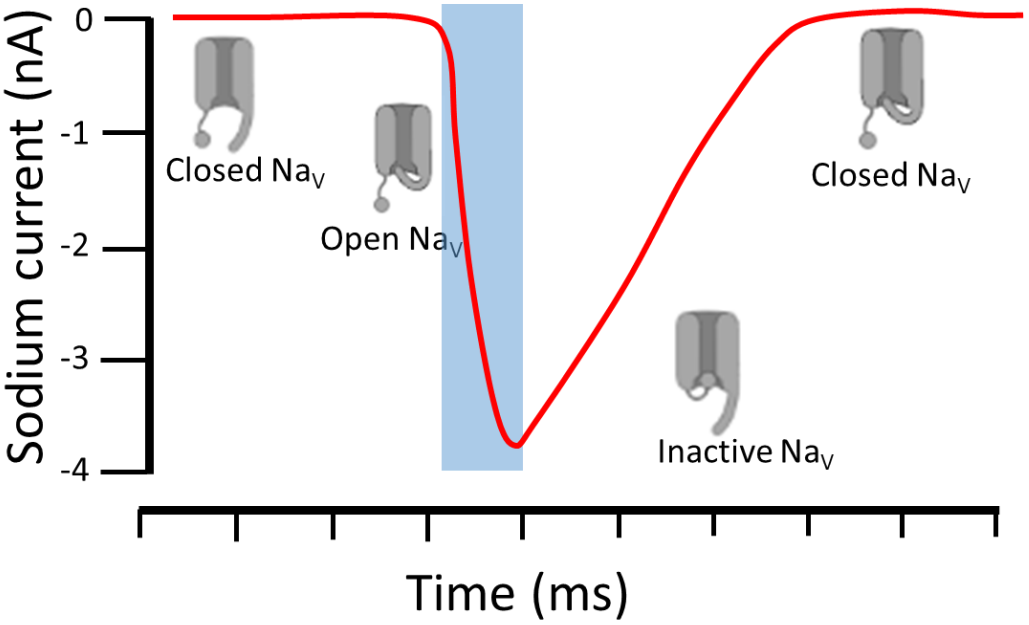

Sodium current (nA)
0
-1
-2
-3
-4
Closed Na_V
Open Na_V
Closed Na_V
Inactive Na_V
Time (ms)

Figure 1.5 Gating model and contribution of Na_V to neuronal action potential

Upper trace depicts an illustration of a whole-cell current clamp recording from a typical neuron. Lower trace is temporally aligned to the upper trace to show changes in sodium current during an action potential. At the resting membrane potential Na_V are closed. A small depolarisation of the neuronal membrane potential in response to a stimulus depolarises the neuronal membrane potential to the threshold for Na_V activation (∼−50 mV). Na_V then activate rapidly (∼1 ms to peak) allowing the influx of sodium (blue pannel) and depolarising the membrane potential further, forming the upstroke of the action potential. After that Na_V inactivate causing a decrease in sodium current and repolarisation of the membrane potential, contributing to the downstroke of the action potential. Recovery from inactivation allows the channels to participate in the next action potential. Adapted from Eijkelkamp et al. (2012). Created with Biorender.

Na_V channels are heteromultimers, consisting of a larger pore-forming α-subunit and smaller auxiliary β-subunits. The α-subunit is essential and sufficient to make a functional channel whereas the smaller auxiliary β-subunits give the channel different functional properties, such as biophysical properties, trafficking and anchoring (Catterall et al., 2005). The α-subunit family consists of 9 members ($Na_V1.1$-$Na_V1.9$) that are voltage gated. The structure of the Na_V channels is closely related to their function. It allows the channels to exist in three voltage-dependent conformational states that determine the channel conductance – open, closed and inactive (**Figure 1.5**). At hyperpolarised membrane potential, the channels will be in their closed conformation, thus not conducting. Upon depolarisation, they open for a short time period (<1 ms), which is followed by their fast inactivation (within ms). In response to prolonged depolarisation, a second form of inactivation, called slow inactivation, can occur (Vilin and Ruben, 2001). With hyperpolarisation, the channels will recover from their inactivated state and return to their closed state (Bennett et al., 2019).

Adult rodent DRG neurons have been shown to express around five Na_V subtypes, including $Na_V1.1$, $Na_V1.6$-$Na_V1.9$ (Dib-Hajj et al., 2009, Dib-Hajj et al., 2010, Bennett et al., 2019). $Na_V1.1$ and $Na_V1.6$ have been localised mostly to non-nociceptive fibres (Fukuoka et al., 2008, Fukuoka and Noguchi, 2011), while $Na_V1.3$ expression has been shown to change with development - it is expressed during embryonic

stages but then it gets downregulated and is no longer present in the adult (Waxman et al., 1994). However, it has been demonstrated that nerve injury could induce expression of $Na_V1.3$ in sensory neurons in adulthood (Waxman et al., 1994, Fukuoka et al., 2008). $Na_V1.7$-$Na_V1.9$ are the major Na_V channels at a peripheral nervous system level, with $Na_V1.8$ reported to be primarily localised to C-fibre nociceptors (Abrahamsen et al., 2008, Akopian et al., 1996, Amaya et al., 2000). More recent evidence has shown that $Na_V1.8$ is also expressed by A-fibres, which are likely to include myelinated LTMR (Shields et al., 2012). Nav1.9 has been shown to be selective for a subset of C-fibres, while $Na_V1.7$ expression has been reported universally in all sensory neurons (Fukuoka et al., 2008, Fukuoka and Noguchi, 2011). However, there is accumulating evidence for a role of $Na_V1.7$ in nociception as demonstrated by mutagenic disorders affecting it, including the gain of function mutations in $Na_V1.7$ resulting in ectopic C-fibre firing in the absence of nerve injury and spontaneous pain conditions (Cox et al., 2006, Dib-Hajj et al., 2010, Faber et al., 2012b).

Na_V channels could also be categorised based on their expression patterns and sensitivity to tetrodotoxin (TTX). The large-diameter DRG neurons of the Aβ-fibres principally express Na_V channels, which are tetrodotoxin-sensitive (TTX-S), including $Na_V1.1$, $Na_V1.6$, and $Na_V1.7$ (Black et al., 1996, Fukuoka and Noguchi, 2011, Ho and O'Leary, 2011). However, a subset of those Aβ-fibres have been shown to also express the tetrodotoxin-resistant (TTX-R) $Na_V1.8$ (Shields et al., 2012) and have been associated with the reported in rat Aβ nociceptors (Djouhri and Lawson, 2004). The small-diameter nociceptors, which includes Aδ- and C-fibre nociceptors, have been shown to express high levels of the TTX-R Na_V channels $Na_V1.8$ and $Na_V1.9$, but also the TTX-S $Na_V1.7$ and $Na_V1.6$ as well (Black et al., 2008).

1.7.1.1 Na_V channels involved in pain processing

1.7.1.1.1 $Na_V1.7$

$Na_V1.7$ expression pattern in the PNS

$Na_V1.7$ expression has been reported to be greater in nociceptive rather than non-nociceptive sensory neurons, with expression along the entire length of these neurons from the free nerve endings in the skin to presynaptic central terminals (Bennett et al., 2019). Specifically, in the DRG, 63% the C-fibre neurons and only 15% of the myelinated A-fibre neurons co-stained for $Na_V1.7$ (Goldstein et al., 1991). In nociceptors, $Na_V1.7$ expression was observed in 65% of the non-peptidergic and 58% of the peptidergic neurons (Black et al., 2012). Within the skin the free nerve terminals of unmyelinated sensory neurons displayed strong $Na_V1.7$ staining and ~27% of the peripheral branches of the unmyelinated fibres showed $Na_V1.7$ immunoreactivity (Black et al., 2012), suggesting that not all peripheral C-fibre axons express $Na_V1.7$. In the small-diameter myelinated fibres, Nav1.7 staining was particularly concentrated at the node of Ranvier. In the central branches, ~30% of the unmyelinated fibres had immunoreactivity to $Na_V1.7$ in the dorsal root (Black et al., 2012). Within the superficial dorsal horn, $Na_V1.7$ was abundantly detected in lamina I, lamina IIo, and lamina IIi within the primary afferent terminals, with all second order neurons within the dorsal horn being negative for $Na_V1.7$, suggesting that $Na_V1.7$ is only expressed in presynaptic terminals (Black et al., 2012).However, recent studies have reported expression of $Na_V1.7$ in dendrites of dorsal horn neurons, with its dorsal horn expression shown to derive from the primary afferent neurons and involve trans-neuronal transport via yet unknown mechanisms (Alles et al., 2020).

$Na_V1.7$ involvement in nociception and pain pathologies

Clinical studies have reported that loss of function mutations in the $Na_V1.7$ encoding gene, *SCN9A*, result in congenital insensitivity to pain in humans (Cox et al., 2006, Dib-Hajj et al., 2008, Ahmad et al., 2007, Drenth and Waxman, 2007). In contrast, gain of function mutations have been implicated in painful conditions like erythromelalgia, proximal extreme pain disorder (Fertleman et al., 2006, Drenth and Waxman, 2007, Estacion et al., 2008, Yang et al., 2004), small fibre neuropathy (Faber et al., 2012a) and painful diabetic neuropathy (Blesneac et al., 2018).

Preclinical studies have shown upregulation of $Na_V1.7$ in different inflammatory pain models (Black et al., 2004, Toledo-Aral et al., 1995, Gould et al., 2000, Dib-Hajj et al., 2007). Nociceptor-specific $Na_V1.7$ knockout mice have been reported to exhibit reduced sensitivity to acute noxious mechanical and to a smaller extent to noxious heat stimuli, and do not develop inflammation-induced hypersensitivity (Nassar et al., 2004). Interestingly, neuropathy-induced hypersensitivity in these mice is unaltered, suggesting involvement of other distinct Na_V channels or another subpopulation of $Na_V1.7$-expressing non-nociceptive neurons contributing to neuropathic pain (Nassar et al., 2005). Subsequent studies in modality-specific sensory neurons have provided further evidence that nociceptors-specific $Na_V1.7$ deletion leads to abolished sensitivity to noxious mechanical stimuli (Minett et al., 2012). However, they have reported unchanged sensitivity to noxious heat and cold stimuli, suggesting that nociceptor specific $Na_V1.7$ is non-essential for mediating noxious heat and cold behaviour. Global $Na_V1.7$ knockout adult mice have been shown to display a reduced sensitivity to noxious heat and mechanical stimuli as well as a reduced formalin–induced hypersensitivity (Gingras et al., 2014). These data highlight the importance of $Na_V1.7$ for the transmission of noxious stimuli to second order neurons. Electrophysiological data have demonstrated that $Na_V1.7$ contributes but is not exclusively responsible for axonal transmission and that the insensitivity to noxious stimuli is not simply due to failure to transduce such stimuli (Gingras et al., 2014).

Thus, the genetic $Na_V1.7$ ablation studies and behavioural assays in mice suggest that $Na_V1.7$ is vital for acute pain sensation and also contributes to sensitization in a number of persistent pain models. The defect in nociceptive processing is proposed to involve a failure of action potential initiation in the peripheral terminals, a failure to transmit action potentials to second order neurons in the spinal cord, and defective neurotransmitter release by presynaptic terminals.

1.7.1.1.2 $Na_V1.8$

$Na_V1.8$ expression in the PNS

$Na_V1.8$ is preferentially expressed in peripheral sensory neurons and has not been detected in non-neuronal tissues or in the CNS (Shields et al., 2012, Novakovic et al., 1998, Sangameswaran et al., 1996, Akopian et al., 1996). $Na_V1.8$ is widely expressed in small-diameter DRG neurons, most of which are functionally identified as nociceptors, but its expression has been detected also in a small percentage of medium- and large-diameter neurons (Agarwal et al., 2004, Djouhri et al., 2003, Stirling et al., 2005). Some studies in rodents have reported $Na_V1.8$ expression in 50-85% of C-fibres and in only 9.5-13% of A-fibres (Abrahamsen et al., 2008, Amaya et al., 2000, Novakovic et al., 1998). However, electrophysiological studies suggest wider $Na_V1.8$ expression in large-diameter neurons (Djouhri et al., 2003, Renganathan et al., 2000). Using reporter mice, it has been demonstrated that $Na_V1.8$ is present in 75% of DRG neurons, with expression in >90% of neurons with nociceptor markers and in a significant population (40%) of myelinated A-fibre neurons, including low-threshold mechanoreceptors essential for touch sensation (Shields et al., 2012). $Na_V1.8$ has been diffusely localised along the entire length of unmyelinated axons, particularly enriched at the free nerve endings in the skin (Rush et al., 2005, Persson et al., 2010, Klein et al., 2017). Although naïve reporter mice show no expression of $Na_V1.8$ outside the PNS (Agarwal et al., 2004), there are studies showing expression in retinal ganglion cells (O'Brien et al., 2008) as well as discrete brain regions.

$Na_V1.8$ involvement in nociception and pain pathologies

Clinical evidence has found mutations in $Na_V1.8$ linked to small-fibre neuropathy (Faber et al., 2012b, Han et al., 2014). The $Na_V1.8$ gain-of-function variants have been shown to cause hyperexcitability in DRG neurons, likely contributing to the pain experienced by the patients carrying those mutations (Huang et al., 2013a). A loss-of function mutation in $Na_V1.8$ has been reported in an erythromelalgia patient, which has been suggested to have a protective role, dampening the pain experienced by the patient, which would have been more severe in the absence of that $Na_V1.8$ mutation (Kist et al., 2016). Interestingly, variants of $Na_V1.8$ have been

found in healthy patients that have been shown to reduce DRG neurons repetitive firing, thus likely contributing to the observed higher mechanical thresholds and lower mechanical pain sensitivity in those patients (Duan et al., 2016).

Preclinical studies in global $Na_V1.8$ knockout mice have shown altered noxious mechanoreception and small deficits in thermoreception, with a delayed development of inflammatory pain hypersensitivity (Akopian et al., 1999). In addition, $Na_V1.8$ seems to be required for perception of cold stimuli, with mice lacking $Na_V1.8$ being insensitive to cold over a wide range of temperatures (Zimmermann et al., 2007). Moreover, mice with $Na_V1.8$ deletion have been shown to exhibit attenuated responses to innocuous and noxious mechanical and cold stimuli along with reduced inflammation-induced mechanical and thermal hypersensitivity (Abrahamsen et al., 2008). Also antisense oligodeoxynucleotides against $Na_V1.8$ or selective pharmacological blockade of $Na_V1.8$ have been able to attenuate CFA-induced inflammatory mechanical and heat hypersensitivity (Jarvis et al., 2007, Joshi et al., 2006). The differences in phenotype between mice lacking $Na_V1.8$ and those mice that had it deleted might reflect the involvement of multiple Na_V channel subtypes in pain transmission.

Studies in rats have reported increased axonal expression of $Na_V1.8$ following nerve injury, likely due to trafficking and axonal translation (Novakovic et al., 1998, Thakor et al., 2009). Interestingly, the changes have been reported to not be restricted to the injured nerves with evidence of $Na_V1.3$ re-expression and increased axonal $Na_V1.8$ levels in neighbouring undamaged fibres (Gold et al., 2003, He et al., 2010) as well as in central nociceptive pathways (Hains et al., 2004, Hains et al., 2005). Moreover, neuropathic pain related symptoms have been shown to be reduced with the use of antisense oligodeoxynucleotides against $Na_V1.3$ and $Na_V1.8$ (Hains et al., 2004, Lai et al., 2002). In contrast, studies in neuron specific conditional $Na_V1.3$, $Na_V1.7$ or $Na_V1.8$ knockout mice have demonstrated no changes in neuropathic pain behaviour (Nassar et al., 2005, Nassar et al., 2006).

Interestingly, there are also reports of reduced $Na_V1.8$ expression in a diabetic neuropathy STZ-induced rat model, which also exhibit mechanical allodynia and thermal hyperalgesia (Hong et al., 2004, Okuse et al., 1997). Blocking of $Na_V1.8$ in diabetic rats with A-803467 has been reported to attenuate mechanical allodynia and thermal hyperalgesia (Mert and Gunes, 2012). However, electrophysiological findings of increased TTX-R current in small diameter DRG neurons, suggest modifications of $Na_V1.8$ in the diabetic condition (Hong et al., 2004). Further evidence suggests modifications of $Na_V1.8$ in the diabetic condition, likely contributing to the hyperexcitability in DRG neurons that underlies the mechanism of metabolically driven hyperalgesia (Bierhaus et al., 2012). In addition, multiple studies have reported inflammation-induced alterations in $Na_V1.8$, including carrageenan-induced increase in $Na_V1.8$ transcript levels as well as TTX-R currents in small DRG neurons (Tanaka et al., 1998) and CFA-induced increase in $Na_V1.8$ levels in both unmyelinated and myelinated nerves (Coggeshall et al., 2004).

1.7.1.1.3 $Na_V1.9$

The characterisation of Nav1.9 has not been as extensive as other Na_V channels due to difficulties in studying its properties in isolation in native neurons.

$Na_V1.9$ expression in the PNS

Similarly to $Na_V1.8$, $Na_V1.9$ is primarily expressed in the PNS. $Na_V1.9$ is found all along the peripheral afferents, from the free nerve terminals in the skin (Coward et al., 2000, Fjell et al., 2000, Liu et al., 2001, Persson et al., 2010), through the DRG and the central terminals within Lamina IIo in the spinal cord (Amaya et al., 2006). The expression of $Na_V1.9$ is tightly regulated with its preferential expression in rodent small diameter DRG neurons, including nociceptors (Benn et al., 2001, Fang et al., 2002, Fang et al., 2006). Although $Na_v1.9$ is predominantly found in unmyelinated C-fibres, it has been also localised to nociceptive Aδ- and Aβ- fibres, including at some nodes of Ranvier (Amaya et al., 2006, Fang et al., 2002, Fjell et al., 2000).

$Na_V1.9$ involvement in nociception and pain pathologies

The role of $Na_V1.9$ in human pain has been supported by findings linking mutations in $Na_V1.9$ and human pain. $Na_V1.9$ mutations have been identified in patients with rare pain diseases and in individuals with painful peripheral neuropathy. Gain-of-function mutations in $Na_V1.9$ have been found in individuals with inherited pain disorder with onset in early childhood (Leipold et al., 2015, Han et al., 2017) or adult-onset peripheral neuropathy (Han et al., 2015, Huang et al., 2014). In addition, mutations in $Na_V1.9$ have been also reported in patients with clinical phenotypes resembling erythromelalgia (Zhang et al., 2014b), with microneurography studies showing increased C-fibre excitability, suggesting these mutations give gain-of-function attributes (Kleggetveit et al., 2016).

Initial preclinical studies in $Na_V1.9$ global knockout mice have demonstrated normal responses to acute noxious mechanical and thermal (both heat and cold) stimuli (Amaya et al., 2006, Leo et al., 2010, Priest et al., 2005). However, they show deficiencies in the development of inflammation-induced noxious thermal (both heat and cold), but not mechanical hypersensitivity (Amaya et al., 2006, Priest et al., 2005, Dib-Hajj et al., 2002). Subsequent studies have shown that these mice can present with a deficit in mechanical pain thresholds if the stimulus is presented at a different location, i.e. tail rather than hindpaw (Minett et al., 2014), or when the stimuli where given at slow ramp, which could reflect the slow $Na_V1.9$ kinetics (Hoffmann et al., 2017). Interestingly, neuropathic pain hypersensitivity in these mice has been shown to develop similarly to controls. In addition to inflammatory pain, $Na_V1.9$ channels have been also shown to be upregulated in large-diameter DRG neurons in the STZ-induced diabetic rat mode (Craner et al., 2002).

1.7.2 C-fibre activity-dependent slowing

The voltage-gated ion channels Na_V, Ca_V, K_V, are involved in the transduction of the receptor potential into action potentials (AP), the basic currency of communication in the nervous system, with different Na_V subtypes also playing an essential role in the transmission of AP along the axon to the spinal cord. Since the action potentials

are all-or-none events, the intensity of a stimulus is encoded by the number of action potentials and the time intervals between them (Adrian and Zotterman, 1926a, Adrian and Zotterman, 1926b, Adrian, 1926). Interestingly, the nociceptive C-fibres display a phenomenon that causes a progressive reduction in the conduction velocity of AP, propagating along the axon, termed activity-dependent slowing (ADS), which occurs following repetitive stimulation at frequencies of 1Hz and above and manifests as a progressive increase in C-fibre response latency (Gee et al., 1996, Serra et al., 1999, Thalhammer et al., 1994, Weidner et al., 1999). This C-fibre ADS phenomenon has been observed in unmyelinated afferent fibres in a number of species, including human (Grafe et al., 1997, Hilliges et al., 2002, Jørum et al., 2007, Orstavik et al., 2003, Schmelz et al., 2000, Serra et al., 1999, Weidner et al., 1999, Shim et al., 2007, Campero et al., 2011, Namer et al., 2009, Obreja et al., 2010), monkey (Ringkamp et al., 2010), pig (Obreja et al., 2010, Obreja et al., 2011b, Obreja et al., 2011a), rabbit (Zhu et al., 2009), rat (Gee et al., 1996, Grafe et al., 1997, Raymond et al., 1990, Thalhammer et al., 1994, Takigawa et al., 1998, Nakatsuka et al., 2000, De Col et al., 2008, Hoffmann et al., 2015) and mouse (Shim et al., 2007, Mazo et al., 2013, Hoffmann et al., 2015, Hoffmann et al., 2017, Hoffmann et al., 2016, De Col et al., 2018). C-fibre ADS can be used as a tool to functionally classify C-fibres subtypes, with mechano-sensitive C-fibres (CM) displaying minimal degree of ADS, while mechano-insensitve (CMi), silent, exhibiting the greatest magnitude of ADS (Weidner et al., 1999, Weidner et al., 2000, Weidner et al., 2002, Bostock et al., 2003, Obreja et al., 2010). C-fibre ADS has been shown to occur in a frequency- and length-dependent manner, with the greatest amount of ADS observed at higher frequencies (Gee et al., 1996, Thalhammer et al., 1994, Serra et al., 1999, Weidner et al., 1999, Dickie et al., 2017) and over longer distances (Zhu et al., 2009, Schmelz et al., 1995, Dickie et al., 2017).

C-fibre ADS has been shown to occur not only in response to repetitive electrical stimulation, but also in response to natural thermal (both heat and cold) stimuli (Thalhammer et al., 1994), suggesting a physiological role for this phenomenon. In addition, ADS has also been proposed to provide a ‘dynamic memory’ of previous

levels of activity, which can influence subsequent responses. Specifically, low-level C-fibre activity, comparable to injury-induced spontaneous C-fibre firing, can induce C-fibre ADS that can thus dynamically influence responses to subsequent higher frequency stimuli (Weidner et al., 2002). Given that the firing frequency of C-fibres encodes pain intensity (Yarnitsky and Ochoa, 1990, Torebjörk et al., 1984), with C-fibre ADS essentially regulating the intervals between successive action potentials being relayed along the C-fibre nociceptive axon, from the periphery to the spinal cord, it has been hypothesised that C-fibre ADS can influence the spinal processing of noxious stimuli as well as the ongoing activity of C-fibres that can drive central sensitisation. As greater ADS magnitude is associated with an increase in conduction failure probability (Nakatsuka et al., 2000, Raymond et al., 1990, Zhu et al., 2009, Obreja et al., 2011a), ADS has also been suggested to act as a form of self-inhibition, potentially limiting nociceptive input to the CNS and influencing central sensitisation (De Col et al., 2012, Mazo et al., 2013). Moreover, studies from this lab have demonstrated that C-fibre ADS can alter the timing of nociceptive inputs being relayed from the periphery to the spinal cord and that ADS can influence spinal summation, with an increased ADS profile limiting spinal summation, suggesting that C-fibre ADS can potentially alter spinal pain processing (Dickie et al., 2017). Interestingly, this study also reported that the magnitude of C-fibre ADS is not only frequency- and length-dependent, but it is also dependent on sex, with female juvenile rats displaying more pronounced baseline C-fibre ADS. Given the demonstrated sex differences in the levels of C-fibre ADS along with the accumulating evidence for sex-differences in acute and chronic pain mechanism (**section 1.4**), it is important to investigate sex differences in C-fibre ADS in different types of pain models and the potential influence of C-fibre ADS on pain hypersensitivity in these models (see thesis aims and hypothesis, **section 1.13**).

1.7.2.1 Injury-induced alterations in C-fibre ADS

1.7.2.1.1 C-fibre ADS alterations in patients

C-fibre ADS has been shown to be altered in chronic pain patients, with majority of the studies showing enhancement of ADS in patients when compared to healthy

subjects (Orstavik et al., 2003, Ørstavik et al., 2006, Kleggetveit et al., 2012a, Serra et al., 2014, Namer et al., 2015, Kist et al., 2016, Namer et al., 2017). Erythromelalgia patients have been shown to exhibit greater C-fibre ADS than control subjects (Orstavik et al., 2003), with studies in erythromelalgia patients with $Na_V1.7$ mutation showing a reduction in ADS in comparison to other erythromelalgia patients and healthy subjects (Namer et al., 2015). Fibromyalgia patients have also been shown to exhibit an abnormally high ADS compared to patients with small fibre neuropathy and controls (Serra et al., 2014). In painful diabetic neuropathy patients it has been shown that there is an enhanced recovery from C-fibre ADS in comparison to patients with non-painful diabetic neuropathy and healthy controls (Ørstavik et al., 2006). Similarly, polyneuropathy patients with pain have been demonstrated to exhibit faster ADS recovery than those without pain (Kleggetveit et al., 2012a). In Fabry disease patients there were no differences in ADS, except for CMiHi (mechano and heat insensitive C-fibres) fibres, which displayed reduced slowing in comparison to healthy patients (Namer et al., 2017). Interestingly, the latter study is the only one conducted in male subjects only, while the remaining studies have been conducted in either females only or in mixed samples of males and females. However, none of the studies have explored the effect of C-fibre ADS based on sex, which given the evidence for sex-dependent C-fibre ADS levels (Dickie et al., 2017) and the accumulating evidence for sex differences in chronic pain mechanisms (**section 1.4**), would be an important factor to be considered when studying disease associated alterations in C-fibre ADS.

1.7.2.1.2 ADS alterations in preclinical pain models

Preclinical studies of inflammatory and neuropathic pain have reported both increased and decreased C-fibre ADS profiles. Our lab (Galley et al., 2017) and others (Wang et al., 2016, Shim et al., 2007, Sun et al., 2012b, Hulse, 2016) have demonstrated that C-fibre ADS is altered in models of neuropathic pain with most studies reporting a reduction in ADS. In the peripheral saphenous nerve ligation injury (PSNI) model, ADS has been shown to be reduced in the mechanosensitive C-fibres (Hulse, 2016). In the paclitaxel-induced neuropathic pain model, C-fibre

activity-dependent showing has also been shown to be reduced following *in vivo* treatment with paclitaxel in comparison to vehicle treatment (Galley et al., 2017). In diabetic neuropathy models specifically it has been shown that there is a reduction in ADS. In sensory neurons from streptozotocin (STZ)-induced diabetic rats it has been demonstrated that there is a reduction in the α-DTX-sensitive K^+ currents, which potentially contributes to the enhanced excitability of the C-fibres accompanied by their decreased ADS (Wang et al., 2016). Moreover, in the same diabetic model it has been demonstrated that diabetic high-firing frequency C-fibres have reduced ADS in comparison to controls (Sun et al., 2012b). In addition, a recent study exploring UVB-irradiation-induced hyperalgesia in pigs has also reported a reduction in ADS (Werland et al., 2021).

In contrast to these studies, other preclinical investigations have contrasting findings, with enhancement of ADS post-injury. In the Zucker Diabetic Fatty rats microneurography studies have shown enhancement of ADS in the mechano-insensitive C-fibres (Garcia-Perez et al., 2018). Also, in the spinal nerve ligation (SNL) neuropathic pain model, where L5 dorsal root is lesioned, ADS is enhanced in the uninjured L4 dorsal root compared to sham operated rats (Shim et al., 2007).

All of these preclinical C-fibre ADS studies have been conducted in male animals only. Interestingly, the sex-dependent C-fibre ADS levels have also been shown to depend on presence of injury, with CFA hindpaw inflammation demonstrated to reduce C-fibre ADS levels in females only males (Dickie et al., 2017). These sex- and injury-dependent C-fibre ADS findings correlated with CFA-induced more pronounced heat but not mechanical hypersensitivity in females compared to males (Dickie et al., 2017). Thus, considering these sex-and injury-dependent differences in C-fibre ADS along with the evidence for sex-differences in acute and chronic pain mechanism (**section 1.4**), it is important to study sex-and injury-dependent differences in C-fibre ADS in different pain models in association with sex-and injury-dependent differences in heat sensitivity, as well as the C-fibre ADS related

mechanism potentially contributing to the observed pain hypersensitivity in these models (see thesis aims and hypothesis, **section 1.13**).

1.7.2.2 Mechanisms underlying C-fibre activity-dependent-slowing

The physiological mechanisms underlying C-fibre ADS have not been fully understood, but early hypotheses have proposed that an increased activity of Na+/K+ ATPase following repetitive stimulation can drive axonal hyperpolarisation, which has been proposed to account for the observed ADS (Kobayashi et al., 1997, Gee et al., 1996). However, direct or indirect Na+/K+ ATPase inhibition was later shown to facilitate rather than attenuate ADS. More recent evidence has suggested involvement of Na_V (De Col et al., 2008, Obreja et al., 2012, Tigerholm et al., 2014b, Tigerholm et al., 2015, Petersson et al., 2014) and/or hyperpolarization-activated cyclic nucleotide-gated (HCN) channels (Grafe et al., 1997, Mazo et al., 2013, Takigawa et al., 1998, Zhu et al., 2009) in the C-fibre ADS phenomenon.

1.7.2.2.1 Na_V channels implicated in C-fibre activity-dependent slowing

C-fibre ADS has been associated with the recovery phase of the action potential, the cumulative inactivation of Na_V and the decrease in their availability (Blair and Bean, 2003, De Col et al., 2008). Pharmacological studies using drugs that at low doses stabilise the slow inactivation state of Na_V, such as the local anaesthetic lidocaine and the anti-convulsant carbamazepine, have demonstrated that the stabilisation of the slow inactivation Na_V can cause an increase in the initial C-fibre response latency and an enhancement of C-fibre ADS (Cardenas et al., 2006, Sandtner et al., 2004). More recent pharmacological studies have suggested that the increasing numbers of Na_V entering a sustained slow inactivated state can explain the ADS phenomenon (De Col et al., 2008, Obreja et al., 2012).

However, subsequent modelling studies proposed that, while Na_V slow inactivation might be involved in ADS, the increased intra-axonal Na^+ concentration likely plays a more important role in ADS (Tigerholm et al., 2014a, Tigerholm et al., 2015). A combined experimental and modelling study comparing the mechanoinsensitive (CMi), displaying greatest amount of ADS (Weidner et al., 1999, Obreja et al., 2010),

and the mechanosensitive (CM), displaying minimal degree of ADS (Weidner et al., 1999, Obreja et al., 2010), C-fibre subtypes revealed that a more pronounced ADS profile is associated with more $Na_V1.8$ and less $Na_V1.7$, more delayed rectifier potassium channels and less Na+/K+ ATPase, a profile consistent with increased intra-axonal Na^+ concentration (Petersson et al., 2014). The study concluded that it is the ratio of $Na_V1.7$ to $Na_V1.8$ rather than absolute levels of either channel that determines the degree of ADS. In line with this, C-fibre ADS has been shown to be altered in $Na_V1.8$ knockout mice (Hoffmann et al., 2016). Furthermore, pharmacological studies using low-dose TTX (De Col et al., 2008, Baker and Waxman, 2012b), that will act on the TTX-sensitive $Na_V1.7$ ion channels, and studies using conditional deletion of $Na_V1.7$ in sensory neurons (Hoffmann et al., 2018), thereby pharmacologically or genetically modifying the $Na_V1.7/Na_V1.8$ ratio, respectively, have demonstrated that these manipulations cause a reduction in the C-fibre conduction velocity and also alter C-fibre ADS (De Col et al., 2008, Obreja et al., 2012, Tigerholm et al., 2014b).

1.7.2.2.2 HCN channels involved on C-fibre activity-dependent slowing

Although majority of the research supports the hypothesis that C-fibre ADS implicates the Na_V channels, there is some evidence suggesting that the HCN channels, which mediate the hyperpolarisation-activated current (I_h), can also influence the C-fibre ADS phenomenon (Grafe et al., 1997, Mazo et al., 2013, Takigawa et al., 1998, Zhu et al., 2009). It has been hypothesised that ADS can be a consequence of the prolonged after-hyperpolarisation, mediated by Ca^{2+}-dependent K^+ currents following repetitive stimulation (Gee et al., 1996, Soleng et al., 2003). Given the findings of altered C-fibre ADS following blockade of the I_h or inhibition of the HCN, the prolonged after-hyperpolarisation has been suggested to be counteracted by I_h (Grafe et al., 1997, Takigawa et al., 1998, Zhu et al., 2009, Soleng et al., 2003, Mazo et al., 2013). In addition, given the CNS evidence of HCN-induced neuronal depolarisation, which modulates Na_V, Kv and Cav functional states, all of which are critical for nerve signal conduction (Ko et al., 2016, George et al., 2009, Hu and Bean, 2018, Huang et al., 2011, Garden et al., 2018), and in the

case of Na_V for C-fibre ADS (see section), the role of HCN in C-fibre ADS can also be indirect via alterations in Na_V.

1.8 Spinal cord processing and organisation of the dorsal horn

The primary afferents terminate in a somatotopic manner in the dorsal horn of the spinal cord, with the majority of the nociceptors terminating in laminae I and II (**Figure 1.4**), where the nociceptive information is relayed to second order dorsal horn neurons that convey nociceptive information to higher brain centres (Basbaum et al., 2009, Todd, 2010, Abraira and Ginty, 2013). The synaptic transmission between the primary afferent fibres and the dorsal horn of the spinal cord is excitatory (Basbaum et al., 2009). The central branches of the primary afferents release glutamate (Glu) as their primary excitatory neurotransmitter, which binds to and activates postsynaptic primarily ionotropic (AMPA, NMDA, kainite) and occasionally metabotropic glutamate receptors (mGluR) on second order neurons, thus generating excitatory post-synaptic currents (EPSCs) (Basbaum et al., 2009, Latremoliere and Woolf, 2009, Larsson and Broman, 2011).

Repetitive or high-frequency stimulation of C-fibres can drive presynaptic release of peptides such as substance P (SP) and calcitonin gene-related peptide (CGRP), which bind to the postsynaptic neurokinin-1 receptor (NK1-R) and CGRP1 receptor (CGRP1-R), respectively, and are important in central pain processing mechanisms in injury-induced conditions (Larsson and Broman, 2011, Latremoliere and Woolf, 2009). For example, SP-induced activation NK1-R has been shown to cause long-lasting membrane depolarisation(Henry, 1976) and to be involved in the temporal summation of C-fibre-evoke synaptic potential (Dougherty and Willis, 1992, Xu et al., 1992a, Xu et al., 1992b). CGRP has been demonstrated to potentiate the SP effects and to be involved in intracellular signalling important for central sensitisation (Sun et al., 2003, Sun et al., 2004) (section 1.10).

The dorsal horn of the spinal cord consists of four major neuronal components essential for sensory information processing: the primary afferents central terminals, projection neurons, interneurons (excitatory or inhibitory) and axons descending from supraspinal regions (Todd, 2010). The incoming afferent information is processed by excitatory and/or inhibitory interneurons as well as by descending input from the brainstem, thus influencing the ascending information being sent to the brain via the projection neurons (Todd, 2010). In addition to transmitting sensory information to higher centres, output from the dorsal horn also mediates the reflex response, which involves the primary afferent fibres synapsing onto interneurons in the dorsal horn, which in turn synapse with motor neurons in the ventral horn, generating a motor reflex to withdrawal from an aversive stimulus (Sherrington, 1910, Stein, 2005).

The central axons of the DRG neurons enter the dorsal horn of the spinal cord via the dorsal root and branch to innervate multiple spinal segments in the rostral and caudal direction, which predominantly terminate on relay neurons and local interneurons important for signal processing in laminae I, II and V (Basbaum et al., 2009). The central terminals of the primary afferents terminate in the dorsal horn of the spinal cord in a distinct pattern (**Figure 1.4**), with different classes of afferents projecting to distinct laminae.

The dorsal horn of the spinal cord includes laminae I-VI, while laminae VII-X comprise the ventral horn of the spinal cord (Rexed, 1952) (**Figure 1.4**). The dorsal horn could be further subdivided into the superficial and deep lamina, which contain laminae I-II and laminae III-VI, respectively. The superficial lamina receives primarily nociceptive inputs from the Aδ- and C-fibre afferents and is thus essential in the central processing of nociceptive information (Todd, 2010), with peptidergic C-fibres projecting predominantly to lamina I and the outer layer of lamina II (IIo) and non-peptidergic C-fibres – to lamina II (Lorenzo et al., 2008). However, there are reports of a small population of non-peptidergic C-fibres targeting lamina I (Saeed and Ribeiro-da-Silva, 2012). The nociceptive Aδ-fibres have been shown to

terminate largely in lamina I (Light and Perl, 1979), with a population of peptidergic Aδ-fibres reported to target lamina I and the outer layer of lamina II (IIo) (Lawson et al., 1997). In addition, some Aδ- and Aβ- hair follicle afferents appear to project to the inner layer of lamina II (IIi) (Brown et al., 1981, Light and Perl, 1979).

Lamina I, also known as the marginal layer, is made of populations of interneurons and projection neurons, with the majority (~80%) of the lamina I projection neurons expressing the neurokinin 1 receptor (NK1-R), which binds SP (Spike et al., 2003, Al-Khater et al., 2008). The lamina I NK1-R-expressing projection neurons (NK1-R+) have been shown to play an important role in chronic pain manifestation (Nichols et al., 1999). Lamina II, which is composed of the inner (Iii) and outer (IIo) layers, is made of excitatory (glutamatergic) and inhibitory (GABAergic and/or glycinergic) interneurons. The inhibitory interneurons and excitatory interneurons play complementary and opposing roles in transmitting somatosensory information, such that excitatory interneurons are essential for cutaneous sensory transmission, while the inhibitory interneurons gate sensory transmission, via both pre- and post-synaptic mechanisms.

Early studies tried to classify laminal II individual neuronal populations based on dendritic morphology into four classes of interneurons, including islet, central, radial and vertical cells (Grudt and Perl, 2002, Light et al., 1979). However, those morphological characteristics did not correlate very well with other neuronal properties and were concluded to be not selective enough to attribute sensory roles to intraneuronal subpopulations (Grudt and Perl, 2002). Subsequent molecular and genetic advances allowed to differentiate intraneuronal subpopulations based on their expression of neurochemical and neuropeptidergic markers (Todd, 2010). Multiple studies have identified molecular markers that differentiate subpopulations of inhibitory and excitatory interneurons that make up the spinal somatosensory circuitry (Abraira and Ginty, 2013, Del Barrio et al., 2013, Wildner et al., 2013, Häring et al., 2018). However, the spinal circuits for nociceptive perception are still poorly understood.

The superficial dorsal horn consists primarily of excitatory interneurons that may be involved in the amplification of nociceptive signals (Koch et al., 2018). These have been identified based on the presence of some neurochemical markers such as cholecystokinin (CCK), gastrin releasing peptide (GRP), and somatostatin (SOM)(Koch et al., 2018). The deep dorsal horn is composed of interneurons and projection neurons, with interneurons comprising majority of lamina III (Todd, 2010). Most of the lamina III interneurons are inhibitory and 40% of these are immunoreactive to GABA (Polgár et al., 2003). Although there are many glycine-positive interneurons, they mostly co-express GABA as well, with only a few inhibitory interneurons expressing glycine alone (Polgár et al., 2003, Todd and Sullivan, 1990). The inhibitory interneurons differentiate into several groups of deep dorsal horn inhibitory interneurons that are defined by the expression neurochemical marker such as neuropeptide Y (NPY), galanin and dynorphin (DYN) (Koch et al., 2018). It has been shown that many excitatory interneurons in lamina III as well as in laminae I-II express vesicular glutamate receptor 2 (VGLUT2) (Maxwell et al., 2007, Todd et al., 2003, Yasaka et al., 2010). In addition, a subgroup of excitatory interneurons that express protein kinase Cγ (PKC) has been identified in laminae III and IIi (Polgár et al., 1999), which have been shown to receive afferent input from A-fibre LTMR (Hughes et al., 2003, Neumann et al., 2008).

Throughout the deeper laminae (laminae III-VI) the number of projection neurons is much less than that in lamina I. Two subgroups of those have been shown to express NK1-R. One subgroup has large dendrites reaching as far as lamina I and has been demonstrated to be targeted by peptidergic C-fibre afferents (Sakamoto et al., 1999, Naim et al., 1997) and thus is considered to be activated by noxious stimuli (Doyle and Hunt, 1999, Mantyh et al., 1995, Polgár et al., 2007). The other subgroup has smaller dendrites and has been reported to respond to non-noxious stimuli (Doyle and Hunt, 1999, Mantyh et al., 1995). These have been suggested to receive predominantly monosynaptic Aβ-fibre inputs (Torsney and MacDermott, 2006).

1.9 Peripheral sensitisation

Diseases, tissue injury or inflammation can alter the properties of the primary afferent fibres, resulting in increased spontaneous activity, altered conduction or altered neurotransmitter properties (Basbaum et al., 2009). These changes usually increase the excitability of the peripheral terminal of the primary afferents and this hyperexcitability is known as peripheral sensitisation (Woolf and Ma, 2007, Latremoliere and Woolf, 2009).

Peripheral sensitisation more commonly results from inflammation-induced changes in the chemical environment of the nerve fibre (McMahon et al., 2006). The injured tissue and/or immune cells release a number of inflammatory mediators, including bradykinin, prostaglandins, adenosine triphosphate (ATP), 5-hydroxytryptamine (5-HT), tumor necrosis factor-α (TNF-α), nerve growth factor (NGF), H^+, interleukin-1β (IL-1β), interleukin-6 (IL-6) and proinflammatory cytokines (Gold and Gebhart, 2010, Woolf and Ma, 2007, Stein et al., 2009). These inflammatory mediators, collectively known as the 'inflammatory soup', activate their corresponding receptors on the peripheral terminals, directly activating or indirectly modulating the activity of ion channels, receptors and second messenger cascades implicated in the transduction of nociceptive signals (Basbaum et al., 2009, Mizumura et al., 2009, Richardson and Vasko, 2002, Rush et al., 2007).

The alterations in the peripheral afferents could occur very fast (within mins) via the activation of intracellular cascade, including protein kinase A (PKA), protein kinase C (PKC), extracellular signal-regulated kinase (ERK) and p38, which can sensitise Na_V and TRP channels in particular via phosphorylation. These rapid changes allow the somatosensory nervous system to dynamically respond to injury (Basbaum et al., 2009, Woolf and Ma, 2007, Cheng and Ji, 2008). Longer-term changes in excitability could also occur. They are the result of changes in gene expression, which can increase the expression of key transduces such as TRP and Na_V channels and the expression of nociceptive neuropeptides such as SP (Basbaum et al., 2009, Woolf and Ma, 2007).

Altogether these changes cause the increased excitability of the peripheral nerve fibres which manifests as reduced activation thresholds, increased spontaneous activity, and enhanced evoked responses (Meyer et al., 2006). The decreased thermal thresholds in the primary area are due in part to sensitisation of TRPV1 and TRPA1 by various inflammatory mediators (Schmidt et al., 2009, Dai et al., 2007). Despite TRPV1 being not the only transducer of acute noxious heat, it is the major contributor to the development of heat hyperalgesia, possibly due to its expression in polymodal neurons involved in neurogenic inflammation and modifying central connections (Caterina et al., 2000, Davis et al., 2000). In addition to reducing the afferent fibres activation thresholds, inflammatory mediators are also able to recruit normally 'silent' C fibres (C-MiHi), making them sensitive to mechanical and heat stimuli via potential long-lasting changes in second messenger signalling cascades and nociceptors sensitisation (Schmidt et al., 1995, Schmelz et al., 2000).

Inflammatory mediators could also alter the activity-dependent slowing (ADS) phenomenon of the C-fibres that could in turn impact spinal processing of the peripheral signals (Dickie et al., 2017). The increased excitability and spontaneous activity of the primary afferent fibres could disrupt the balance between the spinal excitatory and inhibitory inputs, ultimately resulting in the increased spinal cord excitability, known as central sensitisation (Woolf, 1983, Latremoliere and Woolf, 2009). While peripheral sensitisation is thought to underlie thermal hypersensitivity and primary hyperalgesia (hypersensitivity within the injured zone), central sensitisation is thought to be involved in mechanical hypersensitivity and secondary hyperalgesia (hypersensitivity in uninjured tissue around the injured zone) (Lewin et al., 2004).

1.10 Central Sensitisation

Until the 1980s it was believed that pain and its intensity and duration reflect the degree and timing of nociceptor activation. Based on this hypothesis it was suggested that noxious stimuli were required to produce pain, but following injury-induced peripheral sensitisation their sensitivity was increased, and as a result they

were able to respond to less intense (innocuous) stimuli. However, the discovery of central sensitisation, a form of a long-lasting synaptic plasticity in the spinal dorsal horn triggered by nociceptors that facilitate the processing of nociceptive information (Woolf, 1983), has revolutionised this theory (von Hehn et al., 2012). It was realised that the amplification of peripheral nociceptive information within the CNS plays a very crucial role in generating pain hypersensitivity states. Central sensitisation was able to explain how low threshold primary afferents can induce pain, why there is a spread of sensitivity beyond the area of injury, why repeated stimuli at fixed intensity could result in a progressive increase in pain, and why pain could persist beyond the initial peripheral stimulus (Pfau et al., 2011, Seal et al., 2009, Woolf, 2011).

As described (**section 1.9**) injury can induce changes in C-fibres activity and increase their spontaneous activity. These changes in C-fibre function can drive changes in the dorsal horn of the spinal cord, which can ultimately give rise to spinal cord hyperexcitability, i.e. central sensitisation (Woolf, 1983). Spinal cord hyperexcitability is proposed to enable Aβ and Aδ afferents to access nociceptive circuits and thus mediate mechanical allodynia and mechanical hyperalgesia, respectively (Latremoliere and Woolf, 2009, Treede and Magerl, 2000). The altered mechanical sensitivity, unlike the peripherally induced alterations in heat sensitivity, is suggested to be a major feature of central sensitisation (Torsney and MacDermott, 2006, Torsney, 2011). However, the exact circuitry involved in this process is still not fully understood.

Central sensitisation was first discovered by Woolf (1983) when he used electrophysiological recordings from rat α-motoneuron efferent fibres (allowed measuring the flexion-withdrawal reflex) to measure the output of the nociceptive system and showed that repetitive heat stimulation of the hindpaw, which is sufficient to cause inflammation, resulted in long lasting hypersensitivity. The latter was characterised by an increase in the α-motoneurons spontaneous firing, an enlargement of their receptive fields as well as a reduction in their activation

thresholds, with innocuous stimuli now being able to trigger responses. To confirm that the observed changes were due to central rather than peripheral changes in nociceptive processing the following experiments were conducted:

1) Selective activation of Aβ-fibres, which normally would not trigger responses, evoked responses in α-motoneurons after the condition noxious heat stimulation of the hindpaw.
2) Peripheral nerve block using local anaesthetic to the injured hindpaw was not able to reverse the expansion of the receptive fields.
3) Selective C-fibre activation using short low-frequency electrical stimulation was sufficient to elicit similar changes to those seen following the noxious heat stimulation of the hindpaw.

These findings show that C-fibre nociceptors activation drives plasticity in the dorsal horn of the spinal cord, which thereafter allows the nociceptive system to respond to stimuli outside the injured area and to low-threshold afferents, which previously were not able to activate the system. This gave rise to a more general hypothesis that brief trains of nociceptive C-fibre input could produce activity-dependent changes in the function of the dorsal horn neurons, which could lead to a long-lasting sensitisation of the nociceptive system (central sensitisation) and pain hypersensitivity. Subsequent studies have shown that the activity of dorsal horn neurons could be altered in a similar manner to the changes seen in α-motoneurons (Dougherty and Willis, 1992) .

The original Woolf studies referred to central sensitisation as an activity- or use-dependent form of functional synaptic plasticity that resulted in pain hypersensitivity following an intense noxious stimulus. This plasticity is suggested to be triggered by the activity induced in dorsal horn neurons by C-fibre nociceptors input such as repeated noxious thermal stimulation (>49^{o}C) (Woolf, 1983) and repetitive electrical C-fibre activation (1Hz for 10-20secs) (Wall and Woolf, 1984). Subsequent studies have shown that central sensitisation can be induced by various stimuli including capsaicin (acting through TRPV1 channels) (LaMotte et al., 1991, Lin et al., 1999), mustard oil or formalin (acting via TRPA1 channels) (Jordt et al.,

2004, McNamara et al., 2007). It became clear that in order for the noxious stimuli to induce central sensitisation they must be intense, repeated and sustained (Latremoliere and Woolf, 2009). Although peripheral tissue damage is not essential for induction of central sensitisation, the intensity of injury-inducing stimulation in most cases is enough to cause central sensitisation, which is very prominent after surgical injury.

The importance of C-fibre activity in driving pain hypersensitivity has been further supported by genetic studies in mice, showing that lack of $Na_V1.8$-expressing neurons (this includes primarily C- but also A-fibres) leads to no develop of thermal or mechanical hypersensitivity following CFA-induced inflammation and thermal hypersensitivity following carrageenan or NGF injections (Abrahamsen et al., 2008, Shields et al., 2012). However, nerve injury-induced mechanical and thermal hypersensitivity were not altered in these mice, suggesting that the $Na_V1.8$-expressing afferents are important for inflammatory but not neuropathic pain.

1.11 Mechanisms of central sensitisation

Central sensitisation occurs at a number of CNS sites in addition to the spinal dorsal horn, but given this thesis focuses on afferent spinal processing, spinal cord mechanisms of central sensitisation will be introduced in more depth. There are a number of processes in the dorsal horn of the spinal cord that could lead to central sensitisation and these include alterations in sensory inputs, increased excitability or reduced inhibition (Latremoliere and Woolf, 2009).

1.11.1 Altered sensory input to the spinal cord

Activity-dependent central sensitisation is typically induced by a burst of nociceptors activity lasting several tens of seconds and includes establishment of both hetero- and homosynaptic facilitation.

1.11.1.1 Heterosynaptic facilitation of sensory input

A key player in central sensitisation is heterosynaptic facilitation, which reflects the spread of the change in synaptic strength from activated to non-activated

neighbouring synapses (Latremoliere and Woolf, 2009). It has been demonstrated that repetitive activity in one group of C-fibre afferents allowed a different group of Aβ-fibres to novelly drive nociceptive spinal cord circuits (Woolf, 1983). In addition, administration of local anaesthetic (xylocaine) has been reported to not affect the noxious stimuli-induced expansion of the receptive fields, suggesting that the activity in one group of afferents caused central changes that led to the recruitment of a different group of afferents outside the area of injury.

It has been considered that heterosynaptic facilitation is an important determinant in the development of inflammatory and neuropathic pain hypersensitivity. It has been suggested that repetitive activity in the C-fibre nociceptors drives hyperexcitability in the spinal cord, which in turn allows Aβ- and Aδ-fibres to gain access to circuits involved in mediating allodynia and hyperalgesia, respectively (Latremoliere and Woolf, 2009, Treede and Magerl, 2000, Torsney and MacDermott, 2006, Torsney, 2011).

1.11.1.2 Homosynaptic facilitation of sensory input

Homosynaptic facilitation, where stimulation results in potentiation of only those synapses that have been stimulated, is another facilitatory mechanism contributing to central sensitisation (Sandkühler, 2010). While heterosynaptic facilitatory mechanisms have been shown to underlie secondary hyperalgesia and allodynia, primary hyperalgesia has been shown to involve homosynaptic facilitatory mechanisms (Latremoliere and Woolf, 2009). The homosynaptic facilitation of C-fibre input has been suggested to lead to amplification of nociceptive inputs, thus providing a mechanism for primary hyperalgesia. Homosynaptic facilitation has been suggested to have similar features as long term potentiation (LTP) (involved in learning and memory) on cortical neurons (Sandkühler, 2010, Ruscheweyh et al., 2011, Ohnami et al., 2011), but the two processes have unique differences in their underlying mechanisms, duration and purpose, which does not allow for a comparison between the two.

Studies have shown that high frequency (100Hz) C-fibre stimulation significantly potentiates evoked C-fibre potentials in the superficial spinal cord, which points to summation of mainly monosynaptically-evoked postsynaptic currents (Liu and Sandkühler, 1997). In addition, subsequent studies have found that transection- or crush-induced nerve injury (Zhang et al., 2004, Zhou et al., 2010) and capsaicin or formalin hindpaw injections (Ikeda et al., 2006) leads to potentiation of C-fibre-evoked potentials in the rat superficial dorsal horn that is comparable to the electrical stimulation-induced potentiation.

Central sensitisation includes both pre- and post-synaptic change as well as an increase in postsynaptic membrane excitability (Latremoliere and Woolf, 2009). Alterations in postsynaptic calcium levels, which can be the result of calcium influx through ionotropic receptors, voltage-gated ion channels, or the release of intracellular calcium stores on activation of metabotropic receptors or receptor tyrosine kinases (Cheng et al., 2010, Ohnami et al., 2011), are a major driver in initiating changes in synaptic strength. Intracellular Ca^{2+} can trigger downstream signalling cascades, resulting in the post-translational or transcriptional changes in many effector proteins, thus altering their levels, distribution and/or functional activity (Asiedu et al., 2011, Katano et al., 2011, Matsumura et al., 2010, Miletic et al., 2011). The synaptic changes underlying activity-dependent central sensitization also involve the glutamate receptors: NMDA, AMPA, KAR and mGluR as well as the substance P NK1 receptor, BDNF and its TrkB receptor, ephrinB and EphBR, CaMKII, PKA, PKC, src, ERK and CREB and Kv4.2 (Latremoliere and Woolf, 2009).

1.11.2 Increased postsynaptic excitability

Synaptic transmission between the afferent fibres and second order neurons in the dorsal horn of the spinal cord is excitatory, with release of glutamate from primary afferents central terminals. In normal conditions glutamate would activate primarily postsynaptic AMPAR and KAR, generating excitatory postsynaptic currents (EPSCs) in the second order dorsal horn neurons (Latremoliere and Woolf, 2009, Liu and Salter, 2010, Larsson and Broman, 2011). The summation of the sub-threshold

EPSPs in the postsynaptic neuron can lead to action potential firing and transmission of the nociceptive information to higher order neurons. During the early phase of central sensitisation a key underlying mechanism is the increase in the activity and numbers of postsynaptic AMPARs and NMDARs via phosphorylation and insertion of receptors (Ji et al., 2003).

1.11.2.1 AMPARs

AMPARs are heteromeric or homomeric tetramers, assembled from a combination of four subunits – GluA1, GluA2, GluA3 or GluA4 (Hollmann and Heinemann, 1994). The subunit composition determines the cation permeability (Hollmann et al., 1991, Sommer et al., 1990), with GluA2 subunit regulating Ca^{2+} permeability such that only channels without GluA2 are permeable to Ca^{2+} (Burnashev et al., 1992, Hollmann et al., 1991). AMPARs have been detected on DRGs (Sato et al., 1993, Tachibana et al., 1994), with DRG neurons able to translate all four AMPAR subunits. The GluA1 subunit has been localised to both unmyelinated and myelinated afferents (Sato et al., 1993, Lee et al., 2002), while GluA2/3 and GluA4 have been shown to be predominantly localised to myelinated and unmyelinated fibres, respectively (Lu et al., 2002, Willcockson and Valtschanoff, 2008). AMPAR subunits are also transported to the afferents' central terminals and are present in the dorsal horn of the spinal cord. GluA4 has been shown to be predominantly expressed in the superficial laminae (I-III) where the nociceptive peripheral fibres terminate, while GluA2/3 has been detected in the deeper laminae (III-IV)(Lu et al., 2002), on myelinated fibres.

AMPARs are involved in both acute and chronic pain (Dickenson et al., 1997, Garry and Fleetwood-Walker, 2004). They are responsible for mediating fast synaptic transmission between the primary afferent fibres and second order dorsal horn neurons, such that presynaptic release of the excitatory neurotransmitter glutamate from the central terminals of the afferents activates postsynaptic dorsal horn AMPARs, which results in fast EPSPs, with a duration of ms (Yoshimura and Jessell, 1990, Yoshimura and Nishi, 1992).

Evidence for the involvement of AMPARs in central sensitisation includes studies showing that the Ca^{2+} permeable AMPAR antagonist synthetic Joro spider toxin (JSTX) is able to attenuate secondary mechanical hyperalgesia and allodynia following gastrochemius incision (Pogatzki et al., 2003) and first degree burn injury (Jones and Sorkin, 2004), respectively. In addition, GluA2 knockout mice, where there is an enhancement of Ca^{2+} permeable AMPARs, have been shown to exhibit enhanced hypersensitivity following CFA inflammation and hindpaw formalin and capsaicin injections (Hartmann et al., 2004). In addition, those mice have been reported to show no increase in ERK phosphorylation, which is considered a marker of central sensitisation in response to C-fibre stimulation (Ji et al., 1999, Karim et al., 2001, Hartmann et al., 2004), suggesting that the reduction of the Ca^{2+} permeable AMPARs is associated with a reduction in measures of spinal cord potentiation. This supports the role of Ca^{2+} permeable AMPARs in central sensitisation.

1.11.2.2 NMDARs

NMDARs are tetrameric, non-selective cation channels that consist of two essential GluN1 subunits and two regulatory GluN2 (GluN2A, GluN2B, GluN2C or GluN2D) or GluN3 (Glun3A or GluN3B) (Nakanishi, 1992, Paoletti and Neyton, 2007). The subunit composition of the receptors determines its functional characteristics, including ligand affinity, channel conductance and kinetics (Cull-Candy and Leszkiewicz, 2004). Anatomical studies have shown that while the GluN1 subunit is present throughout the dorsal horn, GluN2A-most intensely expressed in laminae III-IV, while GluN2B is most highly expressed in laminae I-II (Nagy et al., 2004). More recent studies suggest that both GluN2A and GluN2B are predominantly localised at synapses in laminae I and II (Tong and MacDermott, 2014), with reports of GluN2B and GluN2D being localised to the pain processing superficial dorsal horn in male rats (Temi et al., 2021). Interestingly, studies in female rats have demonstrated that only GluN2B is present in the superficial dorsal horn, revealing a sex difference in baseline excitatory synaptic signalling within the nociceptive circuits in the spinal cord (Temi et al., 2021).

Early studies presumed that in naïve conditions NMDARs show no involvement in fast excitatory transmission because of the Mg^{2+} block at the channel pore at resting membrane potential (Mayer et al., 1984), but recent studies show that NMDARs can contribute to fast excitatory transmission (Tong et al., 2008, Shiokawa et al., 2010, Tong and MacDermott, 2014, Bardoni et al., 2000). However, repetitive or high frequency C-fibre firing drives the presynaptic release of glutamate, SP or CGRP, which causes sustained depolarisation of the postsynaptic membrane, sufficient to remove the Mg^{2+} block, thus resulting in slow excitatory postsynaptic potentials (EPSPs). The removal of the NMDAR Mg^{2+} block greatly enhances synaptic transmission and allows Ca^{2+} entry into the postsynaptic neuron, which in turn can activate a series of intracellular signalling cascades that are involved in central sensitisation (Latremoliere and Woolf, 2009). Concurrent activation of metabotropic and SP receptors on the postsynaptic membrane can also contribute to sensitisation by enhancing cytosolic Ca^{2+} concentrations. The downstream activation of a series of signalling pathways and second messengers (primarily kinases such as MAPK, PKA, PKC, PI3K, Src) can further increase neuronal excitability in part by modulating NMDAR function (Basbaum et al., 2009).

One of the first findings suggesting involvement of NMDAR in central sensitisation involved the application of NMDAR antagonists, MK-801 or D-CCP, which prevented the induction of and reversed the hyperexcitability associated with central sensitisation (Woolf and Thompson, 1991). Further studies have illustrated using spinal injections of a fragment of Src that disruption of the NMDAR-Src interaction causes a decrease in peripheral injury-induced hypersensitivity, without changes in acute nociception (Liu et al., 2008). Studies looking at NMDAR expression levels have also shown alteration, with nerve injury reported to increase the expression levels of GluN1 and GluN2B, but not GluN2A (Wilson et al., 2005). Sustained phosphorylation of the GluN2B but not GluN2A subunit has been shown following CFA-induced inflammation (Guo et al., 2002). Phosphorylation of GluN1 but not GluN2A or GLuN2B subunits has been shown in carrageenan-induced inflammation,

but no changes in expression levels were reported (Caudle et al., 2005, Seeburg and Hartner, 2003).

1.11.2.3 Intrinsic plasticity of dorsal horn neurons

The intrinsic plasticity of spinal cord dorsal horn neurons refers to changes in their excitability with one proposed mechanism involving phosphorylation of $K_V4.2$, a downstream target for ERKs, which are activated following noxious stimulation (Ji et al., 1999). $K_V4.2$ phosphorylation has been shown to reduce A-type potassium currents, leading to an increase in excitability. However, this mechanism is suggested to involve mainly lamina II interneurons where $K_V4.2$ is highly expressed (Huang et al., 2005). The enhanced excitability of the lamina II interneurons could in turn enhance the activation of lamina I projection neurons through polysynaptic pathway, contributing to the hyperalgesia seen in inflammatory pain states. Moreover, many excitatory interneurons contain somatostatin, but if its release is enhanced this could lead to the hyperpolarisation of nearby inhibitory interneurons ultimately causing a disinhibitory effect (Yasaka et al., 2010, Todd et al., 1998).

1.11.3 Disrupted spinal cord inhibition

GABAergic or glycinergic inhibitory interneurons within the superficial spinal cord are at the basis of the gate control theory of pain, suggesting that loss of function of these inhibitory interneurons (leading to disinhibition) would result in enhanced pain states (Melzack and Wall, 1965). Thus altered inhibition in the dorsal horn of the spinal cord is suggested to also be involved in the development of central sensitisation (Müller et al., 2003, Zeilhofer and Zeilhofer, 2008).

Spinal inhibition has been shown to affect nociceptive processing in the dorsal horn by early studies demonstrating that pharmacologically mimicking disrupted inhibition in naïve animals, using spinal administration of GABA or glycine receptor (GlyR) antagonists, allodynia-like responses could be induced (Sherman and Loomis, 1996, Yaksh, 1989). There is also electrophysiological evidence suggesting that spinal cord inhibition is reduced in central sensitisation. Following CFA-induced inflammation the frequency of the GlyR-mediated miniature inhibitory postsynaptic

current (mIPSC) has been shown to be reduced (Müller et al., 2003), while the frequency of the GABA-mediated mIPSC has been shown to be attenuated along with an increase in the number of neurons displaying no evoked IPSCs (eIPSCs) in neuropathic pain models (Moore et al., 2002). Some studies have also suggested that changes in the projection neurons themselves could be involved in the disinhibition process. For example, downregulation of the potassium-chloride co-transporter (KCC2) following peripheral injury, which is expressed in lamina I projection neurons and essential for maintaining normal K^+ and Cl^- gradients, could result in a shift in the Cl^- gradient such that GABA activation now depolarises rather than hyperpolarises the lamina I projection neurons (Coull et al., 2005, Coull et al., 2003). Further studies have shown that following nerve injury there is a reduction in the spinal dorsal horn levels of GABA and its synthesising enzyme glutamic acid decarboxylase (GAD) (Eaton et al., 1998, Ibuki et al., 1997, Moore et al., 2002), which may be due to death of GABAergic interneurons (Scholz et al., 2005, Moore et al., 2002). However, these findings have not been confirmed by other studies that have reported no loss of dorsal horn neurons following nerve injury (Polgár et al., 2004, Polgár et al., 2005) and no change in the immunoreactivity of GABA, vesicular GABA transporter or GABAR (Polgár and Todd, 2008). Regardless of the aetiology, the resulting decreased tonic inhibition is suggested to enhance projection neurons depolarisation and excitation, thus enhancing spinal cord output in response to noxious and innocuous stimuli, contributing to mechanical allodynia (Keller et al., 2007, Torsney and MacDermott, 2006).

1.12 Spinal circuits involved in nociception and chronic pain

The processing of the afferent nociceptive information in the superficial laminae, prior to its relay to lamina I projection neurons, which then send the information to supraspinal sites, involves the inhibitory and excitatory interneurons within the dorsal horn (Todd, 2010). The interplay between excitation and inhibition is crucial for setting spinal sensitivity, but the circuits for nociceptive processing are not fully

explored. Research has focused on identifying individual neuronal subpopulations within the spinal cord (lamina II) that are involved in pain perception.

1.12.1 Spinal circuits for mechano- and thermoreception

The excitatory SOM+ interneurons within laminae IIo-III have been shown to be recruited within a mechanical pain circuit, with two distinct pathways involved in their recruitment (Christensen et al., 2016, Duan et al., 2014). One involves the direct activation of more superficial SOM+ interneurons via monosynaptic Aδ- and C-fibre inputs, contributing to acute mechanical pain processing. A second pathway involves indirect activation of a second population of SOM+ interneurons in lamina IIi-III, which have been shown to receive polysynaptic Aβ inputs via an intermediate inhibitory interneuron. Studies have shown that ablation of SOM+ interneurons leads to both deficits in mechanical and thermal pain sensitivity as well as deficits in light touch. In addition, direct activation of SOM+ interneurons has been reported to cause both spontaneous pain states and decreased mechanical pain thresholds, consistent with the existence of multiple functionally distinct populations (Christensen et al., 2016, Duan et al., 2014).

The SOM+ interneurons comprise a broad population of excitatory interneurons, some of which have been demonstrated to colocalise with interneurons expressing the excitatory markers calretinin (CR) and vesicular glutamate transporter 3 (VGLUT3), which are suggested to be involved in the amplification of nociceptive signalling (Koch et al., 2018). The subpopulation of Calretinin+/Calbindin 2+ (CR/Calb2) interneurons in laminae I and II, 85% of which reported to display excitatory properties (Smith et al., 2015), has been suggested to act as an amplifier of nociceptive specific inputs because their dorsal spinal ablation has been shown to results in a deficit in thresholds to von Frey filaments and increased withdrawal latency to heat, without the development of innocuous touch phenotype (Duan et al., 2014). The VGLUT3+ interneurons have been suggested to be involved in the processing of similar nociceptive inputs, with ablation studies showing selective deficits in von Frey filament detection in mice (Cheng et al., 2017). Thus, it has been

suggested that the more superficial CR+/Calb2+ and the Aβ-receiving VGLUT3+ interneurons are likely involved in the nociceptive transmission of punctate von Fair stimuli.

The GRP+ population of interneurons in the superficial dorsal horn has been suggested to be involved in gating nociceptive information (Sun et al., 2017). These interneurons have been shown to receive afferent inputs form IB4+, CGRP+ and TRPV1+ primary afferents and to fire at high frequencies in response to nociceptive inputs, with their ablation resulting in hypersensitivity to thermal stimuli and inflammatory mediators. Despite the fact that these neurons respond to nociceptive inputs, it has been shown that their high intensity stimulation leads to postsynaptic recruitment of enkephalinergic interneurons in the superficial dorsal horn, ultimately resulting in inhibition of nocifensive behaviours. This internal gate-mechanism is suggested to be pain-specific as it has been shown that delta opioid receptor antagonists can block nociceptive but not pruritoceptive behaviours in GRP+ interneuron ablated mice (Sun et al., 2017). This polymodal activation of identified populations highlights the plasticity of dorsal horn intraneuronal populations and the adaptability of a single sensory circuit.

1.12.2 Spinal circuits for pathological pain

Inflammatory and neuropathic pain states are proposed to involve distinct mechanisms, including disinhibition or degradation of inhibitory gate pathways via which both touch and pain stimuli are normally silenced, and are thus suggested to recruit additional neuronal populations, not associated with somatosensory phenotype in normal conditions, along with some of the neuronal populations mentioned above (Koch et al., 2018). It must be noted that the role of the neuronal subtypes in pathological pain states could be more complex that our current understanding because of the limited research tools available, which have predominantly utilised ablation techniques to study the role of individual neuronal subtypes, which has been suggested to likely modify spinal circuits (Sandkühler, 2009, Kuner, 2010).

NK1-R$^+$ neurons comprise a major fraction of the projection neurons in lamina I, which are thought to transmit nociceptive information to higher brain centres (Al-Khater et al., 2008, Spike et al., 2003). Since lamina I NK1R$^+$ neurons have been shown to receive monosynaptic C- and Aδ-fibre nociceptive inputs as well as polysynaptic Aβ inputs from LTMR, revealed upon spinal disinhibition (Torsney and MacDermott, 2006), they have been suggested to act as a convergent site for pathological pain with disinhibition of the Aβ fibres likely contributing to the symptoms of allodynia/hyperalgesia. It is the VGLUT3+ interneurons, via their polysynaptic excitatory connectivity to NK1R+ neurons in lamina I, that are thought to transmit the sensation of touch in allodynic pain states when disinhibited because their selective activation has been shown to give rise to spontaneous pain states (Peirs et al., 2015, Cheng et al., 2017, Peirs et al., 2021). They are also suggested to be involved in the amplification of pain sensitivity during pathological pain states following disinhibition. Two separate pathways to allodynia have been proposed depending on the nature of the pathology, with VGLUT3 excitation of CR+/Calb2+ interneurons in inflammatory pain and VGLUT3 excitation of PKCϒ interneurons in neuropathic pain (Peirs et al., 2015, Cheng et al., 2017, Peirs et al., 2021).

Inhibitory interneurons expressing TRPV1 in laminae I and II of the dorsal horn have been also implicated in modulating nociceptive signalling and in mechanical hypersensitivity (Doly et al., 2004, Ferrini et al., 2010, Kim et al., 2012). Studies have found that nerve-injury induced allodynia could be alleviated by TRPV1 antagonists not only via action on the afferents but also via inhibition of a subset of TRPV1-expressing interneurons in the dorsal horn (Woolf et al., 1992, Kim et al., 2012, Coull et al., 2003). TRPV1 knockout mice have been reported to display normal responses to baseline noxious mechanical stimuli, suggesting that TRPV1+ interneurons are involved in a pathology-specific circuit (Caterina et al., 2000, Kim et al., 2012). TRPV1+ interneurons have been suggested to reduce presynaptic expression of GluR2 in excitatory dorsal horn neurons following nerve injury (via mechanisms involving long-term depression), thus causing decreased excitatory

drive to GABAergic interneurons and disinhibition of projection neurons (Kim et al., 2012).

Dorsal horn glutamatergic interneurons expressing PKCγ, which receive afferent inputs from myelinated fibres and are primarily restricted to laminae IIi and III (Abraira et al., 2017, Alba-Delgado et al., 2015, Pham-Dang et al., 2016), have also been implicated in neuropathy-induced mechanical and thermal hypersensitivity (Malmberg et al., 1997, Petitjean et al., 2015, Pham-Dang et al., 2016, Peirs et al., 2015). Studies have shown that strychnine-induced blockade of glycinergic inhibition causes activation of PKCγ-positive neurons in lamina II following innocuous brushing of the hindpaw as well as activation of projection neurons in lamina I (Miraucourt et al., 2007) further confirming that disinhibitory mechanisms lead to their hyperactivation, which could ultimately result in enhanced pain states. The PKCγ-expressing interneurons have been implicated in many disinhibitory allodynia pathways as supported by pharmacological and genetic studies showing that PKCγ manipulation or deletion alters allodynic behaviours (Duan et al., 2014, Peirs et al., 2015, Petitjean et al., 2015, Pham-Dang et al., 2016).

1.13 Thesis aims and hypothesis

Given previous evidence from the lab for sex- and injury-dependent differences in C-fibre ADS, likely influencing spinal summation and potentially contributing to observed sex differences in heat pain thresholds (Dickie et al., 2017), the experiments in this thesis were designed to investigate potential sex differences in C-fibre ADS in different preclinical pain models or different rodent species in association with potential sex differences in thermal and mechanical sensitivity in these models/species. Given C-fibre ADS has been proposed to provide a ’dynamic memory’ of previous levels of activity and also influence responses to subsequent stimuli (Weidner et al., 2002), this thesis also aimed to explore whether there are sex- and/or species dependent differences in C-fibre ’dynamic memory’. This thesis also studied the impact of ADS in monosynaptic C-fibre inputs to spinal neurons involved in noxious heat processing.

1.13.1 Hypothesis

- Chapter 2: C-fibre ADS will be altered in the rat incision model of postoperative pain. This may occur in a sex-dependent manner in association with sex differences in post-incision heat but not mechanical hypersensitivity.
- Chapter 3: C-fibre ADS will be altered in an *ex vivo* model of pain induced by the diabetic pain-associated metabolite methylglyoxal (MG). This may occur in a sex-dependent manner in association with sex differences in an *in vivo* model of MG-induced heat hypersensitivity.
- Chapter 4: Prolonged low-frequency stimulation (manipulation) will alter C-fibre ADS levels post-manipulation, i.e. will induce 'dynamic memory', in a sex-dependent manner in rats. *C57BL/6* mice will also display C-fibre ADS in a sex-dependent manner in association with sex differences in heat pain sensitivity in this species. Manipulation in mice will also alter C-fibre ADS in a sex-dependent manner.
- Chapter 5: Male mice will display more pronounced ADS in monosynaptic C-fibre inputs to noxious heat responsive spinal neurons. The evoked activity – dependent firing of the Fos-EGFP and capsaicin sensitive spinal neurons will depend on the levels of ADS in monosynaptic C-fibre inputs.

1.13.2 Aims

- Chapter 2: Assess C-fibre ADS and sensitivity to mechanical and thermal stimuli in the plantar incision model of postoperative pain in rats of both sexes.
- Chapter 3: Assess C-fibre ADS and sensitivity to mechanical and heat stimuli in an *ex vivo* and *in vivo* MG-induced pain modal in rats of both sexes, respectively.
- Chapter 4: Assess C-fibre ADS in mice of both sexes and explore C-fibre 'dynamic memory' in rats and mice of both sexes
- Chapter 5: Assess the impact of ADS in monosynaptic C-fibre inputs to noxious heat responsive spinal neurons in mice of both sexes

Corresponding chapters discuss the individual aims and hypotheses in greater detail.

Chapter 2

C-fibre activity-dependent slowing and nociceptive behaviour in a rat incision model of postoperative pain

2.1 Chapter summary

- This chapter assessed the incision-induced alteration in C-fibre ADS in the plantar incision model of postoperative pain in male and female juvenile rats.
- Hindpaw plantar incision alters the C-fibre ADS phenomenon in a sex- and frequency-dependent manner, increasing C-fibre latency change in males to the higher baseline levels observed in females. Females show enhanced C-fibre width change post-incision to levels greatly exceeding those in males.
- C-fibre ADS is regulated by the Na_V blocker TTX in an incision- and sex-dependent manner suggesting incision- and sex-dependent differences in the underlying $Na_V1.7/Na_V1.8$ ratio along the axon that may contribute to the incision and sex-dependent changes in ADS.
- Hindpaw incision surgery causes mechanical and heat hypersensitivity in both sexes, with males exhibiting more pronounced peak heat hypersensitivity in comparison to females.
- Consistent with a stronger overall ADS profile post-incision in females that is predicted to limit heat hypersensitivity females displayed less peak heat hypersensitivity than males following incision. Thus, sex-dependent incision-induced C-fibre ADS alterations may be a potential contributory mechanism to post-operative pain.

2.2 Introduction

2.2.1 Postoperative pain

Postoperative pain develops following surgical interventions and often involves on-going pain at rest (non-evoked) and pain during activities, such as walking and coughing (evoked pain) (Brennan, 2011b). Although unpleasant, it can be beneficial in facilitating wound protection, healing, and the resolution of both evoked and non-evoked pain within weeks (Brennan 2001). However, there is a risk of postoperative pain becoming pathological, debilitating, and difficult to treat (Varrassi et al., 2010), with females being more likely to develop chronic pain after injury (Sorge and Strath, 2018). With more than 300 million surgeries performed worldwide each year (Weiser et al., 2015), postoperative pain remains a significant clinical problem with 30-80% of in-patients experiencing moderate or severe pain following surgery (Pöpping et al., 2008, Gan et al., 2014).

Despite an increasing awareness of sex differences in pain sensitivity and responsiveness to analgesia (Mogil and Bailey, 2010, Fillingim et al., 2009, Bartley and Fillingim, 2013b), along with the accumulating evidence for sex differences in chronic pain mechanisms (Mogil, 2018, Sorge and Strath, 2018), impact of sex remains underexplored in the context of postoperative pain. There is clinical evidence for sex differences in postoperative pain with several studies suggesting that women have an increased risk of developing more pronounced pain after surgery (Hah et al., 2019, Bartley and Fillingim, 2013b, Fillingim et al., 2009, Gerbershagen et al., 2014). However, such studies are conflicting, with some reporting male (Chia et al., 2002), and others female (Cepeda and Carr, 2003, Aubrun et al., 2005, Mattila et al., 2005, Gerbershagen et al., 2014) patients as more likely to experience more severe postoperative pain and to require greater analgesic dose to achieve a similar pain-relieving effect. A possible confounding factor in these studies is the variety of surgical procedures carried out, which could also differ by sex (gynaecologic vs prostate surgery). In addition, more recent studies have also investigated and emphasized the impact of other factors,

including age and type of surgery, on postoperative pain (Tighe et al., 2015, Zheng et al., 2017). Thus, in order to fully understand the mechanisms underlying postoperative pain, preclinical investigation is required. However, the preclinical studies in postoperative pain are almost exclusively conducted in male subjects only (Pogatzki-Zahn et al., 2018). Given the current inadequate clinical management of postoperative pain (Brennan, 2011a, Pogatzki-Zahn et al., 2017), it is essential that preclinical investigation be conducted in both sexes to ensure adequate mechanism based development of much needed new therapies that will be effective in both sexes (Segelcke et al., 2019).

2.2.2 Incision model of postoperative pain

The development of a preclinical model for postoperative pain, the plantar hindpaw incision model (Brennan et al., 1996), has contributed greatly to understanding the neural mechanisms underlying postsurgical pain (Segelcke et al., 2019). This model was established in the rat, where under anaesthesia a 1cm longitudinal incision is made through the skin, fascia and underlying muscle of the plantar aspect of the hindpaw, followed by wound closure with two sutures (**Figure 2.1**). Over the last three decades, variations of this model have been developed for studies of specific surgical procedures (Pogatzki-Zahn et al., 2018, Pogatzki-Zahn et al., 2017). However, the original model is used widely in investigative studies of different pain mechanisms. It is well characterised, giving rise to exaggerated evoked pain responses to noxious heat and mechanical stimuli, pain responses to innocuous stimuli, and also non-evoked guarding of the affected limb (Brennan et al., 1996, Scherer et al., 2010a, Zahn and Brennan, 1999c).

2.2.2.1 Behavioural pain phenotype

Guarding pain behaviour is used to assess non-evoked pain behaviours in rats after incision. Typically, it is greatest 2-24h after incision and gradually resolves within 2-3 days (Zahn et al., 1997, Kang and Brennan, 2016, Brennan et al., 1996) in accordance with previous reports in humans (Schulze et al., 1988, Tverskoy et al., 1990). Since guarding pain behaviour can be modified by morphine or ketoprofen (Zahn et al., 1997, Spofford et al., 2009), this suggests that this pain-related

behaviour may be translatable to pain at rest in post-surgery patients. Primary mechanical hypersensitivity is typically assessed using the hindpaw flexion-withdrawal reflex to mechanical stimulation at the incision site, with incision causing a decrease in the withdrawal threshold. Peak mechanical hypersensitivity establishes 1-3 days after incision and gradually returns to baseline by post-surgical day (PSD) 6-7 (Brennan et al., 1996, Cao et al., 2015, Zahn and Brennan, 1999a, Banik et al., 2005). Primary heat hypersensitivity is assessed using the flexion-withdrawal reflex to radiant heat stimulation at the incision site, with incision causing a pronounced decrease in the hindpaw withdrawal latency (Zahn and Brennan, 1999a, Banik et al., 2005, Cao et al., 2015). Peak heat hypersensitivity is observed during the early postoperative period (from 2h to PSD2-3) and gradually returns to baseline levels by PSD 7-10 (Zahn and Brennan, 1999a, Cao et al., 2015, Banik et al., 2005). Secondary mechanical hyperalgesia, an exaggerated response to stimuli applied to the surrounding undamaged area, can be observed for a short period of time (1-2 days after surgery), while secondary hyperalgesia to heat stimuli is absent in the rat plantar incision model (Zahn and Brennan, 1999a, Pogatzki et al., 2002b). These pain behaviours are thought to mimic those seen in patients, with guarding behaviour thought to represent resting pain, and evoked behavioural responses thought to correspond to pain during activities (Brennan, 2011b, Kang and Brennan, 2016) .

To help with the translation of the preclinical work in the plantar incision model, the pain induced by surgical incision of the skin has also been characterised in humans (Kawamata et al., 2002b, Kawamata et al., 2002a). The incision-induced pain behaviours in rats mimic those observed in patients, where patients also reporting spontaneous pain lasting only a few hours and primary mechanical hyperalgesia for the first 2-3 days after incision. Secondary hyperalgesia was present only for 3-6h after incision. The consistency of the incision-induced pain behaviour in the rat and the postoperative pain behaviour in patients, as well as the effectiveness of analgesic drugs in modifying the pain behaviours in this model alongside the clinic (Zahn et al., 1997, Spofford and Brennan, 2012, Zahn et al., 2002), has resulted in

the plantar incision model being widely used in postoperative pain research. It provides an excellent tool for studying the mechanisms underlying postoperative pain, particularly the consequences of wounding on the primary afferent nociceptive neurons, the C-fibre nociceptors. They signal injury and contribute to the incision-induced peripheral sensitisation, with their injury-induced activity also driving central sensitisation.

2.2.3 Mechanism of postoperative pain

The pathophysiological mechanism underlying postoperative pain are increasingly thought to be district from pure neuropathic or inflammatory pain (Kehlet et al., 2006, Whiteside et al., 2004, Hill et al., 2010b, Brennan, 2011b, Segelcke et al., 2019). There are pre-clinical studies investigating peripheral and central sensitisation as main contributors to postoperative pain, with accumulating evidence of differences in the mechanisms of postoperative pain from other pain states.

2.2.3.1 Peripheral sensitisation

Preclinical studies using the plantar incision model of postoperative pain have demonstrated that incision induces peripheral sensitisation, which is suggested to contribute to both ongoing pain and primary hyperalgesia post-incision (Pogatzki et al., 2002a, Banik and Brennan, 2004, Kang and Brennan, 2009a, Xu and Brennan, 2009, Xu and Brennan, 2010, Xu et al., 2010).

2.2.3.1.1 Spontaneous nociceptor activity

Peripheral sensitisation manifests as ongoing spontaneous activity of nociceptors, with *in vivo* studies reporting an increased number of nociceptors with spontaneous activity innervating the plantar incision (Pogatzki et al., 2002a, Xu et al., 2010). *Ex vivo* studies similarly reveal a greater proportion of C-fibres close to the incision site with spontaneous activity when compared to those C-fibres innervating the non-incised plantar skin (Banik and Brennan, 2004, Kang and Brennan, 2009b).

2.2.3.1.2 Nociceptor sensitisation

Sensitisation of the primary afferents innervating the incised area to mechanical stimuli has also been demonstrated (Pogatzki et al., 2002a, Hamalainen et al., 2002, Xu and Brennan, 2010), with Aδ- and C-fibres showing lower thresholds, higher response magnitude and greater receptive field size to mechanical stimuli (Hamalainen et al., 2002). However, the majority of incision-induced alterations in the nociceptive Aδ- and C-fibres have been explored only early after incision surgery (45mins/1day after) and have been found mainly in the Aδ-fibres in response to primarily mechanical stimuli. Incision-induced nociceptor changes in response to thermal stimuli, which will link to C-fibre nociceptor changes, have not been extensively studied. Both mechanical and heat sensitisation have been shown only in cutaneous and muscle nociceptors 1 day after incision (Xu et al., 2010, Banik and Brennan, 2004, Banik and Brennan, 2008).

2.2.3.1.3 Pro-inflammatory mediator nerve growth factor in the incision wound environment

Various chemical pain mediators can lead to nociceptor sensitisation and activation. However, the presence of such mediators in the wound environment after incision has not been extensively studied, but a few studies have explored the role of the pro-inflammatory mediator nerve growth factor (NGF) post-incision (Banik et al., 2005, Wu et al., 2007, Wu et al., 2009, Spofford and Brennan, 2012). NGF has been shown to increase in the wound environment within hours after incision, returning to baseline levels by PSD7 (Banik et al., 2005, Wu et al., 2007, Wu et al., 2009). Moreover, sequestration of NGF (via systemic pre-treatment with anti-NGF antibody or peptibody) has been shown to attenuate guarding behaviours and heat but not mechanical hyperalgesia in a dose-dependent manner (Banik et al., 2005, Wu et al., 2009), further supporting involvement of NGF to ongoing pain and heat hyperalgesia. However, these finding also suggest that the post-incision mechanisms for exaggerated response to heat and mechanical stimuli may differ (Banik et al., 2005, Zahn et al., 2004), but only a few studies have explored the

mechanisms of heat sensitisation in the rat plantar incision model. It has been shown that nociceptors desensitised by capsaicin contribute to the incision-induced guarding pain and heat hyperalgesia (Banik and Brennan, 2009, Kang et al., 2010, Hamalainen et al., 2009). This suggests that the TRPV1-expressing afferent fibres (but not the TRPV1 receptor) are essential contributors to the incision-induced guarding pain. Interestingly, it has also been shown that NGF decreases nociceptor activation threshold in both animals (Hirth et al., 2013) and humans (Obreja et al., 2018) and increases the proportion of mechano-sensitive nociceptors due to potential recruitment of previously silent nociceptors (Hirth et al., 2013). NGF can also affect various nociceptor transducer receptors and voltage-gated ion channels in the peripheral afferents (Aguayo and White, 1992, Zur et al., 1995, Gould et al., 2000, Barker et al., 2020), including the voltage-gated sodium channels (Na_V) (Okuse et al., 1997, Diss et al., 2008, Fang et al., 2005, Fjell et al., 1999, Gould et al., 2000).

2.2.3.2 Central sensitisation

Central sensitisation is characterised by increased neuronal responsiveness in the CNS, with mechanical allodynia and secondary mechancial hyperalgesia post-incision thought to be mainly driven by central sensitisation and potentially linked to increased risk of developing chronic postoperative pain (Kawamata et al., 2002b, Pogatzki et al., 2003).

2.2.3.2.1 Dorsal horn neuronal activity

A few studies have investigated the post-incision activity of spinal dorsal horn neurons, whose receptive fields included at least part of the injury, in order to understand the post-incision changes in spinal sensory processing and its contribution to postoperative pain. The plantar incision resulted in increased spontaneous activity, expansion of receptive fields, enhanced responses to mechanical but not thermal stimuli, and decreased temperature activation thresholds in the immediate period after incision (1h after surgery or PSD1), showing the sensitisation of those neurons (Zahn and Brennan, 1999b, Pogatzki et al., 2002c, Xu and Brennan, 2009, Xu et al., 2009). Local anaesthetics were able to reverse the spinal neuron sensitisation, suggesting it is induced and maintained

largely by the primary afferent inputs from the incision site (Pogatzki et al., 2002c, Xu and Brennan, 2009, Xu et al., 2009). However, 7 days after incision, the sensitisation of the spinal neurons has been shown to resolve, with spontaneous activity and the temperature activation thresholds being comparable to controls (Xu and Brennan, 2009).

2.2.3.2.2 Spinal non-NMDA excitatory amino acid receptors

In contrast to other preclinical models of persistent pain, where spinal N-methyl-D-aspartate receptors (NMDARs) (Fisher et al., 2000, Zhou et al., 2011) or spinal metabotropic glutamate receptors (mGluRs) (Varney and Gereau, 2002, Pereira and Goudet, 2019) have been shown to play a role, in the plantar incision model inhibition of those receptors did not alter incision-induced pain behaviour in rats (Zahn and Brennan, 1998b, Zahn and Brennan, 1998a, Pogatzki et al., 2000). This suggests that NMDARs and mGluRs do not play an important role in postoperative pain models. Instead, non-NMDA receptors seem to play more important role in postoperative pain behaviours. This is supported by studies showing that non-NMDA excitatory amino acid (AMPA or kainate) receptor antagonists are able to attenuate incision-induced pain behaviour (guarding and mechanical) (Zahn et al., 1998, Pogatzki et al., 2000, Jin et al., 2007) and sensitisation of the spinal dorsal horn neurons (Zahn et al., 2005). In addition, compared to other persistent pain models, incision has also been shown to induce a different pattern of amino acid release in the lumbar dorsal horn, with a short-lived increase in glutamate and aspartate (45min) which returns to basal levels within 1h post-incision (Zahn et al., 2002). Furthermore, pre-emptive analgesia, which is based on the idea that treatment before surgery could prevent the establishment of central sensitisation and attenuate the pain experience, assuming that central sensitisation has a major role in postoperative pain, has been shown to have a small effect on postoperative pain behaviour (Pogatzki et al., 2000, Brennan et al., 1997). This further suggests that incisional pain may be initiated and maintained differently from models of persistent inflammatory (Hasani et al., 2011) or neuropathic (Huang et al., 2013b, Burton et al., 1999) pain, where it has been shown that pre-emptive treatment

(initiated before tissue injury to block nociceptive input to the spinal cord) could reduce the subsequent pain behaviours. The findings from the pre-clinical model of postoperative pain support clinical studies, reporting no effect of pre-emptive analgesia (Hariharan et al., 2009).

2.2.4 C-fibre ADS and its potential involvement in the hindpaw incision-induced postoperative pain rat model

Given the involvement of C-fibre nociceptors in the incision model of postoperative pain (Banik and Brennan, 2004, Xu and Brennan, 2009, Kang and Brennan, 2009b) and the fact that their activity is known to drive central sensitisation (Latremoliere and Woolf, 2009, Woolf, 1983) it is important to investigate how the C-fibre nociceptors relay information to the CNS. Previous studies have shown that the temporal relay of pain signals in C-fibre nociceptors is regulated by a phenomenon, specific to these fibres, termed activity-dependent slowing (ADS), which is displayed by both humans (Weidner et al., 1999, Serra et al., 1999) and animals (Gee et al., 1996, Thalhammer et al., 1994). C-fibre ADS manifests as a progressive slowing of action potential conduction velocity in response to repeated stimulation and can thus influence the intervals between successive action potentials being relayed along nociceptor axons, influencing the temporal delivery to and processing of pain signals in the CNS (Dickie et al., 2017). C-fibre ADS has been shown to be altered in preclinical models of neuropathic and inflammatory pain (Galley et al., 2017, Wang et al., 2016, Shim et al., 2007, Sun et al., 2012a, Dickie et al., 2017). Interestingly, in the CFA hindpaw inflammation model it has been demonstrated that C-fibre ADS is altered in a sex-dependent manner, with females showing a reduction in ADS. Given that noxious thermal hypersensitivity is likely associated with behaviour driven by C-fibres rather than A fibres, which more likely drive mechanical hypersensitivity, the inflammation induced reduction in C-fibre ADS in females is in line with the enhanced thermal but not mechanical hypersensitivity in females compared to males (Dickie et al., 2017).

The mechanisms underlying C-fibre ADS remain not fully clear but Na_V, particularly $Na_V1.7$, have been shown to be one of the key channels involved in ADS.

Experimental and modelling studies have revealed that a more pronounced ADS profile, is associated with more $Na_V1.8$ and less $Na_V1.7$, more delayed rectifier potassium channels and less Na+/K+ ATPase, a profile consistent with increased intra-axonal Na^+ concentration (Petersson et al., 2014). The study concluded that it is the ratio of $Na_V1.7$ to $Na_V1.8$ rather than absolute levels of either channel that determines the degree of ADS. Unsurprisingly, low-dose tetrodotoxin (TTX) (De Col et al., 2008, Baker and Waxman, 2012b), acting on the TTX-sensitive $Na_V1.7$ ion channel or conditional deletion of $Na_V1.7$ in sensory neurons (Hoffmann et al., 2018), which pharmacologically or genetically modulate the $Na_V1.7/Na_V1.8$ ratio, respectively, have been shown to alter C-fibre ADS. $Na_V1.8$ knockout mice, which would also show alterations in $Na_V1.7/Na_V1.8$ ratio, have also been shown to display alteration in C-fibre ADS (Hoffmann et al., 2016). Both $Na_V1.7$ and $Na_V1.8$ have been also found to contribute to the development of inflammatory and neuropathic pain (Amir et al., 2006, Levinson et al., 2012), with $Na_V1.7$ also suggested to contribute to postoperative pain (Sun et al., 2018, Eisenried et al., 2017, Barbosa Neto et al., 2019). The gene and protein levels of $Na_V1.7$ in the DRG have been shown to be significantly enhanced following hindpaw incision in male rats (Sun et al., 2018, Barbosa Neto et al., 2019), where pre-treatment with interference RNA (SCN9A-RNAi) was shown to inhibit the increased $Na_V1.7$ expression and to alleviate post-incision pain hypersensitivity (Sun et al., 2018). A similar study in mouse has also demonstrated that silencing $Na_V1.7$ using Herpes-based gene therapy results in attenuated pain hypersensitivity post-incision (Eisenried et al., 2017). Interestingly, both $Na_V1.7$ and $Na_V1.8$ have been shown to be regulated by the pro-inflammatory mediator nerve growth factor (NGF) (Gould et al., 2000, Bennett et al., 2019), which has also been shown to be elevated in the incision model of postoperative pain (Wu et al., 2007, Wu et al., 2009) and to modulate C-fibre ADS (Obreja et al., 2018, Obreja et al., 2011b). Given that the time-course of the NGF-induced ADS alterations parallels the NGF-induced changes in the expression pattern of $Na_V1.7$ and $Na_V1.8$ (Gould et al., 2000), it could be hypothesised that Na_V may have an underlying role in regulating C-fibre ADS in the

incision model of postoperative pain. In addition to the role of $Na_V1.7$ channels in action potential generation these channels also influence the transmission of action potentials to the spinal cord (Bennett et al., 2019).

In summary, given that the ion channels implicated in C-fibre ADS are altered post-incision, it was hypothesised that C-fibre ADS will be altered in the incision model of post-operative pain and that this may occur in a sex-dependent manner in line with sex differences in thermal hypersensitivity. Given the clinical relevance to patients and the role of C-fibre ADS in regulating the temporal relay to and processing of pain signals in the CNS, further investigation of C-fibre ADS following surgical incision is warranted. C-fibre ADS involves the $Na_V1.7$ and $Na_V1.8$ ion channels, which are also key pain pharmaceutical targets, as such further investigations may also provide therapeutic insight.

2.3 Chapter aims and hypothesis

2.3.1 Hypothesis

C-fibre ADS will be altered in the rat incision model of postoperative pain and this may occur in a sex-dependent manner in association with sex differences in heat not mechanical hypersensitivity induced following surgery.

2.3.2 Aims

1. Investigate, using compound action potential recording, whether C-fibre ADS is altered in the plantar incision model of postoperative pain in both sexes.

2. Use low-dose TTX, which targets primarily the TTX-S $Na_V1.7$ channels and thus pharmacologically modulates the $Na_V1.7/Na_V1.8$ ratio and alters ADS, to determine whether any sex- and/or incision-dependent changes in ADS reflect an altered functional $Na_V1.7/Na_V1.8$ ratio.

3. Assess the effect of hindpaw incision surgery on mechanical and thermal sensitivity in juvenile rats of both sexes using behavioural analysis of the nociceptive flexion-withdrawal reflex.

2.4 Methods

2.4.1 Ethical and legal considerations

All studies were carried out in accordance with the UK Animals (Scientific Procedures) Act 1986 and within the confines of project and personal licenses issued by the UK Home Office. The studies were designed to meet the ARRIVE (Animal Research: Reporting *in vivo* Experiments) guidelines, and whenever possible aimed at reducing the number of required animals (Du Sert et al. 2019). Each experiment was reviewed and approved by the University of Edinburgh Named Veterinary Surgeon (NVS) and Named Animal Care and Welfare Officer (NACWO). For all procedures, full training and initial supervision by qualified and experienced personnel was always provided to the investigator until deemed competent to perform tasks independently.

Severity records with the classified animal experiences were kept up to date throughout all the studies, in accordance with the UK Home Office Requirements. Animal health and welfare were a chief priority throughout all studies. With pain sensitivity being a primary outcome measure, animals were monitored daily for any signs of distress, with unusual behaviour being continually recorded. In cases of any concerns, which were infrequent (<2% of subjects), advice was sought from experienced personnel and/or veterinary staff. All animals were handled as gently as possible, and abundant cage bedding provided to ensure a soft environment.

2.4.2 Animals and general animal husbandry

All experiments used juvenile Sprague-Dawley rats (international strain code 400) of both sexes. For the behavioural studies, the rats were aged between postnatal day 15 (P15) to P28. The electrophysiological studies used similar age rats, ranging

between P17 to P24. All animals were bred and supplied by the University of Edinburgh Bioresearch & Veterinary Services (BVS). The juvenile animals remained unweaned throughout the timecourse of the experiments, with the litters housed together with the mother in appropriately sized cages (610x435x215mm). The stock room was maintained at 21°C (range: 19-22°C) with approximately 55% humidity (range: 46-65%), and on a 12-hour light/dark cycle. Food and water were available ad libitum. Bedding consisted of aspen chips and shredded tissue bedding, with cardboard tubes for environmental enrichment.

2.4.3 Rat hindpaw incision model of postoperative pain

The rat plantar incision model developed by Brennan et al. (1996) involves a longitudinal incision made through the skin and underlying deep structures of fascia and the flexor digitorum brevis muscle on the plantar aspect of the hindpaw. The incisional cut extends from the proximal edge of the heel towards the first set of footpads (**Figure 2.1**).

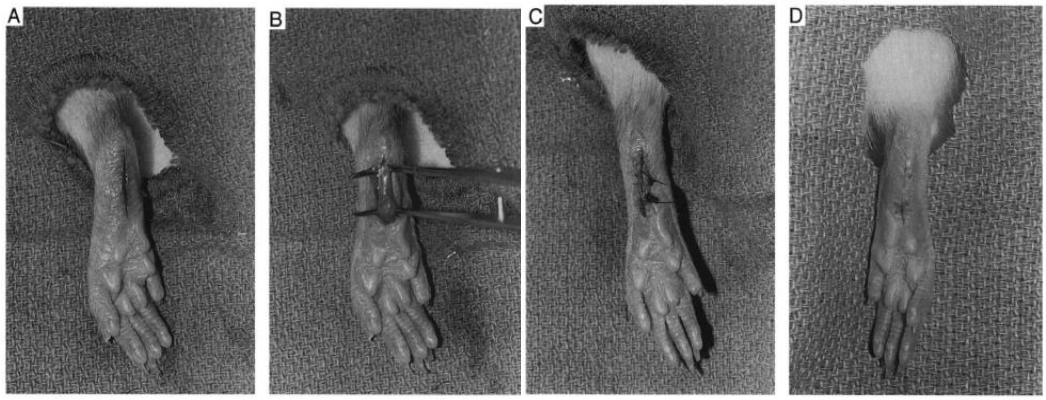


Figure 2.1 Stages of the hindpaw incision surgery.

A longitudinal incision is made through skin (A). The underlying flexor digitorum brevis muscle is elevated and incised longitudinally (B). The incision is closed with two sutures (C). The healed incision after 5 days (D). Adapted from Brennan et al. (1996)

In the presented studies, the incision model was adapted to juvenile rats of both sexes. A cohort of rats from the same litter underwent hindpaw incision surgeries at P19. Prior to the surgical procedure, animals were anaesthetised with 3-4% isofluorane. Adequate level of anaesthesia was confirmed when no tail pinch response was present. Anaesthesia was maintained during the surgical procedure using a facemask, delivering 2-3% isofluorane. Using a number 11 scalpel blade, a longitudinal incision was made through the skin on the plantar aspect of the left hindpaw. The incision extended from the proximal edge of the heel towards the first set of footpads. The underlying flexor digitorum brevis muscle was elevated and incised longitudinally. The wound was closed with two silk non-absorbable sutures using 5-0 needle (Mersilk) (**Figure 2.2**). To prevent wound gaping and suture removal prior to healing, tissue adhesive (Vetbond, 3M) was applied over the incision site. Following recovery from anaesthesia, animals were placed in a half-heated recovery box. After full recovery, the animals were returned to their home cage and supplied with sufficient bedding to ensure a soft environment.

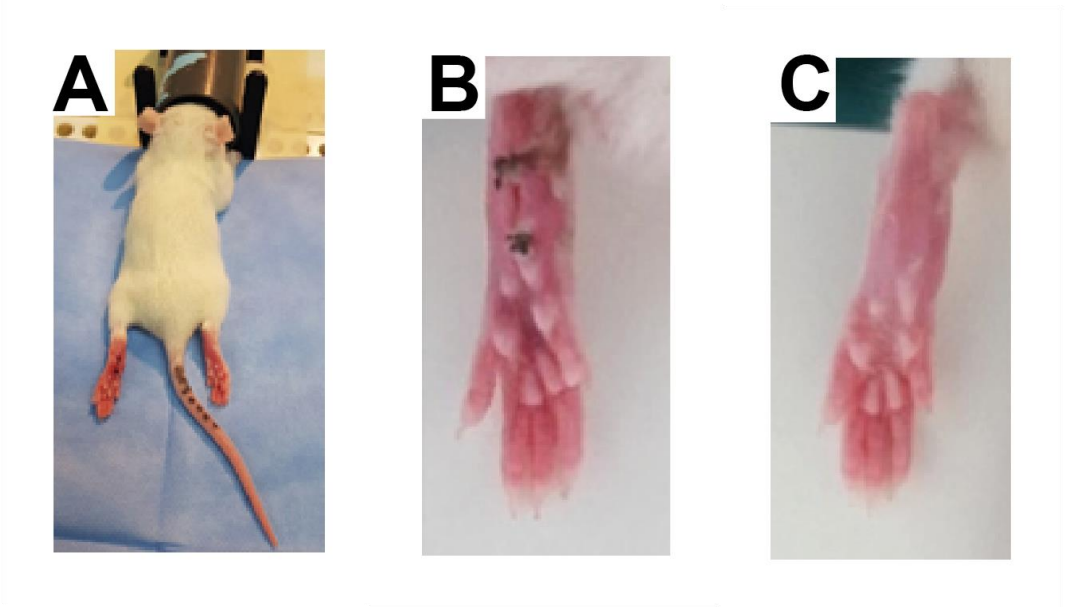


Figure 2.2 Juvenile (P19) rat planar incision surgery.

Anaesthetised animal after completed surgery (A), with a close up image of the incised and sutured left hindpaw at PSD2 (B) and a close up image of the healed hindpaw at PSD7 (**C**).

2.4.4 Behavioural assessment of glabrous hindpaw sensitivity

2.4.4.1 Blinding behavioural measures of sensitivity

To prevent deliberate or unintentional bias, it is important the investigator remains blinded to the examined factors where feasible when performing behavioural measures of sensitivity. Since the incision model involves surgical incision of one hindpaw, which remains visible until around PSD5 when the incision starts to heal, the investigator was blinded only to the sex of each animal. To further ensure consistent and systematic behavioural measures, when learning the techniques for assessing those measures, the personal judgement of withdrawal responses was compared to that of experienced investigators and results did not differ.

To blind the investigator to sex, before the start of the experiment all rats were tail-marked by technical staff and then their sex was identified. The sex corresponding to each tail-marked animal was noted down and known only by one member of the technical staff. Thus, the investigator remained blinded to the experimental animal sex, which at juvenile ages is not obviously apparent when conducting behavioural testing. Tail numbers were noted every day after the behavioural measures were completed. At the end of the experiment the sex of each animal was confirmed by a member of the technical staff. The sex of each tail-marked animal was revealed to the investigator, only after the experiment was finished.

2.4.4.2 Acclimatisation and habituation

For behavioural studies, it is essential that animals are acclimatised to the testing environment so that potential stressors are minimised. Juvenile rats were housed in a cage with the mother in the main rat stock room. They were not weaned for the duration of the behavioural experiment. Each testing day, the animals were

transferred to a behavioural testing room where disruptions were minimised. Behavioural measures were performed roughly at the same each day (approximately starting 9.00am). To allow animals to acclimatise to the testing environment, they were habituated to the testing apparatuses for two consecutive days (P13-P14) before baseline measures (P15-P17) by being placed into each testing apparatus for 30 minutes without stimulus application or sensitivity testing. Thereafter, animals were routinely habituated to the testing apparatus for 20 minutes prior to behavioural assessments of sensitivity.

2.4.4.3 Testing punctate mechanical sensitivity

Mechanical sensitivity was assessed using an electronic von Frey apparatus (eVF) (Ugo Basile). It uses a 0.5mm single un-bending filament, which is applied perpendicularly to the hindpaw with increasing force until a hindpaw withdrawal response is elicited. Using the force ramp and the force at which the withdrawal response occurs, an automatic calculation of the hindpaw withdrawal threshold is recorded and displayed by the apparatus. The withdrawal threshold is used as a measure of mechanical sensitivity, where a decrease in mechanical withdrawal threshold is interpreted as hypersensitivity.

Animals were placed individually in a Perspex box on an elevated metal grid floor that allows access to the glabrous surface of the hindpaws. The metal grid floor contained square holes with approximate surface area of 25mm^2. The eVF stimulation was applied to the plantar region of the hindpaws distal to the incision site (**Figure 2.3**). To allow sufficient time for recovery between subsequent stimuli, first the left paws of all rats were tested. Then testing of the right paws followed in a similar fashion. This allowed sufficient time in between repeated stimulation of any paw. Baseline mechanical sensitivity was assessed two days (at P15 and P17) prior to plantar incision at P19. Post-incision withdrawal thresholds to mechanical stimuli were assessed for 5 days, with testing conducted PSD1 to PSD9.

A B

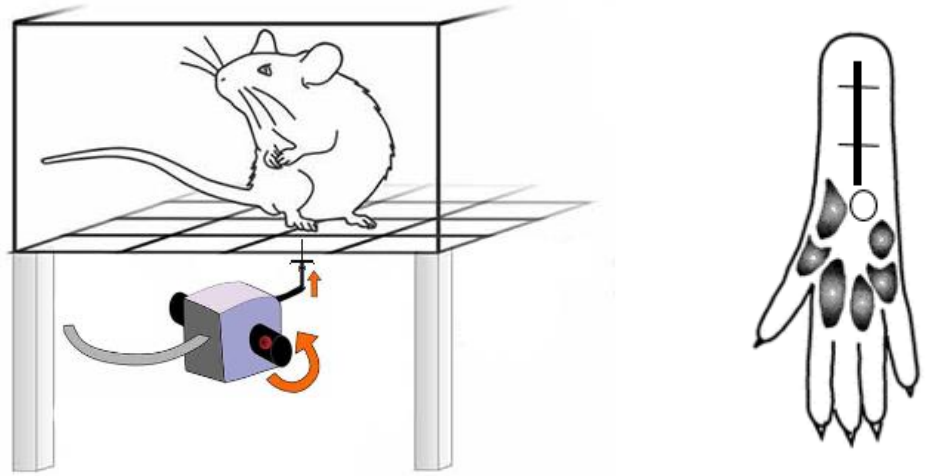


Figure 2.3 Testing punctate mechanical sensitivity using eVF apparatus.

Application of an electronic von Frey stimulus to the plantar surface of the hindpaw (A). Schematic of a rat hindpaw (B). The vertical line marks the site of the surgical incision. The two horizontal lines represent the sites of the two sutures. The white circle marks the site where the mechanical stimulus was applied. Adapted from Deuis et al. (2017).

2.4.4.4 Testing dynamic mechanical sensitivity

Dynamic mechanical allodynia was assessed using the summated withdrawal score to a gentle brush with a paintbrush (**Figure 2.4**). Each hindpaw was stroked three times (~ 1 stroke per sec) in a heel to toe direction and the scores form each response were combined to give a summative score of dynamic mechanical allodynia. The responses were scored as previously described: 0 = no response or walk away, 1= single withdrawal of the paw toward the body; 2 = multiple withdrawals in close succession; 3 = licking or sustained lifting of the paw (Duan et al., 2014). To allow sufficient time for recovery in between successive stimulations, the left paws of all animals were first sequentially tested, followed by the sequential testing of the right paws, ensuring sufficient recovery time before any repeated stimulation of a given paw. Baseline mechanical allodynia was assessed two days (at P15 and P17) prior to plantar incision at P19. Post-incision withdrawal scores to dynamic brush were assessed for 3 days, with testing conducted PSD1 to PSD5.

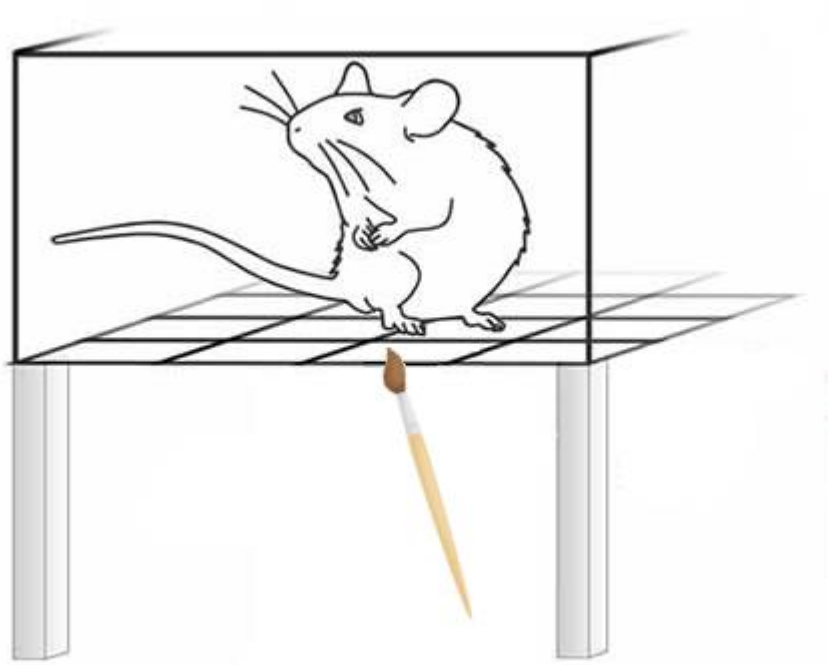


Figure 2.4 Testing dynamic mechanical sensitivity.

A dynamic brush applied stimulus to the plantar surface of the hindpaw. Adapted from Deuis et al. (2017).

2.4.4.5 Testing noxious heat sensitivity

Heat sensitivity was measured using a modified Hargreaves apparatus (IITC Life Science Plantar Test Apparatus) (Hargreaves et al., 1988). Animals were placed within rectangular Perspex containers placed on an elevated glass platform maintained at 30^{o}C. A thermal stimulus was applied by focusing a bright light source, set to 30% of maximal intensity, onto the plantar aspect of the hindpaw (**Figure 2.5A**). When the light source was turned on, a timer automatically started and was manually stopped when a withdrawal response was observed. The site of radiant heat application to the left hindpaw of a rat with respect to the incision site is illustrated in **Figure 2.5B**. The light source was applied for a maximum of 20 seconds to prevent potential tissue damage. The latency to withdrawal response was recorded in seconds. To avoid over-testing, the left paws of all animals were first sequentially tested, followed by the sequential testing of the right paws. This ensured enough time before any repeated stimulation of each paw. The left and the right paws were tested three times, with an average of each paw taken as a thermal

withdrawal latency. Baseline withdrawal latency to radiant heat stimulus was assessed two days (at P15 and P17) prior to plantar incision at P19. Post-incision withdrawal latencies to radiant heat were assessed for 4 days, with testing conducted PSD1 to PSD8.

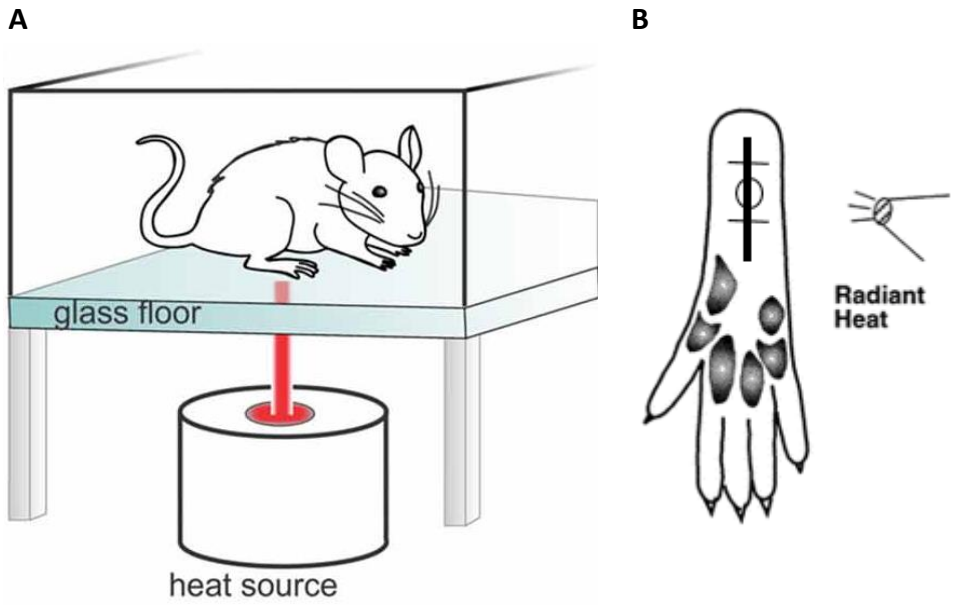


Figure 2.5 Testing noxious heat sensitivity.

Hargreaves apparatus was used to focus a bright light source onto the plantar aspect of the hindpaw (A). The site of application of the radiant heat source onto the plantar aspect of rat hindpaw (B). The vertical line marks the site of the surgical incision. The two horizontal lines represent the sites of the two sutures. The white circle marks the site of the radiant heat application. Adapted from Deuis et al. (2017).

2.4.4.6 Testing noxious cold sensitivity

Noxious cold sensitivity was measured using the cold plantar assay (Brenner et al., 2012, Brenner et al., 2015). Animals were placed within rectangular Perspex containers on an elevated glass platform. A noxious stimulus was delivered by applying a cut off syringe filled with dry ice (5-12^{o}C) to the glass underneath the hindpaw (**Figure 2.6**). The latency to paw withdrawal was recorded in seconds using a stopwatch with a cut off time of 20 seconds to prevent potential tissue damage.

To avoid over-testing, the left paws of all animals were first sequentially tested, followed by the sequential testing of the right paws. This ensured enough time before any repeated stimulation of each paw. The left and the right paws were tested three times, with an average of each paw taken as a noxious cold withdrawal latency. Baseline withdrawal latency to noxious cold stimulus was assessed three days (at P14, P16 and P18) prior to plantar incision at P19. Post-incision withdrawal latencies to noxious cold were assessed for 3 days, with testing conducted PSD1 to PSD5.

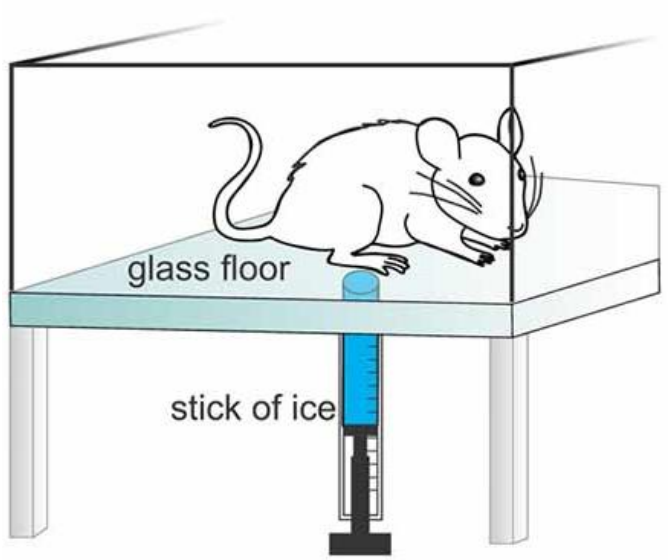


Figure 2.6 Testing noxious cold sensitivity.

The cold plantar assay was used to measure sensitivity to noxious cold, where a cut off syringe filled with dry ice was delivered to the glass surface underneath the hindpaw. Adapted from Deuis et al. (2017).

2.4.5 Isolated dorsal root electrophysiology

2.4.5.1 Isolated dorsal root preparation

Isolated dorsal roots were prepared based on previously established methodologies (Torsney, 2011, Dickie et al., 2017, Bardoni et al., 2004, Labrakakis et al., 2003, Lee et al., 2002). Under isoflurane-induced anaesthesia, naive untreated (control) or rats that received a plantar hindpaw incision (aged from P17 to P24) were decapitated and spinal cords, with attached dorsal roots, were removed in an ice-

cold dissection solution, continuously bubbled with 95% O_2 / 5% CO_2. Lumbar (L4/L5) dorsal roots (DRs) were cut near their entry zone and their dorsal root ganglia (DRG) were removed. The roots were briefly recovered for up to 15mins in 32-34^{o}C oxygenated *N*-methyl-D-glucamine (NMDG) recovery solution and then placed in oxygenated holding solution for 1h at room temperature prior to recording. In case of the rats that received a plantar incision, DRs from the ipsilateral incised (left) side were only prepared, while for control animals – L4 and L5 from both sides were used.

The isolated dorsal roots were transferred to the recording chamber of an upright microscope (Ziess Axiokop II) and perfused with a constant flow (1-2ml/min) of oxygenated recording solution at room temperature. The 95% O_2/5% CO_2-saturated dissection solution contained 3.0mM KCl, 1.2mM NaH_2PO_4, 26mM $NaHCO_3$, 15mM glucose, 251.6mM sucrose, 7mM $MgCl_2$, and 0.5mM $CaCl_2$, pH 7.3-7.4. The NMDG recovery solution comprised 93mM NMDG. 2.5mM KCl, 1.2mM NaH_2PO_4, 30mM $NaHCO_3$ 25mM glucose, 20mM HEPES, 5mM Sodium absorbate, 2mM Thiourea, 3mM Sodium pyruvate,10mM $MgSO_4$, 0.5mM $CaCl_2$, pH 7.3-7.4. The holding solution contained 92mM NaCl, 2.5mM KCl, 1.2mM NaH_2PO_4, 30mM $NaHCO_3$, 25mM glucose, 20mM HEPES, 5mM Sodium absorbate, 2mM Thiourea, 3mM Sodium pyruvate, 2mM $MgSO_4$, 2mM $CaCl_2$, pH 7.3-7.4. The recording solution contained 125.8mM NaCl, 3.0mM KCl, 1.2mM NaH_2PO_4, 26mM $NaHCO_3$, 15mM glucose, 1.3mM $MgCl_2$, and 2.4mM $CaCl_2$, pH 7.3-7.4. A high Mg^{2+}, low Ca^{2+} solutions were used to minimise excitotoxic damage during dissection and recovery. All chemicals were obtained from Sigma.

2.4.5.2 Compound action potential recordings

CAP recordings were used to evaluate the influence of hindpaw incision on the electrically evoked properties of isolated dorsal roots. For incision animals, surgeries were performed on separate cohorts of animals from different litters, aged from P17 to P24, 2-4d before CAP recordings at ~P21. Similarly aged naïve rats, which did not receive anaesthesia or sham surgery, were used as controls. The time window

between PSD2-PSD4 was chosen for electrophysiological recordings as it was established that this is the period of peak hypersensitivity in the incised hindpaw (**Figure 2.20** & **Figure 2.21**) without any change in sensitivity of the contralateral hindpaw, as previously reported in this model (Atianjoh et al., 2010, Cao et al., 2015). The CAP recordings were made using two glass suction electrodes placed at each end of the dorsal root, one connected to an ISO-flex stimulus isolator (A.M.P.I.) and used for electrical stimulation, and the other connected to an extracellular amplifier and used for recording (**Figure 2.7**) (Labrakakis et al., 2003, Lee et al., 2002, Torsney, 2011, Bardoni et al., 2004, Baba et al., 1999). To determine the activation threshold for each primary afferent component, dorsal roots were stimulated 3 times at 0.2Hz at 2.5, 5, 7.5, 10, 15, 20, 25, 30–100 (in steps of 10 μA), and 150–500 μA (in steps of 50 μA), with a 0.1ms pulse width, which replicates the electrical stimuli previously shown to activate the different afferent fibre types in this age of rat (Nakatsuka et al., 2000). Data were acquired and recorded using a Cygnus ER-1 differential amplifier (Cygnus Technologies Inc.) and pClamp 10 software (Molecular Devices). Data were filtered at 10 kHz and sampled at 50 kHz.

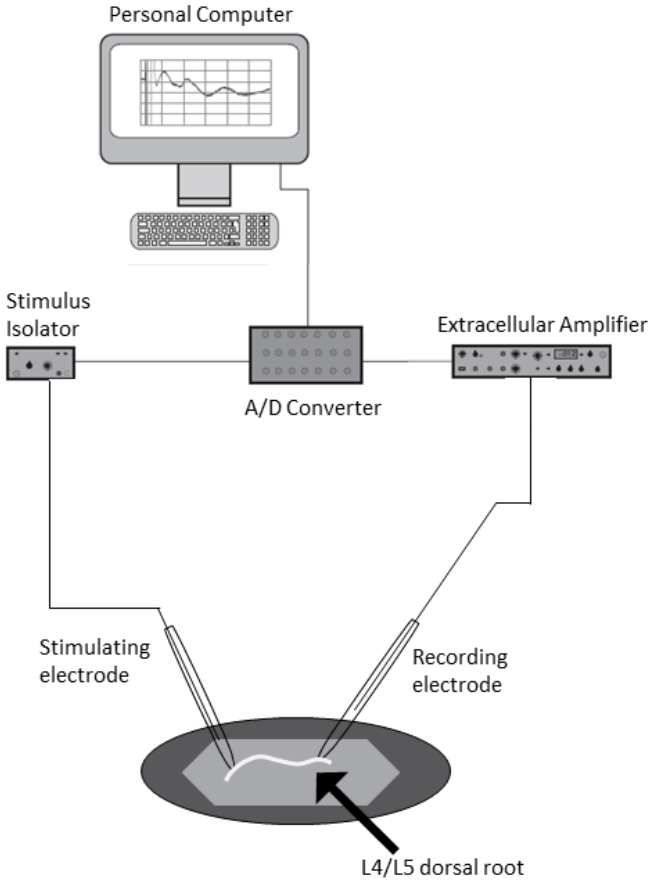


Figure 2.7 Experimental arrangement for ex vivo CAP recordings produced by L4/L5 dorsal root stimulation. Adapted from Dickie (2014)

The three main components of the CAPs could be distinguished based on their activation threshold and conduction velocity as fast (Aβ), medium (Aδ), and slow (C) conducting components, each displaying a characteristic triphasic (positive–negative–positive) response (**Figure 2.8**). Small intermediate components were observed occasionally, as previously reported (Géranton et al., 2009, Torsney, 2011). These, however, were not analysed.

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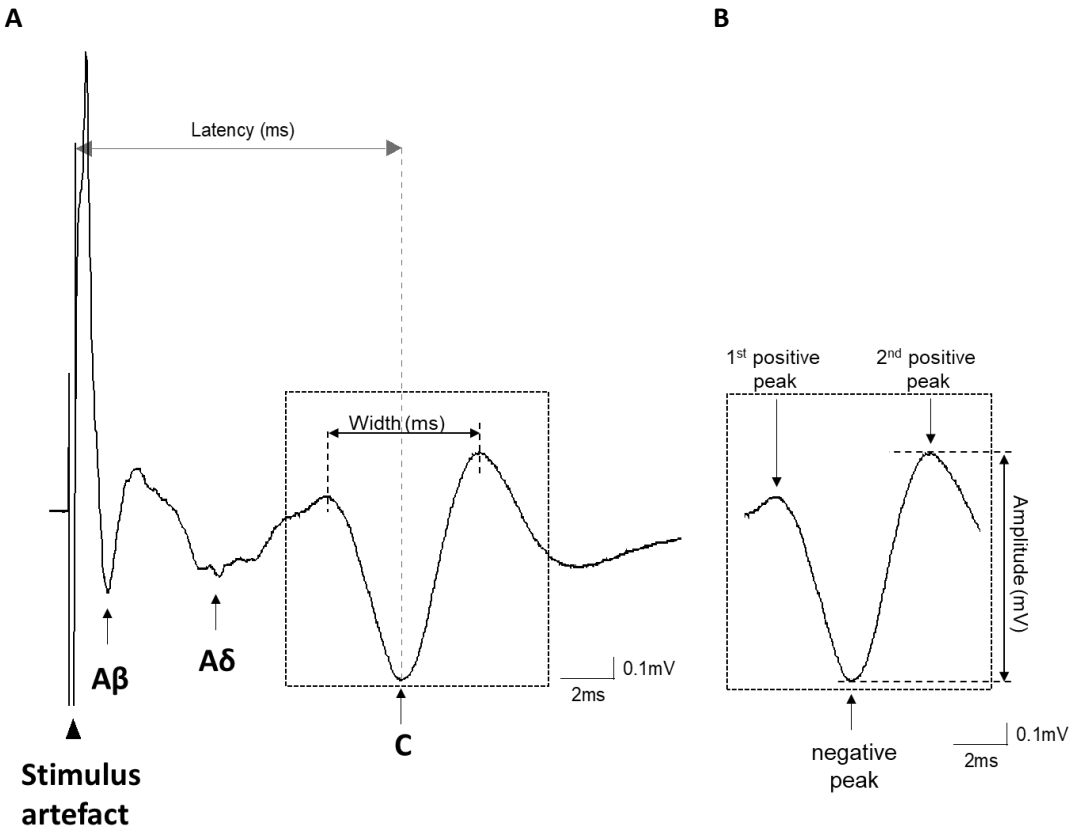


Figure 2.8 Representative population CAP recording.

A) Representative population CAP recording showing the fast (Aβ), medium (Aδ), and slow (**C**) conducting components. This example shows how the C-fibre response latency and width were measured (grey double-headed arrow). Latency was measured as the time distance between the stimulus artefact and the negative peak of the triphasic response. Response width was measured as the time distance from positive-to-positive peaks (black double-headed arrow). The negative peaks of the Aβ, Aδ and C-fibre components are indicated by the arrows. **B**) Example trace shows the C-fibre component denoted by the box in A). The negative and 2 positive peaks of the triphasic response can be clearly seen, denoted by the arrows. Demonstrated is the measurement of the C-fibre response amplitude, measured as the millivolt difference between the negative and second positive peak (double-headed arrow). The trace shown is an average of 3 traces at 500µA.

The activation threshold for each component was defined as the lowest stimulation intensity which produced a clearly identifiable negative component of the triphasic response (**Figure 2.9**). The amplitude and the estimated conduction velocity of the C-fibres response were measured from the averaged traces (average of 3 traces) at 500µA. Amplitude was measured using the voltage difference between the negative and the second positive peaks (**Figure 2.8B**). Estimated conduction velocity was

calculated by dividing the estimated root length by the response latency. The latter was measured as the time distance between the stimulus artefact and the negative peak of the triphasic response (**Figure 2.8A**), while the estimated dorsal root length was measured as the distance between the recording and stimulating electrodes. The width of the C-fibre response was measured as the time distance from positive-to-positive peak of the triphasic response (**Figure 2.8A**). It reflected the range of conduction velocities within the population C-fibre response.

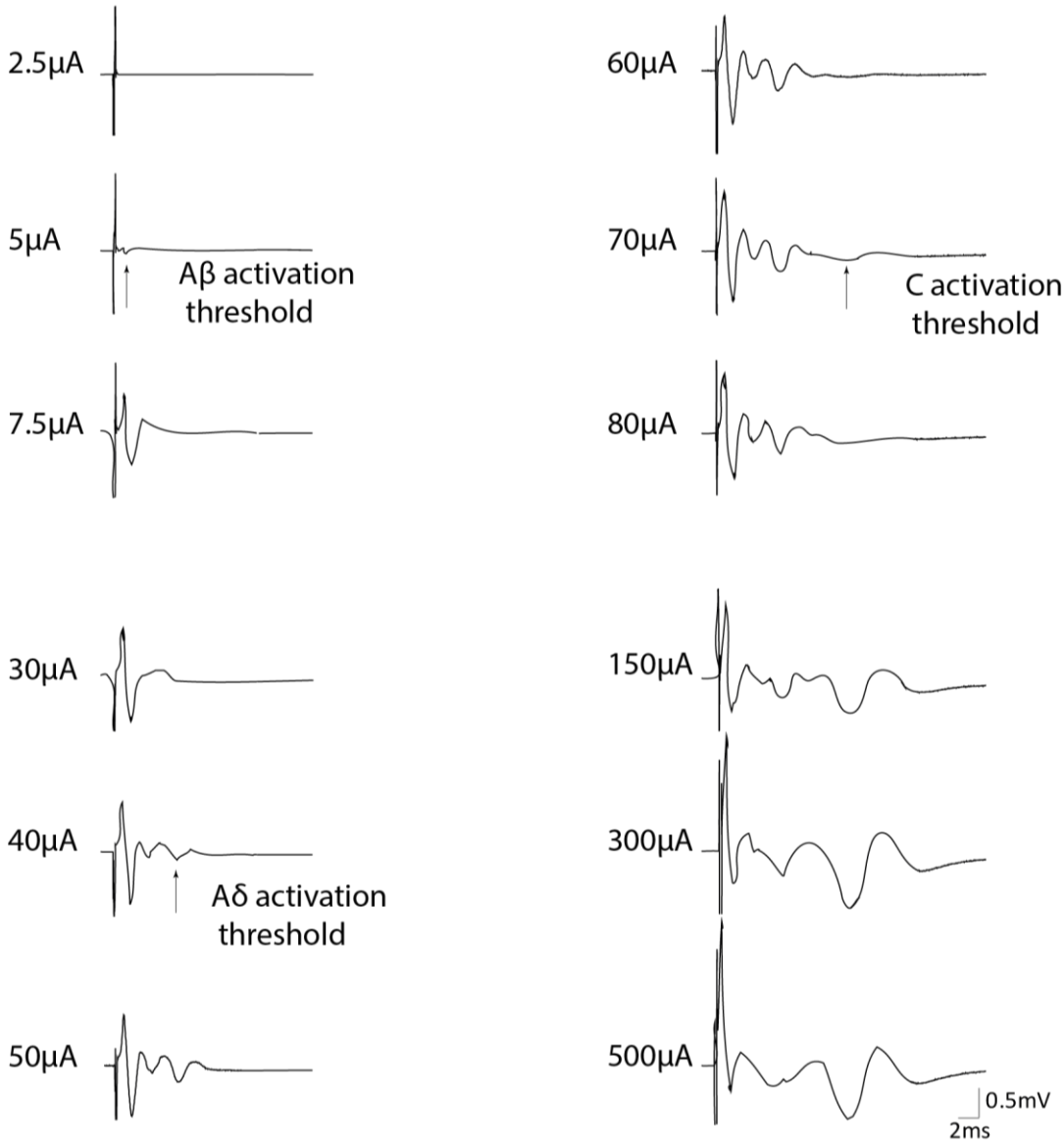


Figure 2.9 Primary afferent activation threshold identification.

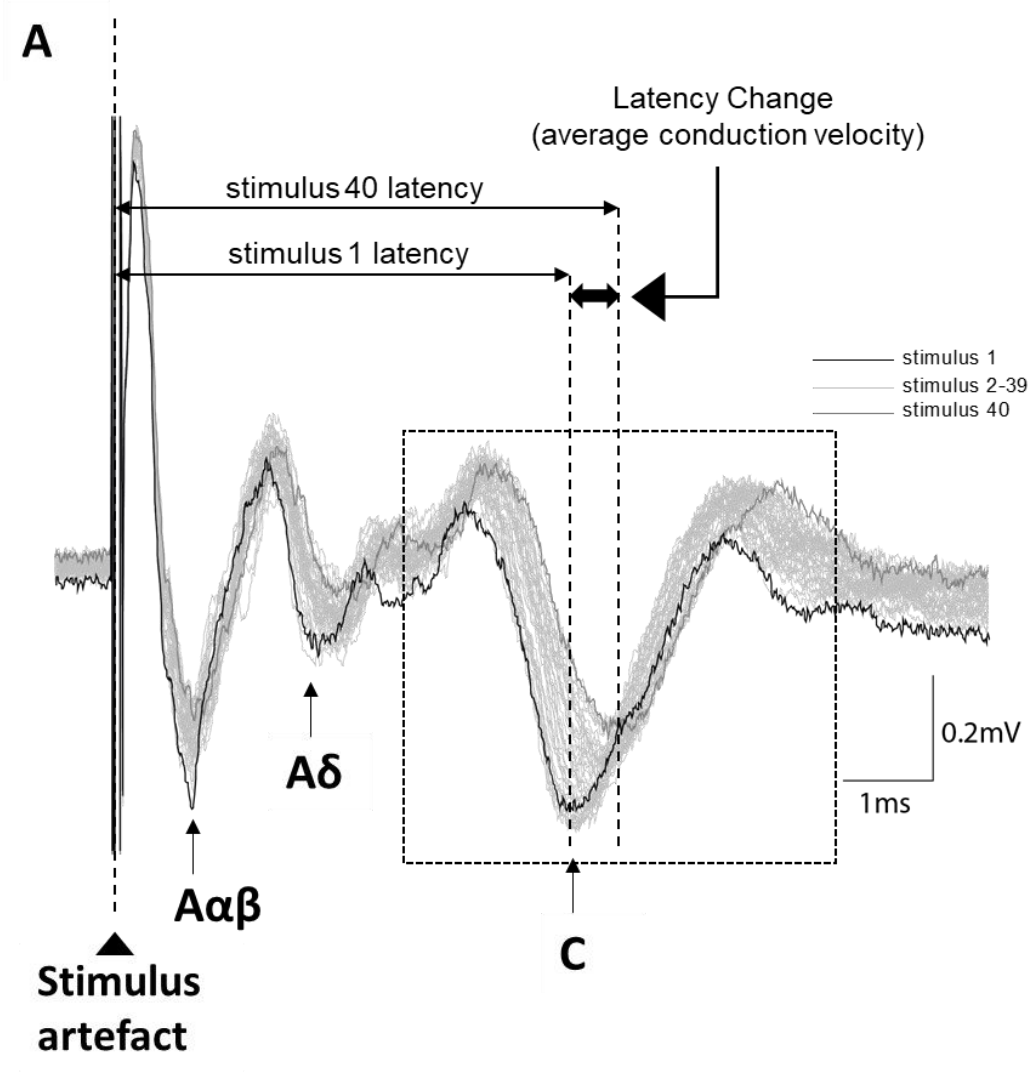
Representative example of how the activation thresholds for Aβ, Aδ and C-fibres were identified in isolated dorsal root CAP recordings. Activation thresholds were defined as the lowest stimulation intensity at which negative peak of the triphasic response is first clearly identifiable (indicated on the traces by arrows). The stimulus artefact is highlighted in the first example recording by the arrowhead. Numbers to the left of traces indicate corresponding stimulation intensity used. The shown traces are the averaged response of 3 traces.

2.4.6 C-fibre activity-dependent slowing

In these experiments some of the analysis on the resulting data was performed by a BSc Neuroscience student – Sophie E Coleman, as part of her honours project under my supervision. All CAP recording analysis was subjected to blinding of sex and incision.

2.4.6.1 C-fibre activity-dependent slowing in CAP recordings from isolated dorsal roots in juvenile rats

To assess C-fibre activity-dependent slowing (ADS), isolated dorsal roots were stimulated 40 times at frequencies of 2Hz and 10Hz at a stimulation intensity of 500μA and stimulus duration 0.1ms. 10-minute-recovery intervals were left between periods of stimulation to allow the fibres to fully recover from ADS (Shim et al., 2007, Weidner et al., 1999). These stimulation frequencies were chosen because they mimic the evoked firing rates of the C-fibres observed following injury (Xiao and Bennett, 2007, Hulse et al., 2010, Matson et al., 2015, Lynn and Carpenter, 1982, Leem et al., 1993). For each stimulus, the latency from the stimulus artefact to the negative peak of the triphasic response was measured and the change in latency from stimulus 1 was calculated **(Figure 2.10A**). This was also done for the remaining 39 stimuli. The change in C-fibre width was analogously calculated (**Figure 2.10A**). Latency change reflects change in the average conduction velocity of the C-fibre population, whereas change in the width of the C-fibre component reflects change in the range of the conduction velocities of C-fibre population. Given that C-fibre ADS is length-dependent (Schmelz et al., 1995, Zhu et al., 2009), the latency/width change was normalised to the length of root stimulated to negate the influence of varying dorsal root lengths on ADS.


A
Latency Change
(average conduction velocity)
stimulus 40 latency
stimulus 1 latency
stimulus 1
stimulus 2-39
stimulus 40
0.2mV
1ms
Aδ
Aαβ
C
Stimulus
artefact

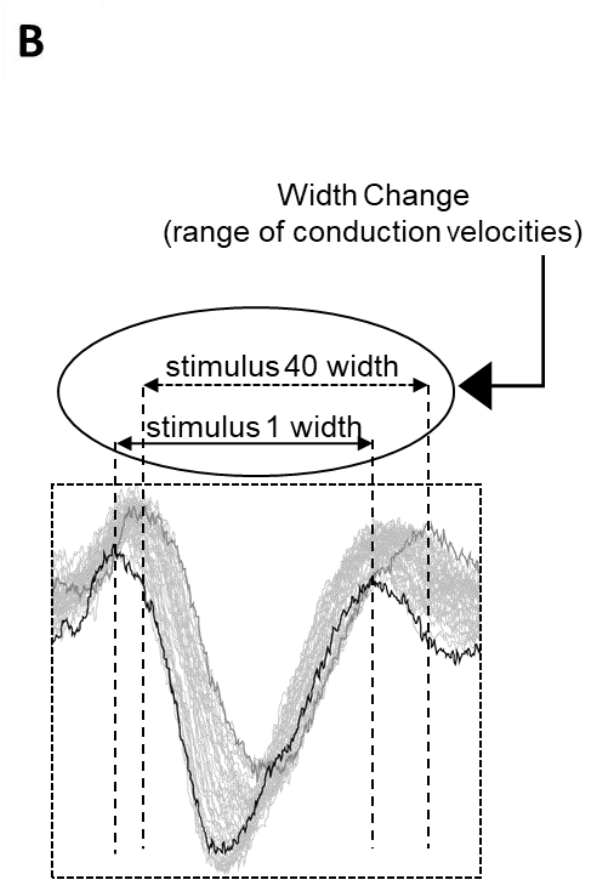

B
Width Change
(range of conduction velocities)
stimulus 40 width
stimulus 1 width

Figure 2.10 Population CAP recording of ADS.

Compound action potential recording of activity-dependent slowing. **A)** Representative population responses to a train of 40 stimuli at 500µA at a frequency of 2Hz. Inset shows the colour coding of the traces for stimulus 1, stimulus 2-39 and stimulus 40. Latency of C-fibre responses was measured as the time distance between the stimulus artefact (black dashed line marked with arrowhead) and the negative peak of the response, as indicated by the arrows and dashed lines. C-fibre latency change is demonstrated by the shift of the 40th response compared to the 1st response (double-headed bold arrow). **B**) Example trace shows the C-fibre component denoted by the box in **A**. Width of C-fibre responses was measured as the distance between the 1st and 2nd positive peaks of the triphasic response. Double-headed arrows mark the distance between the two positive peaks (dashed lines) for the first and last responses. C-fibre width change is demonstrated by the increased width of the 40th response compared to the 1st response.

2.4.6.2 Tetrodotoxin-induced modulation of C-fibre activity-dependent slowing in CAP recordings

To explore the role of TTX-sensitive voltage-gated sodium (Na_V) channels in control and incision tissue, CAP recordings of C-fibres were conducted in dorsal roots isolated from control and incision juvenile rats of both sexes, as previously described in section 2.4.6.1. Dorsal roots were stimulated 40 times at frequencies of 2Hz and 10Hz (500µA, 0.1ms duration) before (baseline) and in the continued presence of low dose (20nM) TTX (Alomone Labs) or vehicle (recording solution), bath-applied for 10mins after baseline recording at 10Hz. The interval of 10mins allowed for TTX to be washed into the recording bath before the CAP recordings in the continued presence of TTX were made at 2Hz and 10-mins after at 10Hz. The interval of 10mins between TTX recordings at 2Hz and 10Hz allowed the C-fibres to recover from ADS. The C-fibre latency and width were measured as previously described in section 2.4.6.1 and demonstrated in **Figure 2.8**. The latency/width change data were assessed by calculating the latency/width change between the response latency/width at stimulus 1 and the subsequent 39 stimuli (**Figure 2.10**). To assess the role of TTX-sensitive channels upon ADS, the extent of the TTX/vehicle effect was calculated for each root individually by subtracting the baseline measurement of latency/width change from the corresponding latency/width change value in the presence of 20nM TTX/vehicle.

The effect of TTX on the initial C-fibre estimated conduction velocity was also investigated since previous studies have shown that TTX affects C-fibre conduction velocity as reflected in the altered initial latency of the C-fibre response (De Col et al., 2008, Baker and Waxman, 2012a). This was done by comparing the latency of the C-fibre response at the first stimulus during baseline recording to the latency of the C-fibre response at the first stimulus during TTX recording.

2.4.7 Statistical Analysis

2.4.7.1 Electrophysiology

In CAP recordings from isolated roots, the effects of incision on the basic properties (activation threshold, initial amplitude and estimated conduction velocity) of the C-fibre response were statistically assessed using two-way ANOVA. Post-hoc multiple comparison tests were run only if a significant interaction between the two investigated factors was detected by the two-way ANOVA. Sidak's multiple comparisons test was performed where there was an interaction between the two factors. To compare the effects of incision and sex on C-fibre ADS at different stimulation frequencies, the ADS area under the curve (AUC) for each group was measured and analysed using three-way ANOVA followed by Sidak's multiple comparisons test if an interaction between factors was observed.

The effects of TTX or vehicle on C-fibre ADS were also measured using AUC for baseline and TTX/vehicle groups. Unpaired t-test were performed for each group to statistically compare baseline ADS versus TTX/vehicle. To compare the magnitude of the TTX effects in control and incision males and females at different stimulation frequencies, the grouped AUC was measured and analysed using three-way ANOVA followed by Sidak's multiple comparisons test if an interaction between factors was observed.

2.4.7.2 Behavioural assessments of sensitivity

Mechanical and thermal sensitivity data, at baseline and following incision, are presented as a percentage of contralateral measures to enable comparison between sexes. These data were analysed using a repeated measures two-way ANOVA followed by Dunnett's multiple comparison test if there was an effect of incision. The extent of peak thermal and mechanical hypersensitivity in males and females, over the time window during which electrophysiological data was collected, was directly assessed using unpaired two-tailed t-tests.

Graphad Prism 9.11 software (GraphPad Software) was used for statistical analysis and graph production. All data are represented as mean ± standard error of the

mean (SEM), with n representing the sample size. P<0.05 was used to indicate statistical significance.

2.5 Results

Some of the data presented in this section were analysed by a BSc Physiology Honours student – Sophie E Coleman, under my supervision.

2.5.1 Incision and sex do not influence basic C-fibre properties.

To assess the impact of hindpaw incision on afferent C-fibre nociceptor function, dorsal roots isolated from control or incision rats were used to record CAPs as demonstrated in **Figure 2.7** and **Figure 2.8**. The three main components of the CAPs were distinguished based on activation threshold and conduction velocity as fast (Aβ), medium (Aδ) and slow (C) conducting components, each displaying a characteristic triphasic (positive-negative-positive) response (**Figure 2.8**). Only the C-fibre components were analysed and their activation thresholds were defined as the lowest stimulation intensity which produced a clearly identifiable negative component of the triphasic response (**Figure 2.9**).The C-fibre response latency and width were measured as shown in **Figure 2.8**. The effect of incision and sex on the basic C-fibre properties, including activation threshold, amplitude and initial average conduction velocity, was investigated. There was no significant effect of incision and sex on C-fibre activation threshold (**Figure 2.11A**; two-way ANOVA: incision, p=0.98, sex, p=0.51), amplitude (**Figure 2.11B**; two-way ANOVA: incision, p=0.81, sex, p=0.44) and initial average conduction velocity (CV) (**Figure 2.11C**; two-way ANOVA: incision, p=0.83, sex, p=0.43).

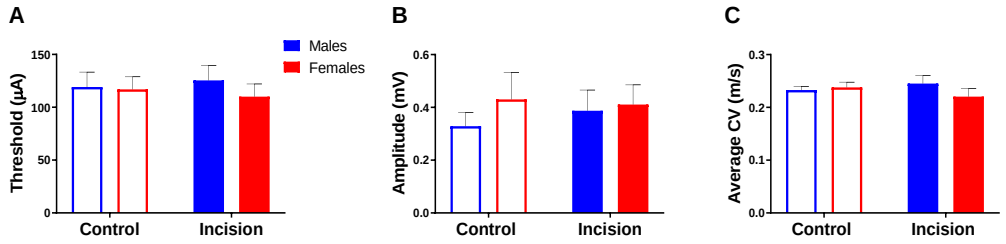


Figure 2.11 Basic C-fibre properties in isolated dorsal roots in control and incision rats of both sexes.

(**A**) C-fibre activation threshold was not significantly different between the control and incision groups in both sexes (Two-way ANOVA: incision, p=0.98, sex, p=0.51, interaction, p=0.62). (**B**) C-fibre initial amplitude was not significantly different between the control and incision groups in both sexes (Two-way ANOVA: incision, p=0.81, sex, p=0.44, interaction, p=0.63). (**C**) C-fibre initial average conduction velocity was not significantly different between the control and incision groups in both sexes (Two-way ANOVA: incision, p=0.83, sex, p=0.43, interaction, p=0.23). All data presented as as mean±SEM. Male: control (n=12), incision (n=13). Female: control (n=13), incision (n=12).

2.5.2 Incision alters frequency-dependent C-fibre ADS in a sex-dependent manner.

To assess C-fibre ADS, dorsal roots from control and incision male and female rats were repetitively stimulated 40x times at 2Hz and 10Hz. This resulted in a clear C-fibre ADS, manifested by a progressive increase in C-fibre response latency (**Figure 2.12**) and width (**Figure 2.13**) changes. The C-fibre ADS phenomenon was confirmed to be frequency-dependent with 10Hz stimulation resulting in greater latency (**Figure 2.12E**; three-way ANOVA: frequency, p<0.0001) and width (**Figure 2.13E**; three-way ANOVA: frequency, p<0.0001) changes than 2Hz, as previously demonstrated in rats (Dickie et al., 2017). In addition, control females showed overall greater latency change increase in comparison to control males (**Figure 2.13E**; three-way ANOVA: sex x incision, p=0.0497). This effect was independent of frequency (**Figure 2.13E**; three-way ANOVA: sex x frequency, p=0.41, frequency x sex x incision, p=0.499), but the statistical analysis showed a significant effect between control males and females only at 10Hz (**Figure 2.12E**; three-way ANOVA

followed by Sidak's multiple comparisons test, control males vs control females, *p*=0.013). Following incision, the progressive increase in C-fibre latency change was enhanced in a sex-dependent manner (**Figure 2.12A-E**; three-way ANOVA: sex x incision, p=0.0497). The male latency change was increased to levels comparable to females with statistically significant post-incision increase in males at 10Hz only (**Figure 2.12E**; three-way ANOVA followed by Sidak's multiple comparisons test, *p*=0.033). The latency change post-incision was not significantly different between males and females both at 2Hz (**Figure 2.12E**; three-way ANOVA followed by Sidak's multiple comparisons test, incision males vs incision females, *p*=0.999) and at 10Hz (**Figure 2.12E**; three-way ANOVA followed by Sidak's multiple comparisons test, incision males vs incision females, *p*=0.999).

When C-fibre ADS was assessed as a progressive increase in response width, in contrast to the latency change data, there was no significant effect of sex on control width change at both 2Hz (*p*=0.998) and 10Hz (*p*> 0.999) (**Figure 2.13E**; three-way ANOVA followed by Sidak's multiple comparisons test, control males vs control females). However, in line with the latency change data, incision also enhanced C-fibre width change in a sex-dependent manner (**Figure 2.13E**; three-way ANOVA: frequency x incision, *p*=0.016, sex x incision, *p*<0.0001). The progressive increase in C-fibre width change post-incision, however, increased only in females (**Figure 2.13E**; three-way ANOVA: frequency x incision, *p*=0.016, sex x incision, *p*<0.0001) with statistically significant increase in female width change at 10Hz only (**Figure 2.13E**; three-way ANOVA followed by Sidak's multiple comparisons test, *p*=0.0003). The width change in incision males was significantly less in comparison to females both at 2Hz (*p*=0.007) and at 10Hz (*p*=0.0001) (**Figure 2.13E**; three-way ANOVA followed by Sidak's multiple comparisons test, incision males vs incision females).

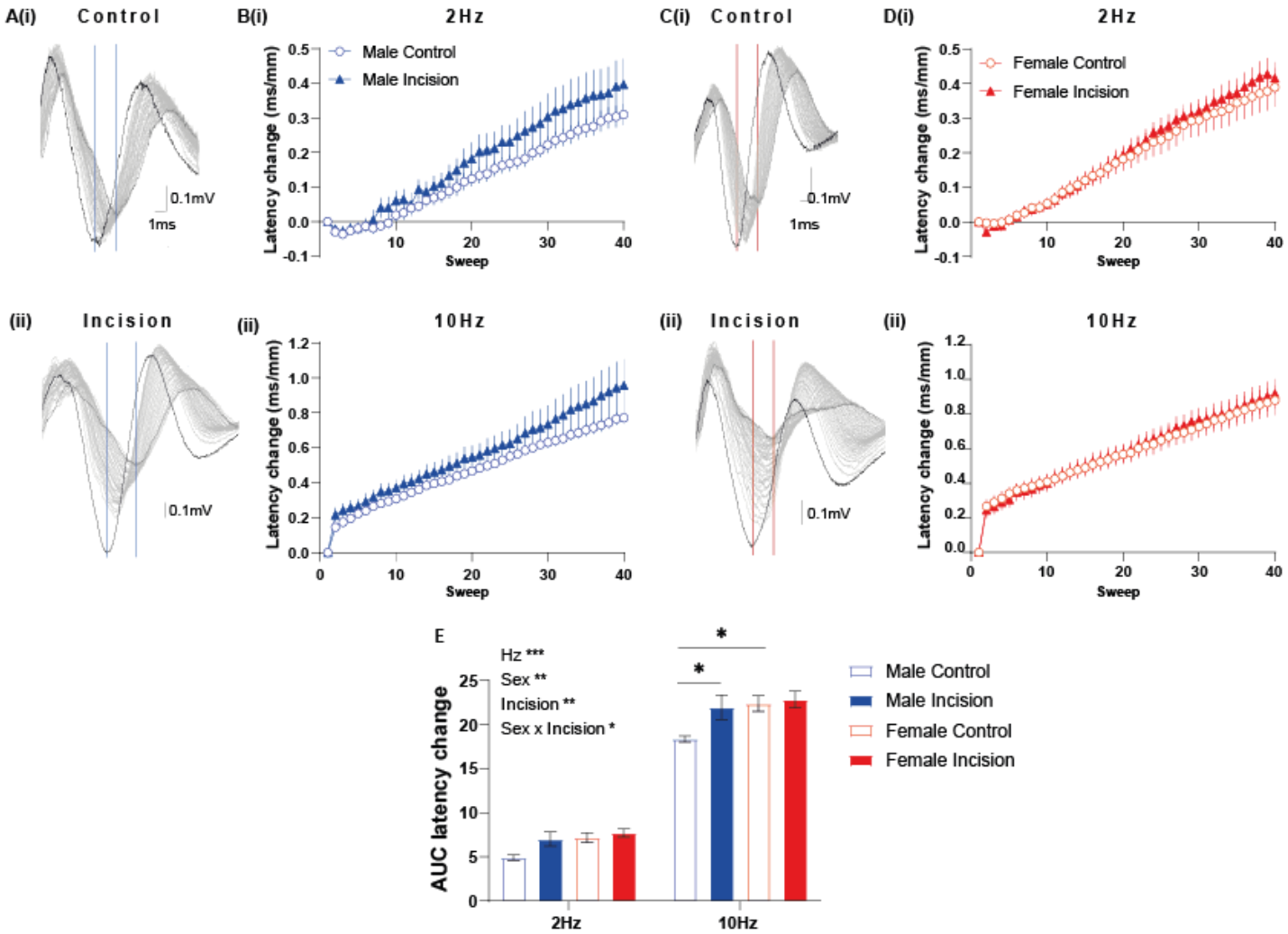

A(i) Control
0.1mV
1ms
B(i) 2Hz
Male Control
Male Incision
Latency change (ms/mm)
Sweep
C(i) Control
0.1mV
1ms
D(i) 2Hz
Female Control
Female Incision
Latency change (ms/mm)
Sweep
(ii) Incision
0.1mV
(ii) 10Hz
Latency change (ms/mm)
Sweep
(ii) Incision
0.1mV
(ii) 10Hz
Latency change (ms/mm)
Sweep
E
Hz ***
Sex **
Incision **
Sex x Incision *
AUC latency change
2Hz
10Hz
Male Control
Male Incision
Female Control
Female Incision

Figure 2.12 Incision increases the progressive C-fibre latency change in males to levels similar to females.

Representative C-fibre CAP recordings from dorsal roots isolated from male (**A**) and female (**C**) control (**i**) and incision (**ii**) rats in response to x40 stimuli at 10Hz; latency change marked by solid lines (response trace 1 black; 2-39 pale grey; 40 dark grey). Repetitive stimulation results in a progressive C-fibre latency increase in control and incision dorsal roots from males (**B**) and females (**D**) at 2Hz (**i**) and 10Hz (**ii**). **E**) AUC analysis reveals a significant effect of frequency (***p<0.001), sex (**p=0.001), incision (**p=0.005) with a sex/incision interaction (*p=0.0497) (Three-way ANOVA followed by Sidak's multiple comparisons test, *p=0.033 at 10Hz). Data presented as mean±SEM. Male: control (n=11), incision (n=13). Female: control (n=12), incision (n=12).

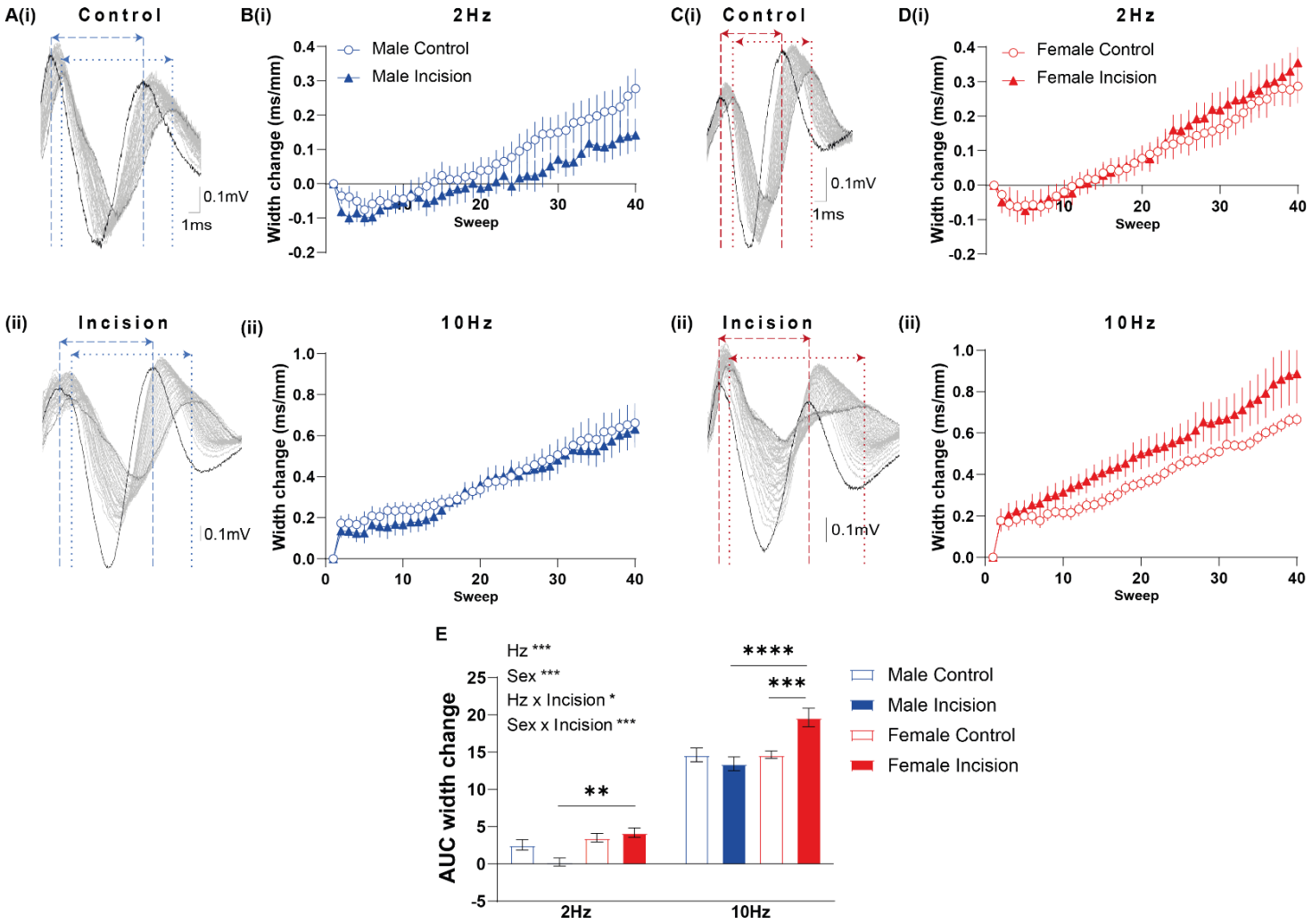

A(i)
Control
0.1mV
1ms
B(i)
2Hz
Male Control
Male Incision
Width change (ms/mm)
Sweep
C(i)
Control
0.1mV
1ms
D(i)
2Hz
Female Control
Female Incision
Width change (ms/mm)
Sweep
(ii)
Incision
0.1mV
(ii)
10Hz
Width change (ms/mm)
Sweep
(ii)
Incision
0.1mV
(ii)
10Hz
Width change (ms/mm)
Sweep
E
Hz ***
Sex ***
Hz x Incision *
Sex x Incision ***
AUC width change
2Hz
10Hz
Male Control
Male Incision
Female Control
Female Incision

Figure 2.13 Incision increases the progressive C-fibre width change in females only.

Representative C-fibre CAP recordings from dorsal roots isolated from male (**A**) and female (**C**) control (**i**) and incision (**ii**) rats in response to x40 stimuli at 10Hz; double-headed arrows mark C-fibre response width, the distance from positive-to-positive peaks for the first (dashed lines) and last (dotted lines) of the x40 responses (response trace 1 black; 2-39 pale grey; 40 dark grey). Repetitive stimulation results in a progressive C-fibre width increase in control and incision dorsal roots from males (**B**) and females (**D**) at 2Hz (**i**) and 10Hz (**ii**). **E**) AUC analysis of C-fibre width change reveals a significant effect of frequency (****p<0.0001) and sex (****p<0.0001) with a sex x incision (****p<0.0001) (three-way ANOVA followed by Sidak's multiple comparisons test, ****p<0.0001 at 10Hz). Data presented as mean±SEM. Male: control (n=12), incision (n=13). Female: control (n=12), incision (n=11).

2.5.3 TTX regulates C-fibre ADS in an incision-, sex- and frequency-dependent manner.

To investigate whether the Na_V blocker TTX can influence C-fibre function and ADS in an incision- and sex-dependent manner to access potential incision- and sex-dependent differences in the underlying $Na_V1.7/Na_V1.8$ ratio, CAP were recorded from control and incision dorsal roots from rats of both sexes before (baseline) and in the continued presence of low dose (20nM) TTX or vehicle. The effects of vehicle and low dose (20nM) TTX on initial average C-fibre conduction velocity were investigated by calculating the difference in the initial average conduction velocity following TTX/vehicle from the baseline initial average conduction velocity. TTX significantly reduced average conduction velocity in comparison to vehicle with no significant effect of either incision or sex **(Figure 2.14**; three-way ANOVA: incision, p=0.26; sex, p=0.756; TTX, p<0.0001; incision x sex, p=0.470; incision x TTX, p=0.127; sex x TTX, p=0.737; incision x sex x TTX interaction, p=0.06).

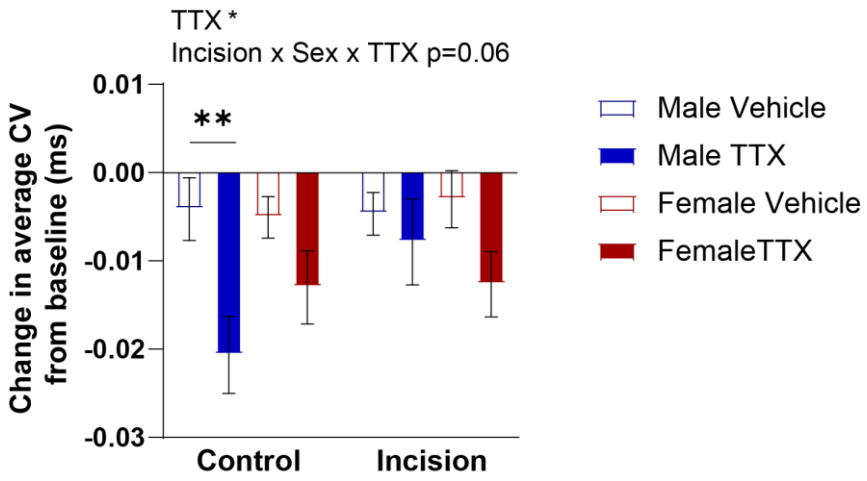


Figure 2.14 Effect of vehicle and TTX on average C-fibre conduction velocity in control and incision dorsal roots in both sexes.

TTX significantly reduces C-fibre average conduction velocity in comparison to vehicle independent of sex and incision (three-way ANOVA: incision, ns, p=0.26; sex, ns p=0.756; TTX, ***p<0.0001; incision x sex interaction, ns p=0.470; incision x TTX interaction, ns p=0.127; sex x TTX interaction, ns p=0.737; incision x sex x TTX

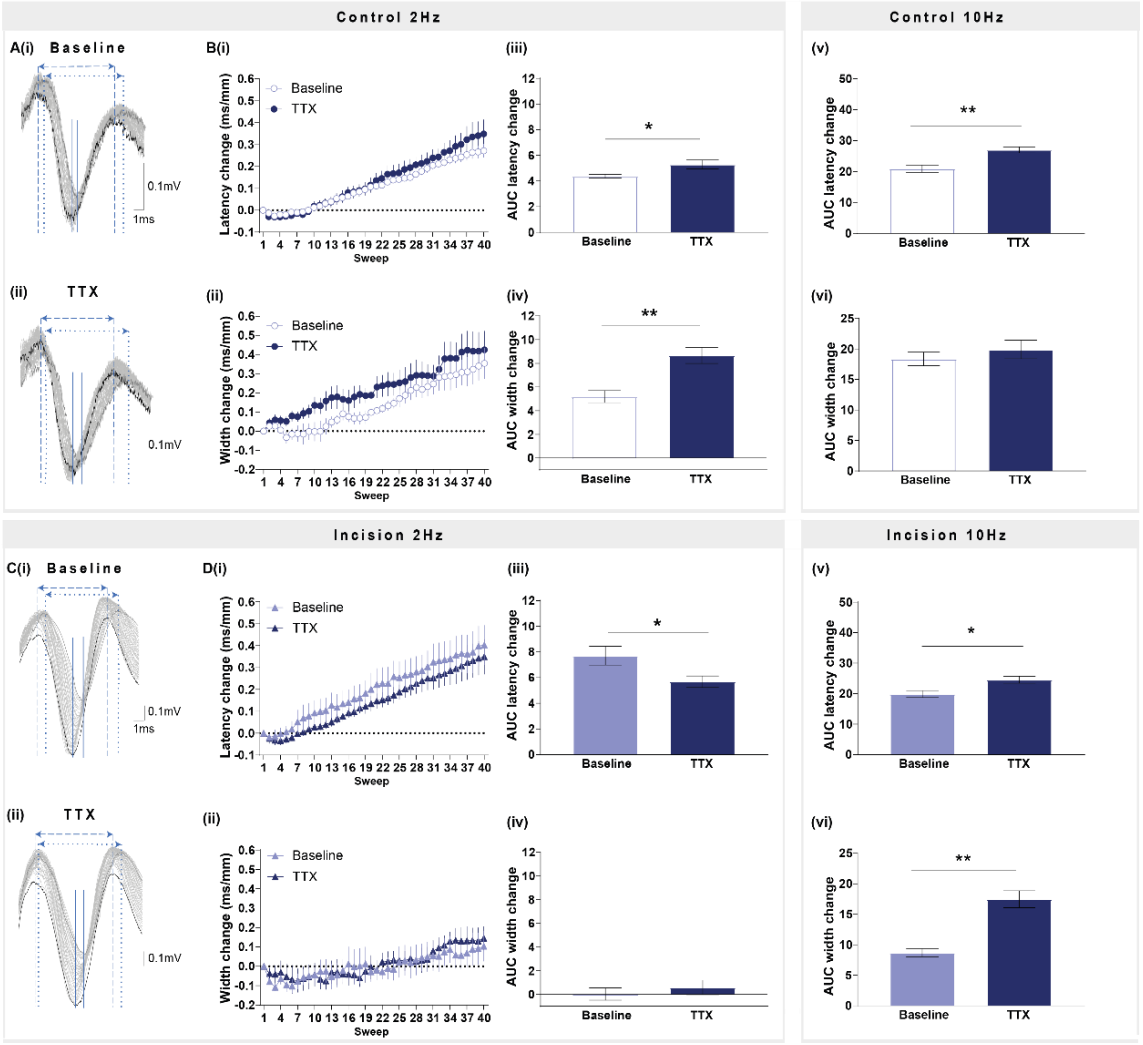
interaction, ns p=0.06). Data presented as mean±SEM. Male: control vehicle (n=6), control TTX (n=6), incision vehicle (n=7), incision TTX (n=6). Female: control vehicle (n=7), control TTX (n=6), incision vehicle (n=6), incision TTX (n=6).

To assess the effect of vehicle or the Na_V blocker TTX on C-fibre ADS in dorsal roots isolated from control and incision animals of both sexes, the dorsal roots were repetitively stimulated with a train of 40x stimuli at 2Hz and 10Hz in the presence of vehicle or TTX. The effects of vehicle and TTX on C-fibre ADS in both sexes are summarised in **Table 2.1**

When C-fibre ADS was assessed as a progressive increase in response latency, in control males the progressive increase in C-fibre response latency was significantly increased following bath application of TTX both at 2Hz (**Figure** 2.15**Bi,iii**; unpaired t-test, p=0.044) and 10Hz **(Figure 2.15Bv**; unpaired t-test, p=0.003). In contrast, following incision TTX had opposite effects at 2Hz, reducing the C-fibre latency change when compared to baseline (**Figure 2.15Di,iii**; unpaired t-test, p=0.040). Following incision at 10Hz, TTX had similar effect to the control 10Hz data, significantly increasing C-fibre latency change but to a lesser degree (**Figure 2.15Dv**; unpaired t-test, p=0.020). Vehicle treatment did not alter the progressive increase in C-fibre response latency at 2Hz for both control (**Figure 2.16Ai,ii**; unpaired t-test, p=0.7591) and incision (**Figure 2.16Ci,ii**; unpaired t-test, p=0.8845) groups and at 10Hz for both control **(Figure 2.16Aiii,iv**; unpaired t-test, p=0.131) and incision (**Figure 2.16Ciii,iv**; unpaired t-test, p=0.734) groups.

When C-fibre ADS was assessed as a progressive increase in response width, TTX treatment in control males significantly enhanced width change at 2Hz only (**Figure 2.15Bii,iv**; unpaired t-test, p=0.002), while at 10Hz TTX had no significant effects when compared to baseline (**Figure 2.15vi**; unpaired t-test, p=0.435). Following incision, TTX also had opposite effects in comparison to the control male data, significantly increasing the progressive C-fibre width change at 10Hz only (**Figure 2.15vi**; unpaired t-test, p=0.001), while causing no significant effect at 2Hz (**Figure**

2.15Bii,iv; unpaired t-test, *p*=0.505). Similarly to the latency change data, vehicle treatment did not alter the progressive increase in C-fibre response width in males. This was observed for both 2Hz control (**Figure 2.16Bi,ii**; unpaired t-test, *p*=0.078) and incision (**Figure 2.16Di,ii**; unpaired t-test, *p*=0.771) data and both 10Hz control (**Figure 2.16Biii,iv**; unpaired t-test, *p*=0.228) and incision (**Figure 2.16Diii,iv**; unpaired t-test, *p*=0.062) data.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0.1mV
1ms
(ii) TTX
0.1mV
B(i)
Baseline
TTX
Latency change (ms/mm)
Sweep
(ii)
Width change (ms/mm)
(iii)
AUC latency change
*
(iv)
AUC width change
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Control 10Hz
(v)
(vi)
Incision 2Hz
C(i) Baseline
0.1mV
1ms
(ii) TTX
0.1mV
D(i)
(iii)
*
(iv)
Incision 10Hz
(v)
*
(vi)
**

Figure 2.15 TTX regulates C-fibre ADS in an incision-dependent manner in males.

Representative C-fibre CAP recordings from dorsal roots isolated from control (**A**) and incision (**C**) male rats in response to 40 stimuli at 2Hz (response trace 1 black; 2-39 pale grey; 40 dark grey) illustrating the C-fibre latency (solid lines) and width (dashed (1^{st} response) and dotted (40^{th} response) double-headed arrows) changes at baseline (**i**) and following low dose (20nM) TTX (**ii**). Repetitive stimulation in control (**B**) and incision (**D**) dorsal roots at baseline and following TTX results in a progressive C-fibre latency (**i**) and width (**ii**) increase. AUC analysis of control (**B**) and incision (**D**) C-fibre latency (**iii,v**) and width (**iv,vi**) changes at 2Hz (**iii,iv**) and 10Hz (**v,vi**). TTX significantly facilitates control C-fibre latency change at 2Hz (**p*=0.044, n=5) and 10Hz (***p*=0.003, n=6), and width change at 2Hz (***p*=0.002, n=6) but not 10Hz (*p*=0.435, n=5) (unpaired two-tailed t-test). Following incision, TTX significantly alters C-fibre latency change at 2Hz (**p*=0.040, n=6) and 10Hz (**p*=0.02, n=5), and width change at 10Hz (***p*=0.001, n=4) but not 2Hz (*p*=0.505, n=6) (unpaired two-tailed t-test). Data presented as mean±SEM.

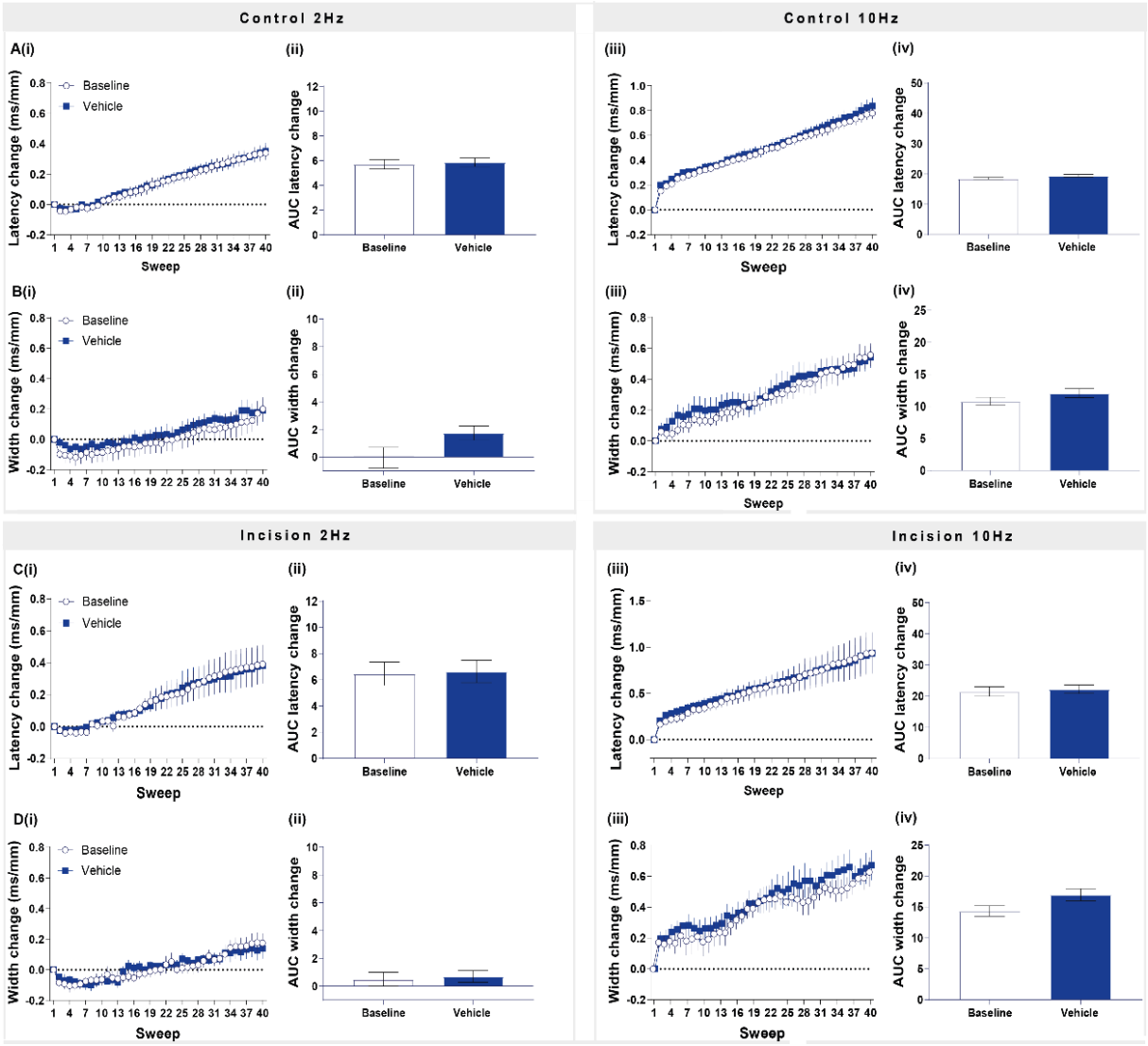
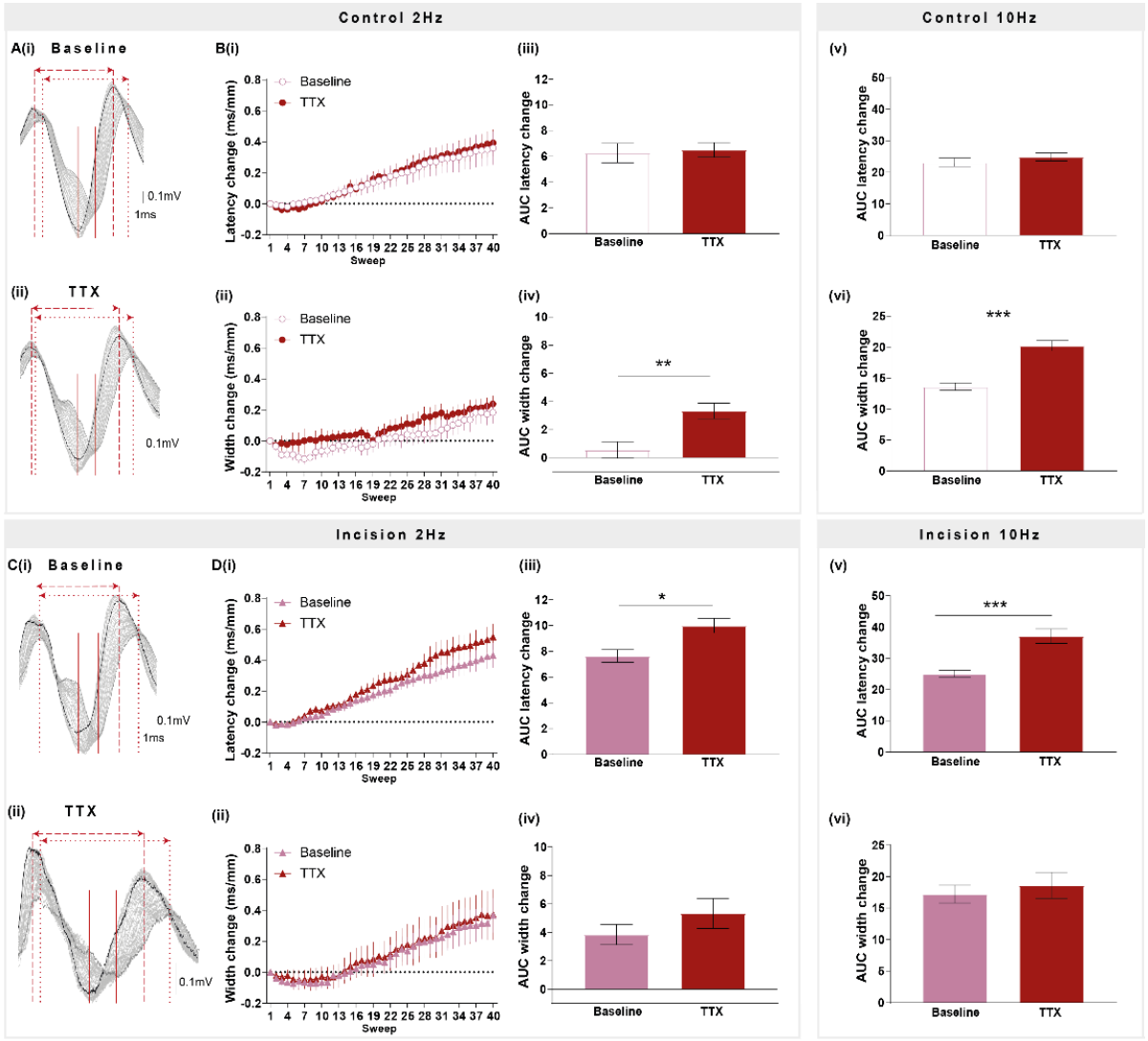

Control 2Hz
Control 10Hz
Incision 2Hz
Incision 10Hz
A(i)
B(i)
C(i)
D(i)
(ii)
(iii)
(iv)
Baseline
Vehicle
Latency change (ms/mm)
Width change (ms/mm)
AUC latency change
AUC width change
Sweep
1 4 7 10 13 16 19 22 25 28 31 34 37 40
Baseline
Vehicle

Figure 2.16 Vehicle has no effect on C-fibre ADS in control and incision tissue in males.

Repetitive stimulation in control **A-B** and incision **C-D** dorsal roots at baseline and following vehicle results in a progressive C-fibre latency **A,C** and width **B,D** increase at 2Hz **i-ii** and 10Hz **iii-iv**. AUC analysis of control **A-B** and incision **C-D** C-fibre latency **A,C** and width **B,D** changes at 2Hz **i-ii** and 10Hz **iii-iv**. Vehicle has no significant effect on control C-fibre latency change at 2Hz (**Aii**, *p*=0.759, n=6) and 10Hz (**Aiv**, *p*=0.131, n=5) and width change at 2Hz (**Bii**, *p*=0.078, n=6) and 10Hz (**Biv**, *p*=0.228, n=5) (unpaired two-tailed t-test). Vehicle does not significantly alter incision C-fibre latency change at 2Hz (**Cii**, *p*=0.885, n=7) and 10Hz (**Civ**, *p*=0.734, n=7) and width change at 2Hz (**Dii**, *p*=0.771, n=7) and 10Hz (**Div**, *p*=0.062, n=7) (unpaired two-tailed t-test).

In control females the progressive increase in C-fibre response latency was unaffected by TTX treatment both at 2Hz (**Figure 2.17Bi,iii**; unpaired t-test, *p*=0.800) and 10Hz (**Figure 2.17Bv**; unpaired t-test, *p*=0.384). In contrast, following incision the C-fibre latency change was increased after TTX application when compared to baseline both at 2Hz (**Figure 2.17Di,iii**; unpaired t-test, *p*=0.011) and 10Hz (**Figure 2.17Dv**; unpaired t-test, *p*<0.001). The vehicle treatment did not affect female C-fibre latency change when compared to baseline at 2Hz for both control (**Figure 2.18Ai,ii**; unpaired t-test, *p*=0.581) and incision (**Figure 2.18Ci,ii**; unpaired t-test, *p*=0.419) and at 10Hz for both control (**Figure 2.18Aiii,iv**; unpaired t-test, *p*=0.362) and incision (**Figure 2.18Ciii,iv**; unpaired t-test, *p*=0.813)

When C-fibre ADS was assessed as a progressive increase in C-fibre response width in females, TTX treatment had opposite effects when compared to the latency change results, with control females showing significantly enhanced progressive width increase after TTX treatment both at 2Hz (**Figure 2.17Bii,iv**; unpaired t-test, *p*=0.006) and 10Hz (**Figure 2.17Bvi**; unpaired t-test, *p*<0.001). Following incision, as opposed to the control data, TTX no longer had a significant effect on the progressive width increase when compared to baseline for both 2Hz (**Figure 2.17Bii,iv**; unpaired t-test, *p*=0.276) and 10Hz (**Figure 2.17Bvi**; unpaired t-test, *p*=0.595). Vehicle treatment did not alter C-fibre width change at 2Hz for both control (**Figure 2.18Bi,ii**; unpaired t-test, *p*=0.878) and incision (Figure 2.18**Di,ii**; unpaired t-test, *p*=0.106) females. At 10Hz vehicle slightly (~17%) enhanced control female C-fibre width change when compared to baseline (**Figure 2.18Biii,iv**; unpaired t-test, *p*<0.001). The effect of vehicle at 10Hz in females is much less extensive than that of TTX (~49%) in the same group. In incision females at 10Hz vehicle still had no effect on the width change (**Figure 2.18Diii,iv**; unpaired t-test, *p*=0.547).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0.1mV
1ms
(ii) TTX
0.1mV
B(i)
Baseline
TTX
Latency change (ms/mm)
Sweep
(ii)
Baseline
TTX
Width change (ms/mm)
Sweep
(iii)
AUC latency change
Baseline
TTX
(iv)
AUC width change
**
Baseline
TTX
Control 10Hz
(v)
AUC latency change
Baseline
TTX
(vi)
AUC width change
***
Baseline
TTX
Incision 2Hz
C(i) Baseline
0.1mV
1ms
(ii) TTX
0.1mV
D(i)
Baseline
TTX
Latency change (ms/mm)
Sweep
(ii)
Baseline
TTX
Width change (ms/mm)
Sweep
(iii)
*
AUC latency change
Baseline
TTX
(iv)
AUC width change
Baseline
TTX
Incision 10Hz
(v)
***
AUC latency change
Baseline
TTX
(vi)
AUC width change
Baseline
TTX

Figure 2.17 TTX regulates C-fibre ADS in an incision-dependent manner in females.

Representative C-fibre CAP recordings from dorsal roots isolated from control (**A**) and incision (**C**) female rats in response to 40 stimuli at 2Hz (response trace 1 black; 2-39 pale grey; 40 dark grey), illustrating the C-fibre latency (solid lines) and width (dashed (1^{st} response) and dotted (40^{th} response) double-headed arrows) changes at baseline (**i**) and following low dose (20nM) TTX (**ii**). Repetitive stimulation in control (**B**) and incision (**D**) dorsal roots at baseline and following TTX results in a progressive C-fibre latency (**i**) and width (**ii**) increase. AUC analysis of control (**B**) and incision (**D**) C-fibre latency (**iii,v**) and width (**iv,vi**) changes at 2Hz (**iii,iv**) and 10Hz (**v,vi**). TTX has no effect on control C-fibre latency change at 2Hz (p=0.800, n=6) and 10Hz (p=0.384, n=5), while significantly facilitating C-fibre width change at 2Hz (**p=0.006, n=6) and 10Hz (***p<0.001, n=5) (unpaired two-tailed t-test). Following incision TTX significantly facilitates C-fibre latency change at 2Hz (*p=0.011, n=6) and 10Hz (**p=0.001, n=6), while having no significant effect on C-fibre width change at 2Hz (p=0.276, n=5) and 10Hz (p=0.595, n=5) (unpaired two-tailed t-test).

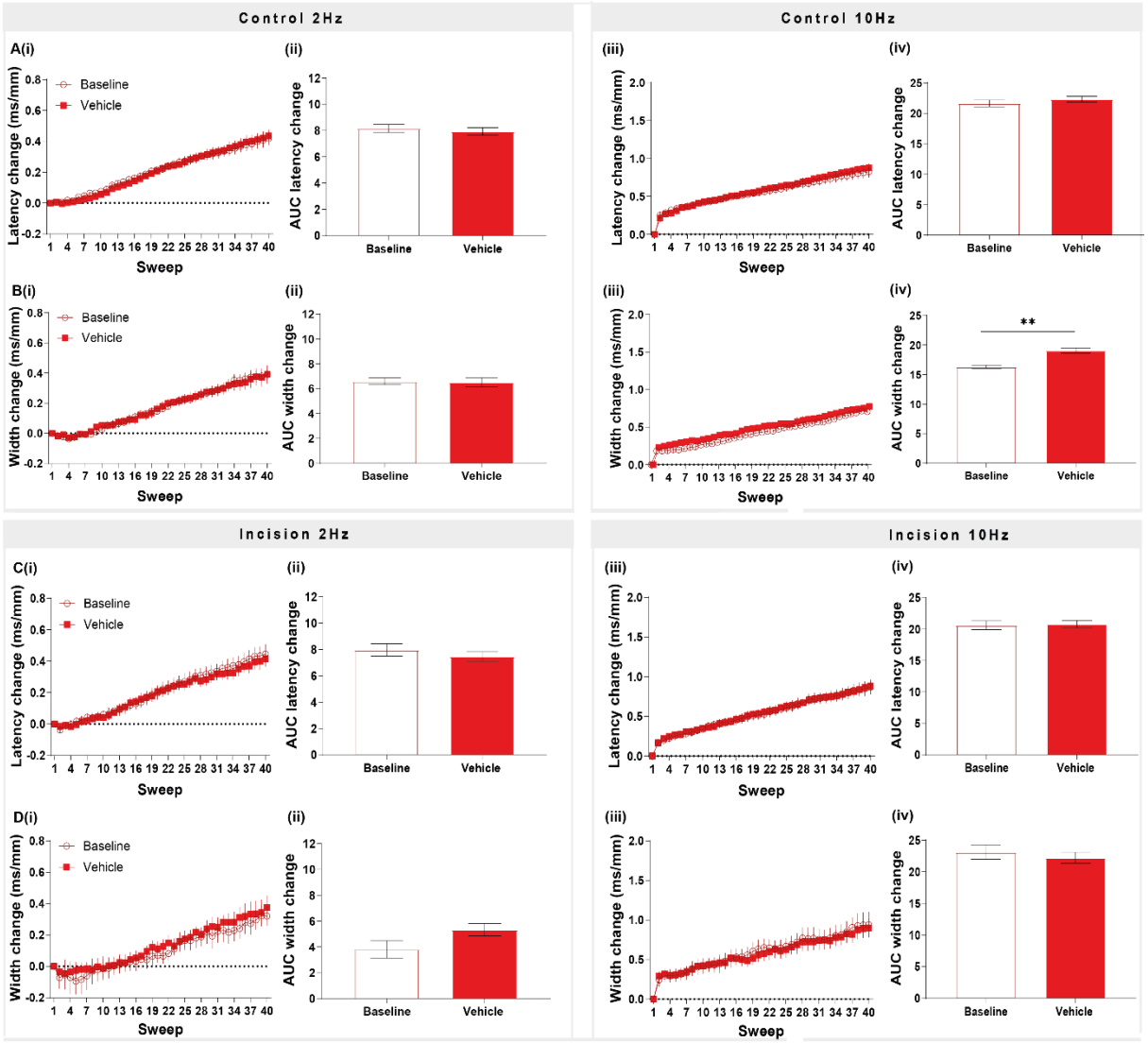

Control 2Hz
Control 10Hz
Incision 2Hz
Incision 10Hz
A(i)
B(i)
C(i)
D(i)
(ii)
(iii)
(iv)
Baseline
Vehicle
Latency change (ms/mm)
Width change (ms/mm)
AUC latency change
AUC width change
Sweep
**

Figure 2.18 Vehicle has no effect on C-fibre ADS in control and incision tissue in females.

Repetitive stimulation in control **A-B** and incision **C-D** dorsal roots at baseline and following vehicle results in a progressive C-fibre latency **A,C** and width **B,D** increase at 2Hz **i-ii** and 10Hz **iii-iv**. AUC analysis of control **A-B** and incision **C-D** C-fibre latency **A,C** and width **B,D** changes at 2Hz **i-ii** and 10Hz **iii-iv**. Vehicle has no significant effect on control C-fibre latency change at 2Hz (**Aii**, *p*=0.581, n=6) and 10Hz (**Aiv**, *p*=0.362, n=6) and width change at 2Hz (**Bii**, *p*=0.878, n=6) but vehicle slightly enhances the width change in control females at 10Hz (**Biv**, ***p*=0.0003, n=6) (unpaired two-tailed t-test). Vehicle does not significantly alter incision C-fibre latency change at 2Hz (**Cii**, *p*=0.419, n=6) and 10Hz (**Civ**, *p*=0.813, n=6) and width change at 2Hz (**Dii**, *p*=0.106, n=5) and 10Hz (**Div**, *p*=0.547, n=5) (unpaired two-tailed t-test).

Table 2.1 Summary comparisons of the effects of TTX and vehicle

Treatment	Sex	Group	2Hz Latency	10Hz Latency	2Hz Width	10Hz Width
TTX	Male	Control	↑	↑	↑	-
		Incision	↓	↑	-	↑
	Female	Control	-	-	↑	↑
		Incision	↑	↑	-	-
Vehicle	Male	Control	-	-	-	-
		Incision	-	-	-	-
	Female	Control	-	-	-	↑
		Incision	-	-	-	-

↑ denotes facilitation of ADS in comparison to baseline; ↓ marks a reduction in ADS compared to baseline; - denotes no significant difference from baseline

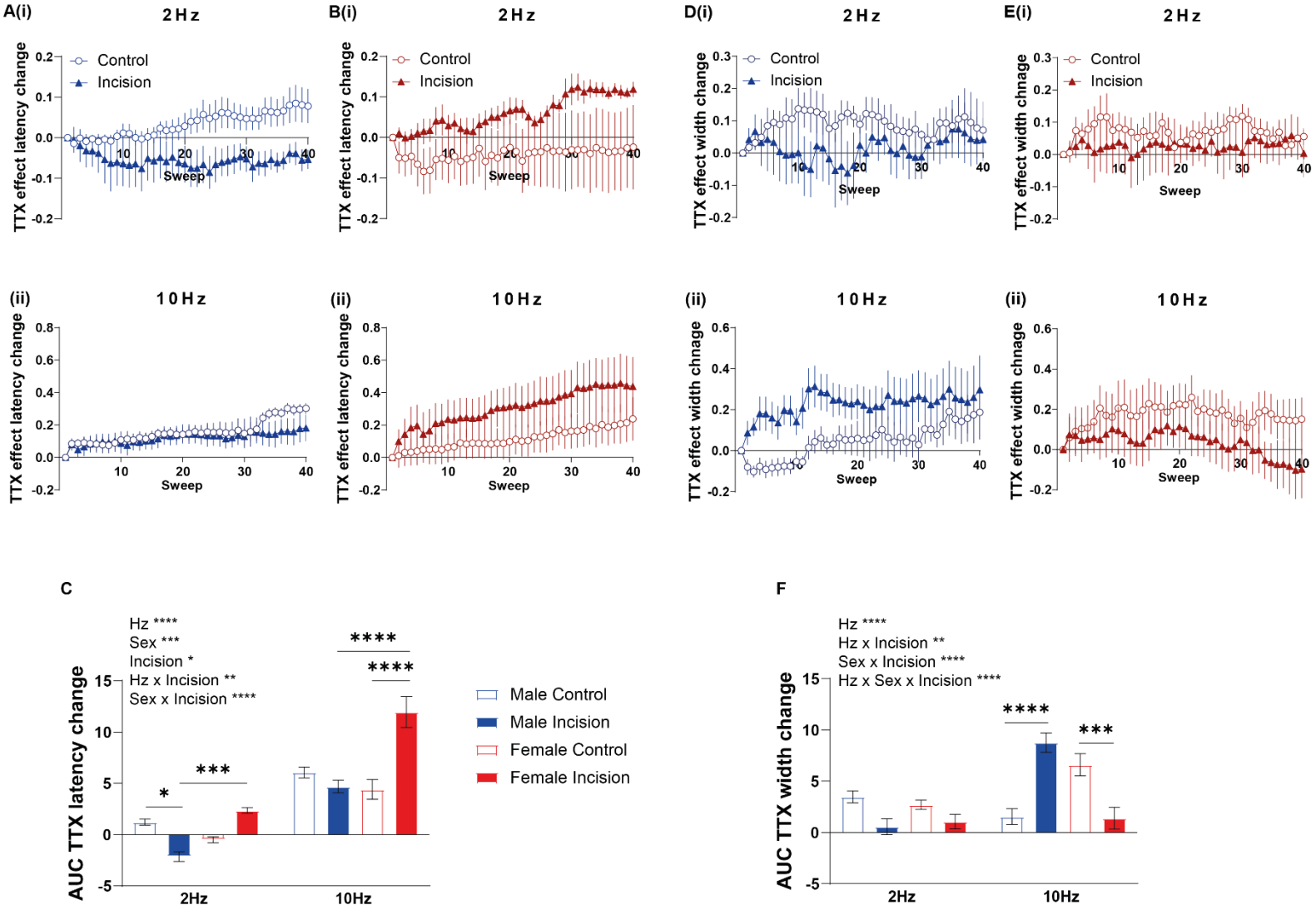
2.5.3.1 TTX effects on post-incision C-fibre ADS are dependent on sex and frequency.

To enable comparison of the TTX effect on C-fibre ADS at 2Hz and 10Hz between the sexes, the C-fibre response latency and width values at baseline were subtracted from the corresponding TTX values to generate 'TTX effect' data over the 40 stimuli for each root (**Figure 2.19**).

AUC analysis of the C-fibre latency change data (**Figure 2.19C**) showed that the effects of TTX were influenced by frequency (p<0.001), sex (p<0.001) and incision (p=0.010), with the effect of TTX on incision being dependent on its interaction with frequency (p=0.003) and sex (p<0.001) (three-way ANOVA). In contrast to the TTX facilitation in control males at 2Hz, incision males displayed a significantly different TTX effect with a reduced C-fibre latency increase (**Figure 2.19Ai and C**; three-way

ANOVA followed by Sidak's multiple comparisons test, p=0.024). In contrast to males, females displayed significantly different TTX effects. At 2Hz control females displayed a small TTX-induced change in C-fibre ADS, with slightly reduced latency change, while the incision females displayed an enhancement of ADS (**Figure 2.19Bi and C**). However the difference in TTX effects between control and incision females at 2Hz was not statistically significant (three-way ANOVA followed by Sidak's multiple comparisons test, p=0.09). However, at 10Hz the females displayed a strong TTX-induced facilitation of C-fibre latency change in incision in comparison to control tissue (**Figure 2.19Aii and C**; three-way ANOVA followed by Sidak's multiple comparisons test, p<0.0001).

AUC analysis of the C-fibre width change data (**Figure 2.19F**) showed that the effects of TTX were influenced by frequency (p<0.001), with the effects of incision depending on its interaction with frequency (p=0.007), sex (p<0.001) and frequency and sex (***p<0.001) (three-way ANOVA). At 2Hz the females also did not show a statistically significant difference between TTX-induced changes in control and incision tissue (**Figure 2.19Ei and F**; three-way ANOVA followed by Sidak's multiple comparisons test, p=0.859). As opposed to the 2Hz data, at 10Hz TTX significantly enhanced the C-fibre latency increase in incision males (**Figure 2.19Dii and F**; three-way ANOVA followed by Sidak's multiple comparisons test, p<0.0001). In incision females the TTX-induced enhancement at 10Hz seen in controls was significantly reduced (**Figure 2.19Eii and F**; three-way ANOVA followed by Sidak's multiple comparisons test, p=0.0009)


A(i)
2 Hz
TTX effect latency change
Control
Incision
Sweep
B(i)
2 Hz
TTX effect latency change
Control
Incision
Sweep
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2 Hz
TTX effect width change
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Incision
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AUC TTX latency change
2Hz
10Hz
Male Control
Male Incision
Female Control
Female Incision
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Hz ****
Hz x Incision **
Sex x Incision ****
Hz x Sex x Incision ****
AUC TTX width change
2Hz
10Hz

Figure 2.19 Incision-dependent TTX effect is influenced by sex and frequency

TTX latency change effect (TTX – baseline latency change) in control and incision males (**A**) and females (**B**) at 2Hz (**i**) and 10Hz (**ii**). **C** AUC analysis of TTX latency change effect reveals a significant influence of frequency (****p*<0.001), sex (****p*<0.001), incision (**p*=0.010) with frequency/incision interaction (***p*=0.003) and sex/incision interaction (****p*<0.001) (three-way ANOVA followed by Sidak's multiple comparisons test, **p*=0.024, ****p*<0.001). Male: control 2Hz (n=6), incision 2Hz (n=6), control 10Hz (n=5), incision 10Hz (n=5). Female: control 2Hz (n=6), incision 2Hz (n=6), control 10Hz (n=5), incision 10Hz (n=6).
TTX width change effect (TTX - baseline width change) in control and incision males (**D**) and females (**E**) at 2Hz (**i**) and 10Hz (**ii**). (**F**) AUC analysis of TTX width change effect reveals a significant influence of frequency (****p*<0.001) with frequency/incision interaction (***p*=0.007), sex/incision interaction (****p*<0.001) and frequency/sex/incision interaction (****p*<0.001) (three-way ANOVA followed by Sidak's multiple comparisons test, *****p*<0.001.Male: control 2Hz (n=6), incision 2Hz (n=6), control 10Hz (n=5), incision 10Hz (n=4). Female: control 2Hz (n=6), incision 2Hz (n=5), control 10Hz (n=5), incision 10Hz (n=5).

2.5.4 Behavioural analysis of the nociceptive flexion-withdrawal reflex in the juvenile incision model of postoperative pain.

Hindpaw incision resulted in the development of mechanical hypersensitivity (**Figure 2.20**) as previously reported in juvenile rats (Walker et al., 2009), with a time course similar to that in adult rats (Brennan, 1999). Both males and females displayed decreased mechanical withdrawal thresholds of the flexion-withdrawal reflex to punctate mechanical stimulus in ipsilateral (incised) hindpaw in comparison to contralateral (control) hindpaw following incision surgery. This hypersensitivity is represented as a negative percentage change from contralateral withdrawal thresholds (**Figure 2.20Ai**). The mechanical hypersensitivity persisted until PSD9 in both males and females (**Figure 2.20Ai**, RM 2-way ANOVA: PSD, $p<0.0001$; sex, $p=0.632$; interaction, $p=0.959$ followed by Dunnett's multiple comparisons test, PSD1,3,5,7, $p<0.0001$, PSD9, $p=0.019$). In addition, both sexes also showed an increase in the withdrawal scores to a dynamic brush stimulus, indicating the development of dynamic mechanical allodynia, which persisted until PSD3 (**Figure 2.20Bi**, RM 2-way ANOVA: PSD, $p=0.0011$; sex, $p=0.372$; interaction, $p=0.884$ followed by Dunnett's multiple comparisons test, PSD1, $p=0.0005$, PSD3, $p=0.037$). The timecourse of punctate and dynamic mechanical hypersensitivity development was similar between the sexes. Given that the dorsal roots used for all the CAP recordings comprising the data (**Figure 2.11-2.17**) were isolated between PSD2-4 the degree of punctate (**Figure 2.20Aii**) and dynamic (**Figure 2.20Bii**) mechanical hypersensitivity was compared between males and females during this time window. This enabled interpretation of the electrophysiological findings in light of the corresponding behavioural hypersensitivity. There was no significant difference in peak punctate (**Figure 2.20Aii**, unpaired two-tailed t-test, p=0.353) and dynamic (**Figure 2.20Bii**, unpaired two-tailed t-test, p=0.325) mechanical hypersensitivity between the sexes.

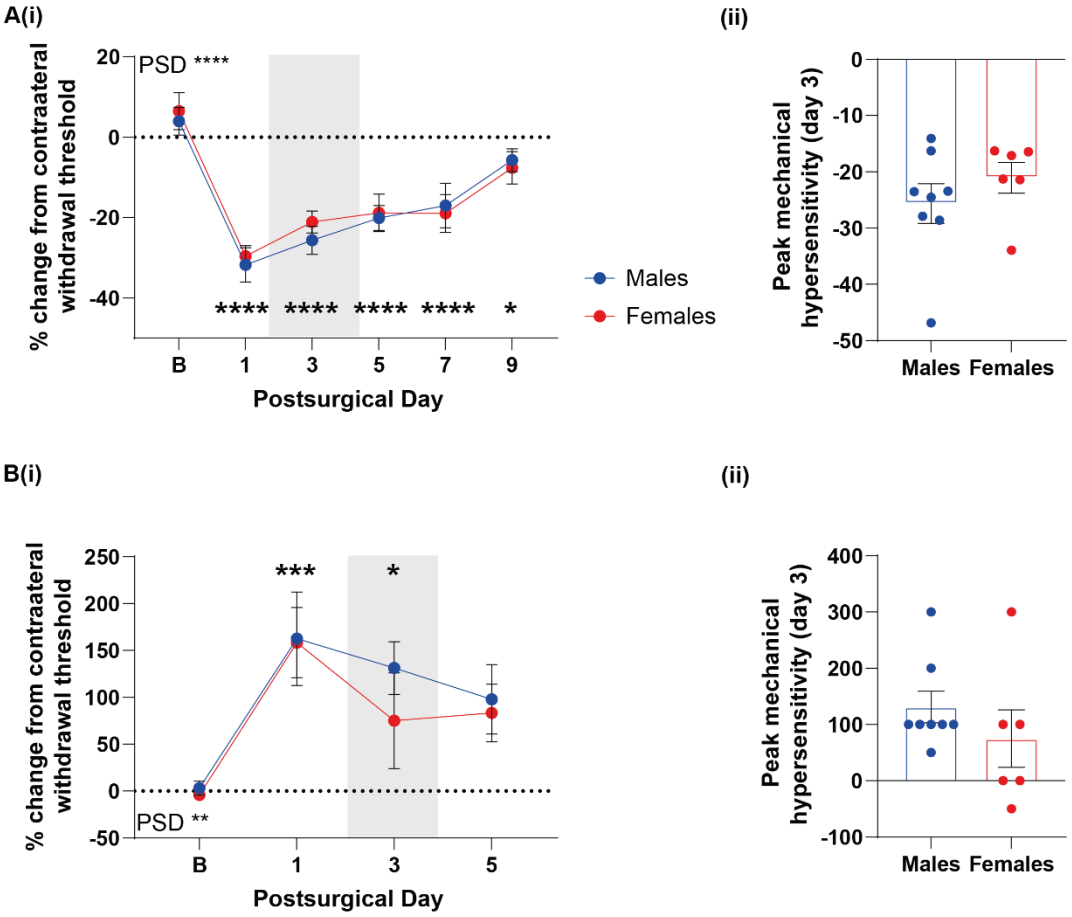


Figure 2.20 Similar timecourse and comparable degree of punctate and dynamic mechanical hypersensitivity following hindpaw incision surgery in male and female juvenile rats.

Ipsilateral hindpaw mechanical withdrawal thresholds (electronic von Frey) presented as a percentage of contralateral values reveal the development of mechanical hypersensitivity over a similar timecourse and to a comparable degree in both sexes (**Ai,** RM two-way ANOVA: PSD, ****p<0.001, sex, p=0.631, interaction p=0.959 followed by Dunnett's multiple comparisons test: ****p<0.0001, *p=0.02). Mechanical hypersensitivity at PSD3, within the time window of tissue collection for electrophysiology, was not significantly different between the sexes (**Aii,** Unpaired two-tailed t-test, p=0.353). Ipsilateral hindpaw withdrawal scores to innocuous dynamic brush stimulus (paintbrush) expressed as a percentage of contralateral values reveal the development of dynamic allodynia over a similar timecourse and to a comparable degree in both sexes (**Bi,** RM two-way ANOVA: PSD, **p=0.0011, sex, p=0.372. interaction, p=0.8837 followed by Dunnett's multiple comparisons test: ***p=0.0005, *p=0.037,). Dynamic allodynia at PSD3, within the time window of tissue collection for electrophysiology, was not significantly different between the sexes (**Bii,** Unpaired two-tailed t-test, p=0.325). Grey boxes mark the timeframe when electrophysiological recordings were conducted. Data in A and B: male (n=8); female (n=6).

Since the plantar incision model has been reported to give rise to evoked pain responses to heat but not cold stimuli in males (Brennan et al., 1996, Scherer et al., 2010b, Zahn and Brennan, 1999a) the effect of hindpaw incision on thermal sensitivity in juvenile male and female rats was also explored.

Both males and females displayed decreased withdrawal latencies of the flexion-withdrawal reflex to a radiant noxious heat stimulus in ipsilateral (incised) hindpaw in comparison to contralateral (control) hindpaw following incision surgery. This hypersensitivity is represented as a negative percentage change from contralateral withdrawal latencies and it persisted until PSD6 (**Figure 2.21Ai**, RM 2-way ANOVA: PSD, $p<0.0001$; sex, $p=0.334$; interaction, $p=0.5504$ followed by Dunnett's multiple comparisons test, PSD2, $p<0.0001$, PSD4, $p=0.0012$, PSD6, $p=0.034$). In contrast, the withdrawal latencies to a noxious cold stimulus were not significantly altered after incision in both males and females, with cold hypersensitivity represented again as a negative percentage change from contralateral withdrawal latencies (**Figure 2.21Bi**, RM 2-way ANOVA: PSD, $p=0.134$; sex, $p=0.122$; interaction, $p=0.737$). The timecourse of heat hypersensitivity development was not statistically different between the sexes (**Figure 2.21Ai**, interaction, $p=0.5504$), but again the degree of peak heat (**Figure 2.21Aii**) and cold (**Figure 2.21Bii**) hypersensitivity was compared between males and females during the time window of tissue collection. Notably, the females displayed significantly less peak heat hypersensitivity when compared to males (**Figure 2.21Aii**; unpaired two-tailed t-test, $p=0.015$), while the withdrawal latency to noxious cold stimuli were not significantly different at the time frame of tissue collection between the sexes (**Figure 2.21Bii**, unpaired two-tailed t-test, $p=0.245$).

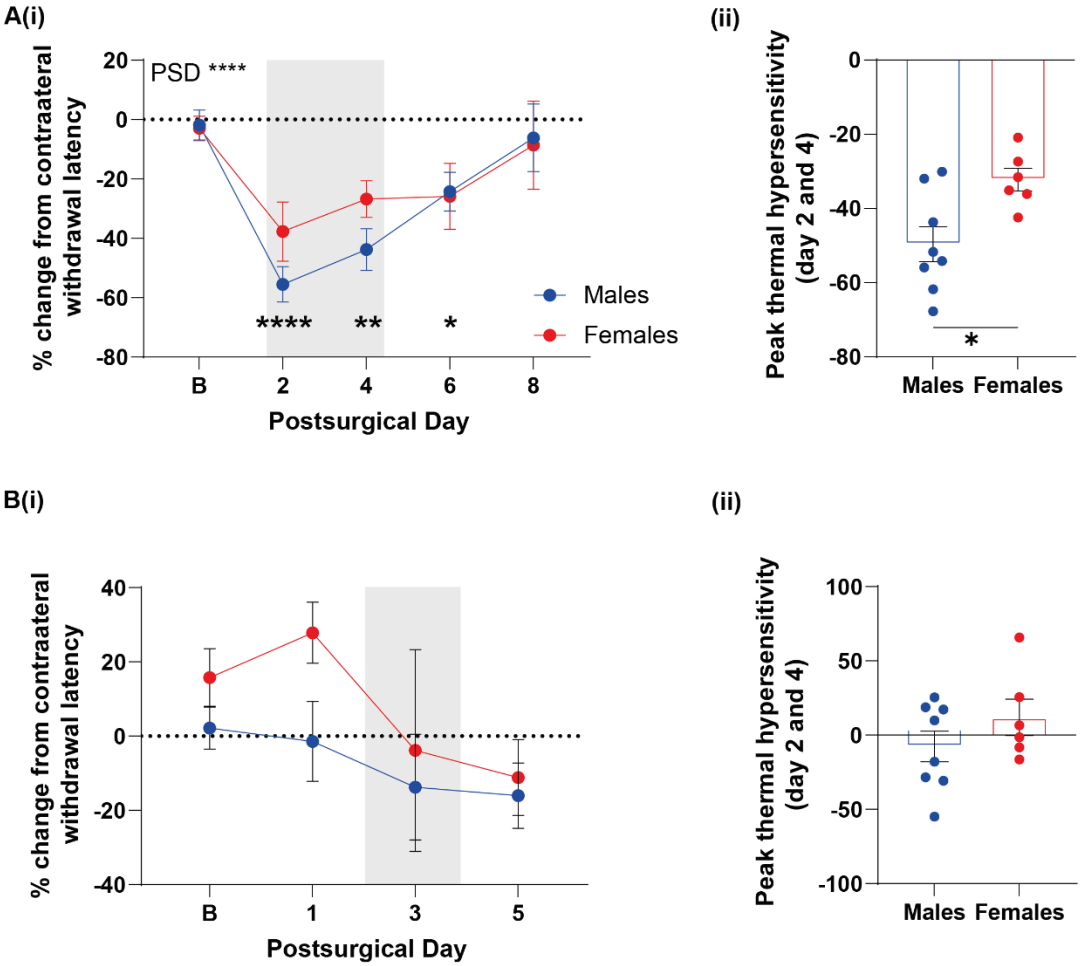


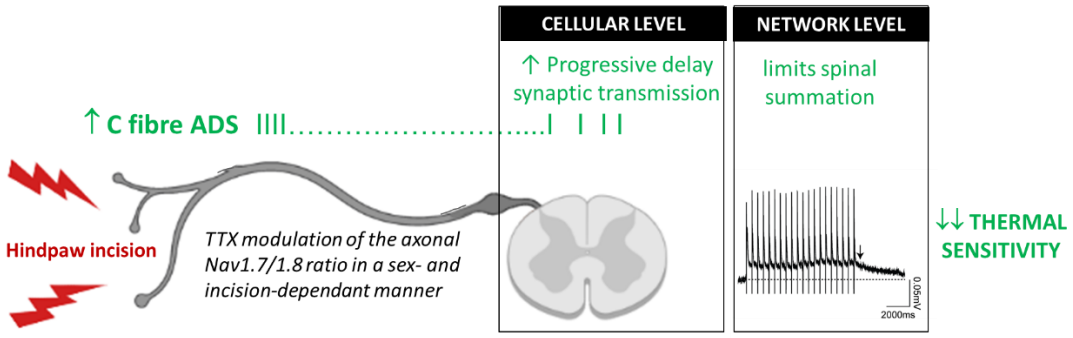
Figure 2.21 Timecourse and degree noxious heat hypersensitivity in juvenile rats of both sexes: peak noxious heat hypersensitivity is enhanced in males following hindpaw incision, while noxious cold hypersensitivity does not develop in both sexes.

Ipsilateral hindpaw withdrawal latencies to noxious radiant heat stimuli (Hargreaves) expressed as a percentage of contralateral values reveal the development of heat hypersensitivity in both sexes (**Ai,** RM two-way ANOVA: PSD, ****p<0.0001; sex, p=0.334, interaction, p=0.550 followed by Dunnett's multiple comparisons test: ****p <0.0001, **p=0.0012, *p=0.034). Thermal hypersensitivity during the time window of tissue collection for electrophysiology is significantly reduced in females compared to males (**Aii,** Unpaired two-tailed t-test, *p=0.015). Ipsilateral hindpaw withdrawal latencies to noxious cold stimuli (cold plantar assay test using dry ice) expressed as a percentage of contralateral values reveal no development of noxious cold hypersensitivity in both sexes (***Bi,*** RM two-way ANOVA: RM two-way ANOVA: PSD, p=0.134, sex, p=0.122, interaction, p=0.737). Noxious cold hypersensitivity at PSD3, within the time window of tissue collection for electrophysiology, was not significantly different between the sexes (***Bii,*** Unpaired two-tailed t-test, p=0.245). Grey boxes mark the timeframe when electrophysiological recordings were conducted. Data in A and B: male (n=8); female (n=6).

2.6 Discussion

2.6.1 Summary findings

This chapter assessed the impact of plantar incision upon C-fibre ADS in male and female juvenile rats, with findings summarised in **Figure 2.22**. Incision did not alter C-fibre basic properties, but the C-fibre ADS phenomenon was altered in a sex- and frequency-dependent manner. In males, C-fibre latency change post-incision was increased to the higher baseline levels observed in females. In females, incision increased the width change of the C-fibre response to levels greatly exceeding those in males. In addition, it was demonstrated that C-fibre ADS is regulated by the Na_V blocker TTX in an incision- and sex-dependent manner suggesting incision- and sex-dependent differences in the underlying $Na_V1.7/Na_V1.8$ ratio along the axon that may contribute to the incision and sex-dependent changes in ADS. Consistent with a stronger overall ADS profile post-incision in females that is predicted to limit heat hypersensitivity (Dickie et al., 2017) females displayed less peak heat hypersensitivity than males following incision. Thus, the findings in this chapter suggested the reported sex-dependent incision-induced C-fibre ADS alterations as a potential contributory mechanism to post-operative pain.



	Basic C-fibre properties	Male ADS	Female ADS	Heat threshold	Mechanical threshold
Incision SD rats	- (M=F)	↑	↑↑	Bigger ↓ Males	M=F

Figure 2.22 Impact of plantar hindpaw incision on C-fibre ADS and on nociceptive behaviour in juvenile SD rats of both sexes

↑ marks an increase; ↓ marks a decrease; M=F denotes equivalent levels in males and females

2.6.2 Incision enhances C-fibre ADS in a sex-dependent manner

In line with previous reports in CFA-induced inflammatory (Dickie et al., 2017) and paclitaxel-induced neuropathic (Galley et al., 2017) pain models, there were no significant changes following plantar incision in basic C-fibre properties, including threshold stimulus intensity, average conduction velocity and amplitude (**Figure 2.11**), which further supports the involvement of C-fibre ADS in the pathology of incision-induced postoperative pain. In addition, electrophysiological studies have shown sensitisation of the Aδ but not the C-fibre afferents to mechanical stimuli at day 1 post-incision, suggesting Aδ sensitisation may contribute to the mechanical sensitivity following incision (Pogatzki et al., 2002a). Interestingly, Pogatzki et al. (2002a) also reported no incision-induced alterations in the response threshold and the stimulus-response function of the C-fibres to mechanical stimuli, which further suggests that there may be other distinct C-fibre alterations post-incision.

Consistent with the observed incision-induced C-fibre ADS changes, other studies have also reported altered C-fibre ADS in both inflammatory (Dickie et al., 2017) and neuropathic (Shim et al., 2007, Wang et al., 2016, Galley et al., 2017, Sun et al., 2012b, Hulse, 2016) pain models. In contrast to the findings of enhanced ADS post-incision, preclinical studies have primarily reported a reduction in ADS in the inflammatory (Dickie et al., 2017), nerve injury (Hulse, 2016), UV sunburn (Werland et al., 2021) and diabetes (Wang et al., 2016, Sun et al., 2012b)- and chemotherapy (Galley et al., 2017)- induced neuropathy models. Reduced ADS has also been reported following administration of the inflammatory mediator NGF in pig (Obreja et al., 2011b) and human (Obreja et al., 2018) skin. However, it must be noted that despite the fact that ADS profiles have been shown to differ based on species (see chapter 4) and sex (Dickie et al., 2017), the majority of the above preclinical studies have been conducted in male subjects only, with some of the studies using different rat strains too, which could potentially also differ in their ADS profiles.

However, similar to the incision model, the spinal nerve ligation (Shim et al., 2007) and Zucker Diabetic Fatty (ZDF) (Garcia-Perez et al., 2018) models of neuropathic pain showed enhanced ADS. In addition, enhanced ADS was also observed using microneurography in chronic pain patients with erythromelalgia (Orstavik et al., 2003) and fibromyalgia (Serra et al., 2014). Studies of patients with erythromelalgia in association with Na_V channel mutations have also reported enhanced C-fibre ADS, specifically in the mechanoinsensitive C fibres that display the greatest ADS (Kist et al., 2016, Kleggetveit et al., 2016). It is suggested that the enhanced ADS may directly result from the mutation, with ADS acting as a negative feedback mechanism that limits longer lasting or high frequency discharge and thus having a protective effect to limit pain in these patients (Kist et al., 2016). In line with this hypothesis, incision could be causing changes in nociceptor profiles, altering expression of various ion channels and/or receptors as suggested by the TTX-induced alterations in C-fibre ADS post-incision. The enhanced C-fibre ADS following incision could thus be similarly acting as a negative feedback mechanism to counteract high firing or longer lasting incision-induced effects. This is consistent with the observation in this chapter of a more enhanced ADS profile in combination with less thermal hypersensitivity following incision in females.

To date, to my knowledge, C-fibre ADS has not been investigated following surgery in preclinical models or in patients with postoperative pain. Given the likely involvement of a neuropathic as well as an inflammatory component in postoperative pain (Kehlet et al., 2006, Hill et al., 2010a) and the fact that its pharmacological management differs from other clinical pain conditions (Brennan, 2011b, Whiteside et al., 2004), suggesting distinct underlying mechanisms, it is not surprising that there are distinct ADS alterations post-incision, which differ from pure inflammatory or neuropathic pain models.

2.6.3 TTX modulates C-fibre ADS in an incision-, sex- and frequency-dependent manner.

Pharmacological studies have suggested that the ADS phenomenon could be explained by the increasing numbers of Na_V channels entering a sustained slow inactivated state (De Col et al., 2008, Obreja et al., 2012). However, subsequent modelling studies proposed that while Na_V channel slow inactivation might contribute, it is increased intra-axonal Na^+ concentration that governs ADS (Tigerholm et al., 2014b, Tigerholm et al., 2015). A combined experimental and modelling study comparing the mechanoinsensitive (CMi) and mechanosensitive (CM) C fibre subtypes revealed that a more pronounced ADS profile, characteristic for the CMi subtype (Weidner et al., 1999, Obreja et al., 2010), is associated with more $Na_V1.8$ and less $Na_V1.7$, more delayed rectifier potassium channels and less Na+/K+ ATPase, a profile consistent with increased intra-axonal Na^+ concentration (Petersson et al., 2014). The study concluded that it is the ratio of $Na_V1.7$ to $Na_V1.8$ rather than absolute levels of either channel that determines the degree of ADS. In line with this, C-fibre ADS is altered in $Na_V1.8$ knockout mice (Hoffmann et al., 2016). Furthermore, pharmacological studies using low-dose TTX (De Col et al., 2008, Baker and Waxman, 2012a), that will act on the TTX-sensitive $Na_V1.7$, and conditional deletion of $Na_V1.7$ in sensory neurons (Hoffmann et al., 2018), thereby pharmacologically or genetically modifying the $Na_V1.7/Na_V1.8$ ratio, showed a reduction in the C-fibre conduction velocity and altered C-fibre ADS.

In line with the above studies, the results in this chapter also found that pharmacological modulation of the $Na_V1.7/Na_V1.8$ ratio with low dose TTX reduced the average C-fibre conduction velocity in both sexes independent of incision. These findings are in line with previous studies exploring the effects of $Na_V1.7$ on conduction velocity, where experiments have been conducted in Wistar rats of both sexes (De Col et al., 2008) or in conditional $Na_V1.7$ KO mice of both sexes (Hoffmann et al., 2018), without investigation of the data based on sex. In further support of the observed sex difference in functional $Na_V1.7/Na_V1.8$ ratio between males and females in normal physiology are the findings of TTX-induced enhancement of ADS

in control males, where ADS was measured as the change in C-fibre response latency and width (2Hz only). In contrast, in females, TTX enhanced C-fibre width change but not latency change measures of ADS. Differences between latency and width results likely reflect ADS in C-fibre subtypes with different baseline conduction velocities; with latency change reflecting medium conducting fibres and width change reflecting the fastest and slowest conducting fibres within the population. Thus, the distinct TTX-induced modulations in control males and females for latency and width change measures of ADS, suggest C-fibre subtype specific sex differences in the functional axonal $Na_V1.7/Na_V1.8$ ratio under normal conditions. This is further supported by studies demonstrating that different C-fibre subpopulations exhibit different amounts of ADS (Gee et al., 1996, Weidner et al., 1999) with pharmacological studies suggesting differential Na_V channel subtype expression profiles (Kankel et al., 2012, Jonas et al., 2020). The fact that vehicle treatment, slightly enhanced the width change at 10Hz (~17%) only in control females, an enhancement that is much less pronounced when compared to the TTX effect in the same group (~49%), but not males, further supports the hypothesis that there are potential sex differences in the $Na_V1.7/Na_V1.8$ ratio in normal physiology.

Incision altered the TTX-induced changes in C-fibre ADS as assessed by latency and width change in both sexes (**Figure 2.14**; **Figure 2.17**). Specifically, in incision tissue the TTX-induced ADS facilitation observed in controls was abolished or converted to TTX-induced ADS reduction (with the exception of male 10Hz latency) (**Table 2.1**). Whereas any absence of TTX-induced effect in control tissue was converted to TTX-induced ADS facilitation following incision (**Table 2.1**). The impact of incision on both TTX-induced latency and width change was influenced by sex (**Figure 2.19**). This suggests there are sex-dependent changes in functional axonal $Na_V1.7/Na_V1.8$ ratio following incision, which could be due to changes in $Na_V1.7$, $Na_V1.8$ or both. The influence of sex on the incision impact on the TTX-induced latency change was notably different from that on the width change. Given that latency change likely reflects ADS in medium conducting C fibres and width change likely reflects ADS in

the fastest and/or slowest conducting C fibres, this suggests that the incision-induced $Na_V1.7/Na_V1.8$ ratio alterations vary dependent on the particular C-fibre subclass. Consistent with this, the CM and CMi C-fibre subtypes that display ADS, but to differing extents (CMi>CM) with differing baseline conduction velocities (CMi<CM) (Weidner et al., 1999, Obreja et al., 2010), both have been reported to show sensitisation following incision but this has only been explored in males (Pogatzki et al., 2002a, Hamalainen et al., 2002).

2.6.4 $Na_V1.7$ and $Na_V1.8$ involvement in post-operative pain

The role of $Na_V1.7$ and $Na_V1.8$ in pain processing has been extensively explored (Bennett et al., 2019) but only a few studies have investigated $Na_V1.7$ and $Na_V1.8$ involvement in postoperative pain. Behavioural studies in rats have shown that silencing $Na_V1.8$ using antisense oligonucleotides does not affect mechanical allodynia post-incision (Joshi et al., 2006). Furthermore, pharmacological studies of a TTX-resistant Na_V channel blocker, suggest only a small contribution of $Na_V1.8$ to mechanical allodynia (Jarvis et al., 2007). However, these behavioural studies have explored the involvement of $Na_V1.8$ for the specific symptom of mechanical allodynia and in male subjects only. Mechanical allodynia likely reflects Aβ-fibre activation of sensitised CNS pain circuits. They have not investigated the role of $Na_V1.8$ in post-incision thermal hypersensitivity in both sexes, which would likely reflect the C-fibre function studied here. More recent behavioural studies using virus-mediated knockdown of $Na_V1.7$ in male mice (Eisenried et al., 2017) and rats (Sun et al., 2018) have demonstrated $Na_V1.7$ involvement in postoperative mechanical and thermal hypersensitivity (Eisenried et al., 2017). In addition, a selective $Na_V1.7$ inhibitor has been shown to limit postsurgical thermal hypersensitivity in male mice (Beckley et al., 2021). These behavioural studies along with the TTX observations in this chapter suggest an involvement of C-fibre $Na_V1.7$, $Na_V1.8$ or both in postoperative thermal hypersensitivity. Specifically, the presented

in this chapter findings propose that incision- and sex-dependent $Na_V1.7$/$Na_V1.8$ ratio changes can occur in parallel with sex differences in peak thermal hypersensitivity post-incision.

Few studies have explored the involvement of $Na_V1.7$ and $Na_V1.8$ in the plantar incision model of postoperative pain. It has been reported that surgical incision increased gene (Barbosa Neto et al., 2019, Sun et al., 2018) and protein (Sun et al., 2018) expression of $Na_V1.7$, but not $Na_V1.8$ gene expression (Barbosa Neto et al., 2019). However, these studies have been exclusively conducted in males and focused only on cell body Na_V expression. This does not take into account, as investigated for $Na_V1.7$, the distribution of channels along the axon (Black et al., 2012) that is likely dynamically regulated (Black et al., 2012, Akin et al., 2019b, Akin et al., 2019a). Given there is evidence suggesting inflammation altered axonal $Na_V1.7$ trafficking (Akin et al., 2019a) and sex differential axonal mRNA transport (Ray et al., 2019) in peripheral nerves, along with the CNS evidence of sex differences in the microRNAs that regulate translation (Morgan and Bale, 2012, McCarthy and Nugent, 2015) that occurs locally in peripheral nerve axons (Price and Géranton, 2009), the demonstrated incision- and sex-induced differences in Na_V functional expression could reflect differences in mRNA and/or protein expression at the axonal level only.

2.6.5 Sex differences in C-fibre ADS and incision-induced pain sensitivity

C-fibre ADS has been shown to regulate the intervals between successive action potentials being relayed by nociceptors to the spinal cord. It has been previously shown that increased ADS levels increase the progressive delay in synaptic transmission of C-fibre inputs that transmit noxious heat to the spinal cord, limiting spinal summation (Dickie et al., 2017). Given the more pronounced C-fibre ADS in females in parallel with higher noxious heat thresholds than males, Dickie et al. (2017) also propose that ADS can potentially influence thermal pain sensitivity in a sex-dependent manner (Dickie et al., 2017). Interestingly, CFA-induced inflammation altered both C-fibre ADS and heat sensitivity in a sex-dependent

manner, with females showing a reduction in ADS to levels similar to males, which would facilitated spinal summation in females, and is proposed to contribute to the exhibited enhanced inflammatory thermal hypersensitivity in females (Dickie et al., 2017). This chapter showed that females have a more pronounced enhancement of ADS post-incision in comparison to males. Therefore, it is predicted that this should enhance the progressive delay in synaptic transmission to the spinal cord, limit spinal summation and consequently reduce heat hypersensitivity in females, as was observed behaviourally.

In line with the behavioural data in juvenile rats of both sexes, preclinical studies have reported a similar timeframe of punctate mechanical and heat hypersensitivity in the incision model of postoperative pain in adult male rats, with peak mechanical hypersensitivity PSD1-PSD4, which resolves by approximately PSD5-PSD7, and peak heat hypersensitivity PSD1-PSD4, which resolves by approximately PSD7-PSD9 (Brennan et al., 1996, Zahn and Brennan, 1999a, Brennan et al., 2005, Whiteside et al., 2004). Similar timeframe of mechanical hypersensitivity has been also shown in juvenile rats of both sexes (~P17) (Walker et al., 2009).

In addition, similar to the findings in this chapter, studies exploring mechanical sensitivity post-incision in both sexes have reported no sex differences in the response to mechanical stimuli in adult rats (Kroin et al., 2003, Song et al., 2018, Cao et al., 2015) and mice (Banik et al., 2006, Patil et al., 2013). Interestingly, sensitivity to dynamic innocuous stimuli, which assesses the development of dynamic mechanical allodynia, has not been investigated in the juvenile plantar incision model of postoperative pain in both sexes. The increased sensitivity to dynamic brush indicated a similar timecourse of development and degree of dynamic mechanical allodynia in both sexes, which is in line with studies investigating punctate mechanical allodynia following plantar incision in males, where alterations were short-lasted and persisted up to PSD3 (Brennan et al., 1996, Zahn and Brennan, 1999a).

However, in contrast to the findings in this chapter of a sex difference in peak heat hypersensitivity, there are studies that have reported no sex difference in heat hypersensitivity in the plantar incision model (Song et al., 2018, Banik et al., 2006, Patil et al., 2013). However, this could be due to differences in age (Song et al., 2018, Banik et al., 2006), strain (Song et al., 2018), species (Banik et al., 2006), testing approach (Song et al., 2018) and the use of a non-heated glass platform (Song et al., 2018, Banik et al., 2006) unlike the heated platform used in the present study. Interestingly, the timing of peak heat hypersensitivity post-incision was altered by sex in adult mice (Patil et al., 2013).

The findings in this chapter of no development of noxious cold hypersensitivity post-incision in juvenile rats of both sexes are in contrast to previous findings in SD rats of both sexes, which were reported to develop cold hypersensitivity following hindpaw plantar incision surgery (Cao et al., 2015). These contrasting findings could be explained by differences in rat age and in noxious cold sensitivity assessment testing approach, where Cao et al. (2015) used the cold plate assay. Interestingly, the incision model of postoperative pain in mice of both sexes also did not report development of cold hypersensitivity (Scherer et al., 2010b), which is in line with the findings in this chapter in rats of both sexes. However, along with the apparent species difference, it must be noted that the noxious cold sensitivity assessment approaches also differed, with noxious cold sensitivity in mice assessed using a cold plate assay. Notably, the absence of an identical sex difference in thermal hypersensitivity in all studies is not surprising given that sex differences in thermal pain sensitivity have been shown to be strain and species dependent (Mogil et al., 2000).

Clinical studies have also reported sex differences in postoperative pain (Hah et al., 2019, Bartley and Fillingim, 2013a, Fillingim et al., 2009) but interestingly these are influenced by age and type of surgery (Tighe et al., 2015, Zheng et al., 2017). This highlights the importance of including both sexes in preclinical pain research to ensure that mechanism-based development of analgesics is sex appropriate.

2.6.6 Limitations

One of the limitations of the work done in this chapter is the method of assessing C-fibre ADS in population CAP recordings. Although previous studies have used extracellular population CAP recordings for assessing the C-fibre ADS phenomenon (Dickie et al., 2017, Galley et al., 2017, Hoffmann et al., 2018), which are a valuable tool for investigating changes at a population level, this method does not allow for differentiation among the different C-fibre subtypes, which display different levels of ADS and could be differentiated based on that, as demonstrated previously (Thalhammer et al., 1994, Serra et al., 1999, Weidner et al., 1999). Despite the fact that the experiments in this chapter tried to assess ADS by measuring both changes in C-fibre latency and width, which would reflect changes in the average conduction velocity and in the range of the conduction velocities within the population, respectively, this allowed to distinguish between differences in ADS in C-fibre subtypes with different baseline conduction velocities only. It did not allow investigation of incision-induced changes in ADS in modality-specific C-fibre subpopulations. It would be beneficial to study the ADS phenomenon in normal physiology and in the plantar incision model in both sexes in specific C-fibre subtypes, which could be done by utilising single-fibre electrophysiology using the skin-nerve preparation. Electrophysiological studies in specific C-fibre subtypes would also allow more detailed pharmacological investigations into the underlying mechanisms of C-fibre ADS in both sexes and the alterations of the ion channels implicated in ADS in different pain pathologies, including incision-induced postoperative pain.

Moreover, the experiments performed in this chapter used low-doses of TTX (20nM) to block the TTX-sensitive Na_V channels along the axon, likely $Na_V1.7$ (De Col et al., 2008, Baker and Waxman, 2012b). This concentration of TTX has been shown to be lower than the IC_{50} of the TTX-resistant Na_V, like $Na_V1.8$ (50µM), and lower than the concentrations required for complete conduction block (Sivilotti et al., 1997, Catterall et al., 2005). However, TTX is a non-selective Na_V blocker, that could act on other TTX-sensitive Na_V channels, like $Na_V1.1$ and $Na_V1.6$, in the peripheral

nerves (Yang et al., 2018). Despite the fact that $Na_V1.7$, $Na_V1.8$ and $Na_V1.9$ have been shown to dominate Na_V expression in small-diameter afferents (Cummins et al., 2007), while $Na_V1.1$ and $Na_V1.6$ have been shown to be confined to the Aβ fibres (Black et al., 1996, Caldwell et al., 2000), an effect of TTX on other TTX-sensitive Na_V other than $Na_V1.7$ could not be excluded. This is particularly important in the incision model, where incision-induced changes in ion channel and receptor expression and function in the peripheral afferents have not been fully explored in both sexes. In addition, it would be optimal to use selective $Na_V1.7$ and $Na_V1.8$ blockers, such as ProTxII and A-803467, respectively, which would allow investigating the contribution of $Na_V1.7$ and $Na_V1.8$ to ADS in both sexes in normal physiology and in the incision model of postoperative pain.

One of the major limitations of this chapter is with regards to the evidence for differences in the $Na_V1.7/Na_V1.8$ ratio along the axon in males and females in normal conditions and post-incision. The experiments in this chapter provided functional evidence using low-dose TTX, likely acting on the TTX-sensitive $Na_V1.7$ along the axons, for sex and incision-induced differences in the $Na_V1.7/Na_V1.8$ ratio in dorsal roots from juvenile rats. However, due to technical and time limitations it was not possible to perform experiments exploring the gene and/or protein expression of $Na_V1.7$ and $Na_V1.8$ in dorsal roots. However, it must be noted that the sex- and injury-dependent differences in the $Na_V1.7/Na_V1.8$ ratio are likely C-fibre subpopulation specific. Given the changes reported in this chapter are at an axonal level (that may not be similarly evident at the cell body level), this would necessitate study of protein ratios within specific C-fibre subpopulations, the axons of which could potentially be visualised in transgenic DRG reporter lines (for example CGRP-expressing). However, inferring ratios from immunostaining might be unreliable due to differences in immunostaining between tissue preparations and was out with the scope of the present study. Nevertheless, it would be important to directly investigate the gene and protein levels of the $Na_V1.7$ and $Na_V1.8$ ion channels along the axon in both sexes in normal physiology and in the pathology of postoperative pain.

Another limitation of the studies conducted in this chapter involves the electrophysiological constraints with regards to age of animals used. All experiments in this chapter were conducted in juvenile rats (P16-P28) due to better preservation and viability of the tissue at this age for electrophysiological studies. However, the fact that age has been shown to be a confounding factor in the development and sex differences in postoperative pain in patients, with reports of lower pain scores but slower rate of resolution in older patients (Tighe et al., 2015) and more pronounced sex differences in older patients (Zheng et al., 2017, Tighe et al., 2015), suggests that the observations in this chapter may well be an underestimate of the sex differences in postoperative pain. Thus, it will be important to conduct similar experiments in adult and aging animals of both sexes. The use of juvenile animals, on the other hand, was beneficial because they remain pre-pubertal until approximately P50 (Sengupta, 2013, Engelbregt et al., 2000), with estrous cycle commencing at approximately P38 in females (Ajayi and Akhigbe, 2020). This suggests that the observed sex differences in these experiments are unlikely a result of hormonal differences in males and females. This is further supported by studies showing no effect of ovariectomy and gonadectomy on postoperative hypersensitivity in adult rats of both sexes (Green et al., 2016). However, previous studies have reported that sex hormones could contribute to sex differences in injury-induced immune responses (Sorge et al., 2011, Sorge et al., 2015). Moreover, recent findings in the incision model have shown sex-differences in posttranscriptional regulation of inflammasomes (Cowie et al., 2019a) and androgen-induced antinociception (Barbosa Neto et al., 2019). Therefore it would be important to investigate the incision-induced changes in ADS and in pain hypersensitivity in fully mature adult animals as well.

Further studies could investigate C-fibre ADS alterations in both sexes throughout the entire timecourse of incision-induced heat hypersensitivity. Although this was out with the scope of the investigation in this chapter, which aimed to first establish potential mechanisms underlying sex- and incision-induced changes in heat hypersensitivity and in C-fibre ADS, studying and correlating the timecourse of

incision-induced heat hypersensitivity with the alterations in ADS post-incision will allow for more conclusive interpretation of the findings.

2.7 Conclusion

The results described in this chapter have shown that C-fibre ADS is altered in the incision model of postoperative pain in a sex- and frequency-dependent manner. The C-fibre latency change, reflective of the change in the average conduction velocity in the population C-fibre response, was increased in males to the higher baseline levels observed in females. The width change of the C-fibre response, reflective of the change in the range of conduction velocities within the population response, was enhanced in the females to levels highly exceeding those in males. C-fibre ADS has been suggested to regulate the intervals of successive action potentials being relayed by nociceptors to the spinal cord, increasing the progressive delay in synaptic transmission of C-fibre inputs that transmit noxious heat to the spinal cord, and thus limiting spinal summation (Dickie et al., 2017). In line with the more pronounced C-fibre ADS profile in females, that should increase the progressive delay in synaptic transmission and limit summation, the data in this chapter also showed a less pronounced peak heat hypersensitivity post-incision in the females. Thus, the presented evidence suggests that C-fibre ADS could influence incision-induced heat sensitivity in a sex-dependent manner.

In addition, the results presented in this chapter have also provide insight into the mechanism underlying ADS in both sexes in normal physiology and in the pathology of incision-induced postoperative pain. The pharmacological studies using TTX have demonstrate that it is able to regulate ADS in a sex- and incision-dependent manner. This functional evidence suggests sex- and incision-dependent differences in the $Na_V1.7/Na_V1.8$ ratio along the axon that may contribute to the sex- and incision-induced changes in ADS following incision. Given these channels are key targets for the pain pharmaceutical industry this information should be considered in the development of sex appropriate pain therapeutics.

Chapter 3

C-fibre activity-dependent slowing and behavioural hypersensitivity in a methylglyoxal pain rat model

3.1 Chapter summary

- This chapter assessed the effect of the diabetic pain-associated metabolite methylglyoxal (MG) upon C-fibre properties and ADS as well as Aβ fibre properties in male and female juvenile rats.
- Chronic, but not acute treatment with MG alters C-fibre ADS in a sex-dependent manner, with chronic MG causing a reduction in C-fibre ADS in males and a pronounced enhancement in females
- Chronic treatment with MG alters initial Aβ fibre amplitude and width in a sex-dependent manner, causing an increase in the Aβ amplitude in males only and a decrease in the Aβ width in females only. In addition, chronic treatment alters Aβ fibre activity-dependent amplitude change in a sex-dependent manner, with chronic MG enhancing the progressive increase in Aβ amplitude in females only.
- Systemic treatment with MG alters noxious heat sensitivity but not mechanical sensitivity in juvenile rats. This occurs in a sex-dependent manner with MG inducing noxious heat hypersensitivity in males only.
- The lack of heat hypersensitivity in females is consistent with a much stronger MG-induced C-fibre ADS profile that is predicted to limit heat hypersensitivity. Thus, sex-dependent MG-induced C-fibre ADS alterations may be a potential contributory mechanism to diabetic neuropathic pain.

3.2 Introduction

3.2.1 Diabetic neuropathy

Diabetes is recognised as the largest global epidemic in the 21st century, with predictions of it affecting approximately 629 million people by 2045 due to rising obesity rates worldwide (Shillo et al., 2019). It is associated with many complications, the most prevalent of which, reported to occur in up to half of diabetes patients (including pre, type 2 and type 1 diabetes), being diabetic neuropathy (Callaghan et al., 2015, Pop-Busui et al., 2017). Diabetic neuropathy (DN) is defined as a group of clinical symptoms resulting from damage to the peripheral and autonomic nervous system (Callaghan et al., 2012). The most common form of DN is distal symmetric polyneuropathy, which affects the peripheral nerves in the upper and lower limbs in a 'glove and stocking' distribution (**Figure 3.1**) and is consistent with systemic metabolic disease (Dellon, 2004). Despite the fact that many patients with distal symmetric polyneuropathy can present without any symptoms, it has been reported that approximately 15-25% of diabetes patients suffer from neuropathic pain symptoms, a condition referred to as painful diabetic neuropathy (PDN) (Daousi et al., 2004, Van Acker et al., 2009, Abbott et al., 2011, Alleman et al., 2015, Binns-Hall et al., 2018). It has been shown that the highly reactive glucose-derived metabolite methylglyoxal (MG) is elevated in diabetes patients and is associated with diabetes complications, including DN and PDN (Shamsaldeen et al., 2016, Brings et al., 2017), with further studies suggesting that plasma MG levels can discriminate between diabetes-affected patients with painful symptoms and those without (Bierhaus et al., 2012, Andersen et al., 2018).

Early on DN primarily affects the sensory nerves, with patients experiencing positive and negative symptoms, pain and numbness/dysesthesia, respectively, while later on the condition could affect the motor nerves, causing motor nerve dysfunction as well (Feldman et al., 2017). The small-diameter nerve fibres appear to be more susceptible and are shown to be the first ones to display diabetes-induced damage (Smith and Singleton, 2008), which is associated with the neuropathic pain symptoms (Sveen et al., 2013). While the diabetes-induced painful symptoms are

thought to be mediated mainly by the small-diameter fibres, the non-painful symptoms (numbness and dysesthesia) have been associated with alterations in the large-diameter A-fibres (Gwathmey and Pearson, 2019). It has been reported that the symptoms in the lower limbs are typically worse than in the upper limbs (Dellon, 2004). The loss in sensation in the lower limbs has been reported to progress quicker and over time to also affect motor neurons, resulting in motor weakness that can lead to loss of balance, falls, numbness and insensate foot, where treatments are limited and might lead to amputations (Margolis et al., 2011, Pop-Busui et al., 2017, Dellon, 2004, Narres et al., 2017). Despite extensive research, it still remains not fully clear why the peripheral sensory nerves are particularly vulnerable to diabetes. Studies have suggested that this is secondary to a combination of factors associated with energy production and ion channel expression, particularly Na_V and the Na/Ca exchanger (NCX2) (Feldman et al., 2017). Interestingly, a few studies have explored whethere there are sex differences in diabetic neuropathy, with the overall findings, showing that diabetic neuropathy is more frequent in men, who also present with diabetic neuropathy-related complication approximately 4 years earlier than women (Albers et al., 1996, Aaberg et al., 2008, Abbott et al., 2011, Abraham et al., 2018, Cardinez et al., 2018, Booya et al., 2005, Brown et al., 2004). However, women have been reported to have greater prevalence, intensity and ~50% increased risk for painful symptoms (Abbott et al., 2011, Cardinez et al., 2018, Abraham et al., 2018). Given the high prevalence of diabetes and the associated PDN along with the currently inadequate therapies for managing the pain symptoms (Javed et al., 2015, Snyder et al., 2016), there is an urgent need for better understanding of the mechanisms underling PDN in both sexes that can help in the discovery and design of more effective treatmetns.

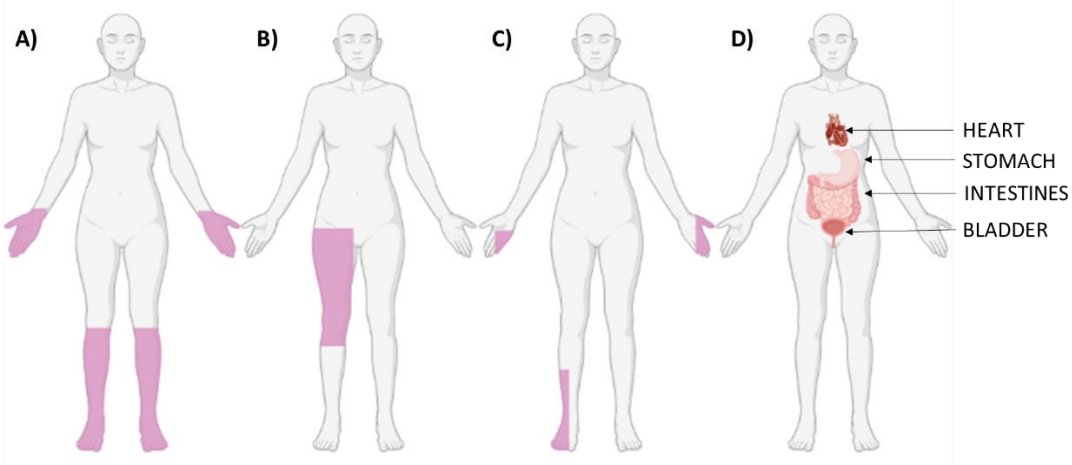


Figure 3.1 Different patterns of diabetes-induced neuropathy

The most common form of diabetic neuropathy is distal symmetric polyneuropathy, which affect predominantly the small-diameter peripheral nerve fibres (A). Other forms of diabetic neuropathy include radiculopathy, which presents with severe unilateral pain but can respond to immunotherapy and, unlike other diabetic neuropathies, usually improves with time (B), mononeuropathy, which involves damage to a specific nerve, usually in the thighs, hips, buttocks and legs (C) and autonomic neuropathy, which is caused by overaggressive glycaemic control and affects the nerves that control involuntary functions of the body (D). Adapted from (Peltier et al., 2014) and created with BioRender

3.2.2 Role of methylglyoxal in DN

3.2.2.1 Methylglyoxal pathway

Methylglyoxal (MG) is a highly reactive dicarbonyl compound, reported as the most potent glycating agent in humans (Glomb and Monnier, 1995, Smuda and Glomb, 2013) that is primarily generated endogenously in cells during the metabolism of glucose and fructose (glycolytic pathways) (Dewanjee et al., 2018) (**Figure 3.2**). The formation and accumulation of MG has been shown to be involved in the pathogenesis of diabetes and its associated complications (Schalkwijk and Stehouwer, 2020) (**Figure 3.3**). Although the plasma levels of glucose have been shown to be much higher than those of MG, the latter has been demonstrate to be up to 50000-fold more reactive with regard to glycation in comparison to glucose (Thornalley, 2005). MG is mainly a by-product of glycolysis and in normal conditions it gets detoxified by conversion to D-Lactate (Sousa Silva et al., 2013) via the

glyoxalase system, which consists of the enzymes glyoxalase 1 (GLO1) and glyoxalase 2 (GLO2) (Racker, 1951) (**Figure 3.2**). GLO1 is the rate-limiting step for MG detoxifications and as such acts as a more critical enzyme (Thornalley, 2003). Formation of MG increases with an increase in glucose metabolism, for instance during hyperglycaemia states, which are a common consequence of diabetes (Ahmed et al., 2003a). Only 1% of MG exists in a free form, while the major part is reversibly bound to macromolecules (Lo et al., 1994). When MG levels exceed cell capacity for detoxification, MG accumulates in the cell and cause a very fast generation of glycation adducts on proteins, lipids and DNA, which leads to the formation of advanced glycation end products (AGEs), specifically methylglyoxal-derived cyclic hydroimidazones (MG-Hs) which are formed as three isoforms MG-H1, MG-H2 and MG-H3. MG-H1 is a MG-derived AGE of particular importance because it has been shown to account for >90% of all MG adducts (Ahmed et al., 2003b, Thornalley et al., 2003). The protein modifications induced by MG can cause loss-of-function and cross-linking of extracellular and cellular proteins as well as cell dysfunction, thus leading to irreversible damage and AGE-associated pathologies such as diabetes and its associated complications (Brownlee, 2005, Maessen et al., 2015).

Despite the fact that many studies have looked at the involvement of glycation in DN (Cullum et al., 1991, Ryle and Donaghy, 1995, Sugimoto et al., 2008, Vlassara et al., 1983), only a few clinical and preclinical studies have investigate the role of MG in the pathology of PDN specifically.

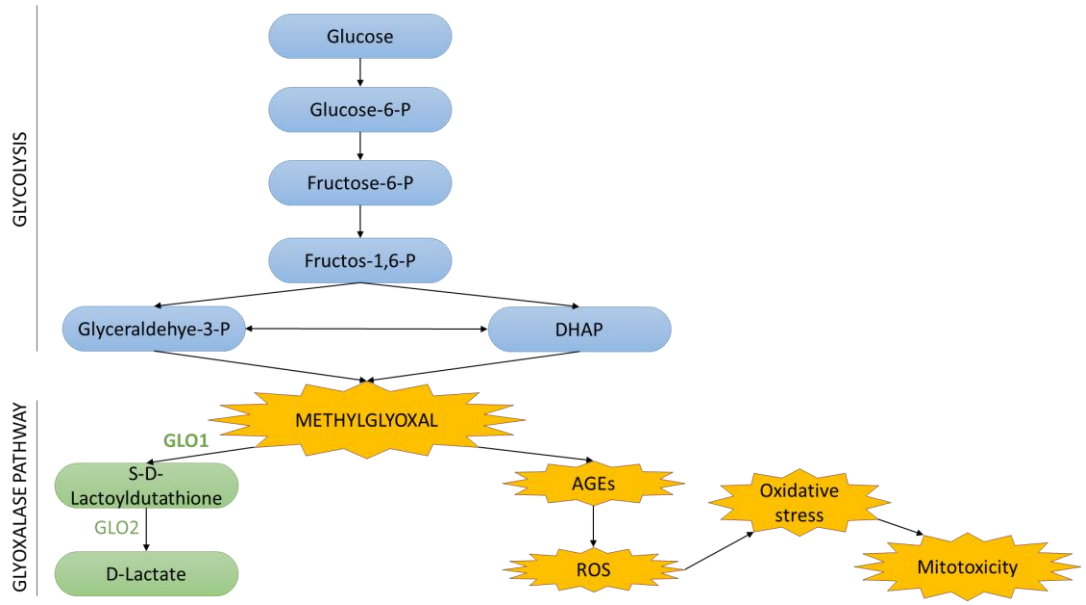


Figure 3.2 Overview of MG formation and degradation

MG is a by-product of glycolysis, formed via the spontaneous degradation of the triosephosphates dihydtroxyacetonephospahte (DHAP) and glyceraldehyde-3-phosphate (G3P), which are intermediate glycolysis products (Degen et al., 2013). MG gets degraded by the glyoxalase system, where glyoxalase 1 (GLO1) catalyses the formation of S-lactoylglutathione, which is hydrolysed by GLO2 to form the end product D-lactate (Schalkwijk and Stehouwer, 2020). MG accumulation in cells causes fast generation of glycation adducts on macromolecules (proteins, lipids and DNA), which leads to the formation of advanced glycation end products (AGEs). AGEs are a source of reactive oxygen species (ROS), contributing to oxidative stress and associated mitotoxicity. This pathway is favoured in DN and though to contribute to the increased nociceptor excitability and pain symptoms. Adapted from Schalkwijk and Stehouwer (2020).

3.2.2.2 Clinical evidence for involvement of MG in PDN

Levels of the highly reactive glucose-derived metabolite methylglyoxal (MG) have been shown to be elevated in diabetes patients and have been suggested to play a significant role in DN and PDN (Shamsaldeen et al., 2016, Brings et al., 2017). A notable difference between diabetes patients with and without pain has been reported in the plasma concentrations of MG, where patients with painful diabetic

neuropathy had higher plasma MG levels when compared to patients with non-painful neuropathy (Bierhaus et al., 2012). In addition, it has also been shown that diabetes patients with PDN have reduced activity of the MG detoxifying enzyme GLO1 compared to patients with painless DN, providing indirect evidence for association of increased MG levels and the painful symptoms in DN (Skapare et al., 2013). Interestingly, experimental findings from patients have also shown that a derivative of MG can alter patients' noxious heat detection threshold, thus supporting the involvement of MG in noxious heat sensitivity (Sveen et al., 2013). MG has also been shown to induce pain and long-lasting hyperalgesia in healthy humans via predominant activation of C-fibre afferents, with involvement of A-fibres too (Düll et al., 2019), further supporting the pain-inducing effect of MG.

3.2.2.3 Preclinical evidence for involvement of MG in PDN

Preclinical studies have shown that sciatic nerves have high levels of MG, and these are even higher in a streptozotocin-induced diabetes rat model (Cullum et al., 1991, Thornalley et al., 2003). Further research into the peripheral sensory nerves in DN has found that the generally lower levels and activity of the MG detoxifying enzyme GLO1 are even further reduced in diabetes conditions, likely contributing to the increased susceptibility of the peripheral sensory nerves to MG accumulation and subsequent damage (Bierhaus and Nawroth, 2009, Jack et al., 2011, Bierhaus et al., 2012). Moreover, additional studies in STZ-induced diabetic mice of different substrains, which have different GLO1 levels, have shown that only the mouse substrain with lower GLO1 levels develops diabetes-associated complications, including loss of mechanical sensitivity (insensate neuropathy), while the mouse strain with higher GLO1 levels has been found to display no signs of neuropathy (Jack et al., 2012). More recently, further evidence for a role of MG in PDN has been demonstrated in diabetes rat models, which have been reported to show diabetes-induced increased plasma MG levels, linked with pain hypersensitivity (Huang et al., 2016). Further studies have suggested that there is not just an association between MG and neuropathic pain but that MG is an important factor in pain etiology because they have found that systemic administration of MG in rodent models

causes pain-like hypersensitivity (Liu et al., 2017, Barragán-Iglesias et al., 2019, Bierhaus et al., 2012).

3.2.3 Mechanisms underlying MG-induced neuropathic pain symptoms in DN

The proposed mechanisms through which MG is thought to induce pain in diabetic conditions involve its direct actions on ion channels and receptors, such as the voltage-gated sodium channels $Na_V1.7$ and $Na_V1.8$ and the TRPA1 channel, as well as its role in the production of AGEs and in the induction of oxidative stress, which likely also contribute to subsequent mitochondrial damage, which can be a hallmark of diabetic and other types of neuropathic pain (Bennett et al., 2014).

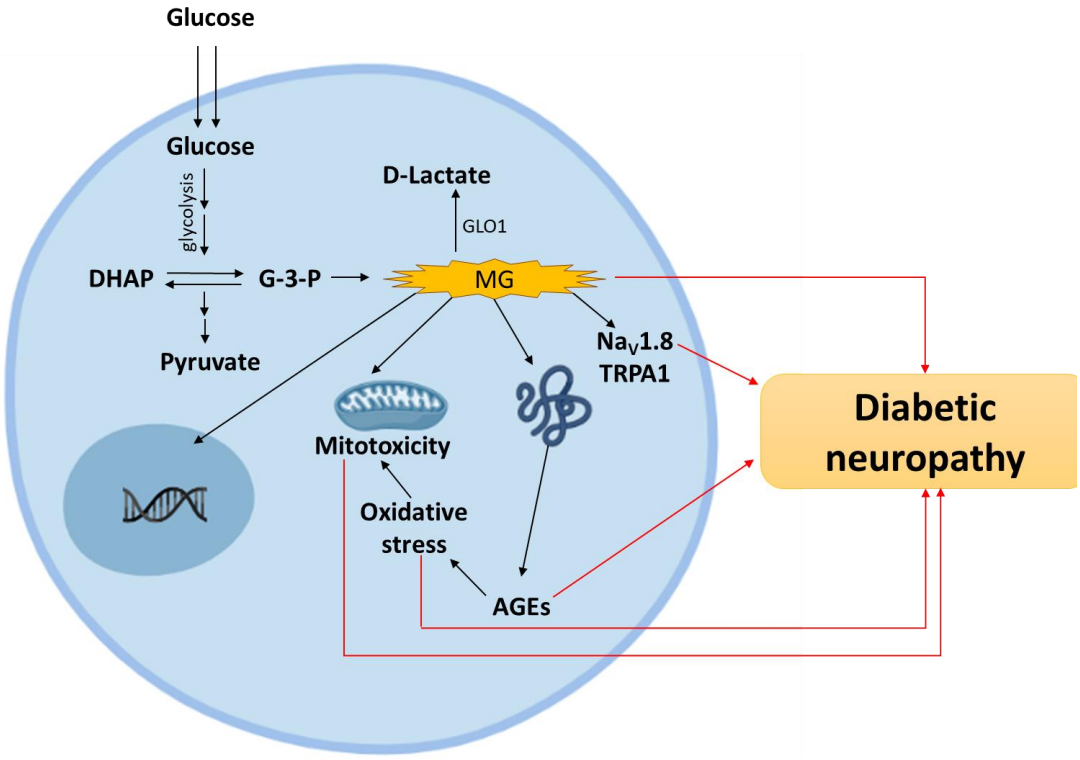


Figure 3.3 The role of MG in diabetic neuropathy

MG is mainly a by-product of glycolysis, which under normal conditions is detoxified by the glyoxalase system, including the GLO1 enzyme. MG is a major precursor of protein and DNA glycation, leading to formation of AGEs. MG and the MG-derived AGEs can affect different tissues and organs, including peripheral neurons, giving rise to diabetic neuropathy and its associated complications. The mechanisms involved in painful diabetic neuropathy involve MG-induced alterations in ion channels and receptor function and production of AGEs, leading to oxidative stress and mitotoxicity. Thus MG-

induced ion channel and receptor function alterations, AGEs formation, oxidative stress and mitochondrial dysfunction are all suggested to be involved in the pathogenesis of painful diabetic neuropathy. Adapted from Schalkwijk and Stehouwer (2020).

3.2.3.1 MG-induced effects on ion channels and receptors

3.2.3.1.1 Acute effects on TRPA1

Studies have shown that MG can act on the transient receptor potential channel subfamily A, member 1 (TPRA1), which has been demonstrated to be involved in diabetic neuropathy and its associated pain symptoms (Andersson et al., 2013, Koivisto et al., 2012, Ohkawara et al., 2012). Further research has shown that high doses of MG (1mM-10mM range) applied extracellularly are able to acutely activate TRPA1 in transfected cells (Eberhardt et al., 2012, Andersson et al., 2013) as well as in DRG neurons (Andersson et al., 2013) and in sciatic nerve C-fibres (Eberhardt et al., 2012). Acute TRPA1 activation causes significant changes in cutaneous nociceptors, stimulating neuropeptide release and action potential firing, while reducing the C-fibre conduction velocity (Eberhardt et al., 2012). However, the high MG concentration required for TRPA1 activation reported in the above studies are much higher than the reported blood levels of MG in diabetic patients, which are in the nanomolar range (~225nM and ~600nM) (Beisswenger et al., 2001, Bierhaus et al., 2012). Although intracellular MG levels have been shown to vary widely and be higher (particularly in neurons) than plasma levels (Ohkawara et al., 2012), the role of TRA1 in mediating the effects of physiologically relevant levels of MG in diabetic neuropathy and its associated pain symptoms remains not fully understood.

3.2.3.1.2 Modification of Na_V channels, specifically $Na_V1.7$ and $Na_V1.8$

Bierhaus et al. (2012) have found that MG concentrations, as found in the plasma of diabetic patients, can result in depolarization of sensory neurons, and induce major slow inactivation of the voltage-gated sodium channel $Na_V1.7$ as well as posttranslational modifications (PTM) of arginine residues within $Na_V1.8$ (Bierhaus et al., 2012). This $Na_V1.8$-specific PTM was shown to account for small but significant changes in the channel's biophysical properties and for the increased

excitability of primary sensory neurons, suggested to contribute to the observed increased pain sensitivity in diabetic mice (Bierhaus et al., 2012). Further behavioural studies have supported the involvement of $Na_V1.8$ in MG-induced pain phenotypes where they have shown that $Na_V1.8$ antagonist can block the local acute MG-induced spontaneous pain (Huang et al., 2016).

Also Bierhaus et al. (2012) have shown that scavengers of MG can reduce the hypersensitivity induced by MG. However, the MG scavengers used in the study were the antioxidant aminoguanidine and alagebrium, which breaks AGE-derived crosslinks in proteins. These findings along with the fact that several common MG scavengers are powerful antioxidants and have analgesic effects in PDN (Ma et al., 2015) suggest that oxidative stress is likely another mechanism via which MG could induce pain hypersensitivity.

3.2.3.2 Oxidative stress and mitochondrial dysfunction

The highly reactive nature of MG facilitates its interactions with different biomolecules, leading to the formation of AGEs (Ahmed et al., 1997, Nagaraj et al., 1996, Shipanova et al., 1997, Schleicher et al., 1997). Many studies have reported increased levels of AGEs in diabetes patients (Meerwaldt et al., 2005, Kilhovd et al., 1999, Schleicher et al., 1997, Schalkwijk et al., 2004, Kilhovd et al., 2003, Sell et al., 2005). In particular, evidence exists for the localisation of AGEs in peripheral nerves of diabetic patients (Sugimoto et al., 1997) and animals (Wada et al., 2001, Karachalias et al., 2003). Studies in rat peripheral nerve have also demonstrated increased levels of the membrane localised receptor for AGES (RAGE), expressed in endothelial and Schwann cells (Wada and Yagihashi, 2004). The interaction of AGEs with their receptor (RAGE) has been shown to trigger various intracellular signalling pathways, resulting in increased oxidative stress, as well as changes in gene expression, in pro-inflammatory molecules and free radicals release, and in local growth factors synthesis (Vincent et al., 2007). The AGEs have been also demonstrated to upregulate the expression of the pro-inflammatory transcription

factor NF-kB, shown to alters gene expression and thereby modulate various cell activities, including inflammation and apoptosis (Ramasamy et al., 2007). Importantly, AGEs have also been shown to lead to stimulation of NAD(P)H oxidase, a potent producer of reactive oxygen species (ROS) thus inducing oxidative stress, which ultimately can cause peripheral nerve damage (Vincent et al., 2007, Jack et al., 2012, Russell et al., 1999). Oxidative stress-induced damage of the peripheral nerves has been shown to cause a state of hyperexcitability in the afferent nociceptors, leading to the generation of spontaneous impulses within the axons and DRG, which likely contributes to the associated neuropathic pain (Ko and Cha, 2012). AGEs has been also shown to affect the expression levels of nitric oxide) synthase, reducing the blood flow to the nerves and inducing a hypoxia state in the peripheral nerve (Amore et al., 1997), resulting in impaired nerve fibre conduction velocity and neurotrophic support system (Yorek et al., 2004), which could also contribute to the associated neuropathic symptoms.

MG-induced AGE formation leading to ROS production and oxidative stress can ultimately result in mitochondrial damage, which is also implicated in the pathology of diabetic neuropathy (Sivitz and Yorek, 2010, Chowdhury et al., 2013). In addition, AGE-induced glycation of mitochondrial proteins can directly alter mitochondrial essential processes, which develop secondary to diabetes (Morcos et al., 2008, Rosca et al., 2005, Brouwers et al., 2011). There is evidence that diabetes-induced oxidative stress can affect mitochondrial membrane potential and permeability (Cameron and Cotter, 1994, van Dam et al., 1995a, Van Dam et al., 1995b) and contribute to progressive mitochondrial degeneration (Russell et al., 1999). This diabetes-induced mitochondrial damage in turn can trigger a cascade of signalling pathways leading to further damage and death of Schwann and neuronal cells (Dewanjee et al., 2018). Given the peripheral afferents of diabetic patients are particularly vulnerable to accumulation of AGEs and oxidative stress due to their low GLO1/2 expression, which facilitates ROS accumulation (Jack et al., 2011), this would suggest that they are very susceptible to diabetes-induced mitochondrial alterations. Other painful neuropathies, including chemotherapy-induced painful

neuropathy (CIPN) and HIV treatment-related painful neuropathy, which share pathology with DPN, also involve altered mitochondrial function (Flatters and Bennett, 2006, Lehmann et al., 2011, Bennett et al., 2014).

It remains controversial whether mitochondrial dysfunction could in turn result in the production of ROS and oxidative stress in sensory neurons. Studies in tissues other than the PNS suggest that the reactive dicarbonyl damage to mitochondria results in oxidative stress (Morcos et al., 2008, Rosca et al., 2005, Rabbani and Thornalley, 2008, Schlotterer et al., 2009). However, other studies have shown that although the DRG neurons axons from diabetic rats have increased ROS and exhibit oxidative stress (Zherebitskaya et al., 2009), the mitochondrial impairments actually resulted in reduced production of ROS (Chowdhury et al., 2010, Akude et al., 2011).

3.2.4 Evidence for altered C-fibre function and ADS in painful diabetic neuropathy

Microneurography studies in humans have demonstrated that spontaneous activity in peripheral nociceptors is typically present in different painful peripheral neuropathies (Kleggetveit et al., 2012a, Ochoa et al., 2005), with reported faster C-fibre ADS recovery in patients with pain than those without pain (Kleggetveit et al., 2012a). Spontaneous nociceptor activity has also been shown in patients with painful diabetic neuropathy (Serra et al., 2015, Ørstavik et al., 2006). Moreover, it has been demonstrated that the peripheral nociceptors of patients with painful diabetic neuropathy show enhanced C-fibre ADS recovery in comparison to patients with non-painful diabetic neuropathy and healthy controls (Ørstavik et al., 2006). These clinical findings suggest alterations in peripheral afferents activity in diabetic neuropathy and specifically in C-fibre ADS in cases of painful diabetic neuropathy.

Preclinical studies in males further support the involvement of C-fibre dysfunction in the pathology of DN (Suzuki et al., 2002, Wang et al., 2016, Sun et al., 2012a, Garcia-Perez et al., 2018). Evidence from studies in diabetic rats have shown enhanced firing frequency of the polymodal C-fibre nociceptors (Suzuki et al., 2002). In addition, research in streptozotocin (STZ)-induced diabetic rats have

demonstrated increased C-fibre excitability (Wang et al., 2016) and a reduced C-fibre ADS profile (Wang et al., 2016, Sun et al., 2012a). Microneurography studies in another diabetic model, the Zucker Diabetic Fatty rat model, have also reported alterations in ADS, specifically in the mechano-insensitive C-fibres, where C-fibre ADS was enhanced (Garcia-Perez et al., 2018).

Given that Na_V (Baker and Waxman, 2012b, De Col et al., 2008, De Col et al., 2012, Obreja et al., 2012, Tigerholm et al., 2014a, Tigerholm et al., 2015, Petersson et al., 2014) and HCN (Grafe et al., 1997, Takigawa et al., 1998, Zhu et al., 2009, Mazo et al., 2013) channels are implicated in C-fibre ADS, further evidence for potential involvement of C-fibre ADS in DN comes from studies demonstrating changes in the Na_V and HCN channels, associated with DN (**Figure 3.4**).

Recent pharmacological and genetic studies in STZ-induced diabetic mice have demonstrated a role of small nociceptor HCN2 channels in diabetes-associated mechanical allodynia (Tsantoulas et al., 2017). Interestingly, this study also showed that pharmacological blockade and genetic deletion of HCN2 in the small nociceptive fibres cause a reduction in c-FOS expression (a marker of neuronal activity (Hunt et al., 1987)) in the superficial dorsal horn of the spinal cord induced by diabetes. These findings support the idea of increased peripheral nociceptive C-fibre activity driving central sensitisation and contributing to the symptoms of mechanical allodynia in diabetic conditions. Earlier studies have investigated specifically the effect of MG on peripheral afferent excitability and have demonstrated upregulation of the I*h* current in MG-treated mice, likely attributed to dysfunctional axonal HCN channels (Shimatani et al., 2015). Similar results were found in patients with diabetes (Shimatani et al., 2015). These findings suggest involvement of HCN channels, potentially driven by MG, in the peripheral nerves hyperexcitability phenotype associated with diabetic neuropathy.

There is also evidence for involvement of Na_V channels in DN. Studies in human diabetic nerves have reported upregulation of persistent Na^+ current (*I*Nap) (Misawa et al., 2006), suggesting a role of Na_V channels in the pathology of diabetic

neuropathy. Studies investigating the effect of MG in particular on $Na_V1.7$ and $Na_V1.8$ channels have shown that MG can cause slow inactivation of $Na_V1.7$ as well as direct posttranslational modifications on $Na_V1.8$ (Bierhaus et al., 2012). Behavioural studies further support the involvement of $Na_V1.8$ in MG-induced pain phenotypes where they have shown that $Na_V1.8$ antagonist can block the local acute MG-induced spontaneous pain (Huang et al., 2016). Given the crucial role of Na_V channels in ADS, particularly $Na_V1.8$ (Tigerholm et al., 2015, Petersson et al., 2014), it is important to investigate the effect of MG on C-fibre ADS.

Oxidative stress and the associated mitochondrial dysfunction, driven by MG-induced AGEs and ROS formation, are also thought to be involved in the pathology of diabetic neuropathy. Oxidative stress and mitochondrial dysfunction are also implicated in other neuropathic pain models, such as the paclitaxel-induced rat model of neuropathic pain, where C-fibre ADS alterations have been also reported (**Figure 3.4**) (Galley et al., 2017). Interestingly, the antioxidant melatonin was found to be effective at limiting the paclitaxel-induced mitochondrial dysfunction, and at protecting against the paclitaxel-induced changes in C-fibre ADS and in mechanical sensitivity (Galley et al., 2017). These findings suggest that oxidative stress can lead to changes in C-fibre ADS, further emphasizing the importance of investigating the effects of highly reactive glycating agent MG on C-fibre ADS.

It must be noted, however, that all of the preclinical studies mentioned here have been conducted in male animals only. Interestingly, in the hindpaw incision model (Velichkova et al., 2021) (**Chapter 2**) and in the CFA hindpaw inflammation model (Dickie et al., 2017), C-fibre ADS has been shown to be altered in a sex-dependent manner, with ADS alterations correlating with thermal but not mechanical hypersensitivity. Given the clinical and preclinical evidence for alterations in C-fibre function and ADS in PDN, along with reported in PDN alteration in ion channels implicated in ADS, it was hypothesised that ADS will be altered in the methylglyoxal-induced rat model of hypersensitivity and that this may occur in a sex-dependent manner in association with sex differences in thermal hypersensitivity. This is

important to assess because ADS regulates the temporal delivery to and processing of pain signals in the CNS (Dickie et al., 2017) and altered ADS profiles have been observed in chronic pain patients. In addition, the $Na_V1.7$ and $Na_V1.8$ ion channels, important pain pharmaceutical targets, are implicated in C-fibre ADS, these studies may also provide therapeutic insight.

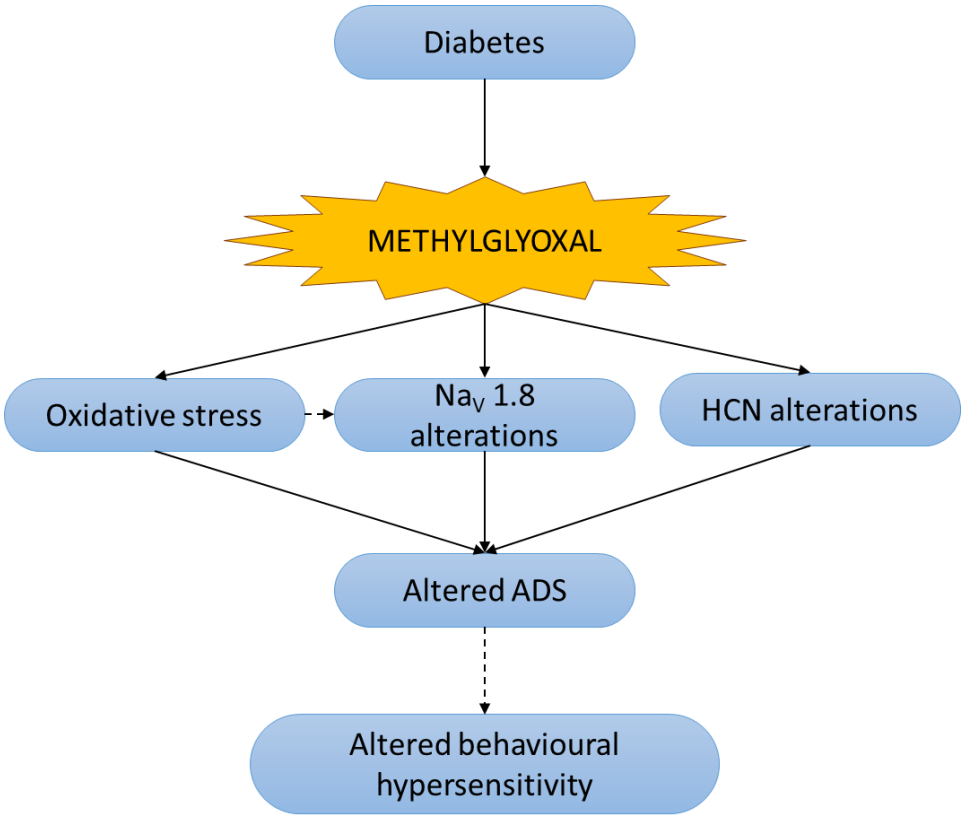


Figure 3.4 Potentials mechanism of MG-induced alterations in C-fibre ADS

Possible routes via which MG may affect C-fibre ADS. One mechanism involves AGEs-induced oxidative stress and mitochondrial dysfunction, which have been shown to also affect C-fibre ADS. Another mechanism involves direct MG action on ion channels implicated in ADS, including $Na_V1.8$ and HCN.

3.3 Aims and hypothesis

3.3.1 Hypothesis

C-fibre ADS will be altered in an *ex vivo* MG-induced model of diabetic hypersensitivity in a sex-dependent manner in association with sex differences in an *in vivo* model of MG-induced thermal hypersensitivity.

3.3.2 Aims

1. Investigate, using compound action potential recording, whether C-fibre ADS is altered in both sexes following chronic incubation with low MG concentration that mimics plasma levels in PDN patients.
2. Compare the effects of physiological chronic levels of MG to the effects of higher acute MG levels, on C-fibre ADS in both sexes.
3. Assess the *in vivo* effects of systemic injection of MG on mechanical and thermal sensitivity in juvenile rats of both sexes using behavioural analysis of the nociceptive flexion-withdrawal reflex

3.4 Methods

3.4.1 Ethical and legal considerations

As described in section 2.4.1.

3.4.2 Animals and general husbandry

All experiments used juvenile naïve Sprague-Dawley rats (international strain code 400) of both sexes, as described in section 2.4.2.

3.4.3 Methylglyoxal-induced model of diabetic behavioural hypersensitivity in juvenile rats

Systemic intraperitoneal (i.p) administration of MG (5µg) has been shown to increase plasma MG concentrations in male mice and to lead to the development of mechanical allodynia and a dose-dependent thermal hyperalgesia within 3h of MG administration, suggesting this as a potential rodent model of diabetes-induced hypersensitivity (Bierhaus et al., 2012).

This model was conducted in juvenile naïve Sprague-Dawley rats (aged from P20 to P25) of both sexes. MG injectable and vehicle solutions were prepared immediately before administration and delivered by intraperitoneal (i.p) injection to the right lower quadrant of the abdomen. Stock MG (100mM) was diluted with sterile 0.9% w/v saline (5µg in 0.2 ml final concentration) and mixed well. Control animals were administered equivalent volume (0.2ml) of vehicle solution (sterile 0.9% w/v saline). The injection solution volume for this age rats (~40g) was decided according to the Recommended Maximum Administration Volumes by the Home Office. Intraperitoneal MG/vehicle injections were administered without anaesthesia in conscious animals, because the injectable solution was a small volume and not expected to be painful. During injections animals were restrained by MSc student Menekše Mutlu-Smith, in a supine position to help avoid accidental puncture of the abdominal viscera. A 1ml syringe with a 25G, 16mm needle was used for the injections, with a fresh needle for each animal. Following injections animals were placed in a recovery box for a brief period of time to allow monitoring and recovery after injections. After full recovery animals were returned to their home cages.

3.4.4 Behavioural assessment of sensitivity

These experiments and the resulting data analysis were performed by Menekše Mutlu-Smith, an MSc Integrative Neuroscience student, under my supervision. All behavioural analysis was subjected to blinding of sex and treatment.

3.4.4.1 Blinding behavioural measures of sensitivity

To prevent deliberate or unintentional bias when performing behavioural measures of sensitivity, it is important the investigator remains blinded to the examined factors and the animals are randomly assigned to a treatment group. To further ensure consistent and systematic behavioural measures the MSc student was closely supervised and their personal judgement of withdrawal responses was compared to that of experienced investigators and results did not differ.

To blind the investigator to treatment group and sex before the start of the experiment I tail-marked and identified the sex of all animals. It must be noted that blinding to sex was possible because at this age the sex of juvenile rats, unlike fully grown adults, is not obvious without inspection. The sex of each animal was further confirmed by a member of technical staff. Males and females were randomly assigned to a treatment group using an online team allocator. The treatment group and sex corresponding to each tail-marked animal were recorded and known only by me. Thus, the investigator performing the behavioural measures of sensitivity remained blinded to both the treatment group and sex of each animal. The investigator recorded tail numbers every day after each set of behavioural measures was completed. At the end of the experiment the sex of each animal was confirmed by a member of the technical staff. The treatment group and sex of each tail-marked animal were revealed to the investigator, only after the experiment was finished.

3.4.4.2 Acclimatisation and habituation

Acclimatisation and habituation were performed as previously described in **section 2.4.4.2**. The animals used for this experiment were from four separate litters at roughly the same age (from P20-P25). On a given experimental day, two litters were tested twice per day (in the morning and in the afternoon) each. Behavioural measures were performed roughly at the same time each day (approximately starting 9.00am for one litter and 11am for the second litter). To allow animals to acclimatise to the testing environment, for two consecutive days before baseline measures, animals were habituated twice daily to each testing apparatus for 30

minutes without stimulus application or sensitivity testing. Thereafter, animals were routinely acclimatised to the testing apparatus for 20 minutes prior to behavioural assessments of sensitivity. Baseline sensitivity was measured in the morning and in the afternoon in the four days prior to MG/vehicle administration to accustom the animals to testing. On the days of MG/vehicle administration, animals were baseline tested in the morning, then 5ug MG was intraperitoneally administered, and the animals were tested again ~3 hours post MG administration.

3.4.4.3 Testing punctate mechanical sensitivity

As described in **section2.4.4.3**.

3.4.4.4 Testing heat sensitivity

As described in **section 2.4.4.5**.

3.4.5 Isolated dorsal root electrophysiology

3.4.5.1 Isolated dorsal root preparation

Dorsal roots from both sexes were isolated as preciously described in **section 2.4.5.1**.

3.4.5.2 Compound action potential recordings from ex-vivo vehicle- and MG-treated dorsal roots

Ex vivo CAP recordings were used to investigate the chronic and acute effects of MG and vehicle (distilled water) on the electrically evoked properties of isolated dorsal roots.

To assess the chronic effects of MG *ex vivo*, after the brief 15-min recovery in 32-34^{o}C oxygenated *N*-methyl-D-glucamine (NMDG) recovery solution, the dorsal roots were incubated at room temperature in oxygenated holding solution with 100µM MG or vehicle control (distilled water) for 3 hours prior to electrophysiological recordings, conducted in the continuous presence of 100µM MG or vehicle. The MG concentration used in these experiments was chosen because it has been previously established that 100µM MG can induce modifications of Na_V channel (Bierhaus et al., 2012). In addition, studies have also demonstrated that shorter incubation at higher MG concentrations are able to produce similar intracellular MG changes as lower concentrations over longer incubation periods (Eberhardt et al., 2012). Also, this was experimentally feasible. Moreover, higher dose for shorter incubation periods has been shown to have similar impact on DRG neurons (Eberhardt et al., 2012). Stock solution of 100mM MG was prepared in distilled water. The control vehicle-treated dorsal roots were incubated with equivalent concentrations of appropriate solvent. The use of naïve animals allowed the preparation of dorsal roots from both the left and right side. Thus, for the chronic MG studies, on a given experimental day both vehicle control and MG-treated dorsal roots from either males or females were used for CAP electrophysiological recordings.

Since previous studies have demonstrated that higher MG concentrations could directly excite nociceptors via TRA1 activation (EC_{50}=744 ± 120µm) (Eberhardt et al., 2012), the acute effects of MG on C-fibre ADS were also investigated. This was done by bath application of 100µM and 1mM MG to vehicle-treated control dorsal roots only. The given MG concentrations were chosen based on the chronic MG studies, where 100µM MG was used for 3h, and based on previous studies investigating the acute effects of MG on TRPA1, which have shown that 1mM MG can activate 20% of cells expressing human TRPA1 and can increase the channel conductance (Eberhardt et al., 2012). Acute MG at 100µM allowed to investigate if the 100µM MG effect was acute, possible acting via the TRPA1 channels, or chronic, potentially via Na_V. And the higher 1mM concentration allowed to further rule out involvement of TRA1 in MG-induced changes in C-fibre ADS.

CAP recordings were made as previously described in **section 2.4.5.2**.The activation thresholds of the primary afferents were determined as previously described in **section 2.4.5.2**. C-fibre amplitude and estimated conduction velocity were measures and calculated as previously described in **section 2.4.5.2**.

This chapter also studies the Aβ fibres. The initial Aβ fibre estimated conduction velocity was calculated from average traces (average of x3) at 20μA, where the Aβ fibre response latency, measured as the time difference between the stimulus artefact and the negative peak of the triphasic response (**Figure 3.5A**), was divided by the estimated dorsal root length, measured as the distance between the recording and stimulating electrodes. The population Aβ fibre response amplitude and width were measured from the first trace of the train of 40x stimuli at 500μA at 1Hz, 2Hz and 10Hz (**Figure 3.5B**). The Aβ fibre response amplitude was measured as the difference in voltage between the first positive and the negative peak of the triphasic response (in mV) as demonstrated in **Figure 3.5B**. The width of the Aβ fibre response, which reflects the range of the conduction velocities, was measured as the time difference from the first positive to the negative peaks of the triphasic response (**Figure 3.5B**).

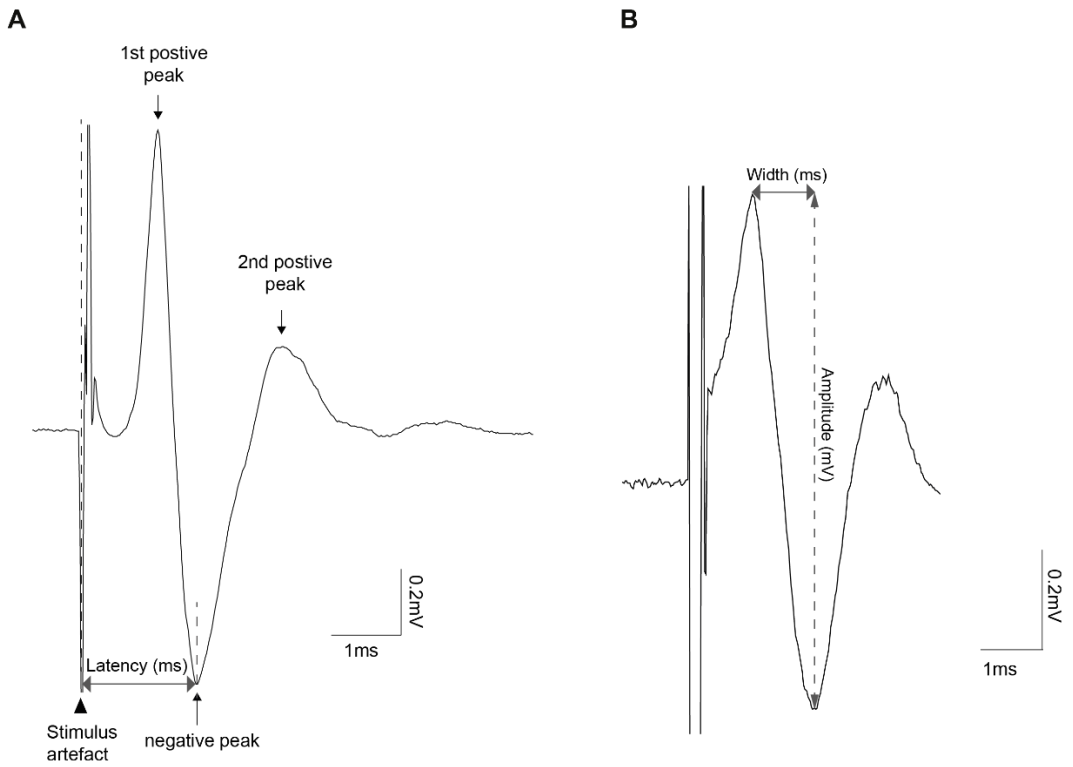


Figure 3.5 Representative Aβ fibre response from CAP recording

(**A**) Representative population CAP recording showing the fast (Aβ) conducting component at 20µA as an average of x3 traces. This example shows the three peaks of the triphasic Aβ response (indicated by black arrows) and the measurement of Aβ latency (double-headed arrows) as the time between the stimulus artefact (black dashed line indicated by arrowhead) and the negative peak of the triphasic response (grey dashed line). (**B**) Example trace showing the Aβ fibre response of the first trace in the train of 40x stimuli at 500µA at 1Hz. This example trace demonstrates the measurement of the Aβ fibre response amplitude, measured as the difference in voltage between the first positive and the negative peaks (grey double-headed dashed arrow) and of the Aβ fibre response width, measured as the time difference between the first positive and the negative peaks (grey solid double-headed arrow).

3.4.6 C-fibre activity-dependent slowing in vehicle/MG-treated dorsal roots

In these experiments some of the electrophysiological recordings were performed by Amy L Hall, a vacation student under the Carnegie Trust, under my supervision.

All CAP recording analysis was subjected to blinding of sex and treatment group.

To explore the effects of the glycolytic metabolite methylglyoxal on the electrically evoked properties of isolated dorsal roots from both sexes, CAP recordings were recorded as described in **section 3.4.5.2**. CAPs were recorded in continued presence of vehicle or 100µM MG.

To investigate activity-dependent changes, isolated dorsal roots were stimulated 40 times at a stimulation intensity for maximal C-fibre response (500µA) at frequencies of 1Hz, 2Hz and 10Hz (0.1ms stimulus width) (Shim et al., 2007, Weidner et al., 1999). A 10-minute interval was left between periods of stimulation to allow the fibres to fully recover from ADS (Shim et al., 2007, Weidner et al., 1999). These stimulation frequencies were chosen because they have been shown to mimic the spontaneous and evoked firing rates of the C-fibres observed following injury (Xiao and Bennett, 2007, Hulse et al., 2010, Matson et al., 2015, Lynn and Carpenter, 1982, Leem et al., 1993). To investigate the activity-dependent changes following acute treatment with MG at higher concentrations, 100µM and 1mM of MG were bath applied to vehicle-treated control dorsal roots only. These were stimulated again at 2Hz only in the continued presence of 100µM/1mM MG, with 10-minute-recovery intervals between periods of stimulation.

C-fibre ADS changes were measured and calculated based on changes in C-fibre latency and width following repetitive stimulation at high frequencies. C-fibre latency was measured as the time between the stimulus artefact and the negative peak of the triphasic response, as demonstrated in **Figure 2.8A**. The width of the C-fibre response was measured as the distance from positive-to-positive peak of the triphasic response **Figure 2.8A**. To access C-fibre ADS, C-fibre latency from the stimulus artefact to the negative peak of the triphasic response was measured and the change in latency from stimulus 1 was calculated **(Figure 2.10A**). This was also done for the remaining 39 stimuli. The change in C-fibre width was analogously calculated (**Figure 2.10A**). C-fibre latency change reflects change in the average conduction velocity of the C-fibre population, whereas change in the width of the C-fibre component reflects change in the range of the conduction velocities of C-fibre

population. Given that C-fibre ADS is length-dependent (Schmelz et al., 1995, Zhu et al., 2009), the latency/width change was normalised to the estimated length of root stimulated to negate the influence of varying dorsal root lengths on ADS. The estimated dorsal root length was measured as the distance between the recording and stimulating electrodes.

3.4.7 Aβ-fibre activity-dependent changes in vehicle/MG-treated dorsal roots

In these experiments some of the data analysis was performed by a BSc Neuroscience student – Quin McGlone-Healey, as part of his honours project under my supervision. All CAP recording analysis was subjected to blinding of sex and treatment group.

To explore the effects of the glycolytic metabolite MG on the electrically evoked properties of the Aβ-fibres of isolated dorsal roots from both sexes, CAP recordings were recorded as described in **section 3.4.5.2**. The basic properties of the Aβ response were defined as described in **section 3.4.5.2**. Dorsal roots were excluded from the analysis if, at any sweep, the first positive or the negative peaks of the triphasic Aβ response could not be identified.

To assess activity-dependent changes of the Aβ fibre response following the 40 stimuli at 500μA at 1Hz, 2Hz and 10Hz, the amplitude and width of the Aβ response were measured after each of the 40 stimuli, at each frequency, as demonstrated in **Figure 3.5B**. To investigate activity-dependent changes in the Aβ response following repetitive stimulation, changes in values from the first stimulus were calculated for the 40x stimuli applied (**Figure 3.6**). The width change was normalised to the estimated length of root stimulated to negate the influence of varying dorsal root lengths.

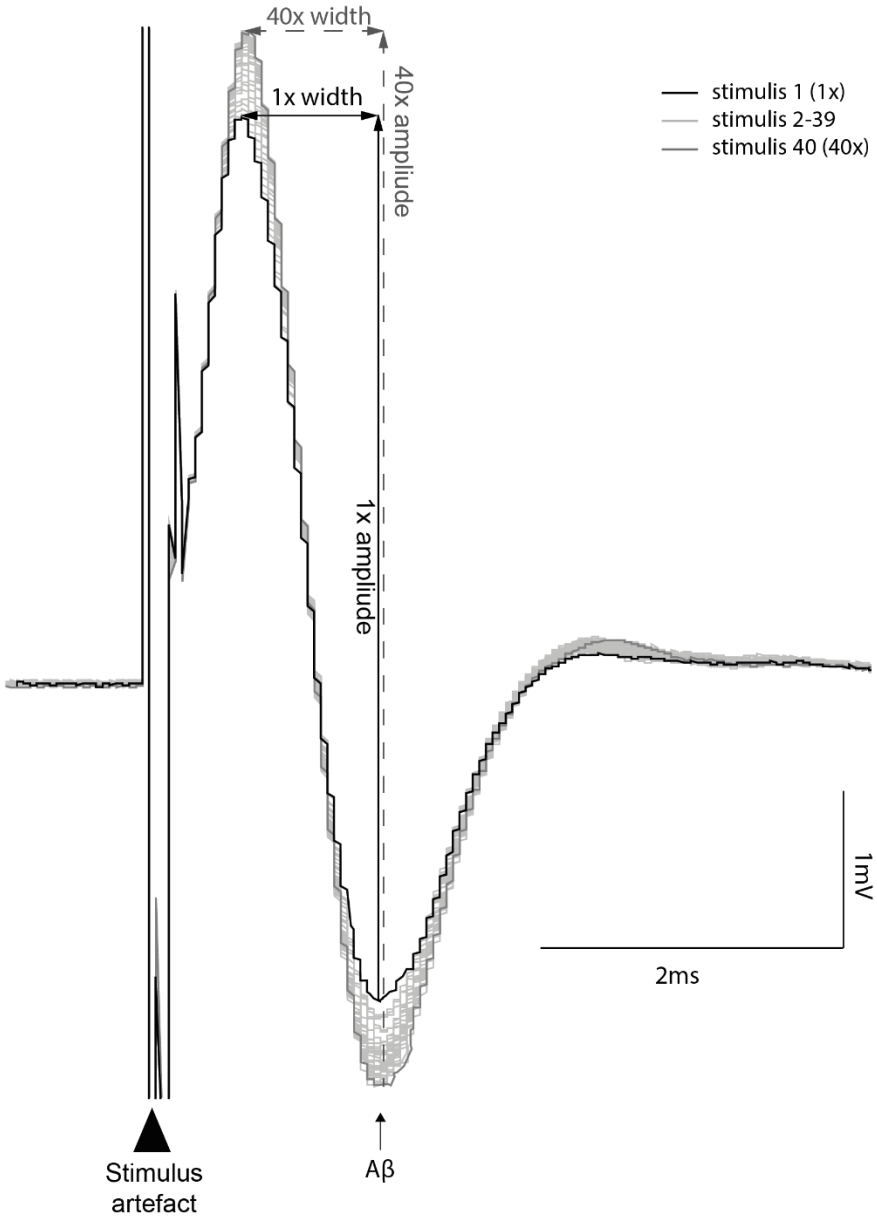


Figure 3.6 Population CAP recording of activity-dependent changes in Aβ response following repetitive stimulation

Example trace shows the Aβ fibre component in a train of 40 stimuli at 500μA at a frequency of 1Hz. Amplitude of the Aβ responses was measured as the voltage difference between the first positive and the negative peaks of the triphasic response. Arrows mark the voltage difference between the positive and the negative peaks for the first (solid black arrow) and last (dashed grey arrow) responses. Width of Aβ responses was measured as the time distance between the first positive and the negative peaks of the triphasic response. Double-headed arrows mark the time distance between the positive and the negative peaks for the first (solid black double-headed arrow) and last (dashed grey double-headed arrow) responses. Aβ responses amplitude change is demonstrated by the increased amplitude of the 40th response compared to the first response.

3.4.8 Statistical Analysis

3.4.8.1 Electrophysiology

In CAP recordings from isolated roots, the effects of MG on basic C-fibre and Aβ fibre properties were statistically assessed using two-way ANOVA. Post-hoc multiple comparison tests were run only if a significant interaction between the two investigated factors was detected by the two-way ANOVA. Sidak's multiple comparisons test was performed where there was an interaction between the two factors. To compare the effects of chronic MG and sex on C-fibre ADS and Aβ fibre activity-dependent changes in amplitude and width at different stimulation frequencies, the ADS area under the curve (AUC) for each group was measured and analysed using three-way ANOVA followed by Tukey's multiple comparisons test if an interaction among factors was observed.

Baseline C-fibre ADS at 2hz and C-fibre ADS following acute 100µM and 1mM MG at 2Hz were also measured using AUC for each group in males and females. One-way ANOVA tests were performed for males and females to statistically compare baseline 2Hz ADS versus 2Hz ADS at 100µM MG and 1mM MG. The acute effects of MG on Aβ fibre activity-dependent changes in amplitude at 2Hz were also measured using AUC for each group in males and females and two-way ANOVA tests were performed to statistically assess the effects of sex and acute MG treatment. Spearman's correlation analysis was used to investigate relationships between continuous variables.

3.4.8.2 Behavioural assessment of sensitivity

For behavioural tests averaged data from three measurements was recorded. To assess the change in punctate mechanical and noxious heat sensitivity the averaged baseline measurements were subtracted from the averaged measurements following systemic MG or vehicle administration for each individual animal (as conducted by Bierhaus et al. (2012). This analysis also enables direct comparison between the sexes. These data sets were analysed using a two-way ANOVA followed by Sidak's multiple comparisons test if an interaction between factors was observed.

Graphad Prism 9.11 software (GraphPad Software) was used for statistical analysis and graph production. All data are represented as mean ± standard error of the mean (SEM), with n representing the sample size. P<0.05 was used to indicate statistical significance.

3.5 Results

3.5.1 *Ex vivo* effect of MG on C-fibre function

3.5.1.1 Chronic MG treatment does not influence basic C-fibre properties

To assess the impact of chronic MG treatment on afferent C-fibre nociceptor function, dorsal roots isolated from control naïve rats were recovered in NaCl holding solution with vehicle (distilled water) or 100µM MG for 3h prior to recordings. CAP recordings were then conducted, as described in the previous chapter (**Figure 2.7** and **Figure 2.8)** in the continued presence of vehicle or 100µM MG. The effect of chronic MG treatment and sex on basic C-fibre properties, including activation threshold, initial amplitude and initial average conduction velocity, was investigated. There was no significant effect of chronic MG treatment and sex on C-fibre activation threshold (**Figure 3.7A**; two-way ANOVA: chronic MG, *p*=0.26, sex, *p*=0.28, interaction, *p*=0.72), initial amplitude (**Figure 3.7B**; two-way ANOVA: chronic MG, *p*=0.64, sex, *p*=0.36, interaction, *p*=0.39) and initial average conduction velocity (**Figure 3.7C**; two-way ANOVA: chronic MG, *p*=0.79, sex, *p*=0.95, interaction, *p*=0.44).

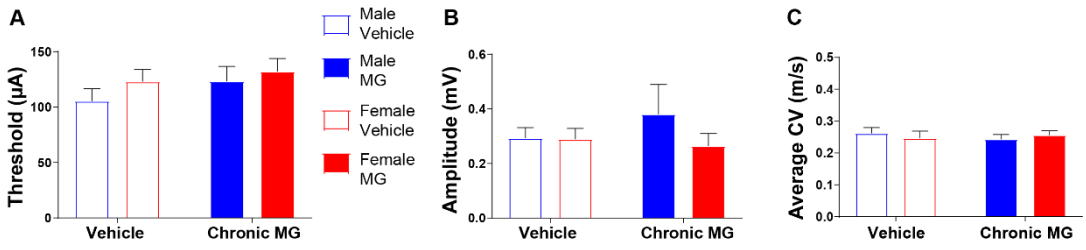


Figure 3.7 Basic C-fibre properties in control and chronic MG-treated dorsal roots of rats of both sexes

(**A**) C-fibre activation threshold was not significantly different between the control and chronic MG groups in both sexes (Two-way ANOVA: chronic MG, *p*=0.26, sex, *p*=0.28, interaction, *p*=0.72). (**B**) C-fibre initial amplitude was not significantly different between the control and chronic MG-treated groups in both sexes (Two-way ANOVA: chronic MG, *p*=0.64, sex, *p*=0.36, interaction, *p*=0.39). (**C**) C-fibre initial average conduction velocity was not significantly different between the control and chronic MG-treated groups in both sexes (Two-way ANOVA: chronic MG, *p*=0.79, sex, *p*=0.95, interaction, *p*=0.44). All data presented as mean±SEM. Male: control (n=9), chronic MG (n=9). Female: control (n=10), chronic MG (n=9).

3.5.1.2 Chronic MG treatment alters C-fibre ADS in a sex-dependent manner

3.5.1.2.1 Chronic MG treatment reduces C-fibre progressive latency increase in males and enhances it in females

To assess C-fibre ADS, vehicle- and MG-treated dorsal roots from naïve rats of both sexes were repetitively stimulated 40x at 1Hz, 2Hz and 10Hz. This resulted in a clear C-fibre ADS, manifested by an increase in C-fibre response latency change from the first stimulus (**Figure 3.8**). The C-fibre ADS phenomenon was confirmed to be frequency-dependent with higher frequencies resulting in greater latency changes (**Figure 3.8G**; Three-way ANOVA: frequency, *p*<0.0001), as previously demonstrated in the lab in similarly aged rats (Dickie et al., 2017). Chronic MG treatment altered the progressive increase in C-fibre latency change in a sex-dependent manner (**Figure 3.8**; Three-way ANOVA: sex x MG, *p*<0.0001). The MG-treated dorsal roots from males showed a reduction in the progressive latency change compared to vehicle-treated roots (**Figure 3.8G**; two-way ANOVA followed by Tukey's multiple comparisons test, males, *p*=0.008). In contrast, the MG-treated dorsal roots from females showed a striking enhancement of the progressive latency change compared to vehicle-treated roots (**Figure 3.8G**; two-way ANOVA followed by Tukey's multiple comparisons test, females, *p*<0.0001). Interestingly, vehicle-treated male tissue showed a greater progressive latency change in comparison to vehicle-treated female tissue and this was statistically significant at all investigated frequencies (**Figure 3.8E**; two-way ANOVA followed by Tukey's multiple

comparisons test, vehicle males vs vehicle females, $p<0.0001$), which is contrary to previous findings from the lab (Dickie et al., 2017). In female dorsal roots, chronic MG treatment increased the progressive latency change to levels exceeding those observed in male dorsal roots after MG treatment (**Figure 3.8E**; two-way ANOVA followed by Tukey's multiple comparisons test, vehicle males/females, $p<0.0001$).

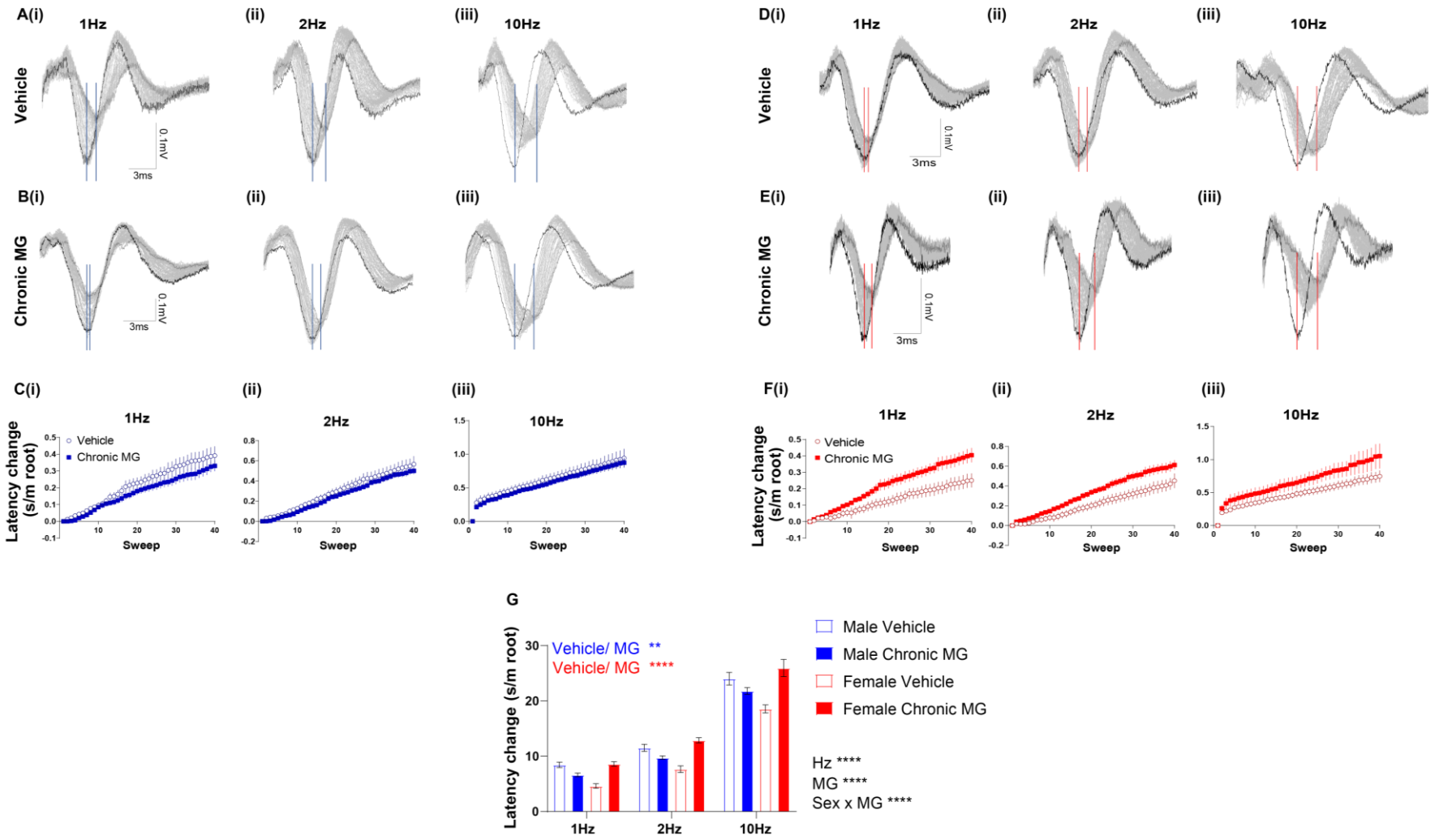

A(i)
1Hz
(ii)
2Hz
(iii)
10Hz
Vehicle
0.1mV
3ms
B(i)
Chronic MG
C(i)
Latency change (s/m root)
Vehicle
Chronic MG
Sweep
D(i)
E(i)
F(i)
G
Vehicle/ MG **
Vehicle/ MG ****
Male Vehicle
Male Chronic MG
Female Vehicle
Female Chronic MG
Hz ****
MG ****
Sex x MG ****

Figure 3.8 Chronic MG enhances frequency dependent C-fibre latency change in females and reduces it in males

Representative C-fibre CAP recordings from 3h vehicle-treated (**A, D**) and MG-treated (**B, E**) dorsal roots isolated from male (**A-B**) and female (**D-E**) naïve rats in response to x40 stimuli at 1Hz (**i**), 2Hz (**ii**) and 10Hz (**iii**). Latency change marked by solid lines (response trace 1 black; 2-39 pale grey; 40 dark grey). Repetitive stimulation results in a progressive C-fibre latency increase in vehicle-treated and MG-treated dorsal roots from males (**C**) and females (**F**) at 1Hz (**i**), 2Hz (**ii**) and 10Hz (**iii**). **G**) AUC analysis reveals a significant effect of frequency (****p<0.0001), chronic MG (****p<0.0001), with a sex x chronic MG interaction (****p<0.001) (Three-way ANOVA). Consolidation of the data by frequency to investigate effects of chronic MG on sex showed a statistically significant effect of chronic MG in males (blue font; two-way ANOVA followed by Tukey's multiple comparisons test, male vehicle/chronic MG, **p=0.008) and females (red font; two-way ANOVA followed by Tukey's multiple comparisons test, female vehicle/chronic MG, ****p<0.0001) and statistically significant effect of sex on C-fibre latency change in vehicle and MG-treated dorsal roots (black font; two-way ANOVA followed by Tukey's multiple comparisons test, vehicle male/female, ****p<0.0001 and MG male/female, ****p<0.0001). Data presented as mean±SEM. Male: vehicle (n=9), MG (n=9). Female: vehicle (n=10), MG (n=9).

3.5.1.2.2 Chronic MG treatment enhances C-fibre progressive width increase in females only

When C-fibre ADS was assessed as a progressive increase in response width, similar to the latency change data, the change in width was demonstrated to be frequency-dependent, with higher frequencies resulting in greater width changes from the first stimulus (**Figure 3.9G**; Three-way ANOVA: frequency, $p<0.0001$). Interestingly, the statistical analysis detected an interaction between the effects of frequency and sex (**Figure 3.9G**; Three-way ANOVA: frequency x sex, $p=0.026$) revealing that the frequency dependent effect is influenced by sex , with width changes following chronic MG treatment shown to be greater in females compared to males at 2Hz and 10Hz (**Figure 3.9G**; Three-way ANOVA followed by Tukey's multiple comparisons test, $p=0.003$, $p<0.0001$). In line with the latency change data, the chronic MG treatment also altered C-fibre width change in a sex-dependent manner (**Figure 3.9G**; Three-way ANOVA: sex x MG, $p<0.0001$). In contrast to the latency change data, chronic MG treatment did not significantly affect width change in males (**Figure 3.9G**; Three-way ANOVA, followed by Tukey's multiple comparisons test: male vehicle vs male MG, 1Hz, p>0.9999, 2Hz, p>0.9999, 10Hz, p=0.995). However, chronic MG enhanced the progressive increase in C-fibre width change in females, but with multiple comparison tests revealing a statistically significant increase in female width change at 2Hz only (**Figure 3.9G**; Three-way ANOVA followed by Sidak's multiple comparisons test, $p=0.0002$). In addition, similar to the latency change data, the width change following chronic MG treatment was shown to be greater in females compared to males (**Figure 3.9G**; Three-way ANOVA followed by Tukey's multiple comparisons test, $p=0.003$ at 2HZ, $p<0.0001$ at 10Hz).

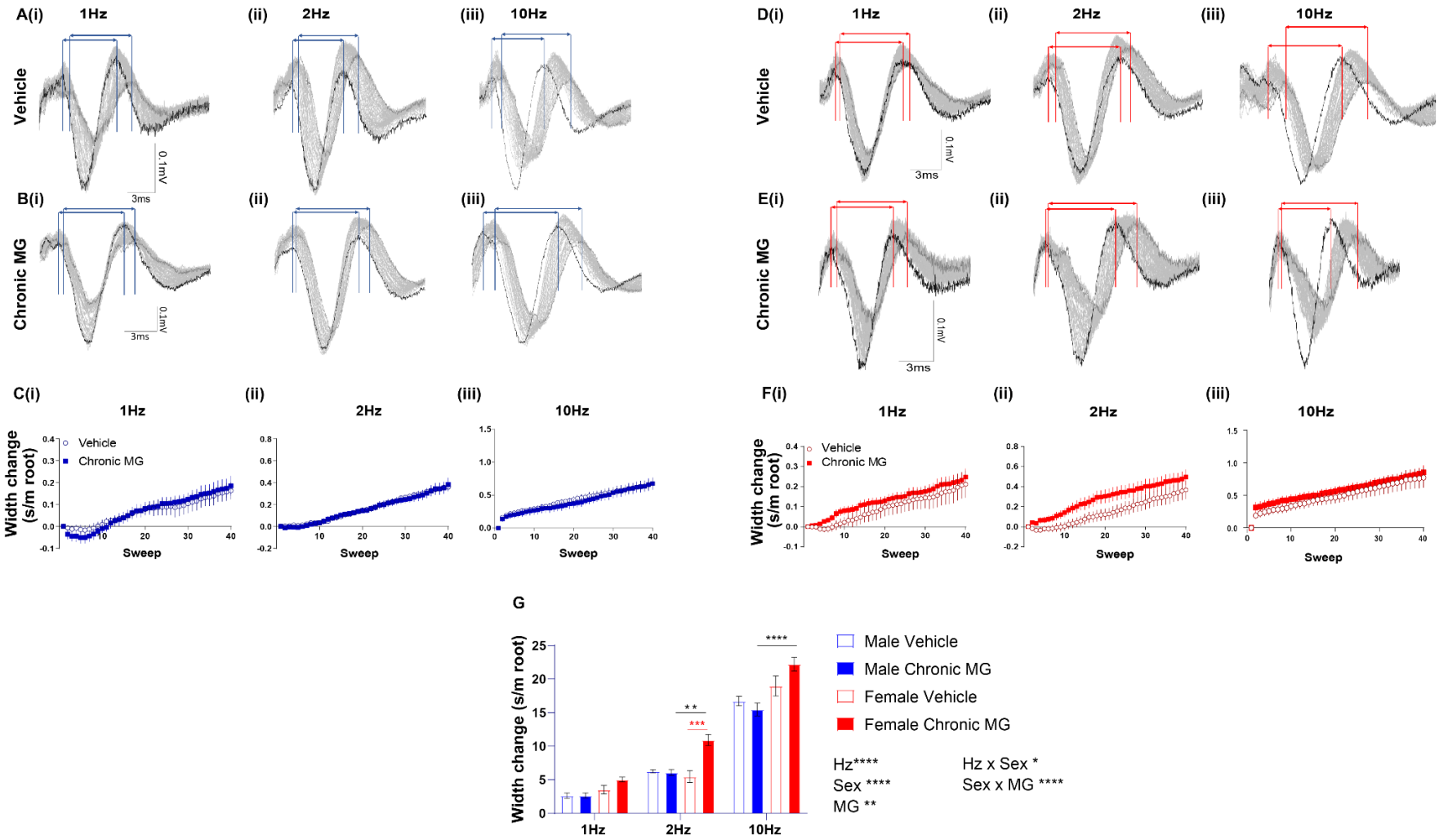

A(i)
1Hz
(ii)
2Hz
(iii)
10Hz
Vehicle
0.1mV
3ms
B(i)
Chronic MG
D(i)
E(i)
C(i)
Width change (s/m root)
Vehicle
Chronic MG
Sweep
F(i)
G
Male Vehicle
Male Chronic MG
Female Vehicle
Female Chronic MG
Hz****
Sex ****
MG **
Hz x Sex *
Sex x MG ****

Figure 3.9 Chronic MG enhances frequency dependent C-fibre width change in females but not in males

Representative C-fibre CAP recordings from 3h vehicle-treated (**A, D**) and MG-treated (**B, E**) dorsal roots isolated from male (**A-B**) and female (**D-E**) naïve rats in response to x40 stimuli at 1Hz (**i**), 2Hz (**ii**) and 10Hz (**iii**). Double-headed arrows mark C-fibre response width, the distance from positive-to-positive peaks for the first (dashed lines) and last (dotted lines) of the x40 responses (response trace 1 black; 2-39 pale grey; 40 dark grey). Repetitive stimulation results in a progressive C-fibre width increase in vehicle-treated and MG-treated dorsal roots from males (**C**) and females (**F**) at 1Hz (**i**), 2Hz (**ii**) and 10Hz (**iii**). **G**) AUC analysis reveals a significant effect of frequency (****p<0.0001), sex, (****p<0.0001) and chronic MG (**p=0.002), with a frequency x sex interaction (*p=0.026) and a sex x chronic MG interaction (****p<0.0001) (Three-way ANOVA followed by Tukey's multiple comparisons test, **p=0.003, ***p=0.0002, ****p<0.0001). There was a statistically significant effect of chronic MG in females only (red lines and asterisks) (Three-way ANOVA followed by Tukey's multiple comparisons test, ***p=0.0002). The statistical analysis showed a significant effect of sex on C-fibre width change in MG-treated dorsal roots at 2Hz and 10Hz (black lines and asterisks) (Three-way ANOVA followed by Tukey's multiple comparisons test, **p=0.003, ****p<0.0001). Data presented as mean±SEM. Male: vehicle (n=9), MG (n=9). Female: vehicle (n=10), MG (n=9).

3.5.1.3 Acute MG treatment does not affect C-fibre ADS

Previous studies have provided evidence for functional TRPA1 ion channel expression along the axons (Eberhardt et al., 2012) and have also demonstrated that higher MG concentrations can acutely excite nociceptors via activation of TRPA1 ion channels (Eberhardt et al., 2012, Andersson et al., 2013). To investigate the possible functional expression of TRPA1 ion channel along the dorsal roots that could have potentially contributed to the reported ADS observations, the acute effects of MG on C-fibre ADS were also studied. To assess C-fibre ADS following acute treatment with different MG concentrations, dorsal roots from vehicle-treated roots from rats of both sexes were repetitively stimulated 40x at 2Hz to record baseline ADS and following 10-min bath application of 100µM and 1mM MG. The repetitive stimulation at 2Hz baseline resulted in a clear progressive increase in C-fibre ADS in both males (**Figure 3.10**) and females (**Figure 3.11**), manifested by the increase in C-fibre response latency and width changes from the first stimulus. Treatment with MG at 100µM and 1mM did not alter the progressive increase in C-fibre latency (**Figure 3.10Bii**; One-way ANOVA, p=0.865) and width (**Figure 3.10*Cii***; One-way ANOVA, p=0.962) changes in males as well as in C-fibre latency (**Figure 3.11Bii**; One-way ANOVA, p=0.469) and width (**Figure 3.11Bii**; One-way ANOVA, p=0.808) changes in females.

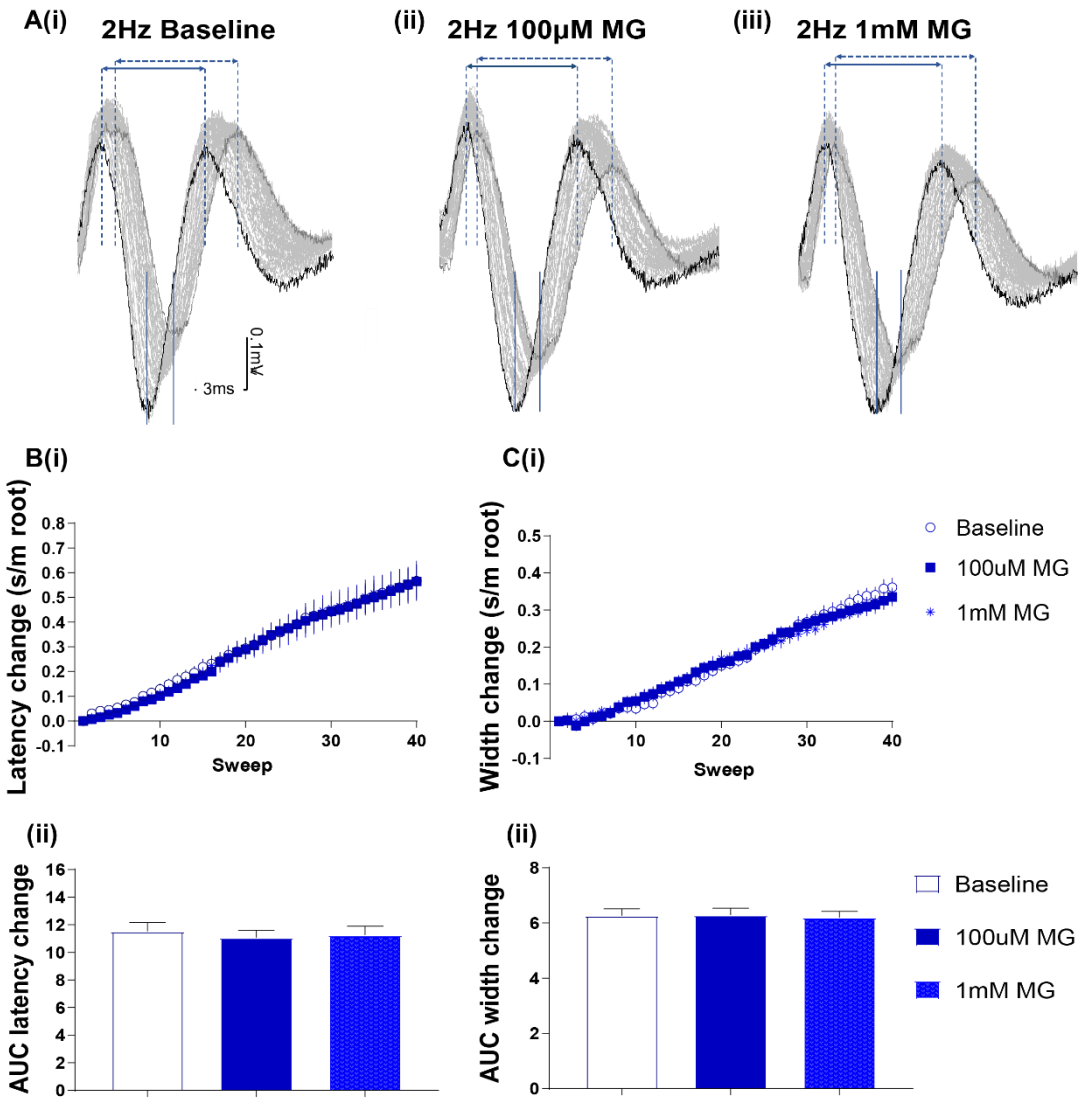


Figure 3.10 Acute MG treatment does not alter C-fibre ADS in males

(**A**)Representative C-fibre CAP recordings from dorsal roots from naïve male rats in response to x40 stimuli at 2Hz at baseline (**i**) and following 10min acute 100µM (**ii**) and 1mM MG (**iii**) application. Latency change marked by solid lines; double-headed arrows mark C-fibre response width, the distance from positive-to-positive peaks for the first (dashed lines) and last (dotted lines) of the x40 responses (response trace 1 black; 2-39 pale grey; 40 dark grey). Repetitive stimulation results in a similar levels of progressive C-fibre latency **B**(**i**) and width **C**(**i**) increase at baseline and following bath application of 100µM and 1mM MG. AUC analysis of C-fibre latency **B**(**ii**) (One-way ANOVA: p=0.865) and width **C**(**ii**) (One-way ANOVA: p=0.962) change reveals no significant effect of acute treatment with different MG concentrations. Data presented as mean±SEM. Sample size: baseline (n=9), 100µM MG (n=9), 1mM MG (n=9).

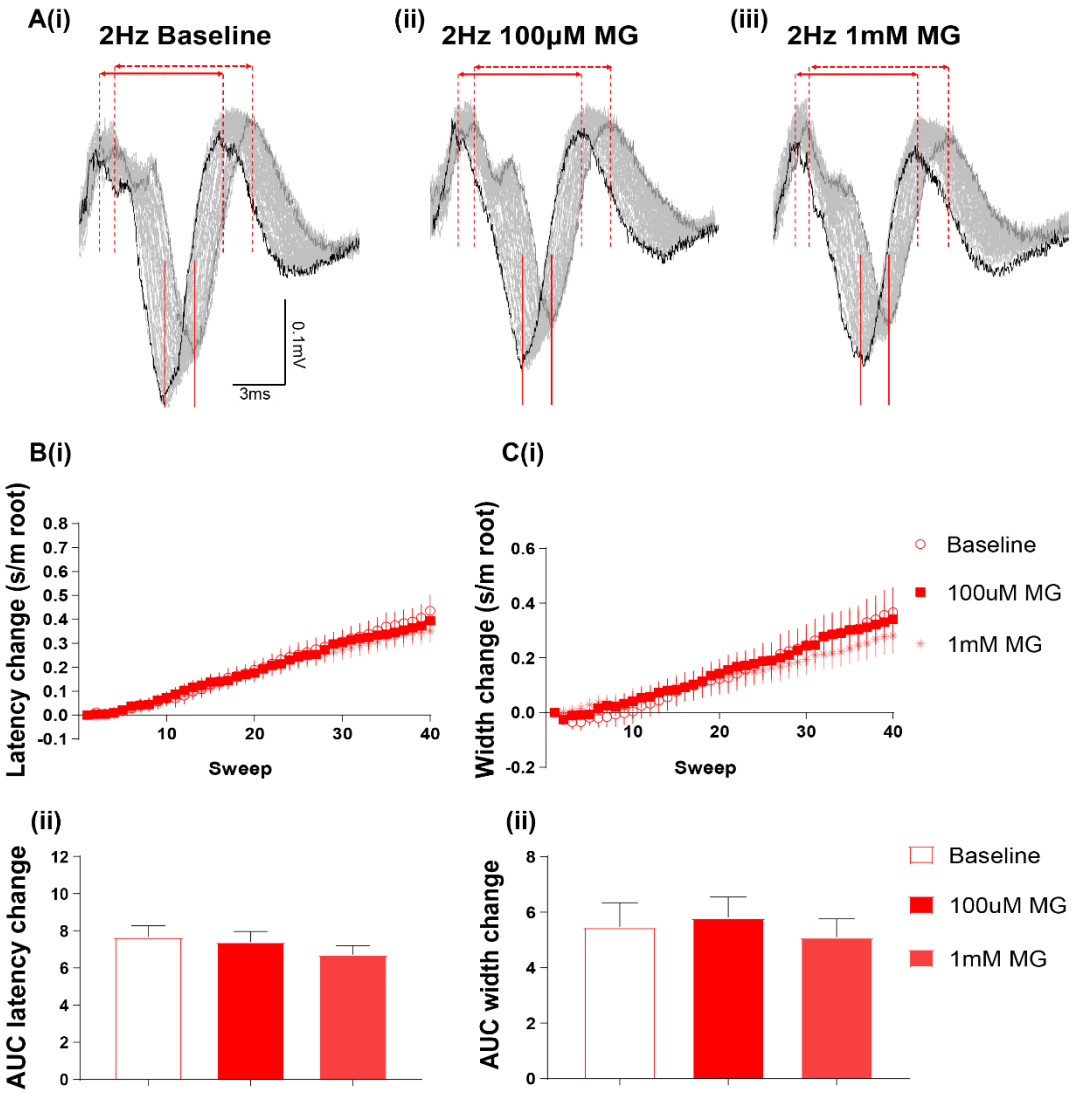


Figure 3.11 Acute MG treatment does not alter C-fibre ADS in females

(**A**)Representative C-fibre CAP recordings from dorsal roots from naïve female rats in response to x40 stimuli at 2Hz at baseline (**i**) and following 10min acute 100µM (**ii**) and 1mM MG (**iii**) application. Latency change marked by solid lines; double-headed arrows mark C-fibre response width, the distance from positive-to-positive peaks for the first (dashed lines) and last (dotted lines) of the x40 responses (response trace 1 black; 2-39 pale grey; 40 dark grey). Repetitive stimulation results in a similar levels of progressive C-fibre latency **B**(**i**) and width **C**(**i**) increase at baseline and following bath application of 100µM and 1mM MG. AUC analysis of C-fibre latency **B**(**ii**) (One-way ANOVA: p=0.469) and width **C**(**ii**) (One-way ANOVA: p=0.808) change reveals no significant effect of acute treatment with different MG concentrations. Data presented as mean±SEM. Sample size: baseline (n=10), 100µM MG (n=10), 1mM MG (n=10).

3.5.2 *Ex vivo* effect of MG on Aβ fibre function

3.5.2.1 Chronic MG treatment does not influence Aβ fibre activation thresholds and initial CV, but alters Aβ fibre initial amplitude and width in a sex-dependent manner

Given diabetes negative sensory symptoms are associated with altered A fibre function (Gwathmey and Pearson, 2019) and changes in the fast (Aβ) conducting component of the CAPs were observed during the repetitive stimulation recordings, the impact of chronic MG upon the Aβ component was also investigated. The Aβ fibre amplitude, latency and width were measured as shown in (**Figure 3.5**). The effect of chronic MG treatment and sex on basic Aβ fibre properties, including activation threshold and initial average conduction velocity, was investigated. There was no significant effect of chronic MG treatment and sex on Aβ fibre activation threshold (**Figure 3.12A**; two-way ANOVA: chronic MG, p=0.095, sex, p=0.134, interaction, p=0.292) and initial average conduction velocity (**Figure 3.12B**; two-way ANOVA: chronic MG, p=0.502, sex, p=0.182, interaction, p=0.537). To investigate the effect of chronic MG and sex on the initial amplitude and width of the Aβ response at the stimulation intensity used for the repetitive stimulation (500µA), the Aβ amplitude and width were measured during the first sweep of the train of 40x stimuli applied at 1Hz, 2Hz and 10Hz. Chronic MG treatment altered the initial Aβ amplitude in a sex-dependent manner (**Figure 3.12C-E;** three-way ANOVA: sex x chronic MG, p=0.021), with chronic MG increasing the initial Aβ amplitude for all frequencies in males only (**Figure 3.12E**; two-way ANOVA of the data consolidated by frequency followed by Sidak's multiple comparisons test, p=0.014). Treatment with chronic MG also altered the width of the initial Aβ response in a sex-dependent manner (**Figure 3.12C,D, F;** three-way ANOVA: sex x chronic MG, * p=0.049) with chronic MG decreasing the initial Aβ width for all frequencies in females only (**Figure 3.12F;** two-way ANOVA of the data consolidated by frequency followed by Sidak's multiple comparisons test, ** p=0.004).

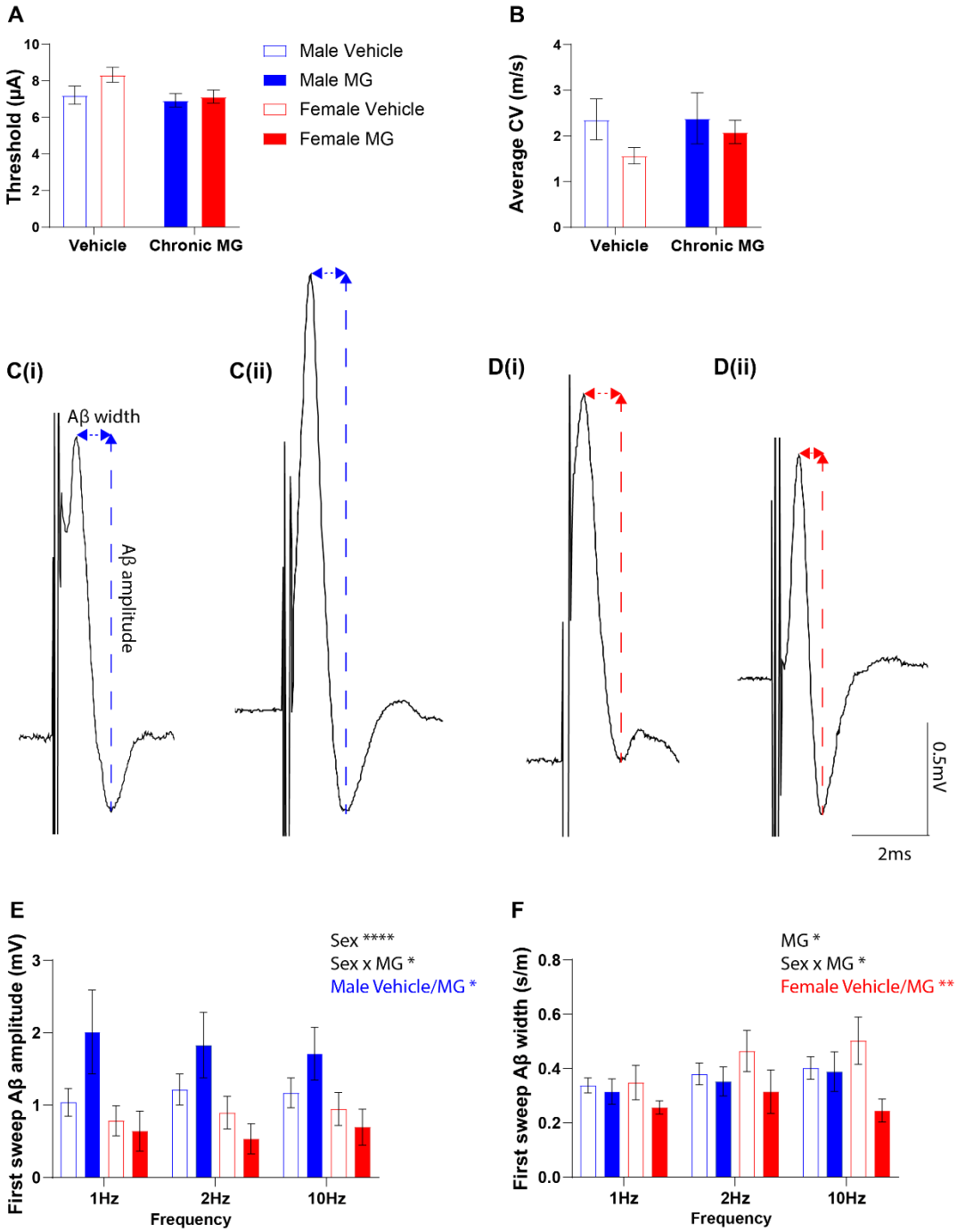


Figure 3.12 Chronic MG does not affect Aβ fibre activation thresholds and initial CV, but alters Aβ fibre initial amplitude and width in a sex-dependent manner

(**A**) Aβ fibre activation threshold was not significantly different between the control and chronic MG groups in both sexes (Two-way ANOVA: chronic MG, p=0.095, sex, p=0.134, interaction, p=0.292). (**B**) Aβ fibre initial average conduction velocity did not differ between the control and chronic MG-treated groups in both sexes (Two-way ANOVA: chronic MG, p=0.502, sex, p=0.182, interaction, p=0.537). Representative Aβ fibre CAP recordings from vehicle- (**i**) and chronic MG-treated (**ii**) dorsal roots isolated from naïve male (**C**) and female (**D**) rats in response to the first stimulus in the train of 40x stimuli at

2Hz, illustrating the initial Aβ response amplitude (dashed arrow) and width, the distance from first positive to negative peaks (dashed double-headed arrow). (**E**) Aβ fibre initial amplitude during the first sweep of the train of 40x stimuli at 1Hz, 2Hz and 10Hz was altered by chronic MG in a sex-dependent manner (Three-way ANOVA: frequency, ns p=0.999, chronic MG, ns p=0.264, sex, *** p=0.0005, sex x chronic MG * p=0.021) with chronic MG increasing the initial Aβ amplitude in males only (Two-way ANOVA of the data consolidated by frequency followed by Sidak's multiple comparisons test, * p=0.014). (**F**) Aβ fibre initial width during the first sweep of the train of 40x stimuli at 1Hz, 2Hz and 10Hz was altered by chronic MG in a sex-dependent manner (Three-way ANOVA: frequency, ns p=0.2069, chronic MG, * p=0.012, sex, ns p=0.8424, sex x chronic MG * p=0.049) with chronic MG decreasing the initial Aβ width in females only (Two-way ANOVA of the data consolidated by frequency followed by Sidak's multiple comparisons test, **p=0.004). All data presented as mean±SEM. Sample size in **A** and **B**: male vehicle (n=9), male MG (n=9), female vehicle (n=9), female MG (n=7). Sample size in **E** and **F**: male vehicle, 1Hz (n=8), 2Hz and 10Hz (n=5); male chronic MG, 1Hz, 2Hz and 10Hz (n=8); female vehicle, 1Hz, 2Hz and 10Hz (n=6); female chronic MG, 1Hz and 2Hz (n=6), 10Hz (n=4).

3.5.2.2 Chronic MG alters the progressive increase in Aβ fibre amplitude in a sex-dependent manner

To assess the effect of chronic MG treatment on Aβ response amplitude following repetitive stimulation in both sexes, the Aβ response amplitude was measured following a train of 40x stimuli at 1Hz, 2Hz and 10Hz. This resulted in a clear progressive increase in Aβ amplitude in males (**Figure 3.13A**) and to a lesser extent in females (**Figure 3.13B**), with vehicle-treated males displaying significantly more enhanced amplitude increase in comparison to the vehicle-treated females (**Figure 3.13E**; three-way ANOVA: sex x MG, p<0.0001 followed by Tukey's multiple comparisons test, vehicle males/females, p=0.0002 at 1Hz and p=0.044 at 10Hz). The progressive increase in Aβ amplitude was shown to be frequency-dependent, with higher frequencies resulting in greater amplitude change (**Figure 3.13E**; three-way ANOVA: frequency, *p=0.046). In addition to increasing the initial Aβ amplitude in males only (**Figure 3.12E**), chronic MG treatment also altered the progressive change in Aβ amplitude in a sex-dependent manner (**Figure 3.13E**; three-way ANOVA: sex x MG, p<0.0001), with chronic MG causing a pronounced enhancement of the Aβ amplitude increase in females only (**Figure 3.13E**; three-way ANOVA followed by Tukey's multiple comparisons test, female vehicle/MG, p=0.0002 at 1Hz and p<0.0001 at 2Hz and 10Hz). Interestingly, chronic MG treatment enhanced the progressive amplitude increase in females to levels comparable to those observed

in vehicle or chronic MG males (**Figure 3.13E**; three-way ANOVA followed by Tukey's multiple comparisons test, MG female/male ns, p=0.999 at 1Hz and 2Hz, p=0.752 at 10Hz; vehicle male vs MG female ns, p>0.999 at 1Hz, p=0.187 at 2Hz and p=0.069 at 10Hz).

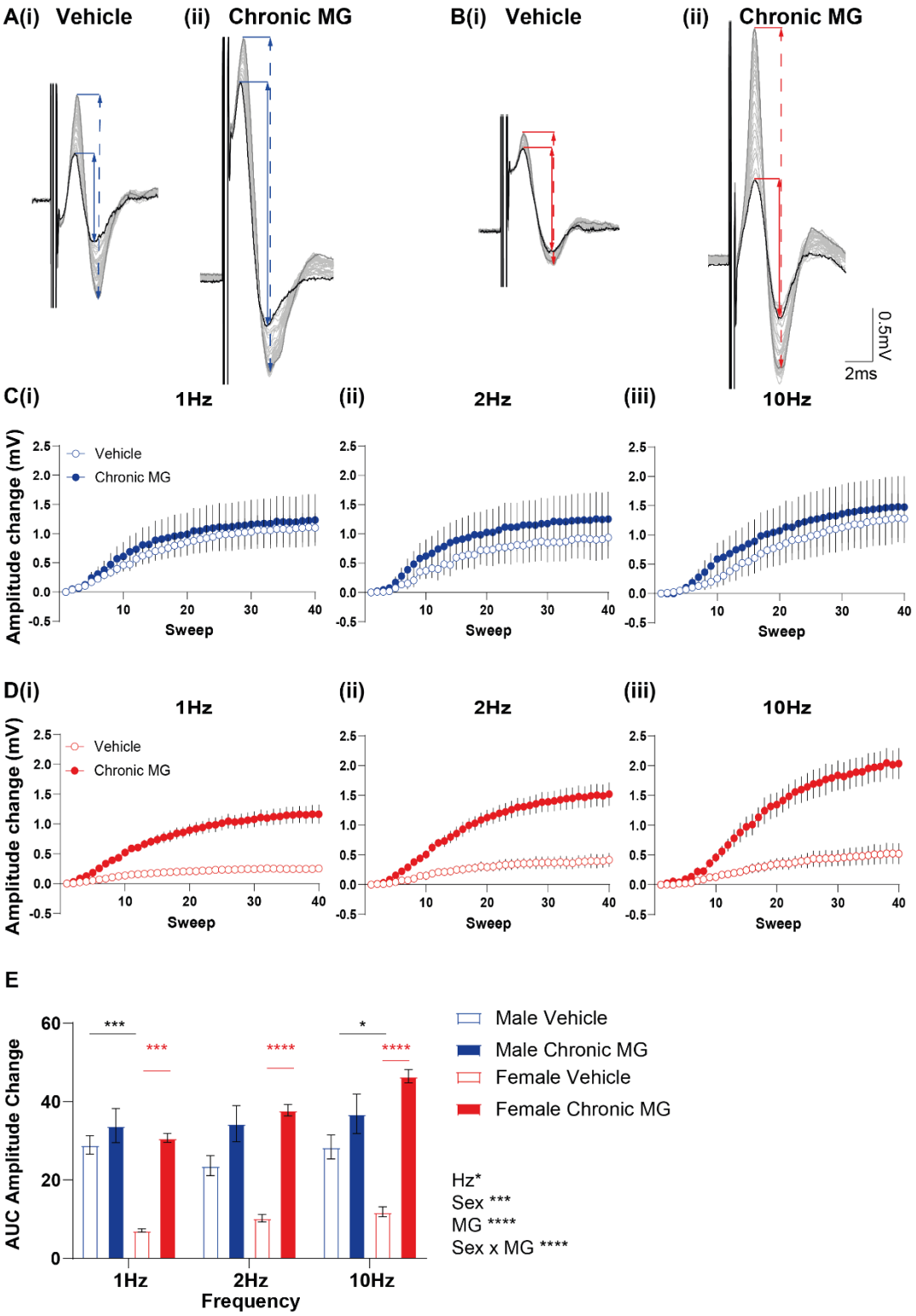


Figure 3.13 Chronic MG alters the progressive increase in Aβ fibre amplitude in a sex-dependent manner, increasing Aβ fibre amplitude change in females only

Representative Aβ fibre CAP recordings from vehicle- (**i**) and chronic MG-treated (**ii**) dorsal roots isolated from male (**A**) and female (**B**) rats in response to 40 stimuli at 2Hz

(response trace 1 black; 2-39 pale grey; 40 dark grey), illustrating the Aβ fibre amplitude of the first (solid double-headed arrows) and last (dashed double-headed arrows) responses. Repetitive stimulation results in a progressive Aβ fibre amplitude increase in vehicle- and chronic MG-treated dorsal roots from males (**C**) and females (**D**) at 1Hz (**i**), 2Hz (**ii**) and 10Hz (**iii**). **E**) AUC analysis reveals a significant effect of frequency (**p*=0.046), sex (****p*=0.005), chronic MG (*****p*<0.0001) with a sex x chronic MG interaction (*****p*<0.0001), with an enhancing effect of chronic MG on the progressive Aβ fibre amplitude increase in females only (Three-way ANOVA followed by Tukey's multiple comparisons test, ****p*=0.0002 at 1Hz and *****p*<0.0001 at 2Hz and 10Hz). The statistical analysis also showed a less pronounced progressive Aβ fibre amplitude increase in vehicle-treated dorsal roots from females when compared to males (Three-way ANOVA followed by Tukey's multiple comparisons test, ****p*=0.0002 at 1Hz and **p*=0.044 at 10Hz). Data presented as mean±SEM. Male: vehicle, 1Hz (n=8), 2Hz and 10Hz (n=5); chronic MG, 1Hz, 2Hz and 10Hz (n=8). Female: vehicle, 1Hz, 2Hz and 10Hz (n=6); chronic MG, 1Hz and 2Hz (n=6), 10Hz (n=4).

3.5.2.3 Chronic MG treatment alters activity-dependent Aβ fibre width change in a sex- and frequency-dependent manner

To assess whether the observed changes in Aβ response amplitude could be associated with the changes in the width of the Aβ response (the range of CVs within the population Aβ response) following repetitive stimulation, the Aβ response width was measured following a train of 40x stimuli at 1Hz, 2Hz and 10Hz. The repetitive stimulation caused a minimal change in the Aβ response width in males (**Figure 3.14A, C**), while there was more variability in the change in Aβ response width in females (**Figure 3.14B, D**). In addition to decreasing the initial Aβ width in females only (**Figure 3.12F**), chronic MG treatment affected Aβ width change in a sex- and frequency-dependent manner (**Figure 3.14E**; three-way ANOVA: frequency x sex, p=0.04, sex x chronic MG, *p*=0.004), with chronic MG causing an increase in Aβ width change at higher frequencies in females only (**Figure 3.14E**; three-way ANOVA followed by Tukey's multiple comparisons test, female vehicle/MG, *p*=0.011 at 2Hz and *p*=0.0007 at 10Hz).

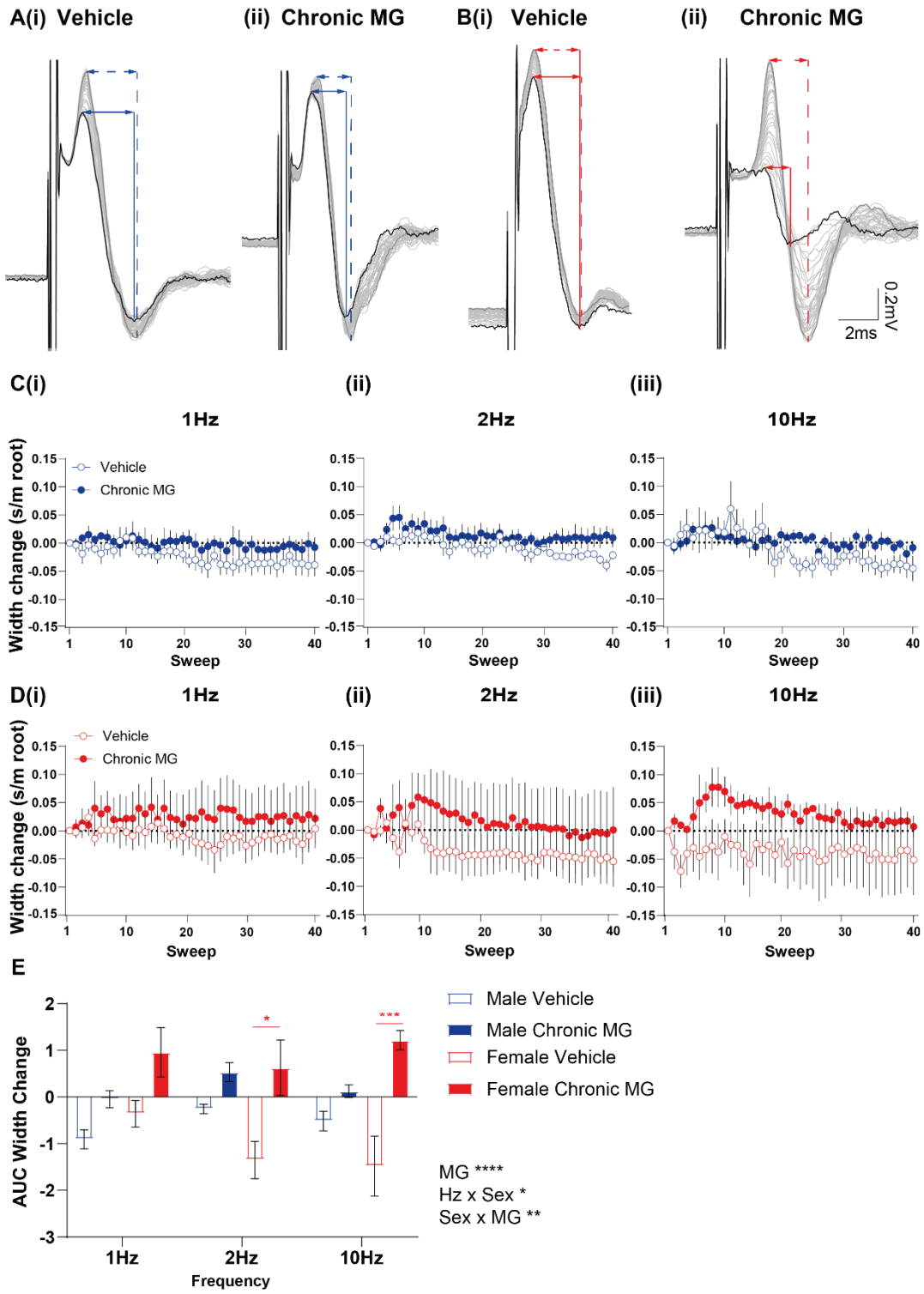


Figure 3.14 Chronic MG alters activity-dependent Aβ fibre width change in a sex- and frequency-dependent manner

Representative Aβ fibre CAP recordings from vehicle- (**i**) and chronic MG-treated (**ii**) dorsal roots isolated from male (**A**) and female (**B**) rats in response to 40 stimuli at 2Hz illustrating the Aβ fibre width, the distance from first positive to negative peaks for the first (solid double-headed arrows) and last (dashed double-headed arrows) of the 40x

responses (response trace 1 black; 2-39 pale grey; 40 dark grey). Repetitive stimulation resulted in a minimal change in the Aβ response width in both vehicle- and chronic MG-treated dorsal roots from males (**C**) at 1Hz (**i**), 2Hz (**ii**) and 10Hz (**iii**). Repetitive stimulation resulted in a small overall decrease in Aβ response width in vehicle-treated dorsal roots from females (**D**) at 1Hz (**i**), 2Hz (**ii**) and 10Hz (**iii**), with chronic MG causing a small increase in the overall width change of the Aβ response at 1Hz (**i**), 2Hz (**ii**) and 10Hz (**iii**). **E**) AUC analysis reveals a significant effect of chronic MG (****p<0.0001) with interaction between frequency x sex (*p=0.40) and sex x chronic MG (**p<0.004), revealing that chronic MG causes an increase in the progressive change in Aβ response width at higher frequencies in females only (Three-way ANOVA followed by Tukey's multiple comparisons test, *p=0.015 at 2Hz and ***p=0.0008 at 10Hz). Data presented as mean±SEM. Male: vehicle, 1Hz (n=8), 2Hz and 10Hz (n=5); chronic MG, 1Hz, 2Hz and 10Hz (n=8). Female: vehicle, 1Hz, 2Hz and 10Hz (n=6); chronic MG, 1Hz and 2Hz (n=6), 10Hz (n=4).

3.5.2.4 Activity-dependent progressive increase in Aβ fibre amplitude is negatively associated with the progressive change in width predominantly in males, but not females

To investigate whether the reported changes in Aβ response amplitude could be influenced by the observed changes in Aβ response width, the association between the two variables was investigated. The amplitude change was plotted against the associated width change for each stimulus in an individual root, which allowed the calculation of a best-fit slope on an individual root basis that showed the magnitude and the direction of the association between Aβ amplitude and width changes at each investigated frequency. Representative graphs for 2Hz are shown for vehicle- and chronic MG-treated males (**Figure 3.15Ai-ii**) and females (**Figure 3.15Bi-ii**), with similar analysis conducted for 1Hz and 10Hz (data not shown). To statistically access the association between the two variables, Spearman's correlation coefficient was calculated for each root at each frequency. There was a sex-dependent difference in the correlation coefficients showing the association between the two variables (**Figure 3.15C**; three-way ANOVA: sex, p=0.011), with significantly more negative correlations in males in comparison to females, suggesting that in males, but not females, the progressive increase in the Aβ amplitude change can be associated with a decrease in the Aβ response width.

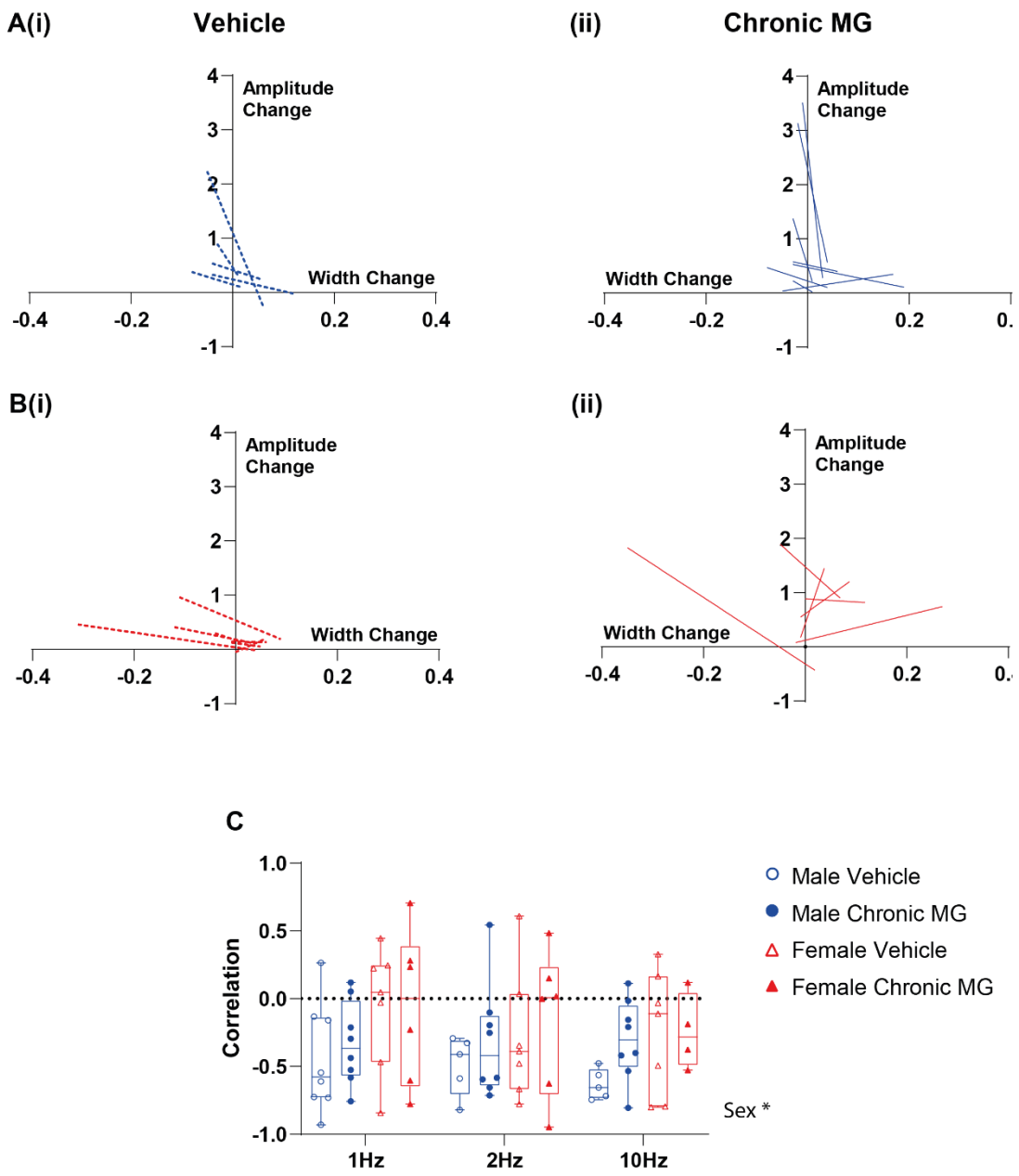


Figure 3.15 Activity-dependent Aβ fibre progressive amplitude increase is negatively associated with width change in most males, but not in females

Linear regression slopes representing the relationship between amplitude and width changes throughout the train of 40x stimuli at 2Hz for each individual root treated with vehicle (**i**) and chronic MG (**ii**) for males (**A**) and females (**B**). (**C**) A summary boxplot representation of the Spearman's correlation coefficients for vehicle- and chronic MG-treated roots from male and female animals at 1Hz, 2Hz and 10Hz, showing a significant effect of sex (*p=0.011) on the association between amplitude and width changes, with significantly more negative association between amplitude and width changes in males than in females (Three-way ANOVA: frequency, ns p=0.487, sex, *p=0.011, chronic MG, ns p=0.188). Male: vehicle, 1Hz (n=8), 2Hz and 10Hz (n=5); chronic MG, 1Hz, 2Hz and

10Hz (n=8). Female: vehicle, 1Hz, 2Hz and 10Hz (n=6); chronic MG, 1Hz and 2Hz (n=6), 10Hz (n=4).

3.5.2.5 Acute MG treatment does not mimic the changes in Aβ fibre properties observed with chronic MG.

To assess the effect of acute MG treatment on Aβ fibre function, 100µM and 1mM MG was bath applied for 10mins prior to and throughout the CAP recordings. The acute effect of MG on basic Aβ fibre properties, including initial amplitude, width, and average conduction velocity, was investigated for the first sweep in the train of 40x stimuli at 500µA. There was a significant sex- and dose-dependent effect of acute MG on Aβ fibre initial amplitude (**Figure 3.16A**; two-way ANOVA: interaction, p=0.006), with acute 1mM (but not 100µM) MG treatment decreasing the initial amplitude in males only (**Figure 3.16A**; two-way ANOVA followed by Sidak's multiple comparison test, *p=0.024). There was no significant effect of acute MG treatment and sex on Aβ fibre initial width (**Figure 3.16B**; two-way ANOVA: acute MG, ns p=0.150, sex, p=0.562, interaction, p=0.311) and average conduction velocity (**Figure 3.16C**; acute MG, ns p=0.117, sex, p=0.298, interaction, p=0.319).

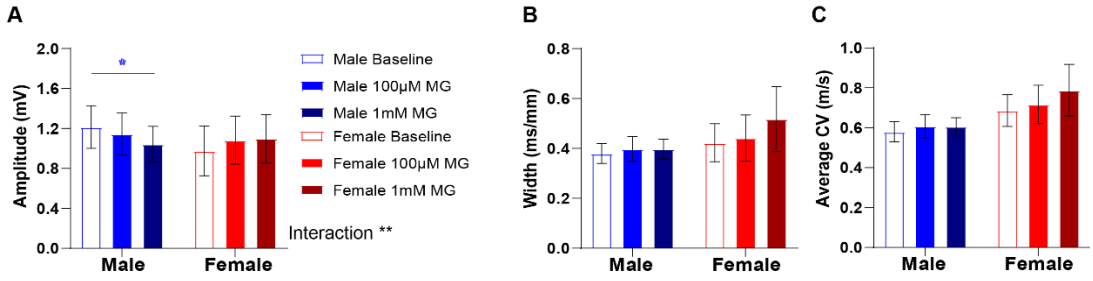


Figure 3.16 Acute MG at high (1mM) not low (100 µM) concentration decreases initial Aβ amplitude in males only, without altering Aβ fibres initial response width and average conduction velocity in both sexes

(**A**) Acute MG treatment altered initial amplitude of the Aβ fibre response in a sex-dependent manner (Two-way ANOVA: acute MG, ns p=0.453, sex, ns p=0.804, interaction, **p=0.006), with acute MG decreasing initial Aβ amplitude in comparison to vehicle at 1mM in males only (Two-way ANOVA followed by Sidak's multiple comparison test, *p=0.024). There was no significant effect of acute MG at different concentration and no significant effect of sex on the initial Aβ fibre response width (**B**, Two-way ANOVA: acute MG, ns p=0.150, sex, p=0.562, interaction, p=0.311) and average conduction velocity (**C**, Two-way ANOVA: acute MG, ns p=0.117, sex, p=0.298, interaction, p=0.319). Data presented as mean±SEM. Male, n=5, female, n= 6.

To assess the Aβ response amplitude change following repetitive stimulation after acute treatment with different MG concentrations, dorsal roots from vehicle-treated roots from naïve rats of both sexes were repetitively stimulated 40x at 2Hz to record baseline Aβ amplitude change. Following 10-min bath application of 100µM and 1mM MG, Aβ amplitude change was assessed again to investigate the acute effects of 100µM and 1mM MG, respectively. The repetitive stimulation at 2Hz resulted in a clear progressive increase in Aβ response amplitude change in both males (**Figure 3.17Ai, C**) and females (**Figure 3.17Bi, D**). Treatment with MG at 100µM and 1mM reduced the progressive increase in Aβ amplitude change in both sexes (**Figure 3.17E**; two-way ANOVA: acute MG, p=0.002), with females showing smaller increase in Aβ response amplitude independent of MG treatment (**Figure 3.17E**; two-way ANOVA: sex, p<0.0001).

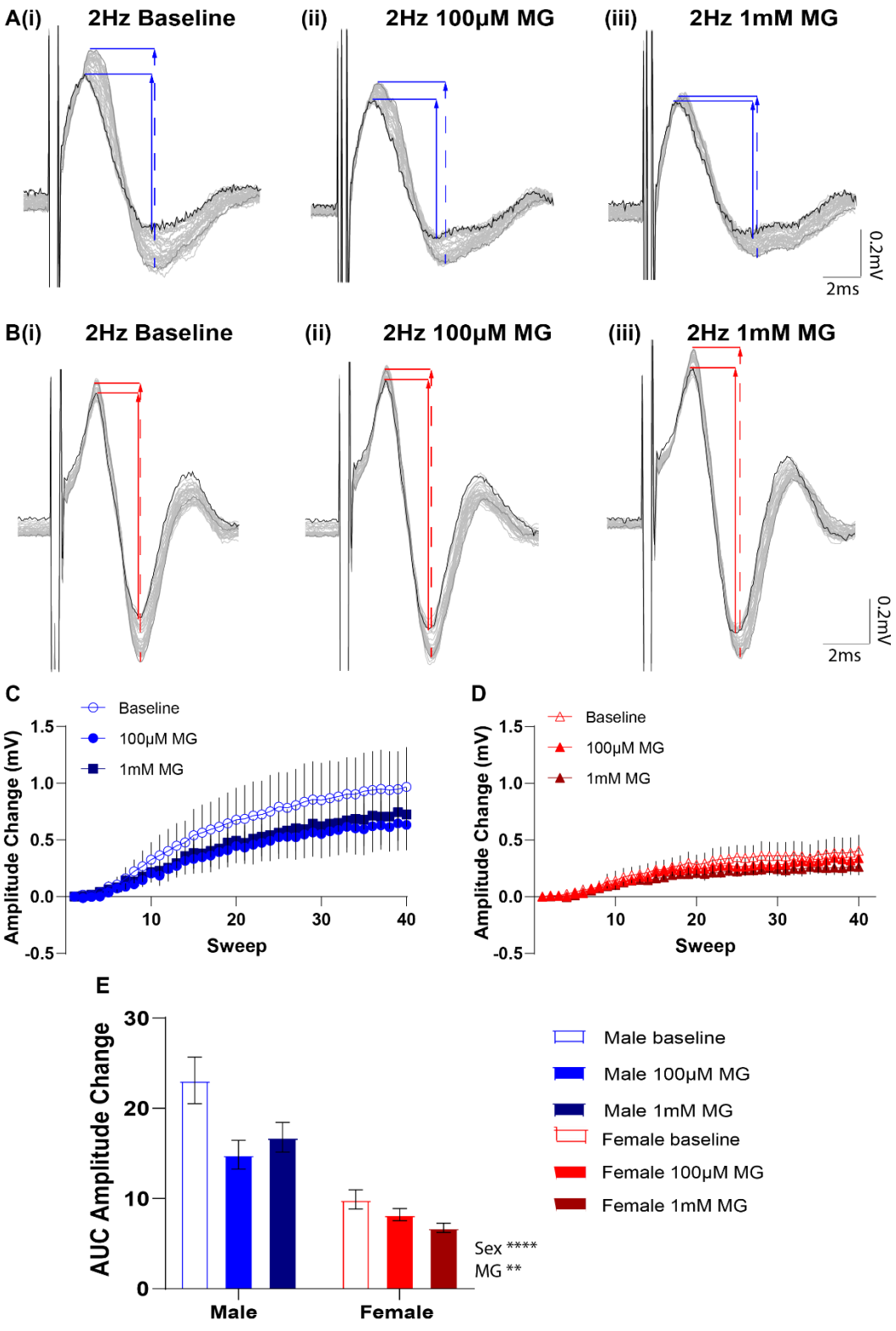


Figure 3.17 Acute MG treatment decreases the activity-dependent progressive increase in Aβ fibre amplitude in both sexes, with females displaying overall a smaller progressive increase in Aβ amplitude.

Representative Aβ fibre CAP recordings from dorsal roots from naïve male (**A**) and female (**B**) rats in response to 40x stimuli at 2Hz at baseline (**i**) and following 10min acute 100μM (**ii**) and 1mM MG (**iii**) application (response trace 1 black; 2-39 pale grey; 40 dark grey), illustrating the Aβ fibre amplitude of the first (solid double-headed arrows) and last (dashed double-headed arrows) responses. Repetitive stimulation results in a progressive Aβ fibre amplitude increase at baseline, with acute MG at both concentrations reducing the progressive increase in Aβ amplitude in dorsal roots from males (**C**) and females (**D**). (**E**) AUC analysis shows a significant effect of acute MG (***p*=0.002) and sex (*****p*<0.0001), with no significant interaction between the two factors (Two-way ANOVA: interaction, *p*=0.087) revealing that acute MG reduces the progressive increase in Aβ amplitude in both sexes, with females showing smaller change in Aβ amplitude compared to males. Data presented as mean±SEM. Male, n=5, female, n= 6.

3.5.3 Effect of systemic MG treatment on the nociceptive flexion-withdrawal reflex in juvenile rats of both sexes

To assess MG-induced behavioural hypersensitivity induced by systemic treatment with MG, the mechanical threshold of the flexion-withdrawal reflex to a punctate mechanical stimulus was measured at baseline and approximate 3hours after intraperitoneal injection with 5μg MG or vehicle in juvenile rats of both sexes (**Figure 3.18**). Analogously, the latency of the flexion-withdrawal reflex to a radiant noxious heat stimulus was also measured (**Figure 3.19**). The systemic administration of MG or vehicle resulted in no significant change in the withdrawal threshold of the flexion-withdrawal reflex to a punctate mechanical stimulus in comparison to baseline measurements prior to MG/vehicle administration in males (**Figure 3.18A**; Two-way ANOVA: vehicle/MG, *p*=0.682, pre/post-injection, *p*=0.334, interaction, *p*=0.514) and in females (**Figure 3.18B**; Two-way ANOVA: vehicle/MG, *p*=0.390, pre/post-injection, *p*=0.985, interaction, *p*=0.592). To compare the data of this experiment to a similar experiment conducted in male adult mice (Bierhaus et al., 2012), the mechanical withdrawal threshold data were also presented as a change from baseline threshold (**Figure 3.18C**). Similar to the results from the analysis conducted in **Figure 3.18A** and **Figure 3.18B**, the mechanical withdrawal change results in juvenile rats indicated no development of mechanical hypersensitivity 3hours after systemic treatment with MG in both males and females (**Figure 3.18C**; Two-way ANOVA: MG, *p*=0.273, sex, *p*=0.727, interaction, *p*=0.770).

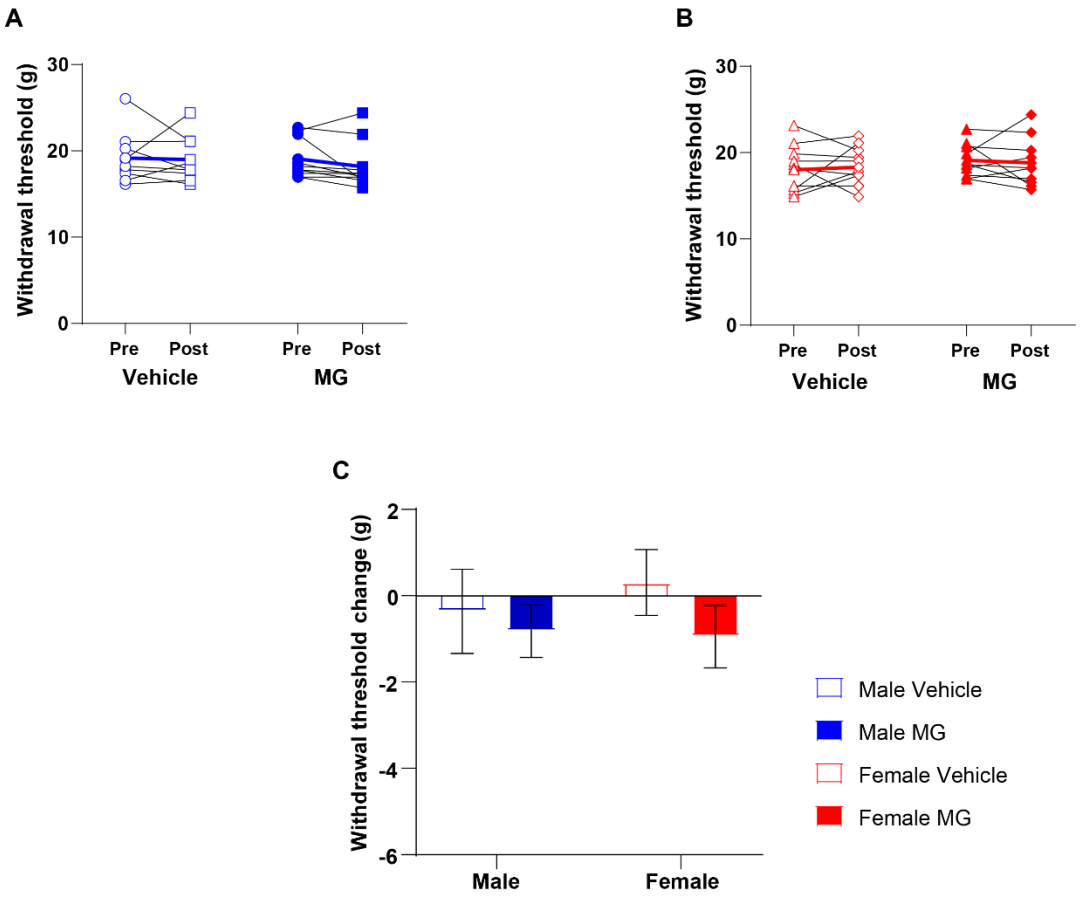


Figure 3.18 Systemic MG treatment does not alter mechanical sensitivity in rats of both sexes.

Hindpaw withdrawal threshold to a punctate mechanical stimulus (electronic von Frey) pre- and post i.p injections with vehicle or 5µg MG in male (**A**) and female (**B**) juvenile rats. Data are presented per individual animal with thicker blue and red lines representing the mean values for males and females, respectively. (**C**) Hindpaw withdrawal threshold to a punctate mechanical stimulus following MG or vehicle administration presented as a change from baseline threshold prior to injections reveals no significant differences in the mechanical withdrawal threshold following MG application when compared to vehicle in males and females (Two-way ANOVA: MG, p=0.273, sex, p=0.727, interaction, p=0.610). Data presented as mean±SEM. Male: vehicle (n=9), MG (n=10). Female: vehicle (n=11), MG (n=10).

Interestingly, the systemic administration of MG resulted in a decrease in the withdrawal latency to a noxious heat stimulus, revealing the development of noxious heat hypersensitivity 3 hour after i.p MG injections in males (**Figure 3.19A**;

Two-way ANOVA: vehicle/MG, *p*=0.064, pre/post-injection, *p*=0.019, interaction, *p*=0.0002 followed by Sidak's multiple comparisons test, pre- vs post-MG, p=0.002), but not in females (**Figure 3.19B**; Two-way ANOVA: vehicle/MG, *p*=0.987, pre/post-injection, *p*=0.367, interaction, *p*=0.191). To compare these data to the previous findings in adult male mice (Bierhaus et al., 2012), the noxious heat withdrawal latency data were also presented as a change from baseline latency (**Figure 3.19C**). Similar to the analysis in **Figure 3.19A** and **Figure 3.19B,** this revealed the development of noxious heat hypersensitivity only in males (**Figure 3.19C**; Two-way ANOVA: MG, *p*=0.048, sex, *p*=0.324, interaction, *p*=0.0002 followed by Sidak's multiple comparisons test, male vehicle vs male MG, *p*=0.0009, female vehicle vs female MG, *p*=0.625).

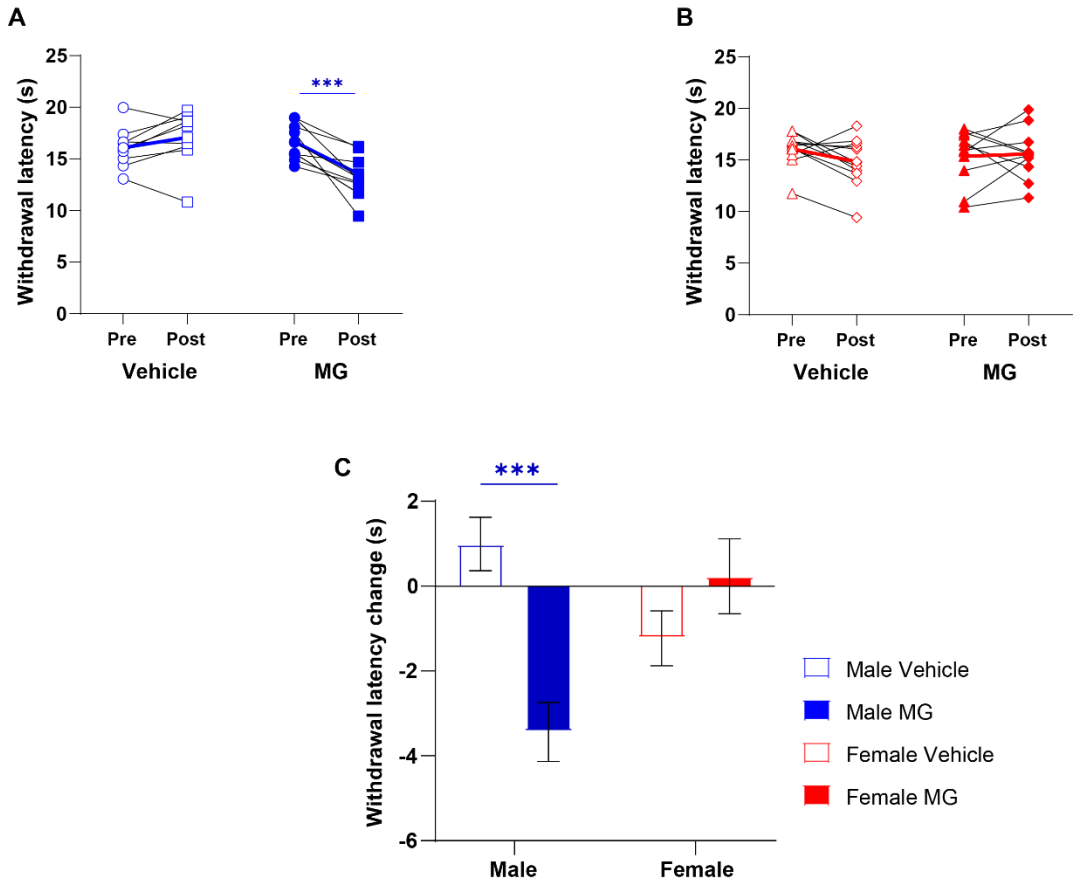


Figure 3.19 Systemic MG administration induces thermal hypersensitivity in male rats only.

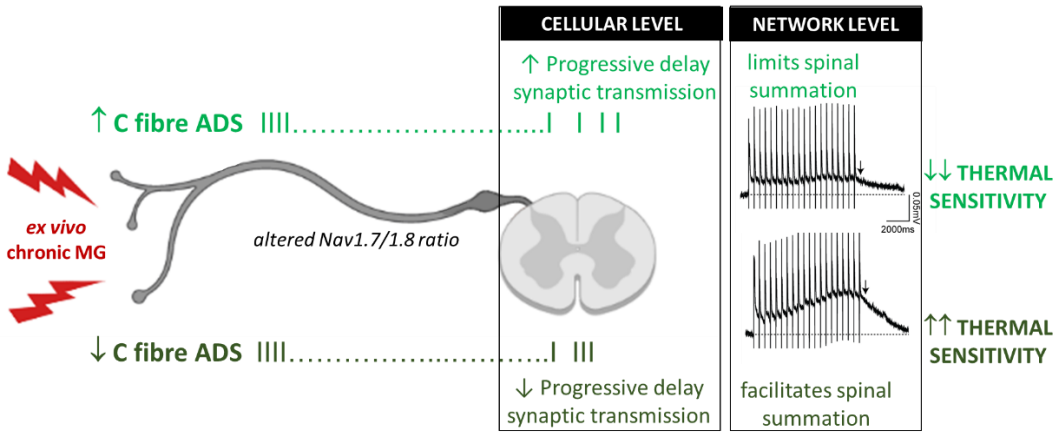
Hindpaw withdrawal latency to a radiant noxious heat stimulus (Hargreaves) pre- and post i.p injections with vehicle or 5μg MG in male (**A**) and female (**B**) juvenile rats. Data

are presented per individual animal with thicker blue and red lines representing the mean values for males and females, respectively. (**C**) Hindpaw withdrawal latency to a radiant noxious heat stimulus following MG or vehicle administration presented as a change from baseline latency prior to injections reveals a significant reduction in the withdrawal latency following MG when compared to vehicle in males but not in females (Two-way ANOVA: MG, *p=0.048, sex, p=0.324, interaction, ***p=0.0002 followed by followed by Sidak's multiple comparisons test, male vehicle vs male MG, ***p=0.0009, female vehicle vs female MG, p=0.625, male vehicle vs female vehicle, p=0.202, male MG vs female MG, **p=0.006). Data presented as mean±SEM. Male: vehicle (n=9), MG (n=10). Female: vehicle (n=11), MG (n=10).

3.6 Discussion

3.6.1 Summary findings

This chapter assessed the effect of the diabetic pain-associated metabolite methylglyoxal (MG) upon C-fibre ADS (**Figure 3.20**) as well as upon Aβ fibre function in male and female juvenile rats (**Table 3.1**). Chronic MG treatment did not affect the basic C-fibre properties but altered C-fibre ADS in a sex-dependent manner. Chronic MG reduced the progressive increase in C-fibre latency, without significantly altering C-fibre width change in males, while causing a pronounced enhancement of the progressive increase in C-fibre latency and width in females. In contrast, acute MG treatment did not cause significant alterations in C-fibre latency and width changes in both sexes. In the behavioural studies, consistent with the reduction in C-fibre ADS in males and the pronounced enhancement of C-fibre ADS in females, that are predicted to respectively enhance and limit heat hypersensitivity (Dickie et al., 2017), it was found that only males displayed heat hypersensitivity following i.p administration of MG *in vivo*. Thus, the experiments in this chapter proposed sex-dependent chronic MG-induced alterations in C-fibre ADS as a potential contributing mechanism to the behavioural hypersensitivity observed in the MG pain rat model.



	Basic C-fibre properties	Male ADS	Female ADS	Heat threshold	Mechanical threshold
Chronic MG	- (M=F)	↓	↑↑↑	↓ M only	M=F
Acute MG	- (M=F)	-	-		

Figure 3.20 Impact of MG on C-fibre ADS and on nociceptive behaviour in juvenile SD rats of both sexes

↑ marks an increase; ↓ marks a decrease; M=F denotes equivalent levels in males and females

Interestingly, alterations in Aβ fibre function were also observed following chronic treatment with MG, as summarised in **Table 3.1**. Chronic MG altered the initial Aβ fibre amplitude and width in a sex-dependent manner, causing an increase in the Aβ amplitude in males only and a decrease in the Aβ width in females only. In addition, chronic treatment altered Aβ fibre activity-dependent amplitude change in a sex-dependent manner, with chronic MG enhancing the limited activity-dependent amplitude change observed in vehicle-treated females to a level comparable with the more pronounced activity dependent amplitude change observed in vehicle- and MG-treated males. The acute MG treatment did not mimic the chronic MG-induced changes in Aβ fibre function.

Table 3.1 I Impact of repetitive stimulation upon Aβ fibre function in vehicle- and chronic MG-treated SD rats of both sexes

↑ marks an increase; ↓ marks a decrease; M=F denotes equivalent levels in males and females

	Initial Aβ amplitude	Initial Aβ width	Male activity-dependent amplitude change	Female activity-dependent amplitude change
Vehicle	-	-	↑↑↑	↑
Chronic MG	↑ in M only	↓ in F only	-	↑↑↑

3.6.2 Chronic MG alters C-fibre ADS in a sex-dependent manner

In line with previous studies investigating C-fibre function in juvenile SD rats of both sexes in a CFA-induced inflammatory pain model (Dickie et al., 2017) and in a plantar incision model of postoperative pain (Velichkova et al., 2021), the experiments in this chapter found no significant effect of sex or chronic MG treatment on C-fibre activation threshold, initial average conduction velocity and amplitude (**Figure 3.7**). Studies in males in the paclitaxel-induced neuropathic pain model (Galley et al., 2017) have further shown no effect of injury on the basic C-fibre properties. These findings suggest distinct alterations in C-fibre function in these models, which do not involve C-fibre basic properties. Interestingly those studies have reported injury-induced alteration in C-fibre ADS (Dickie et al., 2017, Galley et al., 2017, Velichkova et al., 2021), which were also sex-dependent (Dickie et al., 2017, Velichkova et al., 2021). Sex differences in C-fibre ADS have been reported in normal physiology, with females showing more pronounced progressive increase in C-fibre response latency (Dickie et al., 2017, Velichkova et al., 2021). In contrast to these findings, the *ex vivo* MG-induced rat pain model exhibited opposite results, with males having more pronounced baseline C-fibre latency change (**Figure 3.8**). Given the *ex vivo* nature of this experiment, it could potentially be the experimental conditions that can account for that difference in the amount of baseline ADS between the sexes. Normally, the dorsal root preparation involves

only 1h recovery incubation of the roots in NaCl solution. However, the chronic MG experiments required 3h-incubations of the dorsal roots in NaCl with added MG or vehicle (distilled water) solutions. Thus, the vehicle-treated dorsal roots in the MG experiment have been prepared under different conditions in comparison to the dorsal roots from the CFA inflammation (Dickie et al., 2017) or the incision postoperative pain (Velichkova et al., 2021) studies, which were conducted in similarly aged SD rats. These experimental condition differences could potentially account for the difference in the amount of baseline ADS in both sexes in the *ex vivo* MG study.

Other studies have also reported altered C-fibre ADS in different neuropathic pain models (Shim et al., 2007, Wang et al., 2016, Sun et al., 2012b, Hulse, 2016), including different models of diabetes (Wang et al., 2016, Sun et al., 2012b, Garcia-Perez et al., 2018) , which is in line with the findings of C-fibre ADS changes induced by chronic MG treatment. In the STZ-induced diabetes model in male SD rats, which have been confirmed to develop noxious hypersensitivity, C-fibre ADS has been shown to be reduced (Wang et al., 2016, Sun et al., 2012b), which is in line with the findings of reduced C-fibre latency change in males. However, studies that have used the Zucker Diabetic Fatty (ZDF) rat model, which develops spontaneous chronic hyperglycemia and insulin resistance, mimicking type 2 diabetes (Oltman et al., 2005, Noland et al., 2007), have reported enhanced C-fibre ADS (Garcia-Perez et al., 2018), which differs from the findings in males. Although the study in the ZDF model was conducted in males only, and it was confirmed that the animals develop mechanical hypersensitivity, their thermal sensitivity was not assessed. In addition, unlike the ZDF rat model, all the other studies, similarly to the experiments in this chapter, were conducted in SD rats. Interestingly, given that sex differences in noxious heat sensitivity have been shown to depend on animal species and strain (Mogil et al., 2000, Kest et al., 1999), the latter could influence C-fibre ADS profiles given that these have been shown to differ based on sex (Dickie et al., 2017).

It must be also noted that preclinical studies in different pain models have reported alterations in ADS in both direction, with injury-induced reduction (Dickie et al., 2017, Hulse, 2016, Werland et al., 2021, Galley et al., 2017) and injury-induced enhancement (Shim et al., 2007, Velichkova et al., 2021) of C-fibre ADS. In addition, enhanced ADS has also been reported in clinical studies using microneurography in chronic pain patients with erythromelalgia (Orstavik et al., 2003) and fibromyalgia (Serra et al., 2014). Studies of patients with erythromelalgia in association with Na_V channel mutations have also reported enhanced C-fibre ADS (Kist et al., 2016, Kleggetveit et al., 2016), suggesting that the enhanced ADS may be resulting from the mutation and acting as a negative feedback mechanism to limit pain in these patients (Kist et al., 2016). In line with this hypothesis, the chronic treatment with MG could similarly be causing changes in nociceptor profiles in both sexes, but the MG-induced enhancement of C-fibre ADS in females could potentially be acting as a negative feedback mechanism to counteract high firing or longer lasting chronic MG-induced effects. This is consistent with the observation of a pronounced enhancement of C-fibre ADS in combination with no MG-induced noxious heat hypersensitivity in females. Interestingly, early nerve conduction velocity studies, used for diagnosing neuropathy, conducted in patients with mild diabetic neuropathy have demonstrated sex-dependent electrophysiological differences, with women demonstrating better performance for most individual nerve conduction and summary measures (Albers et al., 1996). Given that similar studies in healthy subject have demonstrated no sex differences in nerve conduction measures (Stetson et al., 1992), the sex differences observed in Albers et al. (1996)'s studies are likely due to the early stages of diabetes. These finding suggest that men likely present with neuropathic symptoms early on in the onset of diabetes, earlier than women, which is consistent with the findings of MG-induced enhancement of C-fibre ADS in females, which might have a protective role, and is also in line with the behaviour findings of heat hypersensitivity 3h after *in vivo* MG administration in males but not females.

In addition, this study found that it is the chronic rather than the acute action of MG that can alter C-fibre ADS. These findings are in line with previous studies showing that the same MG treatment as ours (100µM MG for 3h), which has been shown to induce similar intracellular changes and produce similar effects in DRG neurons as lower MG concentrations over longer incubation periods (Eberhardt et al., 2012), can alter $Na_V1.7$ kinetics, promoting its slow inactivation, and induce post-translational modifications in $Na_V1.8$, shifting its steady state inactivation to more depolarised potentials (Bierhaus et al., 2012). Given that $Na_V1.7$ and $Na_V1.8$ are both implicated in C-fibre ADS, with the ratio of $Na_V1.7$ to $Na_V1.8$ determining the extent of C-fibre ADS (Petersson et al., 2014), as also demonstrated by the TTX-induced alterations in C-fibre ADS in a sex-dependent manner pain (Velichkova et al., 2021), it is likely that the chronic MG-induced sex-dependent alterations in C-fibre ADS could be the result of the chronic MG effects on Nav1.7 and/or 1.8. The role of Nav in the MG pain model is further supported by studies reporting MG-induced alterations in Nav1.8 that likely contribute to the observed increased pain sensitivity in diabetic mice (Bierhaus et al., 2012), with further studies in a similar rat model showing that $Na_V1.8$ antagonists can block local MG-induced spontaneous pain (Huang et al., 2016). In addition, the behaviour findings in this chapter further support the chronic nature of the effects of MG on C-fibre ADS, because the timing of the proposed MG-induced $Na_V1.7$ and/or Na_V 1.8 modifications (Bierhaus et al., 2012) parallels the timing of the observed changes in thermal hypersensitivity in the present study.

The acute effects of physiological or higher concentrations of MG on C-fibre ADS were also investigated because previous studies have reported functional expression of TRPA1 ion channels along the axon (Eberhardt et al., 2012). Although there is no evidence so far for involvement of TRPA1 channels in the C-fibre ADS phenomenon, previous studies have reported that higher MG concentrations (1mM-10mM) can affect the TRPA1 ion channels (Eberhardt et al., 2012, Andersson et al., 2013, Ohkawara et al., 2012), which have been suggested to also be implicated in the MG-induced hypersensitivity in rodents (Andersson et al., 2013,

Huang et al., 2016). However, this chapter found no significant effect of acute MG at both 100µM and 1mM on C-fibre ADS in both sexes. This suggests that the observed chronic MG-induced effects on C-fibre ADS are likely not due to action of MG on TRPA1. Given that previous studies have used even higher concentration, much higher than the reported plasma levels of MG in diabetic patients (~225nM and ~600nM) (Beisswenger et al., 2001, Bierhaus et al., 2012), this lack of effect on C-fibre ADS could potentially be because the applied concentrations were not high enough to activate TRPA1 channels. However, this experiment aimed to investigate the acute MG effects on C-fibre ADS at more physiologically relevant concentrations to assess if TRPA1 could be ruled out as a mediator of the chronic MG-induced effects at 100µM. Thus, the unaltered C-fibre ADS following the acute MG application at both concentration can be explained by the insufficient time during acute MG treatment for the induction of posttranslational modifications in Na_V channels, implicated in C-fibre ADS (Petersson et al., 2014), that were, however, shown to occur following 3h incubations with 100µM MG (Bierhaus et al., 2012). This lack of effect of acute MG on C-fibre ADS is not surprising because to my knowledge there are currently no reports of acute effects of MG on Na_V channels.

An alternative mechanism via which chronic MG can induce pain hypersensitivity is suggested to involve the MG-induced formation of advanced glycation end products (AGEs), causing oxidative stress (Vincent et al., 2007, Jack et al., 2012, Russell et al., 1999) and ultimately resulting in mitochondrial damage, which has also been implicated in the pathology of DN (Sivitz and Yorek, 2010, Chowdhury et al., 2013) and other painful neuropathies, including chemotherapy-induced painful neuropathy (CIPN) and HIV treatment-related painful neuropathy (Flatters and Bennett, 2006, Lehmann et al., 2011, Bennett et al., 2014). MG can also cause AGE-induced glycation of mitochondrial proteins and thus directly alter mitochondrial essential processes (Morcos et al., 2008, Rosca et al., 2005, Brouwers et al., 2011). Similarly to the findings of MG-induced alterations in C-fibre ADS, studies in a CIPN rat model, the paclitaxel-induced painful peripheral neuropathy model, which has been shown to develop mechanical hyposensitivity, have reported a reduction in C-

fibre ADS in male SD rats (Galley et al., 2017). Interestingly, the antioxidant melatonin, acting preferentially within mitochondria (Lowes et al., 2011, Reiter et al., 2016), has been shown to be able to limit the paclitaxel-induced reduction in C-fibre ADS and limit the development of mechanical hypersensitivity in this model (Galley et al., 2017). These findings suggest that the mitochondrial dysfunction associated with oxidative stress in peripheral nerves can be another potential mechanism involved in the MG-induced hypersensitivity model in rats.

3.6.3 Chronic MG effects on Aβ fibre function in a sex-dependent manner

3.6.3.1 Activity-dependent changes in Aβ fibre amplitude

Early nerve conduction studies in healthy subjects have demonstrated differences in sensory nerve compound action potential amplitude and conduction velocity which were initially attribute to sex, but after accounting for anatomical factors, such as height and dominant finger circumference, sex was no longer found to be a significant predictor of sensory nerve conduction measures (Stetson et al., 1992, Bolton and Carter, 1980, Soudmand et al., 1982). Although these studies reported variations in human sensory nerve compound action potential amplitude with age, skin temperature, sex and height, the techniques used did not allow for differentiation of different afferent fibre types, including Aβ fibres, and the experimental protocols did not involve repetitive stimulation. Thus, to my knowledge, there are no reports of activity-dependent changes in Aβ fibres response amplitude. There is limited preclinical evidence of activity-dependent changes in Aβ fibre conduction velocity. Early *in vivo* single unit recordings from dorsal roots have demonstrated that at stimulation frequencies ≤25Hz there is limited ADS, which has been shown increases in a frequency-dependent manner at frequencies ≥50Hz (Shin et al., 1997). More recent studies using population CAP recordings in rat dorsal roots have shown that repetitive stimulation at 2Hz causes a negligible reduction (speeding) in the latency of the Aβ fibre response (Dickie et al., 2017). However, the focus of these studies has not been investigation of Aβ fibre

amplitude. The demonstrated in this chapter findings of a clear sex-dependent progressive increase in Aβ fibre amplitude following repetitive stimulation, significantly more pronounced in males, have not been reported before and the mechanisms underlying this phenomenon are currently unknown.

3.6.3.1.1 Potential mechanism underlying activity-dependent changes in Aβ fibre amplitude

Previous studies have reported progressive changes in amplitude for the C-fibres as a result of C-fibre ADS (Hoffmann et al., 2016). Given that different C-fibre subtypes display various degrees of slowing (Weidner et al., 1999, Obreja et al., 2010), the repetitive stimulation has been shown to increase the range of conduction velocities within the population response, i.e. the width of the C-fibre response (Serra et al., 1999). As the response width increases, the number of fibres with average conduction velocity (negative peak of the response) will decrease and so will the amplitude of the response (measured as the distance from negative to positive peaks). Given that repetitive stimulation has been shown to cause progressive change in Aβ fibre latency (Dickie et al., 2017), the progressive increase in the Aβ fibre amplitude could have been due to the decrease in the Aβ response width, i.e. the decrease in the range of Aβ fibre conduction velocities within the population Aβ response. This hypothesis was investigated by calculating the association between width and amplitude changes in the Aβ fibres (**Figure 3.15**). Given that many roots from male animals showed a significant negative correlation between the two variables it is likely that the progressive decrease in the range of Aβ fibre conduction velocities within the population Aβ response can potentially explain the progressive increase in the Aβ response amplitude in males. However, the fact that some roots showed positive or no association between the two variables suggests that an alternative mechanism might be also involved, which is likely to play a more important role in females as roots from females were less likely to display negative association between the two variables.

Given that the amplitude of CAP recordings indicates the number of fibres contributing to the response, the progressive change in Aβ response amplitude

reported here could be the result of changes in the number of recruited fibres. However, it is highly unlikely that this is the explanation for the progressive change in Aβ amplitude because the Aβ fibres have been established primarily as sensitive to weak, innocuous stimuli and having low activation thresholds (Abraira et al., 2017, Handler and Ginty, 2021). In addition, as demonstrated in **Figure 2.9**, in previous studies from the lab (Dickie et al., 2017, Torsney, 2011) and from findings from others (Nakatsuka et al., 2000) using similarly aged SD rats, maximal Aβ response amplitude is reached ~20μA. Considering that the studies in this chapter applied trains of stimuli at stimulation intensities producing maximal C-fibre response, i.e. 500μA, which is well above the suggested intensity for maximal Aβ fibre response, it does not seem feasible that additional Aβ fibres could be recruited at this stimulation intensity. This is because when a stimulation intensity that produces a maximal response amplitude is reached, all fibres have been recruited and further increase in the stimulation intensity, i.e. using supramaximal stimulus, does not produce an increase in the response amplitude. However, given the evidence of myelinated peripheral afferents that have conduction velocities within the Aβ fibre ranges and respond to higher threshold noxious stimuli (Burgess and Perl, 1967, McIlwrath et al., 2007, Woodbury and Koerber, 2003, Djouhri and Lawson, 2004), it is possible that the supramaximal stimulus used in these studies was able to recruited these 'myelinated nociceptors', which have not been studied extensively (Abraira and Ginty, 2013, Lawson et al., 2019). Nevertheless, it would be critical that future studies investigate the Aβ fibre response amplitude following repetitive stimulation using CAP recordings conducted at maximal Aβ fibre stimulation intensities, i.e. at ~20μA. These studies will be essential to confirm whether non-nociceptive low-threshold Aβ fibres exhibit activity-dependent amplitude change at maximal non-nociceptive Aβ fibre stimuli.

Alternative mechanism underlying the progressive change in Aβ amplitude might involve changes in the number of action potentials fired per given stimulus. However, it is unlikely that the progressive increase in the Aβ amplitude is due to an increased number of action potentials per fibre per stimulus because the absolute

refractory period for myelinated rat afferent fibres has been shown to be ~2ms (Segura et al., 2001), which is longer that the pulse width used in these studies (0.1ms). Therefore, each fibre would have been able to fire only once following a stimulus. A possible explanation for the observed progressive increase in Aβ amplitude could involve the Na_V channels, which play a crucial role in action potential initiation and propagation along the axon as they drive the depolarising upstroke of the action potential (**Figure 1.5**) (von Hehn et al., 2012, Catterall et al., 2005). They have also been implicated in other activity-dependent phenomena such as C-fibre ADS (Tigerholm et al., 2015, Tigerholm et al., 2014b, Petersson et al., 2014, Obreja et al., 2012). Activity-induced changes in Na_V kinetics, involving the different gating Na_V states, could allow for altered Na^+ flow, which could potentially affect action potential amplitude. To study the exact mechanisms of the activity-dependent changes in Aβ amplitude, intracellular recordings can be conducted at maximal Aβ fibre stimulation intensities to investigate changes on an individual Aβ fibre level, where changes in action potential shape could give insight of potential changes in Na^+ flow and Na_V gating states.

3.6.3.1.2 Sex differences in the activity-dependent changes in Aβ fibre amplitude

The fact that the progressive change in Aβ amplitude was observed in both sexes but to different degrees, suggest that there are potential sex differences in the mechanisms underlying the phenomenon. Since the mostly likely explanation for this phenomenon involves the Na_V channels, it is possible that the sex differences in Aβ amplitude change reflect differences in Na_V expression profiles along the Aβ fibres that depend on sex. This hypothesis is supported by recent finding showing sex differences in gene expression profiles in peripheral afferents (Ray et al., 2019) and sensory DRG neurons (Mecklenburg et al., 2020). Given that Aβ fibres are principally characterised by expression of $Na_V1.1$, $Na_V1.6$ and $Na_V1.7$ (Black et al., 1996, Fukuoka and Noguchi, 2011), reported in both males (Fukuoka et al., 2008, Fukuoka and Noguchi, 2011) and females (Black et al., 1996), with potential $Na_V1.8$ expression in nociceptive Aβ fibres (Shields et al., 2012, Djouhri and Lawson, 2004,

Djouhri et al., 2003), it would be important to investigate the expression and function of those channels in Aβ fibres in both sexes. To study the protein expression of those channels in Aβ fibres in both sexes immunohistochemical techniques could be applied in peripheral afferents from both sexes, where the Aβ fibres could be differentiated by staining with NF200, a markers of myelinated A fibres. Specific anti- $Na_V1.1$-, $Na_V1.6$ and $Na_V1.7$ antibodies can be utilised to explore the protein expression of those Na_V channels along the axons of Aβ fibres in both sexes. The functional contribution of those channels to the activity-dependent changes in Aβ amplitude can be assessed using CAP recordings where selective $Na_V1.1$, $Na_V1.6$ and $Na_V1.7$ inhibitors, such as ICA 121431 (Osteen et al., 2017), MV1312/4,9-Anhydrotetrodotoxin (Weuring et al., 2020, Rosker et al., 2007), and ProTX II/PF 05089771 (Schmalhofer et al., 2008, Alexandrou et al., 2016), respectively, can be applied and their effect on the Aβ component can be studied.

3.6.3.2 Effect of chronic MG on Aβ fibre function

3.6.3.2.1 Effects of MG on Aβ fibre basic properties

The reported MG-induced alterations on Aβ fibre properties are in line with sensory nerve conduction studies, showing that patients with mild or early diabetic neuropathy display alterations in sensory nerve action potential amplitude (Juan et al., 2008, Zhang et al., 2014a, Albers et al., 1996), with more pronounced changes in amplitude with increased diabetes duration (Bril, 2015). However, these studies in patients have reported decrease in the sensory nerve CAP amplitude and conduction velocity, which is in contrast to the findings in Aβ fibres specifically of MG-induced increase in initial Aβ fibre CAP amplitude in males and no MG-induced alterations in Aβ fibre average conduction velocity. It must be noted that these studies have employed different experimental techniques that did not allow for differentiation of specific afferent fibre types, including Aβ fibres. In addition, although patients of both sexes were included, sex was not accounted for as a factor. Similar nerve conduction studies, where sex was accounted for as a factor, have shown that there are sex differences in sensory nerve CAP measures, with women demonstrating better performance, showing less diabetes-induced

reduction in sensory nerve CAP amplitude and conduction velocity (Albers et al., 1996). However, it must be noted that the experiments in this chapter were conducted in an ex vivo preparation over 3h that mimicked only one aspect of the complex diabetes pathophysiology and thus may not reflect the full multi-factorial diabetes patients profile.

3.6.3.2.2 Effects of MG on Aβ fibre activity-dependent changes

This chapter also reported activity-dependent changes in Aβ fibre amplitude, which to my knowledge have not been reported before, that were altered following chronic treatment with MG in a sex-dependent manner, with chronic MG-induced increase in Aβ fibre amplitude change in females only. Given that treatment with acute MG did not mimic the changes observed with chronic MG, it can be suggested that the mechanisms underlying the sex differences in MG-induced alterations in the activity-dependent changes in Aβ fibre amplitude involve potential chronic rather than acute actions of MG on ion channels in Aβ fibres. Previous studies using the same concentrations of MG for 3h to investigate the chronic effects of MG on Na_V have demonstrated that chronic MG promotes slow inactivation of $Na_V1.7$ as well as posttranslational modifications of $Na_V1.8$ (Bierhaus et al., 2012). Given that $Na_V1.7$ is expressed on Aβ fibres (Black et al., 1996, Fukuoka et al., 2008, Fukuoka and Noguchi, 2011), with potential $Na_V1.8$ expression on nociceptive Aβ fibres (Shields et al., 2012, Djouhri and Lawson, 2004, Djouhri et al., 2003), and both $Na_V1.7$ and $Na_V1.8$ are implicated in activity-dependent changes in C-fibres, it can be proposed that MG-induced alterations in $Na_V1.7$ and/or $Na_V1.8$ might be involved in the MG-induced activity-dependent changes in Aβ fibres.

Given that MG-induced oxidative stress and mitochondrial dysfunction have also been suggested to be involved in the pathology of diabetic neuropathy (Sivitz and Yorek, 2010, Chowdhury et al., 2013), these could also play a role in the MG-induced alterations in the activity-dependent changes in Aβ fibre amplitude. Studies have shown that diabetes-induced mitochondrial damage can trigger a cascade of signalling pathways leading to damage and death the Schwann cells, which wrap

around neuronal axons to form the myelin sheath (Dewanjee et al., 2018). However, studies investigating the effect of physiologically relevant concentrations of MG on myelinating glial cells junctions in the PNS and CNS have demonstrate that overall myelin structures are preserved after exposure to MG but 6h incubations with 100µM or 10mM MG alter paranodal axoglial junctions in the PNS and CNS (Griggs et al., 2018). This MG-induced paranodal disruption has been suggested to lead to impaired action potential propagation along the myelinated axons. Indeed, there is *in vivo* evidence of MG-induced nerve conduction deficits in mice (Bierhaus et al., 2012, Shimatani et al., 2015). However, given that the 6h incubations with 100µM or 10mM MG did not alter the overall myelin structures and only partially affected paranodal axoglial protein clusters, it is unlikely that the 3h incubations with 100µM MG used in the studies in this chapter were enough to induce potential paranodal disruptions that can explain the observations of MG-induced alterations in Aβ fibre amplitude change.

3.6.3.2.3 Sex differences

The observed sex differences in the effects of chronic MG on initial Aβ amplitude, where chronic MG treatment caused a significant change in the initial Aβ amplitude in males, are in support of early findings in mild diabetic neuropathy patients where men showed significantly more pronounced alterations in sensory nerve CAP amplitude, conduction velocity and latency (Albers et al., 1996). However, unlike the findings of the Albers et al. (1996) where men with mild diabetic neuropathy showed lower amplitude and slower conduction velocity than women, which is suggestive of more pronounced degree of peripheral neuropathy in men, the findings in this chapter revealed that chronic MG caused a significant increase in the initial Aβ amplitude in males, while it did not affect the initial amplitude in females. The differences in the direction of amplitude change in the males could be due to the different techniques used, with Albers et al. (1996) conducting sensory nerve CAP measure where there is no differentiation between separate afferent fibre types, including Aβ fibres. Also, the studies in this chapter utilised an *ex vivo* MG-induced pain rat model, which cannot mimic the complex multi-level diabetes-

induced alterations in patients. More importantly, however, the observed MG-induced changes in initial Aβ amplitude in males only, that were in line with patients studies, suggest that the MG-induced pain model can help investigate the mechanisms involved in the diabetes-induced alteration in primary afferents function in both sexes.

This study also reported sex differences in the MG-induced alterations in the progressive Aβ amplitude increase, with a pronounced enhancement of Aβ amplitude change following chronic MG treatment in females, but with no significant Aβ amplitude increase in males. These findings suggest that there are sex differences in the effects of MG on the initial Aβ amplitude and on the mechanisms underlying the activity dependent Aβ amplitude change. Given that MG increased the initial Aβ amplitude in males without significantly altering the activity-dependent Aβ amplitude changes, it is possible that, similarly to inflammatory mediators shown to alter the axonal transport of $Na_V1.7$ (Akin et al., 2019a), MG can potentially also alter the axonal transport of Aβ fibre-specific Na_V, such as $Na_V1.1$, $Na_V1.6$ and $Na_V1.7$ (Black et al., 1996, Fukuoka and Noguchi, 2011), and potentially $Na_V1.8$, expressed in nociceptive Aβ fibres (Shields et al., 2012, Djouhri and Lawson, 2004, Djouhri et al., 2003) and thus affect the amplitude of the Aβ fibres. Interestingly, the correlation analysis of the association between the progressive increase in Aβ amplitude and the progressive change in Aβ width revealed that in males there is a significant negative association between the two variables in both control and MG-treated roots (**Figure 3.15**), suggesting that the progressive increase in Aβ amplitude can potentially result from the progressive decrease in the Aβ fibre width in most males and MG did not alter that association. In contrast to males, MG did not significantly alter the initial Aβ amplitude in females. This suggests that it is likely that alterations in Aβ fibre Na_V axonal transport are involved in the MG-induced changes in the activity–dependent Aβ amplitude change in females. Considering the non-significant association between the progressive increase in Aβ amplitude and the progressive change in Aβ width in both control and MG-treated roots from female roots, the MG-induced increase in

the progressive change in Aβ amplitude cannot be explained by MG-induced alterations in the width of the Aβ response following repetitive stimulation. Given the mostly likely explanation for the activity dependent Aβ amplitude change involves the Na_V channels, it is likely that MG induces changes in the Na_V expressed on Aβ fibres. Indeed, there are reports of MG-induced changes causing $Na_V1.7$ slow inactivation and post translation modifications in $Na_V1.8$, which have been reported to be expressed on Aβ fibres (Black et al., 1996, Fukuoka and Noguchi, 2011), with potential $Na_V1.8$ expression in nociceptive Aβ fibres (Shields et al., 2012, Djouhri and Lawson, 2004, Djouhri et al., 2003).

To study the sex-differences in the MG-induced alterations in initial Aβ fibre amplitude and in the activity-dependent Aβ amplitude increase in will be crucial to first confirm whether there are sex differences in Aβ fibre response amplitude following repetitive stimulation using CAP recordings conducted at maximal Aβ fibre stimulation intensities, i.e. at ~20μA. If the sex differences in the MG-induced alterations in Aβ fibre amplitude are confirmed it would be important to next investigate potential MG-induced changes in the expression of $Na_V1.1$, $Na_V1.6$, $Na_V1.7$ (Black et al., 1996, Fukuoka and Noguchi, 2011), and potentially $Na_V1.8$ (Shields et al., 2012, Djouhri and Lawson, 2004, Djouhri et al., 2003) as described in **section 3.6.3.1.2**. Given that these experiments will be conducted in an *ex vivo* MG-induced pain model, it would be important to investigate whether the results will be similar in an *in vivo* MG-induced pain model and/or in an *in vivo* diabetic model, such as the STZ-induced diabetic model. These *in vivo* models can be used to isolate peripheral nerves for both immunohistochemical and electrophysiological experiments.

3.6.4 Sex differences in C-fibre ADS and MG-induced pain sensitivity

This study reported chronic MG-induced reduction in C-fibre ADS in males and a pronounced chronic MG-induced enhancement of C-fibre ADS in females. Previous

studies have demonstrate that C-fibre ADS can influence spinal summation, with pronounced C-fibre ADS limiting spinal summation, which was consistent with the pronounced ADS profile in females, which showed higher noxious heat, but not mechanical, thresholds than males (Dickie et al., 2017). Thus, it was hypothesised that the chronic MG-induced reduction of C-fibre ADS in males and enhancement of C-fibre ADS in females will respectively enhance and limit spinal summation and consequently enhance heat hypersensitivity in male and reduce heat hypersensitivity in females, as was observed behaviourally, with *in vivo* MG administration causing heat hypersensitivity in males but not in females (**Figure 3.19**).

In line with the behavioural data in this thesis chapter, preclinical studies have reported that systemic administration of 5µg MG incudes heat hypersensitivity ~3h after the i.p injections in adult male mice (Bierhaus et al., 2012). The Bierhaus et al. (2012) study also reported that inhibition of the MG detoxifying enzyme GLO1 increased the plasma MG levels and when those reached comparable levels to the plasma MG levels induced by the 5µg MG i.p injections, this caused a pronounced heat hypersensitivity in male mice whose GLO1 was inhibited. In addition, Bierhaus et al. (2012) also reported similar levels of heat hypersensitivity in STZ-induced diabetic WT male mice and nondiabetic GLO1 knockdown male mice. Additional studies have also reported that STZ-induced diabetes in male Wister rats leads to an increase in plasma levels of MG and to the development of heat hypersensitivity (Huang et al., 2016). These findings support the reported in this chapter MG-induced thermal hypersensitivity in juvenile male SD rats. However, to my knowledge, there have been no preclinical reports so far of the effects of MG on behavioural hypersensitivity in females. Thus, the presented findings of failure of systemic MG administration to induce heat hypersensitivity in females are novel. The lack of MG-induced heat hypersensitivity in females is in line with the pronounced MG-induced enhancement of C-fibre ADS in females, which based on previous findings has been predicted to limit spinal summation and reduce heat hypersensitivity (Dickie et al., 2017). In addition, the presented findings in females

are in line with early sensory nerve CAP studies in mild diabetic neuropathy patients showing that women demonstrate better sensory nerve measures, indicative of no or milder degree of peripheral neuropathy than men (Albers et al., 1996). Furthermore, more recent clinical studies have also demonstrated that diabetic neuropathy is less common in women than men, who present with diabetic neuropathy-related complications much earlier than women (Albers et al., 1996, Aaberg et al., 2008, Abbott et al., 2011, Abraham et al., 2018, Cardinez et al., 2018, Booya et al., 2005, Brown et al., 2004).

Interestingly, the behavioural studies in this chapter found no development of mechanical hypersensitivity following systemic MG administration in juvenile male and female rats. These findings are in contrast to previous studies that have reported development of mechanical hypersensitivity ~3h after administration of 5µg MG in male mice (Bierhaus et al., 2012). Elevated plasma MG levels and pronounced mechanical hypersensitivity have also been found in studies in STZ-induced diabetic male mice (Bierhaus et al., 2012), GLO1 deficient male mice (Bierhaus et al., 2012) and STZ-induced diabetic male rats (Barragán-Iglesias et al., 2019, Huang et al., 2016). Interestingly, intraplantar as well as intrathecal MG administration have been shown to also induce mechanical hypersensitivity in male mice and rats (Barragán-Iglesias et al., 2019, Huang et al., 2016, Liu et al., 2017). However, it must be noted that all of the above studies conducted behavioural test exclusively in adult males and therefore, to my knowledge, there are currently no investigations of the MG-induced effects on female nociceptive behaviour. In addition, all of the above studies employed von Frey filament tests to measure changes in mechanical sensitivity, which is different from electronic von Frey test used for assessing mechanical sensitivity in this thesis. Arguably, these two tests are very different, with electronic von Frey allowing a consistent standardised measure of mechanical withdrawal threshold in response to single application of force, whereas the manual von Frey filaments require multiple applications of different fibres. Therefore, it is not surprising that these two tests can result in very different readouts and can actually provide measure of two different phenomena, with

electronic von Frey providing distinct reproducible measure of mechanical withdrawal threshold, while manual von Frey filaments can potentially be measuring sensitisation and irritability. It must also be noted that the experiments in this chapter were based on the Bierhaus et al. (2012) study, which used adult mice rather than juvenile rats for behavioural experiments. Given that there are species- and strain-dependent differences in behaviour sensitivity (Mogil et al., 2000), it is possible that the differences in species and assessment technique could have contributed to the observed differences in MG-induced mechanical hypersensitivity reported in this chapter and in the Bierhaus et al. (2012) study. In must be also noted that in the study in this chapter as predicted the observed MG-induced changes in C-fibre ADS did not correlate with observed MG-induced behavioural sensitivity to mechanical stimuli, which likely reflects behaviour driven by A fibre rather than C-fibre activation of sensitised spinal pain circuits.

3.6.5 Limitations

The electrophysiological studies in this chapter used an *ex vivo* MG-induced pain model, where dorsal roots from juvenile rats of both sexes were incubated with 100μM MG for 3h. This has been previously established to induce comparable intracellular MG changes to longer exposures at lower MG concentrations (similar to those of diabetic) (Eberhardt et al., 2012) and to cause alterations in $Na_V1.7$ and $Na_V1.8$ and to induce behavioural hypersensitivity (Bierhaus et al., 2012). However, plasma MG levels have been previously shown to differ from and likely underestimate peripheral nerve levels (Phillips et al., 1993, Cullum et al., 1991, Thornalley et al., 2003), with effects of MG not restricted to peripheral nerves (Schalkwijk and Stehouwer, 2020). Thus, *ex vivo* exposure to MG cannot mimic its effect on the whole body and the impact that could have on peripheral nerves. One way to improve this would be to use an *in vivo* MG model, where equivalent concentrations of MG can be administered intraperitoneally and 3h later dorsal roots can be isolated for electrophysiological experiments in the same manner as described for the experiments in this chapter. This can further confirm whether *in vivo* MG affects C-fibre ADS and Aβ fibre activity-dependent changes in amplitude in

a similar manner as the *ex vivo* application of MG. Another way to improve the *ex vivo* model would be to use an *in vivo* diabetes model, such as the STZ-induced diabetes rodent model , where the drug streptozotocin (STZ), which damages the beta cells of the pancreas, is administered (Rakieten et al., 1963). Given that previous studies have reported that increased plasma MG levels and behavioural hypersensitivity in the STZ-induced diabetes model (Bierhaus et al., 2012, Huang et al., 2016), it would be important to investigate if this model also displays similar changes in Aβ and C-fibre function. These *in vivo* models will allow the study of the effects of MG not only in the periphery but also in the CNS, which could give further insight into the diabetes-induced neuropathic complications, including the numbness/dysesthesia symptoms, which recent studies have suggested to involve actions of MG in the spinal cord (Cheng et al., 2019).

This study concluded that chronic MG induces sex-dependent changes in C-fibre ADS, which potentially involve the $Na_V1.7$ and $Na_V1.8$ channels (Petersson et al., 2014), given that those have been implicate the C-fibre ADS phenomenon. However, these experiments provide functional evidence for sex-differences in MG-induced alterations in $Na_V1.7$ and/or $Na_V1.8$ channels. Thus, it would be important to explore this in more detail and investigate the contribution of $Na_V1.7$ and $Na_V1.8$ to the MG-induced alterations on C-fibre ADS in both sexes by applying selective $Na_V1.7$ and $Na_V1.8$ blockers, such as ProTxII and A-803467, or by using tissue from $Na_V1.7$ or $Na_V1.8$ knockout animals. Similar experiments can be conducted to study the potential involvement of the Na_V channels in the Aβ fibre activity-dependent changes in amplitude in normal physiology and in the pathophysiology of diabetes in both sexes. However, given that Aβ fibres have been shown to express primarily $Na_V1.1$, $Na_V1.6$, $Na_V1.7$ (Black et al., 1996, Fukuoka and Noguchi, 2011), and potentially $Na_V1.8$ (Shields et al., 2012, Djouhri and Lawson, 2004, Djouhri et al., 2003), specific $Na_V1.1$ and $Na_V1.6$ inhibitors, such as ICA 121431 and MV1312 or 4,9-Anhydrotetrodotoxin, can be used to differentiate the potential contribution of these channels in the activity-dependent changes in Aβ amplitude in normal physiology and in the pathophysiology of diabetes.

Since the electrophysiological studies in this chapter were designed to investigate C-fibre function, the stimulation parameters were optimised for C-fibres rather than Aβ fibres. The frequencies used were 1Hz, 2Hz and 10Hz because the C-fibre rarely fire above those frequencies(Van Hees and Gybels, 1981, Hotta et al., 2010). However, although previous studies in models of peripheral neuropathy have found that Aβ fibres can spontaneously discharge at 1.6Hz (Xiao and Bennett, 2008), similar to the stimulation frequencies applied in this study, Aβ fibres have been shown to fire at frequencies of 30Hz and above in response to stimuli. Thus, it would be important to investigate the activity-dependent changes in Aβ amplitude at higher frequencies in normal conditions and in models of diabetic neuropathy. In addition, in this study the repetitive stimulation was delivered at 500µA, since it has been established that this is the maximal stimulation intensity for C-fibres in juvenile rats (Torsney, 2011, Nakatsuka et al., 2000, Dickie et al., 2017). This is a supra-maximal stimulation intensity for the low threshold Aβ fibres, which has been shown to be display maximal response at stimulation intensities of ~20µA in similarly aged rats (Torsney, 2011, Nakatsuka et al., 2000, Dickie et al., 2017). Thus, it would be critical that future studies investigate the Aβ fibre response amplitude following repetitive stimulation using CAP recordings conducted at maximal Aβ fibre stimulation intensities. These studies will be essential to confirm whether low-threshold Aβ fibres exhibit activity-dependent changes in amplitude at maximal Aβ fibre response stimulation intensity and whether MG could alter these changes in males and females.

Another limitation of the studies conducted in this chapter involves the electrophysiological constraints with regard to age of animals used. All experiments in this chapter were conducted in juvenile rats (P17-P24) due to better preservation and viability of the tissue at this age for electrophysiological studies. However, the fact that painful symptoms have been shown to increase in prevalence with the patients increase in age (Abbott et al., 2011), it will be important to conduct similar experiments in adult and potentially aging animals of both sexes. However, the use of juvenile animals was beneficial because they remain pre-pubertal until

approximately P50 (Sengupta, 2013, Engelbregt et al., 2000), with oestrous cycle commencing at approximately P38 in females (Ajayi and Akhigbe, 2020). This suggests that the sex differences observed in the studies in the chapter are unlikely to due to hormonal differences in males and females. However, it is important to investigate the effect of hormones in fully mature adult animals on the observed MG-induced changes in C-fibre ADS as well as on the activity-dependent changes in Aβ fibre amplitude in normal conditions and following systemic administration of MG.

3.7 Conclusion

The results described in this chapter have shown that the diabetic pain-associated metabolite methylglyoxal (MG) alters C-fibre ADS and Aβ fibre function in the *ex vivo* MG pain model. Chronic, but not acute treatment with MG altered C-fibre ADS in a sex-dependent manner. In males, chronic MG caused a reduction in the progressive increase in C-fibre latency, reflective of the change in the average conduction velocity in the population C-fibre response, without an effect on C-fibre width change, reflective of the change in the range of conduction velocities within the population response. In females, chronic MG treatment caused a pronounced enhancement in the progressive increase in C-fibre latency and width. In line with the proposed role of C-fibre ADS as a regulator of the intervals between successive action potentials being relayed along nociceptors to the spinal cord, increasing the progressive delay in synaptic transmission of C-fibre inputs that transmit noxious heat to the spinal cord, thus limiting spinal summation (Dickie et al., 2017), the MG-induced pronounced enhancement of C-fibre ADS in females, is suggested to limit summation and reduce heat hypersensitivity. This is supported by the behavioural data in this chapter, showing that MG does not cause heat hypersensitivity in females, but only in males, which showed MG-induced reduction in C-fibre ADS. Thus, the presented evidence suggests that C-fibre ADS can potentially influence MG-induced heat sensitivity in a sex-dependent manner.

In addition, the results presented in this chapter have also provide insight into the mechanism underlying MG-induced hypersensitivity. Given that the mechanisms underlying C-fibre ADS involve $Na_V1.7$ and $Na_V1.8$, with evidence in this chapter of sex- and MG-dependent differences in C-fibre ADS along with evidence from Bierhaus et al. (2012) of functional alterations in $Na_V1.7$ and $Na_V1.8$ following treatment with MG, it is likely that MG-induced hypersensitivity involves changes in those channels.

Interestingly the studies in this chapter reported that chronic MG treatment also alters initial Aβ fibre amplitude and width in a sex-dependent manner, causing an increase in the Aβ amplitude in males only and a decrease in the Aβ width in females only. Moreover, chronic MG treatment altered the reported here Aβ fibre activity-dependent amplitude change in a sex-dependent manner, with chronic MG enhancing the progressive increase in Aβ amplitude in females only. Given that mechanical hypersensitivity is likely associated with behaviours driven by A fibre activation spinal pain circuits (Hsieh et al., 2015, Ziegler et al., 1999), with proposed involvement of A fibres primarily in the negative diabetic neuropathy symptoms (characterised by positive and negative symptoms) like numbness and/or dysesthesia (Baron, 2009, Gwathmey and Pearson, 2019), the reported in this chapter sex-dependent MG-induced alterations in Aβ fibre function may potentially influence in a sex-dependent manner the negative symptoms of numbness and/or dysesthesia in diabetic neuropathy.

Chapter 4

C-fibre dynamic memory: manipulation of C-fibre activity-dependent slowing in rats and mice of both sexes

4.1 Chapter summary

- This chapter aimed to investigate, using compound action potential recordings, whether prolonged low-frequency stimulation can alter C-fibre ADS in a sex-dependent manner in rats and whether *C57BL/6* mice also display C-fibre ADS, whether its levels are sex-dependent and whether prolonged low-frequency stimulation can also alter C-fibre ADS in mice and whether this occurs in a sex-dependent manner.
- Prolonged low-frequency stimulation alters C-fibre ADS in juvenile rats giving rise to two distinct post-manipulation profiles: an increased C-fibre ADS profile and a decreased C-fibre ADS profile.
- The enhancement of ADS in the increased post-manipulation C-fibre ADS profile is comparable between the sexes whereas there is a sex difference in the decreased post-manipulation C-fibre ADS profile. There is a more pronounced reduction in C-fibre latency change and a less pronounced reduction in C-fibre width change in the females compared to the males. This suggests that the post-manipulation-induced changes in C-fibre ADS depend on C-fibre subtype and sex. Interestingly, the direction and the magnitude of the post-manipulation-induced changes in C-fibre ADS showed strong negative association with the degree of baseline C-fibre ADS in both sexes.
- The C-fibre ADS phenomenon was also observed in CAP recordings from adult mice of both sexes. In contrast to previous rat C-fibre ADS findings, the male mice showed more pronounced C-fibre ADS than the females. There were no significant sex differences in basic C-fibre properties in mice, as observed in rats, but the primary afferent fibre activation thresholds in mice were lower than those in rats, with peak C-fibre amplitude induced by lower stimulation intensities than in rat tissue.
- Prolonged low-frequency stimulation in mice also altered C-fibre ADS giving rise to an increased C-fibre ADS profile post-manipulation. There was a more

pronounced enhancement of C-fibre latency change in female compared to male mouse tissue.

4.2 Introduction

4.2.1 C-fibre ADS and dynamic memory

C-fibre ADS is proposed to provide a 'dynamic memory' of previous levels of activity and to influence responses to subsequent inputs. Specifically, low level firing, which is comparable to the injury-induced spontaneous C-fibre firing, can induce ADS, which in turn can dynamically influence the responses to subsequent, higher frequency stimuli (Weidner et al., 2002) (**Figure 4.1**). Thus, C-fibre 'dynamic memory' is dependent on ADS, with the amount of previous slowing determining the level of dynamic memory (Weidner et al., 2002). Surprisingly beyond this publication, this phenomenon has been little explored despite the potential implications for pain processing. In addition, given that the amount of slowing can influence the extent of dynamic memory it is important to explore whether there are sex differences in dynamic memory given the previously demonstrated sex-dependent differences in ADS (Dickie et al., 2017, Velichkova et al., 2021)(**Chapter 3**).

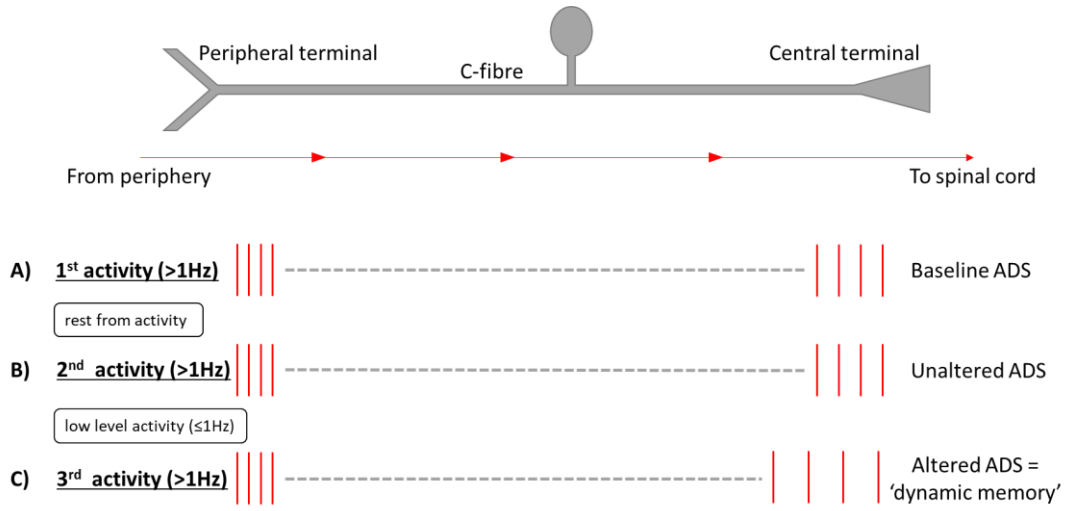


Figure 4.1 C-fibre dynamic memory

Repetitive stimulation at higher frequencies (≥1Hz) induces C-fibre ADS (**A**) and when there is a rest period between bouts of repetitive stimulation C-fibre ADS remains at baseline levels (**B**). Lower-frequency stimulation for prolonged periods of time can also induce ADS in C-fibres and alter their ADS levels following subsequent higher frequency stimulation, a phenomenon known as C-fibre 'dynamic memory' (**C**).

4.2.2 Potential sex-dependent influence of C-fibre ADS on heat pain sensitivity

Previous studies in the lab have shown sex- and injury-dependent changes in C-fibre ADS in population CAP recordings, where there is more pronounced baseline C-fibre ADS in female compared to male juvenile naïve Sprague-Dawley rats, with CFA-induced inflammation causing a reduction in the C-fibre ADS in females only, to a level comparable to males (Dickie et al., 2017). The pronounced C-fibre ADS profile in females, correlated with an observed higher noxious heat, but not mechanical, thresholds than males (Dickie et al., 2017). This sex-dependent difference in the noxious heat thresholds of Sprague-Dawley rats has also been reported by other studies (Mogil et al., 2000). Interestingly, the comparable noxious heat thresholds following CFA inflammation in males and females are in line with the comparable C-fibre ADS profiles in both sexes following CFA inflammation (Dickie et al., 2017). These findings proposed C-fibre ADS as a potential mechanism that can influence

noxious heat drive to spinal pain circuits driving the flexion-withdrawal reflex in a sex- and injury-dependent manner.

Additional studies from the lab provide further support for this hypothesis. Baseline C-fibre ADS has been shown to be more pronounced in female compared to male naïve juvenile Sprague-Dawley rats (**Figure 2.12**) (Velichkova et al., 2021), with hindpaw incision-induced postoperative pain causing an overall more pronounced C-fibre ADS in females, compared to males (**Figure 2.13)** (Velichkova et al., 2021). Consistent with the incision-induced overall more pronounced C-fibre ADS profile in females, which is suggested to limit heat hypersensitivity (Dickie et al., 2017), there was less peak heat hypersensitivity in females compared to males following incision (**Figure 2.21**) (Velichkova et al., 2021). Additional evidence comes from studies of the diabetic pain-associated metabolite methylglyoxal (MG) and its effect on C-fibre ADS in *ex vivo* dorsal root preparations from naïve juvenile Sprague-Dawley rats of both sexes, where chronic MG treatment caused a reduction in C-fibre ADS in males and a pronounced enhancement in females (**Figure 3.8; Figure 3.9**). Consistent with the MG-induced reduction in C-fibre ADS in males, MG induced thermal hypersensitivity in male rats, but in contrast failed to induce thermal hypersensitivity in female rats (**Figure 3.19**), in line with the pronounced enhancement of C-fibre ADS in females, which is predicted to limit heat hypersensitivity. These findings support the hypothesis that sex differences in ADS occur in C-fibres relaying heat pain signals to the spinal cord thereby potentially influencing the spinal processing of heat pain. Interestingly, sex differences in noxious heat sensitivity are dependent on animal species and strain (Mogil et al., 2000). Female *C57BL/6* mice have been shown to exhibit lower heat thresholds (Kest et al., 1999, Leo et al., 2008), which is similar to observed sex differences in humans (Mogil, 2012), but is in contrast to the higher heat thresholds observed in female Sprague-Dawley rats (Dickie et al., 2017, Mogil et al., 2000).

Given the fact that noxious heat thresholds have been associated with more pronounced population C-fibre ADS profiles, this hypothesis was further explored

by investigating whether the more pronounced heat thresholds in male *C57BL/6* mice are associated with more pronounced C-fibre ADS profiles. In addition, given the prediction that C-fibre ADS may exhibit both sex- and species-dependent differences and that it has been shown that ADS levels can influence subsequent higher frequency responses, thereby providing a 'dynamic memory', this chapter aimed to also investigate whether C-fibre dynamic memory is also influenced by sex and species.

4.3 Aims and hypothesis

4.3.1 Hypothesis

C-fibre ADS levels will be altered following prolonged low-frequency stimulation in a sex-dependent manner in juvenile SD rats. *C57BL/6* mice will display C-fibre ADS, with male mice showing higher levels of ADS, which will also be altered following prolonged low-frequency stimulation in a sex-dependent manner.

4.3.2 Aims

- Investigate, using compound action potential recording, whether prolonged low-frequency stimulation can alter C-fibre ADS in a sex-dependent manner in rats.

- Assess whether *C57BL/6* mice display C-fibre ADS and whether there are sex differences in the levels of C-fibre ADS.

- Investigate, using compound action potential recording, whether prolonged low-frequency stimulation can also alter C-fibre ADS in mice and assess whether that is dependent on sex.

4.4 Methods

4.4.1 Ethical and legal considerations

As described in section 2.4.1.

4.4.2 Animals and general husbandry

The experiments used juvenile naïve Sprague-Dawley rats (international strain code 400) of both sexes aged between P14 to P23, as described in **section 2.4.2**, and adult wild type (WT) *C57BL/6* and WT (non-carriers) Fos-EGFP mice of both sexes aged between 5-11 weeks. The hemizygous FosEGFP mouse line (Jackson Laboratory, B6.Cg-Tg(Fos/EGFP)1-3Brth/J) was made in the Barth lab (Barth et al., 2004) and was then maintained by breeding to WT mice (non-carriers) from the colony or *C57Bl/6J* inbred stock mice. WT Fos-EGFP mice were used in these CAP studies in order to establish C-fibre basic properties and ADS in this strain, which were then used in subsequent patch-clamp studies exploring spinal processing of thermal inputs in these mice (**Chapter 5**). This mouse line has been established in the literature with no reported side effects related to the transgenic modification (Barth et al., 2004). All animals were bred by the University of Edinburgh Bioresearch & Veterinary Services (BVS).

Mice were housed in individually ventilated cages (IVC) (391x199x160mm) in a stock room with a partial barrier. The room was maintained at 21°C (range: 19-22°C) with approximately 55% humidity (range: 46-65%), and on a 12-hour light/dark cycle. Animals were kept on a diet of standard mouse chow, and had free access to food and drinking water at all times. Bedding consisted of aspen chips shredded tissue bedding, with cardboard houses and wooden chew sticks for environmental enrichment and plastic tubes for both environmental enrichment and animal handling.

4.4.3 Isolated dorsal root electrophysiology in rats and mice.

4.4.3.1 Isolated dorsal root preparation from rats and mice.

Dorsal roots from naïve (control) juvenile rats (P14-P23) and adult mice (5-11 weeks) were isolated and prepared as previously described for rats in section 2.4.5.1.

4.4.3.2 Compound action potential recordings from isolated dorsal roots from rats and mice.

To investigate ADS in rats and mice and whether it can be modulated using prolonged low frequency electrical stimulation in both species, CAP recordings were recorded as described for rats in **section 2.4.5.2**.

In rat tissue, primary afferent fibre activation thresholds and C-fibres basic electrophysiological properties (amplitude, latency and conduction velocity) were defined and measured as previously described in **section 2.4.5.2**.

In mouse tissue, to identify the activation threshold for each primary afferent component, the dorsal roots were stimulated 3 times at 0.2Hz at 1-10μA (in steps of 1μA), 15–30μA (in steps of 5μA), 40–100 μA (in steps of 10μA), and 150–500μA (in steps of 50μA). A pulse width of 0.1ms was used as conducted in rat tissue. Data were acquired and recorded using a Cygnus ER-1 differential amplifier (Cygnus Technologies Inc.) and pClamp 10 software (Molecular Devices). Data were filtered at 10 kHz and sampled at 50 kHz. In mice the three main components of the CAPs could also be distinguished based on their activation threshold and conduction velocity as fast (Aβ), medium (Aδ), and slow (C) conducting components, each displaying a characteristic triphasic (positive–negative–positive) response (**Figure 4.2**). As previously reported, small intermediate components can be occasionally observed (Géranton et al., 2009, Torsney, 2011), but these were not analysed. The activation threshold for each component was defined as the lowest stimulation intensity which produced a clearly identifiable negative component of the triphasic response (**Figure 4.3**). The C-fibre response amplitude was measured from averaged traces (x3) (**Figure 4.2B**) at each stimulus intensity applied to determine the stimulation intensities that generate the maximal C-fibre response that would be optimal for C-fibre ADS recordings. The conduction velocity of the C-fibres response was calculated by dividing the estimated root length by the response latency from the averaged traces (average of x3) at 300μA. The latency was measured as the time distance between the stimulus artefact and the negative peak of the triphasic

response as demonstrated in **Figure 4.2A**, while the dorsal root length was measured as the distance between the recording and stimulating electrodes.

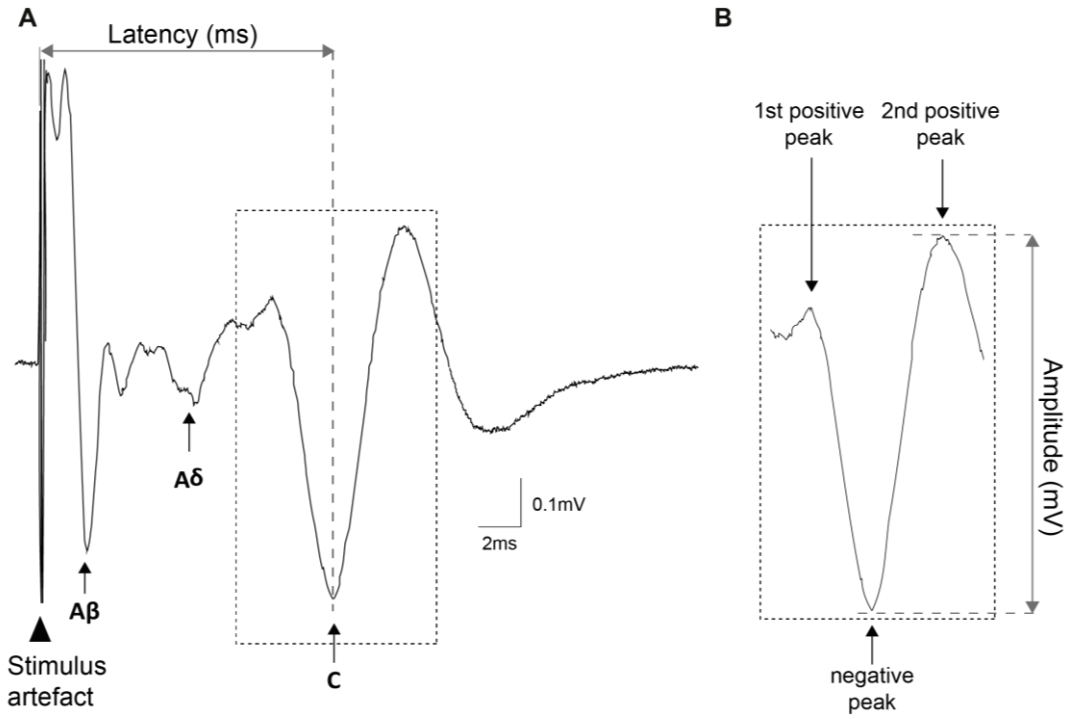


Figure 4.2 Representative mouse CAP recording

A) Representative population CAP recording from mouse dorsal root showing the fast (Aβ), medium (Aδ), and slow (C) conducting components. This example shows how the C-fibre response latency was measured as the time between the stimulus artefact and the negative peak of the triphasic response (solid double-headed arrow). The negative peaks of the Aβ, Aδ and C-fibre components are indicated by the arrows. **B**) Example trace shows the C-fibre component denoted by the box in **A**). The negative and 2 positive peaks of the triphasic response are can be clearly seen, denoted by the arrows. Demonstrated is the measurement of the C-fibre response amplitude, measured as the voltage difference between the negative and second positive peak (double-headed arrow). The trace shown is an average of x3 traces.

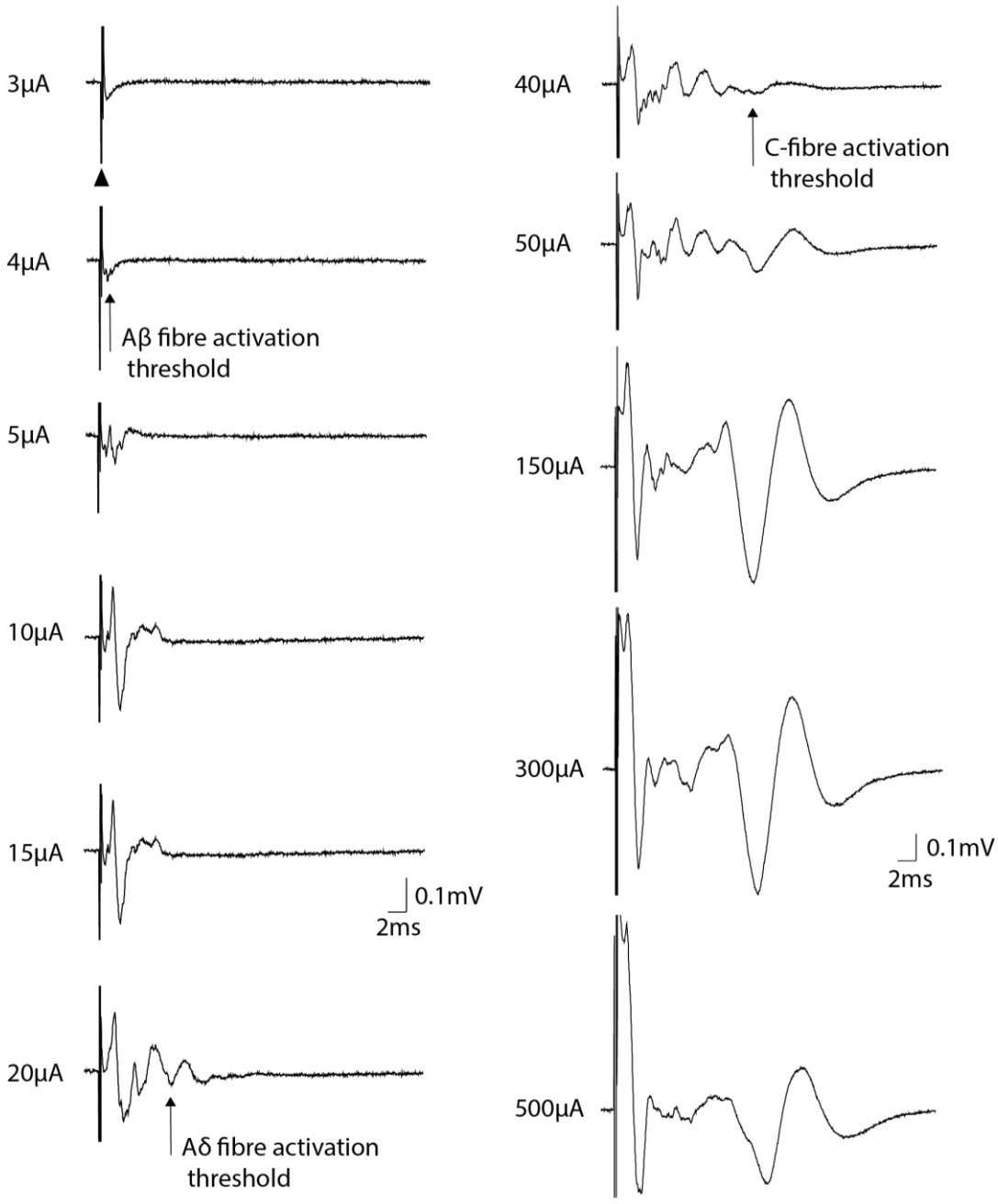


Figure 4.3 Primary afferent fibre activation threshold identification in mouse.

Representative example of how the activation thresholds for Aβ, Aδ and C-fibres were identified in isolated dorsal root CAP recordings in mouse. Activation thresholds were defined as the lowest stimulation intensity at which a negative peak of the triphasic response is first clearly identifiable (indicated on the traces by arrows). The stimulus artefact is highlighted in the first example recording by the arrowhead. Numbers to the left of traces indicate corresponding stimulation intensity used. The traces are the averaged response of x3.

4.4.3.3 Manipulation of C-fibre activity-dependent slowing in isolated dorsal roots from rats and mice.

Dorsal roots isolated from naïve juvenile SD rats or adult WT *C57BL/6* mice of both sexes were stimulated 40 times (500µA intensity for rat and 300µA intensity for mice, 0.1ms duration) at 10Hz (for rats) or 2Hz (for mice) to record baseline ADS. Following a 10-minute recovery time, prolonged low-frequency stimulation with a 2-minute train of 120x stimuli at 1Hz (for rats) or a 1-minute train of 60x stimuli at 1Hz (for mice) (referred to as 'manipulation') was applied to manipulate C-fibre ADS levels. Immediately after, another train of 40 stimuli at 10Hz (for rats) or 2Hz (for mice) was applied to allow for the assessment of ADS post-manipulation (**Figure 4.4**). Ten minutes after the post-manipulation, the roots were stimulated again with a train of 40 stimuli at the respective frequency for each species to allow for assessment of C-fibre ADS recovery. For the control manipulation experiments in rats, instead of applying a 2min train of 120x stimuli at 1Hz, 2min was added to the 10min break following baseline ADS recording, when no stimulation was applied, and after this 12min 'break', 'control' post-manipulation C-fibre ADS at 10Hz was recorded and a further 10min later C-fibre ADS recovery was recorded.

Prolonged low-frequency stimulation gave rise to both an increased and a decreased post-manipulation C-fibre ADS in rats. For analysis the data were subdivided into x2 analysis groups based on the direction (increased/decreased) profile of the C-fibre ADS change at post-manipulation with respect to baseline C-fibre ADS. This was done for each root individually by calculating the AUC for baseline C-fibre ADS and subtracting it from the AUC measurement for post-manipulation C-fibre ADS. This analysis grouping approach was applied for both the manipulation and the control manipulation experiments. Specifically, if the change in the AUC C-fibre ADS post-manipulation was positive, the roots were categorised as having an increased C-fibre ADS post-manipulation whereas if the change in the AUC C-fibre ADS post-manipulation was negative, the roots were categorised as having a decreased C-fibre ADS post-manipulation. Thus, roots in the increased C-fibre ADS post-manipulation profile, which had positive AUC change, were

compared between the manipulation and control manipulation conditions. Analogously, roots in the decreased C-fibre ADS post-manipulation profile, which had negative AUC change, were compared between the manipulation and control manipulation conditions.

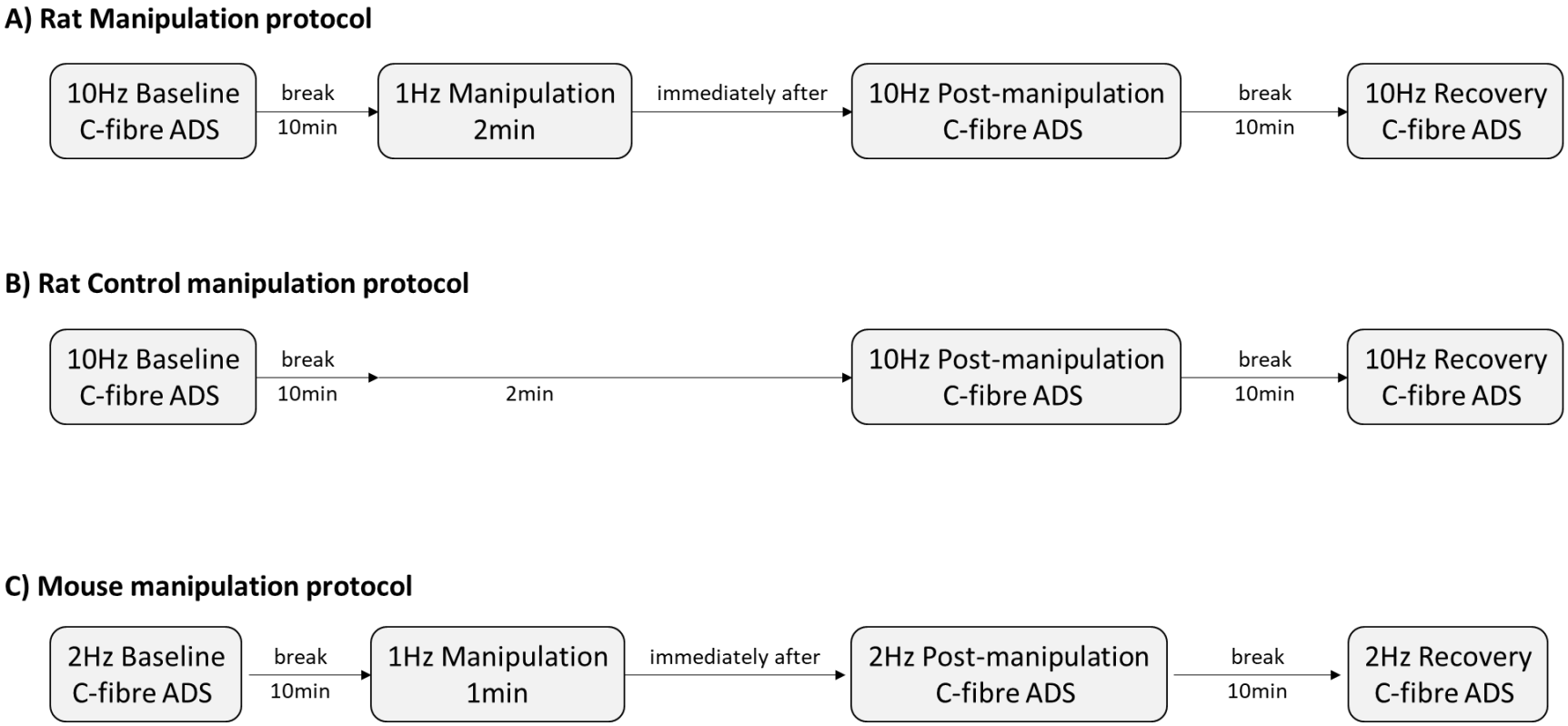


Figure 4.4 Manipulation protocols for rat and mouse CAP recordings

A) Rat manipulation protocol where C-fibre ADS was assessed in response to a train of 40x stimuli at 500µA (0.1ms duration) at 10Hz in a dorsal root from a juvenile rat. Prolonged low-frequency stimulation with a 2-minute train of 120x stimuli at 1Hz, applied 10min after baseline ADS recording and just before post-manipulation ADS recording, was used to manipulate C-fibre ADS levels. **B**) Rat control manipulation protocol where C-fibre ADS was assessed in response to a train of 40x stimuli at 500µA (0.1ms duration) at 10Hz in a dorsal root from a juvenile rat.

Instead of the prolonged low-frequency stimulation for 2min, a 2min rest period was added to the 10min break after baseline ADS recording, thus a 12min break in total was applied before control post-manipulation ADS recording. **C**) Mouse manipulation protocol where C-fibre ADS was assessed in response to a train of 40x stimuli at 300µA (0.1ms duration) at 2Hz in a dorsal root from an adult mouse. Prolonged low-frequency stimulation with a 1-minute train of 60x stimuli at 1Hz, applied 10min after baseline ADS recording and just before post-manipulation ADS recording, was used to manipulate C-fibre ADS levels.

4.4.4 Statistical analysis

In CAP recordings from isolated roots from juvenile rats, to compare the effect of manipulation on C-fibre ADS at baseline, post-manipulation and recovery, the ADS area under the curve (AUC) for each group was measured and analysed using two-way ANOVA followed by Sidak's multiple comparisons test if an interaction between factors was observed. Similar analysis was performed when the manipulation data was separated by sex. The effect of manipulation on C-fibre latency and width changes in manipulated dorsal roots from male and female rats was also measured using the AUC for each group and statistically analysed using a two-way ANOVA with Sidak's multiple comparisons test performed only if a significant interaction between the two investigated factors was detected by the two-way ANOVA.

The association between baseline C-fibre ADS and the direction and magnitude of the post-manipulation C-fibre ADS change in rats and mice was assessed by measuring the AUC of baseline C-fibre ADS and the percentage change in the AUC of the post-manipulation C-fibre ADS from baseline AUC of C-fibre ADS. The correlation between the two factors was statistically assessed using Pearson's correlation coefficient.

The effect of sex on the basic primary afferents' properties (activation threshold and conduction velocity) of the C-fibre response in mice was statistically assessed using two-way ANOVA. Post-hoc multiple comparison tests were run only if a significant interaction between the two investigated factors was detected. To compare levels of C-fibre ADS in mice of both sexes, the ADS area under the curve (AUC) for each sex was measured and analysed using an unpaired two-tailed t-test. As for the rat C-fibre ADS manipulation data, to compare the effect of manipulation and sex on C-fibre ADS in mice at baseline, post-manipulation and recovery, the ADS AUC for each group was measured and data were analysed using two-way ANOVA followed by Sidak's multiple comparisons test if an interaction between factors was observed. The effect of manipulation on C-fibre latency changes in male and female

mice was also measured using the AUC for each group and statistically analysed using an unpaired two-tailed t-test.

Graphad Prism 9.11 software (GraphPad Software) was used for statistical analysis and graph production. All data are represented as mean ± standard error of the mean (SEM), with n representing the sample size. P<0.05 was used to indicate statistical significance.

4.5 Results

4.5.1 C-fibre dynamic memory in rats of both sexes.

4.5.1.1 Prolonged low frequency stimulation (manipulation) results in both increased and decreased C-fibre ADS profiles

Dynamic memory was assessed by measuring C-fibre ADS following a train of x40 stimuli at 10Hz 10min before (baseline) and immediately after a prolonged low frequency stimulation, termed 'manipulation' (a train of 120stimuli for 2min at 1Hz) (**Figure 4.4A**), or after an equivalent control period (control manipulation) (**Figure 4.4B**), and following a 10min recovery period. The manipulation resulted in two distinct ADS profiles – an increased (**Figure 4.5**) and a decreased (**Figure 4.6**) profile. To enable comparison of the data, first all the 'manipulation' and 'control manipulation' data were separated into data with an increased profile or a decreased profile (as described in **section 4.4.3.3**) and then the manipulation and control manipulation treatment groups within the appropriate profile were compared. The repetitive stimulation at 10Hz resulted in a clear progressive increase in C-fibre response latency (**Figure 4.5A, Bi, C, Di, E** and **Figure 4.6A, Bi, C, Di, E**) and width (**Figure 4.5A, Bii, C, Dii, F** and **Figure 4.6A, Bii, C, Dii, E**) changes.

In the dataset showing an increased C-fibre ADS (**Figure 4.5**), the prolong low frequency stimulation, termed 'manipulation', enhanced the progressive increase in C-fibre latency change post-manipulation in the manipulation treatment group in comparison to post-manipulation in the control manipulation treatment group and in comparison to baseline in the manipulation treatment group (**Figure 4.5A, Bi, C, Di, E;** two –way ANOVA: manipulation, p<0.0001, treatment, p<0.0001,

manipulation x treatment, p=0.0006 followed by Sidak's multiple comparisons test, p<0.0001 at post-manipulation between treatment groups and p<0.0001 in the manipulation treatment group: post-manipulation vs baseline and post-manipulation vs recovery). Similarly, the progressive increase in C-fibre response width was also enhanced post-manipulation in the manipulation treatment group in comparison to post-manipulation in the control manipulation treatment group and in comparison to baseline in the manipulation treatment group (**Figure 4.5A, Bii, C, Dii, F;** two –way ANOVA: manipulation, p=0.0008, manipulation x treatment, p=0.023 followed by Sidak's multiple comparisons test, p=0.012 at post-manipulation between treatment groups and in the manipulation treatment group, p<0.0001 post-manipulation vs baseline, p=0.0004 post-manipulation vs recovery and p=0.002 baseline vs recovery). In the manipulation treatment group, interestingly, the progressive increase in C-fibre response width remained more enhanced in the 'recovery' recording when compared to baseline C-fibre ADS (**Figure 4.5A, Bii, F;** two–way ANOVA followed by Sidak's multiple comparisons test, p=0.002 baseline vs recovery atIn t manipulation conditions).

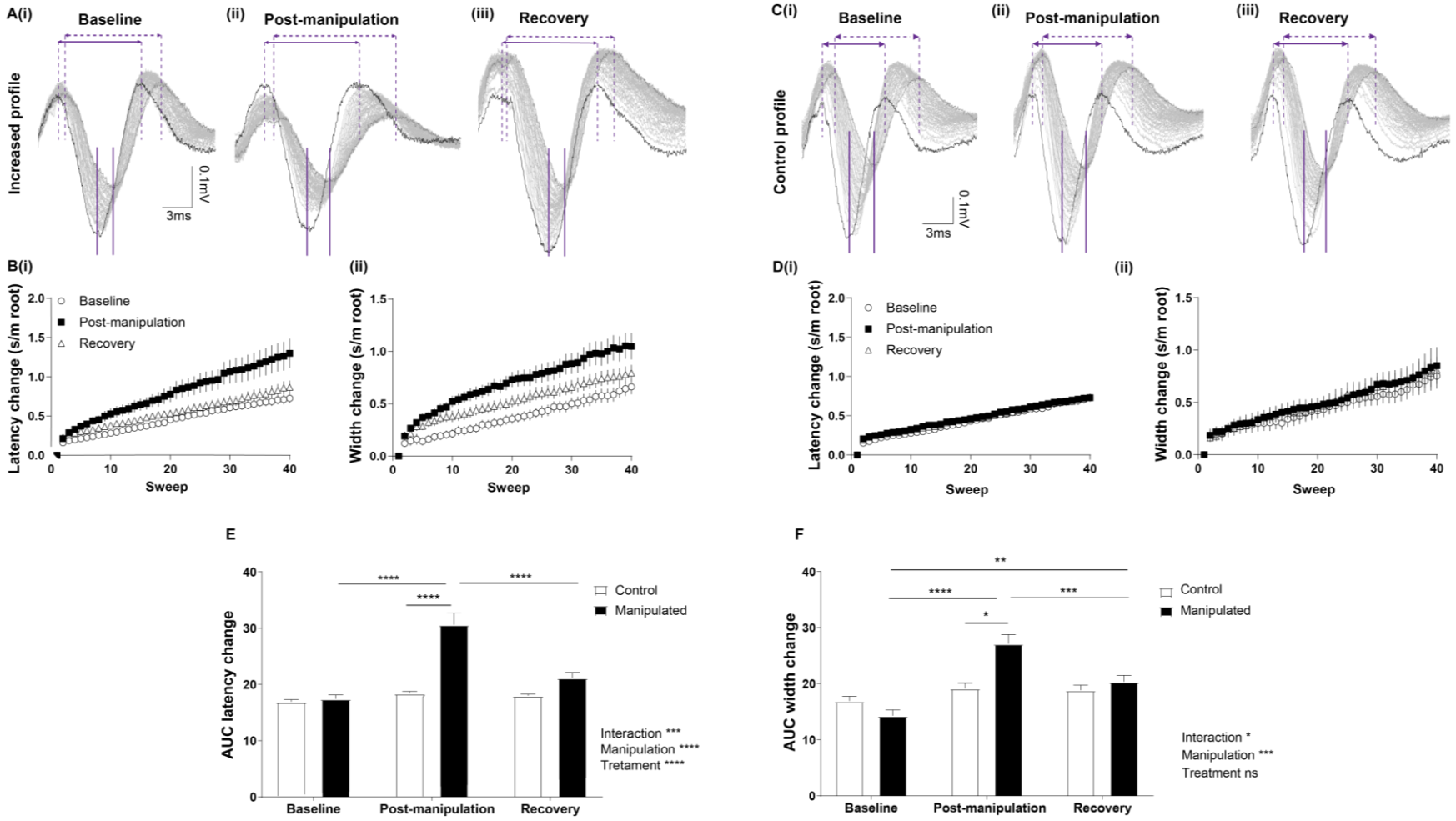


Figure 4.5 Prolonged low-frequency stimulation increases C-fibre ADS

Representative C-fibre CAP recordings from dorsal roots isolated from naïve juvenile rats of both sexes that show increased C-fibre ADS following manipulation (**A**) and unaltered C-fibre ADS following control manipulation (**C**). Baseline (**i**), post-manipulation (**ii**) and recovery (**iii**) recordings in response to x40 stimuli at 10Hz displayed. Solid lines mark C-fibre latency change and double-headed arrows mark C-fibre response width, the distance from positive-to-positive peaks for the first (solid lines) and last (dashed lines) of the x40 responses; the width change is the difference between the two double-headed arrows (response trace 1 black; 2-39 pale grey; 40 dark grey). Repetitive stimulation in manipulation (**B**) and control manipulation (**D**) conditions results in a progressive C-fibre latency (**i**) and width (**ii**) increase at baseline, post-manipulation and recovery. **E**) AUC analysis of C-fibre latency change reveals a significant effect of manipulation (****p<0.0001) and treatment group (****p<0.0001) with an interaction between the two factors (***p=0.0006) (two-way ANOVA followed by Sidak's multiple comparisons test, ****p<0.0001 at post-manipulation between treatment groups and ****p<0.0001 post-manipulation vs baseline and post-manipulation vs recovery in the manipulation treatment group). **F**) AUC analysis of C-fibre width change reveals a significant effect of manipulation (***p=0.0008) with an interaction between manipulation and treatment (*p=0.023) (two-way ANOVA followed by Sidak's multiple comparisons test, *p=0.012 at post-manipulation between the treatment groups; in the manipulation treatment group: ****p<0.0001 post-manipulation vs baseline, ***p=0.0004 post-manipulation vs recovery and **p=0.002 baseline vs recovery). Data presented as mean±SEM. Latency change: control (n=7), manipulated (n=16). Width change: control (n=8), manipulated (n=20).

In the dataset showing a decreased C-fibre ADS profile (**Figure 4.6**) the repetitive stimulation at 10Hz at baseline also resulted in a clear progressive increase in C-fibre response latency (**Figure 4.6A, Bi, C, Di, E**) and width (**Figure 4.6A, Bii, C, Dii, E**) changes. However, here the prolonged low-frequency stimulation resulted in a reduction in the progressive increase in C-fibre latency change post-manipulation in comparison to baseline in the manipulation treatment group (**Figure 4.6A, Bi, C, Di, E;** two –way ANOVA: manipulation, p=0.0005, treatment, p<0.0001, manipulation x treatment, p=0.016 followed by Sidak's multiple comparisons test, p<0.0001 in the manipulation treatment group: post-manipulation vs baseline and post-manipulation vs recovery). Interestingly, there was no significant difference in post-manipulation C-fibre latency change between the control and manipulation treatment groups (**Figure 4.6E**; two –way ANOVA followed by Sidak's multiple comparisons test, p=0.9231), but the baseline and recovery C-fibre latency changes were significantly different between the control and manipulation treatment groups (**Figure 4.6E**; two –way ANOVA followed by Sidak's multiple comparisons test: between treatment groups, baseline, p=0.0005, recovery, p=0.0002). When C-fibre ADS was assessed as the progressive increase in response width, here the manipulation also reduced the progressive increase in C-fibre width change in comparison to baseline (**Figure 4.6A, Bii, F;** two –way ANOVA: manipulation, p<0.0001, treatment, p<0.0001, manipulation x treatment, p<0.0001 followed by Sidak's multiple comparisons test, p<0.0001 post-manipulation vs baseline). Similarly to the latency change data for the decreased C-fibre ADS profile (**Figure 4.6E**), here there was also no significant difference in post-manipulation C-fibre width change between the treatment groups (**Figure 4.6F**; two –way ANOVA followed by Sidak's multiple comparisons test, p=0.149), but the baseline and recovery C-fibre width changes were significantly different between the treatment groups (**Figure 4.6F**; two –way ANOVA followed by Sidak's multiple comparisons test: baseline, p<0.0001, recovery, p<0.0001 between treatment groups).

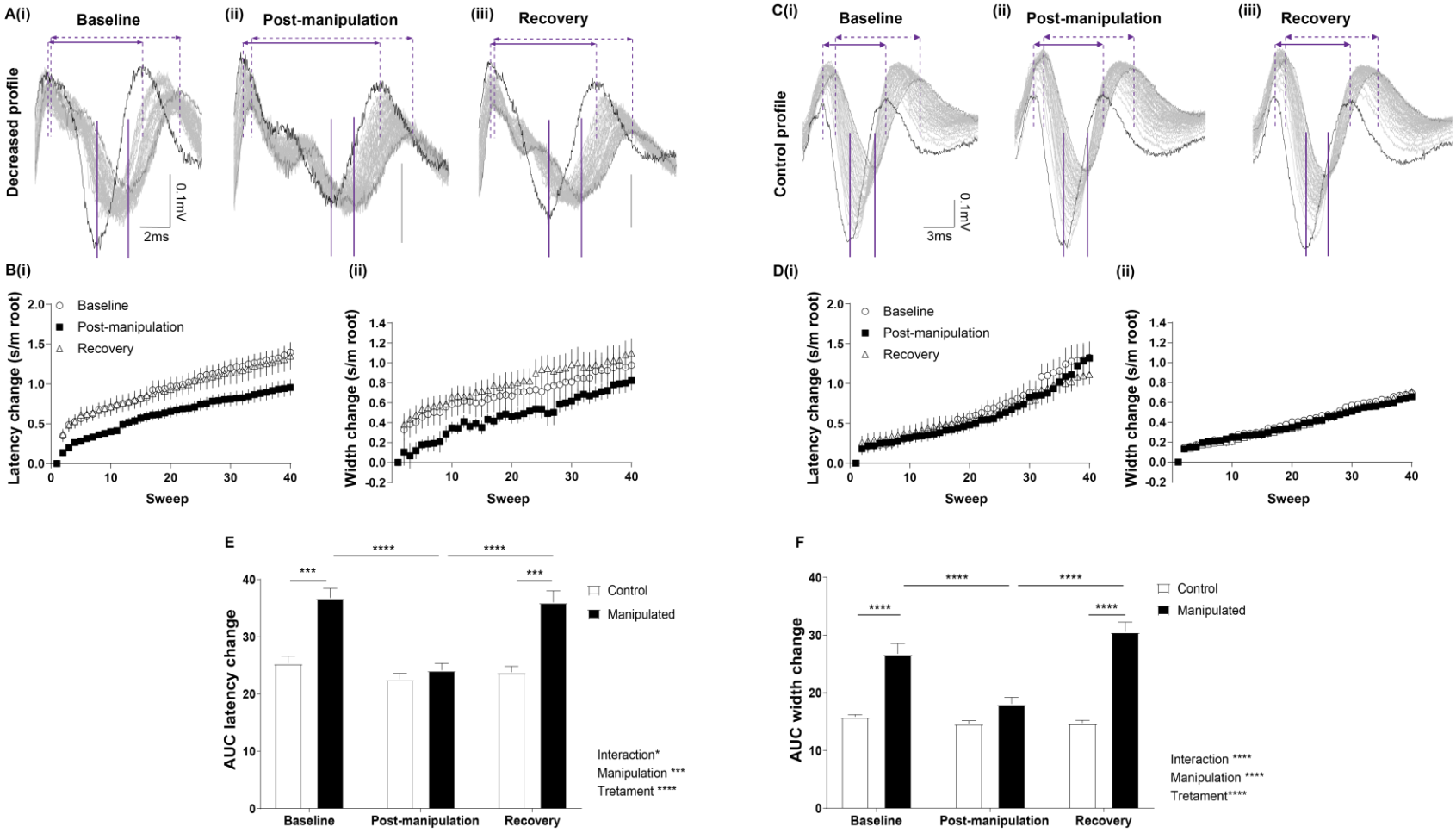


Figure 4.6 Prolonged low-frequency stimulation decreases C-fibre ADS

Representative C-fibre CAP recordings from dorsal roots isolated from naïve juvenile rats of both sexes that show decreased C-fibre ADS following manipulation (**A**) and the control manipulation (**C**). Baseline (**i**), post-manipulation (**ii**) and recovery (**iii**) recordings in response to x40 stimuli at 10Hz displayed. Solid lines mark C-fibre latency change and double-headed arrows mark C-fibre response width, the distance from positive-to-positive peaks for the first (solid lines) and last (dashed lines) of the x40 responses; the width change is the difference between the two double-headed arrows (response trace 1 black; 2-39 pale grey; 40 dark grey). Repetitive stimulation in manipulation (**B**) and control manipulation (**D**) conditions results in a progressive C-fibre latency (**i**) and width (**ii**) increase at baseline, post-manipulation and recover. **E**) AUC analysis of C-fibre latency change reveals a significant effect of manipulation (***p=0.0005) and treatment (****p<0.0001) with an interaction between the two factors (*p=0.016) (two-way ANOVA followed by Sidak's multiple comparisons test, ***p=0.0005 at baseline and ***p=0.0002 at recovery between treatment groups; in the manipulation treatment group ****p<0.0001 post-manipulation vs baseline and post-manipulation vs recovery). **F**) AUC analysis of C-fibre width change reveals a significant effect of manipulation (****p<0.0001) and treatment (****p<0.0001) with an interaction between the two factors (****p<0.0001) (two-way ANOVA followed by Sidak's multiple comparisons test, ****p<0.0001 at baseline and recovery between the treatment groups; in the manipulation treatment group: ****p<0.0001 post-manipulation vs baseline and ****p<0.0001 post-manipulation vs recovery). Data presented as mean±SEM. Latency change: control (n=5), manipulated (n=13). Width change: control (n=8), manipulated (n=9).

Given the observed two distinct C-fibre ADS profiles (increased and decreased) following the prolonged-low frequency stimulation, the data have been treated as two populations by an 'exploratory approach' for the ADS analysis, highlighted in **Figure 4.7.** The magnitude and the direction of the manipulation-induced changes in latency (**Figure 4.7A**) and width (**Figure 4.7B**) were summarised by treatment group (manipulation/control manipulation) and sex. The data from control manipulation treatment group for males (dark grey) and females (light grey) showed negligible changes (close to ~0%) in latency (**Figure 4.7A**) and width (**Figure 4.7B**) in both positive and negative directions. The data from the manipulation treatment group for males (blue) and females (red) displayed much more

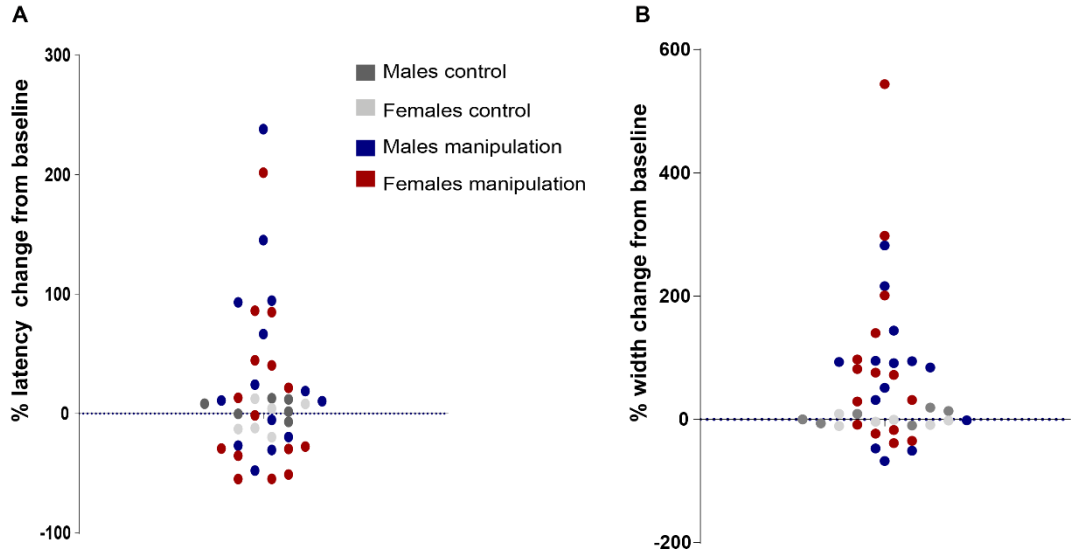


Figure 4.7 Manipulation-induced percentage change in C-fibre ADS profile in both sexes

Latency (**A**) and width (**B**) change data from both sexes for an individual root presented as a percentage change from the baseline latency and width changes, respectively. The percentage latency/width change from baseline is calculated from the AUC baseline and post-manipulation data for an individual root.

pronounced changes in latency (**Figure 4.7A**) and width (**Figure 4.7B**) in both positive and negative directions.

4.5.1.2 Influence of sex and C-fibre subtype on the post-manipulation C-fibre ADS effect (effect of manipulation on C-fibre ADS)

To investigate the effect of sex on the manipulation-induced changes in C-fibre ADS in both the increased and the decreased ADS profiles, the data for each profile were separated and analysed by sex.

For the manipulation-induced increased C-fibre ADS profile, the grouped AUC analysis of the latency change data at baseline, post-manipulation and recovery for males and females confirmed the observed enhancing effect of the manipulation with no significant effect of sex and showed no significant influence of sex on the manipulation (**Figure 4.8A*i*;** two –way ANOVA: manipulation, $p<0.0001$, sex, $p=0.415$, manipulation x sex, $p=0.431$). Similarly, the AUC analysis of the width change data in the increased C-fibre ADS profile supported the observed enhancing effect of the manipulation and also revealed an effect of sex, which was not influenced by the manipulation, showing overall less pronounced C-fibre width change increase in the females compared to the males (**Figure 4.8B*i*;** two –way ANOVA: manipulation, $p<0.0001$, sex, $p<0.0001$, manipulation x sex, $p=0.173$). To directly compare the effect of the manipulation on C-fibre ADS between the sexes, the C-fibre response latency and width change values at baseline were subtracted from the corresponding post-manipulation latency and width change values to generate 'manipulation effect' data over the 40x stimuli for each root for the latency (**Figure 4.8A*ii*)** and width (**Figure 4.8B*ii***) change. Given that the latency change reflects the ADS within the medium-conducting C-fibres, while the width change reflects the ADS within the slowest- and fastest-conducting C-fibres, the latency and width change data were used to differentiate between C-fibre subtypes. The AUC analysis of the 'manipulation effect' latency and width change data revealed no significant effect of sex in the different C-fibre subtypes (**Figure 4.8C***;*

two –way ANOVA: C-fibre subtype, p=0.250, sex, p=0.137, C-fibre subtype x sex, p=0.396).

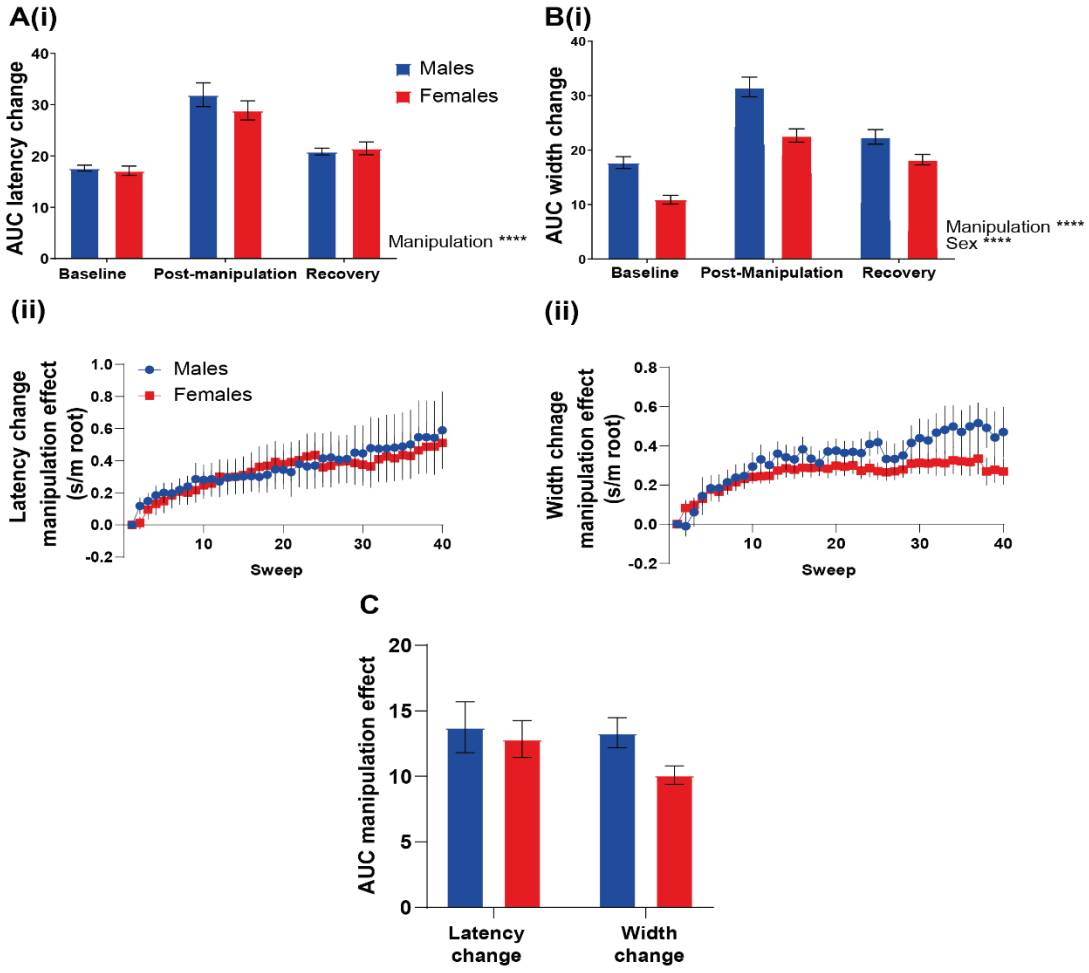


Figure 4.8 No effect of sex and C-fibre subtype on the increased post-manipulation ADS

Latency (**A**) and width (**B**) change data following manipulation for males and females. The AUC analysis of C-fibre latency change (**Ai**) reveals a significant effect of manipulation (****p<0.0001) (two-way ANOVA). The AUC analysis of C-fibre width change (**Bi**) reveals a significant effect of manipulation (****p<0.0001) and sex (****p<0.0001) (two-way ANOVA). Males and females display enhancing effect of manipulation on post-manipulation C-fibre ADS, represented by the progressive increase in the change in latency (**Aii**) and width (**Bii**) from corresponding baseline recordings. **C**) AUC analysis of the increase in C-fibre latency and width change from baseline for males and females reveals no significant effect of sex (p=0.137) and C-fibre subtype (p=0.25) with no interaction between the two factors (p=0.396) (two-way ANOVA). Data presented as

mean±SEM. Latency change: males (n=9), females (n=7). Width change: males (n=10), females (n=10).

For the manipulation-induced decreased C-fibre ADS profile, the grouped AUC analysis of the latency change data at baseline, post-manipulation and recovery for males and females confirmed the observed limiting effect of manipulation on post-manipulation C-fibre latency change, with a significant effect of sex that was not influenced by the manipulation (**Figure 4.9A*i*;** two –way ANOVA: manipulation, $p<0.0001$, sex, $p=0.0005$, manipulation x sex, $p=0.071$). Since the *p* value for interaction between sex and manipulation was close to significance, multiple comparisons tests were conducted on this dataset, revealing sex-dependent differences in C-fibre ADS at baseline and recovery, with a more pronounced latency change in females (**Figure 4.9Ai***;* two –way ANOVA followed by Sidak's multiple comparisons test, baseline, $p=0.004$, recovery, $p=0.019$). Interestingly, there was no significant difference in post-manipulation C-fibre latency change between the sexes (**Figure 4.9A*i*;** two –way ANOVA followed by Sidak's multiple comparisons test, post-manipulation, $p=0.987$). Similarly, the AUC analysis of the width change data in the decreased C-fibre ADS profile supported the observed limiting effect of the manipulation on post-manipulation C-fibre width change and also revealed an effect of sex, which was not influenced by the manipulation and showed overall more pronounced C-fibre width change increase in the females compared to the males (**Figure 4.9B*i*;** two –way ANOVA: manipulation, $p<0.0001$, sex, $p<0.0001$, manipulation x sex, $p=0.455$). To directly compare the effect of the manipulation on C-fibre ADS between the sexes, the C-fibre response latency and width change values at baseline were subtracted from the corresponding post-manipulation latency and width change values to generate 'manipulation effect' data over the 40x stimuli for each root for the latency (**Figure 4.9*Aii*)** and width (**Figure 4.9B*ii***) change. The AUC analysis of the 'manipulation effect' latency and width change data revealed a significant interaction between sex and C-fibre subtype (**Figure 4.9C***;* two –way ANOVA: sex x C-fibre subtype, $p<0.0001$) where the

effect of the manipulation was influenced by sex and C-fibre subtype such that in the medium-conducting C-fibres the females showed a significantly more pronounced decrease in ADS, while in the range of slowest-fastest conducting C-fibres the females showed significantly less pronounced decrease in ADS (**Figure 4.9C**; two –way ANOVA followed by Sidak's multiple comparisons test, latency change, p=0.0005, width change, p=0.005).

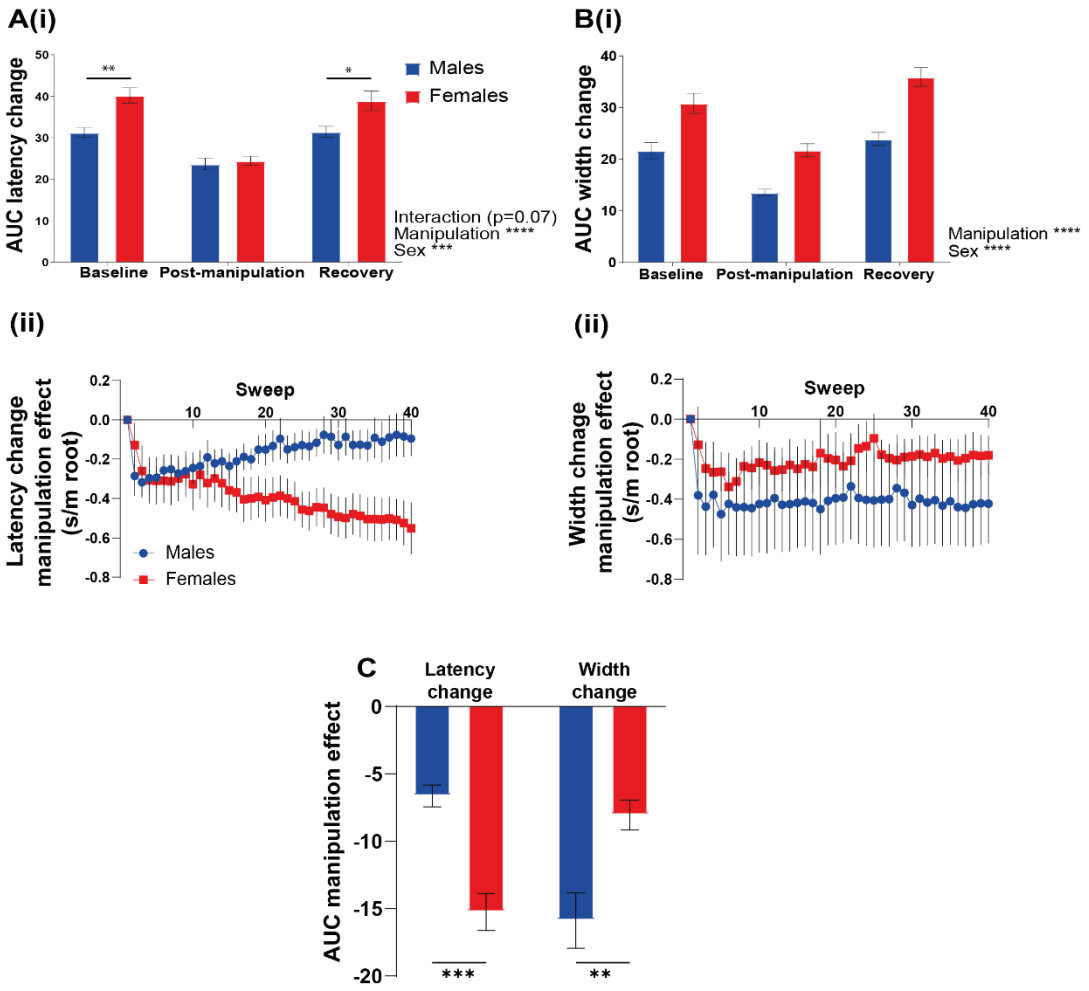


Figure 4.9 Effect of sex and fibre type on the decreased post-manipulation ADS

Latency (**A**) and width (**B**) change data following manipulation for males and females. The AUC analysis of C-fibre latency change (**Ai**) reveals a significant effect of manipulation (****p<0.0001) and sex (***p=0.0005) with no significant interaction between the two factors (p=0.071) (two-way ANOVA). Sidak's multiple comparisons test were conducted on this dataset, given the p value for interaction (p=0.071) was close to significance level, revealing sex-dependent differences in baseline (**p=0.004) and recovery (*p=0.019) C-

fibre ADS. The AUC analysis of C-fibre width change (**Bi**) reveals a significant effect of manipulation (****p<0.0001) and sex (****p<0.0001) with no significant interaction between the two factors (p=0.455) (two-way ANOVA). Males and females display a limiting effect of manipulation on post-manipulation C-fibre ADS, represented by the decrease in the change in latency (**Aii**) and width (**Bii**) from corresponding baseline recordings. **C**) AUC analysis of manipulation effect on C-fibre latency and width change for males and females reveals a significant interaction between effect of sex and C-fibre subtype (****p<0.0001) (two-way ANOVA followed by Sidak's multiple comparisons test, ***p=0.0005, **p=0.005). Data presented as mean±SEM. Latency change: males (n=5), females (n=8). Width change: males (n=4), females (n=5).

The baseline AUC data in both sexes for the increased (**Figure 4.8**) and the decreased (**Figure 4.9**) post-manipulation C-fibre ADS profiles revealed that the degree of baseline C-fibre latency and width changes in the both profiles seem to be different, with dorsal roots displaying the decreased profile showing a higher degree of baseline ADS when compared to baseline ADS levels in the increased profile. To statistically explore the possible association between baseline C-fibre ADS and the direction and magnitude of the post-manipulation C-fibre ADS change, the baseline C-fibre ADS AUC was plotted against the post-manipulation percentage change from baseline C-fibre ADS AUC for each individual root and the correlation between the two factors was investigated (**Figure 4.10A**). The correlation analysis for the C-fibre latency change in both sexes revealed a significant negative association between the baseline C-fibre latency change and the post-manipulation percentage change from baseline C-fibre latency change (**Figure 4.10Ai**; Pearson r=-0.548, r2=0.3, p=0.002). This suggests that as the baseline C-fibre latency change increases, the manipulation-induced enhancement of the latency change decreases until baseline C-fibre latency change reaches a point from which its increase begins to be associated with a reduction of the latency change post-manipulation. To investigate this association in each sex, the data was separate for females (**Figure 4.10Aii**) and males (**Figure 4.10Aiii**). In line with the data in both sexes, the separate female data showed significant negative association between baseline C-fibre latency change and post-manipulation percentage change from baseline C-fibre latency change (**Figure 4.10Aii**; Pearson r=-0.653, r2=0.427, p=0.008). The male data

showed no significant association between baseline C-fibre latency change and post-manipulation percentage change from baseline C-fibre latency change (**Figure 4.10Aiii**; Pearson r=-0.395, r2=0.156, p=0.162), which could be due to the narrower range of baseline C-fibre latency change in males.

Similarly to the correlation analysis for the latency change data, the correlation analysis for the C-fibre width change in both sexes revealed a significant strong negative association between baseline C-fibre width change and post-manipulation percentage change from baseline C-fibre width change (**Figure 4.10Bi**; Pearson r=-0.617, r2=0.381, p=0.0004). This suggests that as the baseline C-fibre width change increases, the manipulation-induced enhancement of the width change decreases until baseline C-fibre width change reaches a point from which its increase begins to be associated with a reduction of the width change post-manipulation. To investigate this association in each sex, the data was separate for females (**Figure 4.10Bii**) and males (**Figure 4.10Biii**). In line with the data in both sexes, the separate female data showed a significant negative association between baseline C-fibre width change and post-manipulation percentage change from baseline C-fibre width change (**Figure 4.10Bii**; Pearson r=-0.597, r2=0.357, p=0.019). The separate male data also showed significant negative association between baseline C-fibre width change and post-manipulation percentage change from baseline C-fibre width change (**Figure 4.10Biii**; Pearson r=-0.661, r2=0.437, p=0.010).

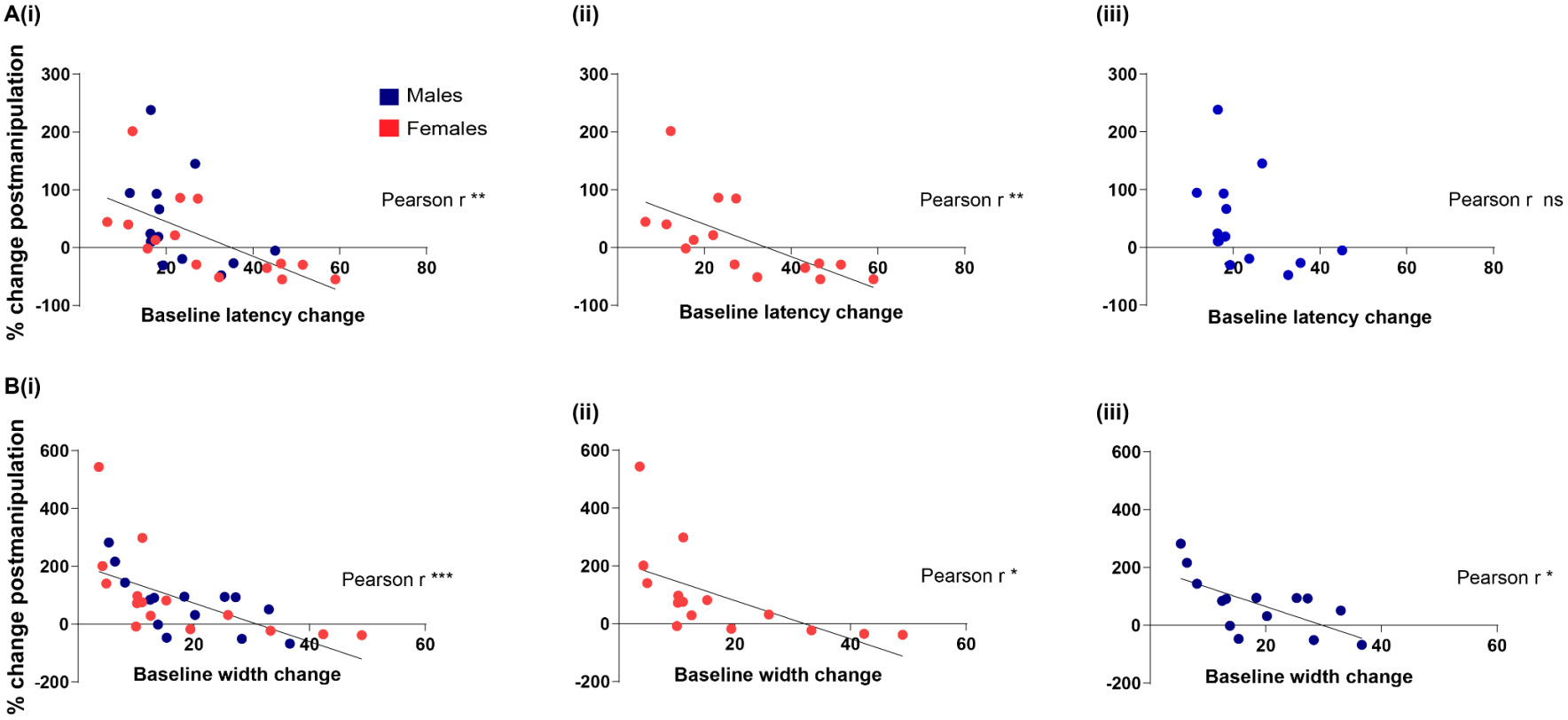

A(i)
(ii)
(iii)
% change postmanipulation
Males
Females
Pearson r **
Pearson r **
Pearson r ns
Baseline latency change
B(i)
(ii)
(iii)
Pearson r ***
Pearson r *
Pearson r *
Baseline width change

Figure 4.10 Direction and magnitude of post-manipulation ADS changes are associated with the degree of baseline ADS

Baseline C-fibre latency change AUC plotted against the post-manipulation percentage change from baseline C-fibre latency change AUC for each individual root for both sexes (**Ai**) and plotted separately for females (**Aii**) and males (**Aiii**). The correlation analysis between the two factors for the C-fibre latency change in both sexes revealed a significant negative association between baseline C-fibre latency change and post-manipulation percentage change from baseline C-fibre latency change (Pearson r=-0.548, r2=0.3, **p=0.002). The separate female latency change data (**Aii**) showed significant negative association between baseline C-fibre latency change and post-manipulation percentage change from baseline C-fibre latency change (Pearson r=-0.653, r2=0.427, **p=0.008). The separate male latency change data (**Aiii**) showed no significant association between baseline C-fibre latency change and post-manipulation percentage change from baseline C-fibre latency change (Pearson r=-0.395, r2=0.156, p=0.162). Baseline C-fibre width change AUC plotted against the post-manipulation percentage change from baseline C-fibre width change AUC for each individual root for both sexes (**Bi**) and plotted separately for females (**Bii**) and males (**Biii**). The correlation analysis between the two factors for the C-fibre width change in both sexes revealed a significant strong negative association between baseline C-fibre width change and post-manipulation percentage change from baseline C-fibre width change (Pearson r=-0.617, r2=0.381, ***p=0.0004). The separate female width change data (**Bii**) showed significant negative association between baseline C-fibre width change and post-manipulation percentage change from baseline C-fibre width change (Pearson r=-0.597, r2=0.357, *p=0.019). The separate male width change data (**Biii**) also showed significant negative association between baseline C-fibre width change and post-manipulation percentage change from baseline C-fibre width change (Pearson r=-0.661, r2=0.437, *p=0.010). Latency/width data: both sexes (n=29), females (n=15), males (n=14).

4.5.2 Electrophysiological properties and C-fibre ADS of mouse dorsal roots in both sexes.

In order to investigate C-fibre ADS and dynamic memory it was first important to determine the activation thresholds and the conduction velocities of Aβ, Aδ, and C-fibres and the optimal stimulation intensity for maximal C-fibre response amplitude in CAPs recorded from dorsal roots isolated from adult male and female mice. These data allowed the identification of appropriate stimulation intensifies to activate the different primary afferent components and this information was used in subsequent dorsal root evoked EPSC (eEPSC) patch clamp recordings in spinal slice studies (see **Chapter 5**). In addition, these data also allowed the identification of appropriate stimulation intensifies to recruit maximal number of C-fibres, which were used in subsequent C-fibre ADS CAP studies and in subsequent eEPSC studies (see **Chapter 5**).

It has been well established that the main three primary afferent components can be differentiated based on their activation threshold (Aβ < Aδ < C) and conduction velocity (Aβ > Aδ > C) (Meyer et al., 2006), as demonstrated in **Figure 4.3 and Figure 4.2A**. At maximum Aβ fibre stimulation intensity (~15μA) only a single component with a characteristic triphasic response is visible in the CAP recordings from dorsal roots from mice (**Figure 4.3**). Since this is the first component following the stimulus artefact, it is the fastest conducting and can be thus considered to be the Aβ fibre component (**Figure 4.3**). With increasing stimulation intensity, the activation thresholds for the Aδ and C-fibres are reached at ~20μA and ~40μA, respectively, when a second (Aδ) and then third (C) slower component first become identifiable, with maximal Aδ and C-fibre responses at ~40μA and ~300μA, respectively (**Figure 4.3**). The activation thresholds for the different primary afferent components in mice were significantly different, with no significant effect of sex and no interaction between sex and fibre type (**Figure 4.11A***;* two –way ANOVA: fibre type, $p<0.0001$, sex, p=0.847, sex x fibre type, p=0.115). Interestingly, the here presented activation thresholds for adult mice were lower than the activation thresholds for juvenile rats reported in previous chapters, with CAP recordings conducted in identical fashion

and under the same conditions (**Table 4.1**). There was also a significant effect of fibre type on the conduction velocity of the primary afferents with no significant effect of sex and no significant interaction between the two factors (**Figure 4.11B***;* two –way ANOVA: fibre type, $p<0.0001$, sex, p=0.390, sex x fibre type, p=0.390). The mouse C-fibre conduction velocity was similar to the C-fibre conduction velocity in juvenile rats reported in previous chapters, with CAP recordings conducted in similar fashion and under similar conditions (**Table 4.1**). Since the similarly conducted CAP studies in rat described in this thesis did not aim to investigate the properties and function of other primary afferents fibres other than the C-fibres, the conduction velocity of the Aβ and the Aδ primary afferents was not measured. Thus to compare the conduction velocity of the mouse A fibres to that of juvenile rat A fibres, with CAP recordings conducted in the same manner under the same conditions, the mouse A fibre conduction velocity data was compared to previous conduction velocity data from the lab, conducted in juvenile rat under the same conditions (Dickie, 2014). Similarly to the activation thresholds, the conduction velocities for the Aβ and the Aδ primary afferents in adult mice were lower that the previously reported in the lab Aβ and Aδ fibres conduction velocities in juvenile rats (**Table 4.1**). In order to identify the stimulation intensity for maximal C-fibre response amplitude to allow the selection of appropriate stimulation intensifies to recruit maximal number of C-fibres for subsequent C-fibre ADS studies, a stimulus-response curve for C-fibre amplitude in male and female mice was generated (**Figure 4.11Ci**). It demonstrates the progressive increase in C-fibre response amplitude with increasing stimulus intensity until a plateau is reached at ~200-300μA, after which the C-fibre amplitude starts to decrease (**Figure 4.11Ci**). AUC analysis of the stimulus response curve in males and females revealed no significant effect of sex on the C-fibre response amplitude across the different stimulation intensities applied (**Figure 4.11Ci**; unpaired two-tailed t-test, $p=0.877$).

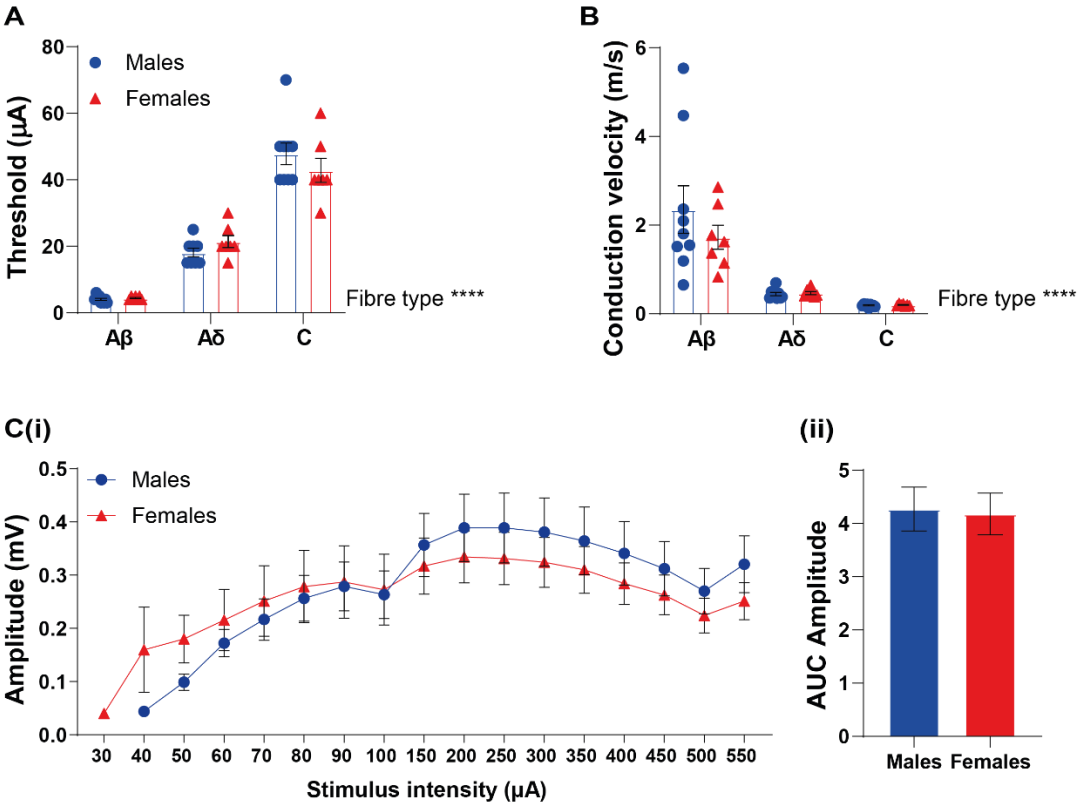


Figure 4.11 Basic electrophysiological properties of primary afferent fibres in population CAP recordings from adult mouse dorsal roots from both sexes

A) The primary afferent fibre activation thresholds in mice were significantly different based on afferent fibre type in both sexes, with no significant effect of sex and no significant interaction between sex and fibre type (Two-way ANOVA: fibre type, ****p<0.0001, sex, p=0.847, sex x fibre type, p=0.115). **B**) The primary afferent fibre conduction velocities were significantly different based on afferent fibre type in both sexes, with no significant interaction between sex and fibre type (Two –way ANOVA: fibre type, p<0.0001, sex, p=0.390, sex x fibre type, p=0.390). **Ci**) A stimulus-response curve for C-fibre amplitude in male and female mice showing maximal C-fibre response amplitude at 200-300µA. **Cii**) AUC analysis of the stimulus response curve in males and females showing no significant effect of sex on the C-fibre response amplitude across the different stimulation intensities (Unpaired two-tailed t-test, p=0.877). Data presented as mean±SEM. Males (n=9), females (n=7).

Table 4.1 Summary of the primary afferent fibre activation thresholds and conduction velocities in adult mice and juvenile rats

		Threshold (µA)		Average conduction velocity (m/s)	
	Fibre type	Mean	Range	Mean	Range
Mice	Aβ	4.19	3-6	2.08	0.65-5.54
	Aδ	19.67	15-30	0.46	0.35-0.7
	C	45.63	30-70	0.2	0.13-0.24
Rats	Aβ	6.88	5-10	*4.6**	*4.1-5.1***
	Aδ	52.5	20-80	*0.9**	*0.8-1.0***
	C	137.5	70-300	0.24	0.17-0.3

*denotes data from previous studies in the lab (Dickie, 2014) ** marks SEM instead of range

4.5.3 C-fibre ADS in mice of both sexes

To assess whether repetitive stimulation at higher frequencies can induce C-fibre ADS in extracellular electrophysiological population CAP recordings from dorsal roots isolated from adult mice and whether there are sex difference in C-fibre ADS in mice, dorsal roots from male and female mice were stimulated with a train of 40x stimuli at 2Hz. This resulted in a clear progressive increase in C-fibre response latency change (**Figure 4.12**). The AUC analysis of the C-fibre latency change in both sexes revealed that the C-fibre ADS phenomenon is more pronounced in male than in female adult mice (**Figure 4.12B**: Unpaired two-tailed t-test, $p<0.0001$).

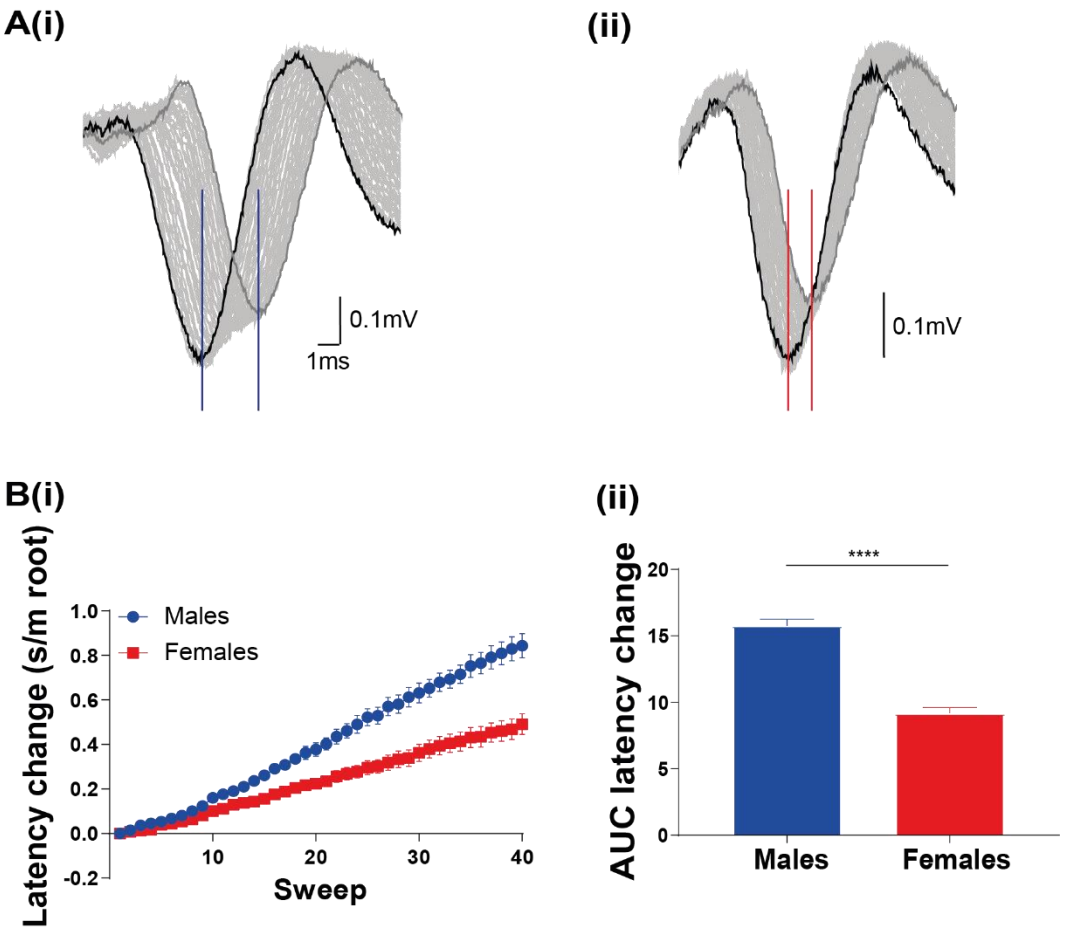


Figure 4.12 Sex differences in C-fibre ADS in mice

Representative C-fibre CAP recordings from dorsal roots isolated from male (**Ai**) and female (**Ai**) naïve mice in response to x40 stimuli at 2Hz; latency change marked by blue solid lines in males and by red solid lines in females (response trace 1 black; 2-39 pale grey; 40 dark grey). **Bi**) Repetitive stimulation results in a progressive C-fibre latency increase in dorsal roots from males and females. **Bii**) AUC analysis reveals sex-dependent difference in the degree of C-fibre latency change (****p<0.0001) with more pronounced latency change increase in males compared to females (Unpaired two-tailed t-test). Data presented as mean±SEM. Males (n=11), females (n=11).

4.5.4 Mouse C-fibre dynamic memory in both sexes.

Dynamic memory in mice was assessed by measuring C-fibre ADS following a train of x40 stimuli at 2Hz 10min before (baseline) and immediately after a prolonged low frequency stimulation, termed 'manipulation' (a train of 60stimuli for 1min at 1Hz), and following a 10min recovery period (**Figure 4.4C**). The repetitive stimulation at

2Hz resulted in a clear progressive increase in C-fibre response latency change in both males (**Figure 4.13B**) and females (**Figure 4.13C**). After applying prolonged low-frequency stimulation, the progressive increase in C-fibre latency change was enhanced post-manipulation in both sexes in comparison to baseline C-fibre latency change, with males showing more pronounced C-fibre latency change across all stimulations (**Figure 4.13C;** two –way ANOVA: manipulation, $p<0.0001$, sex, $p<0.0001$, manipulation x sex, $p=0.037$). The statistical analysis of the AUC latency change data in both sexes revealed an effect of manipulation that is dependent on sex (**Figure 4.13C;** two –way ANOVA: manipulation x sex, $p=0.037$ followed by Sidak's multiple comparisons test for males vs females - baseline, $p<0.0001$, post-manipulation, $p=0.006$, recovery, $p=0.0002$). To directly compare the effect of manipulation between the two sexes, the C-fibre latency change values at baseline were subtracted from the corresponding post-manipulation latency change values to generate 'manipulation effect' data over the 40x stimuli for each root for, with **Figure 4.13Ei** showing the manipulation effect on C-fibre latency change in males and females. The AUC analysis of the manipulation effect on C-fibre latency change revealed a significant difference between the sexes, with females displaying a more pronounced effect of manipulation than males (**Figure 4.13Eii;** Unpaired two-tailed t-test: $p=0.021$).

The association between the baseline C-fibre latency change and the direction and magnitude of the post-manipulation C-fibre latency change was investigated in mice (**Figure 4.13F**), as conducted for rat (**Figure 4.10A**). The mouse baseline C-fibre latency change AUC was plotted against the post-manipulation percentage change from baseline C-fibre latency change AUC for each individual root in both sexes (**Figure 4.13Eii**). The statistical analysis of the correlation between the two factors revealed a negative association between the two factors that was not significantly different (Pearson r=-0.631, r2=0.398, p=0.051). Given the small sample size for this correlation analysis and the *p* value being close to significance along with the results from the rat correlation analysis, it can be suggested that the mouse results follow a

similar trend of a negative association between baseline C-fibre latency change and post-manipulation percentage change from baseline C-fibre latency change.

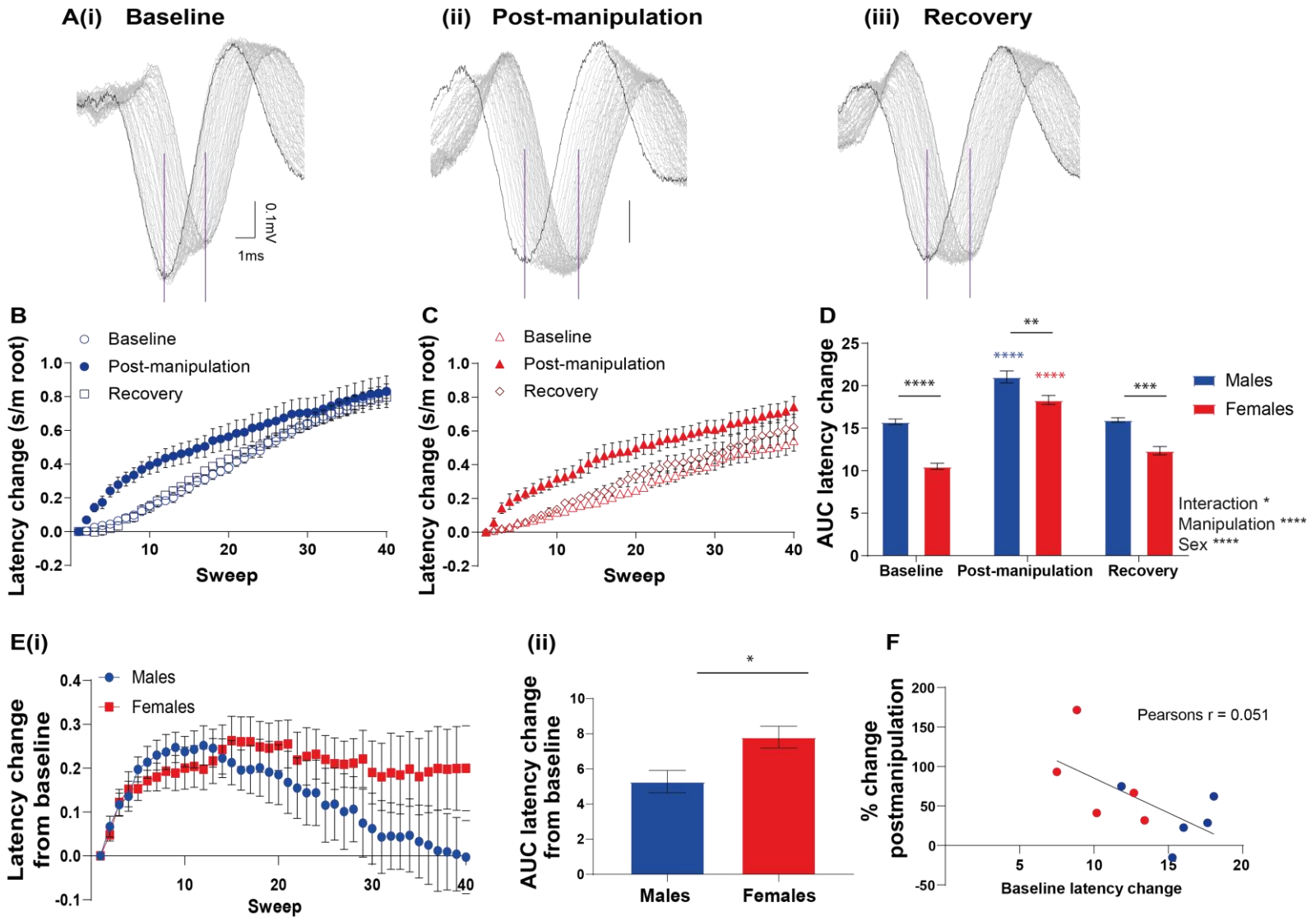

A(i) Baseline
(ii) Post-manipulation
(iii) Recovery
0.1mV
1ms
B
Baseline
Post-manipulation
Recovery
Latency change (s/m root)
Sweep
C
Baseline
Post-manipulation
Recovery
Latency change (s/m root)
Sweep
D
AUC latency change
Males
Females
Interaction *
Manipulation ****
Sex ****
Baseline
Post-manipulation
Recovery
E(i)
Males
Females
Latency change from baseline
Sweep
(ii)
AUC latency change from baseline
Males
Females
F
% change postmanipulation
Pearsons r = 0.051
Baseline latency change

Figure 4.13 Sex differences in manipulation-induced changes in C-fibre ADS

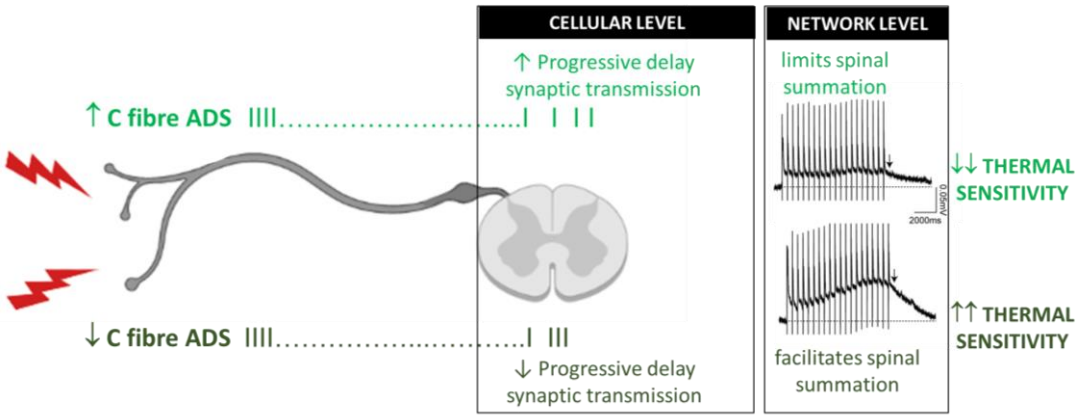
A) Representative C-fibre CAP recordings from dorsal roots isolated from naïve mice at baseline (i), post-manipulation (ii) and recovery (iii) in response to x40 stimuli at 2Hz; latency change marked by solid lines (response trace 1 black; 2-39 pale grey; 40 dark grey). Repetitive stimulation results in a progressive C-fibre latency increase in dorsal roots from males (**B**) and females (**C**). **D**) AUC analysis reveals a significant effect of manipulation (*****p*<0.0001) and sex (*****p*<0.0001), with a sex x manipulation interaction (**p*=0.037) (Two-way ANOVA followed by Sidak's multiple comparisons test, ***p*=0.006, ****p*=0.0002, *****p*<0.0001. Blue and red asterisks denote significant differences between post-manipulation latency change and latency change at baseline and recovery for males and females, respectively). **Ei**) The grouped male and female effect of manipulation data represented as the change in post-manipulation C-fibre latency change from the corresponding baseline C-fibre latency change in an individual root basis. **Eii**) AUC analysis of the effect of manipulation on C-fibre latency from baseline for males and females reveals a significant difference between the two sexes (**p*=0.021), with females showing more pronounced effect of manipulation than males (Unpaired two-tailed t-test). **F**) Baseline C-fibre latency change AUC plotted against the post-manipulation percentage change from baseline C-fibre latency change AUC for each individual root for both sexes, with correlation analysis between the two showing a negative association between the two factors that was not significantly different (Pearson r=-0.631, r2=0.398, p=0.051). Males (n=5), females (n=5).

4.6 Discussion

4.6.1 Summary findings

This chapter assessed whether prolonged low-frequency stimulation, i.e. manipulation, can alter C-fibre ADS in rats of both sexes and whether C-fibre ADS can also be observed and altered following manipulation in CAP recordings from *C57BL/6* mice of both sexes, with findings summaries in **Figure 4.14**. Following manipulation in rats, C-fibre ADS was altered, resulting in two distinct post-manipulation profiles in both sexes: an increased C-fibre ADS profile and a decreased C-fibre ADS profile. In the increased post-manipulation C-fibre ADS profile the amount of enhancement of C-fibre ADS, i.e. C-fibre dynamic memory, was comparable between the sexes. However, in the decreased C-fibre ADS profile the amount of reduction of C-fibre ADS, i.e. C-fibre dynamic memory, differed between the sexes, with a more pronounced reduction in C-fibre latency change and a less pronounced reduction in C-fibre width change in females compared to males. This suggests that in the decreased C-fibre ADS profile dynamic memory is dependent on sex and is influenced by C-fibre subtype. Interestingly, this study found that the direction and the magnitude of the manipulation-induced changes in C-fibre ADS showed strong negative association with the degree of baseline C-fibre ADS in both sexes. Additional studies in *C57BL/6* mice have confirmed that the C-fibre ADS phenomenon can be also observed in CAP recordings from adult mice in a sex-dependent manner, with male mice showing more pronounced C-fibre ADS than females. Sex, similarly to findings in rats, was not a significant factor in basic C-fibre properties in mice. However, compared to findings in rat, the primary afferent fibre activation thresholds in mice were lower than those in rats, with peak C-fibre amplitude induced by lower stimulation intensities than in rat tissue (**Table 4.1**). In mice, the prolonged low-frequency stimulation (manipulation) also altered C-fibre ADS, resulting in an increased C-fibre ADS profile post-manipulation. The amount of enhancement of C-fibre ADS, i.e. C-fibre dynamic memory, differed between the sexes with a more pronounced enhancement of C-fibre latency change in females

compared to males, suggesting that in mice C-fibre dynamic memory is dependent on sex.



		Male ADS	Female ADS	PM ADS (Dynamic memory⁺)	Heat threshold	Mechanical threshold
Naive	SD rats*	↑	↑↑	F>M	F>M	M=F
	C57BL/6 mice	↑↑	↑	F>M	M>F**	M=F**

Figure 4.14 C-fibre dynamic memory and species-dependent sex differences in C-fibre ADS levels in association with nociceptive behaviour in both sexes

↑ marks an increase; ↓ marks a decrease; M=F denotes equivalent levels in males and females ⁺for medium conducting C-fibres * Findings from Dickie et al. (2017) ** Findings from Kest et al. (1999) and Leo et al. (2008)

4.6.2 C-fibre dynamic memory in rats of both sexes

Previous studies have proposed that C-fibre ADS can provide a 'dynamic memory' of previous levels of activity that can influence response to subsequent inputs. Specifically, low level firing, comparable to injury-induced spontaneous C-fibre firing, can induce C-fibre ADS, which can dynamically influence response to higher frequency inputs (Weidner et al., 2002). The findings in this chapter show that in rats, low level firing (manipulation) can alter the post-manipulation C-fibre ADS profile, causing either an enhancement or a reduction in C-fibre ADS levels, when compared to baseline C-fibre ADS levels, with post-manipulation C-fibre ADS levels returning to levels comparable to baseline when C-fibres were allowed to recover. This property of the C-fibres to remember previous levels of activity and alter their higher frequency-induced ADS profiles suggests that the C-fibres display 'dynamic memory'. This C-fibre 'dynamic memory' was observed when C-fibre ADS was

measured as both a progressive increase in C-fibre latency and as a progressive increase in C-fibre response width. Therefore, the fact that C-fibre latency change reflects the change in average conduction velocity in the population C-fibre response, while C-fibre width change reflects the change in the range of conduction velocities within the population C-fibre response, suggests that different C-fibre subtypes, which can be differentiated based on the amount of ADS they display (Schmelz et al., 2000, Gee et al., 1996, Raymond et al., 1990, Thalhammer et al., 1994, Obreja et al., 2010, Serra et al., 1999, Weidner et al., 1999), can exhibit 'dynamic memory'.

Given that C-fibre ADS has been shown to be sex-dependent (Dickie et al., 2017) it was important to investigate whether both males and females display C-fibre 'dynamic memory' and whether its levels differed between the sexes. Separation of the data by sex revealed that both males and females displayed 'dynamic memory' to varying degrees (**Figure 4.7**). Interestingly, although in the increased C-fibre ADS profile both sexes showed similar levels of dynamic memory, measured as both latency and width change manipulation effects, in the decreased C-fibre ADS profile females showed more pronounced latency change manipulation effect and less pronounced width change manipulation effect. These findings suggest that in females with pronounced baseline C-fibre ADS, assuming baseline C-fibre ADS can predict the direction of manipulation-induced change (**Figure 4.10**), the average conducting C-fibres may display more pronounced 'dynamic memory', while the range of the slowest and fastest conducting C-fibres may display less pronounced 'dynamic memory' in comparison to males. In addition, the more pronounced latency compared to width change manipulation effects in females may suggest that in females with pronounced baseline C-fibre ADS, given that baseline C-fibre ADS can predict the direction of manipulation-induced change (**Figure 4.10**), the average conducting C-fibres may display more pronounced 'dynamic memory' in comparison to the range of the slowest and fastest conducting C-fibres. Similarly, in males the more pronounced width compared to latency change manipulation effects may suggest that the in males with pronounced baseline C-fibre ADS, given

that baseline C-fibre ADS can predict the direction of manipulation-induced change (**Figure 4.10**), the range of the slowest and fastest conducting C-fibres may display more pronounced 'dynamic memory' in comparison to the average conducting C-fibres. Thus, there could be sex-difference in dynamic memory of different C-fibre subtypes. However, in the increased C-fibre ADS profile, the similar latency and width change manipulation effects suggest that that the different C-fibre subtypes display similar levels of 'dynamic memory' in both sexes.

Interestingly, the previously reported sex difference in baseline C-fibre ADS, with more pronounced increase in females (Dickie et al., 2017), was not always observed when the data were separated into post-manipulation 'increased C-fibre ADS' and 'decreased C-fibre ADS' profiles. This, however, is not surprising given that the correlation analysis graphs for latency change (**Figure 4.10A**) reveal that some females have higher baseline ADS. Interestingly, at lower baseline ADS levels there are similar numbers of males and females. Thus, it is not surprising that this chapter showed no sex differences in baseline C-fibre ADS for the increased profile, which based on the correlation analysis is associated with lower baseline ADS levels change (**Figure 4.10**). However, in the decreased C-fibre ADS profile, which is associated with higher baseline ADS levels, there were significant sex differences, as observed by the previous studies in the lab (Dickie et al., 2017, Velichkova et al., 2021). In addition, it must be noted that when the data were separated by post-manipulation C-fibre ADS profile and by sex, the sample size in some of the groups was reduced and thus this data should be interpreted with caution.

Interestingly, when the manipulation data were separated by post-manipulation C-fibre ADS profile it was noticed that the grouped data in the increased post-manipulation C-fibre ADS profile had less pronounced baseline C-fibre ADS latency and width changes in comparison to the grouped data in the decreased post-manipulation C-fibre ADS profile. Thus, the association between the baseline ADS levels and the manipulation-induced changes in the direction and magnitude of C-fibre ADS on an individual root basis was investigated. There was a strong negative

association between baseline latency and width changes and post-manipulation changes from baseline latency and width levels for the combined male and female data. This suggests that the baseline C-fibre ADS levels can potentially predict the direction and the magnitude of the post-manipulation change in C-fibre ADS, such that high levels of C-fibre ADS at baseline predicted pronounced manipulation-induced reduction in C-fibre ADS post-manipulation. When the data were separated by sex, the significant negative association between two factors remained for all data, except for the male latency change data. However, as shown in change **Figure 4.10C**, the range of baseline ADS in males was revealed to be much narrower, emphasizing again the sex differences in C-fibre ADS. This narrow range of baseline C-fibre ADS in males likely contributed to lack of significant association between baseline ADS and the manipulation-induced changes in the direction and magnitude of C-fibre ADS. Thus, in order to explore the association between the baseline ADS levels and the manipulation-induced changes in the direction and magnitude of C-fibre ADS, i.e. C-fibre dynamic memory, future studies might need to employ bigger sample sizes in male to assess whether baseline C-fibre ADS can be used to predict the direction and the magnitude of the manipulation-induced changes in C-fibre ADS.

4.6.1 C-fibre properties and ADS in mice of both sexes

The findings in this chapter showed that the different primary afferent components in mice also display different activation thresholds (C > Aδ > Aβ) and estimated conduction velocities (Aβ > Aδ > C), similarly to previous electrophysiological findings in rats and mice (Dickie et al., 2017, Torsney, 2011, Bardoni et al., 2019, Daniele and MacDermott, 2009, Nakatsuka et al., 2000, Park et al., 1999, Meyer et al., 2006). There was no significant effect of sex on the primary afferent fibre thresholds and estimated conduction velocities, which, to my knowledge, have not previously been compared directly between the sexes in similar experimental conditions. Interestingly, the data in this chapter revealed that the activation thresholds for adult mice primary afferents were lower than the activation thresholds for juvenile rats reported in previous chapters (Velichkova et al., 2021)

(**Chapter 3**), with CAP recordings conducted in an identical manner and under the same conditions (**Table 4.1**). These were also lower than the activation thresholds for juvenile rats previously reported in the literature (Nakatsuka et al., 2000, Dickie et al., 2017). The lower activation thresholds in mice are, however, in line with those previously reported for mice in the literature (Daniele and MacDermott, 2009, Bardoni et al., 2019), which suggests that mice exhibit lower primary afferent activation thresholds compared to rats. In terms of estimated conduction velocity, the data in this chapter showed that mouse C-fibre conduction velocity is similar to the C-fibre estimated conduction velocity in juvenile rats reported in previous chapters (Velichkova et al., 2021), with CAP recordings conducted in a similar manner and under similar conditions (**Table 4.1**). In addition, mouse C-fibre conduction velocity was similar to that previously reported in rat (Torsney, 2011, Dickie et al., 2017) and mouse (Daniele and MacDermott, 2009). The mouse A fibre conduction velocities in this chapter were also similar to previously reported A fibre conduction velocities in mice of different ages (Daniele and MacDermott, 2009). However, the conduction velocities of the mouse A fibres in this chapter and in Daniele and MacDermott (2009) study were slower than the A fibre conduction velocities in previously reported rat studies, conducted in a similar manner and under similar conditions (Dickie et al., 2017, Torsney, 2011). Therefore, it is likely that the conduction velocities of the fast-conducting, lower-threshold myelinated A fibres in mice are slower than those in rats. Given the species differences in primary afferent activation thresholds and conduction velocities, this chapter also investigated stimulation intensities for maximal C-fibre response amplitude to allow the selection of appropriate stimulation intensities to recruit maximal numbers of C-fibres for subsequent population C-fibre ADS studies. In contrast to previous reports in rats, where maximal C-fibre response was triggered and studied at 500µA (Dickie et al., 2017, Torsney, 2011, Torsney and MacDermott, 2006), the mouse findings in this chapter revealed that maximal population C-fibre response amplitude was reached at ~200-300µA, which is lower than findings in rats but within the range of intensities used in other studies in mice (Bardoni et al., 2019, Daniele and

MacDermott, 2009). The fact that there were no sex differences in mouse C-fibre stimulus-response curve further supports the findings of similar C-fibre activation thresholds in this chapter and the similar maximal C-fibre responses in rats of both sexes (Velichkova et al., 2021) (**Chapter 3**).

Given that previous studies have shown that sex differences in noxious heat sensitivity in rats can be associated with sex differences in C-fibre ADS profiles (Dickie et al., 2017, Taguchi et al., 2010, Velichkova et al., 2021) (**Chapter 3**), it was important to investigate C-fibre ADS in *C57BL/6* mice, where males have been shown to display higher heat threshold (Kest et al., 1999, Leo et al., 2008), similar to the observed sex differences in humans (Mogil, 2012). In line with these findings, the reported in this chapter more pronounced C-fibre latency change in male *C57BL/6* mice is consistent with the behavioural findings of higher heat thresholds in male *C57BL/6* mice (Kest et al., 1999, Leo et al., 2008). Given that sex differences in noxious heat sensitivity have been shown to be species- and strain-dependent (Mogil et al., 2000), the sex differences in C-fibre ADS reported in this chapter provide further support for a possible role of ADS in C-fibres relaying heat pain signals to the spinal cord, which could potentially influence spinal cord processing of heat pain signals and thus heat pain sensitivity.

4.6.2 C-fibre dynamic memory in mice of both sexes

Taking into account that C-fibre 'dynamic memory' is dependent on ADS, with the amount of previous slowing determining the level of 'dynamic memory' (Weidner et al., 2002), it was important to explore whether *C57BL/6* mice, similarly to SD rats, also display dynamic memory and whether it is sex-dependent, given the findings of sex (Dickie et al., 2017, Velichkova et al., 2021)(**Chapter 3**) and species (**section 4.5.3**) differences in C-fibre ADS. The prolonged low frequency stimulation (manipulation) in mice resulted in a distinct increased post-manipulation C-fibre ADS profile, unlike findings in rats, where the manipulation caused two distinct C-fibre ADS profiles – an increased and a decreased C-fibre ADS (**section 4.6.2**). The absence of observed manipulation-induced decreased C-fibre ADS profile in mice

can be explained by the more than three times smaller sample size of the mouse data compared to the rat data. Also, the slight differences in the manipulation protocols between the two species could have attributed to the differences in the observed post-manipulation C-fibre ADS profiles between mice and rats. In addition, the fact that C-fibre ADS in mice was measured as a change in the progressive increase in C-fibre latency only, without measuring the width change in the C-fibre response, could have also contributed to the decreased probability of reporting manipulation–induced decrease in C-fibre ADS. In contrast to the manipulation-induced increase in C-fibre latency change in rats, where there was no significant effect of sex on C-fibre latency change, the manipulation-induced increase in C-fibre latency change in mice showed an effect of sex, with males showing significantly more pronounced baseline C-fibre latency change. Interestingly, the effect of the manipulation in mice was dependent on sex, with further analysis of sex differences in latency change manipulation effect, i.e. 'dynamic memory' revealing that females displayed more pronounced dynamic memory in comparison to males. This is in contrast to the 'increased C-fibre ADS' rat profile where both sexes exhibited similar levels of dynamic memory. This species-dependent sex difference in dynamic memory is not surprising given that dynamic memory has been shown to depend on baseline ADS (**Figure 4.10**) and this chapter also revealed sex differences in baseline C-fibre ADS that is different between SD rats and *C57BL/6* mice. It must be noted that in male mice there was an initial progressive increase in latency change manipulation effect, i.e. 'dynamic memory', up to ~stimulus 12, after which there was a progressive decrease in latency change manipulation effect, which by the last stimulus of the train of 40x stimuli returned to ~0 latency change manipulation effect. This suggests that 'dynamic memory' in male mice could be short-lasting. In contrast to males, in female mice the latency change manipulation effect, i.e. 'dynamic memory', increased progressively up to ~stimulus 12, when it reached levels similar to males and plateaued, but unlike in males, in females the 'dynamic memory' levels remained stable throughout the remainder of the stimuli, suggesting that 'dynamic memory' in females is longer-

lasting. The findings of negative association between baseline C-fibre latency change levels and manipulation-induced changes in the direction and magnitude of C-fibre latency change in mice further support the findings in rats. Although in mice the association between the two variables was not statistically significant, it must be noted that the sample size was small for conducting a correlation analysis. Thus, the fact that the *p* value for the Pearsons correlation test was very close to significance, suggests that in mice it is also likely that baseline C-fibre ADS levels can potentially predict direction and magnitude of the manipulation-induced changes in C-fibre ADS. However, to confirm the association between baseline C-fibre ADS and manipulation-induced changes in the direction and magnitude of C-fibre ADS in mice, it would be important that future studies employ bigger sample sizes. In addition, it would be interesting to investigate if this association is sex-dependent, so it would be important to conduct the experiments in a big enough sample that could allow correlation analysis based on sex.

Given that C-fibre 'dynamic memory' is dependent of C-fibre ADS, with the amount of previous slowing determining the level of dynamic memory (Weidner et al., 2002) along with the reported in this chapter correlation between baseline ADS and C-fibre 'dynamic memory', it can be hypothesised that the sex- and species-dependent differences in C-fibre 'dynamic memory' might be associated with the reported sex- and species-dependent differences in levels of C-fibre ADS (Dickie et al., 2017, Velichkova et al., 2021) (**Chapter 3**; **Figure 4.12**). Thus, the sex- and species-dependent differences in C-fibre 'dynamic memory' can potentially involve the ion channels implicated in C-fibre ADS, such as the Na_V ion channels and/or the HCN channels, as discussed in **section 1.7.2.2**.

4.6.3 Limitations

One of the limitations of the work done in this chapter is the small sample size of some of the experimental groups. The experiments investigating C-fibre dynamic memory in rats had a bigger sample size, but the fact that the manipulation resulted in two distinct post-manipulation profiles for both males and females required the

data to be separated not only by sex but also by manipulation profile. Therefore, the male and female data in the decreased post-manipulation C-fibre ADS profile, which was observed less often, had a smaller sample size. Sample size was of particular importance for the correlation analysis of the association of baseline C-fibre ADS and the manipulation-induced changes in the direction and magnitude of C-fibre ADS. This was clear when the data were separated by sex and the baseline latency change in males was revealed to cluster around similar levels of baseline ADS. This was thought to contribute to the statistical results of the Pearsons correlation test, which showed a *p* value close to significance, but the study was found to be underpowered, with power analysis (G*Power 3.1, Universität Kiel, Germany) revealing that a sample size of 33 would be able to provide 80% power to detect the association. The correlation analysis of the association of baseline C-fibre ADS and the manipulation-induced changes in the direction and magnitude of C-fibre ADS in mice had the same limitation. The dataset was also shown to be underpowered, with power analysis (G*Power 3.1, Universität Kiel, Germany) showing that a sample size of 23 would be sufficient to provide 80% power to detect significant association of baseline C-fibre ADS and the manipulation-induced changes in the direction and magnitude of C-fibre ADS. The fact that the low frequency manipulation in mice resulted in an increased post-manipulation C-fibre latency change profile only, further emphasises the need to increase the sample size, because if the sample size is increased that would increase the probability of detecting a decreased post-manipulation C-fibre latency change profile if it was also happening in mice. Thus, it would be essential to conduct the C-fibre dynamic memory experiments in adequately powered studies for both mice and rats.

This study concluded that low-frequency stimulation is able to cause changes in subsequent higher-frequency-induced C-fibre ADS, i.e. is able to induce ‘dynamic memory’ in both rats and mice of both sexes, with levels of baseline C-fibre ADS likely determining the direction and the magnitude of the change in post-manipulation C-fibre ADS compared to baseline C-fibre ADS. However, given that previous studies have shown that low-level firing induced-ADS can dynamically

influence response to higher-frequency inputs (Weidner et al., 2002), it would be important for future studies to investigate the levels of C-fibre ADS during the prolonged low-frequency manipulation and how they relate to the post-manipulation and baseline C-fibre ADS.

In addition, for the experiments investigating C-fibre ADS in mice it would be important to assess C-fibre ADS by measuring the progressive increase in C-fibre repose width, similarly to what has been done for the rat data, but due to time limitations this was not undertaken for the C-fibre dynamic memory experiments in mice. Given the fact that C-fibre width change reflects the range of C-fibre conduction velocities within the population C-fibre response, it would be interesting to investigate whether there are manipulation-induced changes in the progressive increase in C-fibre width and whether those are also sex-dependent. Moreover, assessing C-fibre ADS as changes in both C-fibre repose width and latency will allow the comparison of manipulation-induced changes in different C-fibre subtypes and will provide additional information for the dynamic memory of different C-fibre subtypes and whether these are dependent on sex. It must be also noted that assessing C-fibre ADS by measuring the C-fibre width change would increase the probability of detecting a decreased post-manipulation C-fibre ADS profile if, similarly to rats, it is also characteristic of mice. It could be possible that in mice, unlike in rats, the manipulation-induced decrease in C-fibre ADS is more commonly observed for the range of the fastest and slowest conducting C-fibre, reflected by the width measure of the C-fibre response. Thus, it would be essential to investigate the low-frequency manipulation-induced changes in C-fibre ADS in mice, measured as changes in C-fibre response width.

The chapter also reported species differences in C-fibre 'dynamic memory' (post-manipulation induced C-fibre ADS), with rats displaying either an increased post-manipulation increased C-fibre ADS profile or a decreased one, unlike mice which exhibited only an increased post-manipulation C-fibre ADS profile, and rats displaying no sex differences in the latency change manipulation effect, i.e 'dynamic

memory', in the increased post-manipulation C-fibre ADS profile , which was in contrast to findings in mice, where females displayed more pronounced latency change manipulation effect, i.e 'dynamic memory'. These species differences in C-fibre 'dynamic memory' likely reflect the demonstrated in this chapter species differences in the sex-dependent C-fibre ADS. However, there were slight differences in the manipulation protocols between species (**Figure 4.4**), which might have contributed to the observed species differences in 'dynamic memory'. It is possible that the more prolonged manipulation train in rats (2mins), induced more pronounced changes in the C-fibres, which contributed to distinguishing two distinct post-manipulation C-fibre ADS profiles in rats. The brief manipulation train in mice (1min) may not have been long enough to trigger change in C-fibres that can result in two distinct post-manipulation C-fibre ADS profiles. It must be also noted that the stimulation frequencies at which C-fibre ADS was assessed were different between the two species, with higher frequencies used to assessed C-fibre ADS in rats. Preliminary data in mice showed that the stimulation frequencies used in rat did induced reliable manipulation changes in ADS and therefore different stimulation frequencies were used in mice. However, the combination of more prolonged manipulation train and higher frequencies used to assess C-fibre ADS in rats could have both potentially contributed to the observed species differences in C-fibre 'dynamic memory'. Therefore, it would be important for future studies to assess C-fibre 'dynamic memory' using exactly the same manipulation protocols in both rats and mice of both sexes.

4.7 Conclusion

The results in this chapter have shown that prolonged low-frequency stimulation, i.e. manipulation, can alter C-fibre ADS resulting in two post-manipulation profiles in rats of both sex: an increased and a decreased manipulation profiles. The amount of change in C-fibre ADS post-manipulation from baseline, i.e. dynamic memory, was dependent on sex and differed between different C-fibre subtypes. Interestingly, the baseline levels of C-fibre ADS in both sexes were demonstrated to

be predictive of the direction and the magnitude of C-fibre 'dynamic memory' (of the manipulation-induced changes in C-fibre ADS).

In addition, adult *C57BL/6* mice, similar to juvenile SD rats, were also shown to display C-fibre ADS in a sex-dependent manner, with a more pronounced C-fibre ADS in males, in striking contrast to findings in SD rats. Sex, however, was not a significant factor in basic C-fibre properties in mice, similarly to findings in juvenile rats. However, there were species-dependent difference, with lower primary afferent fibre activation thresholds and maximal C-fibre stimulus intensity in mice compared to rats. Furthermore, mice exhibited sex differences in manipulation-induced changes in C-fibre latency change, with more pronounced 'dynamic memory' in females.

The fact that the demonstrated sex-dependent C-fibre ADS in mice is consistent with previously established behavioural findings of higher heat thresholds in males (Kest et al., 1999, Leo et al., 2008), contrary to findings in SD rats, where females show more pronounced C-fibre ADS in line with higher heat thresholds (Dickie et al., 2017), further supports the hypothesis that sex-dependent differences in C-fibre ADS can influence spinal heat pain processing , potentially leading to sex differences in heat pain sensitivity.

Chapter 5

Spinal impact of C-fibre ADS and dynamic memory in mice of both sexes

5.1 Chapter summary

- Capsaicin injection *in vivo* lead to Fos-EGFP expression in spinal cord neurons with capsaicin sensitive inputs
- Natural noxious heat stimulus also induces Fos-EGFP expression in spinal cord neurons, including superficial dorsal horn neurons with monosynaptic C-fibre input, displaying ADS. Proportion of these Fos-EGFP expressing spinal cord neurons were used to test capsaicin sensitivity to confirm that they have heat-capsaicin sensitive inputs.
- The Fos-EGFP heat and WT capsaicin sensitive spinal cord neurons displayed sex differences in ADS in monosynaptic C-fibre input, with males showing significantly more pronounced ADS in the monosynaptic C-fibre inputs. These results are in line with the CAP findings where there is more pronounced C-fibre ADS in male compared to female mice.
- The Fos-EGFP heat and WT capsaicin sensitive spinal cord neurons also showed sex differences in action potential firing following a train of stimuli, with females showing initial more pronounced firing and more pronounced wind-down phenomenon.
- Prolonged low-frequency stimulation increased the number of input failures and also increased ADS in monosynaptic C inputs to Fos-EGFP heat and WT capsaicin sensitive spinal cord neurons in both sexes.
- Using the length-dependency of ADS, it was demonstrated that stimulation distance alters ADS in monosynaptic C-fibre input to the Fos-EGFP heat and WT capsaicin sensitive spinal cord neurons to similar extent in both sexes. Stimulation distance also altered action potential firing following a train of stimuli in both sexes and influenced the degree of wind-down in females only.
- Stimulation distance did not affect the initial amplitude and the change in amplitude following a train of stimuli in monosynaptic C-fibre inputs to the Fos-EGFP heat and WT capsaicin sensitive spinal cord neurons in both sexes.
- Stimulation distance also had no impact on the initial net charge and the change in net charge following a train of stimuli in monosynaptic C-fibre inputs to the Fos-EGFP heat and WT capsaicin sensitive spinal cord neurons in both sexes.

5.2 Introduction

5.2.1 C-fibre ADS

Repetitive firing of C-fibre nociceptors is known to drive central sensitisation and plasticity (Latremoliere and Woolf, 2009, Treede and Magerl, 2000). While mechanical hypersensitivity likely reflects behaviour driven by A fibre activation of spinal pain circuits, sensitised by repetitive C-fibre firing, thermal hypersensitivity has been postulated to reflect the altered nociceptive C-fibre function (Koerber et al., 2010, Lenoir et al., 2018, Uta et al., 2019, Hsieh et al., 2015, Kobayashi et al., 2005, Taguchi et al., 2010, Ziegler et al., 1999, Latremoliere and Woolf, 2009). C-fibre nociceptors have been associated with the slow, burning, poorly localised pain (Ochoa and Torebjörk, 1989, Magerl et al., 2001). They are thought to signal ongoing tissue injury (Craig, 2003), unlike the A fibre nociceptors, which are proposed to signal acute tissue damage, associated with well-localised pain (Ochoa and Torebjörk, 1989, Magerl et al., 2001), and are thus suggested to have more of a protective function (Cervero, 2009). Since the early findings suggesting that the firing frequency in afferent fibres in the somatosensory system encodes intensity of a sensation (Adrian and Zotterman, 1926b, Adrian, 1926, Adrian and Zotterman, 1926a), studies in humans have directly demonstrated that the firing frequency of the C-fibre nociceptors can encode pain intensity, with stronger noxious stimuli causing shorter intervals between successive action potentials in nociceptive C-fibres and more intense pain (Torebjörk et al., 1984, Yarnitsky and Ochoa, 1990). Interestingly, the C-fibre activity-dependent slowing (ADS) phenomenon, whereby repetitive stimulation at high frequencies (≥1Hz) causes a progressive slowing of action potential conduction velocity (Gee et al., 1996, Thalhammer et al., 1994), has been suggested as a potential regulator of the timing of successive action potentials being relayed along nociceptor axons and reaching the spinal cord, where the processing of nociceptive information occurs. C-fibre ADS manifests as a progressive increase in C-fibre response latency, which has been shown in both human (Weidner et al., 1999, Serra et al., 1999) and animal studies (Gee et al., 1996, Thalhammer et al., 1994). Levels of C-fibre ADS has been shown to dependent on

stimulation frequency and stimulation length, with more pronounced ADS occurring at higher frequencies (Weidner et al., 1999, Serra et al., 1999, Gee et al., 1996, Thalhammer et al., 1994, Dickie et al., 2017) and over longer lengths (Zhu et al., 2009, Schmelz et al., 1995, Dickie et al., 2017). Thus, given that C-fibre ADS occurs at both evoked (↑~20Hz) and spontaneous (≤2Hz) firing ranges (Thalhammer et al., 1994, Djouhri et al., 2006) and in response to not only electrical but also to noxious thermal stimulation (Thalhammer et al., 1994), it could influence the timing of action potentials reaching the spinal cord and thus impact the spinal processing of noxious stimuli and the ongoing C-fibre activity that drives central sensitisation (Latremoliere and Woolf, 2009, Treede and Magerl, 2000).

5.2.2 C-fibre ADS can potentially influence noxious heat sensitivity

My thesis findings (Velichkova et al., 2021) (**Chapter 3, 4**) and prior studies from the lab (Dickie et al., 2017) have suggested that C-fibre ADS can influence noxious heat sensitivity of the flexion-withdrawal reflex in a sex- and injury-dependent manner. A more pronounced C-fibre ADS profile in juvenile naïve female rats was shown to be consistent with their higher noxious heat threshold (Dickie et al., 2017). Interestingly, following CFA inflammation both sexes were demonstrated to display comparable noxious heat thresholds, which was in line with the comparable post-inflammation C-fibre ADS profiles in both sexes (Dickie et al., 2017). Moreover, following hindpaw incision-induced postoperative pain juvenile female rats displayed an overall more pronounced C-fibre ADS profile (**Figure 2.12**; **Figure 2.13**) (Velichkova et al., 2021), which was consistent with the less pronounced peak heat hypersensitivity in females post-incision (**Figure 2.21**) (Velichkova et al., 2021). Further studies investigating the effects of the diabetic pain-associated metabolite methylglyoxal (MG) on C-fibre ADS in *ex vivo* dorsal root preparations, have shown that in line with the MG-induced reduction in C-fibre ADS in males and pronounced enhancement in females (**Figure 3.8**; **Figure 3.9**), MG induced thermal hypersensitivity in males only (**Figure 3.19**). Interestingly, sex-differences in thermal pain sensitivity have been shown to be strain- and species-dependent (Mogil et al.,

2000), with male *C57BL/6* mice shown to have higher noxious heat thresholds (Leo et al., 2008, Kest et al., 1999), similar to the sex difference observed in humans (Mogil, 2012), but opposite to findings in Sprague-Dawley rats (Mogil et al., 2000, Dickie et al., 2017). In line with the reported higher noxious heat threshold in male *C57BL/6* mice, the results in this chapter revealed a more pronounced C-fibre ADS profile in male compared to female *C57BL/6* mice (**Figure 4.12**). These findings suggest that C-fibre ADS can potentially influence in a sex- and injury-dependent manner the noxious heat drive to spinal pain circuits.

5.2.3 C-fibre ADS regulates timing of pain signals delivered to the spinal cord and can influence spinal summation

The injury- and sex-dependent changes in ADS demonstrated by Dickie et al. (2017) have been shown to be present in monosynaptic C-fibre inputs to lamina I NK1R+ output neurons, known to process noxious thermal inputs (Allard, 2019, Andrew, 2009, Bester et al., 2000). These changes have been also demonstrated to alter the timing of synaptic transmission of the monosynaptic C-fibre inputs to the lamina I NK1R+ output neurons (Dickie et al., 2017), which process noxious thermal inputs (Allard, 2019, Andrew, 2009, Bester et al., 2000) and are crucial for plasticity (Ikeda et al., 2006, Torsney, 2011, Li and Baccei, 2016, Ikeda et al., 2003). In addition, utilising different stimulation lengths to experimentally manipulated levels of ADS using *ex vivo* dorsal root-ventral root potential recordings it has been demonstrated that C-fibre ADS can influence spinal summation, with longer stimulation lengths (increased ADS) limiting spinal summation, which is consistent with the more pronounced C-fibre ADS profile in females, which also displayed higher noxious heat, but not mechanical, thresholds (Dickie et al., 2017). Given that juvenile naïve female rats displayed pronounced C-fibre ADS and higher noxious heat threshold, also observed by others in the same rat strain (Mogil et al., 2000), it has been suggested that C-fibre ADS can influence heat nociceptive drive to spinal circuits involved in noxious heat sensation. Interestingly, in line with the proposed sex-dependent ADS regulation of spinal summation relevant for noxious heat sensation,

previous studies have also reported sex differences in temporal summation of thermal pain in humans (Fillingim et al., 2009). Thus, in order to specifically investigate the impact of C-fibre ADS at an individual spinal neuron level an activity marker transgenic strategy was employed to identify noxious heat responsive spinal neurons to enable study of the impact of C-fibre ADS on synaptic transmission and spinal neuron activity.

5.2.1 Fluorescent reporters of neuronal activity (Fos-EGFP mice)

Noxious heat responsive neurons for studying the impact of C-fibre ADS on synaptic transmission and spinal neuron activity were identified using an activity marker transgenic strategy employing *c-fos* expression. Immediate early genes (IEGs), such as *c-fos*, can be used as markers of neuronal activity because they can be induced very rapidly (within minutes) (Greenberg et al., 1986) in response to neuronal activity usually triggered by external stimuli. *C-fos* encodes a transcription factor that can regulate the activity of different effector genes and also drive the transcription of engineered fluorescence-based reporters, such as enhanced green fluorescent protein (EGFP), which produces detectable fluorescence in living cells without need for signal amplification (Barth et al., 2004). Expression of *c-fos* is a commonly used method for identification of neurons activated by a wide array of somatosensory stimuli, including noxious thermal, mechanical and chemical stimuli (Jinks et al., 2002, Yi and Barr, 1995, Cavanaugh et al., 2009, Gutierrez-Mecinas et al., 2017). *C-Fos* has been used as a marker for neuronal activation since 1987, when it was first used to map seizure-induced neuronal activation in the brain (Morgan et al., 1987). Early studies of Fos expression in the rat spinal cord have shown that it can be induced by both low- and high-threshold sensory stimuli, with very low baseline expression levels (Hunt et al., 1987), but pronounced expression levels following high-threshold noxious stimulation. Given that Fos is a specific, rapid, and robust marker for neuronal activation after noxious stimulation and tissue injury, it has been widely used tool for studying of neural correlates of nociception (Harris, 1998) and different fos-based techniques have been used to

explore spinal circuits involved in pain processing (Coggeshall, 2005). Similarly, the studies in this chapter employed the hemizygous FosEGFP mouse line (Jackson Laboratory), made in the Barth lab that allows the identification of recently activated neurons without destroying cell viability and with no reported side effects related to the transgenic modification (Barth et al., 2004). These Fos-EGFP mice were used to identify recently activated neurons by a noxious stimulus in an *ex vivo* preparation that could be employed in electrophysiological studies to facilitate studying the impact of C-fibre ADS upon spinal neurons sensitive to a specific stimulus.

5.3 Aims and hypothesis

5.3.1 Hypothesis

Fos-EGFP heat and WT capsaicin sensitive neurons will display sex differences in ADS in monosynaptic C-fibre input, with males showing significantly pronounced ADS. ADS in monosynaptic C-fibre input will influence the activity of Fos-EGFP heat or/ WT capsaicin sensitive neurons in a sex-dependent manner. Prolonged low-frequency stimulation will alter ADS in monosynaptic C-fibre inputs to Fos-EGFP heat and WT capsaicin sensitive neurons and will affect their activity in a sex-dependent manner. ADS in monosynaptic C-fibre inputs to heat-capsaicin sensitive spinal neurons will be length-dependent with more ADS at longer stimulation lengths. The stimulation distance-induced changes in ADS levels in monosynaptic C-fibre inputs will differentially alter the activity of heat-capsaicin sensitive spinal neurons.

5.3.2 Aims

- Assess whether an *in vivo* intraplantar stimulation with capsaicin, an agonist of the ligand- and heat-sensitive TRPV1 ion channel, expressed primarily in C-fibres, and with noxious heat stimulation can induce Fos-EGFP expression in spinal neurons with capsaicin sensitive inputs in Fos-EGFP mice.

- Investigate, using patch-clamp evoked excitatory post-synaptic current (eEPSC) and evoked excitatory post-synaptic potential (eEPSP) recordings, whether there are sex differences in ADS in monosynaptic C-fibre inputs to Fos-EGFP heat and WT capsaicin sensitive spinal neurons and whether the activity of the Fos-EGFP heat neurons is also altered in a sex-dependent manner.
- Assess whether prolonged low-frequency stimulation can be used as a tool to manipulate ADS in monosynaptic C-fibre inputs to and affect the activity of heat-capsaicin sensitive neurons in a sex-dependent manner.
- Access whether ADS in monosynaptic C-fibre inputs to Fos-EGFP heat and WT capsaicin sensitive spinal neurons is dependent on stimulation length and whether the stimulation distance-induced changes in ADS levels in monosynaptic C-fibre inputs can differentially alter the activity of Fos-EGFP heat and WT capsaicin sensitive spinal neurons in mice of both sexes

5.4 Methods

5.4.1 Ethical and legal considerations

As described in section 2.4.1.

5.4.2 Animals and general husbandry

As described for mice in section 4.4.2.

5.4.3 Identification of noxious heat or capsaicin responsive spinal neurons in Fos-EGFP mice

In these studies hemizygous Fos-EGFP mice of both sexes were used to enable the selective targeting of noxious heat sensitive neurons for electrophysiological investigation *ex vivo* (Hunt et al., 1987). As previously established*, in vivo* intraplantar capsaicin injections and hot water bath hindpaw immersion, applied

under anaesthesia, were used to selectively activate spinal neurons receiving TRPV1 expressing inputs and noxious heat sensitive inputs, respectively. This pre-identification of spinal neurons allowed for targeted patch-clamp recordings in spinal cord slices.

5.4.3.1 Capsaicin injections

To investigate whether the *in vivo* intraplantar capsaicin stimulus can induce Fos-EGFP expression in spinal neurons, capsaicin injections were administered subcutaneously into the plantar surface of the left hindpaw (0.25mg/ml, 10µL, Sigma, **Figure 5.1**) of 5-7week old Fos-EGFP mice, briefly anaesthatised with inhlation of isoflurane. Animals were allowed to recover from the anaesthesia and 2 hours later, when c-Fos expression has been shown to be maximal (Bullitt, 1990) animals were terminated to allow preparation of *ex vivo* spinal cord slices as described in **section 5.4.4.1**, except all dorsal roots were removed prior to embedding the spinal cord in agar. Whole-cell patch-clamp mEPSC recordings were conducted approximately 4hours after the intraplantar capsaicin injections.

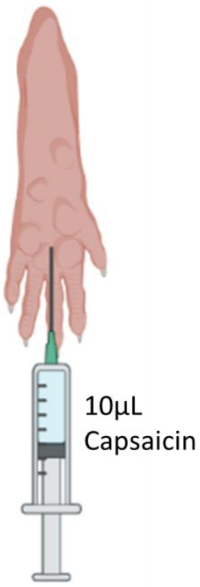


Figure 5.1 Hindpaw injection with capsaicin, showing injection site

5.4.3.2 Hot (52°C) water bath stimulation

To investigate whether *in vivo* noxious heat stimulation can induce Fos-EGFP expression in spinal neurons, a noxious heat stimulus was applied under isoflurane-induced anaesthesia. This was done by immersing one hindpaw of 5-7week old Fos-EGFP mice in 52°C water bath (**Figure 5.2**) two times for 15sec with a 2 minute resting interval in between the two stimulations, similar to what was done by Abbadie et al. (1994). Following stimulus application under anaesthesia, the animals were allowed to recover and ~2 hours later, when c-Fos expression has been shown to be maximal (Bullitt, 1990), spinal cord slices with attached dorsal roots were prepared as described in **section 5.4.4.1**. WT control animals did not receive any stimulation.

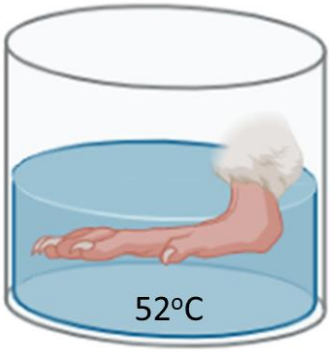


Figure 5.2 Hot water bath hindpaw stimulation

One hindpaw of Fos-EGFP mice, which were briefly anaesthetised using isofluorane inhalation, was immersed in 52°C water bath for 15sec twice with a 2-minute resting interval in between stimulations.

5.4.4 Mouse spinal cord slice electrophysiology

5.4.4.1 Mouse spinal cord preparation

Mouse spinal cord slices with attached dorsal roots were prepared using previously established methodologies (Bardoni et al., 2004, Bardoni et al., 2000, Nakatsuka et al., 1999, Tong et al., 2008, Tong and MacDermott, 2006, Torsney and MacDermott, 2006, Torsney, 2011). Spinal cords with attached thoracic, lumbar and sacral spinal

nerves were removed from 5-8-week-old naïve WT *C57BL/6* and noxious-heat treated Fos-EGFP mice of both sexes (~2hours after noxious heat stimulation) and placed in ice-cold dissection solution, continuously bubbled with 95% O2 / 5% CO2. The ventral roots, DRGs, dura mater and arachnoid membrane were carefully removed, leaving only the lumbar spinal cord with L4 or L5 attached. The latter was embedded in 3% low-melting-point agar (Invitrogen) to allow for slicing of the spinal cord with the attached dorsal roots. Using a vibrating blade microtome (Leica Biosystems) parasagittal/transverse slices (450-500µm) with dorsal roots attached were cut and briefly recovered for up to 15mins in 32-34^{o}C oxygenated *N*-methyl-D-glucamine (NMDG) recovery solution and then placed in oxygenated holding solution for 1h at room temperature prior to recording. The spinal cord slices with attached dorsal roots were transferred to the recording chamber of an upright microscope (Zeiss Axiokop II) equipped with infrared-differential interference contrast (ir-DIC) (Hamamatsu) for electrophysiological recordings and with fluorescence (Cairn Research) for identification of Fos-EGFP labelled (Fos-EGFP +ve) neurons. The spinal cord slices were fully submerged and held in place with a small anchor made of sliver wire and continually perfused with oxygenated recording solution at a flow rate of 1-2ml/min at room temperature. The 95% O_2/5% CO_2-saturated dissection solution contained 3.0mM KCl, 1.2mM NaH_2PO_4, 26mM $NaHCO_3$, 15mM glucose, 251.6mM sucrose, 7mM $MgCl_2$, and 0.5mM $CaCl_2$, pH 7.3-7.4. The NMDG recovery solution comprised 93mM NMDG. 2.5mM KCl, 1.2mM NaH_2PO_4, 30mM $NaHCO_3$ 25mM glucose, 20mM HEPES, 5mM Sodium absorbate, 2mM Thiourea, 3mM Sodium pyruvate,10mM $MgSO_4$, 0.5mM $CaCl_2$, pH 7.3-7.4. The holding solution contained 92mM NaCl, 2.5mM KCl, 1.2mM NaH_2PO_4, 30mM $NaHCO_3$, 25mM glucose, 20mM HEPES, 5mM Sodium absorbate, 2mM Thiourea, 3mM Sodium pyruvate, 2mM $MgSO_4$, 2mM $CaCl_2$, pH 7.3-7.4. The recording solution contained 125.8mM NaCl, 3.0mM KCl, 1.2mM NaH_2PO_4, 26mM $NaHCO_3$, 15mM glucose, 1.3mM $MgCl_2$, and 2.4mM $CaCl_2$, pH 7.3-7.4. A high Mg^{2+}, low Ca^{2+} recording solution was used to minimise excitotoxic damage during dissection and recovery. All chemicals were obtained from Sigma.

5.4.4.2 Patch-clamp electrophysiology

Whole-cell patch-clamp recordings were conducted in Fos-EGFP heat and WT capsaicin sensitive spinal neurons visualised under fluorescence (Fos-EGFP) and/or infrared differential interference contrast (ir-DIC) microscopy on an upright Zeiss Axiokop II microscope. To conduct evoke excitatory post-synaptic currents (eEPSC) or evoked excitatory post-synaptic potentials (eEPSP) one or two (for the ADS length-dependency experiments described in section 5.4.4.2.3) glass suction electrodes were used to electrically stimulate the attached dorsal root and activate primary afferent inputs (**Figure 5.3**).

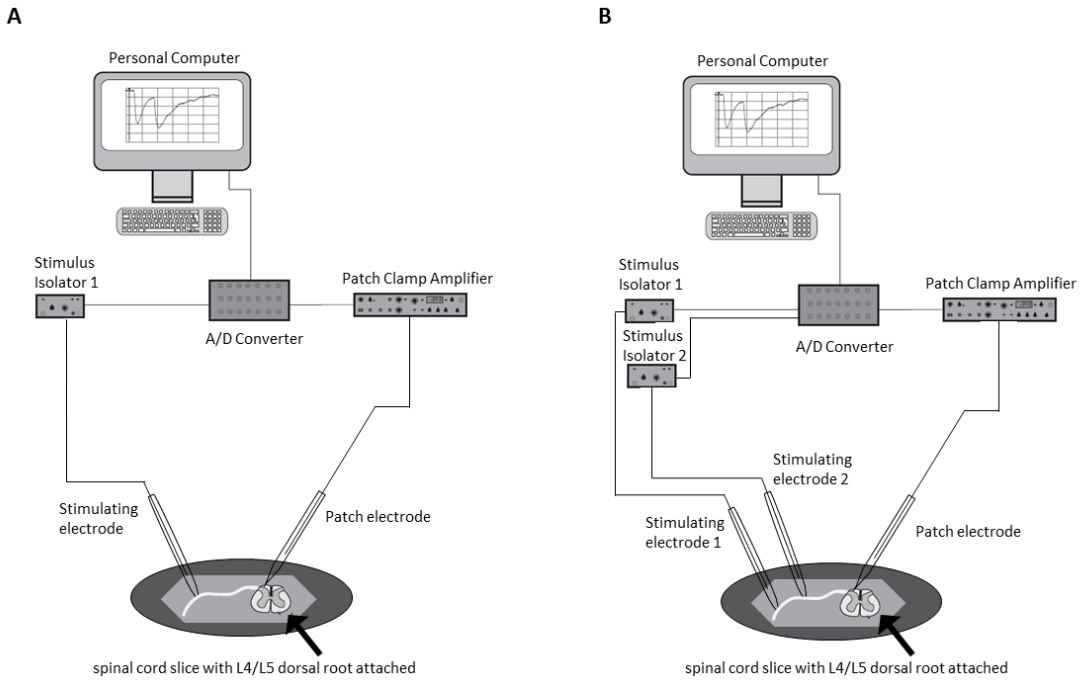


Figure 5.3 Set up for patch-clamp recordings in spinal cord slices with attached dorsal roots

A) Set up for used for conducting eEPSC and eEPSP recordings for assessing and manipulating ADS in monosynaptic C-fibre inputs to Fos-EGFP heat or WT capsaicin sensitive neurons. One suction electrode was used to electrically stimulate the attached dorsal root and activate the primary afferent inputs. **B**) Set up for used for conducting eEPSC and eEPSP recordings for assessing the length-dependency of ADS in monosynaptic C-fibre inputs to spinal neurons with noxious heat/capsaicin sensitive inputs. Two glass suction electrodes were attached to the same dorsal root. One was attached near the DRG end of the dorsal root, giving maximal root length, and the other one was attached at least 4mm away from the root entry zone to the spinal cord, giving a shorter root length.

All the targeted cells were located in the superficial (lamina I/II region) region of the dorsal horn of the spinal cord. The lamina II region was visually identified as the distinguishable translucent band across the spinal cord dorsal horn, while lamina I region was identified as the thin area between the white matter and lamina II (**Figure 5.4**). To confirm that the recorded neurons were located in the superficial laminae, images were obtained with X5 objective, showing the spinal cord dorsal horn and the position of the patch electrode (**Figure 5.4**).

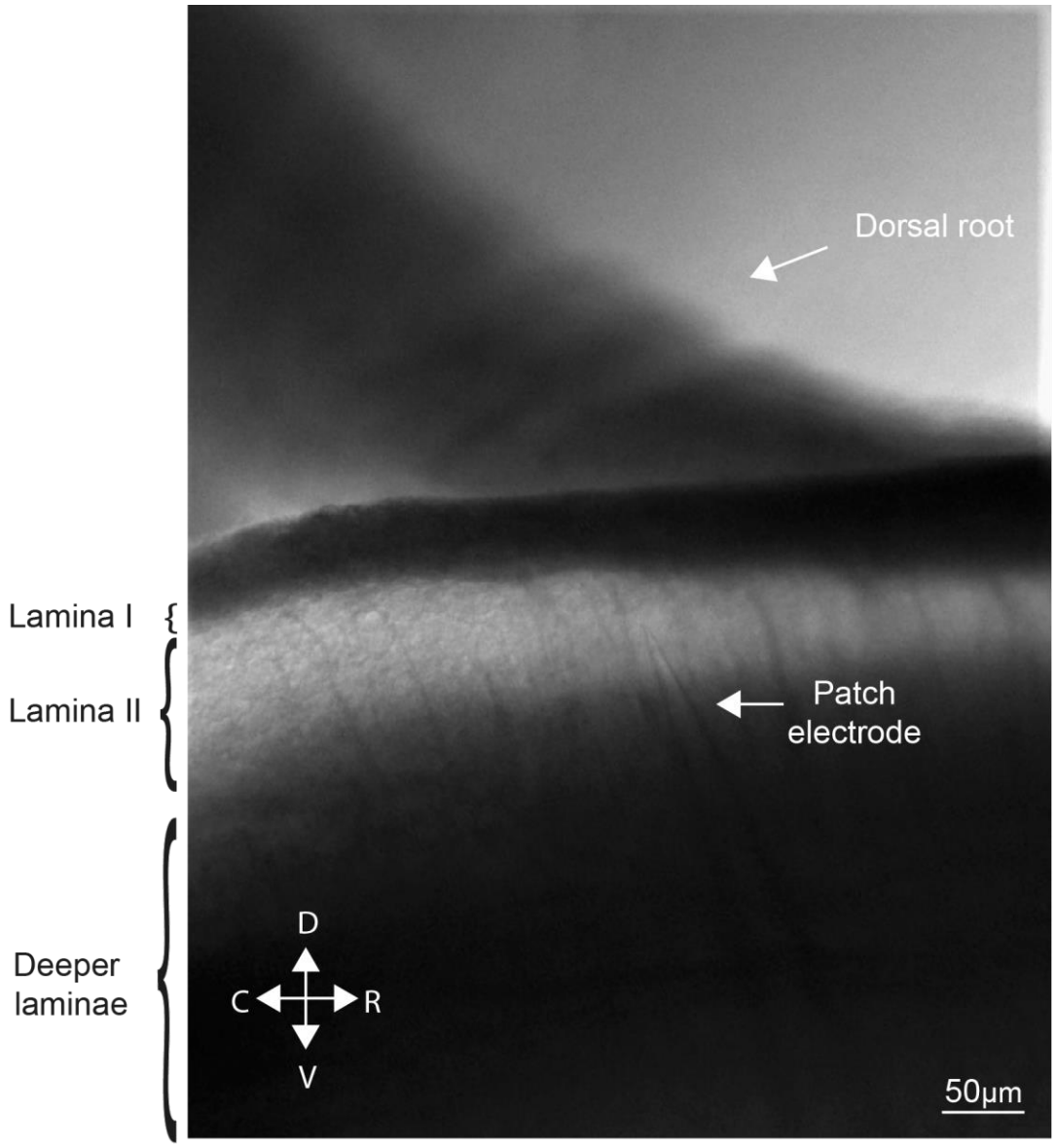


Figure 5.4 Parasagittal spinal cord slice with attached dorsal root showing location of neurons targeted for patch-clamp recordings

Example image of a parasagittal spinal cord slice with attached dorsal root at x5 magnification taken after conducting patch-clamp recordings to show example location of the patch electrode, illustrating the approximate location of the targeted spinal neuron.

Patch electrodes were pulled from thick wall borosilicate glass (GC150F-7.5, Harvard Apparatus) using a Flaming/Brown model P-97 microelectrode puller (Sutter Instruments) with a tip resistance of 4-6MΩ when filled with intracellular solution. The intracellular solution used in all voltage-clamp recordings contained (in mM): 120 Cs-methanesulfonate, 10 Na-methanesulfonate, 10 ethylene glycol tetraacetic acid (EGTA), 1 CaCl2, 10 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 5 2-(triethylamino)-N-(2,6-dimethylphenyl) acetamine chloride (QX-314-Cl), 2 Mg-ATP, pH adjusted to 7.2 with CsOH, osmolarity ~290mOsm. The intracellular solution used in all current-clamp recordings contained (in mM): 120 K-gluconate, 10 KCl, 0.5 ethylene glycol tetraacetic acid (EGTA), 2 MgCl2, 10 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 2.0 Na2-ATP, 0.5 Na-GTP, pH adjusted to 7.2 with KOH. All chemicals were obtained from Sigma, except from QX-314-Cl (Alomone Labs). Before start of recordings, 1µM Alexa Fluor 555 hydrazide (Thermo Fisher Scientific) was added to the intracellular solution, which filled the neuron recorded from, allowing for conformation that it was the targeted Fos-EGFP +ve neuron (**Figure 5.5**). Following successful configuration of whole-cell mode, cells were voltage clamped at a holding potential of -70mV, which blocks inhibitory inputs onto these neurons (Coull et al., 2003). This allowed the study of mEPSCs and dorsal root evoked EPSCs, without the influence of inhibition. Current-clamp mode was established following successful configuration of whole-cell mode in voltage-clamp. In current-clamp mode, recordings of dorsal root-evoked action potential discharge were obtained at resting membrane potential of ~ -60mV. If the membrane potential of the sampled neuron was ± 10mV out with the resting membrane potential, it was injected with continuous bias currents to give

membrane potential of ~ -60mV. Neurons with membrane potential <-70mV and >-50mV were not included. Junction potential was always corrected prior to recording. Data were recorded and acquired with an Axopatch 200B amplifier and pClamp 10 software (Molecular Devices). Data were filtered at 5kHz and digitised at 10kHz. Cells' exclusion criteria in voltage-clamp recordings were any of the following: access resistance > 30MΩ; holding current (>0pA or <-100pA); membrane resistance <300MΩ. Membrane resistance was monitored by measuring current responses to a -20mV voltage step.

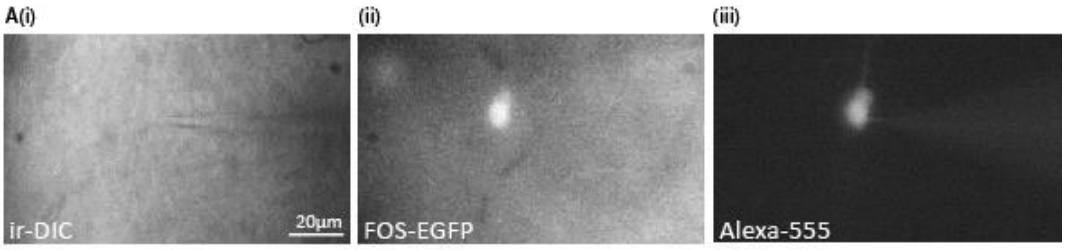


Figure 5.5 Identification of a noxious heat-induced Fos-EGFP-expressing neuron

Spinal neuron visualised with ir-DIC (**i**), EGFP fluorescence (**ii**) and by filling with Alexa Fluor 555 hydrazide (**iii**).

5.4.4.2.1 Characterising capsaicin mEPSC frequency potentiation in intraplantar capsaicin-induced Fos-EGFP-expressing neurons

To verify the reliability of Fos-EGFP identification of noxious heat processing neurons, proof of principle experiments were conducted to show that spinal neurons with intraplantar capsaicin-induced Fos-EGFP expression receive capsaicin sensitive inputs. Fos-EGFP mice that have received intraplantar capsaicin injections (as described in **section 5.4.3.1**) 2h prior to termination were used. Spinal cords were removed from the Fos-EGFP mice that received a capsaicin intraplantar stimulation and used to prepare spinal cord slices as described in **section 5.4.4.1**, except all dorsal roots were removed prior to embedding the spinal cord in agar. Whole-cell patch clamp configuration (as described in **section 5.4.4.2**) was used to

record miniature mEPSCs. These were recorded in the presence of 0.5µM TTX (Alomone Labs), 10µM bicuculline (Tocris) and 1µM strychnine (Sigma). TTX was used to block action potential dependent events. Bicuculline and strychnine were used to block inhibitory events, as they are antagonists of the inhibitory receptors $GABA_A$ ($GABA_AR$) and the glycine receptor (GlyR), respectively. Inhibitory events were also blocked by recording at a holding potential of -70mV, the anion reversal potential for spinal cord neurons (Coull et al., 2003). The internal solution composition was as follows (in mM): 120 Cs-methanesulfonate, 10 Na-methanesulfonate, 10 ethylene glycol tetraacetic acid (EGTA), 1 CaCl2, 10 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 5 2-(triethylamino)-N-(2,6-dimethylphenyl) acetamine chloride (QX-314-Cl), 2 Mg-ATP, pH adjusted to 7.2 with CsOH, osmolarity ~290mOsm.

Following baseline mEPSCs recordings for 5min, the TRPV1 agonist capsaicin (1µM, Sigma) was bath applied for 5min to pharmacologically potentiate the TRPV1-expressing primary afferent inputs (Yang et al., 1998). TRPV1 is highly expressed on primary afferent terminals in the spinal cord (Caterina et al., 1997, Cavanaugh et al., 2011, Tominaga et al., 1998), with recent reports of only limited TRPV1 expression in lamina II inhibitory interneurons (Kim et al., 2012). Given the high levels of TRPV1 expression on the primary afferents central terminals (Cavanaugh et al., 2011), the application of capsaicin to spinal cord slices should potentiate primary afferent input only by increasing the release of glutamate from the central terminals (Kim et al., 2009). Potentiation was assessed by measuring the mEPSC frequency at baseline, during capsaicin application and following 10min wash out of capsaicin (recovery) in Fos-EGFP +ve neurons. These data were compared to equivalent recordings in nearby Fos-EGFP –ve neurons. In all recordings mEPSC frequency and amplitude were measured during the final 2 min of all baseline, capsaicin and recovery recordings. Detection threshold for mEPSCs was 10pA so only mEPSC with amplitude ≥10pA were included in the analysis.

5.4.4.2.2 Primary afferent input characterisation

To investigate primary afferent input to Fos-EGFP heat and WT capsaicin sensitive spinal neurons, eEPSCs were recorded in spinal cord slices with attached dorsal roots from noxious-heat treated (as described in **section 5.4.3.2**) and naïve WT mice of both sexes, respectively, in response to dorsal root stimulation. Following successful configuration of whole-cell mode, cells were voltage-clamped at -70mV and the dorsal root was stimulated with a suction electrode. To determine the afferent fibre types providing input to the targeted neurons, dorsal roots were initially electrically stimulated at low frequency (0.05Hz, 0.1ms stimulus duration) with progressively increasing intensities using an ISO-Flex stimulus isolator (AMPI Intracell). Three stimuli were applied at intensities of 10μA for Aβ fibres, 30μA for Aδ fibres, and 100μA and 500μA for C-fibres. Both monosynaptic and polysynaptic primary afferent inputs display a stable latency at this stimulation intensity, with responses classified as 'evoked' when they were stable and time locked with the stimulus. Responses that were unstable and did not coincide with the stimulus were considered 'spontaneous' (Nakatsuka et al., 2000, Torsney, 2011, Torsney and MacDermott, 2006). To determine the monosynaptic versus polysynaptic nature of stable afferent inputs, the dorsal root was stimulated 20 times, with 0.1ms stimulus duration at the intensities and frequencies detailed in **Table 5.1**, in line with previously established and described methodology (Nakatsuka et al. 2000, Torsney 2011, Torsney & MacDermott 2006).

Table 5.1 Electrical stimulation parameters used to differentiate the monosynaptic versus polysynaptic nature of primary afferent inputs to heat- capsaicin sensitive Fos-EGFP neurons.

Fibre Input	*Stimulation intensity (μA)*	*Stimulation frequency (Hz)*
Aβ	10	20
Aδ	30	2

C	500	1

The indicated intensities were chosen based on the findings for the primary afferent fibres activation thresholds from the CAP experiments in mice (**Figure 4.11**) showing the mean activation threshold for the Aβ, Aδ- and C-fibres are ~5, 20 and 50µA, respectively, with the range of activation thresholds indicated in **Table 4.1**. Stimulation intensities which were higher that the activation threshold of the component of interest, but did not activate higher threshold components (e.g. activated all Aβ, but no Aδ-fibres), were selected. It must be noted that in the mice CAP recordings there seemed to be some overlap between the activation threshold for some Aδ and C- fibres (~30µA) (**Table 4.1**) and to avoid mischaracterisation of the primary afferent inputs in eEPSCs patch-clamp studies additional recording at 100µA were conducted to help identify the primary afferent inputs. Thus, to activate the Aβ fibres 10µA were used which is above the activation threshold for the Aβ fibres but below the activation threshold for the Aδ fibres (**Table 4.1**). To activate the Aδ-fibres, 30µA and 100µA were used because 30µA was not above the activation threshold of some Aδ-fibres (**Table 4.1**). To activate the C-fibres 30µA, 100µA, and 500µA were used because the activation threshold for some of the C-fibres was 30µA (**Table 4.1**), but maximal response amplitude was not reached before 500µA. Higher stimulation intensities than threshold were chosen to also avoid synaptic failures in control conditions. Thus, majority of the low-stimulus intensity-evoked responses (10-30µA) were classified as likely A fibre-driven, while the higher intensity-evoked responses (100-700µA) were classified as likely to be C-fibre-driven. These values are consistent with those previously reported in mice of comparable age (Daniele and MacDermott, 2009, Bardoni et al., 2019). The stimulation frequencies were chosen based on previous findings (Nakatsuka et al., 2000) showing that Aδ- and C-fibre action potential firing fails at stimulation frequencies >2Hz and >1Hz, respectively, whereas Aβ-fibres show no failures at frequencies of up to 20Hz. The use of frequencies greater than these would not

allow for the differentiation between synaptic failures and a failure of action potential firing in the primary afferent inputs. A failure was defined as having occurred when a stimulus did not result in a clear eEPSC, an example of which is shown in **Figure 5.6C**. A-fibre eEPSCs were classified as monosynaptic on the basis of a stable latency (≤2ms) and an absence of synaptic failures, while A-fibre eEPSCs were classified as polysynaptic on the basis variable latency change (>2ms) and/or occurrence of synaptic failures (**Figure 5.6A**). C-fibre eEPSCs were classified as monosynaptic if there were no synaptic failures, irrespective of the variability in latency (**Figure 5.6A-B**), and as polysynaptic if synaptic failures occurred (**Figure 5.6C**) (Nakatsuka et al., 2000, Torsney, 2011, Torsney and MacDermott, 2006). Only Fos-EGFP-expressing neurons that receive monosynaptic C-fibre input or WT neurons that received monosynaptic C-fibre input that was capsaicin sensitive, regardless of their polysynaptic input, were used for experiments and analysis. Fos-EGFP +ve neurons or WT neurons with polysynaptic input, regardless of whether this was polysynaptic Aβ-, Aδ- or C-fibre input, were excluded from the experiment.

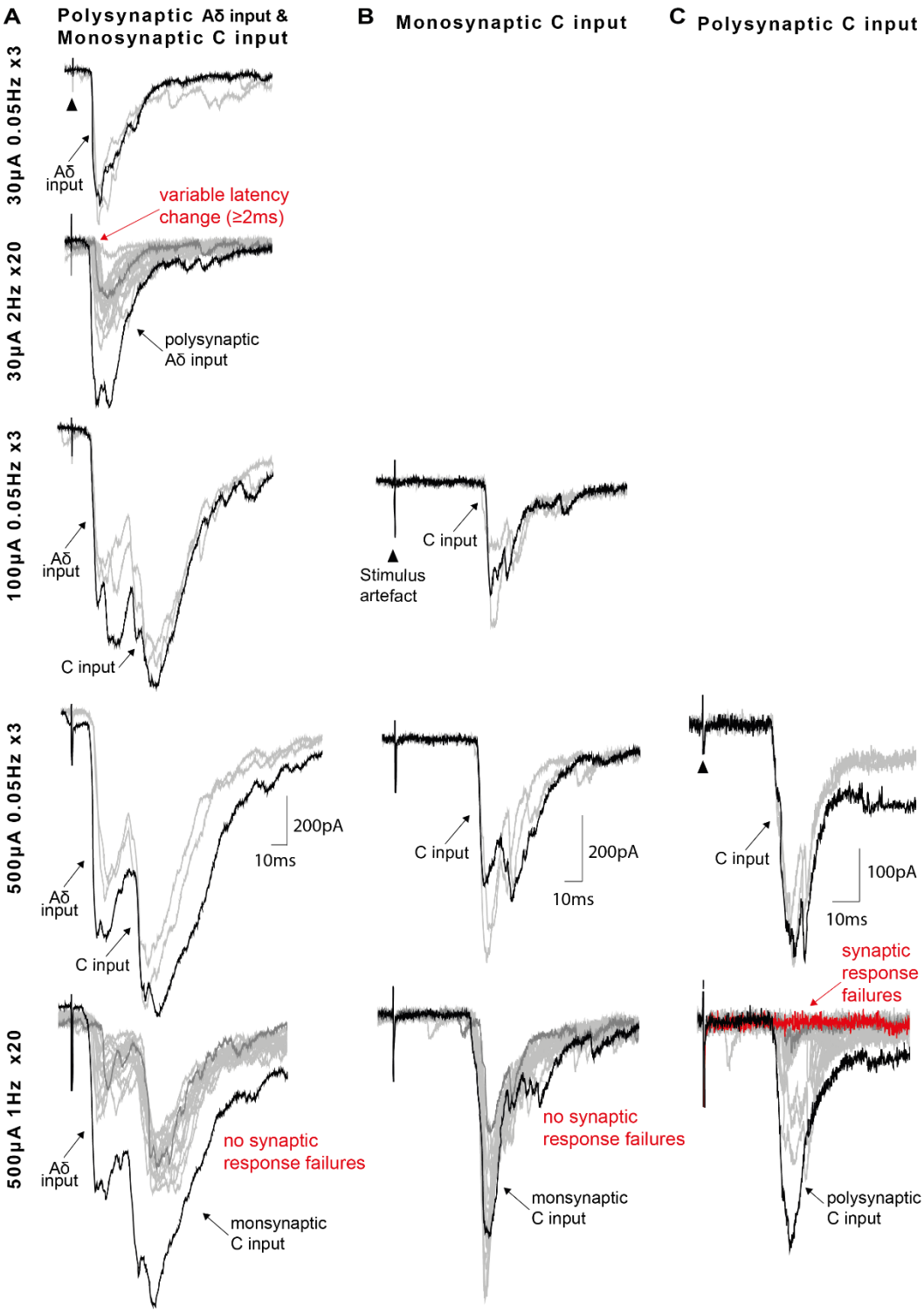


Figure 5.6 Classification of monosynaptic and polysynaptic Aδ and C-fibre inputs

Representative eEPSC recordings from spinal cord dorsal horn neurons receiving polysynaptic Aδ and monosynaptic C-fibre inputs (**A**), monosynaptic C-fibre inputs (**B**) or polysynaptic C-fibre inputs (**C**). The first row shows eEPSCs in response to Aδ fibre

stimulation at 0.05Hz at 30μA, the average activation threshold for Aδ inputs in eEPSCs in mice. The second row shows eEPSCs in response to repetitive Aδ fibre stimulation 20x at 2Hz at 30μA, which allowed differentiation of polysynaptic (failures were present and/or ≥2ms change in latency) versus monosynaptic (no failures and <2ms latency change) Aδ fibre inputs. The third row shows eEPSCs in response to C-fibre stimulation at 0.05Hz at 100μA, the average activation threshold for C-fibre inputs in eEPSCs in mice. The forth row shows eEPSCs in response to C-fibre stimulation at 0.05Hz at 500μA, an intensity that evoked a strong response as observe in rat. The fifth row shows eEPSCs in response to repetitive C-fibre stimulation (120x at 1Hz at 500μA), which allowed differentiation of polysynaptic (failures were present irrespective of latency change) versus monosynaptic (failures were not present irrespective of latency change) C-fibre inputs. The first row in each example trace illustrates the intensity at which a response was observed for the first time. No responses were observed at lower intensities. Response trace 1 black; 2-19 pale grey; 20 dark grey, Red response trace illustrates example of synaptic response failure. Latency change is illustrated by the difference in input latency of the 20th stimulus (dark grey response trace) from the first stimulus (black response trace).

The amplitude of the monosynaptic C-fibre inputs was calculated by measuring the ampere difference between the start of the monosynaptic C-fibre eEPSC and the negative peak of the response (**Figure 5.7**). The latency of the monosynaptic C-fibre response was measured as the time distance between the stimulus artefact and the onset of the monosynaptic eEPSC (**Figure 5.7**). The length of the dorsal root was estimated based on the distance between the stimulating electrode and the dorsal root entry point to the dorsal horn of the spinal cord. The conduction velocity of the monosynaptic eEPSC was calculated by dividing the monosynaptic C-fibre response latency by the estimated root length. It must be noted that this method of determining conduction velocity provides a highly imperfect estimate, because it does not take into account the spinal cord portion of the conduction pathway and the synaptic delay. However, given the difference within the conduction velocities of the different primary afferent inputs (Géranton et al., 2009, Nakatsuka et al., 2000, Torsney, 2011) this measure is still informative. To quantify the amount of polysynaptic C-fibre input onto the Fos-EGFP +ve neurons and the WT neurons with capsaicin sensitive inputs, the polysynaptic C-fibre-evoked net charge influx was measured using Clampfit 10.7.0.3 software (Molecular devices). This was measured after the peak of the monosynaptic C-fibre input until the end of the recording with

respect to baseline before stimulus artefact. Initial latency, estimated conduction velocity, amplitude and net charge were calculated using the first trace in the train of 16x stimuli at 2Hz at 700µA.

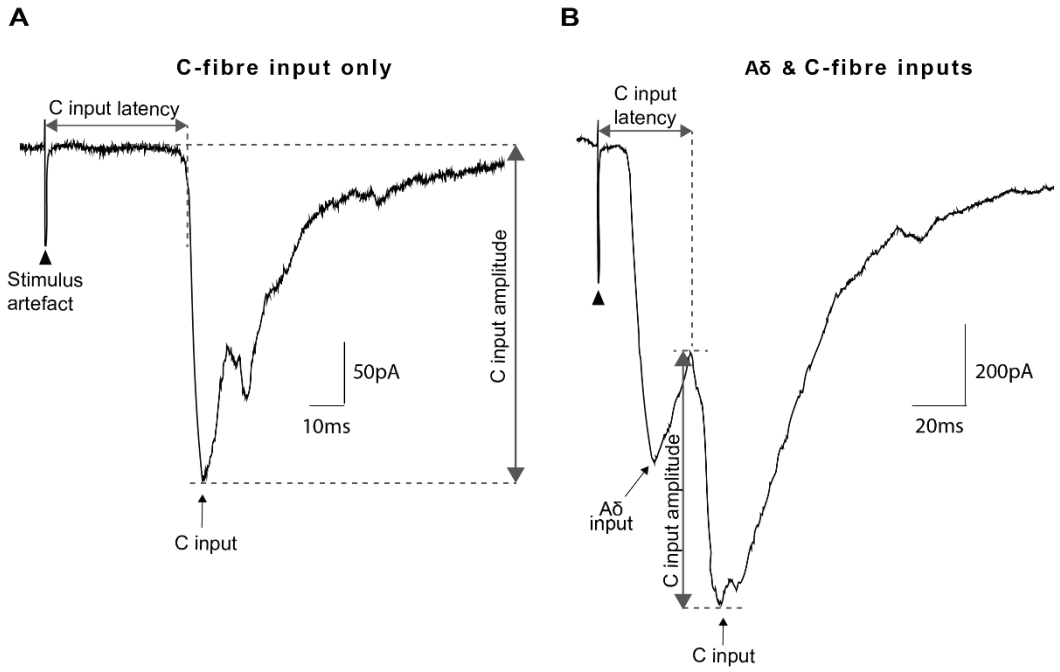


Figure 5.7 Amplitude and latency measures of monosynaptic C-fibre input in eEPSC recordings from spinal cord dorsal horn neurons.

Representative traces showing how C-fibre eEPSC peak amplitude and latency were measured in eEPSC recordings with C-fibre inputs only (**A**) and with both Aδ and C-fibre inputs (**B**). The C-fibre eEPSC peak amplitude was measured as the ampere differece between the onset and the positive peak of the monosynaptic C-fibre eEPSC (dashed vertical double-headed arrow). The C-fibre eEPSC latrency was measured as the time differece between the stimulus artefact and the onset of the monosynaptic C-fibre eEPSC (dashed horizontal double-headed arrow). The C-fibre eEPSC latency was also used for calculating estimated conduction velocity of the monosynaptic C-fibre eEPSC.

5.4.4.2.3 Activity-dependent slowing in monosynaptic C-fibre input to noxious heat sensitive Fos-EGFP-expressing neurons and to capsaicin-sensitive WT neurons

Fos-EGFP mice that received noxious heat stimulation (as described in **section 5.4.3.2**) 2h prior to termination or naïve WT *C57BL/6* mice were used to isolate

spinal cords to prepare spinal cord slices with attached dorsal roots, as described in **section 5.4.4.1**. Whole-cell patch clamp configuration (as described in **section 5.4.4.2**) was used to record eEPSC in response to repetitive stimulation of the dorsal roots in order to investigate ADS in monosynaptic C-fibre eEPSC to the patched noxious heat-induced Fos-EGFP +ve or WT neurons in spinal cord slices with attached dorsal roots.

Monosynaptic C-fibre input to the targeted neurons was identified as described in **section 5.4.4.2.2** (**Figure 5.6)**. Voltage- and current-clamp recordings were used to assess ADS in monosynaptic C-fibre inputs to and its impact on noxious Fos-EGFP heat or WT capsaicin sensitive spinal neurons, respectively. To assess ADS, the dorsal root was stimulated 16x times at 2Hz at an intensity of 700μA and a stimulus duration of 0.1ms. A train of 16x stimuli instead of 40x stimuli (as per CAP recordings) was used to prevent overstimulation of the cell. The frequency used was chosen to mimic the C-fibre evoked firing rate (Thalhammer et al., 1994, Yeomans and Proudfit, 1996). In an individual targeted neuron 10min resting intervals in between periods of repetitive dorsal root stimulation were left to allow the fibres to recover from ADS (Shim et al., 2007, Weidner et al., 1999).

For the ADS manipulation experiment, prolonged low-frequency stimulation was used as a tool to manipulate ADS, i.e. induce dynamic memory, in monosynaptic C-fibre inputs to Fos-EGFP heat or WT capsaicin sensitive spinal neurons. This was to enable investigation of the impact of different levels of ADS on the activity of spinal neurons with heat/capsaicin sensitive inputs. Voltage-clamp recordings of C-fibre eEPSCs were conducted to confirm that manipulation altered the ADS profile (monosynaptic C-fibre latency change), i.e. induce dynamic memory, in monosynaptic C-fibre inputs to spinal neurons with heat/capsaicin sensitive inputs. Specifically, ADS in monosynaptic C-fibre input was recorded at baseline and post-manipulation (or control manipulation), similarly to the CAP manipulation ADS mouse data (**section 4.4.3.2.**). In the 'manipulation' treatment group, baseline ADS was recorded by repetitively stimulating the dorsal root in the spinal cord slice

preparation 16x times at 2Hz. Following 10min rest interval to allow recovery from ADS, a 1min train of 60x stimuli at 1Hz was applied to manipulate ADS. After 30sec rest interval a train of 16x stimuli at 2Hz was applied to assess ADS post-manipulation. In the 'control manipulation' treatment group, instead of applying a 1min train of 60x stimuli at 1Hz, a 1min period was added to the 10min break following baseline ADS recording, when no stimulation was applied, and after overall 11min 30s break, post-manipulation C-fibre ADS at 2Hz was recorded.

To assess the impact of monosynaptic C-fibre ADS manipulation upon the activity of Fos-EGFP heat or WT capsaicin sensitive spinal neurons, eEPSP recordings were conducted in Fos-EGFP heat or WT capsaicin sensitive neurons from spinal cord slices with attached dorsal roots. Similarly to the eEPSC recording for assessing the effect of manipulation on C-fibre input, the dorsal roots in the spinal cord slice preparations were repetitively stimulated 16x times at 700µA at 2Hz to assess baseline ADS. Following 10min rest interval to allow recovery from ADS, a 1min train of 60x stimuli at 1Hz was applied to manipulate ADS. After 30sec rest interval a train of 16x stimuli at 2Hz was applied to assess ADS post-manipulation. Control manipulation treatment group was not included in I-clamp experiments.

Since previous studies have shown that ADS occurs in a length-dependent manner, with greater ADS over longer length (Schmelz et al., 1995, Zhu et al., 2009) the length dependency of ADS was also used as a tool to experimentally alter ADS in monosynaptic C-fibre inputs and assess the impact of different levels of ADS on the activity of spinal neurons with heat/capsaicin sensitive inputs. To assess the effects of long (increased ADS) versus short (decreased ADS) root stimulation lengths on ADS in monosynaptic C-fibre inputs and their impact on the activity of Fos-EGFP heat or WT capsaicin sensitive spinal neurons, two glass suction electrodes were attached to and used for stimulating the same dorsal root, one at a shorter distance and one at a longer distance (**Figure 5.3**). In voltage- and current-clamp recordings, ADS in monosynaptic C-fibre input was recorded in response to 16x stimuli at 2Hz at

a short and at a long stimulation distance with a 10min recovery period in between stimulations.

ADS in monosynaptic C-fibre inputs in eEPSC recordings was reflected by the change in monosynaptic C-fibre response latency, where the latter was measured as the time between the stimulus artefact and the onset of the monosynaptic C-fibre response (**Figure 5.8**). To assess the change in monosynaptic C-fibre response latency following the train of 16x stimuli, the latency following each stimulus was normalised to the latency recorded at stimulus 1. Since ADS is length-dependent (Schmelz et al., 1995, Zhu et al., 2009), to negate the influence of varying root lengths, the latency change in all experiments was normalised to the estimated length of the stimulated root, except for the experiment utilising the length dependency of ADS. For the length-dependent experiment, latency change differences were not normalised to root length to enable investigation of the impact of different levels of C-fibre ADS in monosynaptic C-fibre inputs to the spinal neurons.

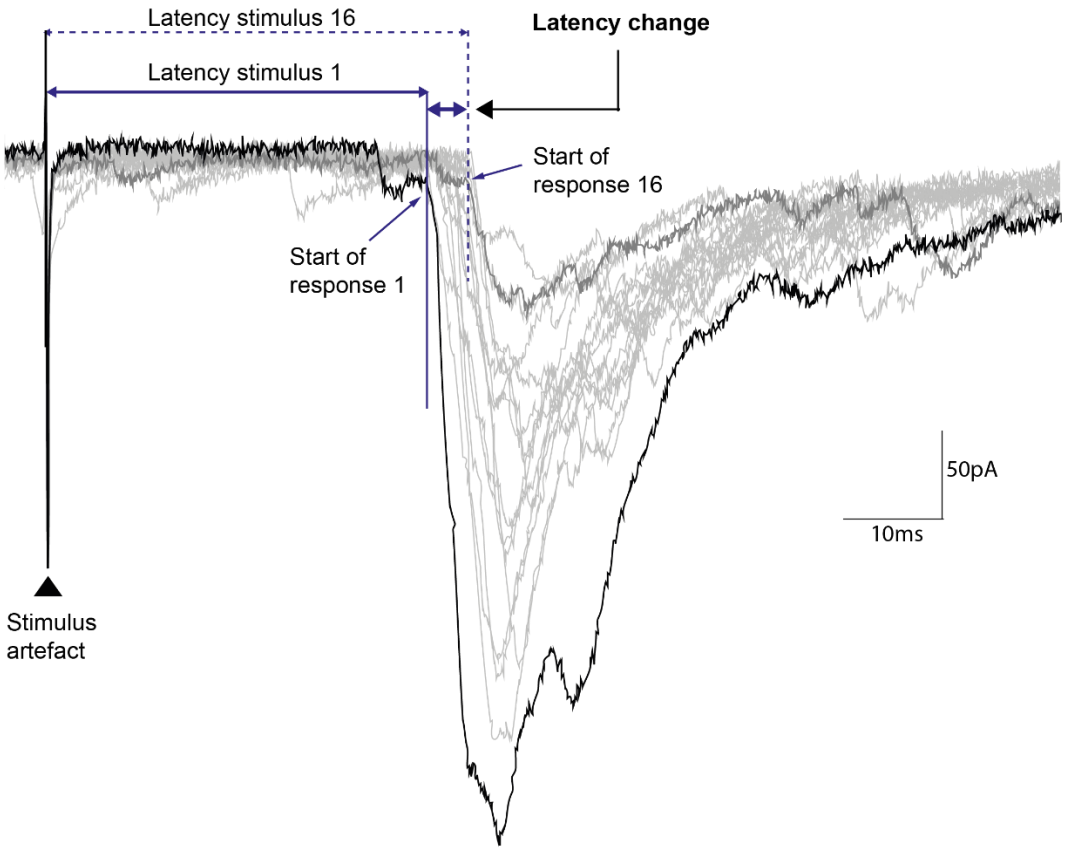


Figure 5.8 Voltage-clamp eEPSC recording of ADS in monosynaptic C-fibre input

Representative monosynaptic C-fibre eEPSC recording from a Fos-EGFP heat sensitive spinal neuron in response to 16x stimuli at 700µA at 2Hz, which results in a progressive increase in the response latency. The ADS phenomenon is illustrated by the latency change of the 16th response when compared with the first response (thicker double-headed arrow). Response trace 1 black; 2-39 pale grey; 40 dark grey. Monosynaptic C-fibre eEPSC in response to the first (vertical solid line) and the 16th (vertical dashed line) stimulus is mark by arrows, indicating the latency of the monosynaptic C-fibre input. The ADS phenomenon is represented by the latency change (thicker double-headed arrow), illustrated by the difference in latency of the 16th stimulus (dashed double-headed arrow) from the first stimulus (solid double-headed arrow).

5.4.4.2.4 Characterising capsaicin sensitivity in Fos-EGFP heat sensitive and WT spinal neurons

At the end of the experiments exploring ADS in monosynaptic C-fibre input in spinal neurons from naïve WT mice or in a subset of noxious heat-induced Fos-EGFP +ve neurons from Fos-EGFP mice (**section 5.4.4.2.4**), eEPSC were recorded at 0.05Hz at

700µA following continuous bath application of 1µM capsaicin for 15mins. This allowed the assessment of the effect of bath-applied capsaicin on the monosynaptic C-fibre input amplitude. The amplitude of the monosynaptic C-fibre input was measured as described in **Figure 5.7** before capsaicin application (at 0mins) and at 5, 10 and 15mins following bath capsaicin application. The recordings in noxious heat-induced Fos-EGFP +ve neurons with monosynaptic C-fibre input were used to confirm that these cells indeed receive heat/capsaicin sensitive inputs, while the recordings in spinal neurons with monosynaptic C-fibre input from WT mice were used to limit analysis to neurons with monosynaptic C-fibre inputs that are heat/capsaicin sensitive.

5.4.5 Statistical analysis

In mEPSC recordings from *in vivo* capsaicin-induced Fos-EGFP expressing spinal neurons capsaicin sensitivity was measured by counting the number of mEPSCs following bath capsaicin application and statistically assessed using two-way ANOVA followed by Sidak's multiple comparisons test because an interaction between factors was observed. To assess whether *in vivo* noxious heat stimulation could drive Fos-EGFP expression in spinal dorsal horn neurons, the number of Fos-EGFP +ve neurons in the ipsilateral and the contralateral side of the dorsal horn of the spinal cord in *ex vivo* spinal cord slices was counted and statistically assessed using a paired two tailed t-test.

In eEPSC recordings from spinal cord slices with attached dorsal roots, to statistically compare the activation threshold, initial average conduction velocity and latency of the monosynaptic C-fibre inputs to spinal neurons with heat/capsaicin sensitive inputs in both sexes, unpaired two-tailed t-tests were used. In the same preparation, to compare ADS in monosynaptic C-fibre inputs to Fos-EGFP heat or WT capsaicin sensitive neurons in both sexes, latency change area under the curve (AUC) for males and females was measured and analysed using unpaired one-tailed t-test. One-tailed t-test was used because of the prediction that in line with the CAP ADS data in mice, males will have more pronounced ADS in

monosynaptic C-fibre inputs to spinal neurons. Similarly, in the manipulation and stimulation distance experiments ADS in monosynaptic C-fibre inputs to Fos-EGFP heat or WT capsaicin sensitive neurons for each group in both sexes was measured using latency change AUC and statistically analysed using two-way ANOVA in the control manipulation and the manipulation treatment groups followed by Sidak's multiple comparisons test if an interaction between factors was observed.

To assess the impact of ADS in monosynaptic C-fibre input on the activity of Fos-EGFP heat or WT capsaicin sensitive neurons in both sexes, eEPSP recordings were conducted in Fos-EGFP heat or WT capsaicin sensitive neurons from spinal cord slices with attached dorsal roots from mice of both sexes. The monosynaptic C input-induced action potentials were estimated based on the latency of the monosynaptic C input in preceding voltage-clamp recordings. The number of monosynaptic C input-induced action potentials was counted for each stimulus and the AUC of action potentials per stimulus for males and females was measured and statistically assessed using unpaired two-tailed t-test. Similarly, in the manipulation and stimulation distance experiments the impact of ADS in monosynaptic C-fibre input on the activity of spinal neurons was measured by counting the number action potentials evoked at C-fibre strength for each stimulus. The percentage change of monosynaptic C input-induced action potentials from the first stimulus was plotted for each of the 16x stimuli and the AUC for each group in males and females was measured and statistically assessed using two-way ANOVA followed by Sidak's multiple comparisons test if an interaction between factors was observed. The initial number of monosynaptic C input-induced action potentials (at stimulus 1) and the wind-down in both sexes were assessed using unpaired two-tailed t-tests, while for the manipulation and the stimulation distance experiments these were assessed for each group in males and females using repeated-measure two-way ANOVA followed by Sidak's multiple comparisons test if an interaction between factors was observed.

To assess potential effects of amplitude and net charge on the stimulation distance-induced changes in activity of Fos-EGFP heat sensitive spinal neurons, initial amplitude and net charge were measure in both sexes and assessed using two-way ANOVA followed by Sidak's multiple comparisons test if an interaction between factors was observed unpaired two-tailed t-tests. To assess activity-dependent changes in amplitude and net charge in both sexes, the percentage change from initial amplitude/net charge for the 16x stimuli was measured using the AUC. The data was statistically assessed using two-way ANOVA followed by Sidak's multiple comparisons test if an interaction between factors was observed.

Graphad Prism 9.11 software (GraphPad Software) was used for statistical analysis and graph production. All data are represented as mean ± standard error of the mean (SEM), with n representing the sample size. $P<0.05$ was used to indicate statistical significance

5.5 Results

5.5.1 Intraplantar capsaicin- and noxious heat-induced Fos-EGFP expression in spinal dorsal horn neurons in mice

Capsaicin is an agonist of the ligand- and heat-sensitive TRPV1 ion channel (Caterina et al., 1997, Caterina et al., 2000), that has been established to be expressed primarily in C-fibres, with only a small proportion of the A fibres expressing the channel (Amaya et al., 2003, Kobayashi et al., 2005, Michael and Priestley, 1999, Yu et al., 2008). Further studies have demonstrated that TRPV1 is not expressed on spinal cord dorsal horn neurons but is only expressed in the central terminals of primary afferent fibres in the spinal cord (Cavanaugh et al., 2011). Thus, to assess whether an *in vivo* intraplantar stimulation with capsaicin, which is selective for the TRPV1-expressing afferent fibres, can lead to Fos-EGFP expression in spinal neurons with capsaicin sensitive inputs, spinal cord slices were prepared from Fos-EGFP mice, which had received an intraplantar hindpaw capsaicin injection under anaesthesia 2 hours prior to termination. The impact of bath applied capsaicin upon

mEPSC frequency in these neurons was also assessed. This allowed the identification of intraplantar capsaicin-induced Fos-EGFP positive (Fos-EGFP +ve) neurons, and assessment of capsaicin sensitivity of inputs (**Figure 5.9**). The *in vivo* intraplantar capsaicin injections led to expression of Fos-EGFP in dorsal horn neurons from spinal cord slices (**Figure 5.9Ai,ii**). These Fos-EGFP+ve neurons displayed a significant increase in mEPSC frequency in response to bath applied capsaicin (**Figure 5.9Aiii,iv**), while nearby Fos-EGFP–ve neurons showed no change in mEPSC frequency (**Figure 5.9B**; two-way ANOVA: Fos-EGFP x capsaicin, p=0.01, followed by Sidak's multiple comparisons test, *p*=0.001 (baseline vs capsaicin), and *p*=0.002 (capsaicin vs recovery)).

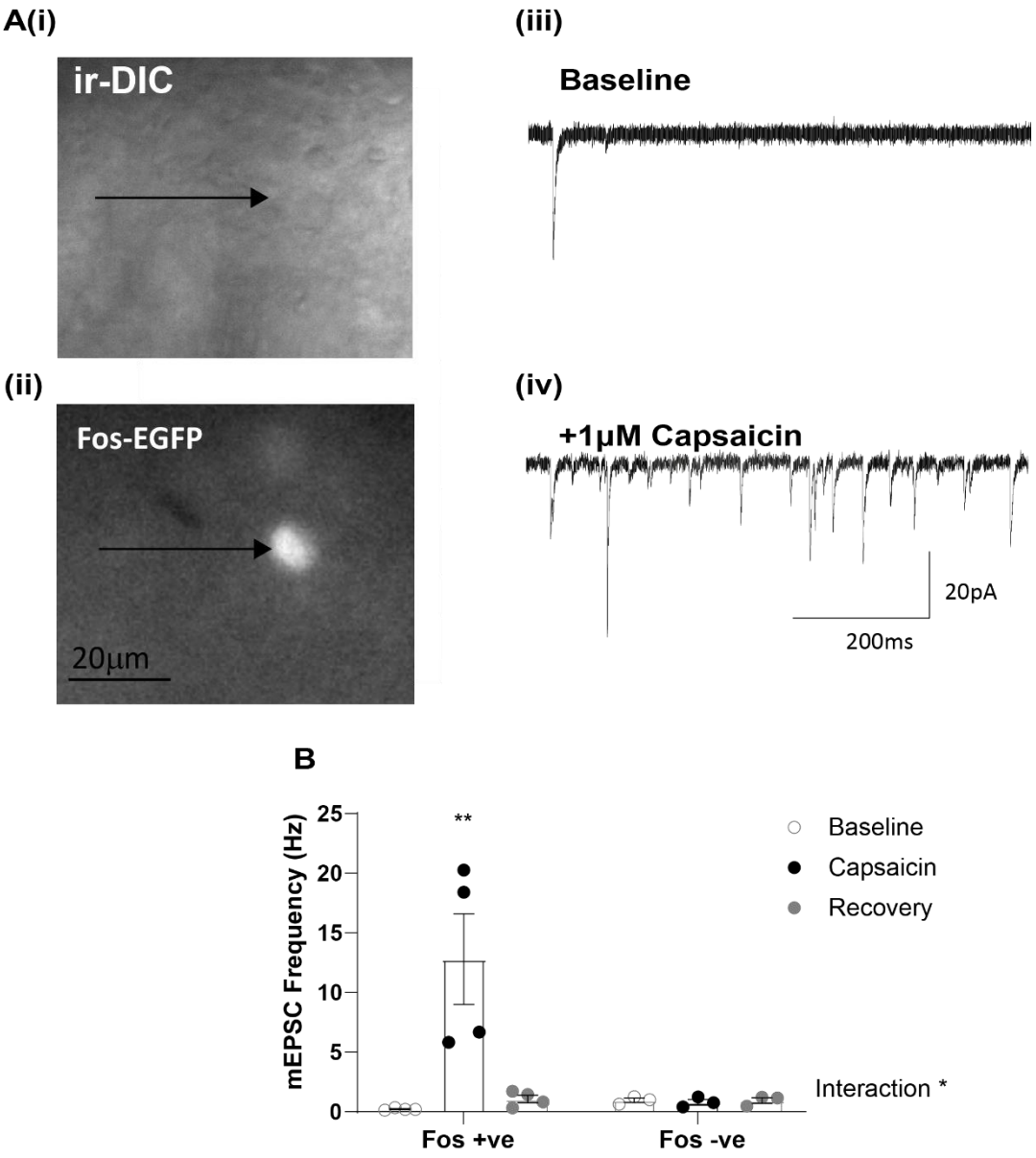


Figure 5.9 Intraplantar capsaicin injections in vivo lead to Fos-EGFP expression in spinal neurons with capsaicin sensitive inputs

A) Example image of Fos-EGFP expressing neuron, induced by intraplantar capsaicin injection 2h prior, in an *ex vivo* spinal cord slice preparation, visualised with fluorescence (**i**) and ir-DIC (**ii**) microscopy employed to record mEPSCs, with representative mEPSC traces recorded at baseline (prior to bath capsaicin application) (**iii**) and during bath application of 1µM capsaicin (**iv**). **B**) Analysis of the mEPSC frequency prior to (baseline), during and after wash out (recovery) of capsaicin in Fos-EGFP positive neurons and in nearby Fos-EGFP negative neurons revealed an effect of capsaicin that is dependent on Fos-EGFP (**p*=0.01), with capsaicin increasing mEPSC frequency in Fos-EGFP positive neurons but not in nearby Fos-EGFP negative neurons (Two-way ANOVA followed by Sidak's multiple comparisons test, ***p*=0.001 (baseline vs capsaicin), ***p*=0.002

(capsaicin vs recovery)). Data presented as mean±SEM. Fos-EGFP positive cells, n=4; Fos-EGFP negative cells, n=3.

In addition, to assess whether *in vivo* noxious heat stimulation (brief hindpaw immersion in a hot 52^{o}C water bath, while under anaesthesia), could also lead to Fos-EGFP expression in spinal neurons, spinal cord slices with attached dorsal roots were prepared from Fos-EGFP mice, which had received the noxious heat stimulation 2 hours prior to termination. This allowed the identification of noxious heat-induced Fos-EGFP +ve neurons, which could be patch-clamped for eEPSC and eEPSP recordings for assessing ADS in the monosynaptic C-fibre inputs and its impact on action potential firing in these neurons, respectively.

The noxious heat stimulation successfully induced Fos-EGFP expression in spinal neurons in lumbar (L4/5) spinal cord slices (**Figure 5.10Ai**), prepared ~2h after the noxious heat stimulus. There were significantly more Fos-EGFP +ve neurons in the dorsal horn of the spinal cord, ipsilateral to the applied stimulus compared to the contralateral spinal cord (**Figure 5.10Aii**; paired two-tailed t-test, p=0.027). Moreover, the noxious heat-induced Fos-EGFP neurons, present in spinal cord slices with attached dorsal roots (**Figure 5.10Bi,ii**), were successfully recorded from, with eEPSC recordings showing that these spinal neurons receive monosynaptic C-fibre input (**Figure 5.10Biii**) , which also display ADS (**Figure 5.10Biv**).

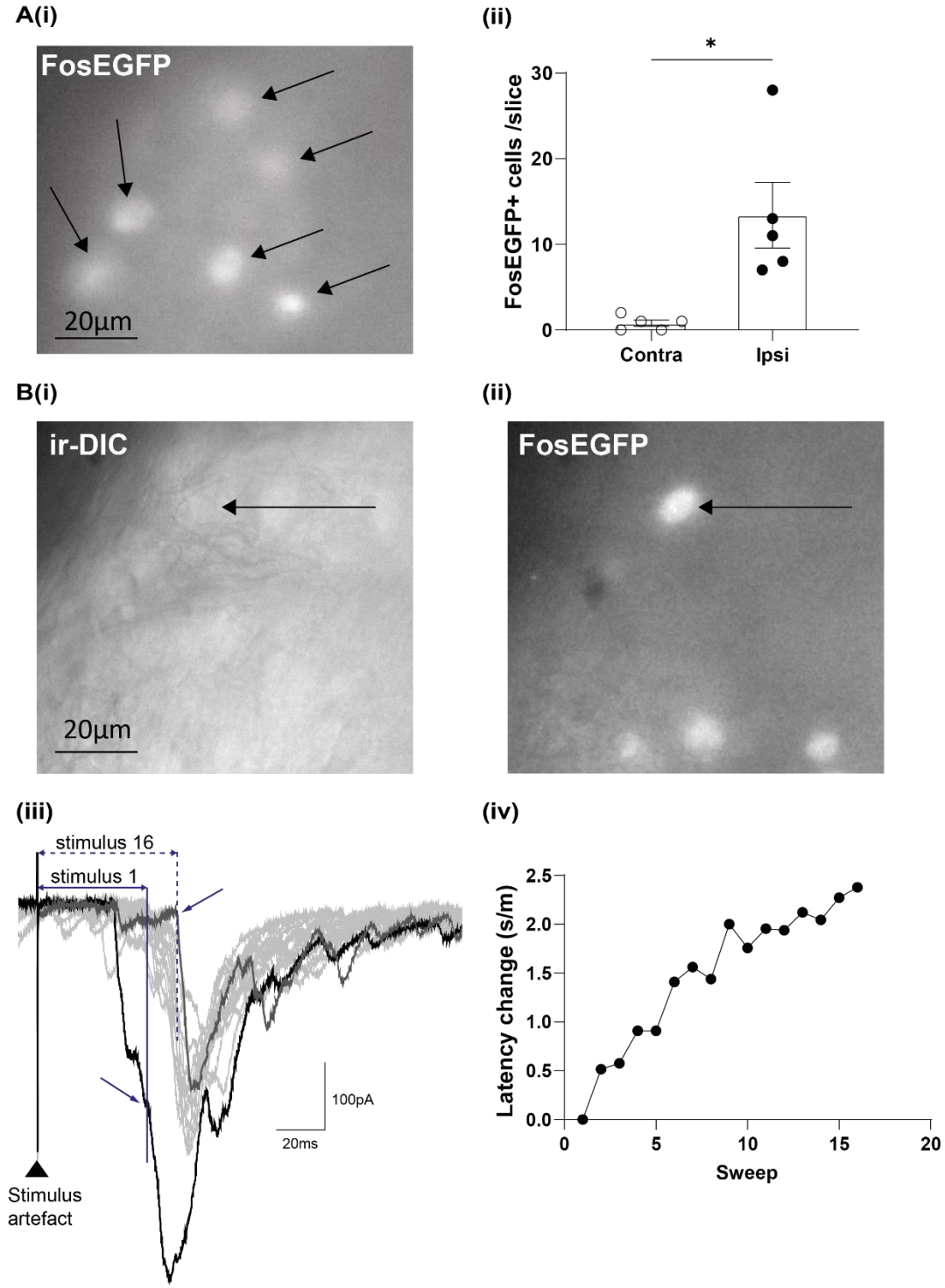


Figure 5.10 Noxious heat stimulation induces Fos-EGFP expression in spinal neurons, including neurons with monosynaptic C-fibre input, displaying ADS

A) Noxious heat stimulation of the hindpaw by brief immersion in a 52°C water bath causes the induction of Fos-EGFP expression in neurons (marked by arrows) in the dorsal horn of the lumbar (L4/5) spinal cord (**i)** with statistical analysis (**ii**) revealing significantly more Fos-EGFP-expressing neurons in the superficial dorsal horn of the spinal cord, ipsilateral to

given stimulus (*p=0.027) when compared to the equivalent contralateral spinal cord (paired two tailed t-test). Data presented as mean±SEM, n=5 slices, N=2 animals. **B**) Images under ir-DIC (**i**) and fluorescence (**ii**) microscopy of a noxious heat-induced Fos-EGFP neuron with the corresponding monosynaptic C-fibre eEPSC recording from that neuron (**iii**) in response to 16x stimuli at 700μA at 2Hz, which results in a progressive latency change from the first monosynaptic C input (**iv**), demonstrating the ADS phenomenon in monosynaptic C-fibre input to the Fos-EGFP heat sensitive spinal neurons. Response trace 1 black; 2-39 pale grey; 40 dark grey. Monosynaptic C-fibre eEPSC in response to the first (vertical solid line) and the 16th (vertical dashed line) stimulus is mark by arrows, indicating the latency of the monosynaptic C-fibre input. Latency change is illustrated by the difference in latency of the 16th stimulus (dashed double-headed arrow) from the first stimulus (solid double-headed arrow).

Previous studies have demonstrated that the TRPV1 ion channel agonist capsaicin (Caterina et al., 1997) causes a depolarisation of C-fibre cell bodies and a suppression of the C-fibre-mediated CAP (Agrawal and Evans, 1986). Moreover, capsaicin-induced activation of the TRPV1 ion channel has been shown to block C-fibre mediated eEPSC (Yang et al., 1999, Tong and MacDermott, 2006), leading to a reduction of their amplitude due to depolarisation of the primary afferent fibres, which probably prevents action potential invasion of the terminal. Thus, to assess whether the noxious heat-induced Fos-EGFP-expressing neurons receive monosynaptic C-fibre inputs that are also capsaicin-sensitive, i.e. TRPV1 expressing, eEPSC were recorded prior to and during the continued presence of 1μM capsaicin in a subset of the Fos-EGFP heat sensitive neurons in spinal cord slices with attached dorsal roots. Capsaicin application led to a progressive reduction in the peak amplitude of monosynaptic C-fibre eEPSCs (**Figure 5.11Ai, ii**) as demonstrated in the stimulus-response plot of the C-fibre eEPSC peak amplitude over time with capsaicin (**Figure 5.11B**). Following 5min of capsaicin application there was ~30% decrease in the C input amplitude as compared to the baseline (prior to capsaicin) C-fibre eEPSC amplitude, while following 10mins of capsaicin application the decrease reached ~50% (**Figure 5.11B**). Interestingly, in the presented data capsaicin caused maximum ~50% decrease in the C-fibre eEPSC amplitude after 10mins of its application, which is when the effect of capsaicin has plateaued.

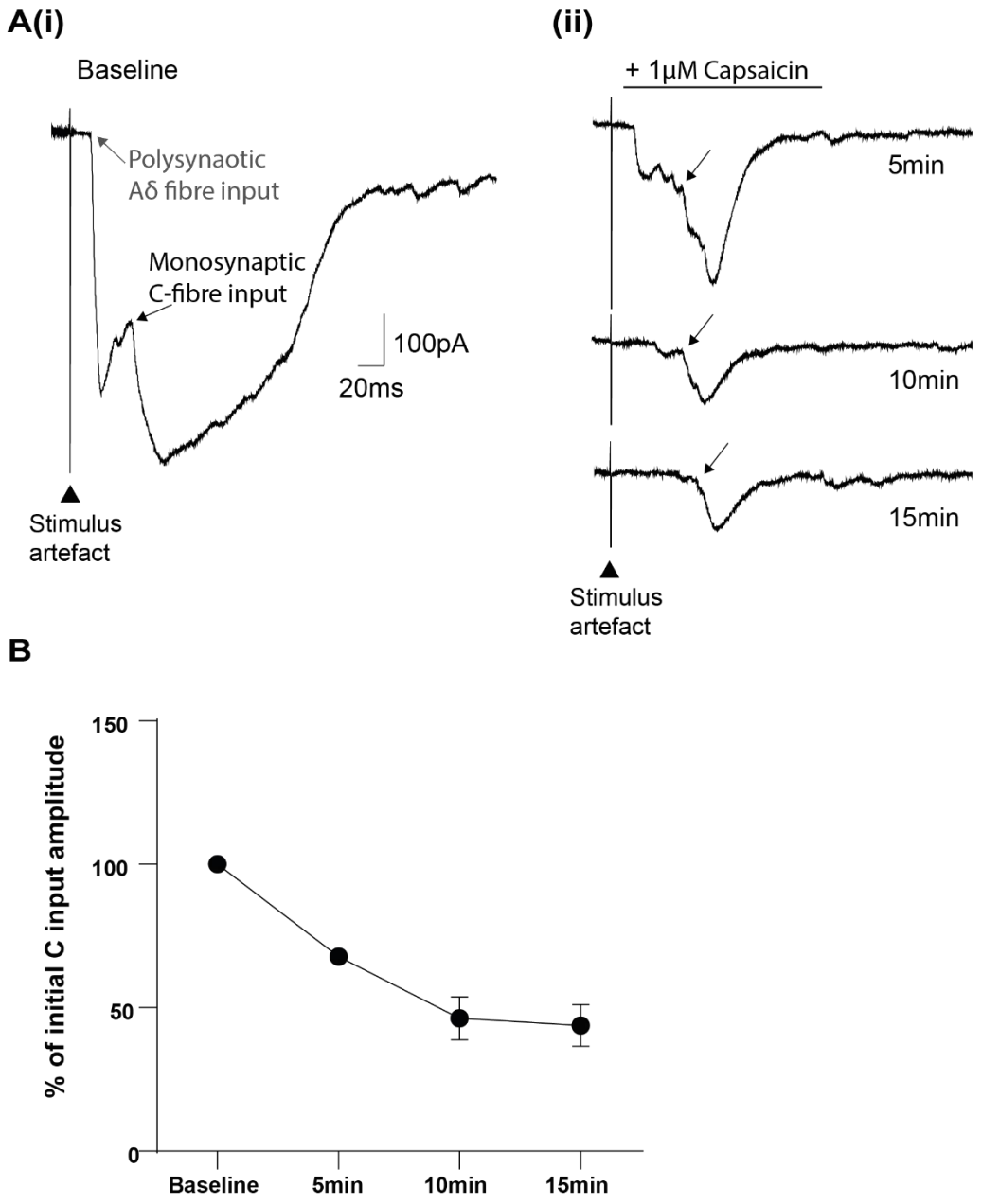


Figure 5.11 Noxious heat-induced Fos-EGFP expressing neurons show capsaicin sensitivity

A) Representative eEPSC recordings (averaged from 3x) at 500μA at 0.05Hz showing the monosynaptic C-fibre eEPSC (black arrow) amplitude at baseline (**i**) and following 5min, 10min and 15mins bath application of 1μM capsaicin (**ii**). **B**) Stimulus-response plot of the C-fibre eEPSC peak amplitude as a percentage of the initial C-fibre eEPSC peak amplitude at different time points following capsaicin application. Data presented as mean±SEM, n=4 cells.

5.5.2 ADS in monosynaptic C-fibre input and its impact on Fos-EGFP heat and WT capsaicin sensitive spinal neurons in mice of both sexes

eEPSC recordings were conducted in Fos-EGFP heat and WT capsaicin sensitive spinal neurons from spinal cord slices with attached dorsal roots from male and female mice to assess basic properties and ADS in the monosynaptic C-fibre inputs to these neurons in both sexes. The primary afferent inputs were differentiated based on activation threshold and conduction velocity, with repetitive stimulation at higher frequencies used to differentiate between polysynaptic and monosynaptic inputs (**Figure 5.6**). Only neurons with monosynaptic C-fibre input were analysed. There was no significant effect of sex on activation threshold (**Figure 5.12A**; unpaired two-tailed t-test: p=0.243), initial average conduction velocity (CV) (**Figure 5.12B**; unpaired two-tailed t-test: (p>0.999) and initial latency (**Figure 5.12C**; unpaired two-tailed t-test: p=0.949) of monosynaptic C-fibre inputs.

Repetitive dorsal root stimulation (16x times at 700µA at 2Hz) resulted in a clear ADS in monosynaptic C-fibre input to Fos-EGFP heat and WT capsaicin sensitive spinal neurons, analysed collectively, reflected by a progressive increase in the C-fibre eEPSC latency (**Figure 5.12D, E**). In line with the mouse CAP data, where the males displayed significantly more pronounced C-fibre ADS, in the eEPSC recordings from males there was also significantly more pronounced ADS in monosynaptic C-fibre inputs to Fos-EGFP heat and WT capsaicin sensitive spinal neurons, analysed collectively (**Figure 5.12E, D**; Unpaired one-tailed t-test: *p*=0.028).

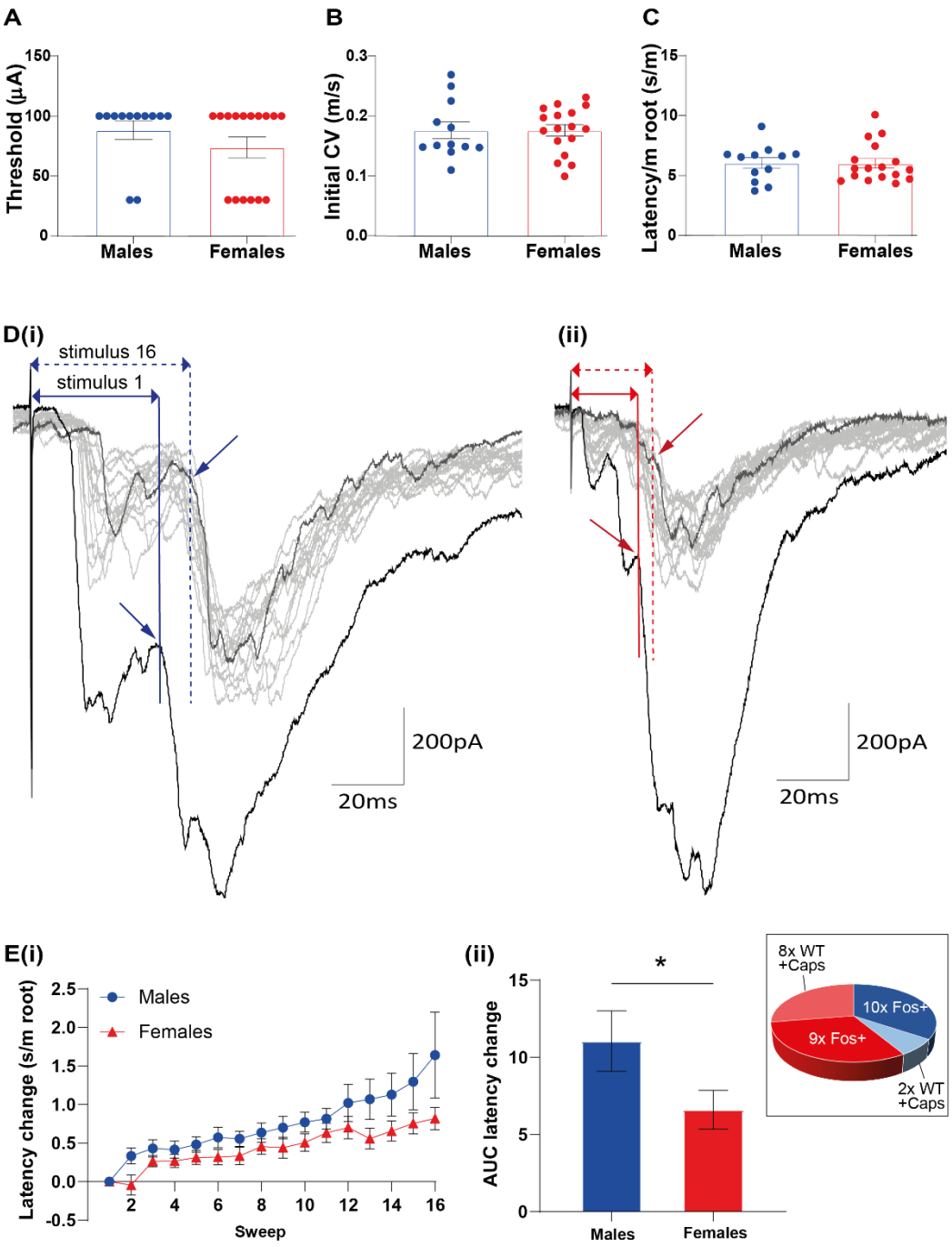


Figure 5.12 Sex differences in ADS in monosynaptic C-fibre input to Fos-EGFP heat and WT capsaicin sensitive spinal neurons, analysed collectively.

The activation threshold (**A**) (p=0.243), initial conduction velocity (**B**) (p>0.999) and the latency (**C**) (p=0.949) of monosynaptic C-fibre eEPSCs to Fos-EGFP heat and WT capsaicin sensitive spinal neurons, analysed collectively, were not significantly different between both sexes (Unpaired two-tailed t-test). **D**) Representative monosynaptic C –fibre eEPSC recordings from spinal cord slices with attached dorsal roots isolated from male (**i**, annotated in blue) and female (**ii,** annotated in red) mice in response to x16 stimuli at 2Hz at 700µA (response trace 1 black; 2-39 pale grey; 40 dark grey). Monosynaptic C-fibre eEPSC in response to the first (vertical solid line) and the 16th (vertical dashed line)

stimulus is mark by arrows, indicating the latency of the monosynaptic C-fibre input. Latency change is illustrated by the difference in latency of the 16th stimulus (dashed double-headed arrow) from the first stimulus (solid double-headed arrow). **Ei**) Repetitive stimulation results in a progressive increase in the latency of monosynaptic C-fibre input in both males and females. **Eii**) AUC analysis reveals that male mice display more pronounced ADS in monosynaptic C-fibre input to Fos-EGFP heat or WT capsaicin sensitive spinal neurons, analysed collectively (Unpaired one-tailed t-test (based on CAP mouse data): **p*=0.028). Pie chart inset at top right corner illustrates the proportion of noxious heat-induced Fos-EGFP spinal neurons and WT spinal neurons with capsaicin-sensitive inputs that comprise the presented data. Data presented as mean±SEM. Males, n=12 (10x Fos-EGFP and 2x WT capsaicin-sensitive); females, n=17 (9x Fos-EGFP and 8x WT capsaicin-sensitive).

To explore the impact of ADS in monosynaptic C-fibre input upon the activity of spinal neurons with heat sensitive inputs, current clamp eEPSP recordings were conducted in Fos-EGFP heat sensitive spinal neurons from spinal cord slices with attached dorsal roots from mice of both sexes. Repetitive stimulation (16x times at 700µA at 2Hz) resulted in a progressive decrease in action potential firing, induced at C-fibre stimulation strength, which was more pronounced in females in the Fos-EGFP heat sensitive spinal neurons (**Figure 5.13A-Ci**). The statistical analysis revealed a significantly more pronounced monosynaptic C-fibre-evoked activity in the Fos-EGFP heat sensitive spinal neurons in females compared to males (**Figure 5.13Cii**; unpaired two-tailed t-test: p=0.03). Given that there were evidently more monosynaptic C-fibre-evoked action potentials to start with in females, the effect of sex on the initial number of monosynaptic C-fibre-evoked action potentials was statistically assessed, revealing significantly more C-fibre-evoked action potentials in response to the first stimulus in females (**Figure 5.13D**; unpaired two-tailed t-test: p=0.04). To compare the amount of wind-down in both sexes, wind-down was calculated based on the formula for wind-up calculation (Dickenson et al., 1997), where the overall number of action potentials induced by all the applied stimuli is subtracted from the number of action potentials induced by the first stimulus multiplied by the overall number of stimuli applied. The statistical analysis of these data revealed a significantly more pronounced wind-down in females compared to males (**Figure 5.13E**; unpaired two-tailed t-test: p=0.043).

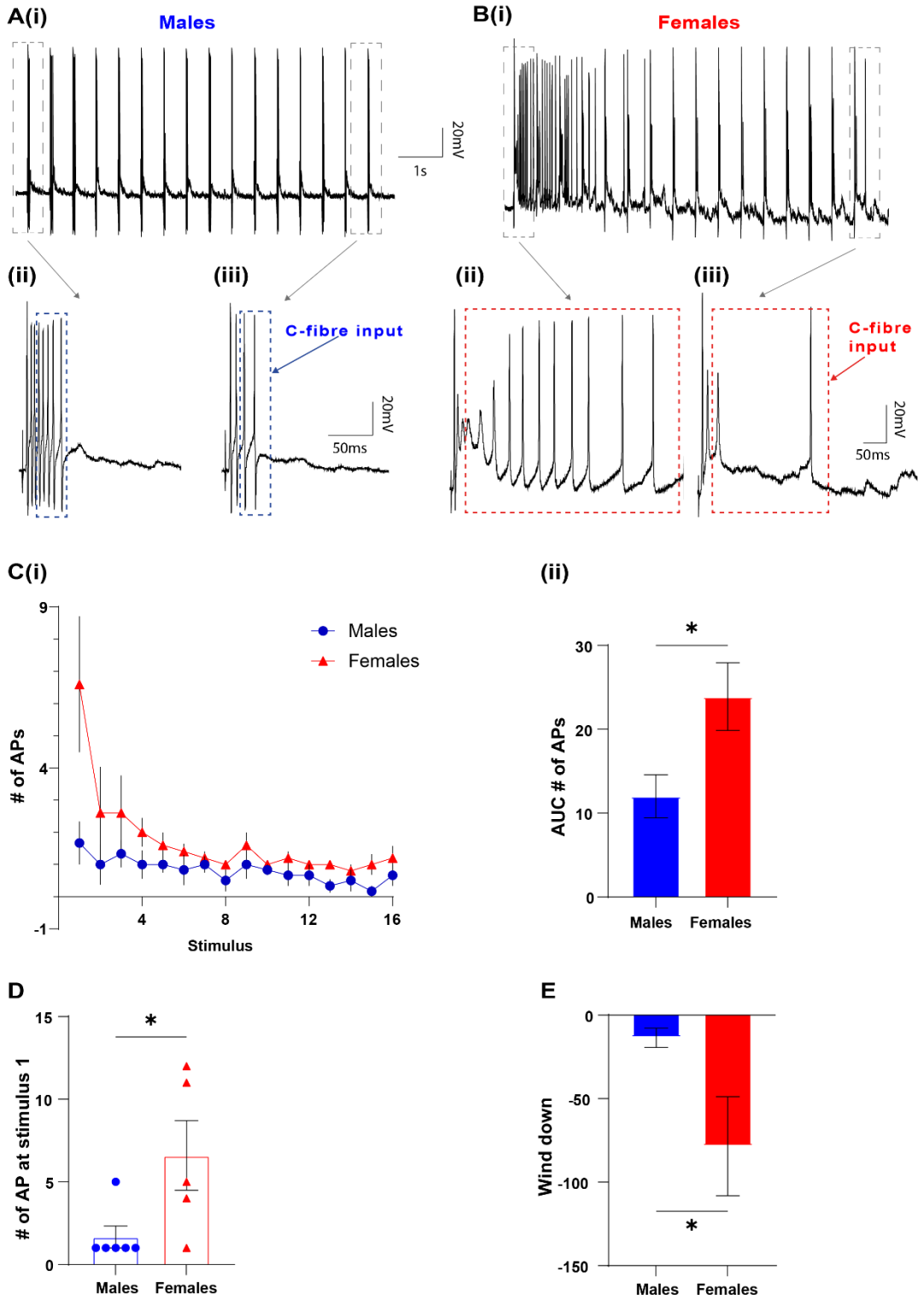


Figure 5.13 Noxious heat sensitive spinal neurons show sex differences in C-fibre-induced AP firing in a train of stimuli, with females showing more pronounced AP firing initially and a more pronounced wind down

Representative eEPSP recordings from spinal cord slices with attached dorsal roots isolated from male (**A**, annotated in blue) and female (**B,** annotated in red) mice in

response to x16 stimuli at 2Hz at 700µA, with an expanded timescale of the area marked by black dashed boxes (**i**), showing the number of action potentials following the first (ii) and the 16th (**iii**) stimulus. The C-fibre –induced action potentials, identified based on the latency of the C-fibre response in preceding eEPSC recordings, are marked by the dashed boxes in blue and red for males (**A**) and females (**B**), respectively. **C**) Repetitive stimulation results in a progressive decrease in the number of action potentials firing that was more pronounced in females (**i**), with AUC analysis (**ii**) showing that females display significantly more pronounced action potential firing following repetitive stimulation in comparison to males (Unpaired two-tailed t-test: **p*=0.03). **D**) Comparison of the number of action potentials at stimulus 1 in both sexes revealed that the females show significantly more action potential firing in response to the first stimulus in comparison to the males (Unpaired two-tailed t-test: **p*=0.04). **E**) Comparison of the overall number of action potentials following the 16x stimuli with respect to the initial number of action potentials at stimulus 1 revealed a significantly more pronounced wind-down in females than in males (Unpaired two-tailed t-test: **p*=0.043). Data presented as mean±SEM. Males, n=6 cells (Fos-EGFP); females, n=5 cells (Fos-EGFP).

5.5.3 Effect of prolonged low-frequency stimulation on monosynaptic C-fibre inputs and its impact on Fos-EGFP heat and WT capsaicin sensitive spinal neurons in mice of both sexes

Prolonged low-frequency stimulation was used as a tool to manipulate ADS in monosynaptic C-fibre inputs to Fos-EGFP heat and WT capsaicin sensitive spinal neurons given that prolonged low-frequency stimulation altered C-fibre ADS in population CAP recordings in mice (**Chapter 4**). This allowed to investigate the effect of different levels of ADS on monosynaptic C-fibre inputs and their impact on the activity of spinal neurons with heat/capsaicin sensitive inputs. To assess the effect of manipulation on ADS in monosynaptic C-fibre inputs to spinal neurons with heat/capsaicin sensitive inputs, ADS in monosynaptic C-fibre input was recorded at baseline and post-manipulation, similarly to the CAP manipulation ADS mouse data (**Chapter 4**).

Experimental low-frequency manipulation, but not control manipulation, caused synaptic response failures during post-manipulation repetitive stimulation of monosynaptic C-fibre input. In the control manipulation treatment group 2Hz repetitive stimulation of monosynaptic C-fibre input resulted in minimal synaptic response failures both at baseline and post-manipulation, with no significant

differences in overall number of synaptic response failures to Fos-EGFP heat and WT capsaicin sensitive spinal neurons, analysed collectively, in both sexes (**Figure 5.14Ci**; two-way ANOVA: control manipulation, $p>0.999$, sex, $p=0.233$, control manipulation x sex, $p>0.999$). There was also no change in the number of synaptic response failures per stimulus during repetitive stimulation of monosynaptic C-fibre input at 2Hz at baseline and post- manipulation (**Figure 5.14Cii**). However, in the manipulation treatment group, the prolonged low-frequency stimulation for 1min at 1Hz caused a significant increase in the overall number of synaptic response failures during post-manipulation repetitive stimulation of monosynaptic C-fibre input to Fos-EGFP heat and WT capsaicin sensitive spinal neuron, analysed collectively, in both sexes (**Figure 5.14Di**; two-way ANOVA: manipulation, $p<0.0001$, sex, $p=0.658$, manipulation x sex, $p=0.658$). There was also a progressive increase in the number of synaptic response failures per stimulus during post-manipulation repetitive stimulation of monosynaptic C-fibre input to Fos-EGFP heat and WT capsaicin sensitive spinal neurons, analysed collectively (**Figure 5.14Dii**).

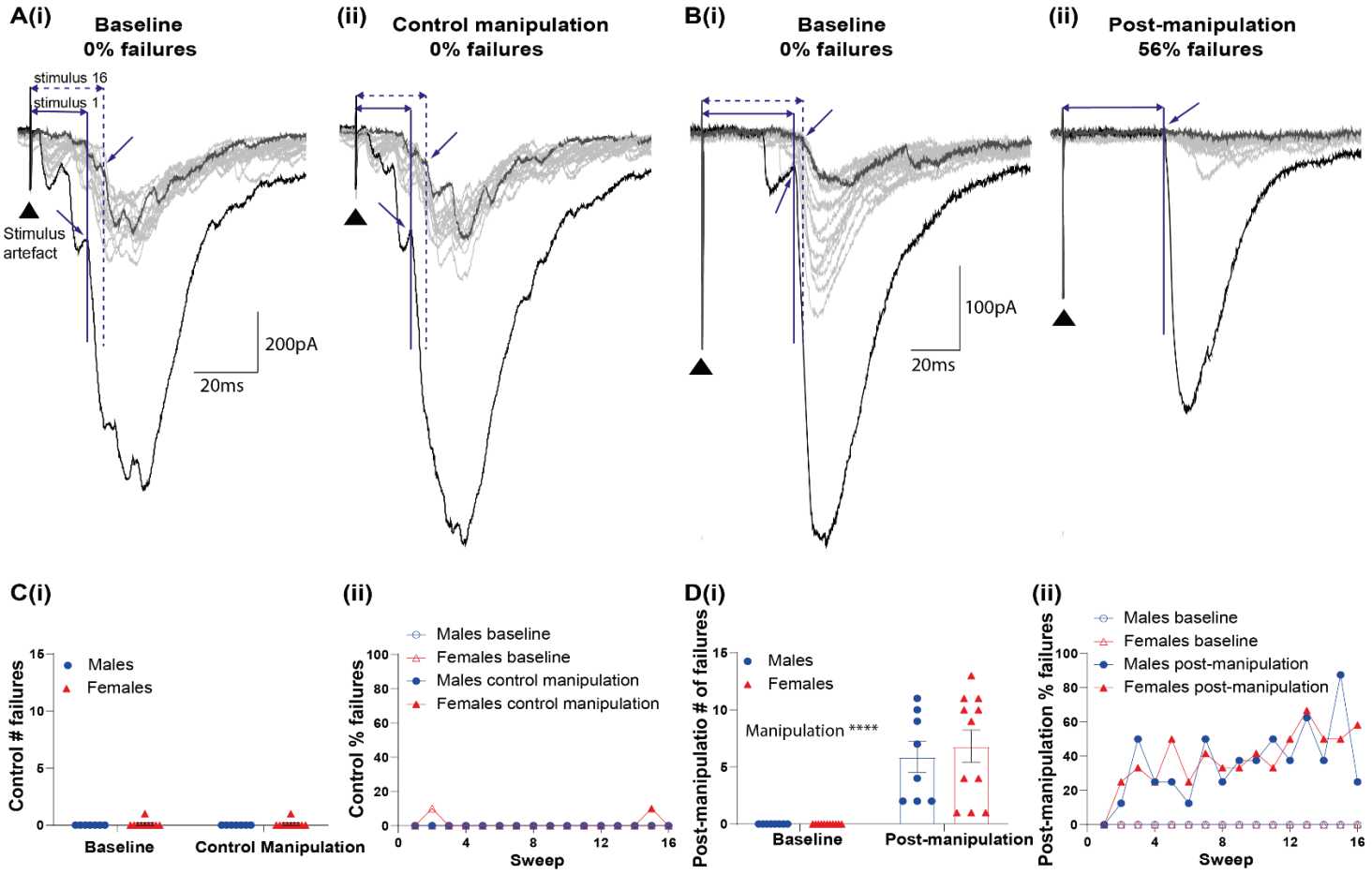


Figure 5.14 Prolonged low frequency stimulation increases the number of synaptic response failures in monosynaptic C-fibre inputs to Fos-EGFP heat and WT capsaicin sensitive spinal neurons, analysed collectively, in both sexes

Representative monosynaptic C-fibre eEPSC recordings in the control manipulation (**A**) and the manipulation (**B**) conditions in response to x16 stimuli at 2Hz at baseline (**i**) and following manipulation (**ii**) (response trace 1 black; 2-39 pale grey; 40 dark grey). Monosynaptic C-fibre eEPSC in response to the first (vertical solid line) and the 16^{th} (vertical dashed line) stimulus is mark by arrows, indicating the latency of the monosynaptic C-fibre input. Latency change is illustrated by the difference in latency of to the 16th stimulus (dashed double-headed arrow) compared with the first stimulus (solid double-headed arrow). **C**) In the control manipulation treatment group there is no significant difference in the overall number of synaptic response failures in both sexes (**i**) (manipulation, $p>0.999$, sex, $p=0.233$, manipulation x sex, $p>0.999$) with no progressive increase in the number of synaptic response failures per stimulus (**ii**) during repetitive stimulation of monosynaptic C-fibre input to Fos-EGFP heat and WT capsaicin sensitive spinal neurons, analysed collectively. **D**) Following prolonged low-frequency stimulation for 1min at 1Hz there is a significant increase in the overall number of synaptic response failures in both sexes (**i**) (manipulation, ****$p<0.0001$, sex, $p=0.658$, manipulation x sex, $p=0.658$) with a progressive increase in the number of synaptic response failures per stimulus (**ii**) during post-manipulation repetitive stimulation of monosynaptic C-fibre input to Fos-EGFP heat and WT capsaicin sensitive spinal neurons, analysed collectively (two-way ANOVA). Data presented as mean±SEM. Control manipulation: males, n=7 (5x Fos-EGFP and 2x WT capsaicin-sensitive; females, n=10 (5x Fos-EGFP and 5x WT capsaicin-sensitive). Manipulation: males, n=8 (6x Fos-EGFP and 2x WT capsaicin-sensitive; females, n=11 (5x Fos-EGFP and 6x WT capsaicin-sensitive).

After quantification of the synaptic response failures during repetitive stimulation of monosynaptic C-fibre input, ADS in monosynaptic C-fibre input to Fos-EGFP heat or WT capsaicin sensitive spinal neurons at baseline and post-manipulation was assessed in the control manipulation and the manipulation treatment groups. In the manipulation treatment group, ADS during post-manipulation was assessed based on the synaptic responses that did not fail during repetitive stimulation of monosynaptic C-fibre input. The repetitive stimulation of monosynaptic C-fibre input resulted in a clear progressive increase in ADS in monosynaptic C-fibre input in males (**Figure 5.15B**) and females (**Figure 5.15C**) in both the control manipulation (i) and the manipulation (ii) treatment groups, reflected by the progressive increase in the C-fibre eEPSC latency. The prolonged low-frequency stimulation caused a significant increase in post-manipulation ADS in monosynaptic C-fibre inputs in both males (**Figure 5.15Biii**: two-way ANOVA: interaction, p=0.006, followed by Sidak's multiple comparison test, p=0.0006 baseline vs post-manipulation in the manipulation treatment group) and females (**Figure 5.15Cii**i: two-way ANOVA: interaction, p=0.005, followed by Sidak's multiple comparison test, p=0.001 baseline vs post-manipulation in the manipulation treatment group). To compare the effect of the manipulation on ADS between the sexes, the change in post-manipulation monosynaptic C-fibre eEPSC latency change from the corresponding baseline monosynaptic C-fibre eEPSC latency change was calculated on an individual cell basis in the control manipulation (**Figure 5.15Di**) and the manipulation (**Figure 5.15Dii**) conditions. This revealed a significantly more pronounced increase in ADS post-manipulation in the manipulation compared to control manipulation that was comparable between both sexes (**Figure 5.15Diii**; Two-way ANOVA: manipulation, p=0.0001, sex, p=0.399, manipulation x sex, p=0.214).

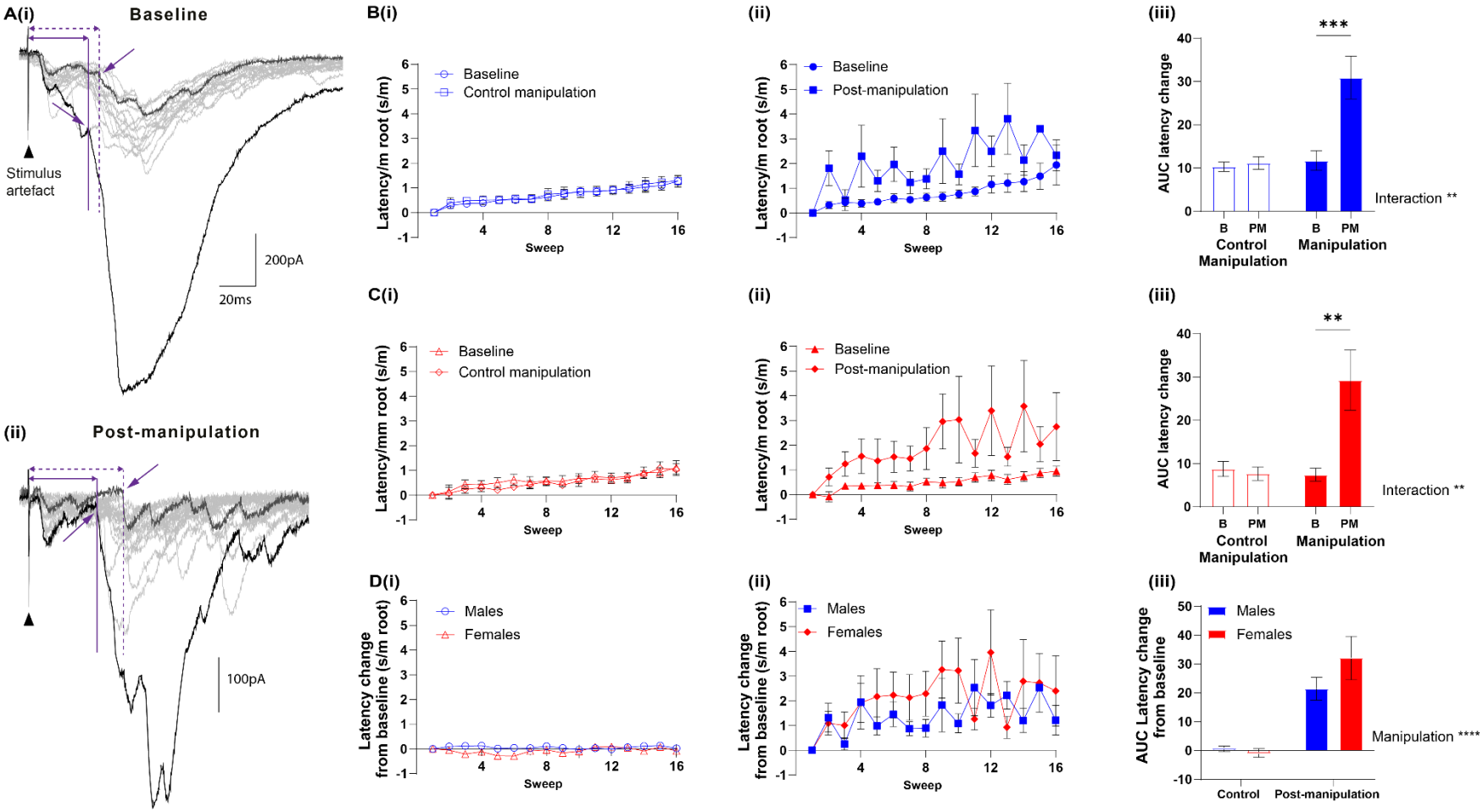


Figure 5.15 Prolonged low frequency stimulation increases ADS post-manipulation in monosynaptic C-fibre inputs to Fos-EGFP heat and WT capsaicin sensitive spinal neurons, analysed collectively, in both sexes

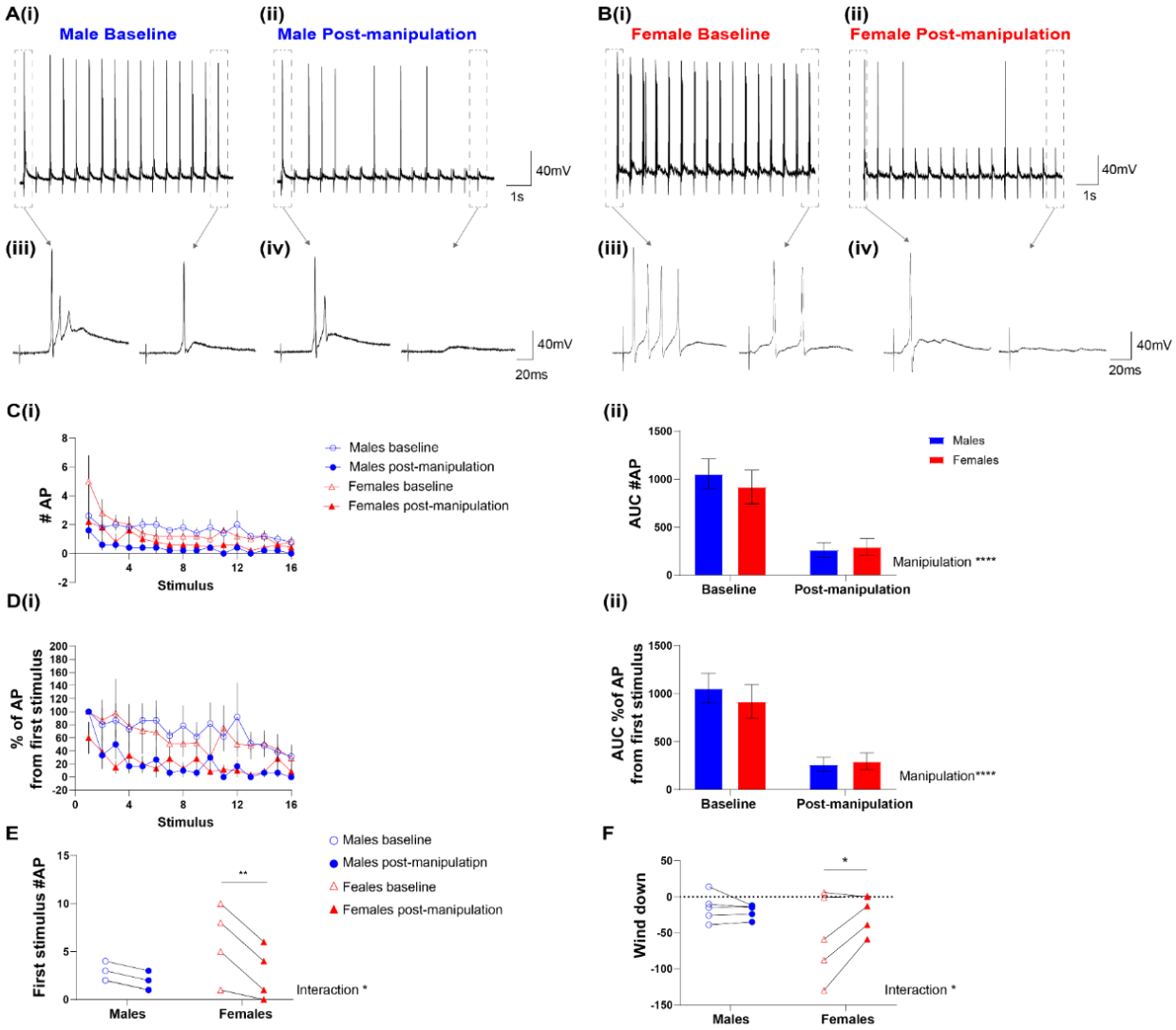
A) Representative eEPSC recordings of monosynaptic C-fibre input in the manipulation conditions in response to x16 stimuli at 2Hz at baseline (**i**) and post-manipulation (**ii**) (response trace 1 black; 2-39 pale grey; 40 dark grey). Monosynaptic C-fibre eEPSC in response to the first (vertical solid line) and the 16^{th} (vertical dashed line) stumus is mark by arrows, indicating the latency of the monosynaptic C-fibre input. Latency change is illustrated by the difference in latency of the 16th stimulus (dashed double-headed arrow) compared with the first stimulus (solid double-headed arrow). Progressive increase in the latency change in males (**B**) and females (**C**) in the control manipulation (i) and the manipulation (**ii**) conditions at baseline and post-manipulation during repetitive stimulation of monosynaptic C-fibre input to Fos-EGFP heat and WT capsaicin sensitive spinal neurons, analysed collectively. AUC analysis (**iii**) revealed a significant effect of manipulation that is dependent on the experimental condition (**p=0.006 in males, **p=0.005 in females) (control manipulation vs manipulation) (Two-way ANOVA followed by Sidak's multiple comparison test, males: ***p=0.0006 baseline vs post-manipulation in the manipulation treatment group; females: **p=0.001 baseline vs post-manipulation in the manipulation treatment group). **D**) The grouped male and female effect of manipulation data represented as the change in post-manipulation monosynaptic C-fibre input latency change from the corresponding baseline monosynaptic C-fibre input latency change on an individual cell basis in the control manipulation (**i**) and manipulation (**ii**) conditions. AUC analysis (**iii**) revealed a significant effect of manipulation (****p<0.0001) to a comparable degree in both sexes (p=0.399) with no interaction between the two factors (p=0.214) (Two-way ANOVA). Data presented as mean±SEM. Control manipulation: males, n=7 (5x Fos-EGFP and 2x WT capsaicin-sensitive; females, n=10 (5x Fos-EGFP and 5x WT capsaicin-sensitive). Manipulation: males, n=8 (6x Fos-EGFP and 2x WT capsaicin-sensitive; females, n=11 (5x Fos-EGFP and 6x WT capsaicin-sensitive).

To assess the impact of ADS manipulation/dynamic memory in monosynaptic C-fibre input upon the activity of spinal neurons with heat-capsaicin sensitive inputs in both sexes, eEPSP current clamp recordings were conducted in Fos-EGFP heat and WT capsaicin sensitive spinal neurons from spinal cord slices with attached dorsal roots from mice of both sexes. Given the eEPSC data showed no effect of control manipulation on ADS in monosynaptic C-fibre input, the control manipulation was not conducted in I-clamp experiments.

Repetitive stimulation (16x times at 700µA at 2Hz) of monosynaptic C-fibre input to Fos-EGFP heat and WT capsaicin sensitive spinal neurons, analysed collectively, resulted in a decrease in the number of C-fibre-evoked action potentials at baseline and post-manipulation (**Figure 5.16A-Ci**). There was significantly more pronounced C-fibre-evoked action potential firing at baseline compared to post-manipulation in both sexes (**Figure 5.16Ci**i; two-way ANOVA: manipulation, $p<0.0001$, sex, $p=0.409$, manipulation x sex, $p=0.143$). In addition, normalising the number of evoked action potentials to the number of action potentials evoked at the first stimulus in a train of 16x stimuli revealed a reduced action potential firing post manipulation when compared to baseline in both sexes (**Figure 5.16Di**). The statistical analysis revealed overall less C-fibre strength-evoked action potential firing post-manipulation compared to baseline in both sexes (**Figure 5.16Dii**; two-way ANOVA: manipulation, $p<0.0001$, sex, $p=0.692$, manipulation x sex, $p=0.531$).

As evident in **Figure 5.16A-Ci** there appeared to be differences in the initial number of monosynaptic C input-evoked action potentials. Indeed, analysis of the initial number of monosynaptic C input-evoked action potentials revealed significantly more C input-evoked action potentials during the first stimulus at baseline compared to post-manipulation in females only (**Figure 5.13E**; Two-way ANOVA: manipulation x sex, $p=0.04$ followed by Sidak's multiple comparison test, $p=0.001$). To compare the amount of wind-down at baseline and post-manipulation in both sexes, wind-down was calculated based on the formula for wind-up calculation (Dickenson et al., 1997), where the overall number of action potentials induced by

all the applied stimuli is subtracted from the number of action potentials induced by the first stimulus multiplied by the overall number of stimuli applied. The statistical analysis of these data revealed a significantly more pronounced wind-down post-manipulation compared to baseline in females, but not in males (**Figure 5.13F**; two-way ANOVA: manipulation x sex, p=0.047 followed by Sidak's multiple comparison test, *p=0.04).


A(i)
Male Baseline
(ii)
Male Post-manipulation
B(i)
Female Baseline
(ii)
Female Post-manipulation
40mV
1s
(iii)
(iv)
40mV
20ms
C(i)
# AP
Stimulus
Males baseline
Males post-manipulation
Females baseline
Females post-manipulation
(ii)
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Baseline
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Interaction *
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Wind down
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Interaction *
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Figure 5.16 Repetitive stimulation decreases C-fibre-evoked action potential firing post-manipulation in both sexes, with females showing more pronounced action potential firing initially and more pronounced wind down.

Representative current clamp eEPSP recordings from spinal cord slices with attached dorsal roots isolated from male (**A**) and female (**B**) mice at baseline (**i**) and post-manipulation (**ii**) during repetitive stimulation (x16 stimuli at 2Hz) of monosynaptic C-fibre input to Fos-EGFP heat sensitive spinal neuron, with an expanded timescale of the area marked by black dashed boxes, showing the number of action potentials following the first and the 16th stimulus at baseline (**iii**) and post-manipulation (**iv**). All action potentials were induced by dorsal root stimulation at C-fibre strength in Fos-EGFP heat sensitive spinal neurons with monosynaptic C-fibre input. **C**) Repetitive stimulation results in a decrease in the number of action potentials at baseline and post-manipulation in both sexes (**i**). AUC analysis (**ii**) showed significantly more pronounced action potential firing at baseline compared to post-manipulation (****p<0.0001) in both sexes (p=0.409) following repetitive stimulation at C-fibre strength in Fos-EGFP heat sensitive spinal neurons with monosynaptic C-fibre input (Two-way ANOVA: interaction, p=0.143). **D**) Repetitive stimulation causes a progressive decrease in the normalised number of action potentials at baseline and post-manipulation in both sexes (**i**). AUC analysis (**ii**) revealed significantly more pronounced action potential firing at baseline compared to post-manipulation (****p<0.0001) in both sexes (p=0.692) following repetitive stimulation at C-fibre strength in Fos-EGFP heat sensitive spinal neurons with monosynaptic C-fibre input (Two-way ANOVA: interaction, p=0.531). **E**) Comparison of the number of action potentials at stimulus 1 at baseline and post-manipulation in both sexes revealed a significant effect of manipulation that is dependent on sex (*p=0.04) with a more pronounced action potential firing in response to the first stimulus at baseline compared to post-manipulation in females only (Two-way ANOVA followed by Sidak's multiple comparison test, **p=0.001). **F**) Comparison of the overall number of action potentials following the 16x stimuli with respect to the initial number of action potentials at stimulus 1 at baseline and post–manipulation in both sexes revealed a significant effect of manipulation that is dependent on sex (*p=0.047) with a more pronounced wind-down post-manipulation in females (Two-way ANOVA followed by Sidak's multiple comparison test, *p=0.04). Data presented as mean±SEM. Males, n=5; females, n=5.

5.5.4 Effect of stimulation distance on ADS in monosynaptic C-fibre inputs and its impact on Fos-EGFP heat or WT capsaicin sensitive spinal neurons in mice of both sexes

Although the prolonged low-frequency manipulation enabled assessment of the impact of dynamic memory upon spinal pain processing, it was not an appropriate tool to experimentally manipulate levels of ADS in monosynaptic C-fibre inputs to assess the impact on of ADS on the activity of spinal neurons with heat/capsaicin sensitive inputs due to the high number of synaptic response failures post-manipulation (see **Section 5.5.3**). Thus, the length dependency of ADS (Schmelz et al., 1995, Zhu et al., 2009) was used to investigate the impact of different levels of ADS upon the activity of spinal neurons with heat/capsaicin sensitive inputs. Repetitive stimulation (16x times at 700µA at 2Hz) resulted in a clear ADS in monosynaptic C-fibre input at both short and long stimulation distances, reflected by the progressive increase in the C-fibre eEPSC latency (**Figure 5.17A-Bi**). As hypothesised, longer stimulation distances resulted in a significantly more pronounced progressive latency change in monosynaptic C-fibre eEPSC, with no significant difference in the amount of ADS between males and females at short and long stimulation distances (**Figure 5.17Bii**; Two-way ANOVA: distance, $p<0.0001$, sex, $p=0.371$, distance x sex, $p=0.675$).

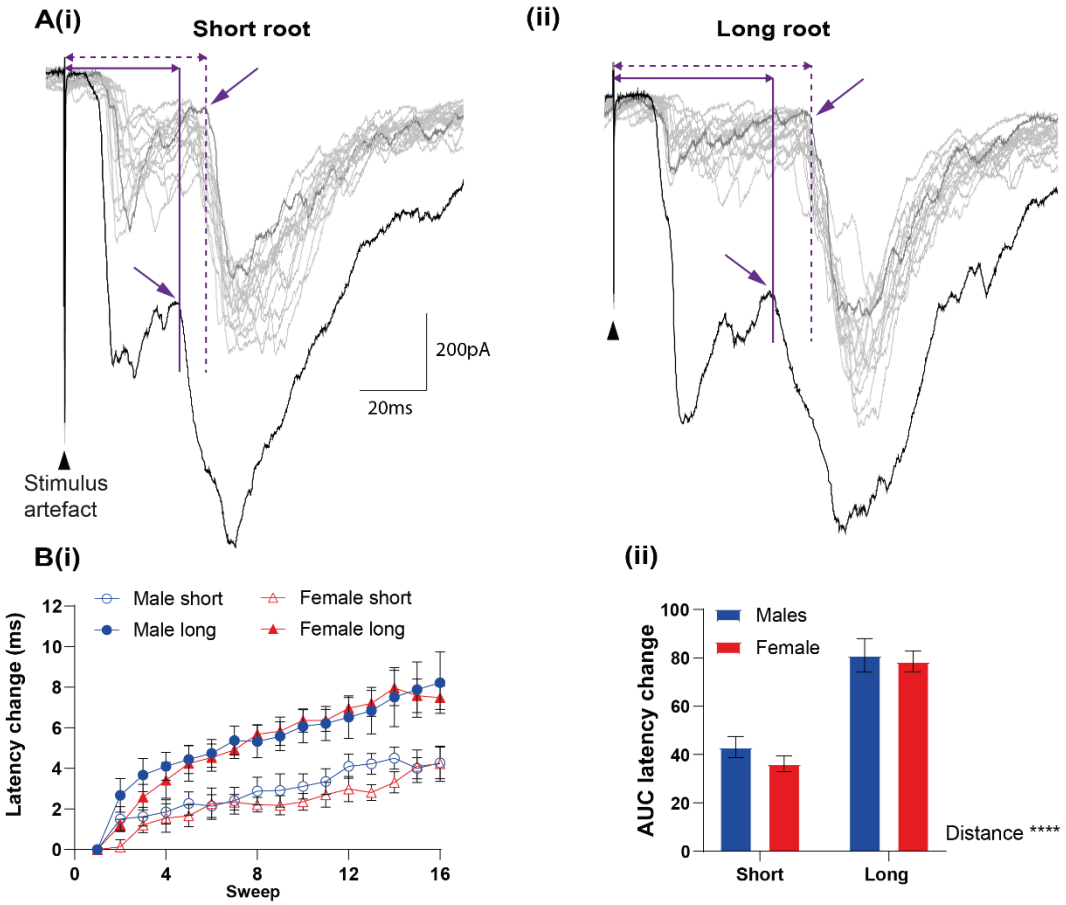


Figure 5.17 Stimulation distance affects ADS in monosynaptic C-fibre input to noxious heat sensitive spinal neurons in both sexes

A) Representative eEPSC recordings of monosynaptic C-fibre in response to x16 stimuli at 2Hz at 700µA at short (**i**) and long (**ii**) stimulation distance (response trace 1 black; 2-39 pale grey; 40 dark grey). Monosynaptic C-fibre eEPSC in response to the first (vertical solid line) and the 16th (vertical dashed line) stimulus is mark by arrows, indicating the latency of the monosynaptic C-fibre input. Latency change is illustrated by the difference in latency of the 16th stimulus (dashed double-headed arrow) compared with the first stimulus (solid double-headed arrow). **B**) Progressive increase in latency change at short and long stimulation distances in both sexes during repetitive stimulation of monosynaptic C-fibre input to Fos-EGFP heat or WT capsaicin sensitive spinal neurons (**i**). AUC analysis (**ii**) showed a significant effect of stimulation distance (****p<0.0001), with more pronounced ADS at long stimulation distances in both sexes (p=0.371) and no interaction between the two factors (p=0.675) (Two-way ANOVA). Data presented as mean±SEM. Males, n=7 (6x Fos-EGFP and 1x WT capsaicin-sensitive); females, n=6 (5x Fos-EGFP and 1x WT capsaicin-sensitive).

To assess the impact of the degree of ADS in monosynaptic C-fibre input upon the activity of spinal neurons with heat/capsaicin sensitive inputs in both sexes, eEPSP

recordings were conducted in Fos-EGFP heat sensitive spinal neurons from spinal cord slices with an attached dorsal root stimulated at a short and long distance (**Figure 5.3B**). To enable comparison of distance and sex data were normalised to the number of action potentials induced by the first stimulus in a given condition to account for any potential differences in action potential number between conditions. The repetitive stimulation (16x times at 700µA at 2Hz) of monosynaptic C-fibre input to Fos-EGFP heat sensitive spinal neurons, progressively reduced the number of C-fibre-evoked action potentials at short and long stimulation distances in both sexes (**Figure 5.18A-Ci**). This progressive decrease in C-fibre-evoked action potential firing was greater at short compared to long stimulation distances in both sexes (**Figure 5.18Cii**; two-way ANOVA: distance, p=0.038, sex, p=0.178, manipulation x sex, p=0.71). Notably, there was significantly more pronounced wind-down at shorter compared to longer stimulation lengths in females, but not in males (**Figure 5.18D**; two-way ANOVA: manipulation x sex, p=0.047 followed by Sidak's multiple comparison test, *p=0.04). Given the previously observed differences in the initial number of monosynaptic C input-evoked action potentials in the manipulation experiment (**Figure 5.16**), the effects of sex and manipulation on the initial number of monosynaptic C input-evoked action potentials were assessed. The statistical analysis revealed significantly more C-fibre-evoked action potentials during the first stimulus at shorter stimulation lengths in females compared to males **(Figure 5.18E**; Two-way ANOVA: distnce x sex, p=0.003 followed by Sidak's multiple comparison test, p=0.042).

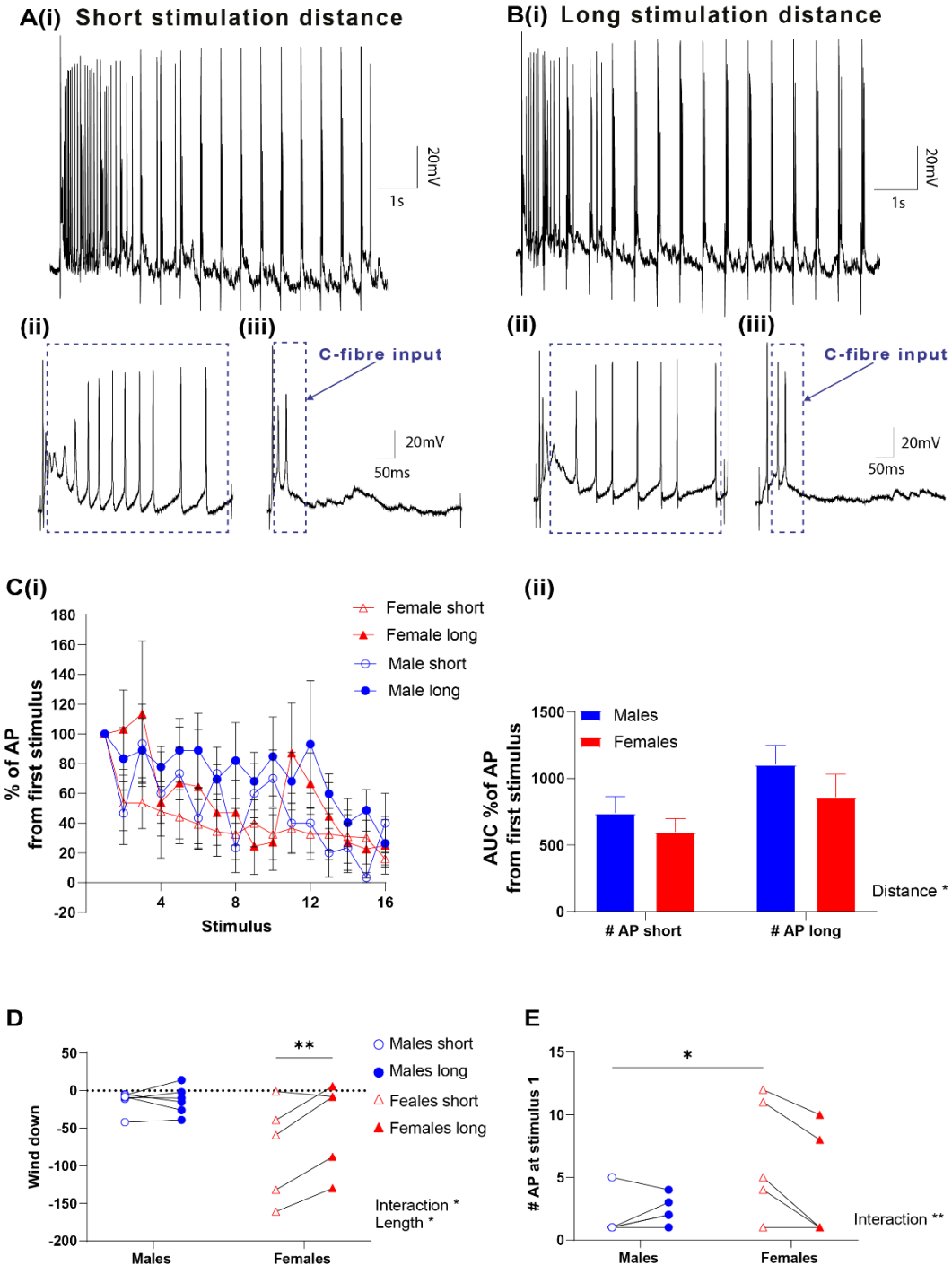


Figure 5.18 Stimulation distance modulates AP firing in a train of stimuli in both sexes and influences degree of wind-down in females only

Representative eEPSP recordings from Fos-EGFP heat sensitive spinal neurons in spinal cord slices with attached dorsal roots, repetitively stimulated at C-fibre strength (x16 stimuli at 2Hz at 700µA) at a short (**A**) and at a long (**B**) dorsal root distance. An expanded timescale shows the number of action potentials following the first (**ii**) and the 16th (**iii**) stimulus. The C-fibre –induced action potentials, identified based on the latency of the C-fibre response in preceding eEPSC recordings, are marked by the dashed boxes. **C**) There

is a progressive decrease in the normalised number of action potentials at short and long stimulation distances in both sexes (**i**) following repetitive stimulation of monosynaptic C-fibre input to Fos-EGFP heat sensitive spinal neurons. AUC analysis (**ii**) showed significantly more pronounced action potential firing at long compared to short stimulation distances (*p=0.038) in both sexes (p=0.178), with no interaction between the two factors (p=0.71) (Two-way ANOVA). **D**) Comparison of wind-down calculations revealed an effect of stimulation distance that is dependent on sex (*p=0.018) with a more pronounced wind-down at short distances in females (Two-way ANOVA followed by Sidak's multiple comparison test, **p=0.007). **E**) Comparison of the number of action potentials at stimulus 1 at short and long stimulation distances in both sexes revealed a significant effect of stimulation distance that is dependent on sex (**p=0.003), with more pronounced action potential firing in response to the first stimulus at short stimulation distance in females compared males (Two-way ANOVA followed by Sidak's multiple comparison test, *p=0.042). Data presented as mean±SEM. Males, n=6; females, n=5.

To assess whether the observed impact of the degree of ADS in monosynaptic C-fibre input on the activity of spinal neurons with heat-capsaicin sensitive inputs in both sexes could be explained by activity-dependent changes in the monosynaptic C-fibre eEPSC peak amplitude, this was measured following each of the 16x applied stimuli at 2Hz in both sexes. There was no significant effect of stimulation distance or sex on the initial peak amplitude of the monosynaptic C-fibre eEPSC (**Figure 5.19A**; two-way ANOVA: distance, p=0.928, sex, p=0.079, distance x sex, p=0.361). In addition, there was no significant effect of distance and sex on the normalised peak amplitude change following repetitive stimulation (**Figure 5.19B-C**; two-way ANOVA: distance, p=0.150, sex, p=0.673, distance x sex, p=0.654).

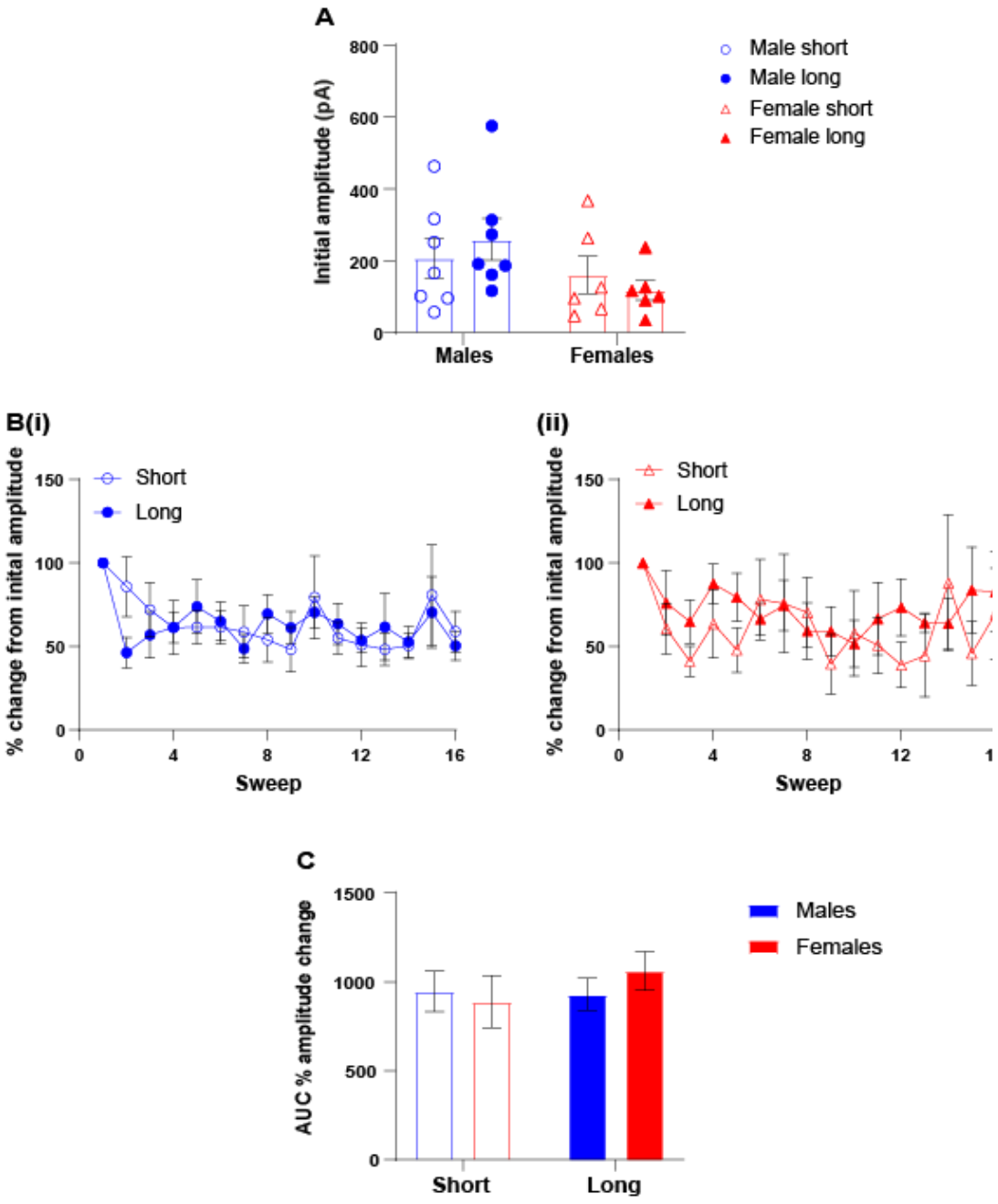


Figure 5.19 Stimulation distance does not alter initial C-fibre eEPSC peak amplitude or the change in normalised C-fibre eEPSC peak amplitude following repetitive stimulation in both sexes

A) Comparison of the initial amplitude at stimulus 1 at short and long stimulation distances in both sexes revealed no significant effect of stimulation distance (p=0.928) and sex (p=0.079), and no interaction between the two factors (p=0.361) (Two-way ANOVA). **B**) Normalised peak amplitude per stimulus at short and long stimulation distances in males (**i**) and females (**ii**) following repetitive stimulation of monosynaptic C-fibre input to Fos-EGFP heat sensitive spinal neurons. **C**) AUC analysis revealed no significant effect of stimulation distance (p=0.505) and sex (p=0.756), and no interaction

between the two factors (p=0.418) (Two-way ANOVA). Data presented as mean±SEM. Males, n=7; females, n=6.

To assess whether the observed impact of the degree of ADS in monosynaptic C-fibre input on the activity of spinal neurons with heat-capsaicin sensitive inputs in both sexes, could be explained by activity-dependent changes in polysynaptic C-fibre-evoked net charge, this was measured following each of the 16x applied stimuli at 2Hz in both sexes. There was no significant effect of stimulation distance or sex on the initial net charge (**Figure 5.20A**; two-way ANOVA: distance, p=0.457, sex, p=0.127, distance x sex, p=0.563). In addition, there was no significant effect of distance and sex on normalised net charge following repetitive stimulation (**Figure 5.20B-C**; two-way ANOVA: distance, p=0.150, sex, p=0.673, distance x sex, p=0.654).

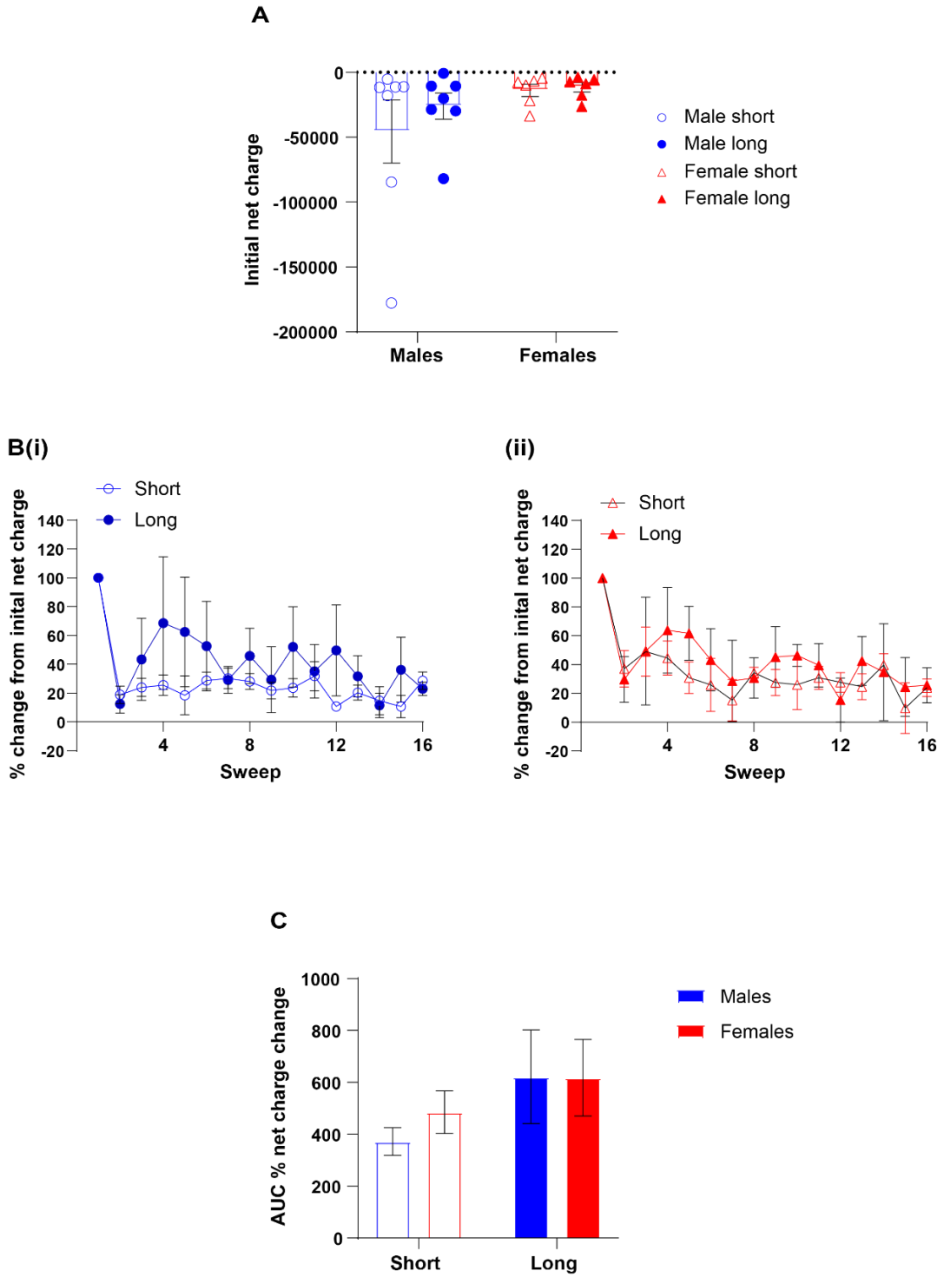


Figure 5.20 Stimulation distance does not alter the polysynaptic C-fibre-evoked initial net charge or the normalised net charge following repetitive stimulation in both sexes

A) Comparison of the initial net charge at stimulus 1 at short and long stimulation distances in both sexes revealed no significant effect of stimulation distance (p=0.457) and sex (p=0.127), and no interaction between the two factors (p=0.563) (Two-way ANOVA). **B**) Normalised net charge at short and long stimulation distances in males (**i**) and females (**ii**) following repetitive stimulation of monosynaptic C-fibre input to Fos-EGFP heat or WT capsaicin sensitive spinal neurons. **C**) AUC analysis revealed no significant effect stimulation distance (p=0.150) and sex (p=0.673), and no interaction

between the two factors (p=0.654) (Two-way ANOVA). Data presented as mean±SEM. Males, n=7; females, n=6.

5.6 Discussion

5.6.1 Summary findings

This chapter studied the processing of monosynaptic C-fibre input in Fos-EGFP spinal neurons responsive to noxious heat and WT spinal neurons with capsaicin sensitive input. It was demonstrated that Fos-EGFP heat sensitive spinal neurons receive monosynaptic C-fibre inputs, which displayed ADS following repetitive stimulation of the primary afferent fibres at C-fibre stimulation strength. Fos-EGFP heat sensitive spinal neurons were confirmed to have capsaicin sensitive inputs. The results in this chapter were obtained by combining data from Fos-EGFP heat and WT capsaicin sensitive spinal neurons and will be referred to as 'Fos-EGFP heat/WT capsaicin sensitive spinal neurons' for remainder of the discussion.

In line with sex differences in C-fibre ADS in CAP recordings, there were also sex differences in ADS in monosynaptic C-fibre input to Fos-EGFP heat/WT capsaicin sensitive spinal neurons, with males showing significantly more pronounced ADS compared to females (**Table 5.2**). When studying the impact of repetitive C-fibre stimulation upon the activity of Fos-EGFP heat sensitive neurons, females were revealed to display more pronounced overall activity following repetitive stimulation, which was likely the result of the significantly more pronounced initial firing in females. These sex differences in the evoked activity of the Fos-EGFP heat sensitive neurons may potentially contribute to the observed sex differences in heat sensitivity in this mouse strain.

Table 5.2 Sex differences in ADS and its impact on heat pain processing on a single spinal neuron level

**findings from Kest et al. (1999), Leo et al. (2008)

	C-fibre ADS	Monosynaptic C input	Initial evoked activity	Overall activity repetitive stimulation	Heat threshold	Mechanical threshold
C57BL/6 mice	M>F	M>F	F>M	F>M	M>F**	M=F**

Interestingly, this chapter also found that prolonged low-frequency manipulation similarly affects both sexes by increasing the number of synaptic input failures and ADS in monosynaptic C inputs to the Fos-EGFP heat/WT capsaicin sensitive spinal neurons (**Table 5.3**). The prolonged low frequency manipulation also reduced the activity of the Fos-EGFP heat sensitive neurons to a similar level in both sexes, but females displayed a significant manipulation-induced reduction in initial AP firing and in wind-down.

Table 5.3 Prolong low frequency stimulation (manipulation)-induced changes in ADS and their impact on heat pain processing on a single spinal neuron level

PM denotes post-manipulation

	PM ADS	PM monosynaptic C input	PM Initial activity	PM activity repetitive stimulation
C57BL/6 mice	F>M	↑ ADS (M=F) ↑ Input failures (M=F)	↓ F only	↓ (M=F)

Utilising the length-dependency of ADS it was demonstrated that stimulation distance alters ADS levels independent of sex, with longer stimulation distances causing more pronounced ADS in monosynaptic C-fibre inputs to the Fos-EGFP heat/WT capsaicin sensitive spinal neurons (**Table 5.4**). These increased levels of ADS in the monosynaptic C-fibre inputs to the Fos-EGFP heat/WT capsaicin sensitive spinal neurons were associated with less pronounced reduction in the activity of the Fos-EGFP heat/WT capsaicin sensitive spinal neurons following repetitive

stimulation. The more pronounced reduction in the activity of the Fos-EGFP heat/WT capsaicin sensitive spinal neurons in both sexes was shown to not relate to changes in amplitude or net charge in monosynaptic C-fibre inputs to the Fos-EGFP heat/WT capsaicin sensitive spinal neurons.

Table 5.4 Stimulation distance-induced changes in ADS and their impact on heat pain processing on a single spinal neuron level

L denotes longer-stimulation lengths; S denotes shorter-stimulation lengths

	ADS	Monosynaptic C input	Reduction in activity-dependent firing
C57BL/6 mice	L>S	L>S	S>L

5.6.2 Spinal cord Fos-EGFP expression induced by intraplantar capsaicin injections and noxious heat hindpaw stimulation

Previous studies have shown that the chemical stimulus capsaicin, which activates primarily C-fibres (Amaya et al., 2003, Kobayashi et al., 2005, Michael and Priestley, 1999, Yu et al., 2008), induces expression of Fos protein in dorsal horn spinal cord neurons (Wu et al., 2000, Zou et al., 2001, Palecek et al., 2003, Zou et al., 2002, Coggeshall, 2005) in a dose-dependent manner, with higher doses of capsaicin correlating with increased c-Fos expression (Jinks et al., 2002). Although these studies have demonstrated capsaicin-induced expression of Fos protein using different immunohistochemical techniques, the findings in this chapter have also shown that intraplantar capsaicin injection can lead to Fos-EGFP expression in superficial dorsal horn neurons in an *ex vivo* spinal cord slice preparation from Fos-EGFP mice. It was further demonstrated that the intraplantar capsaicin-induced Fos-EGFP expressing superficial spinal cord dorsal horn neurons received capsaicin-sensitive inputs, with bath applied capsaicin potentiating mEPSC frequency in these neurons, as demonstrated for other unidentified spinal cord neurons (Baccei et al.,

2003, Wrigley et al., 2009, Spicarova and Palecek, 2009) and for lamina I NK1R+ spinal neurons (Labrakakis and MacDermott, 2003, Dickie and Torsney, 2014). Interestingly, the findings of this experiment also showed that the effects of 1μM capsaicin on mEPSC frequency in the intraplantar capsaicin-induced Fos-EGFP neurons were reversible, which unlike findings reported by Baccei et al. (2003) and Dickie and Torsney (2014) for a proportion of the studied neurons. However, the reported reversible effect of capsaicin on mEPSC frequency in this chapter can be due to the lower concentrations of capsaicin used (1μM vs 2μM) and/or the small sample size of the experiment in this chapter. The fact that bath application of capsaicin did not alter mEPSC frequency of nearby Fos-EGFP –ve neurons further supports the findings that intraplantar capsaicin can induce Fos-EGFP in spinal neurons specifically receiving capsaicin–sensitive inputs. Given that the capsaicin receptor TRPV1 has been shown to be expressed only on the primary afferents' terminals in the spinal cord (Caterina et al., 1997, Tominaga et al., 1998, Cavanaugh et al., 2011) these capsaicin–induced effects are suggested to be presynaptic. However, this chapter did not access capsaicin-induced effects on mEPSC amplitude, which typically indicates a change in the postsynaptic response to a neurotransmitter (Engelman and MacDermott, 2004), so a postsynaptic effect of bath applied capsaicin on the intraplantar capsaicin-evoked mEPSC cannot be ruled out. Nevertheless, previous reports have attributed duel increase in mEPSC frequency and amplitude following bath application of capsaicin at ≥1μM to summation of high frequency events (Baccei et al., 2003, Wrigley et al., 2009, Dickie and Torsney, 2014), which further suggests that the capsaicin-induced potentiation of mEPSC frequency is most likely due to presynaptic effects of bath applied capsaicin.

In line with previous studies showing that hot water bath hindpaw immersion leads to Fos-like immunoreactivity in rat lumbar spinal cord neurons localised primarily to the lamina I-II region (Buritova and Besson, 2000, Abbadie et al., 1994, Huang and Simpson, 1999, Jones, 1998, Bullitt, 1990), the findings of this chapter have also shown that hot water bath hindpaw stimulation, similar to the intraplantar

capsaicin injections, is able to induce Fos-EGFP expression in superficial spinal cord dorsal horn neurons. Earlier studies have tried different water bath temperatures ranging from 52 to 60 ^{o}C to study Fos protein expression using immunohistochemical techniques. However, Abbadie et al. (1994) have demonstrated that the number of Fos-like immunoreactive neurons increased with increasing temperatures of the water bath from 46 to 52^{o}C, however 65 ^{o}C stimulation, caused expression of Fos in fewer spinal neurons compared to 52^{o}C. Therefore, 52^{o}C hot water stimulation has been established as optimal for activating maximal number of spinal cord neurons (Abbadie et al., 1994), with increasing stimulus duration leading to increased numbers of Fos-like immunoreactive neurons (Abbadie et al., 1994, Bullitt et al., 1992). Thus, although studies have shown spinal cord Fos immunoreactivity following stimulation of one hindpaw at 52^{o}C for 15s only, the *ex vivo* patch-clamp experiments in this chapter employed a stimulation at 52^{o}C twice for 15s with a 2-minute interval between stimulations. The slightly increased duration of the hot water bath stimulation led to an increased number of spinal neurons expressing Fos-EGFP, which increased the numbers of available Fos-EGFP neurons for electrophysiological recording. This in turn increased the probability of establishing successful patch-clamp recordings from Fos-EGFP heat sensitive spinal neurons, receiving monosynaptic C-fibre input so that the ADS phenomenon and its impact on those neurons could be investigated.

It was important to confirm whether the monosynaptic C-fibre input to the Fos-EGFP heat sensitive spinal neurons was also TRPV1-expressing, i.e. sensitive to capsaicin, the agonist of the ligand- and heat-sensitive TRPV1 ion channel (Caterina et al., 1997, Caterina et al., 2000, Tominaga et al., 1998). Previous studies have showed that capsaicin-induced activation of TRPV1 can block C-fibre medicated eEPSC (Yang et al., 1999, Tong and MacDermott, 2006) likely due to capsaicin-mediated TRPV1 activation, causing depolarisation of the primary afferent fibres, which is thought to prevent action potential invasion of the terminals. In line with these studies, the results in this chapter demonstrated that bath application of capsaicin onto spinal cord slices with attached dorsal roots reduced the amplitude

of the C-fibre mediated eEPSC to Fos-EGFP heat sensitive spinal neurons. This further confirms that these spinal neurons receive direct inputs from capsaicin- and heat-sensitive C-fibre nociceptors. Similar to Tong and MacDermott (2006), where the bath application of capsaicin was shown to inhibit monosynaptic eEPSC from primary afferent Aδ fibres to NK1R+ and NK1R- lamina I spinal neurons with an average depression of ~68% and ~85%, respectively, this chapter also reported a capsaicin–induced inhibition of monosynaptic C-fibre eEPSC to Fos-EGFP heat sensitive spinal neurons with an average depression of ~50%. The slightly less pronounced capsaicin-induced reduction in the amplitude of monosynaptic C-fibre eEPSC to the Fos-EGFP heat sensitive spinal neurons reported in this chapter can be explain by the fact that the experiments in this chapter were conducted in adult mice, unlike the Tong and MacDermott (2006) study, which used juvenile rats. In addition, the different type of primary afferent fibre input investigated could have also contributed to these slight differences in capsaicin-induced reduction in the amplitude of monosynaptic C-fibre eEPSC. Unlike Tong and MacDermott (2006) study, which investigated effect of capsaicin superfusion on monosynaptic eEPSC from primary afferent Aδ fibres, this chapter assessed the effect of capsaicin superfusion on monosynaptic eEPSC from primary afferent C fibres. However, this is unlikely because, in contrast to the Tong and MacDermott (2006) study, earlier studies have reported that only the amplitude of C-fibre eEPSC to lamina II spinal cord neurons is affected by capsaicin, while the amplitude of Aδ fibre eEPSC has been shown to remain unaffected (Yang et al., 1999). However, this study did not assess the mono/polysynaptic nature of the primary afferent inputs and therefore the absence of capsaicin-induced effect on the amplitude of A fibre eEPSC to lamina II spinal neurons should be interpreted with caution. Another factor that could have contributed to the slight differences in the degree of capsaicin-induced reduction in the amplitude of monosynaptic eEPSC from primary afferent fibres could have been the type and location of the spinal neurons receiving the primary afferent inputs. This is supported by the demonstrated by Tong and MacDermott (2006) ~68% and

~85% average depression of monosynaptic eEPSC from primary afferent Aδ fibres to NK1R+ and NK1R- lamina I spinal neurons, respectively.

5.6.3 ADS in monosynaptic C-fibre input to Fos-EGFP and WT capsaicin sensitive neurons in mice of both sexes

The results in this chapter have also novelly revealed that monosynaptic C-fibre input to Fos-EGFP heat/WT capsaicin sensitive spinal neurons in adult mice displays sex-dependent ADS in response to repetitive stimulation at 2Hz, similar to findings in lamina I NK1R+ spinal neurons in juvenile rats (Dickie et al., 2017). The sex differences in ADS in monosynaptic C-fibre input to the Fos-EGFP heat/WT capsaicin sensitive spinal neurons (**Figure 5.12**) were in line with sex differences in mouse C-fibre ADS in population CAP recordings (**Figure 4.12**), with males displaying more pronounced C-fibre ADS in monosynaptic C-fibre eEPSC to the Fos-EGFP heat/WT capsaicin sensitive spinal neurons. In contrast to these findings in mice, studies in juvenile rats have demonstrated the opposite sex difference in ADS, with females displaying a more pronounced C-fibre ADS in population CAP recordings and a more pronounced ADS in monosynaptic C-fibre eEPSC to lamina I NK1R+ spinal neurons (Dickie et al., 2017). These species-dependent sex differences in C-fibre ADS at a population level and in ADS in monosynaptic C-fibre input to individual spinal cord neurons are consistent with behavioural findings of noxious heat sensitivity, with male *C57BL/6* mice shown to have higher noxious heat thresholds (Leo et al., 2008, Kest et al., 1999), in contrast to SD rats, where females have been demonstrated to exhibit higher noxious heat thresholds (Mogil et al., 2000, Dickie et al., 2017). Interestingly, Dickie et al. (2017) have also demonstrated that ADS can influence summation at a spinal network levels, with an increased ADS profile limiting spinal summation, which was consistent with the ADS profile in female SD rats, which also displayed higher noxious heat, but not mechanical, thresholds. This study also reported that following CFA inflammation both males and females displayed comparable ADS profiles and comparable CFA-induced noxious heat threshold, with females therefore exhibiting a more pronounced reduction in noxious heat thresholds, i.e. more pronounced noxious heat hypersensitivity. This suggested that

sex- and injury-dependent ADS can regulate noxious heat drive to spinal pain circuits, influencing spinal summation relevant for noxious heat sensation and the flexion-withdrawal reflex. Consistent with this proposal, reports in humans have also demonstrated sex difference in temporal summation of thermal pain (Fillingim et al., 2009).

To further investigate the impact of sex-dependent ADS on the activity of individual spinal neurons, responsible for noxious heat processing, the experiments in this chapter employed current-clamp recordings in Fos-EGFP heat sensitive spinal neurons with monosynaptic C-fibre input in mice of both sexes. The fact that repetitive stimulation at C-fibre strength caused a progressive decrease in C-fibre-induced action potential firing is consistent with the suggested role of ADS acting as a protective mechanism to limit nociceptive input to the spinal cord (De Col et al., 2012, Mazo et al., 2013, Kist et al., 2016). In line with this proposal, the reported in males more pronounced C-fibre ADS in population CAP recordings (**Figure 4.12**) is consistent with the reported in males more pronounced ADS in monosynaptic C-fibre eEPSC to the Fos-EGFP heat/WT capsaicin sensitive spinal neurons (**Figure 5.12**) and less pronounced C-fibre-induced AP firing in Fos-EGFP heat sensitive spinal neurons (**Figure 5.13C**). Interestingly, these findings were also consistent with previous reports of more pronounced noxious heat threshold in *C57BL/6* male mice (Leo et al., 2008, Kest et al., 1999). However, the C-fibre-induced AP firing in Fos-EGFP heat sensitive spinal neurons in both sexes (**Figure 5.13C**) does not reflect alterations induced by C-fibre ADS only because the data has not been normalised to the number of action potentials induced by the first stimulus in the train. In addition, the fact that females showed significantly more pronounced initial AP firing and more pronounced wind-down, further supports the idea that the observed sex differences in C-fibre-induced AP firing in the Fos-EGFP heat sensitive spinal neurons likely reflect other sex differences in these neurons. The more pronounced initial AP firing in females can potentially result from pre- and/or postsynaptic differences that can allow their Fos-EGFP heat sensitive spinal neurons to fire more C-fibre-induced AP in response to an electrical stimulation.

Previous studies have suggested that the temporal summation of nociceptive inputs could be influenced by the wind-up phenomenon, which is a form of short-term potentiation (STP), which enhances synaptic strength and plays an important role in spinal neurons and in the development of central sensitisation (McMahon et al., 1993, Arendt-Nielsen and Petersen-Felix, 1995). Wind-up describes the frequency-dependent increase in the response of spinal neurones following repetitive stimulation of peripheral C-fibres (Herrero et al., 2000, Mendell, 1966, Mendell and Wall, 1965). This form of facilitation has been demonstrated in a variety of spinal neurons, including wide dynamic range (WDR) neurons in the deep dorsal horn, motor neurons in the ventral horn as well as unidentified and spinoparabrachial neurons in the superficial dorsal horn (Schouenborg and Sjölund, 1983, Woolf and Wall, 1986, Zhang et al., 1991, Hachisuka et al., 2018, Greenspon et al., 2019). Both wind-up and temporal summation are suggested as physiological mechanisms of amplification that have the potential to contribute to hypersensitivity and following injury, as suggested by inflammatory pain models showing magnified wind-up (Hedo et al., 1999, Herrero et al., 2000, Thompson et al., 1995, Traub, 1997) and in pain patients displaying heightened temporal summation (Eide et al., 1996, Nikolajsen et al., 1996, Price et al., 1992, Vase et al., 2013). Therefore, this chapter also investigated whether repetitive stimulation at C-fibre strength, shown to induce ADS in monosynaptic C-fibre inputs to the Fos-EGFP heat/WT capsaicin sensitive spinal neurons, could influence the temporal summation of the nociceptive inputs to and the wind-up phenomenon in the Fos-EGFP heat sensitive spinal neurons, in a sex-dependent manner.

Interestingly, contrary to suggested induction of wind-up following repetitive stimulation of peripheral C-fibres, the findings in this chapter reported a progressive reduction in AP firing in response to repetitive stimulation of peripheral C-fibres, which was referred to as a ‘wind-down’ and was used as a measure of short-term depression (STD). STD is another form of STP thought to be involved in information processing, where there is a transient reduction in synaptic strength, which can last from hundreds of milliseconds to seconds, causing a dynamic reduction in

postsynaptic responses (Zucker and Regehr, 2002, Fioravante and Regehr, 2011, Deperrois and Graupner, 2020). This chapter reported a more pronounced wind-down in females, which is in contrast to predictions, based on the demonstrated less pronounced ADS in monosynaptic C-fibre eEPSC to the Fos-EGFP heat/WT capsaicin sensitive spinal neuron in females, expected to decrease timing between successive nociceptive inputs reaching the spinal cord, likely enhancing wind-up or in this case limiting wind-down in females. However, these findings further support the idea that the observes sex differences in C-fibre-induced AP firing in the Fos-EGFP heat sensitive spinal neurons likely reflect other sex differences in these neurons rather than sex differences in C-fibre ADS. Given that STD has been attributed to primarily presynaptic mechanisms, mainly involving depletion of some pool of vesicles and likely release site and calcium channel inactivation (Zucker and Regehr, 2002, Fioravante and Regehr, 2011). Therefore, the reported sex differences in wind-down suggest sex differences in the mechanism underling STD, likely involving differences in the depletion of the readily reversible pool. Given that the number of vesicles within the readily reversible pool is limiting, if a large fraction of those are released upon a stimulus, there will be fewer vesicle for release upon subsequent stimuli before the readily reversible pool is replenished (Zucker and Regehr, 2002). This modal predicts an increase in depression with an increase in initial release probability and frequency of activation, which has been shown for corticothalamic synapses and synapses in the auditory brainstem (Zucker and Regehr, 2002, Ran et al., 2009, Schneggenburger et al., 2002). Given the demonstrated in this chapter increased initial firing in females and more pronounced wind-down, it is possible that this prediction is also true for the studies synapses in the spinal cord, with females potentially having an increased initial release probability. However, further analysis will need to be conducted to further investigate this hypothesis. To investigate the initial release probability, it would be important to analyse the initial amplitude of monosynaptic C-fibre eEPSC to the Fos-EGFP heat sensitive spinal neurons and compare it between the sexes.

5.6.4 Effect of manipulation on ADS in monosynaptic C-fibre input to and its impact on Fos-EGFP and WT capsaicin sensitive neurons in mice of both sexes

Given that previous chapters have shown that prolonged low-frequency stimulation can enhance C-fibre ADS in population CAP recordings in mice (SEE CHAPTER 4), it was used as a tool to manipulate levels of ADS in monosynaptic C-fibre inputs to Fos-EGFP heat and WT capsaicin sensitive spinal neurons. This method allowed investigation of the effect of different levels of ADS in monosynaptic C-fibre inputs to and their impact on the activity of Fos-EGFP heat/WT capsaicin sensitive spinal neuron. Previous studies have shown that higher stimulation frequencies correlate with a greater number of C-fibre response conduction failures during repetitive stimulation (Nakatsuka et al., 2000, Raymond et al., 1990, Zhu et al., 2009, Sun et al., 2012b) along with increased incidences of synaptic response failures at higher frequencies (Dickie et al., 2017). The results in this chapter have shown that at 2Hz there are minimal incidences of synaptic response failures in monosynaptic C-fibre eEPSC recordings following repetitive stimulation in mice, similarly to findings in rats at 2Hz (Dickie et al., 2017). Importantly there were no sex differences in the number of synaptic response failures in mice at 2Hz. The prolonged low-frequency stimulation caused a pronounced increase in the number of synaptic response failures at 2Hz stimulation, with no differences between the sexes. These synaptic response failures are unlikely due to C-fibre conduction failures because the C-fibre component of the compound action potential, specifically its amplitude, does not seem to be altered at 2Hz (**Figure 4.12- Figure 4.13**). However, it must be noted that the negative peak of the triphasic population response, used for measuring the amplitude (**Figure 2.8**), reflects the average conducting C-fibres and therefore might not reliably reflect conduction failures of the slowest/fastest conducting C-fibres. The prolonged low-frequency stimulation-induced failures at 2Hz are more likely associated with neurotransmitter release, as suggested by early studies showing the synapse can fatigue rapidly (Bullock, 1948, Hagiwara and Tasaki, 1958, Katz and Miledi, 1967). Given that chemical synapses are the main conduits of information in the nervous system (Abbott and Regehr, 2004), where a presynaptic action

potential causes docked vesicles, packed with neurotransmitters, to release with a certain probability (Kandel et al., 2000), which is not constant but displays short-term dynamics (Zucker and Regehr, 2002, Dittman et al., 2000), it is possible that the prolonged stimulation followed immediately by a higher frequency stimulation likely caused neurotransmitter release at a higher rate than reuptake can recycle it, thus resulting in a depletion of readily reversible pool of vesicles with neurotransmitter at the presynaptic terminal. The prolonged 1Hz stimulation for 1min followed by the immediate stimulation with a train of 16x stimuli at 2Hz is likely to deplete the readily-reversible pool of vesicles with neurotransmitter, resulting in a STD, which could lead to a transient reduction in synaptic strength (Zucker and Regehr, 2002, Fioravante and Regehr, 2011, Deperrois and Graupner, 2020), as discussed in **section 5.6.3**. This depletion of neurotransmitter in the presynaptic terminal is likely to explain the observed increase in incidences of synaptic response failures in mice at 2Hz immediately following the prolonged 1Hz stimulation for 1min. However, although STD has been thought to involve primarily presynaptic mechanisms, most likely vesicle depletion as well as inactivation of both release sites and calcium channels (Zucker and Regehr, 2002, Fioravante and Regehr, 2011), the precise nature of the changes in the presynaptic terminal during prolonged repetitive stimulation cannot be determined from the present experiments and further experiments need to be conducted to determine the type of changes involved.

The reported prolonged low-frequency stimulation-induced increase in post-manipulation ADS in monosynaptic C-fibre inputs to Fos-EGFP heat/WT capsaicin sensitive spinal neuron in both sexes, with similar latency change manipulation effects in both sexes (**Figure 5.15**), suggests that the levels of ADS in monosynaptic C-fibre inputs to the combination of Fos-EGFP heat and WT capsaicin sensitive spinal neurons were altered to similar degree in both sexes. This is in contrasts to the population CAP data, showing that the prolonged low-frequency stimulation caused more pronounced increase in the C-fibre latency change in females compared to males (**Figure 4.13**). These differences could arise from the different

techniques used for measuring C-fibre ADS in the dorsal roots and in monosynaptic inputs to spinal cord neurons. Specifically, the CAP recorded population C-fibre responses, while the whole-cell voltage-clamp technique was used to record monosynaptic C-fibre eEPSC to Fos-EGFP heat/WT capsaicin sensitive spinal neurons. Given that different C-fibres subtypes display varying degrees of ADS (Thalhammer et al., 1994, Serra et al., 1999, Weidner et al., 1999), it is possible that the Fos-EGFP heat/WT capsaicin sensitive spinal received monosynaptic inputs form C-fibre subtypes were different from the medium-conducting C-fibres, used for assessing C-fibre latency changes in the population CAP recordings. In addition, it might be possible that the combination of Fos-EGFP heat and WT capsaicin sensitive spinal neurons received monosynaptic inputs form different C-fibre subtypes, especially given the fact that the proportion of Fos-RGFP heat to WT capsaicin senstiove neurons differed between the sexes, with more WT capsaicin sensitive spinal neurons in the female sample. It must also be noted that the CAP C-fibre ADS was recorded in response to a train of 40x stimuli in comparison to the eEPSC, recorded in response to a train of 16x stimuli. A train of 16x stimuli was used for the eEPSC recordings due to concerns regarding neuronal overstimulation and synaptic fatigue (described above) and previous studies in the lab have shown that a train of 16x stimuli can induced ADS in monosynaptic C-fibre input to lamina I NK1R+ neurons (Dickie et al., 2017). Moreover, x16 stimuli are commonly employed to study the spinal wind-up phenomenon (Herrero et al., 2000, Guan and Raja, 2010, Guan et al., 2006). Interestingly, from the CAP C-fibre latency change manipulation effect data in both sexes (**Figure 4.13**) it is clear that up to ~stimulus 20 there is no obvious difference in manipulation-induced latency change between the sexes. This would be consistent with the manipulation-induced increase in ADS in monosynaptic C-fibre eEPSC to Fos-EGFP heat/WT capsaicin sensitive spinal neurons data.

However, given the manipulation-induced high number of synaptic failures in ADS in monosynaptic C-fibre inputs to the Fos-EGFP heat/WT capsaicin sensitive spinal neurons in both sexes, this the manipulation-induced increase in ADS in

monosynaptic C-fibre input data should be interpreted with caution. Given that the synaptic response failures potentially involve fatigue of the presynaptic terminal, with likely depletion of the presynaptic neurotransmitter vesicle pool, which would potentially interfere with neurotransmitter release kinetics (Zucker and Regehr, 2002, Fioravante and Regehr, 2011) and thus with EPSC and potentially eEPSC kinetics, it is possible that this presynaptic fatigue could interfere with eEPSC latency, which is what was measured to assess ADS in monosynaptic C-fibre input. Thus, if the synaptic response failures could be attribute to synaptic fatigue, the observed post-manipulation changes in ADS in monosynaptic C-fibre input could be not entirely due to activity-dependent changes in conduction velocity. Thus, further experiments need to be conducted to determine whether manipulation-induced changes in ADS in monosynaptic C-fibre input to the combination of Fos-EGFP heat and WT capsaicin sensitive spinal neurons in both sexes are attributed to manipulation-induced changes in synaptic response failures in combination with manipulation-induced changes in ADS or solely to the manipulation-induced changes in C-fibre ADS.

Despite the nature of the changes, however, the increased incidences of synaptic failures following repetitive stimulation and the manipulation-induced changes in ADS in monosynaptic C-fibre input to Fos-EGFP heat/WT capsaicin sensitive spinal neurons can clearly alter the synaptic drive to the Fos-EGFP heat and WT capsaicin sensitive spinal neurons. Whole-cell current-clamp recordings of eEPSP of Fos-EGFP heat sensitive spinal neurons in mice of both sexes showed that indeed following the prolonged low frequency stimulation there were changes in the number of eEPSP's evoked at C-fibre stimulation strength. The repetitive stimulation at baseline induced similar level of action potential firing in the Fos-EGFP heat sensitive spinal neurons in both sexes but following the prolonged low-frequency manipulation the level of eEPSP firing was reduced to a similar degree in both sexes. These findings can suggest that dynamic memory via the synaptic response failures may reflect an intrinsic self-inhibition mechanism to limit overdrive of nociceptive pathways. Given the observed manipulation-induced changes in synaptic response

failures, the reduced firing of the Fos-EGFP heat sensitive spinal neurons in both sexes could have been influenced by the similar increase in the number of synaptic response failures in eEPSC evoked at C-fibre stimulation strength between the sexes. However, the prolonged low frequency stimulation-induced increase in post-manipulation ADS in monosynaptic C-fibre eEPSC to the Fos-EGFP heat/WT capsaicin sensitive spinal neurons reported in both sexes could have also contributed to the observed reduced firing of the Fos-EGFP heat sensitive spinal neurons in both sexes. Interestingly, despite the observed similar levels of action potential firing in both sexes at baseline and post-manipulation, females were found to display more pronounced initial AP firing and more pronounced wind-down. These findings support the idea that other sex differences might be driving the more pronounced initial AP firing and more pronounced wind-down in females as described in **section 5.6.3**. It must be also noted, however, that the experiments investigating ADS in monosynaptic C-fibre eEPSC to heat sensitive spinal neurons comprised a bigger sample size with both Fos-EGFP heat and WT capsaicin sensitive spinal neurons, while the experiments investigating the impact of C-fibre synaptic response and ADS on the heat sensitive spinal neurons comprised a small sample size of only Fos-EGFP heat sensitive spinal neurons, which could have contributed to these opposing findings. Considering that the wind-up phenomenon has been demonstrated only in a subpopulation of neurons (Zhang et al., 1991, Hachisuka et al., 2018), and the fact that wind-up was not observed in any of the Fos-EGFP heat sensitive spinal neurons, it would be interesting to investigate the effect of the manipulation on ADS in monosynaptic C-fibre inputs to spinal neurons that display wind-up. These experiments will allow to explore the effect of dynamic memory as well as the effect of different levels of ADS in synapses with repetitive-stimulation-induced enhanced synaptic strength.

5.6.5 Effect of stimulation distance on ADS in monosynaptic C-fibre input to Fos-EGFP and WT capsaicin sensitive neurons in mice of both sexes

The prolonged low-frequency manipulation enabled assessment of synaptic response failures in monosynaptic C-fibre input and their impact upon heat sensitive spinal neurons. However, due to the manipulation-induced increase in incidences of synaptic response failures in monosynaptic C-fibre inputs to the Fos-EGFP heat/WT capsaicin sensitive spinal neurons, the prolonged low frequency stimulation did not provide an appropriate tool to experimentally manipulate levels of ADS in monosynaptic C-fibre inputs to and assess their impact on the activity of spinal neurons with heat/capsaicin sensitive inputs. Therefore, given that previous studies have established that C-fibre ADS is length-dependent (Schmelz et al., 1995, Zhu et al., 2009), this length dependency was utilised to assess the impact of different levels of ADS upon the activity of Fos-EGFP heat sensitive spinal neurons in mice of both sexes. The resultant more pronounced progressive increase in monosynaptic C-fibre eEPSC latency to the Fos-EGFP heat/WT capsaicin sensitive spinal neurons over longer stimulation lengths when compared to stimulations at shorter lengths further supported previous findings of C-fibre ADS being length-dependent (Schmelz et al., 1995, Zhu et al., 2009). However, unlike the previously reported in this chapter sex differences in levels of ADS in monosynaptic C-fibre inputs to the Fos-EGFP heat/WT capsaicin sensitive spinal neurons (**Figure 5.12**) the levels of ADS over the short and long stimulation distances were similar between the sexes (**Figure 5.17**). In must be noted, however, that these two experiments employed different sample sizes with the length-dependency experiment having almost half the sample size of the experiment investigating sex differences in ADS in monosynaptic C-fibre eEPSC to the Fos-EGFP heat/WT capsaicin sensitive spinal neurons (**Figure 5.12**). Also, majority of the neurons in the length dependency experiment were Fos-EGFP heat sensitive, with only one WT capsaicin sensitive spinal neuron in each sex. Given that different C-fibres subtypes display varying degrees of ADS (Thalhammer et al., 1994, Serra et al., 1999, Weidner et al., 1999), it may be possible that different subtypes of C-fibres provided monosynaptic inputs to

the combination of Fos-EGFP heat and capsaicin sensitive spinal neurons in the length-dependency experiment (majority were Fos-EGFP heat sensitive) when compared to the experiment investigating ADS sex differences in monosynaptic C-fibre inputs (**Figure 5.12**), which had more even proportion of Fos-EGFP heat to WT capsaicin sensitive spinal neurons, especially in females. Thus, it would be important for future length dependency experiments of ADS in monosynaptic C-fibre input to heat sensitive spinal neurons to be conducted separately in Fos-EGFP heat and capsaicin sensitive spinal neurons, with optimised sample sizes.

Similarly to the length-dependent differences in ADS in monosynaptic C-fibre inputs to the Fos-EGFP heat/WT capsaicin sensitive spinal neurons in both sexes, there were length-dependent differences in action potential firing evoked by repetitive C-fibre stimulation in Fos-EGFP heat sensitive spinal neurons in both sexes, with no differences in eEPSP firing between the sexes. However, in contrast to the prediction that pronounced ADS would limit summation/limit wind-up, the longer stimulation length induced more pronounced ADS in monosynaptic C-fibre input to the Fos-EGFP heat/WT capsaicin sensitive spinal neurons in both sexes (**Figure 5.17**) but evoked less pronounced reduction in action potential firing of Fos-EGFP heat sensitive spinal neurons in both sexes (**Figure 5.18**). The findings of no sex differences in ADS in monosynaptic C-fibre eEPSC to the Fos-EGFP heat/WT capsaicin sensitive spinal neurons independent of stimulation length (**Figure 5.17**) are thus in line with the demonstrated no sex differences in C-fibre evoked eEPSP firing independent of stimulation length (**Figure 5.18**). However, it would be important to investigate ADS in monosynaptic C-fibre input to and its impact on the activity of the same heat sensitive spinal cord neurons such that both eEPSC and eEPSP recordings are conducted in Fos-EGFP heat or WT capsaicin sensitive spinal neurons.

Notably, the findings in this chapter at a single neuron level are in contrast to the previously demonstrated sex-dependent effect of ADS in juvenile rats acting to increase the timing between successive inputs along the C-fibre nociceptors

reaching the spinal cord, likely limiting the temporal summation of C-fibre evoked synaptic activity at a spinal network level (Dickie et al., 2017). Thus, this chapter has demonstrated that at a single neuron level higher levels of ADS in monosynaptic C-fibre inputs to Fos-EGFP heat sensitive spinal neurons may be associated with less pronounced reduction in activity of those neurons following repetitive stimulation. This could be influenced by a potential involvement of ADS in the recovery kinetics of the presynaptic terminal, which might have undergone repetitive-stimulation-induced depletion of the readily reversible pool of vesicles, as discussed in **section 5.6.3**, such that the more pronounced ADS following repetitive stimulation might allow for the reuptake and recycling of the presynaptic vesicles pool. Higher levels of ADS may increase the time between successive stimuli thus likely allowing the presynaptic terminal to recover from the preceding stimulus, replenish the presynaptic vesicle pool and therefore potentially release enough neurotransmitter necessary for the generation of postsynaptic responses. The fact the amplitude of the monosynaptic C-fibre eEPSC to the Fos-EGFP heat sensitive spinal neurons showed fluctuations in the normalised eEPSC amplitude across the applied 16x stimuli (**Figure 5.19**) and the fact that desensitisation of the post-synaptic receptors has been shown to usually have little contribution to STD at frequencies <10Hz (Wong et al., 2003), further suggest that the demonstrated STD is likely a result of presynaptic rather than post-synaptic mechanisms. However, considering the length-dependency of ADS and the fact that the normalised peak amplitude of the monosynaptic C-fibre eEPSC showed no dependency on stimulation length in both sexes (**Figure 5.19**) it is highly unlikely that ADS in monosynaptic C-fibre input to the Fos-EGFP heat sensitive spinal neurons can influence presynaptic vesicle depletion and thus STD.

Dickie et al. (2017) has demonstrated that levels of ADS can impact upon spinal summation in a sex-dependent manner in juvenile rats, with more pronounced ADS in monosynaptic C-fibre inputs to NK1R+ spinal neurons in females, which also showed greater change in facilitation of spinal summation between short and long stimulation lengths at a spinal network level, studied with field potential recordings.

In contrast to those findings, the results of the length-dependency experiment in this chapter showed length-dependent differences in ADS in monosynaptic C-fibre eEPSC to the Fos-EGFP heat/WT capsaicin sensitive spinal neurons, with no effect of sex at both short and long stimulation lengths (**Figure 5.17**). Based on the field potential recordings investigating the impact of different levels of ADS on summation at a spinal network level (Dickie et al., 2017), it was predicted that longer stimulation length-induced more pronounced ADS would limit summation and thus the wind-up in heat sensitive spinal neurons in both sexes. However, considering the fact that this experiment aimed to investigate the impact of ADS on an individual neuron level and the fact that none of the recorded heat sensitive spinal neurons displayed wind-up, it is not surprising that the data in this chapter did not fit with that prediction. Interestingly, females demonstrated significantly more pronounced initial AP firing at shorter root lengths compared to males. This data suggests that there are likely other neuronal sex differences, but not ADS, that drive these sex differences in initial firing of the Fos-EGFP heat sensitive spinal neurons. The more pronounced initial AP firing in females can potentially result from pre- and/or postsynaptic differences that can allow their Fos-EGFP heat sensitive spinal neurons to fire more C-fibre-induced AP in response to an electrical stimulation. Moreover, it must be noted that the experiments in this chapter did not explore the identity of the recorded cells. Therefore, it might be possible that the Fos-EGFP heat sensitive neurons in females were not identical to those in males, which could also explain the sex-dependent differences in initial AP firing of those cells. This chapter showed length- and sex-dependent differences in repetitive stimulation-induced wind-down of individual Fos-EGFP heat sensitive spinal neurons. In females, shorter stimulation lengths enhanced wind-down, while longer-stimulation lengths limited it, whereas stimulation length did not affect the wind-down in males. Given that the wind-up/-down phenomena depend on the number of starting responses, the demonstrated significantly higher number of starting responses in females (**Figure 5.18E**) likely contributed to the observed sex differences in wind-down (**Figure 5.18C**). A potential mechanism for the induced

wind-down phenomenon in Fos-EGFP heat sensitive spinal neutrons may involve reduced reverberatory activity of a network of spinal excitatory interneurons evoked by afferent C-fibre input as it has previously been demonstrated that their increased reverberatory activity promotes wind-up in spinoparabrachial neurons (Hachisuka et al., 2018). However, this chapter showed no stimulation length- and sex-dependent changes on the normalised net charged following repetitive stimulation, suggesting that the reverberatory activity of spinal interneurons evoked by the afferent C-fibre input likely do not influences the reported here stimulation length- and sex-dependent wind-down phenomenon in Fos-EGFP heat sensitive spinal neurons. These findings are not surprising as the afferent C-fibre-evoked reverberating activity in spinal interneurons has been postulated to influence wind-up (Mendell, 1966, Hachisuka et al., 2018), which similarly to the employed in this chapter wind-down to quantify STD, is a form of STP, but as established these two phenomena have different underlying mechanisms. Wind-up has been shown be mediated primarily by the NMDARs (Davies and Lodge, 1987, Dickenson, 1990, Dickenson and Sullivan, 1987) and the increased reverberatory activity of a network of spinal excitatory interneurons evoked by afferent C-fibre input (Hachisuka et al., 2018), while STD has been shown to primarily involve depletion of some pool of vesicles and likely release site and calcium channel inactivation (Zucker and Regehr, 2002, Fioravante and Regehr, 2011). In addition, the Fos-EGFP heat sensitive spinal neurons may receive polysynaptic input from different spinal interneurons than those whose reverberatory activity was evoked by the afferent C-fibre stimulation and influenced the spinoparabrachial neurons. Therefore, it would be important for future studies to further investigate the length- and sex-dependent differences in wind-down in Fos-EGFP sensitive spinal neurons following repetitive stimulation at C-fibre strength as well as the impact of different levels of C-fibre ADS on the activity of spinal neurons displaying wind-up.

5.6.6 Limitations

It would be important that future studies investigate the identity of the noxious heat hot water bath-induced Fos-EGFP superficial dorsal horn neurons in the *ex vivo* spinal cord preparation in both sexes. Previous studies have shown that hot water bath hindpaw immersion leads to Fos-like immunoreactivity in rat lumbar spinal cord dorsal horn neurons localised primarily to the lamina I-II region (Buritova and Besson, 2000, Abbadie et al., 1994, Huang and Simpson, 1999, Jones, 1998, Bullitt, 1990), with the studies in this chapter demonstrating that hot water bath hindpaw immersion also leads to expression of Fos in lumbar dorsal horn neurons in *ex vivo* spinal cord slices from mice of both sexes that were localised primarily to the lamina I-II region. However, it would be important for future studies to investigate the identity of those heat sensitives spinal neurons in both sexes by exploring their neuropeptides expressing profiles as well as their morphological, electrophysiological, and pharmacological properties. Although the majority of the neurons (~99%) within the superficial laminae have been shown to be interneurons (Abraira and Ginty, 2013, Todd, 2010), large proportion of which have been shown to be glutamatergic excitatory neurons (Todd, 2017) it would be important to differentiate the Fos-EGFP heat sensitive spinal neurons as either excitatory or inhibitory. This could be done by utilising *post hoc* immunohistochemical techniques for the inhibitory marker Pax2 (Cheng et al., 2004). In addition, it would be important to investigate the morphology of the Fos-EGFP heat sensitive spinal neurons, for example by simply by adding Neurobiotin to the intracellular solution to allow labelling of the Fos-EGFP heat sensitive spinal neuron that has been recorded from in the electrophysiological experiments. The Neurobiotin labelled Fos-EGFP heat sensitive spinal neurons can thus be examined to allow for the reconstruction of their morphology. The morphological analysis could provide further information about neuronal subtypes because there are morphologically distinct classes of excitatory interneurons, some of which can be differentiated based on the orientation and density of their dendrites (Dickie et al., 2019). Given that neuronal morphology has been shown to associated with the functional

properties of the neurons (Grudt and Perl, 2002), investigation of the firing properties of the Fos-EGFP heat sensitive spinal neurons could also help identify the subtypes of these neurons. Furthermore, it would also be important to quantify the proportion of Fos-EGFP heat sensitive spinal neurons receiving different types of afferent fibre inputs in both sexes. These further experiments and analysis can provide additional to help build a more detailed picture of the Fos-EGFP heat sensitive spinal neurons in both sexes.

One of the biggest limitations in this chapter is the fact that although C-fibre ADS has been shown to influence spinal summation at a network level in a sex-dependent manner, with pronounced levels of ADS limiting spinal summation to a higher degree in females, which have also been demonstrated to displayed higher noxious heat, but not mechanical, thresholds (Dickie et al., 2017), noxious heat and mechanical sensitivity were not assessed in the Fos-EGFP *C57BL/6* mice of both sexes. Despite the fact that several studies have reported that female *C57BL/6* mice display lower noxious heat thresholds (Kest et al., 1999, Leo et al., 2008), this chapter did not assess that for the hemizygous Fos-EGFP backcrossed with *C57BL/6* mice. Therefore, future studies should conduct those behavioural experiments to confirm if the electrophysiological C-fibre ADS findings reported in this chapter can be associated with the previously observed behavioural findings.

Another limitation of the work done in this chapter is the smaller sample sizes of the experiments assessing the effects of manipulation and stimulation length on ADS in monosynaptic C-fibre eEPSC to the combination of Fos-EGFP heat and WT capsaicin sensitive spinal neurons and their impacts on the activity of Fos-EGFP heat sensitive spinal neurons in both sexes. In addition, it must be noted that the voltage-clamp experiments assessing the effects of manipulation and stimulation length on ADS in monosynaptic C-fibre eEPSC were conducted in a combination of both Fos-EGFP heat and WT capsaicin sensitive spinal neurons. However, the current clamp experiments assessing the impact of the manipulation- or stimulation distance-induced changes in ADS levels on the C-fibre stimulation strength-evoked

eEPSP firing were conducted in Fos-EGFP heat sensitive spinal neurons only. Considering that the patch-clamp recordings were conducted in different heat sensitive spinal neuron subtypes, receiving monosynaptic C-fibre inputs, and that different C-fibres subtypes display varying degrees of ADS (Thalhammer et al., 1994, Serra et al., 1999, Weidner et al., 1999), it may be possible that the different heat sensitive spinal neuron subtypes potentially received monosynaptic inputs from different subtypes of C-fibres, displaying different levels of ADS. The fact that C-fibre ADS has been shown to be also sex-dependent (Dickie et al., 2017), may have further contributed to the differences in levels of ADS in different subtypes of C-fibre sending monosynaptic inputs to the different heat sensitive spinal neuron subtypes. Therefore, it would be important that future studies explore the manipulation- and stimulation distance-induced alterations in levels of ADS in monosynaptic C-fibre inputs to either Fos-EGFP heat or capsaicin sensitive spinal neurons separately in both sexes.

5.7 Conclusion

The results in this chapter have shown that intraplantar capsaicin injections and hindpaw immersion in hot water bath are both able to induce Fos-EGFP expression in spinal cord neurons from Fos-EGFP mice. The Fos-EGFP heat sensitive spinal neurons received monosynaptic C-fibre input, which displayed the characteristic C-fibre ADS phenomenon. In addition, the Fos-EGFP heat sensitive spinal neurons, were confirmed to have capsaicin sensitive inputs, i.e. TRPV1 expressing inputs, suggesting that both the Fos-EGFP and the WT capsaicin sensitive inputs were heat sensitive.

In line with the C-fibre ADS CAP findings, with more pronounced C-fibre ADS in male compared to female mice, the Fos-EGFP heat/WT capsaicin sensitive spinal neurons also displayed similar sex differences in ADS in monosynaptic C-fibre input to those neurons, with males showing significantly more pronounced ADS in the monosynaptic C-fibre inputs to the Fos-EGFP heat/WT capsaicin sensitive spinal neurons. Interestingly, the males also showed less pronounced C-fibre-induced AP

firing in Fos-EGFP heat sensitive spinal neurons, which, however, was unlikely to reflect alterations induced by sex-dependent C-fibre ADS only. This idea that the observed sex differences in C-fibre-induced AP firing in the Fos-EGFP heat sensitive spinal neurons likely reflect other sex differences in these neurons was further supported by the findings of sex-dependent differences in initial AP firing and wind-down, which were more pronounced in females

Using the prolonged low-frequency manipulation to alter levels of ADS, it was demonstrated that manipulation followed by a repetitive stimulation at higher frequencies causes an increase in the number of C-fibre evoked input failures and also an increase in ADS in monosynaptic C inputs to Fos-EGFP heat/WT capsaicin sensitive spinal neurons in both sexes. The increased incidences of synaptic failures post-manipulation and the manipulation-induced changes in ADS in monosynaptic C-fibre input to Fos-EGFP heat/WT capsaicin sensitive spinal neurons were associated with similar level of eEPSP firing in the Fos-EGFP heat sensitive spinal neurons in both sexes. Following the prolonged low-frequency manipulation the levels of eEPSP firing were reduced to a similar degree in both sexes. These findings can suggest that dynamic memory via the synaptic response failures may reflect an intrinsic self-inhibition mechanism to limit overdrive of nociceptive pathways.

Using the length-dependency of ADS, it was demonstrated that stimulation distance alters ADS in monosynaptic C-fibre input to the Fos-EGFP heat/WT capsaicin sensitive spinal neurons to similar extent in both sexes. These findings were in line with the length-dependent alterations in action potential firing that were similar between the sexes following a train of stimuli. However, the longer stimulation length-induced more pronounced ADS in monosynaptic C-fibre input to the Fos-EGFP heat/WT capsaicin sensitive spinal neurons was associated with a more pronounced repetitive C-fibre stimulation strength-evoked firing of the Fos-EGFP heat sensitive spinal neurons. These ADS-induced alterations in the activity of individual heats sensitive spinal neurons do not reflect the findings of the impact of C-fibre ADS on the coordinated spinal network activity, where increased levels of C-

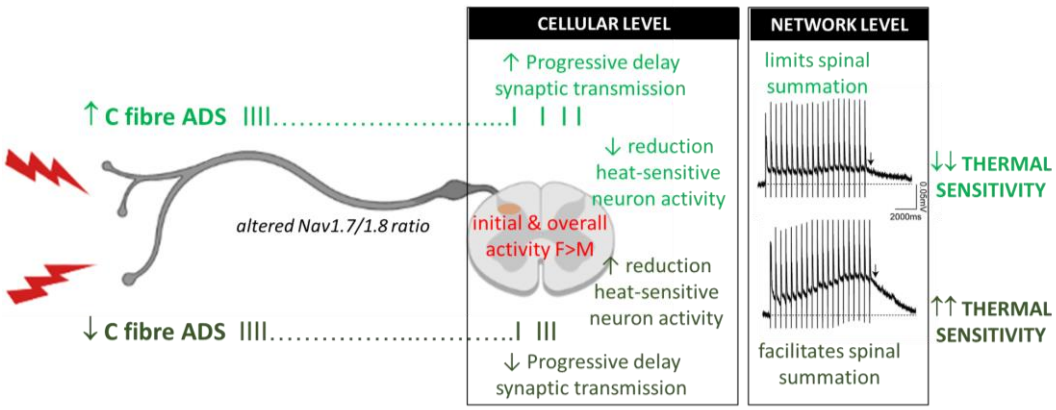
fibre ADS have been shown limit the temporal summation of C-fibre-evoked synaptic activity, likely influencing the noxious heat sensitivity of the flexion-withdrawal reflex (Dickie et al., 2017). However, it is likely that the impact of coordinated network activity at spinal level overrides the impact of the activity at an individual spinal neuron level in terms of the noxious heat flexion-withdrawal reflex responses, i.e the noxious heat thresholds. Interesting, it must be noted that this chapter also demonstrated sex differences in the repetitive stimulation-induced overall AP firing and in the initial AP firing of the Fos-EGFP heat sensitive spinal neurons, which was higher in females. These sex-differences in the C-fibre-induced firing of the Fos-RGFP heat sensitive spinal neurons, likely involving mechanisms unrelated to C-fibre ADS, could potentially be associated with the previously demonstrated sex differences in the noxious heat thresholds in *C57BL/6* mice, with females showing lower noxious heat thresholds than males (Kest et al., 1999, Leo et al., 2008). To further investigate potential mechanism underling the activity-dependent changes in the firing of the Fos-EGFP heat sensitive spinal neurons, the length- and sex-dependent alterations in amplitude or net charge change were assessed following the repetitive stimulation at C-fibre stimulation strength. The Fos-EGFP heat sensitive spinal neurons length- and sex-dependent activity evoked by the repetitive stimulation at C-fibre stimulation strength was found to not be associated with the repetitive stimulation-induced alterations in the amplitude change or the net charge change, suggesting that presynaptic fatigue and reverberating activity of spinal interneurons evoked by the afferent C-fibre inputs likely to do not contribute to the length- and sex-dependent activity changes in Fos-EGFP heat sensitive spinal neurons. Thus, further studies would need to be conducted to investigate the repetitive stimulation-induced length- and sex-dependent activity changes in Fos-EGFP heat sensitive spinal neurons and their potential contribution to the sex differences in noxious heat sensitivity.

Chapter 6

Final discussion and conclusions

6.1 Final discussion and conclusions

The aim of this thesis was to investigate the afferent nociceptive C-fibre activity-dependent slowing (ADS) phenomenon, which has been observed in both humans (Weidner et al., 1999, Serra et al., 1999) and animals (Gee et al., 1996, Thalhammer et al., 1994). It has been shown to be altered in chronic pain patients (Kleggetveit et al., 2012a, Serra et al., 2014, Namer et al., 2015, Kist et al., 2016, Namer et al., 2017) and in animal pain models (Sun et al., 2012a, Hulse, 2016, Galley et al., 2017, Garcia-Perez et al., 2018), with reports of injury- and sex-dependent differences in levels of C-fibre ADS, which have been shown to influence the temporal relay of inputs along the nociceptive C-fibres to individual spinal neurons and is proposed to alter the temporal summation of nociceptive signals contributing to injury- and sex-dependent differences in heat pain sensitivity (Dickie et al., 2017). Thus, given the accumulating evidence of sex differences in acute and chronic pain mechanisms (Mogil, 2018, Sorge and Strath, 2018, Mogil, 2020), this thesis aimed to study C-fibre ADS in pain models and in different species in both sexes in association with noxious heat hypersensitivity. Given that C-fibre ADS is proposed to provide a 'dynamic memory' (Weidner et al., 2002), this was also explored in rats and mice of both sexes. In addition, this thesis aimed to explore the levels of ADS in monosynaptic C-fibre input to heat sensitive spinal neurons and investigate their impact on the activity of those spinal neurons in mice of both sexes. **Figure 6.1** presents summary thesis findings and how they associate with the proposed influence of C-fibre ADS on heat pain sensitivity.



		Male ADS	Female ADS	Heat threshold	Mechanical threshold
Naïve	SD rats*	↑	↑↑	F>M	M=F
	C57BL/6 mice	↑↑	↑	M>F**	M=F**
Pain model	Incision SD rats	↑	↑↑	Bigger ↓ Males	M=F
	MG SD rats	↓	↑↑↑	↓ M only	M=F

Figure 6.1 Summary potential influence of C-fibre ADS on heat pain sensitivity

Previous findings and findings of this thesis suggest that C-fibres displaying pronounced ADS (↑ C-fibre ADS) will show an increase in the progressive delay in synaptic transmission, which can alter the temporal relay of noxious heat pain signals reaching the spinal cord, shown to limit spinal summation (Dickie et al., 2017), thus likely altering the processing of noxious heat inputs, potentially causing a decrease in thermal sensitivity. C-fibre ADS findings in naïve female SD rats and male mice support this profile, with female SD rats and male *C57BL/6* mice exhibiting higher noxious heat thresholds. Females were also shown to display this profile in the incision and MG models, where they exhibited less pronounced or no noxious heat hypersensitivity, respectively. However, if C-fibres display limited ADS (↓ C-fibre ADS) this will decrease the progressive delay in synaptic transmission, which has been shown to enhance spinal summation (Dickie et al., 2017), potentially causing an increase in thermal sensitivity. Findings in naïve male SD rats and female *C57BL/6* mice support this profile, with male SD rats and female *C57BL/6* mice exhibiting lower noxious heat thresholds. Males were also shown to display this profile in the incision and MG models, where they exhibited pronounced noxious heat hypersensitivity. These altered ADS profiles potentially reflect altered ratios of $Na_V1.7/Na_V1.8$ along the C-fibre axons. The fact that none of the C-fibre ADS profiles correlated with mechanical sensitivity supports the involvement of A fibre rather than C-fibre driven synaptic circuits in mechanical sensitivity. The demonstrated ADS regulation of temporal summation at a spinal network level (Dickie et al., 2017), however, was not observed at the level of individual Fos-EGFP noxious heat sensitive spinal neurons in the Fos-EGFP *C57BL/6* mice. More pronounced C-fibre ADS, induced at longer stimulation lengths, resulted in a less pronounced reduction in activity of the Fos-EGFP noxious heat sensitive spinal neurons in both sexes. Sex differences, however, were observed in the activity of the Fos-EGFP heat sensitive spinal neurons, with females showing more pronounced initial AP firing and repetitive stimulation-induced overall AP firing. These sex differences at an individual neuron level along with the previously demonstrated limited ADS-induced enhanced spinal summation (Dickie et al., 2017) could potentially contribute to the previously demonstrated lower noxious heat thresholds in female *C57BL/6* mice (Kest et al., 1999, Leo et al., 2008). However it must

be noted that the identity of the noxious heat sensitive spinal neurons was not investigated, but evidence suggests they may be inhibitory interneurons (Leitner et al., 2013, Heinke et al., 2004, Schoffnegger et al., 2006, Hantman et al., 2004, Sullivan and Sdrulla, 2022). Thus ↑ C-fibre ADS may potentially act to maintain the activity of inhibitory spinal interneurons, facilitating spinal inhibition and potentially contributing to less thermal sensitivity. *findings from Dickie et al. (2017)**findings from Kest et al. (1999), Leo et al. (2008).

In the incision model of postoperative pain (**Figure 6.1**; table, row 3), juvenile female rats displayed an overall stronger C-fibre ADS profile post-incision, that was proposed to increase the progressive delay in synaptic transmission, limit summation and thus likely reduce heat hypersensitivity (Dickie et al., 2017). Consistent with this, female juvenile rats exhibited less pronounced incision-induced peak heat hypersensitivity compared to males. The sex-dependent incision-induced alteration in ADS likely reflected the sex-dependent incision-induced alterations in the $Na_V1.7/Na_V1.8$ ratio along the axon, which has been proposed as an underlying mechanism of C-fibre ADS (Petersson et al., 2014).

In the *ex vivo* pain model, induced by the diabetic pain-associated metabolite methylglyoxal (MG) (**Figure 6.1**; table, row 4), juvenile male rats displayed chronic MG-induced reduction in C-fibre ADS, while females showed a pronounced enhancement of C-fibre ADS. Consistent with these C-fibre ADS profiles, predicted to enhance summation in males and limit summation in females (Dickie et al., 2017), the behavioural data showed that *in vivo* MG treatment induced heat hypersensitivity in males only. Thus, the presented evidence from rats suggested that C-fibre ADS can potentially influence injury-induced heat sensitivity in a sex-dependent manner**.** Interestingly, chronic MG treatment also induced changes in Aβ fibre function, causing an increase in the initial Aβ amplitude in males and a decrease in the initial Aβ width in females. Repetitive stimulation at C-fibre strength caused activity-dependent changes in Aβ fibre amplitude in a sex-dependent manner, with chronic MG enhancing the progressive increase in Aβ amplitude in females only. Given that mechanical hypersensitivity is likely associated with behaviours driven by A fibre activation of C-fibre sensitised spinal pain circuits (Hsieh et al., 2015, Ziegler et al., 1999), with proposed involvement of A fibres

primarily in the diabetes-induced negative symptoms (Baron, 2009, Gwathmey and Pearson, 2019), it is possible that the demonstrated MG-induced sex-dependent alterations in Aβ fibre initial amplitude and width as well as in the activity-dependent changes in Aβ amplitude may potentially contribute to the negative diabetes-induced symptoms of numbness and/or dysesthesia (Feldman et al., 2017). However, the studies in this chapter assessed the MG-induced alterations in the flexion-withdrawal reflex in response to punctate mechanical stimuli only and thus cannot provide information about the negative symptoms associated with diabetes, which would be difficult to measure in rodents. Nevertheless, it would be important for future studies to further explore the MG-induced effects on Aβ fibre function and its impact on cellular and network activity at a spinal level. In addition, it would be essential to study these in association with MG-induced alterations in response to different mechanical stimuli, particularly dynamic brush, which is typically used to assess dynamic mechanical allodynia, which is a more clinically relevant symptom reported by patients with peripheral neuropathies (Fisher et al., 2020). Therefore, dynamic brush could potentially be useful in assessing symptoms of dysesthesia in an *in vivo* MG-induced model of hypersensitivity.

The studies in naïve *C57BL/6* mice demonstrated that mice also display C-fibre ADS in a sex-dependent manner, as previously demonstrated for SD rats (Dickie et al., 2017), but unlike the SD rats, the male *C57BL/6* mice displayed more pronounced C-fibre ADS, consistent with previous studies reporting higher heat thresholds in *C57BL/6* male mice (Kest et al., 1999, Leo et al., 2008) (**Figure 6.1**; table, row 2), which again was in contrast to the previously reported higher heat thresholds in female SD rats (Dickie et al., 2017) (**Figure 6.1**; table, row 1). These findings have suggested that levels of C-fibre ADS are not only sex-dependent, but also influenced by species. Moreover, these findings suggest that sex- and species-dependent C-fibre ADS may be able to influence spinal heat pain processing in a sex- and species-dependent manner, thus potentially playing a role in the sex- and species-dependent differences in heat pain sensitivity.

Given previous reports in humans of C-fibre ADS being able to provide a 'dynamic memory' of previous levels of activity that can influences response to subsequent responses to higher frequency stimuli (Weidner et al., 2002), it was demonstrate that prolonged low frequency stimulation (manipulation) can alter subsequent levels of C-fibre ADS induced by repetitive stimulation at higher frequency in a species-and sex-dependent manner. Interestingly it was demonstrated that the direction and the magnitude of the manipulation-induced changes in C-fibre ADS had a strong negative association with the level of baseline ADS in rats of both sexes, with similar trends for the mouse data, but the small sample size of the mouse data may have contributed to the insufficient power of this experiment to detect significant associations. Altogether these findings suggests that sex- and species-dependent levels of C-fibre ADS may be able to influence dynamic memory and also spinal heat pain processing in a sex- and species-dependent manner, thus potentially playing a role in the sex- and species-dependent differences in heat pain sensitivity. In addition, these data confirmed that prolonged low frequency stimulation can be used as a tool to manipulate levels of C-fibre ADS, which can be used to assess the impact of levels of C-fibre ADS on an individual spinal neuron level.

Further studies of the effect of C-fibre ADS on an individual spinal neuron level have shown that consistent with the observed sex differences in C-fibre ADS in CAP recordings in mice, males also showed significantly more pronounced ADS in monosynaptic C-fibre input to Fos-EGFP heat/WT capsaicin sensitive spinal neurons. The less pronounced ADS in females occurred in tandem with a more C-fibre stimulation strength-evoked firing in Fos-EGFP heat sensitive spinal neurons in females. However, females also showed a more pronounced initial firing and a more pronounced wind-down, suggesting that sex differences other that the ones observed in C-fibre ADS might contribute to the more pronounced repetitive stimulation-induced firing, initial firing and wind-down in females. Interestingly, it was also shown that manipulation of the C-fibre ADS levels with prolonged low frequency stimulation caused a pronounced increase in synaptic input failures as

well as an increase in ADS in monosynaptic C-fibre inputs to Fos-EGFP heat/WT capsaicin sensitive spinal neurons. These manipulation-induced changes in both sexes were associated with a reduced firing of the Fos-EGFP heat sensitive spinal neurons in both sexes. These findings can suggest that dynamic memory via the induction of synaptic response failures may act as an intrinsic self-inhibition mechanism to limit the activity of the heat sensitive spinal neurons. In addition, longer stimulation lengths were confirmed to lead to increased levels of ADS in monosynaptic C-fibre inputs to Fos-EGFP heat/WT capsaicin sensitive spinal neurons and were associated with a less pronounced reduction in action potential firing following repetitive stimulation in Fos-EGFP heat sensitive spinal neurons in both sexes. However, none of the Fos-EGFP heat sensitive spinal neurons displayed summation following repetitive stimulation at C-fibre strength, which did not allow the assessment of the impact of C-fibre ADS on summation on an individual spinal neuron level. Thus, the previous findings of higher C-fibre ADS levels leading to a reduced temporal summation of C-fibre-evoked synaptic activity at a spinal network level in a sex-dependent manner suggested to influence the noxious heat flexion-withdrawal reflex (Dickie et al., 2017) could not be investigated further on an individual spinal neuron level. However, the demonstrated less pronounced reduction in the activity of the Fos-EGFP heat sensitive spinal neurons in response to the increased levels of ADS in monosynaptic C-fibre inputs in both sexes is in contrast to the previous findings of the impact of C-fibre ADS on the coordinated spinal network activity, where higher C-fibre ADS levels were associated with a reduced temporal summation of C-fibre-evoked synaptic activity in a sex-dependent manner(Dickie et al., 2017). These findings suggest that the impact of coordinated network activity at spinal level is likely to supersede the impact of the activity at an individual spinal neuron level in terms of the noxious heat flexion-withdrawal reflex responses, i.e the noxious heat threshold. However, it must also be noted that the identity of the noxious heat sensitive spinal neurons was not investigated. Previous studies have shown that a greater proportion of the inhibitory spinal neurons express noxious heat-induced c-Fos (Leitner et al., 2013), with findings of many

inhibitory superficial dorsal horn interneurons receiving direct monosynaptic C-fibre input (Heinke et al., 2004, Schoffnegger et al., 2006, Hantman et al., 2004) and with recent Ca^{2+} imaging studies revealing that noxious heat sensitive inhibitory spinal neurons show a Ca^{2+} profile similar to the repetitive stimulation-induced profile observed in **Chapter 5** (Sullivan and Sdrulla, 2022). Thus, it is possible the noxious heat-evoked Fos-EGFP spinal neurons may be inhibitory, with higher C-fibre ADS potentially acting to maintain the activity of these inhibitory spinal interneurons, facilitating spinal inhibition and potentially contributing to less thermal sensitivity. Thus, C-fibre ADS may impact differently the excitatory vs the inhibitory spinal neurons, with high levels of ADS potentially limiting excitatory drive and facilitating inhibitory drive, suggesting C-fibre ADS as a filter to peripheral drive to the spinal cord, acting to magnify spinal inhibition following repetitive stimulation, facilitating the balance between spinal excitation and inhibition.

The more pronounced repetitive stimulation-induced overall AP firing, initial AP firing and wind-down of Fos-EGFP heats sensitive spinal neurons in female *C57BL/6* mice suggests that there are likely sex differences in mechanisms unrelated to C-fibre ADS that could potentially be associated with the previously demonstrated lower noxious heat thresholds in female *C57BL/6* mice (Kest et al., 1999, Leo et al., 2008). Further investigation of potential mechanisms contributing to activity-dependent changes in the firing of the Fos-EGFP heat sensitive spinal neurons have shown no association of repetitive stimulation-induced length- and sex-dependent neuronal activity changes and the repetitive stimulation-induced alterations in amplitude or net charge change, suggesting that presynaptic fatigue and reverberating activity of spinal interneurons evoked by the afferent C-fibre inputs likely to do not contribute to the length- and sex-dependent activity changes in Fos-EGFP heat sensitive spinal neurons. However, other presynaptic inhibitory mechanisms could potentially have contributed to the observed stimulation distance activity-dependent changes in the firing of the Fos-EGFP heat sensitive spinal neurons. However, the findings of this thesis suggest that although C-fibre ADS could influence progressive delay in synaptic transmission at an individual

spinal neuron level, coordinated spinal activity at a network level can likely override impact at an individual spinal neuronal level, thus likely being a better reflection of the processing of noxious heat pain at a spinal network level. More importantly, the demonstrated sex differences in initial AP firing and repetitive stimulation-induced overall AP firing and wind-down might reflect sex-dependent mechanisms that could play a more important part in influencing the demonstrated sex-dependent differences in noxious heat thresholds in *C57BL/6* mice.

6.2 Future Perspectives

This thesis provided evidence for sex- and species-dependent differences in levels of C-fibre ADS that can be altered by injury and that can be associated with sensitivity to heat pain, but future work should focus on investigating the mechanisms underlying these sex-, species- and injury-dependent differences in nociceptive C-fibre ADS levels. Given that C-fibre ADS has been proposed to involve voltage-gated sodium channels - $Na_V1.7$ and $Na_V1.8$ (Petersson et al., 2014), which are key targets for the pain pharmaceutical industry (Bagal et al., 2015, Emery et al., 2016), it would be important to investigate the sex-, species- and injury-dependent differences in the expression levels of $Na_V1.7$ and $Na_V1.8$ ion channels in the noxious heat peripheral fibres. Given the recent evidence of sex differences in axonal mRNA transport in peripheral nerves (Ray et al., 2019) along with CNS evidence of sex differences in microRNAs (Morgan and Bale, 2012) regulating local translation at a peripheral nerve level (Price and Géranton, 2009), it would be interesting to explore the mRNA and/or protein expression levels of $Na_V1.7$ and $Na_V1.8$ at a 'cell body and axonal' level specifically in the noxious heat afferent fibres. In addition, considering the physiological relevance of the C-fibre ADS phenomenon, with studies showing that it occurs not only following electrical stimulation but also in response to noxious thermal stimulation (Thalhammer et al., 1994) with reports of ADS alterations in chronic pain patients (Kleggetveit et al., 2012b, Serra et al., 2014, Namer et al., 2015, Kist et al., 2016, Namer et al., 2017), it

would be important for future clinical studies performing microneurography to account sex as a factor when investigate ADS levels in different C-fibre subtypes. Given the evidence of sex differences in heat sensitivity in humans, with women showing lower noxious heat thresholds (Mogil, 2012), similarly to the sex differences reported in mice (Kest et al., 1999, Leo et al., 2008), it would be important to explore whether, in line with predictions in this thesis, women may display lower levels of ADS in noxious heat afferent fibres. Thus, this can provide information for potential differences in the function and/or expression of $Na_V1.7$ and $Na_V1.8$ ion channels that would be important to be considered in the development of sex appropriate pain therapeutics.

Considering the findings of sex difference in the initial AP firing and in the repetitive stimulation-induced overall AP firing and wind-down in the Fos-EGFP heat sensitive neurons it would be important for future studies to identify and characterise the noxious heat sensitive spinal neurons activated by hot water bath hindpaw stimulation in both sexes. These studies will allow to assess whether the noxious heat activate spinal neuron are the same neuron subtypes in both sexes and whether their functional properties are the same. In addition, given the demonstrated differences of the impact of C-fibre ADS at the level of individual spinal neuron activity vs spinal network activity, it would be important to also investigate the impact of C-fibre ADS at a spinal network level based on sex, species and injury. Thus, calcium imaging studies using genetically encoded calcium indicator in either excitatory or inhibitory spinal neurons can allow to investigate the temporal and spatial extent of the network response in both neuronal subpopulations at different levels of ADS in order to fully understand the extent to which ADS can regulate summation of noxious heat inputs at a network level. These studies will be essential to understand the role of ADS in regulating the temporal relay of nociceptive heat inputs and the processing of noxious heat stimuli in normal physiology and in injury-induced states in both sexes.

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