The processing of inflammatory joint pain in the developing spinal cord

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DECLARATION

The work presented in this thesis was carried out in the Department of Neuroscience, Physiology and Pharmacology at University College London between January 2010 and January 2013. I, Luke Paul Affricain la Hausse de Lalouvière, confirm that the work presented here is my own. Where information has been derived from other sources, I confirm that this has been indicated in the text.

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List of abbreviations

GlyR	glycine receptor
5-HT	5-hydroxytryptamine, serotonin
ATP	adenosine tri-phosphate
ANOVA	analysis of variance
AMPA	alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
BDNF	brain-derived neurotrophic factor
BF	biceps femoris
cAMP	cyclic adenosine monophosphate
Ca^{2+}	calcium ion
CaMKII	calmodulin dependent protein kinase II
CFA	complete Freund's adjuvant
CGRP	calcitonin gene-related peptide
CNS	central nervous system
COX	cyclo-oxygenase
DH	dorsal horn (of spinal cord)
DRG	dorsal root ganglion
E	embryonic day
EMG	electromyography/electromyographic
FWR	flexion withdrawal reflex
GABA	γ-aminobutyric acid
Hz	hertz
IASP	International Association for the Study of Pain
IB4	isolectin B4
ILAR	International League of Associations for Rheumatology
IL-1β	interleukin-1β
IL-6	interleukin-6
JIA	juvenile idiopathic arthritis
KA	kainate
L	lumbar (spinal cord segment)
LII_i	lamina II inner
LII_o	lamina II outer
mEPSC	miniature excitatory postsynaptic current
mIPSC	miniature inhibitory postsynaptic current

Mg^{2+}	magnesium ion
Mrgpr	mas-related G-protein coupled receptors
ms	millisecond
Na^{+}	sodium ion
NGF	nerve growth factor
NK-1	neurokinin-1
NMDA	N-methyl-D-aspartate
NO	nitric oxide
NSAIDs	non-steroidal anti-inflammatory drugs
P	postnatal day
PAG	periaqueductal grey
PG	prostaglandin
PGE_2	prostaglandin E2
PCA	post-conceptional age
PKC	protein kinase C
RMS	root mean squared
RVM	rostro-ventral medulla
s	second
SEM	standard error of the mean
SP	substance P
SPA	stimulus-produced analgesia
TLR	toll-like receptor
TNFα	tumour necrosis factor alpha
TrkA	neurotrophic tyrosine kinase receptor type 1
TRPV1	transient receptor potential cation channel subfamily V member 1
TTX	tetrodotoxin
vFH	von Frey hair
WDR	wide dynamic range

Abstract

Approximately 10 in 100,000 children develop inflammatory arthritis every year and a large proportion of those are diagnosed as having Juvenile Idiopathic Arthritis (JIA). A major cause of suffering in the disease is pain, and indeed it contributes significantly to the morbidity of this condition when assessed by various disability scores. Pain from affected joints causes sleep disturbance, limits normal activities, disrupts school attendance and results in considerable psychosocial stress. Very little is understood about arthritic pain processing in the immature nervous system. Both clinical and neurobiological studies in animal models show that CNS nociceptive connections differ in juveniles and adults and that the normal maturation of these connections depends upon early life stress and pain experience. The immaturity of synaptic connections and integrated circuits means that children's pain experience is different from that of adults and may impact upon pain in later life. It was our aim to develop and characterise a rodent model of joint inflammation to better understand the neurobiological basis of joint pain in early life and to establish whether joint inflammation in childhood influences joint pain sensitivity as an adult.

In the first results chapter (Chapter 2), the normal development of joint evoked and cutaneous reflexes were mapped out over the postnatal period. In the second Results chapter (Chapter 3), monoarthritis of the ankle was induced in Sprague-Dawley rats of different postnatal ages using complete Freund's adjuvant (CFA) and the effect of this inflammation upon spinal circuits was studied using behavioural and electrophysiological measures. Electromyographic (EMG) recordings show that inflammation leads to widespread reflex hypersensitivity to mechanical stimuli in young animals that differs significantly from the effects of adult joint inflammation. In adults, a significant attenuation of reflexes, or 'protective inhibition' phase was observed at 24 hours and 4 days post-inflammation, followed by a 'hypersensitivity phase' at 10 days when reflexes to pinch were dramatically enhanced. These effects were not detected with simple behavioural observation. In the third results chapter (Chapter 4), the long-term effects of joint inflammation (6 weeks) were investigated and shown to be highly dependent upon the age at which the inflammation occurred. Baseline nociceptive reflexes were enhanced in animals that had experienced joint inflammation when young (postnatal day (P) 8) but slightly reduced if the

inflammation had occurred at the same time interval, but in adult life. The effects of a second injury in adulthood also depended on the past history of the animal. Animals first inflamed in early life, displayed a significantly greater 'protective inhibition' than adult inflamed controls, while animals first inflamed in adult life displayed enhanced hypersensitivity to joint inflammation.

The results here describe previously unknown characteristics and mechanisms of joint pain in early life which will contribute to a better understanding and treatment of pain in JIA.

Chapter 1 General Introduction

1.1 Introduction

Pain is a sensation that is almost universal to the human experience. Without it, one is unable to detect threatening or tissue damaging influences. In the human, pain promotes health and wellbeing yet, when pathological can simultaneously be the cause of a great deal of suffering. Pain associated with arthritis is a case of the latter. Inflammation, pain and joint destruction are the chief features of this condition and the sheer incidence of arthritic pain has prompted a great deal of interest in its aetiology.

Action potentials from the damaged or inflamed joint pass first through the peripheral nerves to the spinal dorsal horn (DH), the first site of sensory processing in the mammalian central nervous system (CNS) (Todd, 2010). Within the DH the peripheral signal is modulated, refined and passed on to second order neurones whose axons form ascending spinal tracts on their way to higher centres. Neurones within the brainstem, limbic areas, thalamus and cortex receive input either directly or indirectly from the spinal cord. These centres in turn may exert descending control on the processing of sensory information in the DH (Fields et al., 1977).

Sensory input is also processed within the segment of the spinal cord it enters. Segmental neuronal circuits are key to the genesis of reflex activity. The archetypal reflex of this nature is the flexion withdrawal reflex (FWR), which involves the coordinated, yet unconscious, withdrawal of a limb from harm. This reflex has provided neuroscientists a window into the CNS and particularly the function of spinal cord circuits in response to painful stimulation.

Attention has shifted in recent years from the study of pain as an acute phenomenon to unravelling the chronic effects of painful experiences on the nervous system. This is particularly important in the developing nervous system, as early life is an important time for the shaping of nociceptive circuits (Fitzgerald, 2005). It has become apparent that the long-term consequences of pain in early life may persist into adulthood (Fitzgerald and Walker, 2009).

Juvenile arthritis is an example of a chronic inflammatory condition that causes pain in early life. A better understanding of arthritic pain processing in early life is important. A major cause of suffering in juvenile joint inflammation is pain and indeed pain is the most commonly reported symptom in the disease. Pain from affected joints causes sleep disturbance, limits normal activities, disrupts school attendance and results in considerable psychosocial stress (Kimura and Walco, 2007). Very little is understood about arthritic pain processing in the immature nervous system.

This thesis aims to explore the consequences of inflammatory joint pain on developing nociceptive circuits as well as to examine the differences between the processing of inflammatory pain in the young and adult spinal cord. Nothing is currently known about the processing of inflammatory joint pain in the developing nervous system and how it may affect nociceptive circuitry in later life.

This introduction outlines the sensory circuitry required for the processing of inflammatory joint pain in the adult spinal cord. It will provide an overview of the transmission of nociceptive information from the peripheral to central nervous system and the mechanisms that underlie chronic joint pain. Experiments contained within this thesis employ the FWR as a tool to assess the excitability of spinal sensory circuits, thus functional neuroanatomy of spinal reflex circuits will also be described. What is known and understood of the development of sensory circuits in postnatal life is then examined. As this thesis is concerned with the processing of inflammatory joint pain in the immature nervous system, the clinical literature on pain in patients with juvenile arthritis will be outlined as well as the pre-clinicial and clinical evidence for long-term changes in pain processing following pain or injury in early life.

1.2 Pain and nociception

The ability to feel pain is crucial for survival and avoidance of harm. Pain is defined by the International Association for the Study of Pain (IASP) as,

An unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage

Nociception, as defined by IASP, is,

The neural process of encoding noxious stimuli

The study of nociception is, therefore, concerned with the passage of neural activity through the peripheral and central sensory systems to the brain where nociceptive information may become pain. This section will outline the neural substrate and functional components of nociceptive circuitry in so far as it pertains to this thesis.

1.3 Anatomy of the adult spinal cord and its inputs

The DH of the spinal cord is the first site of the integration of sensory information in the CNS. The spinal cord grey matter is divided into ten parallel laminae which were originally described by Rexed from cytoarchitectural Nissl stains of the spinal cords of young and adult cats (Rexed, 1952, 1954) (Figure 1-1). This highly conserved lamination is also found in the rat (Molander et al., 1984). The dorsal horn can be further divided into superficial laminae (I and II) and deep laminae (III-VI). The peripheral afferent input to the spinal cord is highly somatotopically organised such that areas on the skin in the periphery have specific representations within their central terminal fields (Molander and Grant, 1985). Furthermore, afferents carrying different modalities of sensory information provide specific input to different laminae of the dorsal horn. This information is then relayed locally, to reflex circuits, or onwards via projection neurones to higher brain centres.

1.3.1 Spinal cord lamination

Lamination of the spinal cord was originally a cytoarchitectural observation. In the intervening years a greater, but as of yet, incomplete understanding of the subtypes of neurones, their connections within the spinal cord and their roles in sensory processing has emerged. An overview of the laminar organisation and various functions of the DH are outlined below and summarised in Figure 1-1.

1.3.1.1 Laminae I and II

Laminae I and II (LI and LII) together comprise the superficial DH and are the primary site of nociceptive afferent terminals. LI, also known as the marginal layer, covers the dorsal surface of the spinal cord and curves, to a variable extent, around its lateral edge. It is a thin sheet of neurones which contains the highest numbers of projection neurones in the DH (Spike et al., 2003). These cells project to higher centres such as the thalamus, periaqueductal grey (PAG), parabrachial area, nucleus of the solitary tract and medullary reticular formation via the spinoreticular and spinothalamic tracts (Todd, 2002). The vast majority of these projection neurones express the neurokinin-1 (NK-1) receptor for substance P (SP). Peripheral afferents positive for the excitatory neurotransmitter SP richly innervate these neurones. Projection neurones are surrounded in LI by numerous interneurones whose dendrites largely remain within the laminae (Gobel, 1978). Functional studies of LI/II neurones reveal multiple subtypes of interneurones with distinct functional and morphological characteristics (Grudt and Perl, 2002). This area of the DH can also modulate the excitability of spinal circuits via projection neurones that reach areas of the brainstem responsible for descending control of spinal excitability. These areas can then directly feedback onto superficial and deep DH circuits to modulate receptive field size as well as sensitivity to mechanical and thermal stimuli (Suzuki et al., 2002). NK-1 expressing projection neurones also play a key role in the tuning of mechanical sensitivity in adulthood. Ablation of these neurones in early life can result in abnormal sensory thresholds later in life (Man et al., 2012). This lamina is also the termination site of thinly myelinated Aδ fibres, which carry information from hair-follicles as well as pin-prick.

LII, by contrast, mainly contains small equally sized interneurones. The substantia gelatinosa, as it is also known, is the key termination site for unmyelinated C-fibre afferents and can be divided into two sub-laminae based on the termination patterns of two neurochemically distinct populations of C-fibres. Lamina II inner (LII_i) contains the terminals of non-peptidergic C-fibres that are isolectin-B4 positive ($IB4^+$) whilst Lamina II outer (LII_o) contains the terminals of peptidergic C-fibres that express calcitonin gene-related peptide (CGRP). C-fibres are high-threshold afferents that carry nociceptive information from the periphery such as noxious mechanical, thermal and chemical stimuli.

1.3.1.2 Laminae III-VI

The deep DH receives input from cutaneous mechanoreceptors carrying innocuous brush or touch known as Aβ fibres (Schneider, 2008). These laminae contain large interneurones that have extensive dendritic trees extending both dorsally and ventrally (Gobel, 1978; Woolf and King, 1987; Grudt and Perl, 2002; Schneider, 2008). Some cells also receive input from afferents, Aδ fibres, carrying noxious information; these cells are known as wide-dynamic range neurones (WDRs) as they respond to both noxious and innocuous peripheral stimuli. Lamina VI, Rexed claims, is not found outside of the cervical and lumbar enlargements. In the rat it is found from L3 to S1 (Molander et al., 1984).

This area also acts as a sensory relay to ventral horn motor pools. There is evidence for a 'musculotopic' organisation of reflex circuitry that lies across lamina V in L4 and L5 (Schouenborg et al., 1995). In this area, DH interneurones, receiving convergent polysynaptic A and C-fibre inputs coordinate the spatial outputs to ventral horn motor neurone pools (Schouenborg and Sjölund, 1983)

1.3.1.3 Laminae VII and VIII

These areas do not receive much attention from sensory physiologists. These ventral parts of the spinal cord grey matter contain neurones of variable size and are important for the coordination of motor output, particularly locomotors circuits but also reflex coordination (Jankowska and Edgley, 2010; Kiehn, 2011).

1.3.1.4 Lamina IX

Lamina IX is not a true layer, rather it is the collection of the sizeable cell bodies of motor neurones and can be seen at the most ventral aspect of the spinal cord grey matter. In the cervical and lumbar enlargements additional motor neurone pools can be seen laterally. Motor pools are arranged rostro-caudally throughout the spinal cord in a column-like fashion (Romanes, 1951; Swett et al., 1986).

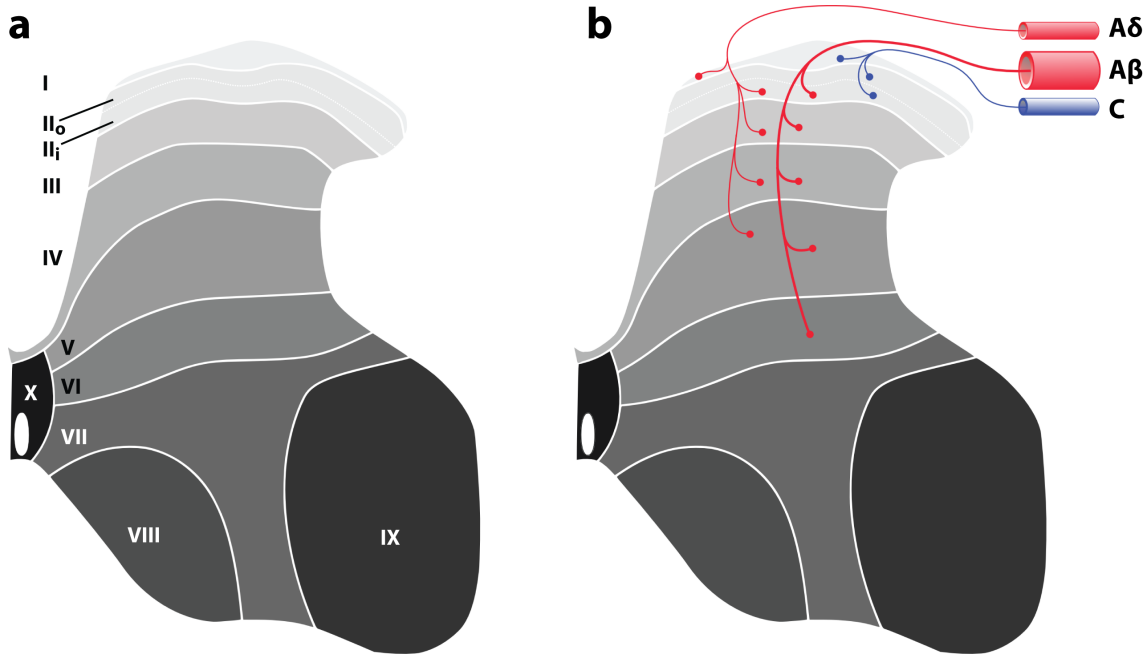


Figure 1-1 Lamination of the spinal cord and peripheral inputs from different fibre types
(a) Shows the lamination of a lumbar segment of spinal cord as originally described by Rexed and detailed in the preceding section. **(b)** Shows the peripheral afferent input to the spinal cord and its lamina distribution, though connections are indicated, this does not bear any relation as to the relative density of the innervation in any given lamina. Aδ and C-fibres terminate in LI and II outer and carry nociceptive specific information to WDR neurones in this area. LII inner receives input from C-fibres exclusively. C-fibre input to the deep dorsal horn is polysynaptic (shown as blue dotted line **(b)**. Inputs converge on alongside more thickly myelinated A-fibres on WDR neurones found in LV.

1.3.2 Anatomy and physiology of descending pathways

Although the role of descending circuits is not the subject of this thesis it is important to understand how these systems are organised and how the descending control modulates incoming sensory information. Observations that microinjection of morphine into certain brainstem nuclei can result in analgesia provided evidence of descending pathways which have specific modulatory effects on DH activity (Tsou and Jang, 1964). *Stimulation-produced analgesia* (SPA) can be elicited by electrical stimulation of the PAG and periventricular structures (Mayer et al., 1971). If stimulated, the PAG, which surrounds the aqueduct of Sylvius connecting the third and forth ventricles, inhibits reflex responses to noxious stimuli such as tail flick and paw withdrawal.

The PAG receives input from the limbic forebrain, amygdala, diencehphalon as well as ascending input from DH projections neurones (Fields et al., 1991). The amygdala also receives input from spinal cord nociceptive sources directly as well as indirect connections via projection neurone terminals in the parabrachial nucleus (Gauriau and Bernard, 2004).

Another important brainstem site of descending modulation is the rostro-ventral medulla (RVM). Indeed it is the relay through which most of the PAG's actions are mediated. Other nuclei that give rise to projections include the parabrachial nucleus, dorsal reticular nucleus, ventrolateral medulla and the locus coeruleus. The PAG itself has few monosynaptic connections with the spinal DH. The injection of excitatory amino acids into this area, reduces DH responses to noxious stimulation and produces analgesia. SPA from the PAG can be abolished by the microinjection of local anaesthetic into the RVM (Fields et al., 1991).

The action of descending systems can also facilitate the transmission of nociceptive information. Recordings from RVM neurones have characterised three distinct cell types which are thought to modulate nociceptive behaviours. These are 'ON', 'OFF' and 'NEUTRAL' cells (Fields and Heinricher, 1985; Fields et al., 1991). 'ON' cells fire immediately preceding a tail-flick reflex to noxious stimuli. 'OFF' cells cease firing immediately prior to the tail-flick and 'NEUTRAL' cell firing rates are indifferent to the presence or not of a noxious reflex (Fields and Heinricher, 1985). The temporal correlation of 'ON' cell firing with the onset of reflex activity has led to the suggestion that they are pro-nociceptive, whilst the converse has been suggested about the 'OFF' cell population. These observations were consistent with others that showed bi-directional modulation of nociceptive inputs by the RVM. Indeed, spinal cord transection prevents the development of secondary, but not primary, mechanical and thermal hyperalgesia after topical mustard oil application, carrageenan inflammation or nerve-root ligation (Urban and Gebhart, 1999). Similarly, low intensity electrical or chemical stimulation inhibits nociceptive responses whilst higher intensities facilitate it (Urban and Gebhart, 1999; Hathway et al., 2009).

1.4 Afferent input to the spinal cord

1.4.1 Nociceptors

Sherrington proposed that specific sensory fibres existed for the detection of tissue damaging stimuli (Sherrington, 1903). Many years later, Burgess and Perl identified a subset of thinly myelinated primary afferent neurones that responded exclusively to noxious mechanical stimulation (Burgess and Perl, 1967). Unmyelinated peripheral afferents that were more slowly conducting were found to be sensitive to a range of stimuli including heat, mechanical and chemical stimuli (Bessou and Perl, 1969).

The current classification of afferent input to the spinal cord broadly defines afferents by the size and conduction velocity of the respective fibres. Each subtype of fibre shows different laminar termination patterns in the grey matter of the spinal cord and each generally carry different sensory modalities. A schematic representation of the anatomy of central afferent terminals is found in Figure 1-1 and their functional characteristics are summarised below.

- C-fibres – thin unmyelinated fibres that have a conduction velocity at approximately 0.5-2ms^{-1}. C-fibre terminals are predominantly found in LII, but are also found in laminae I and VI. They carry information about noxious heat, mechanical and chemical stimuli.
- Aδ fibres – small and thinly myelinated fibres that conduct at approximately 12-30ms^{-1}. Terminals are found predominantly in Laminae I, III and V. They carry information about noxious inputs such as pin-prick as well as non-noxious input from hair follicles.
- Aβ fibres – large myelinated fibres that conduct at approximately 30-100ms^{-1}. These fibres carry information about light touch and brush stimuli and terminate in laminae III and IV.

The following section discusses these different afferent fibre classes in more detail.

1.4.2 C-fibres and mechanisms of signal transduction

C-fibres are unmyelinated slowly conducting fibres that are commonly associated with the slow dull component of pain. C-fibres are a diverse set of primary afferents that can respond to many noxious stimuli. Many of them are polymodal, that is, they respond, albeit to different degrees, to thermal, mechanical and chemical stimuli. One system of classification relies on the differential expression of peptides and receptors. C-fibres, by this system, can be non-peptidergic or peptidergic. The non-peptidergic class express Isolectin-B4 positive (IB4) as well as the purinergic receptor $P2X_3$, while the peptidergic class of C-fibres express the peptide neurotransmitter SP as well neurotrophic tyrosine kinase receptor type 1(TrkA), the high affinity receptor for nerve growth factor (NGF) (Julius and Basbaum, 2001). Peptidergic and non-peptidergic C-fibre terminals are found in distinct bands across the substantia gelantinosa with morphologically distinct synaptic endings (Hunt and Mantyh, 2001).

Though there may be distinct morphological differences between these populations, functional differences have been more difficult to tease out. Transduction of a particular sensory modality is dependent on the presence of receptors and ion channels that can turn a certain stimulus, such as heat, into a train of action potentials. In rat C-fibres the transient receptor potential cation channel subfamily V member 1 (TRPV1) which responds to heat, capsaicin and protons is found in half of peptidergic and non-peptidergic neurones (Caterina et al., 1997; Caterina et al., 1999; Meyer et al., 2013). Interestingly, *TRPV1*$^{-/-}$ mice have dramatically reduced sensitivity to heat, but retain some thermosensation, indicating that there are other transducers of heat stimuli of different intensities (Caterina et al., 2000). Importantly, TRPV1 deficient mice do not develop heat hyperalgesia following peripheral inflammation (Caterina et al., 2000).

As well as heat, C-fibres can transduce signals from multiple stimuli. Acid sensing ion channels are a family of chemo-electrical transducers particularly sensitive to changes in tissue pH (Deval et al., 2010). Cold sensation in the skin is thought to be mediated by C-fibres expressing the ion-channel TRPM8, whilst in the viscera the TRPA1 channel may be responsible (McKemy et al., 2002; Fajardo et al., 2008). The exact mechanism of mechano-transduction in the periphery is not well

understood. The recent identification of Piezo as a mechano-transducer in Drosophila may lead to a better understanding of these processes in mammalian cells (Kim et al., 2012). Piezo, however, remains a candidate mechanotransducer among many (see Delmas et al., 2011 for review).

Recently, several studies have identified a group of C-fibres that express *mas-related G protein-coupled receptors* (mrgpr) (Dong et al., 2001). Expression of different isoforms of these receptors has led to the identification of four distinct neuronal subpopulations within the dorsal root ganglion (DRG) (Dong et al., 2001; Zylka et al., 2003). Indeed the distribution of mrgpr$^+$ afferents is highly specific. Not only do they have different terminal fields in the skin, where they are exclusively found, they have specific termination fields within the spinal cord (Zylka et al., 2005). Recent work has demonstrated that the absence of the mrgprd$^+$ isoform results in selective mechanical deficits (Cavanaugh et al., 2009). Intriguingly, neurones expressing another isoform, mrgprb4, are responsible for the detection of massage-like stroking in the skin (Vrontou et al., 2013). A neuronal population coding specifically for itch has long been elusive, however, it may be that another mrgpr$^+$ subset of neurones is responsible for cutaneous responses to pruritogens. Animals in which mrgprA3$^+$ neurones are ablated show dramatically reduced scratching behaviour than controls, and when the TRPV1 receptor is expressed in mrgprA3$^+$ neurones, animals show itching behaviour in response to the cutaneous application of the TRPV1 agonist capsaicin (Han et al., 2013).

1.4.3 A-fibres

A-fibres are more rapidly conducting peripheral afferents than C-fibres and can be divided into several classes. Myelinated low-threshold mechanoreceptors enter the spinal cord and bifurcate to terminate in LII$_i$-V. Many of these fibres are associated with down-hairs and those that conduct in the Aδ range, provide low-threshold input to LII$_i$ (Light and Perl, 1979). Myelinated high-threshold afferent fibres are thought to mediate fast pricking pain. These constitute afferents in the Aδ through to the Aβ range and are responsive to mechanical and thermal stimuli (Burgess and Perl, 1967; Fitzgerald and Lynn, 1977). These afferents are mainly found to terminate in LI and LII (Todd, 2010). Unlike C-fibres, the neurochemical characterisation of A-fibres is not as extensive. There is evidence, however of SP-

like and CGRP-like immunoreactivity in A-fibre neurones in the DRG (Lawson et al., 1997; Lawson, 2002).

1.5 Functional organisation of reflex circuits

Previous sections have considered the anatomy of nociceptive afferent systems. This section will focus on the functional organisation of spinal circuits with specific reference to the organisation of reflex behaviour and reflex receptive fields.

The FWR is a polysynaptic multi-segmental reflex that serves to withdraw a limb from a noxious or potentially tissue damaging stimulus. This reflex, which has been widely studied both in animal and in man, provides a window for investigators to examine the function of spinal sensorimotor circuits.

Using spinalised and decerebrate cats C.S. Sherrington was the first to describe this phenomenon systematically (Sherrington, 1910). He made some key observations on the genesis, nature and coordination of reflexes of which the most pertinent are detailed below.

1) 'The term receptive field may be conveniently applied to designate the total assemblage of receptive points whence by suitable stimuli a particular reflex movement can be evoked'

Sherrington explored the areas of both hind and fore limbs that would evoke reflex movements. Reflex hind-limb withdrawal can be evoked from the whole hind limb both from cutaneous and deep structures including joints and muscles. Whilst stimulation throughout the limb may elicit a reflex, a response is more readily evoked from the paws and particularly the toes.

2) 'The stimuli especially effective are of nocuous quality'

Sherrington observed a strong positive correlation between the intensity of stimulation and strength of the resultant contraction. He noted, that high or noxious intensity stimulation evokes a response most reliably.

3) 'The stimulus which evokes the flexion-reflex in the stimulated limb commonly evokes at the same time certain reflex movements elsewhere.'

In the event of reflex flexion of the limb ipsilateral to the stimulus the resulting postural disturbance is accounted for by extension of the contralateral limb. This phenomenon is termed the crossed-extension reflex. Additional reflexes can include head rotation, mouth opening and vocalisation.

Much has been added to the understanding of reflex coordination and circuitry in the period following Sherrington's initial characterisation of the FWR. The mechanisms underlying his observations will be expounded here as the basis of a discussion of reflex physiology.

1.5.1 Receptive fields and input/output relationships

Woolf and Swett undertook a detailed study of the input output relationship between cutaneous sensory and motor circuits (Woolf and Swett, 1984). They recorded from single α-motorneurones from the nerve to the posterior head of the biceps femoris muscle and the principal head of the semitendinosus muscle in a decerebrate rat preparation. All α-motorneurones had cutaneous receptive fields on the ipsilateral foot that were responsive to noxious stimulation (pinch of the foot, temperatures of <10°C or >49°C and application of the chemical irritant mustard oil), but no activity was seen in response to non-noxious stimuli (light touch, brush or vibration).

In order to ascertain which sensory afferents were responsible for eliciting flexion reflexes Woolf and Swett electrically stimulated the sural nerve (Woolf and Swett, 1984). Aβ strength stimulation, confirmed by recording compound action potentials from the dorsal root, produced a short latency response in α-motorneurones, with after-discharge lasting for 10 milliseconds (ms). Increasing stimulus intensity resulted in the recruitment of Aδ afferents which produced a longer latency to response and an after-discharge of up to 1400ms. Increasing the stimulus strength further recruited high-threshold C-fibre afferents resulting in the longest latency responses and most substantial after-discharge of 7s.
Unlike Sherrington, who claimed that stimulation of entire ipsilateral limb might give rise to reflex movements, Woolf and Swett established that individual flexor

motorneurones responded to the stimulation of sometimes small and specific areas of skin.

The effective protection of an area of skin from a damaging or potentially damaging stimulus requires coordinated contraction of muscles leading to an appropriate movement away from a stimulus. This is achieved by the modular organisation of reflex circuits. In a key study, Schouenborg and Kalliomäki set down three important components of reflex input-output relationships (Schouenborg and Kalliomäki, 1990). Firstly, each muscle has a highly organised cutaneous receptive field which, if stimulated, normally causes the withdrawal of that receptive field if it were on the ground. Secondly, the most sensitive part of skin is also the most effectively withdrawn by that muscle. Thirdly, activation thresholds and time course of reflex activation is different between muscles, but muscles that have similar actions have similar activation thresholds and reflex time courses (Schouenborg and Kalliomäki, 1990; Schouenborg and Weng, 1994). Alongside excitatory receptive fields, stimulation of inhibitory receptive fields helps to ensure that inappropriate reflex movements are avoided (Weng and Schouenborg, 1996).

At the spinal cord level coordination is mediated by convergence of input on reflex encoding neurones in the deep DH (LV) (Schouenborg et al., 1995). These so-called *reflex encoder neurones* are passed somatotopically organised sensory information from laminae III-IV (Levinsson et al., 2002). They receive convergent A and C fibre input and their firing patterns are highly correlated with response patterns of the withdrawal reflexes in single muscles (Schouenborg et al., 1995).

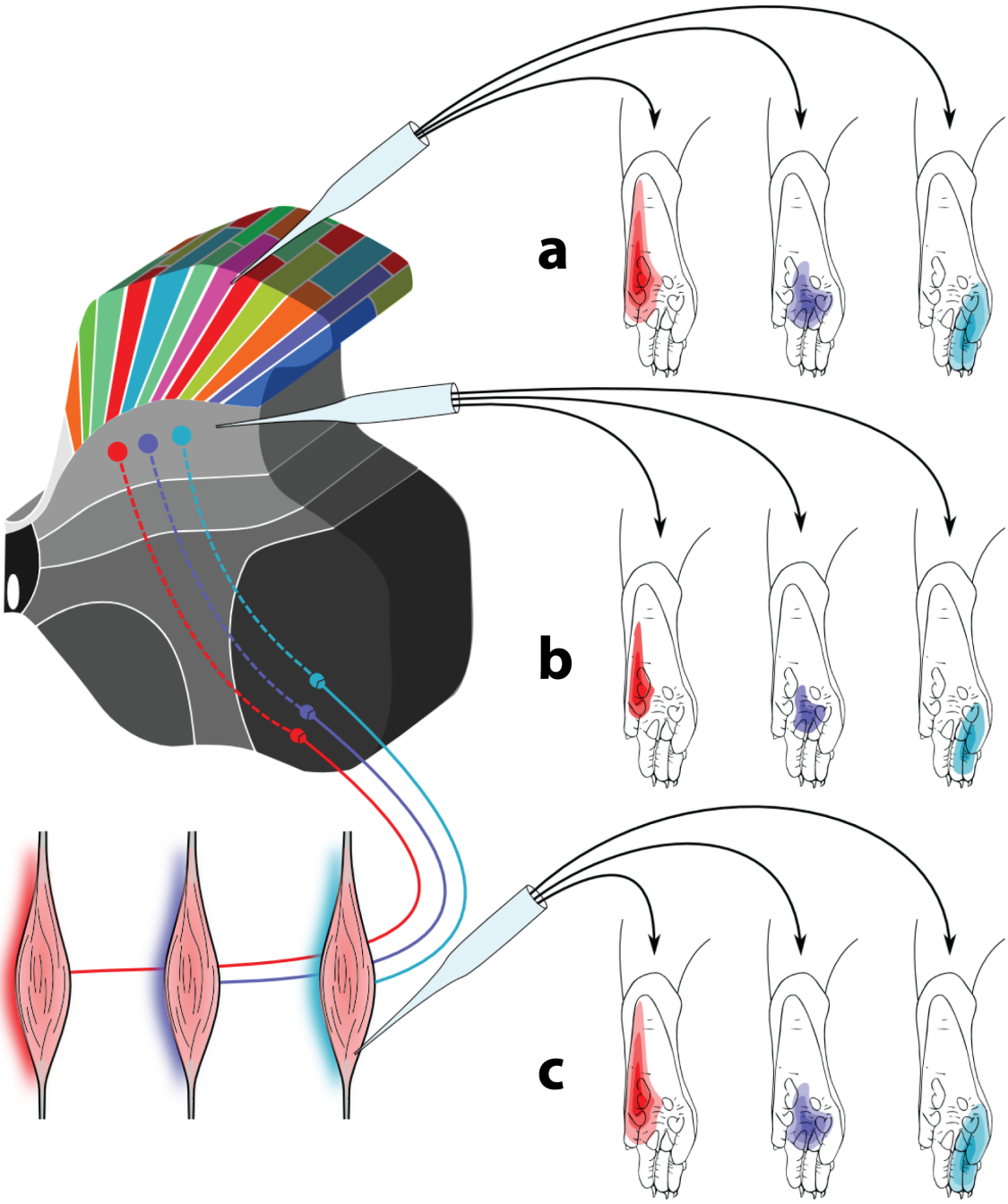


Figure 1-2 Modular Organisation of reflex pathways in the spinal cord
(a) Columns of the DH receive cutaneous input that has a specific weighting which is somatotopically organised in the superficial DH, these inputs converge onto neurones found in lamina V. **(b)** At the level of lamina V, a musculotopic organisation of reflex encoding (Re) neurones lies medio-laterally across the lamina. Re neurones are thought to project to single muscles and the weighting of the projection is dependent on the withdrawal efficacy of a particular muscle. **(c)** The modular organisation of reflex circuits is preserved at the level of the muscles, where the ability of a muscle to withdraw the stimulated skin area away determines the extent of its activation. Connections between LV and motorneurones are polysynaptic and are therefore shown as dotted lines. Illustration based on diagram in (Schouenborg, 2004).

Re neurones are not antidromically driven by stimulation of the cervical spinal cord, suggesting that they are interneurones. These interneurones are laid out medio-laterally across LIV and V in what appears to be a *musculotopic* fashion (Schouenborg et al., 1995). Neurones in this area are known to project to more ventral laminae (Woolf and King, 1987). The modular organisation of reflex circuits is summarised in Figure 1-2.

1.5.2 Supraspinal controls of reflex activity

One of the key observations that Sherrington made was on the effect of spinalisation on reflex circuits (Sherrington, 1910). Spinalisation is performed by transecting the spinal cord at the thoracic or cervical level. This leaves the lumbar cord intact but free from of the influence of descending control. Reflex responses to noxious stimulation of the hindlimb are dramatically enhanced following spinalisation. Furthermore, spinalisation lowers cutaneous mechanical thresholds and expands receptive fields (Woolf and Swett, 1984; Schouenborg et al., 1992). Spinalised preparations are often used to test the actions of analgesics on reflex circuits with the absence of descending influences (Kehne et al., 1985). In a study in humans, Willer and colleagues found that electrically evoked flexion reflexes are dampened by intravenously administered morphine. Higher doses of morphine resulted in greater inhibition of flexion reflexes which could be reversed by naloxone (Willer and Bussel, 1980b, a). Descending modulation is therefore a key part of activity within reflex circuits.

1.5.3 Sensitivity in reflex circuits

The study of reflex circuits has played an instrumental role in understanding the genesis, time course and mechanisms of post-injury hypersensitivity. In his original description of central sensitisation Woolf used the discharge of α-motorneurones to monitor the progressive increase in DH responses to a thermal injury of the hind paw (Woolf, 1983). It was demonstrated that wind-up, when repetitive stimulation results in progressive enhancement of DH neuronal responses to further stimuli, of nociceptive responses in the DH are associated with a concomitant increase in flexor motor neurone discharge. The biceps femoris α-motorneurones on the injured side develop receptive fields on the contralateral uninjured paw. Importantly, enhanced responses continue in the absence of peripheral input leading him to conclude that this hypersensitivity

was mediated at the level of the spinal cord and is not dependent on peripheral input (Woolf, 1983). Furthermore, it has been subsequently established that the state of central sensitisation was not due to changes in the excitability of the motor output (Cook et al., 1986).

Post-injury, the mechanical threshold to elicit a reflex is lower. Suprathreshold stimuli result in sustained reflex responses whilst motor units show increased spontaneous activity, prolonged after-discharge and slowly adapting responses to sustained stimulation (Woolf and McMahon, 1985). A state of central excitability can be elicited by the electrical stimulation of afferent nerves. It appears that a large component of this flexor reflex excitability is dependent on the input of C-fibres. Application of capsaicin to the sciatic nerve desensitises C-fibres and abolishes the facilitation of reflexes following a conditioning electrical stimulation (Woolf and Wall, 1986). In addition, the origin of the C-fibres can determine the degree of post-injury facilitation. When the chemical irritant mustard oil is injected into the ankle joint, flexor α-motorneurones have a greater and more long lasting response to noxious pinch than when mustard oil is injected into the skin (Woolf and Wall, 1986).

1.5.4 The flexion withdrawal reflex as a measure of pain

The FWR has been used extensively as a window onto nociceptive processing in the spinal cord in man due to the well-characterised input-output relationship between cutaneous stimulation and motor activity as well as its sensitivity to well-known analgesics. Studies in man have demonstrated that the perception of pain, stimulus intensity and magnitude of reflex responses are closely related (Willer, 1977; Bromm and Treede, 1980; Chan and Dallaire, 1989). The electrical stimulation of the skin at an intensity sufficient to evoke a reflex is the same intensity for the perception of pain by the subject (Willer, 1977). This fact is of particular clinical importance in non-verbal patients whose communication of pain may be compromised.

1.6 Sensitisation in pain states

The previous section outlined how the study of reflex circuits has revealed key neuronal processes that underlie sensitivity following injury. This section will address the mechanisms by which peripheral and central neuronal activity is enhanced following inflammation and tissue damage.

1.6.1 Peripheral sensitisation

In normal circumstances nociceptors respond only to high intensity stimulation. Following tissue injury or inflammation, the adaptations in the peripheral terminals of sensory nerves result in responses to previously non-nocuous stimuli and enhanced responses to noxious ones. Molecules released from damaged cells activate toll-like receptors (TLRs) expressed on the membranes of immune cells (Ren and Dubner, 2010). Once activated a plethora of cytokines and small molecules are released into the tissue surrounding nociceptors. These include 5-hydroxytryptamine (5-HT), prostaglandin-E_2 (PGE_2), interleukin-1 beta (IL-1β), interleukin-6 (IL-6) and tumour necrosis factor alpha (TNFα). The cytokines TNFα, IL-6 and IL-1β play a pivotal role in the expression of a key mediator of inflammatory hypersensitivity, NGF and thus the establishment of peripheral hypersensitivity associated with inflammation (Woolf et al., 1997). Indeed, TNFα, IL-6 and IL-1β will sensitise peripheral terminals in their own right. Intraplantar injection of IL-1β results in a detectable drop in mechanical thresholds within a minute, suggesting a direct action on peripheral terminals (Ferreira et al., 1988; Fukuoka et al., 1994). Furthermore, intraplantar administration of TNFα results in an increase in thermal sensitivity lasting 3-6 hours that can be reversed by the administration of anti-NGF serum (Woolf et al., 1997). All of these pro-nociceptive cytokines are released from the tissues surrounding the inflamed area, as well as by infiltrating immune cells (Chiu et al., 2012).

Many of the rapid changes in mechanical sensitivity following peripheral inflammation or injury can be explained by changes in the kinetics of various ion channels in the membranes of nociceptors. Released NGF binds its high affinity receptor on nociceptors. This results in downstream phosphorylation of Src kinase which can directly phosphylate TRPV1 leading to TRPV1 insertion into the

membrane (Zhang et al., 2005). When TRPV1 opens, calcium (Ca^{2+}) and other cations pass into the cell, depolarising it. Many other factors can influence the phosphylation of TRPV1 in a similar way including ATP, glutamate, PGE_2 and proteases (Schaible et al., 2011). Indeed, animals lacking the TRPV1 receptor do not develop enhanced response to thermal stimuli following inflammation (Caterina et al., 2000).

Changes to sodium (Na^+) channels are also powerful mediators of peripheral sensitisation following injury (Linley et al., 2010). Voltage-gated Na^+ channels are essential for the generation and propagation of action potentials. Subtypes $Na_v1.8$ and $Na_v1.9$ are only found in the sensory afferents and are thought to be responsible for action potential propagation and action potential initiation, respectively. Following inflammation they are upregulated and show enhanced Na^+ currents (Linley et al., 2010).

Changes in the sensitivity of peripheral afferents provide an important, but by no means complete, picture of hypersensitivity following injury. These mechanisms would only account for increased sensitivity at the site of damage, that is, a primary hypersensitivity. Inflammatory pain however, leads to increased sensitivity in areas that were not affected by the tissue damage, referred to as secondary hypersensitivity. The mechanisms that underlie this phenomenon are outlined below.

1.6.2 Central sensitisation

Changes at the central terminals of primary afferents also contribute to the hypersensitivity following injury. This process is not initiated by the activation of Aδ or Aβ fibres, but by the selective and repeated activation of C-fibres. The wind-up of the flexor reflex following peripheral injury is due to changes in the central synapses of incoming afferent neurones and their post-synaptic targets (Latremoliere and Woolf, 2009). If a peripheral nerve is repeatedly stimulated one can observe a progressive increase in action potential discharge and long lasting changes to the excitability of neurones in the spinal cord that persist in the absence of peripheral input (Woolf, 1983; Woolf and Thompson, 1991). Administration of the non-competitive N-methyl-D-aspartic acid (NMDA) receptor antagonist MK-801 results in an attenuation of the reflex facilitation seen

following a conditioning sural nerve stimulation or cutaneous application of mustard oil (Woolf and Thompson, 1991).

The substrate of these changes is altered excitatory neurotransmission in the spinal DH. Depolarisation of the central terminals of primary afferents results in the release of glutamate, SP and CGRP into the synaptic cleft and depolarisation of the post-synaptic membrane that is sufficient to remove the magnesium (Mg^{2+}) ion block of the NMDA receptor allowing an inward Ca^{2+} current (Mayer et al., 1984). Release of glutamate is a key step in this process. Activation of the NMDA receptor as well as the amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA) receptor is necessary for the establishment of central sensitisation (Woolf and Thompson, 1991; Latremoliere and Woolf, 2009). Additionally, the binding of SP, released by unmyelinated peptidergic C-fibres, to the NK1 receptor causes long-lasting membrane depolarisation which increases the likelihood of C-fibre evoked temporal summation (Latremoliere and Woolf, 2009). CGRP acts to potentiate the actions of SP, through binding to the CGRP1 and CGRP2 receptors (Woolf and Wiesenfeld-Hallin, 1986). An increase of intracellular Ca^{2+} in the post-synaptic cell activates numerous intracellular Ca^{2+} signalling cascades and is critical in the establishment of central sensitisation. This is achieved by the release of intracellular stores as well as influx through multiple ion channels. Particularly important are the Ca^{2+} dependent kinases protein kinase C (PKC) and calcium calmodulin-dependent-protein kinase II (CaMKII). These kinases can phosphorylate multiple receptors and ion channels both increasing their insertion into the post-synaptic membrane and changing channel kinetics so as to enhance excitatory neurotransmission in the DH (Latremoliere and Woolf, 2009). As well as changes in excitatory neurotransmission, reduced inhibitory drive is also apparent in central-sensitisation. Activation of PKC decreases tonic inhibition mediated by the inhibitory neurotransmitters γ-Aminobutyric acid (GABA) and glycine (Lin et al., 1996).

Inflammation itself can have particular effects on the sensitisation of central circuits. The cell bodies of afferents in the dorsal root ganglion (DRG) begin to express SP and brain-derived neurotrophic factor (BDNF), whilst receptors for these transmitters are unregulated post-synaptically (Mannion et al., 1999). BDNF signalling interferes with normal chloride homeostasis, resulting in enhanced excitability in LI neurones (Coull et al., 2005). Peripheral inflammation also allows

greater access of Aβ inputs to LII which may explain, in part, why light touch and brush can become painful in inflammatory pain states (Baba et al., 1999). The changing balance of the enzyme cyclo-oxygenase (COX) expression and production of prostaglandins (PG) are further important contributors to post-inflammation hypersensitivity. COX-1 and COX-2 are constitutively expressed in the CNS both in neuronal and glial cells (Vanegas and Schaible, 2001). The release of COX products PGE2 and PGI2 which bind to EP receptors on spinal neurones and in primary afferents, causing the downstream release of cyclic-adenosine monophosphate (cAMP) and PKC pathway activation. Enhanced tetrodotoxin (TTX) resistant Na^+ currents, reduced voltage dependent K^+ currents and increased $Ca2^+$ currents all contribute to enhanced neurotransmission (Vanegas and Schaible, 2001). Prostaglandins can also directly reduce inhibitory glycinergic transmission in the DH via interactions with the glycine receptor (GlyR) subunit α3 (Harvey et al., 2004).

1.6.3 Non-neuronal contributions to central sensitisation

While neuronal processes are critical for the induction and maintenance of central sensitisation, non-neuronal processes can also alter the excitability of spinal cord circuits. Microglia, resident immunocompetent cells of the nervous system, also contribute to central sensitisation in inflammatory pain. Microglial activation results in the release of cytokines including IL-6, IL-1β and TNFα in the DH (Raghavendra et al., 2004). When pro-inflammatory cytokine expression is reduced by the microglial inhibitor minocycline, the onset of central sensitisation is prevented (Ledeboer et al., 2005). Microglial recruitment and activation is initiated by the release of nitric oxide (NO) as well as numerous chemokines and adenosine tri-phosphate (ATP) released by damaged neurones, astrocytes and microglia themselves (Latremoliere and Woolf, 2009).

1.7 Mechanisms of joint pain

Inflammatory joint pain is a common clinical problem. Rheumatoid arthritis, a chronic inflammatory arthritis of adulthood, is estimated to affect 1% of the UK population (Symmons et al., 2002). Pain in the affected joint as well as in non-affected areas is associated with long-term joint destruction and disability (Morris et al., 1997). Pain in joint inflammation is often chronic in nature, requiring long-term treatment with analgesics including non-steroidal anti-inflammatory drugs (NSAIDs) and opiates (Kidd et al., 2007). This section will outline what is understood of the mechanisms that underlie the generation and maintenance of joint pain in adult joint disease.

1.7.1 The peripheral innervation of joints

Cutaneous inputs to the spinal cord are well described, and much of the understanding of primary afferent physiology has come from the study of cutaneous nociceptors. Less is known about the spinal cord input from joints. Much of the input from joints to the spinal cord is from proprioceptive afferents that are important for coordinated movement. Joint articular surfaces are devoid of peripheral nerve endings whilst the tendons and ligaments surrounding the joints are richly innervated by specialised nerve endings and free nerve endings including nociceptive afferents. The innervation of the joint is outlined in Table 1-1.

Subtype	Morphological Features	Size (µm)	Location	Afferent Fibre	Function
1	Globular or ovoid corpuscle with thin capsule	100 x 40	Joint capsule, periosteum, ligaments, tendons	Small myelinated (5-8µm)	Mech R, LT, SA
2	Cylindrical or conical corpuscle with thick laminated capsule	280 x 120	Deep joint capsule	Medium myelinated (8-12µm)	Mech R, LT, RA
3	Fusiform corpuscle with thin capsule	600 x 100	Ligaments, tendons	Large myelinated (13-17µm)	Mech R, HT, SA
4	(a) Unmyelinated plexuses	<1.5	Fibrous capsule, ligaments fat pads, adventitia of blood vessels, sub-synovial tissue	Fine myelinated (2-5 µm)	Nociception, HT
	(b) Unmyelinated free nerve endings	0.5 – 1.5	Ligaments and tendons	Unmyelinated (<2µm)	Nociception, HT
	(c) Unmyelinated nerve terminals	<1.0	Walls of small arteries and arterioles	Unmyelinated (<2µm)	Vasomotor, efferent

Table 1–1 Classification of joint mechanoreceptor terminals
This table summarises the innervation of the normal joint. **Mech R** = mechanoreceptor, **LT** = low threshold, **HT** = high threshold, **SA** = slowly adapting, **RA** = rapidly adapting. Size refers to the average dimensions of the end organ described. Modified from the original in (Freeman and Wyke, 1967).

Sensory innervation of joints is carried in the branches of main nerve trunks or their muscular, cutaneous or periosteal branches (Schaible and Grubb, 1993). Specialised joint nerve endings were originally classified into four subgroups (Table 1-1), dependent on the afferent fibre type and the appearance of the nerve ending. This nomenclature should not be confused with Group I, II, III, IV afferents, which refers to subtypes of afferent fibres that can originate, both in the joint muscle and skin. Joints are also richly innervated sympathetic fibres (Schaible and Grubb, 1993). The following paragraphs contain further detail of specialised articular nerve endings.

1.7.1.1 Type 1 endings

These endings are mostly found in the superficial parts of the fibrous joint capsule and cluster together in groups of approximately six corpuscles innervated by the same axon (Freeman and Wyke, 1967). In articular tissues, the afferents supplying these endings have close associations with blood vessels. In the skin these are similar to Ruffini endings, are low-threshold and are thought to signal stretch (Hogervorst and Brand, 1998).

1.7.1.2 Type 2 endings

These endings are elongated conical capsules that are thicker at the base and narrow towards the top. The capsule is made up of multiple layers (up to 12) of elongated connective tissue surrounding an unmyelinated nerve ending at the centre. They are found in the fibrous capsules of joints and may intermingle with Type I endings, but on the whole are found deeper within the capsular tissue (Freeman and Wyke, 1967). These endings resemble Meissner's corpuscles in the skin which are responsible for signalling dynamic deformation of the skin and are served by (Delmas et al., 2011).

1.7.1.3 Type 3 endings

These endings are anatomically distinct from Type I and II endings and are not found in the joint capsule, rather they innervate the intrinsic and extrinsic ligaments of the joint (Freeman and Wyke, 1967). Type III endings are surrounded by a large fusiform body that lies plate-like on the surface of ligaments. They are the largest end organs in articular tissues and are associated with the largest afferent fibres. Unlike Type I and II endings, the fibres supplying them have no relation to blood vessels. They are similar in functional properties, size and innervation to Golgi tendon organs (Freeman and Wyke, 1967).

1.7.1.4 Type 4 endings

Type IV endings are unmyelinated free nerve endings that are found in articular tissues and can be subdivided into (a) unmyelinated plexuses, (b) free nerve endings and (c) unmyelinated nerve terminals. Unmyelinated plexuses are found throughout articular tissues, excluding articular surfaces and ligaments, in a

mesh-like network and function as nociceptive afferents. Free nerve endings are found in close association with small arteries and arterioles but are also found as independent nerve fibres in all ligaments and around the joint capsule. The third type of unmyelinated fibre in joints is responsible for smooth muscle tone in small arterioles and arteries, but is found in the tunica media of vessels rather than the adventitia where free nerve endings are usually found (Freeman and Wyke, 1967).

1.7.2 Models of joint pain

No animal models of juvenile joint pain exist. In adult rodents there are several interesting and potentially useful arthritic models which may be adapted to suit younger animals. Arthritic models, generally, cause swelling of the affected joint, behavioural hypersensitivity and impairment of movement. There are many rodent models of inflammatory arthritis that have been used to study the pathogenesis of arthritis from an immunological perspective (see Lindqvist et al., 2002). Others models are used to study mechanisms of pain underlying osteoarthritis (OA). These models induce cartilage destruction and bone erosion which results in a mechanical destruction of the joint, secondary to which inflammation results; these models are not considered here. Since one of the principal aims of this thesis is to determine how inflammatory pain is processed in the developing nervous system, it is necessary to consider models of inflammatory joint pain currently used in adult animals. Furthermore we wanted a well-characterised model used in the study of adult arthritis, with which to frame our findings.

Collagen-induced arthritis (CIA) is widely used to assess the pathogenesis and treatment of rheumatoid arthritis. Systemic immunisation with type II collagen along with an adjuvant promotes T and B-cell dependent destruction of cartilage and generalised inflammation which develops 21-28 days post immunisation (Brand et al., 2007). This model has been shown to induce significant reductions in sensory withdrawal thresholds and induces spontaneous behaviours in genetically susceptible adult mice consistent with animals suffering arthritic pain (Inglis et al., 2007).

One of the most widely used models of adult arthritic pain utilises a suspension known as complete Freund's adjuvant (CFA). T-cells that are reactive to both cartilage and the active component of CFA – the cell walls of mycobacterium butyricum are recruited to the CFA injected joint (van Eden et al., 1985). Joint swelling and immune cell infiltration are apparent within hours of injection, after which cell mediated destruction of articular cartilage occurs. Joint injection of CFA limits damage to the affected joint and prevents any systemic effects that CFA administration would have on other tissues and behaviour of the animal (Butler et al., 1992; Seino et al., 2006). Intra-articular CFA has also been shown to induce hyperalgesia and reduced weight bearing in the affected limb that is apparent within days following injection (Cruz et al., 2005). In contrast to CIA models which induce systemic long-term illness, CFA induced monoarthritis remains localised, is apparent soon after injection and resolves in a matter of weeks rather than months.

Another widely used model of inflammatory arthritis uses a mixture of kaolin, a fine clay, which acts as an adjuvant and carrageenan, a seaweed extract, to induce joint inflammation (Schaible and Schmidt, 1985b; Sluka et al., 1994). Affected animals develop an intense inflammatory response that resolves more acutely than the models discussed above. Other adjuvant induced arthritis models include injection of non-immunogenic compounds such as pristane, avridine, squalene and mineral oil, although these models are more widely used in immunological fields than in pain research (see Holmdahl et al., 2001).

1.7.3 Peripheral mechanisms of joint pain

Inflammation is a key component in the development and maintenance of arthritic pain (Schaible et al., 2002). By contrast, osteoarthritis is a condition that is primarily driven by degenerative and mechanical changes in the joint. Once damage is present, pain associated with this condition is maintained and enhanced by the presence of inflammatory and neuropathic disease processes (McDougall et al., 2009).

Physiologically, the immune system has an integral role in the protection and repair of tissues. Inflammation after injury promotes increased vascular permeability and thus infiltration of immune cells from the blood into peripheral

tissues. This results in an acute response that is typified by the five cardinal signs of inflammation: dolor, rubor, calor, tumor and functio laesa or pain, redness, heat, swelling and loss or reduction of function respectively (Medzhitov, 2008). This response is generally short-lived and kept in check, by a variety of mechanisms such as anti-inflammatory cytokines which modulate the amplitude and duration of the immune response (Xu et al., 2010b). In many autoimmune conditions such as inflammatory arthritis, however, inflammation becomes chronic and rarely resolves completely. Destruction of articular cartilage, fibrosis of peri-articular structures, proliferation of the synovial membrane (pannus formation), loss of movement and pain are key features of this disease process. The pain associated with arthritis is typified by hyperalgesia (enhanced responses to painful stimulation) and pain at rest (spontaneous pain). In addition, previously innocuous stimulation such as the movement of joints becomes painful (Schaible et al., 2002b).

Joint pain is commonly associated with mechanical hyperalgesia, that is, enhanced responses to noxious mechanical stimuli (Schaible et al., 2009). This can be mediated by the following peripheral mechanisms. Peripheral sensitisation of joint mechanoreceptors occurs in the first hours following inflammation and can persist in the presence of ongoing inflammation (Guilbaud et al., 1985; Schaible and Schmidt, 1985b). The mechanical thresholds of high threshold Aδ and C-fibres originating from the inflamed joint and of low threshold receptors that conventionally respond to movement within the normal range of joint movement are reduced (Grigg et al., 1986; Schaible and Schmidt, 1988b). In normal joints it is difficult to elicit responses from Type IV afferents, however, following inflammation, responsive afferents are more frequently observed suggesting the sensitisation of a population of silent nociceptive neurones (Grigg et al., 1986). There is also evidence that myelinated Aβ fibres increase their responses to peripheral mechanical stimuli which may be mediated via Type I and II joint endings as a result of the stretching of the fibrous capsule of joints (Andrew and Dodt, 1953; Schaible and Schmidt, 1988b).

Many of the mechanisms of peripheral sensitisation are shared by articular and cutaneous tissues. Like the skin, a plethora of cytokines, peptides and growth factors have been shown to influence the excitability of joint afferents. Local

release of cytokines can also drive peripheral joint sensitivity. IL-1β, IL-6 and TNFα have been shown to not only sensitise joint afferents in the first instance, but also maintain hypersensitivity of sensory terminals peripherally (Richter and Schaible, 2007; Kawasaki et al., 2008; Richter et al., 2010).

Furthermore, neuropeptides including SP, CGRP and vasoactive intestinal peptide (VIP) have been found within inflamed joints (McDougall, 2006). VIP, for instance, found in post-ganglionic sympathetic fibres and some primary afferents, lowers the mechanical thresholds of joint afferents when injected intra-articularly (Abramovici et al., 1991; Catre and Salo, 1999; Schuelert and McDougall, 2006).

Other factors that can change the excitability of primary afferents include many of the products derived from arachidonic acid such as prostaglandins, leukotrienes, lipoxins, thromboxanes and endocannabinoids (Gauldie et al., 2001; McDougall, 2006). PGE_2, for example, has a profound ability to change the excitability of primary afferents (Schaible and Schmidt, 1988a). COX, in particular, is the main target of NSAIDs which can be effective in alleviating some of the pain associated with joint inflammation in a clinical setting. Pain relief from such drugs, however, is limited due to other mechanisms that maintain joint pain in CNS circuits.

1.7.4 Central neuronal mechanisms of joint pain

Trains of impulses from the arthritic joint are also a driver of hypersensitivity mediated by central sensitisation at the spinal cord level. This sensitisation is reinforced by the direct action of cytokines on spinal cord neurones both by increasing excitatory synaptic transmission and reducing inhibitory synaptic transmission (Kawasaki et al., 2008). These mechanisms have been outlined in section 1.6.2. This section will introduce neuronal mechanisms underlying central sensitisation in joint inflammation.

Dorsal horn neurones that receive joint input are rendered excitable within the first hours following joint inflammation (Schaible et al., 1987; Neugebauer et al., 1993). Input from uninflamed tissue surrounding the joint is also sensitised (Neugebauer and Schaible, 1990). Following inflammation the descending control of DH neuronal activity is enhanced. Spinalisation results in an increase of DH neuronal responses to joint input (Schaible et al., 1991; Herrero and Cervero, 1996).

This strongly suggests that rapid and powerful descending control of nociceptive processing is brought to bear on the DH following the induction of arthritis. Chronic arthritis results in long-term expansion of receptive fields, both ipsilateral and contralateral, as well as enhanced responses to joint input that are associated with the enhanced responses to A and C-fibre inputs (Grubb et al., 1993; Martindale et al., 2007). As is true with cutaneous inflammation, there is also a reduction of tonic inhibition at the segmental level (Calvino et al., 1987b). The changes in spinal excitability observed following joint inflammation, are thought to be mediated by changes to excitatory neurotransmission in the DH. Spinal application of an NMDA or AMPA antagonist prevented the development of central sensitisation in a rodent model of inflammatory arthritis (Neugebauer et al., 1993). Clinically, central sensitisation in the presence of an inflamed joint, can explain widespread tenderness around the affected joint as well as deep pain following mechanical stimulation at unaffected sites (Arendt-Nielsen et al., 2010).

1.8 Development of sensory networks in the postnatal period

Sensory systems undergo profound developmental changes in the postnatal period such that the processing of nociceptive input differs in young and adult animals (see Fitzgerald, 2005; Fitzgerald and Walker, 2009). So far this introduction has focused on mechanisms that have been well characterised in the adult nervous system. While much is known about how inflammatory joint pain is processed in the adult nervous system, almost nothing is known of the processing of inflammatory joint pain in the immature nervous system. This section will outline how sensory systems develop over the postnatal period with particular attention to the development of cutaneous reflexes and what little is known of inflammatory pain processing in the context of development.

Rats are born prematurely compared to humans and develop rapidly so that by the age of 8 weeks they are considered adult. This makes direct comparisons with human development difficult. The importance of understanding postnatal development in neuroscience cannot be underestimated. Many studies conducted on the immature nervous system whose findings are extrapolated into adult systems may not be valid (Mccutcheon and Marinelli, 2009). This was starkly illustrated when it was shown that the expression of a key glutamate receptor

thought to be involved in 'tripartite' synapses between neurones and glia was highly developmentally regulated. Metabotropic glutamate receptor 5 dependent increases in cytosolic Ca^{2+} in astrocytes leading to the release of gliotransmitters only occurred up until the third postnatal week in mice and was completely absent in adult preparations (Sun et al., 2013).

1.8.1 Development of nociceptive connections to the spinal cord

In the rat, much of the nociceptive circuitry required for the discrimination of noxious inputs is in place by birth (postnatal day 0 - P0). Sensory neurones grow out from the dorsal root ganglia both toward the spinal cord and the periphery. The protein GAP-43 found in high concentrations in the growth cones of axons, is integral to the presynaptic terminals, neuronal growth and plasticity. GAP-43^{+} fibres are seen in the developing limb bud and reach the epidermis of the toes by embryonic day (E) 19 (Reynolds et al., 1991). At the same time connections are being formed between primary afferents and the DH of the lumbar spinal cord so that by E20 terminals extend as far as LII_i (Fitzgerald, 1987b). *In vivo* DH recordings from the rat foetus at E16-20 show that newly formed cutaneous terminals sensitive to chemical, heat and mechanical stimuli exist and can evoke activity in the spinal cord (Fitzgerald, 1987a). This is also consistent with functional data showing that reflexes can be evoked by mechanical stimulation of the hindlimbs and rump by E18 (Angulo y Gonzalez, 1932).

1.8.2 Development of afferent organisation in the spinal cord

Newborns are highly sensitive to tactile sensory inputs which is illustrated by the enhanced sensitivity of reflex circuits present in animals and humans (Ekholm, 1967b; Andrews and Fitzgerald, 1994). Mechanically evoked FWR have lower thresholds in the postnatal period, which progressively increase over the first 3 postnatal weeks in rats. Reflex receptive fields are enlarged and disorganised in the early postnatal period and are gradually refined over the postnatal period (Holmberg and Schouenborg, 1996a; Waldenström et al., 2003). This is also reflected in the properties of DH neurones, which show large receptive fields to mechanical stimuli (Fitzgerald, 1985, 1988; Torsney and Fitzgerald, 2002).

The pattern of afferent terminals in the immature spinal cord undergoes substantial postnatal reorganisation. During this period myelinated fibres are

found in areas not seen in the adult spinal cord. The immature superficial DH is dominated by A-fibre inputs. The marker of neuronal activation, c-fos, is found in LII following Aβ-fibre strength stimulation or touch in young animals but is not seen after P21 (Jennings and Fitzgerald, 1996). Furthermore, anatomical tracing studies have found the terminations of myelinated and unmyelinated afferents overlap in the early postnatal period and undergo a progressive separation over the postnatal period (Coggeshall et al., 1996; Granmo et al., 2008). As the animal grows, A-fibres begin to withdraw from superficial DH in an activity dependent manner (Beggs et al., 2002). The withdrawal of A-fibres can be prevented by the blockade of excitatory neurotransmission in the spinal cord. By P21 A-fibre terminals reach a near-adult pattern (Beggs et al., 2002; Granmo et al., 2008).

As outlined above A-fibres overlap with C-fibres during postnatal development and this partly explains the sensitivity of young animals to low-threshold inputs. C-fibres also undergo substantial changes in the postnatal period which are important both for the normal development of nociceptive processing and also low-threshold input processing. Indeed, the withdrawal of A-fibre terminals from superficial laminae does not occur in animals treated with capsaicin in the neonatal period which destroys C-fibre terminals (Torsney et al., 2000). Although neonates show strong reflexes to noxious stimuli, these are likely due to A-fibre inputs, since, C-fibres in the first three postnatal weeks are functionally immature compared to adults (Fitzgerald, 1988; Fitzgerald and Jennings, 1999). Topical application of capsaicin can activate TRPV1 expressing fibres at P0 but evokes weaker responses in young compared to adult animals (Baccei et al., 2003; Walker et al., 2007). In the second postnatal week, C-fibre evoked spiking in the DH becomes more pronounced as inputs mature (Baccei et al., 2003).

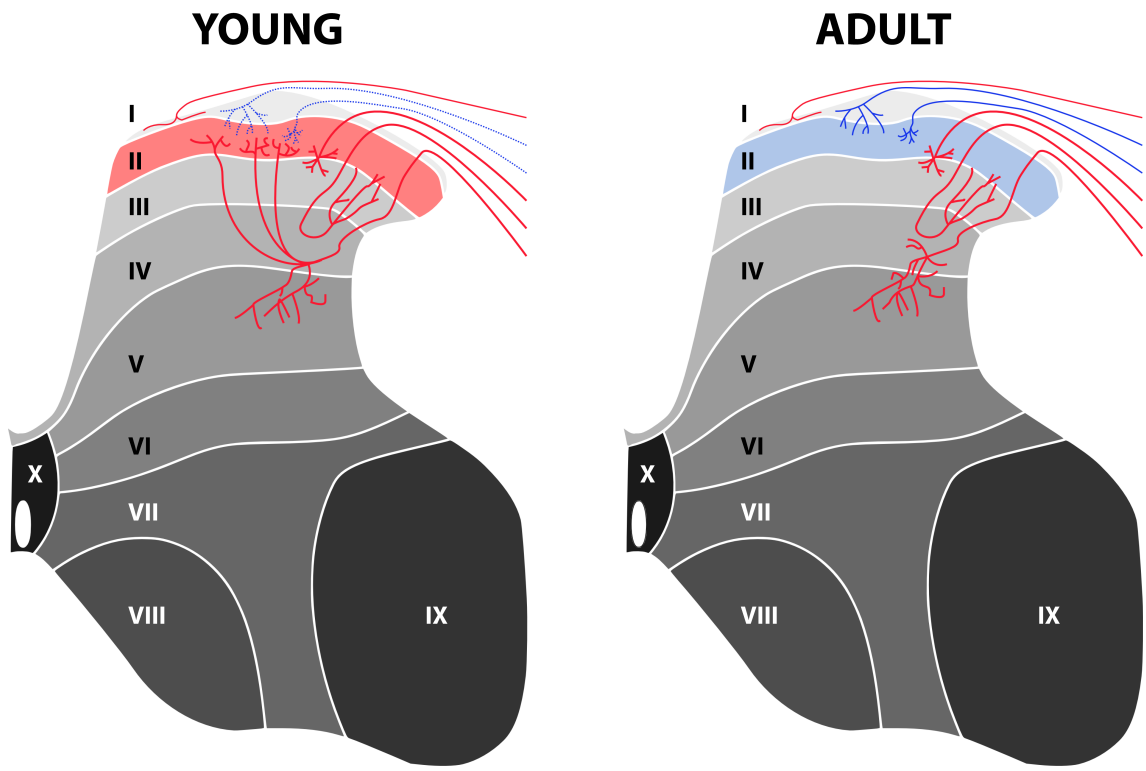


Figure 1-3 Differences in afferent termination patterns between the neonatal and adult spinal cords
In the young cord afferent input from A-fibres (shown in red) dominates the input the superficial laminae of the spinal cord. C-fibre (shown in blue) evoked activity is weak at this stage. By the end of the 3rd postnatal week, C-fibre evoked potentials reach maturity, whilst the withdrawal of Aβ-fibres from the superficial DH is well underway. LII becomes dominated by input from C-fibres and A-fibre touch inputs are restricted to deeper laminae. (Modified from (Todd, 2010))

1.8.3 Excitatory and inhibitory neurotransmission in immature systems

Alongside the changing architecture of the spinal cord in the first postnatal weeks, a parallel shift in the inhibitory control of spinal sensory networks occurs, from a facilitation of synaptic transmission in the early period to greater inhibition as an adult. The intrinsic excitability of spinal DH neurones does not change in the postnatal period, although there are substantial alterations in excitatory neurotransmission (Baccei et al., 2003; Pattinson and Fitzgerald, 2004). AMPA, kainate (KA) receptors and NMDA receptors mediate fast excitatory transmission within the spinal cord. High levels of expression of AMPA, NMDA and KA receptors are seen in the immature cord but the expression of these receptors becomes increasingly anatomically restricted to the superficial DH over the first postnatal weeks (Jakowec et al., 1995; Brown et al., 2002; Pattinson and Fitzgerald, 2004). The progressive restriction of expression of these receptors, as well as their changing subunit composition and falling density over the postnatal period coincides with a dampening of general spinal cord excitability. The mature profile of AMPA and NMDA receptor expression seems to be in place at P28 and

P21 respectively, although the subunit composition of these receptors continues to develop until at least P35 (Jakowec et al., 1995; Brown et al., 2002).

Like excitatory transmission, fast inhibitory transmission, mediated by GABA and glycine undergoes maturation in the postnatal period. Evidence would suggest that unlike glycinergic inhibition, GABAergic inhibition is more mature at birth and GABA acts as an inhibitory neutrotransmitter postsynaptically in young animals. Differences in GABAergic transmission between neonates and adults are subtle. The blockade of GABAergic transmission lead to enlarged receptive fields and increased activity following cutaneous stimulation (Bremner, 2006). Others have suggested that the immaturity of GABAergic transmission may underlie the large and poorly directed reflexes seen at early postnatal ages (Cordero-Erausquin, 2005). Whilst their GABAergic inhibition may be of lower fidelity in the early postnatal weeks, the most striking deficit appears to be in glycinergic inhibition (Ingram et al., 2008). Recent evidence would suggest a lack of glycinergic inhibitory control in the developing spinal cord (Koch et al., 2012). In the more mature P21 cord, glycinergic blockade results in enhanced behavioural sensitivity and neuronal responses in the DH *in vivo*, whilst application of the glycine receptor antagonist strychnine, has no effect on the P3 cord (Koch et al., 2012). The combination of enhanced excitatory neurotransmission and the lack of glycinergic inhibition in the first postnatal weeks seems to play a pivotal role in the 'excitable' state of the spinal cord at this stage.

1.8.4 Development of descending modulation of pain

In adult spinalised cats, Sherrington demonstrates that when the spinal cord is cut at the thoracic level, mechanical reflex thresholds fall and responses to noxious stimuli become exaggerated (Sherrington, 1910; Sherrington and Sowton, 1915). Fitzgerald and Koltzenberg showed that electrical stimulation in the dorsolateral funiculus, through which descending fibres run, dampened DH responses to stimulation of the hind paw, but only from P9 onwards (Fitzgerald and Koltzenburg, 1986). By P12, stimulation weakly inhibits around 50% of cells and by P18, stimulation causes widespread inhibition in the DH but requires a more intense stimulation protocol than in adults. Similarly, van Praag and Frenk, using direct electrical stimulation of PAG, were only able to elicit analgesia from P21 onwards and showed that the stimulus intensity required for analgesia was

greater than that in adults (van Praag and Frenk, 1991). More recently, Hathway et al., showed that descending systems in the immature brainstem and spinal cord are facilitatory. By contrast, electrical stimulation of the adult (P40) RVM leads to inhibition of reflex responses to a graded mechanical stimulus at low stimulation intensity and facilitation at higher intensities. At P21 stimulation at all currents (5-200μA) is facilitatory. By P25 a current of greater than 50μA inhibits reflex responses, which would be the expected result in adult animals (Hathway et al., 2009). Interestingly, the pharmacological ablation of the RVM in animals up to the age of P21 results in a rise in mechanical thresholds. The same treatment in adults results in a fall in mechanical thresholds (Hathway et al., 2009).

1.8.5 Characteristics of immature reflexes

Reflex activity, in the forelimbs and the trunk, can be evoked in prenatal rats as early as E16 and in the hindlimbs and rump by E18 (Angulo y Gonzalez, 1932). These movements are uncoordinated, weak and can be evoked at multiple sites across on the skin surface (Angulo y Gonzalez, 1932). The former is associated with the growth of peripheral sensory afferents into the cervical and upper thoracic cord and the latter into the lumbo-sacral cord a few days subsequently (Vaughn and Grieshaber, 1973).

Generally, it is observed that young animals are more sensitive to cutaneous stimulation. In neonatal decerebrate kittens, substantially lower mechanical force than that required for similar adult cats, is required to elicit whole hindlimb withdrawal (Weed, 1917; Ekholm, 1967b). Thermal thresholds in rats are similarly low in neonates and reach adult levels between P18 and P21, with the most rapid change occurring between P3-15 (Falcon et al., 1996; Jiang and Gebhart, 1998). The same study showed that repeated thermal stimulation, with an inter-stimulus interval of three minutes, produced significant desensitisation of the tail flick reflex that was not seen in older animals (Falcon et al., 1996). Adult reflexes are well directed and are elicited from spatially well-organised receptive fields. By comparison, reflexes in the first postnatal week are elicited from wide areas of the hindlimb and are often inappropriate, sometimes even moving the limb toward the stimulus (Holmberg and Schouenborg, 1996b).

A similar pattern of reflex development has been mapped out for tail-flick responses to noxious heat stimulation (Waldenström et al., 2003). A stimulus was applied to the tail and the direction of movement, either toward or away from the stimulus was recorded. In newborns the response was poorly coordinated and often toward the stimulus, which is considered to be inappropriate (see Figure 1-4). With increasing postnatal age there was a progressive refinement of the reflex such that by P28 the tail-flick was almost universally away from the stimulus. Following a week of sensory deprivation by application of a local anaesthetic cream to the tail and hair removal during the period between P14 and P21, coordinated reflex activity fails to develop. This demonstrates the importance of sensory input from low-threshold fibres for the development of normal reflex responses to noxious stimuli (Waldenström et al., 2003). Additionally, there are important contributions from descending systems. Neonatal spinalisation results in disorganised reflex responses in adulthood (Levinsson et al., 1999). There is a close temporal correlation between the development of reflex responses to noxious stimuli and the appearance of functional descending control of the spinal cord reflex circuitry (Fitzgerald and Koltzenburg, 1986; Hathway et al., 2009).

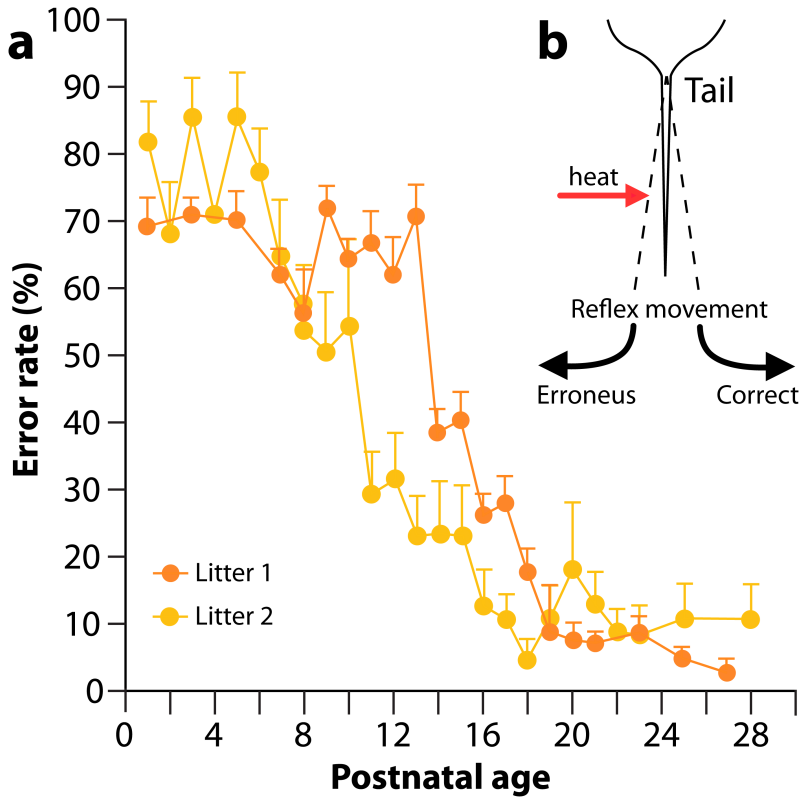


Figure 1-4 Postnatal development of nociceptive withdrawal reflex
Error rate of tail flick in response to a noxious heat stimulus (CO_2 laser – indicated by red arrow in **(b)**) is reduced with increasing postnatal age. Different colours show results from two separate litters, Litter 1 (n=13) and Litter 2 (n=12). **(b)** Tail flicks toward the laser were considered erroneous and those away from the stimulus were considered correct, (adapted from Waldenström et al., 2003).

Fitzgerald and Gibson examined the neurochemical and physiological properties of C-fibres over the first three postnatal weeks in rats (Fitzgerald and Gibson, 1984). It was demonstrated that flexor reflex activity could be elicited over the entire developmental period in question using mechanical, thermal or electrical stimulation. Reflex responses were prolonged compared to those of adults until the end of the second postnatal week. The same pattern of activity could not be evoked in the adult even with more intense stimulation, which the authors argue, precludes this effect being dependent on thickening of the skin that occurs concurrently. The application of mustard oil, a chemical irritant and specific activator of C-fibre nociceptors, to the skin of the hind paw, readily induces substantial withdrawal reflex activity in the adult rat. Up until P10-11, however, such chemical stimulation does not evoke a reflex even though nociceptors terminals can be visualised in the DH of the spinal cord in the first few postnatal days (Fitzgerald and Gibson, 1984). Jiang and Gebhart, in contrast, were able to evoke a low level of spontaneous activity in P2 and P6 animals, albeit much less than that observed in adults (Jiang and Gebhart, 1998).

In summary, the nature of the FWR changes substantially over the first three postnatal weeks. The general properties of immature reflexes are as follows:

- Early nociceptive reflexes are both poorly coordinated and poorly directed, which at times, may lead to movement toward a potentially tissue damaging stimulus (Holmberg and Schouenborg, 1996a; Waldenström et al., 2003).

- The strength of stimulation required to evoke a reflex increases with postnatal age, independent of changes in skin thickness (Fitzgerald and Gibson, 1984).

- Cutaneous reflexes are exaggerated and have larger disorganised receptive fields compared to adults (Holmberg and Schouenborg, 1996b; Holmberg et al., 1997; Levinsson et al., 1999; Waldenström et al., 2003; Schouenborg, 2004)

- The maturation of nociceptive reflexes parallels that of descending systems but is also dependent on peripheral inputs from low-threshold afferent fibres (Levinsson et al., 1999; Waldenström et al., 2003).

1.9 Painful early life experience

Both clinical and neurobiological studies in animal models show that CNS nociceptive processing and connections differ in juveniles and adults (Fitzgerald, 2005; Slater et al., 2007). The immaturity of the synaptic connections and integrated circuits mean that children's pain experience is different from that of adults. The normal development of pain processing in young mammals is dependent on a 'normal' sensory experience, particularly non-noxious stimulation such as touch (Fitzgerald, 2005). Development can be altered by patterns of sensory inputs arising as the result of tissue injury and may lead to changes in somatosensory processing, pain signalling and future analgesic responsiveness.

Hind paw inflammation, for instance, is known to produce long-term structural changes in nociceptive pathways (Walker, 2003; Ren et al., 2004). Milder hind paw inflammation can produce generalised hypoalgesia and an increased hyperalgesic response if the previously injured paw is re-inflamed in adulthood (Ren et al., 2004). Similarly enhanced sensitivity to repeat injury in adulthood has been documented in a surgical incision model (Walker et al., 2009a). Early immune stress can also affect future pain processing in rats. When young rats are exposed to intraperitoneal lipopolysaccharide (LPS), persistent changes are reported in centrally mediated inflammatory responses and increased sensitivity to mechanical and thermal stimuli as adults (Boissé et al., 2004; Boissé et al., 2005).

The experience of pain in early life has also been explored in the context of visceral hypersensitivity which, in adults, is typified by irritable bowel syndrome a condition whose aetiology remains unknown. Al-Chaer and colleagues demonstrated long-lasting hypersensitivity in a rat model following neonatal mechanical or chemical colonic irritation in the absence of permanent tissue damage (Al-Chaer et al., 2000). Animals whose colon was repeatedly mechanically stimulated in the neonatal period remained more sensitive to colon distension than non-stimulated controls up to 3 months following the initial stimulation (Al-

Chaer et al., 2000). This sensitivity was associated with increased background firing in primary afferent neurones coupled with lower thresholds to mechanical stimulation up to 3 months after the initial pain (Lin and Al-Chaer, 2003). Interestingly, somatic stimuli can also affect visceral pain processing. The enhanced hyperalgesic response in adulthood following mild hind paw inflammation in the neonatal period extends both to somatic and visceral sites (Wang et al., 2004). A summary of the findings of multiple studies into the long-term effects on sensory processing of early pain or injury are summarised in Figure 1-5.

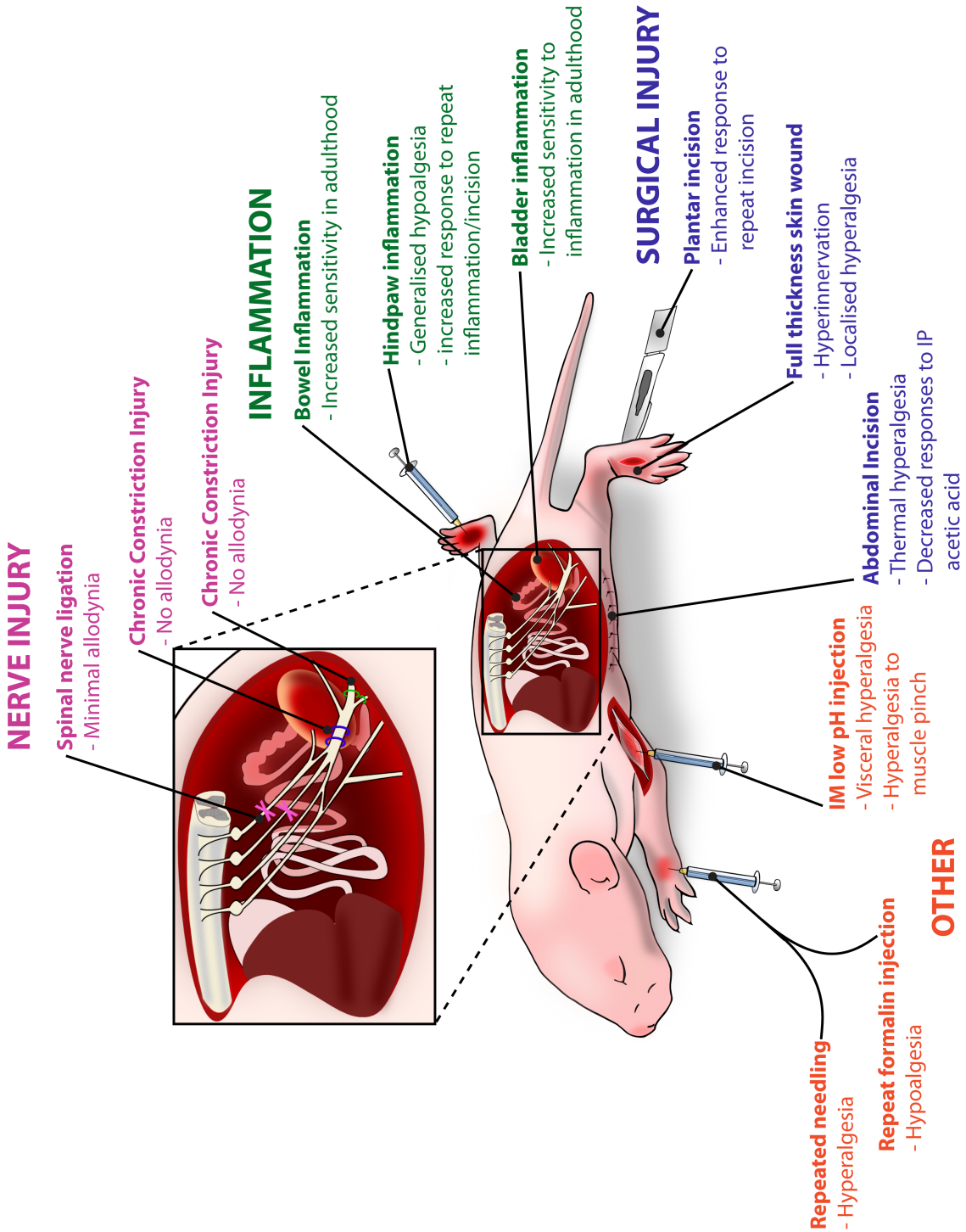


Figure 1-5 The effects of early injury, inflammation and nerve damage
Many studies have investigated the effects of early injury on nociceptive processing later in life. They are summarised above. Nerve injury in the neonatal period is associated with little allodynia (Vega-Avelaira et al., 2012). Bladder or bowel inflammation in the first 3 postnatal weeks results in increased sensitivity to visceral stimulation in adulthood (Al-Chaer et al., 2000; Randich et al., 2006), and hind paw inflammation results in long-term reorganisation of afferent terminals in the dorsal horn, baseline hypoalgesia and enhanced responses to repeat inflammation (Ruda et al., 2000; Tachibana et al., 2001; Ren et al., 2004). Surgical injuries such as full thickness skin wounding result in long-lasting changes in innervation of the wound as well as localised sensitivity (Moss et al., 2005; Beggs et al., 2012b). Early plantar incision results in more prolonged and profound sensitivity following repeat injury in adulthood (Beggs et al., 2012a). Laparotomy in neonatal mice results in reduced sensitivity to adulthood visceral and thermal stimuli (Sternberg et al., 2005). Daily injection of low-pH saline in the first 3 postnatal weeks results in enhanced responses to mechanical stimulation of the muscle and viscera (Miranda et al., 2006). Repeated needling in the neonatal period results in increased sensitivity in adulthood (Anand et al., 1999). **IM** = intramuscular.

1.10 The clinical profile of juvenile arthritis

Chronic musculoskeletal pain is common and affects between 4-40% of people in childhood or adolescence especially girls (Goodman and McGrath, 1991; King et al., 2011). Despite the suffering of children and adolescents the processing of pain in childhood and adolescence is poorly understood and managed (Walker, 2008). This section will briefly outline the extent and nature of chronic joint pain in childhood, as well as the treatment strategies commonly employed to control it.

The International League of Associations for Rheumatology (ILAR) classification delineates homogenous groups of patients with JIA that have mutually exclusive clinical and laboratory features, the details of which can be found in Table 1-2 (Petty et al., 2004).

Subtype	Definition	Details
Systemic Arthritis	Arthritis in one or more joints with or preceded by fever of at least 2 weeks duration, that is documented for 3 consecutive days.	One of the following: 1) Non-fixed erythematous rash 2) Generalised lymph node enlargement 3) Hepatomegaly and/or spenomegaly 4) Serositis
Oligoarthritis	Arthritis affecting one to four joints during the first 6 months of disease.	Can be either: **Persistent** (affecting not more than four joints throughout the disease course), or **Extended** (affecting a total of more than four joints after the first six months of disease).
Polyarthritis (RF –ve)	Affecting five or more joints during the first six months of disease	Tests for RF are negative.
Polyarthritis (RF +ve)	Affecting five or more joints during the first six months of disease	Two or more tests for RF are positive, at least 3 months apart during the first six months of disease.
Psoriatic Arthritis	Arthritis and psoriasis	Or arthritis and at least 2 of the following: 1)Dactylitis 2) Nail pitting or onycholysis 3) Psoriasis in a first-degree relative
Enthesitis Related Arthritis	Arthritis and enthesitis	Or arthritis or enthesitis with at least two of the following: 1) The presence of, or a history of sacroiliac joint tenderness and/or inflammatory lumbosacral pain 2) The prescence of HLA-B27 antigen 3) Onset in male over six years of age 4) Acute (symptomatic) anterior uveitis 5) History of ankylosing spondylitis, enthesitis related arthritis, sacroiliitis with inflammatory bowel disease. Reiter's syndrome or Acute anterior uveitis in a first-degree relative.
Undifferentiated Arthritis	Arthritis that fulfils criteria in no category or in two or more of the above categories.	None.

Table 1-2 ILAR classification of JIA

This table shows the ILAR classification of JIA (as set down in Petty et al., 2004).

Juvenile idiopathic arthritis (JIA) is an umbrella term which refers to chronic inflammatory arthritis of unknown cause in children. It is important to remember that there are numerous types of inflammatory joint disease in childhood for which a cause is established. These cases would not fall into the JIA category. A diagnosis of JIA is made in the presence of persistent joint swelling following the exclusion of other important differential diagnoses. The cause of JIA remains unknown, although there is evidence that certain subtypes of the disease are associated with the expression of different human leucocyte antigen (HLA) classes (Thomson et al., 2002).

What is important here is that joint inflammation in JIA and other arthritic disease of childhood result in pain and disability, the mechanisms of which are poorly understood.

In a recent large multicentre cohort study in the United States, the median age of onset of inflammation was 4.7 years though some infants developed inflammation when they were as young as 4 months old (Oen et al., 2002). Due to the heterogeneity of the condition, varying nomenclature and diagnostic criteria, the true burden of juvenile arthritic disease is difficult to establish (Helmick et al., 2008). However, it has been estimated that 10 in 100,000 children develop inflammatory arthritis a year and a large proportion of those are diagnosed as having JIA (Oen et al., 2003). A major cause of suffering in the disease is pain, and indeed it contributes significantly to the morbidity of this condition when assessed by various disability scores. Pain from affected joints causes sleep disturbance, limits normal activities, disrupts school attendance and results in considerable psychosocial stress (Kimura and Walco, 2007). Ongoing disease and biomechanical pathology contribute to the long-term effects of the condition, which can persist into adulthood (Oen et al., 2002). Interestingly, pain appears to be a more important factor in disability than the progression of the disease itself and just a small decrease in pain intensity has been shown to have an appreciable effect on these children's well-being (Wolfe, 2000a; Dhanani et al., 2002).

Early studies have highlighted some important and interesting features of pain in JIA. Children with chronic inflammatory arthritis have lower pain thresholds to pressure at the joint capsules of the wrists, elbows, knees, ankles and in paravertebral soft tissues than their healthy peers. This is true of both children with active inflammation and children without detectable inflammation, although the former showed significantly lower thresholds (Hogeweg, et al., 1995a; Hogeweg et al., 1995b). The enhanced sensitivity to noxious stimuli in all measured body areas suggesting that it is not just the nociceptors in and around the affected joints that are sensitized but that changes in central pain processing have occurred in this condition. The fact that the increased pain sensitivity persists even when the inflammation is resolved further suggests that the central sensitization is maintained in the CNS of these children after periods of active disease (Hogeweg, et al., 1995a; Hogeweg et al., 1995b).

In one of a limited number of studies into the long-term effects of juvenile arthritis, of 246 patients with a mean disease duration of 28.3 years, 32.9% percent had severe pain (over 50 on a visual analogue scale of 1-100) whilst 22.8% felt that their pain was poorly controlled (Packham et al., 2002). Interestingly, pain was not related to disease activity. Patients in this study also report higher persistent pain than children (Packham et al., 2002). A disconnect between disease severity and pain is also seen in paediatric patients, where the degree of inflammation accounts for between 1-10% in the variability of pain ratings (Ilowite et al., 1992; Schanberg et al., 1997).

1.11 Aims of thesis

The aims of the experiments were as follows,

I. To characterise the development of joint evoked flexion withdrawal reflexes.
II. To investigate the effect of postnatal age on the processing of inflammatory joint pain.
III. To understand the effects of joint inflammation in early life on nociceptive processing in adulthood as well as the effect of early joint inflammation on re-inflammation in adulthood.

Chapter 2 Postnatal development of nociceptive hindlimb flexion reflex muscle activity evoked from the hind paw and ankle joints of naive rats

2.1 Introduction

The study of nociceptive reflexes both in the adult and developing nervous system has largely focused on those evoked from cutaneous structures. Stimulation of skin in the noxious range evokes a reflex contraction of specific groups of muscles, demonstrated in classical physiological studies by Sherrington in the cat and has since been extended to rats (Swett and Woolf, 1983) and man (Willer et al., 1978). Noxious mechanical, thermal and chemical stimuli applied to the skin of the rat hind paw readily evoke reflexes *in vivo* (Fitzgerald and Gibson, 1984; Holmberg and Schouenborg, 1996b). However, reflex responses evoked by noxious stimulation of joint mechanoreceptors and nociceptors are less well understood. Investigators have examined the sensory afferent outflow of joints in several ways, notably from joint nerve preparations in anaesthetised rodents. This technique records action potentials originating from articular nerves (typically from the knee) following various stimuli including innocuous and noxious flexion or rotation of the joint as well as direct mechanical and electrical stimuli (Schaible and Schmidt, 1985a; McDougall et al., 2009; Richter et al., 2010). Other studies have explored the properties of DH and trigeminal complex neurones that receive joint afferent inputs suggesting the involvement of these neurones in the detection of acute articular pain, and in the hyperalgesia and poor localization, spread, and referral of pain that characterize many painful conditions of joints and other deep structures (Sessle & Hu, 1991). However, noxious inputs from joints also evoke strong muscle reflexes and yet little is known about nociceptive reflex physiology in relation to noxious joint stimulation.

In addition all existing studies have been in the adult and even less is known about these reflexes in relation to joint input.

This chapter is concerned with the normal development of nociceptive reflexes evoked by ankle joint and cutaneous hind paw stimulation. The following section will review studies on the maturation of withdrawal reflexes and compare and contrast the reflex physiology of the young and adult nervous systems.

2.1.1 The flexion withdrawal reflex

The FWR is a polysynaptic multi-segmental reflex that serves to withdraw a limb away from a noxious or potentially tissue damaging stimulus. This reflex, which has been widely studied both in animal and in man, provides a window for investigators to examine the function of spinal sensorimotor circuits. Using spinal and decerebrate adult cats C.S. Sherrington was one of first to describe this phenomenon systematically (Sherrington, 1910). In this study he made some key observations on the genesis, nature and coordination of reflexes. Sherrington noted that the receptive fields of limb withdrawal reflexes, defined as the cutaneous area that, when stimulated, evoke a reflex, were frequently large and could be elicited from the whole limb but were most readily evoked from areas on the paws, more specifically, the toes. He observed that these movements are often accompanied by extension of the non-stimulated limb, thus introducing the notion of a crossed flexion reflex. The most pertinent finding of his investigation for subsequent investigation of nociceptive processing in the spinal cord was that noxious stimuli most reliably evoked reflex withdrawal (Sherrington, 1910).

In young and infant humans and animals cutaneous reflex circuitry is immature and undergoes substantial refinement over the postnatal period (Andrews and Fitzgerald, 1994, 1999; Fitzgerald, 2005). Cutaneous flexion reflexes in the newborn rat, kitten, and human are exaggerated compared with the adult (Ekholm, 1967b; Issler and Stephens, 1983; Fitzgerald and Gibson, 1984; Andrews and Fitzgerald, 1994; Holmberg and Schouenborg, 1996a). Additionally, thresholds to cutaneous mechanical stimulation are lower and the reflex contractions more synchronized and long lasting (Marsh et al., 1999a).

2.1.2 Spinal flexion reflexes and receptive fields in young and adult mammals

Receptive fields in infant humans and rodents alike undergo substantial postnatal reorganisation. In premature infants of 27 weeks post-conceptional age (PCA) a reflex withdrawal of the lower limb can be evoked by stimulation of areas up to and including the thigh and buttock (Andrews and Fitzgerald, 1994). Furthermore, at this age the sensitivity of this receptive field is uniform, that is to say, the likelihood of evoking a reflex is the same across the sensitive area. With

increasing PCA the sensitivity becomes progressively more localised to the skin covering the toes and progressively less sensitive further up the limb (Andrews and Fitzgerald, 1994).

The work of Schouenborg and colleagues has led to a greater understanding of how sensorimotor systems develop, are organised and allow for coordinated movement (reviewed in (Schouenborg, 2004)). Holmberg and Schouenborg, using behavioural and EMG measures, have characterised the development of withdrawal reflexes in the developing rat nervous system (Holmberg and Schouenborg, 1996b). In the youngest animals (postnatal day (P) 1 to 3) reflexes are typified by long latency and inappropriate reflex movements that are evoked from variable cutaneous receptive fields that have multiple foci. They found no clear spatial organisation of receptive fields within the first postnatal days and reflexes often failed to withdraw the limb from the stimulus. By P20-P25 receptive fields show a similarity with those found in adults. Concurrent with the development of receptive fields the early postnatal period also sees a substantial increase in the mechanical threshold required to evoke a reflex, a finding that has been corroborated by human studies (Andrews and Fitzgerald, 1994, 1999).

Adult (P60-80) reflexes, by contrast, are localised and effectively withdraw the stimulated limb from potential harm. This phenomenon is a function of a so-called input-output relationship between the stimulated skin and the movement that contraction of the muscle evokes (Schouenborg and Kalliomäki, 1990; Schouenborg and Weng, 1994). For instance, noxious stimulation of the toes will evoke a contraction in the biceps-femoris-posterior (BF), which, due to the action of the muscle across the knee joint will remove the toes away from the noxious stimulus (Schouenborg and Kalliomäki, 1990). The idea of a specific input-output relationship in nociceptive reflex organisation was developed further in subsequent investigations (Schouenborg et al., 1995) which provided evidence for a 'musculotopic' organisation of reflex circuitry that lies across lamina V of the DH in the lumbar spinal cord segments 4 and 5 (L4 and L5). In this area, DH interneurones, receiving convergent polysynaptic A and C-fibre inputs coordinate the spatial outputs to ventral horn motor neurone pools (Schouenborg and Sjölund, 1983). This subset of neurones in the deep DH, termed reflex encoders (Re), is activated by both innocuous mechanical and nociceptive inputs and tend

to have high thresholds. The relative strength of an input to a Re neurone is determined by the location within the receptive field from which the input is derived. Thus, inputs at the centre of a receptive field form a larger contribution to Re inputs than inputs found originating from its periphery. In the context of development, it is hypothesised that spontaneous bursting within these neurones may contribute to the refinement of immature reflex circuitry (Schouenborg, 2002). It follows that the balance of excitatory and inhibitory input to Re neurones, both in the early postnatal period and in adulthood, will determine whether or not a reflex is evoked.

In order to understand the maturation of spinal nociceptive reflexes evoked by noxious joint stimulation it is important to understand how these reflexes develop over the postnatal period and how they may differ from cutaneous nociceptive reflexes. To date there are no such data available.

2.2 Aim of the experiments

The primary aim of this Chapter was to study the postnatal development of the hindlimb flexion reflex in rats using electromyographic recordings (EMG) from the biceps femoris in response to noxious stimulation of the ankle joint and hind paw. The key objectives were:

I. To establish a reliable, repeatable and quantitative method of recording flexor reflex activity in response to noxious mechanical ankle joint and hind paw stimulation in rats of different postnatal ages.
II. To reliably record simultaneous bilateral EMG activity from the biceps femoris muscles from both hindlimbs in postnatal rats.
III. To provide a quantitative analysis of the changing postnatal properties in naive healthy rodents.

2.3 Methods

2.3.1 Animals

Male Sprague-Dawley rats were obtained from the UCL Biological Services Unit where they had been bred on site. All animal procedures and experimenters were licensed by the UK Home Office and performed in accordance with the Animals (Scientific Procedures) Act 1986. As part of this process, the relevant authorities granted ethical approval for the work. Rats were housed with their littermates and mother up until weaning at P21. Animals older than P21 were housed in cages of six age-matched animals with free access to food and water. The room in which animals were housed had a 12 h–12 h light–dark cycle. In total, 47 Sprague-Dawley rats were used for the data presented in this section. The breakdown by age is shown in Table 2–1. In addition to the animals detailed in the table, two animals (P21) provided data for section 2.5.1.1.

Postnatal Age	**9**	**12**	**18**	**22**	**23**	**31**	**41**
n	5	10	6	6	6	6	6

Table 2–1 Ages and numbers of animals used to characterise the development of naïve flexion reflex

2.3.2 EMG recording

EMG recordings from the BF muscle were performed as previously described (Hathway and Fitzgerald, 2006) with some modifications. Briefly, animals were anaesthetised with isoflurane (2-4% (vol/vol) in medical O_2) and an endotracheal cannula inserted for artificial ventilation during the experiment. A bipolar concentric needle electrode was then inserted into the posterior part of the biceps femoris muscle of both hind limbs through a small, 3mm, incision in the overlying skin. Both hind paws were secured to fixed platforms and held in slight extension and plantar flexion. Isoflurane anaesthesia was then reduced to 1.1% (MSS Isoflurane Vaporiser, Harvard Apparatus, UK) and then left to equilibrate for at least 10 minutes prior to recording. The sampling rate was 2000Hz and signals were amplified by using a head-stage amplifier (NL100; Neurolog, Digitimer, Digitimer Ltd, Welwyn Garden City, UK), pre-amplified and filtered (NL104, NL125). The signal was fed to an analogue-to-digital signal converter into MacLab

software (PowerLab 4S; AD Instruments, Castle Hill, Australia). Data were saved for further offline analysis.

2.3.3 Stimulation protocol

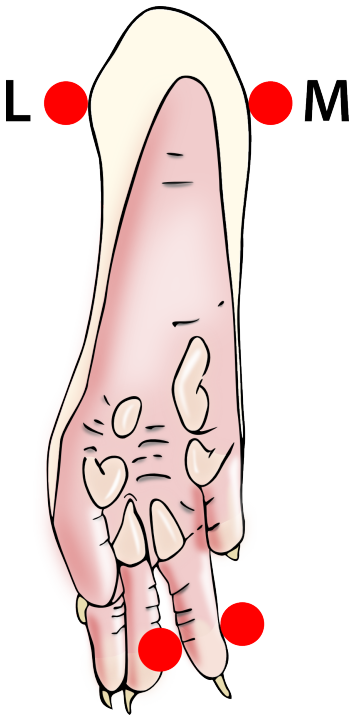


Figure 2-1 Pinch Sites
Red dots indicate where pinch forceps were placed. **M** – medial, **L** - lateral

Pinch was used in all experiments. This consisted of the application of pair of forceps across the ankle joint, thus including the subcutaneous tissue of the medial and lateral ankle (see Figure 1-1). This was compared to the pinch across the toe (Figure 1-1). These stimuli were chosen as they closely reflected the way in which joint pain is assessed clinically. Repeated noxious input from the periphery is known to cause sensitisation, which is an increased response to subsequent stimuli and so a protocol was introduced to minimise this effect. As these experiments required repeated pinching of the hind paw it was decided to devise a pinch protocol that would reduce the effect of sensitisation. Responses to pinch were time locked to the beginning of the pinch stimulus in post-processing in Matlab. Strain gauges mounted on the arms of the forceps provided a constant readout of force applied. When not pinching, strain gauges read '0'. Beginning of the stimulus was considered as the point when the strain gauge read above zero. Onset was confirmed visually once the automatic detection code had run.

The side at which the pinch was started was alternated, such that animals received a series of three pinch stimuli in the following order: either, right ankle (RA), left ankle (LA), right toe (RT) and left toe (LT) or LA, RA, LT, RT. In each experimental group half the animals were stimulated first on the right ankle and half on the left ankle. The series of stimulation consisted of three pinches of 0.5s duration at 10s intervals.

2.3.4 EMG analysis

Saved raw EMG data was exported to Matlab software (Mathworks, Cambridge, UK). The onset of pinch was used to mark the beginning of the responses. For

each response, data from 1s pre-stimulus and 5s post-stimulus was extracted and saved. The root mean squared (RMS) was then calculated for each of the three EMG responses to pinch. The average of these three responses was then used as the EMG response from that site.

2.4 Statistical analysis

The EMG RMS data was divided into 250ms time-bins for graphical representation and for statistical analysis. Data were plotted in these time-bins from 1s pre-stimulus and 5s post-stimulus. Significance testing was performed as follows:

EMG comparisons were made using a 2-way analysis of variance (ANOVA) with a Bonferroni post-hoc test to compare differences at individual time-points (250ms time bin). These were performed to establish differences between left and right-sided responses.

2.5 Results

2.5.1 General properties of rat hindlimb nociceptive flexion reflex activity

2.5.1.1 Establishing optimal hind paw receptive field sites

In this study biceps femoris EMG activity was recorded in naive animals in response to pinch of the ankle joint and the toes. Pilot experiments were undertaken to map the hind paw cutaneous receptive field of the biceps femoris muscle and establish which areas when pinched, reliably evoke the most robust flexion reflex. To do this the hind paw was divided into 13 regions as shown in Figure 2-2a.

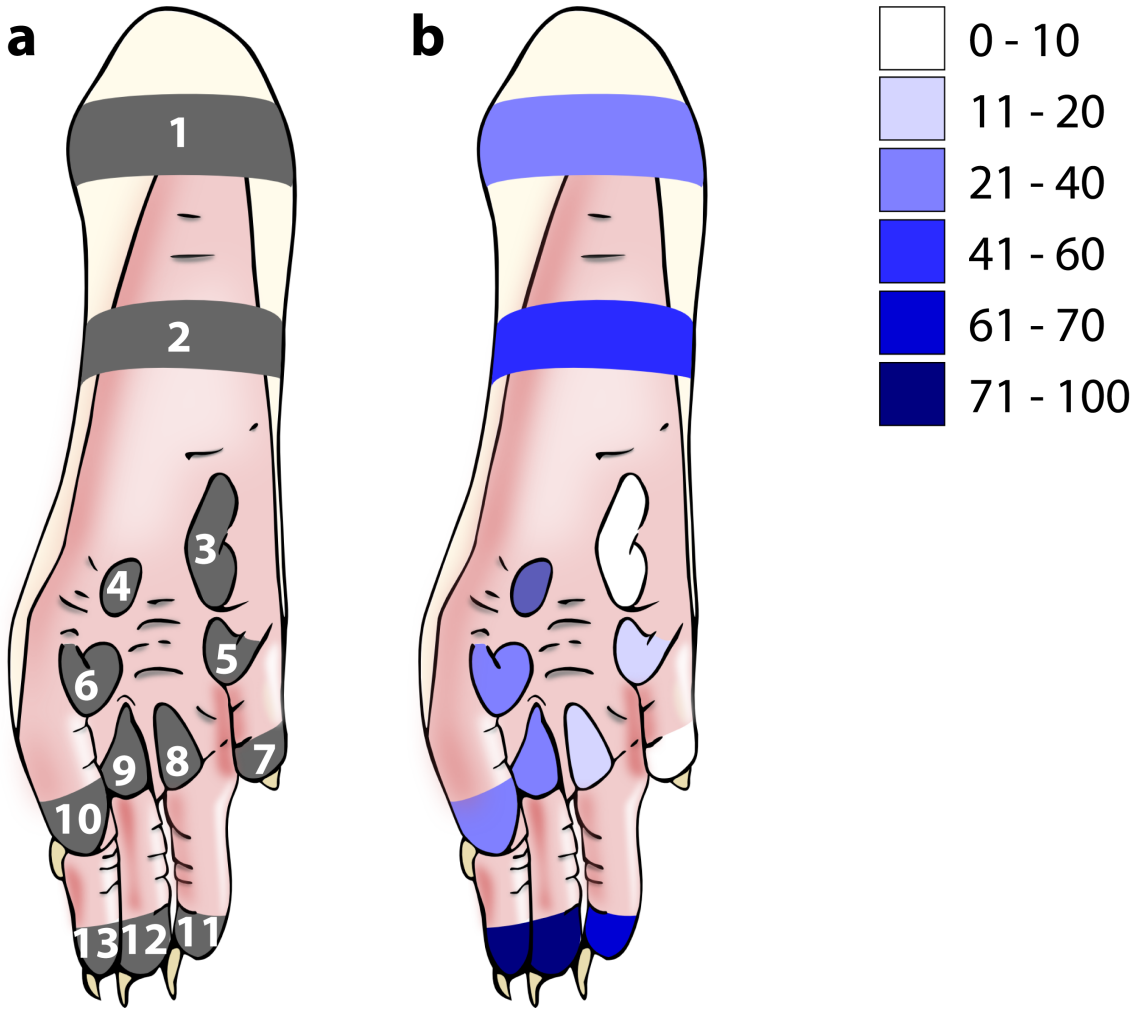


Figure 2-2 Schematic representation of the rat hind paw showing pinch sites
(a) 1 – across the ankle joint, 2 across the midfoot, 3-6, 8 and 9 represent skin protrusions, 7 and 10-13 across the distal tarsal-metatarsal joint. (b) Flexor reflex activity generated from the 13 sites is represented as a percentage of the maximal evoked activity. A representation of activity generated in the biceps femoris muscle following pinch at that site (n=3, P21). Different colours represent activity at a given site as a percentage of the largest response.

Figure 2-2b illustrates the relative sensitivity of each area expressed as a percentage of the maximum reflex activity from any of the 13 sites in a given animal. While a strong reflex was readily evoked from all of the distal toes, toes 2 and 3 (11 and 12) were the most sensitive. Thus it was decided that toe 2 and the ankle would be stimulated to evoke the reflex throughout this study. Pinch of the ankle joint also evoked robust responses in all animals.

2.5.1.2 EMG activity evoked by noxious hind paw stimulation in naïve animals

Flexion reflex activity was evoked by pinching the toe and the ankle in naive animals at P9, P22 and P41. Figure 2-3a,b show raw recordings of ipsilateral biceps femoris EMG activity evoked in naïve animals by pinching the ankle and toe, respectively, at the three ages. Individual reflex responses varied, particularly in younger animals, but at postnatal day P9, evoked EMG activity was typified by large amplitude, prolonged responses, lasting over 4s. With increasing postnatal age the pattern of response became briefer, with the maximal reflex amplitude occurring in the first second post stimulation. By P40 the reflex was markedly reduced in amplitude and duration compared to younger ages.

Flexion reflex EMG responses were recorded simultaneously from the biceps femoris muscles of both hind limbs throughout this study. Simultaneous recording from the biceps femoris on both sides revealed a contralateral as well as an ipsilateral flexion reflex response (Figure 2-3). A contralateral reflex was more commonly recorded in P9 animals and decreased in size and incidence with age.

Since one of the aims of the work presented in this thesis was to compare inflamed and non-inflamed ankles, it is essential to establish that there is no laterality in the flexion reflex at each age. For this reason much of the data presented in this chapter is presented with the left and the right side responses separately.

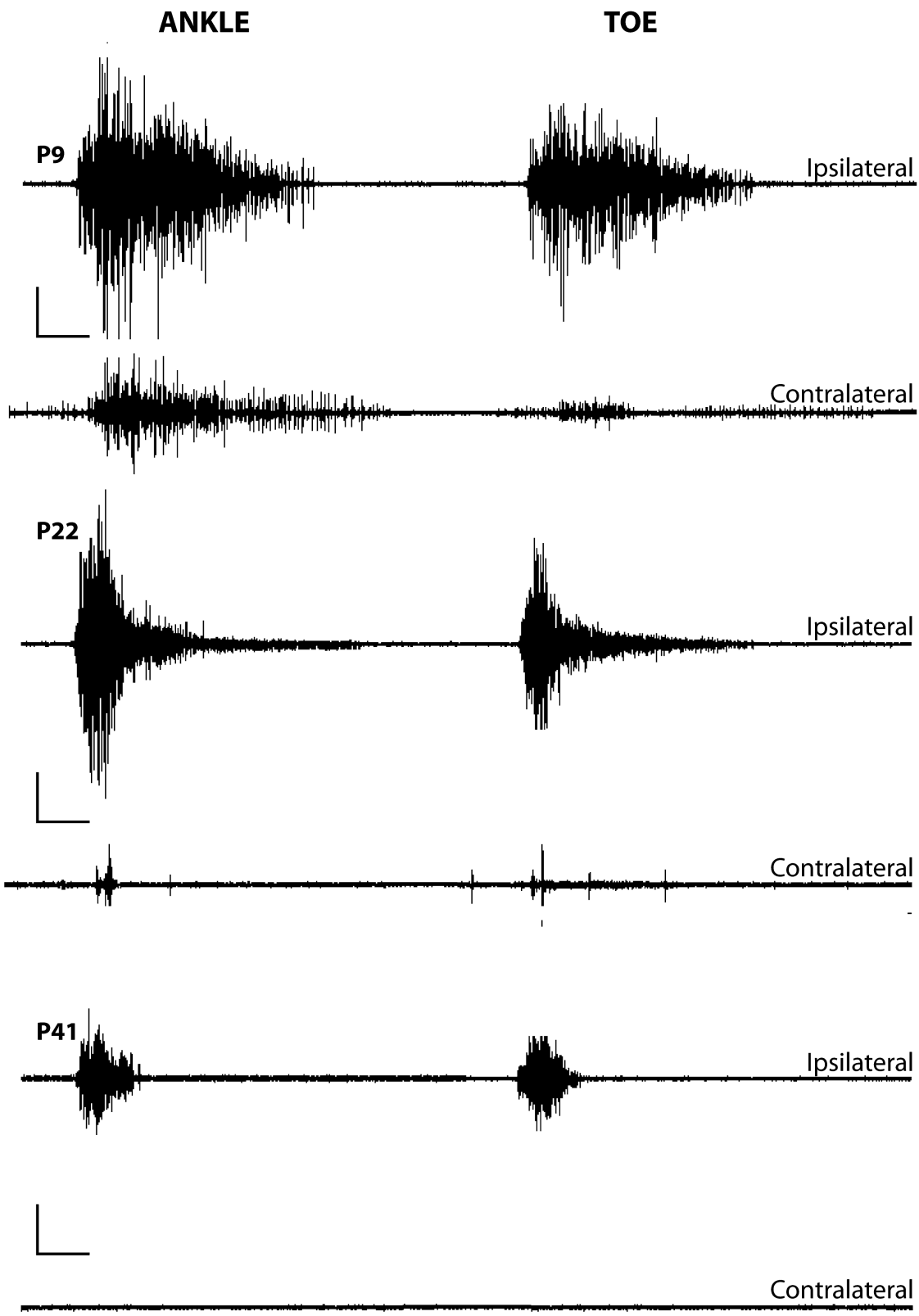


Figure 2-3 Representative EMG responses from P9, P22 and P41 naïve animals
Raw recordings from a single animal at each age from the biceps femoris ipsilateral and contralateral to the pinched ankle and toe. Vertical scale bar = 5mV, horizontal scale bar = 1s.

2.5.2 The pattern of noxious evoked flexion withdrawal reflex activity in naive animals at P9, P22 and P41

The mean ipsilateral and contralateral flexor reflex activity evoked by noxious pinch of the toe and ankle were plotted in naive animals at P9, P22 and P41. RMS of EMG activity was calculated in 250 ms time bins over 5s for each animal, where 0s represents the onset of the noxious stimulus. Left and right limbs are plotted separately to demonstrate absence of laterality in the developing reflex. Error bars represent standard error of the mean (SEM).

2.5.2.1 Postnatal Day 9

Figure 2-4 shows the mean ipsilateral flexion reflex EMG response at P9 (n=5) to pinch of the ankle (a) and toe (b). Pinch of the ankle and the toe evoked a large, 8-10 fold increase in EMG over baseline, which remains above baseline for at least 4s. No significant difference was observed in ipsilateral activity evoked from the left and the right foot.

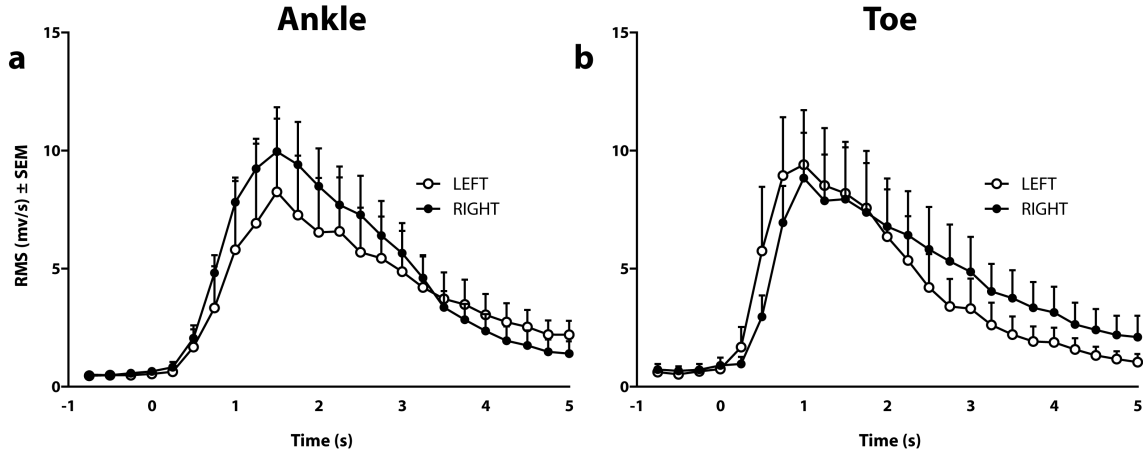


Figure 2-4 Ipsilateral EMG responses from naïve P9 animals following pinch at the ankle and toe
Data shown here represent the average responses evoked from pinch of **(a)** the ankle and **(b)** the toe in 5 P9 animals. Data is presented from both the left and right side to check for laterality. Time 0 on the x-axis indicates the onset of the noxious pinch. Activity is shown in time bins of 250ms.

In contrast to the ipsilateral reflex, contralateral reflexes were not observed in all P9 animals and this variability is reflected in Figure 2-5. When present (in 3 of 5 animals), mean contralateral responses evoked by pinch of the ankle and the toe are smaller than the ipsilateral response (note scale in Figure 2-5). The rate of rise in both ipsilateral and contralateral responses from the toe appeared to be greater than from the ankle although this was not tested for significance here.

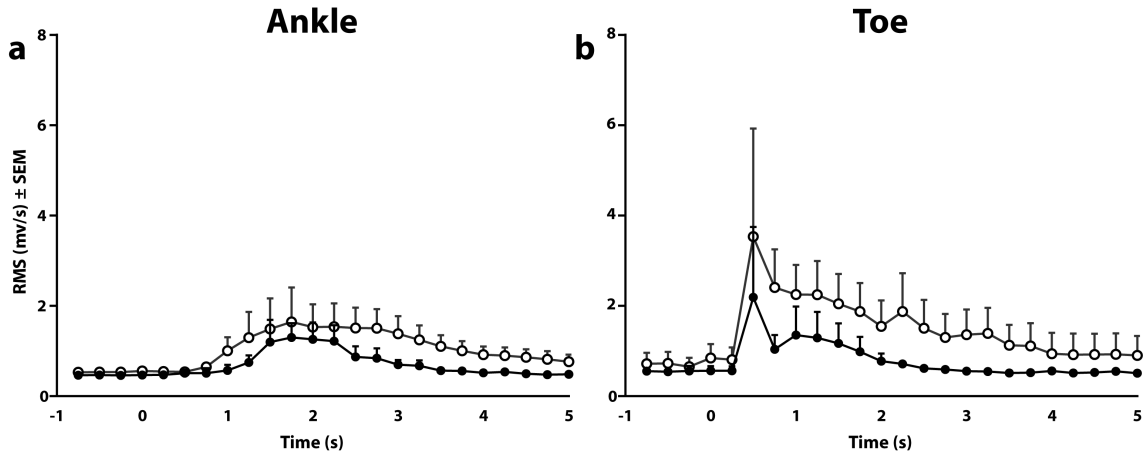


Figure 2-5 Contralateral EMG responses following pinch at the ipsilateral ankle and toe
Data shown here represent the average responses in the contralateral muscle evoked from pinch from **(a)** the ankle and **(b)** the toe in 5 P9 animals. White circles represent the activity evoked in the left limb by pinching the right hind paw, and vice versa. Data is presented from both left and right side to check for laterality. Time 0 on the x-axis indicates the onset of the noxious pinch. Activity is shown in time bins of 250ms.

2.5.2.2 Postnatal Day 22

Figure 2-6 shows the mean ipsilateral flexion reflex EMG response at P22 (n=6) to pinch of the ankle (a) and toe (b). Pinch of the ankle and the toe evokes a large, 8-10 fold increase in EMG over baseline, which remains above baseline for at least 1.5s. No significant difference was observed in ipsilateral activity evoked from the left and the right foot.

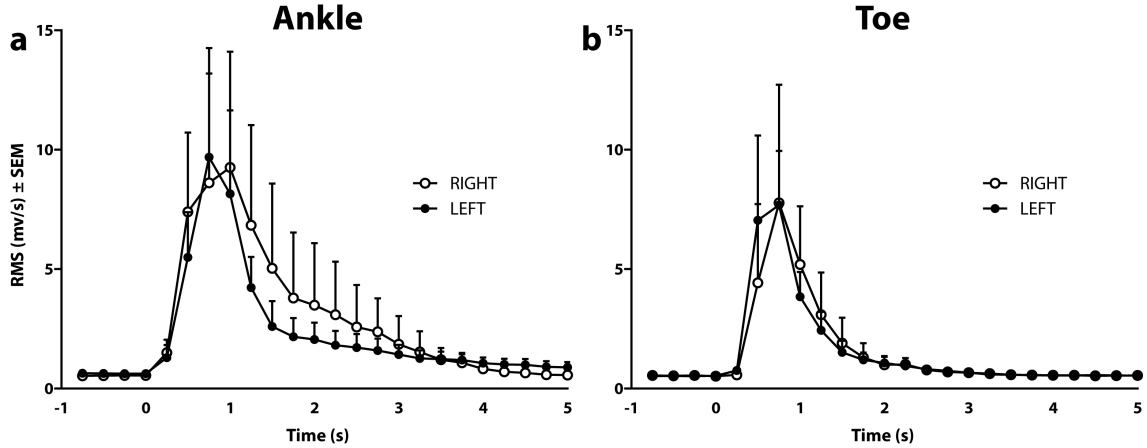


Figure 2-6 Ipsilateral EMG responses from naïve P22 animals following pinch at the ankle and toe
Data shown here represent the average responses evoked from pinch of **(a)** the ankle and **(b)** the toe in 6 P22 animals. Data is presented from both left and right side to check for laterality. Time 0 on the x-axis indicates the onset of the noxious pinch. Activity is shown in time bins of 250ms.

As at P9 and in contrast to the ipsilateral reflex, contralateral reflexes were not observed in all P22 animals. When present (in 2 of 6 animals) mean contralateral responses evoked by pinch of the ankle and the toe are smaller than the ipsilateral response (Figure 2-7). Figure 2-7 shows that the contralateral response from the toe is notably smaller than that from the ankle.

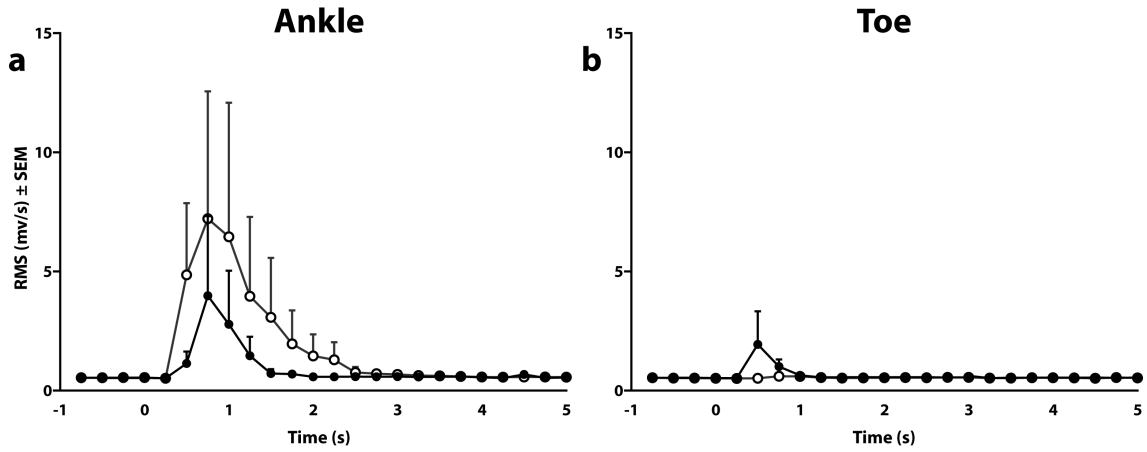


Figure 2-7 Contralateral EMG responses following pinch at the ipsilateral ankle and toe
Data shown here represent the average responses in the contralateral muscle evoked from pinch from **(a)** the ankle and **(b)** the toe in 6 P22 animals. White circles represent the activity evoked in the left limb by pinching the right hind paw, and vice versa. Data is presented from both left and right side to check for laterality. Time 0 on the x-axis indicates the onset of the noxious pinch. Activity is shown in time bins of 250ms.

2.5.2.3 Postnatal Day 41

Figure 2-8 shows the mean ipsilateral flexion reflex EMG response at P41 (n=6) to pinch of the ankle (a) and toe (b). Pinch of the ankle and the toe evokes a large, 5-8 fold increase in EMG over baseline, which remains above baseline for at least 1s. No significant difference was observed in ipsilateral activity evoked from the left and the right foot.

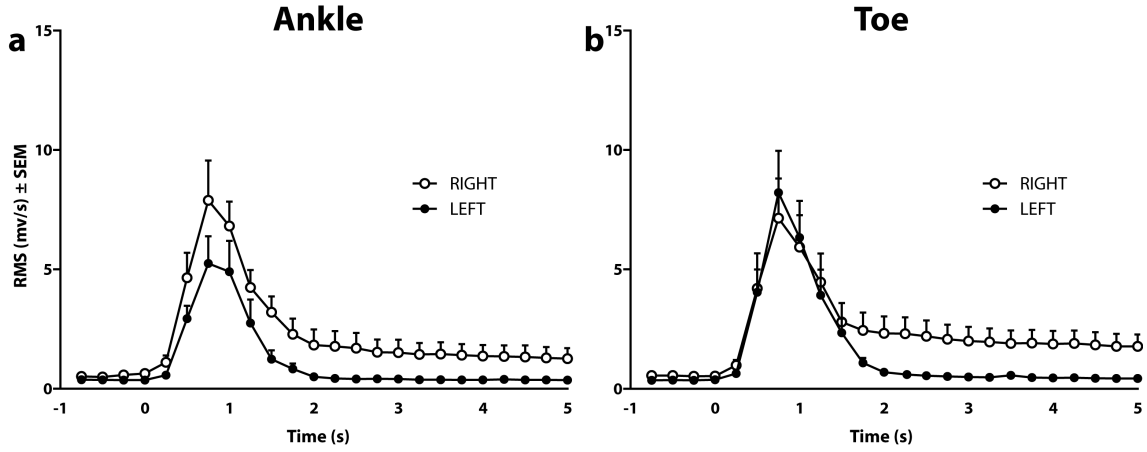


Figure 2-8 Ipsilateral EMG responses from naïve P41 animals following pinch at the ankle and toe
Data shown here represent the average responses evoked from pinch of **(a)** the ankle and **(b)** the toe in 6 P41 animals. Data is presented from both left and right side to check for laterality. Time 0 on the x-axis indicates the onset of the noxious pinch. Activity is shown in time bins of 250ms.

2.5.3 Postnatal development of rat hindlimb nociceptive flexion reflex activity

The data suggest that the hindlimb nociceptive flexion reflex is strongly developmentally regulated between the P9 and P22 leading to a marked decrease in reflex excitability over this period. To establish more precisely when this change takes place, the pattern of reflex activity was studied across six ages in total: P12, P18, P22, P30 and P41. Figure 2-9 shows the mean responses to pinch from animals aged P9 through to P41. Inspection of the data shows that there is a clear fall in amplitude and duration over the period in question. At P9 reflexes are prolonged compared to older animals. At P12 reflexes begin to shorten and notably it is the rate of increase in EMG activity that increases as postnatal age increases. This continues until P22 at which reflexes are of the greatest amplitude and appear to have a more adult-like appearance. The pattern of reflex refinement continues to progress between P22 and P41 as the peak amplitude of responses falls with increasing postnatal age.

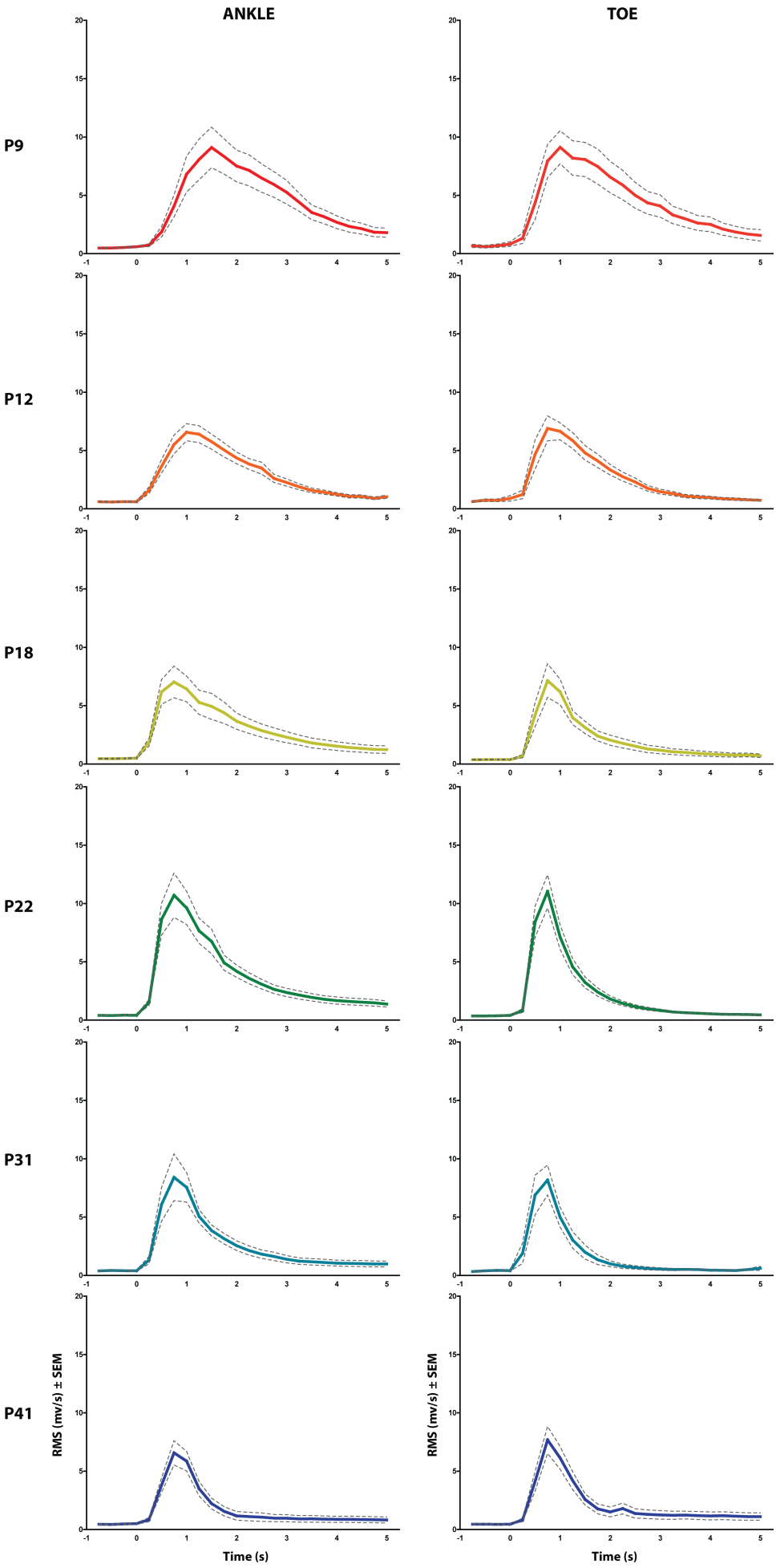

ANKLE
TOE
P9
P12
P18
P22
P31
P41
RMS (mv/s) ± SEM
Time (s)

Figure 2-9 Normal developmental profile of EMG responses to pinch (i)
Plots on the **previous page** show pooled (left and right sides) responses from naïve animals aged P9 (n=5), P12 (n=6), P18 (n=6), P22 (n=6), P31 (n=6) and P41 (n=6). These plots illustrate the general developmental patterns that are observed over the postnatal period. For all graphs the middle line indicates the mean EMG response, either side of the central line the SEM is shown in grey.

To test the statistical significance of these observed age differences the EMG responses to pinch from animals aged P9, P12, P18, P22, P31 and P41 were compared in a 2-way ANOVA. The responses evoked from the ankle and toe were considered separately. Tables 2-2 and 2-3 provide an overall summary of the significant differences that arose from these comparisons. It should be noted, however, that the exact post-stimulus time-bin at which significant differences exist varied according to the comparison made.

VS.	P12	P18	P22	P31	P40
P9	Yes	Yes	Yes	Yes	Yes
P12		No	Yes	No	Yes
P18			Yes	No	No
P22				No	Yes
P31					No

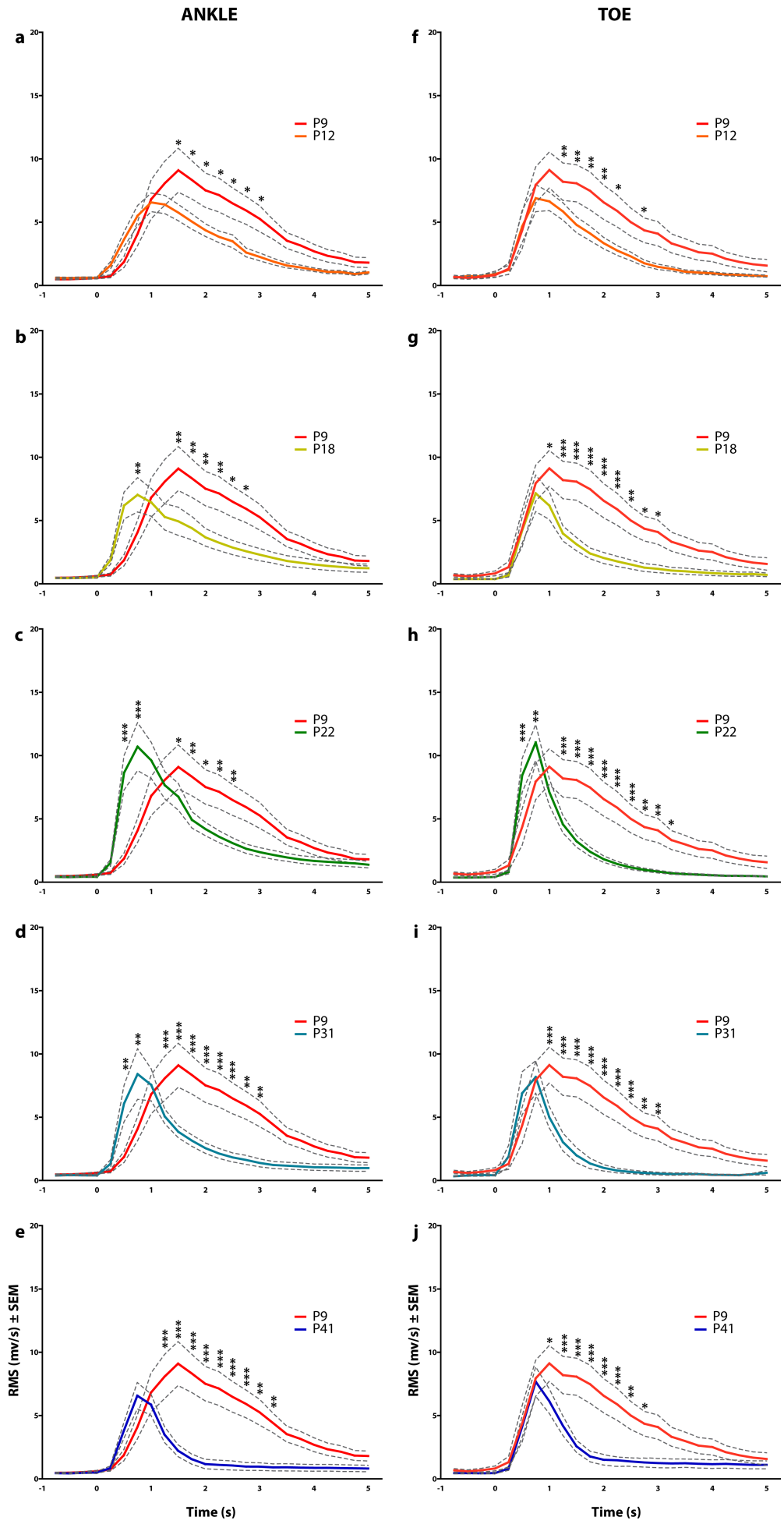
Table 2–2 Results of 2-way ANOVA comparing responses of all ages to each other at the ankle
Results of post-hoc (Bonferroni) tests between the responses to pinch at different postnatal ages. Yes indicates that significant differences exist between the responses between the ages compared. These results are represented graphically in Figure 2-10 (P9 vs. all other ages) and Figure 2-11 (P22 vs. all other ages).

VS.	P12	P18	P22	P31	P40
P9	Yes	Yes	Yes	Yes	Yes
P12		No	Yes	Yes	No
P18			Yes	No	No
P22				No	Yes
P31					No

Table 2–3 Results of 2-way ANOVA comparing responses of all ages to each other at the toe
See caption above for detail

Two examples have been chosen below to illustrate these comparisons between P9 and all other ages and P22 and all other ages are shown in Figures 2-10 and 2-11 respectively (on pages 65 and 66). Figure 2-10 illustrates the significantly longer duration and rate of rise of the P9 reflex compared to all other ages, while Figure 2-11 illustrates that the reflex at P22 peaks in amplitude and that a small but significant difference exists between this age and P41 animals.

Figure 2-10 Normal developmental profile of EMG responses to pinch (ii)
Plots on the following page show pooled (left and right sides) responses from naïve animals aged P9 (n=5), P12 (n=6), P18 (n=6), P22 (n=6), P31 (n=6) and P41 (n=6). Each plot consists of responses of P9 animals compared to other ages. Post-hoc significance is indicated on the plot (2-way ANOVA, all ages together, Bonferroni post-hoc ***p<0.001, **p<0.01, *P0.05). Solid coloured line shows the mean EMG response, SEM is shown in grey.


ANKLE
TOE
a
b
c
d
e
f
g
h
i
j
P9
P12
P18
P22
P31
P41
RMS (mv/s) ± SEM
Time (s)

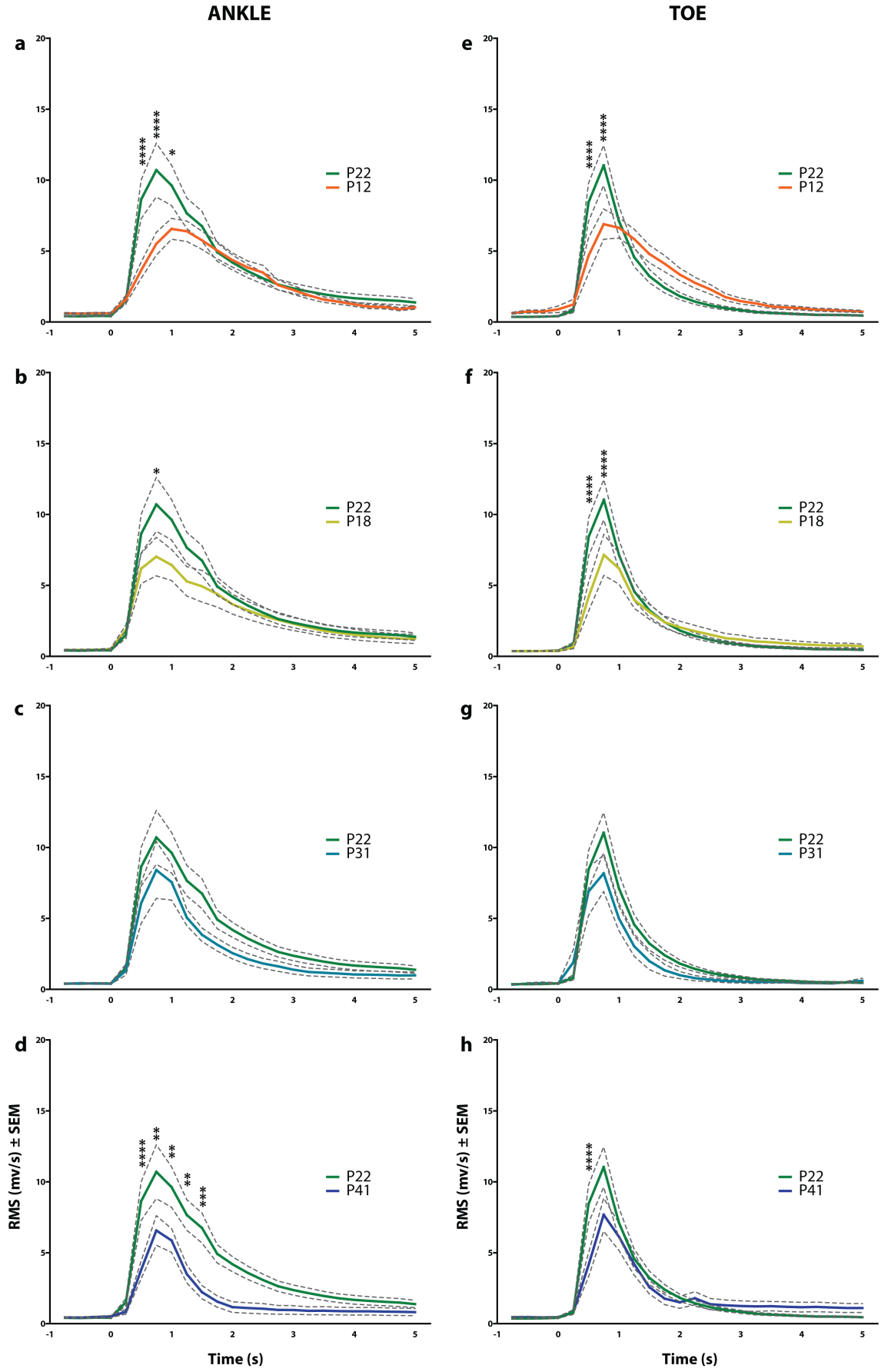


Figure 2-11 Normal developmental profile of EMG responses to pinch (iii)
Plots show pooled responses from naïve animals (same as previous figures) All ages compared to P22 Post-hoc significance is indicated on the plot (2-way ANOVA, all ages together, Bonferroni post-hoc ****p<0.0001, ***p<0.001, **p<0.01, *P0.05). Solid coloured line shows the mean EMG response, SEM is shown in grey.

2.6 Summary of results

The data presented above can be summarised as follows:

- Noxious pinch stimulation of the ankle and toe evokes robust reflex EMG responses in the BF ipsilateral to the stimulus at all ages tested (P9, P12, P18, P22, P31, P41).
- At P9 ipsilateral reflex responses to pinch are larger and of longer duration than at older ages, lasting at least 5s. Pinch stimulation at this age also evokes contralateral reflex BF responses, which although significantly smaller than ipsilateral responses are greater than those seen at older ages.
- At P22 flexion reflex activity following ankle pinch is significantly longer in duration than those evoked by toe pinch. In contrast to P9 responses, EMG activity evoked from both ankle and toe in P22 animals return to baseline levels within 5s.
- At P41 reflexes are shorter duration than at P9 or P22. Activity returns to baseline levels by 2s following stimulation of the ankle and the toe.
- Maturation of reflex activity occurs rapidly between P9 and P22. Increasing postnatal age from P9, 12, 18, and 22 is associated with an increased rate of rise of flexion reflex activity evoked from ankle pinch, although this not the case at the toe.
- At P22 nociceptive reflexes are larger than those evoked at P31 or P41 from both the toe and the ankle.
- These data provides the first quantitative analysis of nociceptive reflexes evoked from the ankle.

2.7 Discussion

The results presented above describe the postnatal development of the nociceptive withdrawal reflex from P9 to P41. This section will first consider the advantages and limitations of the study and secondly examine the results in the context of previous studies on the development of nociceptive networks. Importantly, these results are consistent with previous studies of the development of nociceptive reflexes in rodents (Ekholm, 1967a; Fitzgerald and Gibson, 1984; Fitzgerald et al., 1988; Holmberg and Schouenborg, 1996b).

2.7.1 Methodological considerations

2.7.1.1 EMG recording

The first aim of this chapter was to develop an appropriate method for evoking flexion reflexes from the ankle across different age groups.

The methods of analysis and experimental protocols that have been employed in this study have been novel to our group. This is particularly true of the EMG recording setup. In pilot experiments it was noted that the pinch stimulus used had a profound sensitising effect on reflexes and therefore the quicker the protocol is performed the less variable and more reliable the results. It was decided, therefore, to minimise the number of stimulation sites to two. Sites of stimulation were chosen on the basis of sensitivity to noxious pinch.

The advantages of a double electrode setup are substantial. The experimenter can move easily between stimulating one side or the other. Previously, in similar recordings, anaesthesia was increased temporarily to reposition the recording electrode in the opposite muscle before lowering it again and leaving time for anaesthetic equilibration (Hathway and Fitzgerald, 2006). This protocol introduces unnecessary variability in the level of anaesthesia over the recording period. There was concern that intra-animal variability might cloud results. There was, at times, great variability between responses evoked from the left and right hind paw. Alternating the side that was stimulated first eliminated this bias.

Proprioceptive input is known to profoundly affect flexion withdrawal. Balance and the stepping cycling have been shown to modulate the amplitude of withdrawal to noxious stimuli (Crenna and Frigo, 1984; Andersen et al., 1995; Spaich et al., 2004). During EMG recording of the flexion reflex little or none of the animal's weight is borne on the hind paws. It is reasonable to assume that the level of afferent proprioceptive input at rest would be minimal and would not affect the recorded responses (Sabbahi et al., 1990). The absence of proprioceptive input is likely to account, in part, for the presence of contralateral reflex withdrawal seen in the youngest animals; something that is not observed in behavioural studies.

The strength of a muscle contraction and the extinction of reflex activity are dependent on a multitude of factors. Importantly among them is the angle at which the joint is held as well as the load and strain that are applied across the muscle before a contraction takes place. In this instance the tension on the biceps femoris muscle could not be directly measured but the angle at which the joint was held was standardised by virtue of the platforms that the hind paw was fixed to for the recording. To ensure that there was no axial rotation of the hips the height of the animals' left and right paws were fixed at the same level. It is probable that the preload on the biceps femoris was variable between animals, and may have contributed to variability, particularly in younger animals.

2.7.1.2 EMG analysis

In order to compare and contrast the responses of animals of different postnatal ages it was necessary to analyse EMG data in a biologically meaningful way. This technique allowed quantitative assessment nociceptive circuit sensitivity across different ages with little post-hoc manipulation of data. This fulfilled a key aim of this chapter. The signal processing that was developed and employed here is outlined in the methods section of this chapter. The advantages of this technique are three-fold. Firstly, this method of analysis considers the shifts in the amplitude, time-course, variability and general morphology of reflex responses to pinch that occurs over the postnatal period. Secondly, stimuli can be time locked to the stimulus, which with further analysis can provide data on the latency of reflex responses. Finally, the intuitive way in which the data is presented allows a

straightforward comparison of reflex responses in many different experimental conditions.

There are also inevitable limitations to this technique. The 6s period of interest limits the data collected to 1s pre-stimulus and 5s post-stimulus. In most, but not all, instances this proved sufficient to observe the onset and extinction of a reflex. Additionally, this technique takes no account of the way in which different muscle fibres are recruited throughout development and which types of muscle fibres are responsible for the reflex. Related to this, whilst the recording sampling frequency used was sufficient to identify individual multi-unit action potentials in raw recordings, this technique does not differentiate number or size of a motor unit involved in a reflex contraction. Further analysis of such data may provide a greater understanding of the postnatal development of reflex responses to nociceptive stimuli. A key assumption made here is that the intra-animal variability, in a statistical sense, is greater than that of the inter-animal variability.

2.7.2 The development of the nociceptive circuitry

The rat nervous system is immature at birth and undergoes widespread changes in early postnatal weeks. Evidence would suggest that inhibitory circuitry found in the adult spinal cord and brainstem is profoundly different to that found in the immature one. Furthermore, it has been hypothesised that given the immaturity of inhibitory control, long-term consequences may result from exposure to intense sensory inputs early in life. The latter point shall be discussed in detail in Chapter 4. This section will discuss the results presented above in the context of the developmental changes known to be occurring over the postnatal period in question.

2.7.2.1 Reflexes of P9 animals

Data presented above show that in P9 animals reflexes are longer, larger and reach peak amplitude later than those in older animals. Additionally, noxious pinch evokes activity both in the biceps femoris ipsilateral and contralateral to the stimulus. The developmental regulation of reflexes has been studied in preterm human infants, (Issler and Stephens, 1983; Andrews and Fitzgerald, 1994, 1999; Andrews et al., 2002), rat pups (Fitzgerald and Gibson, 1984; Fitzgerald et al., 1988; Holmberg and Schouenborg, 1996b) and kittens (Ekholm, 1967a). This work has in large part concentrated on the earliest part of reflex development;

premature infants in humans and the first postnatal days in rats and kittens. During this period the reflex response to noxious input changes profoundly. In premature human infants and rat pups repeated stimulation of the hind limb results in a facilitation of reflex activity. By contrast, in adult rats habituation results (Fitzgerald et al., 1988). In this study repeated stimulation facilitated reflex activity progressively less until P20 and by P26 the mechanical force required to evoke a reflex pre- and post-repeat stimulation was the same. The lack of reflex habituation in young animals, in part, reflects the immaturity of sensory and motor circuitry within the spinal cord. Compared to adults, juvenile sensory networks lack the same inhibitory interneuronal control as well as being intrinsically more excitable (Fitzgerald, 2005).

The development of spinal sensory systems does not occur in isolation. Over early postnatal weeks there are concurrent changes in motor systems. The neonatal spinal cord is a widely used model to study spinal locomotor circuits (Hochman et al., 2012). The circuitry required for the generation of fictive locomotion is in place in the neonatal spinal cord and indeed the stepping patterns observed in these preparations are adult-like (Hochman et al., 2012). The changing amplitude of reflex responses to pinch may be attributable to the way in which motor units are recruited in the neonatal rodent. In the early postnatal period striated muscle, for instance that found within the biceps femoris, is known to be polyaxonally innervated, thus a single muscle fibre may receive input from multiple axons (Redfern, 1970; Benoit and Changeux, 1975; Brown et al., 1976; Rosenthal and Taraskevich, 1977). In adulthood a single motor neurone axon will innervate a single muscle fibre and cause the contraction of that and any other fibre within the functionally related motor unit. Polyaxonal innervation may lead to greater likelihood of muscle fibre contraction following motor neuronal activity. The transition between multiple inputs and single inputs occurs at approximately P14. Between P8 and P14 there is a daily loss of functionally active axons and by the end of the second postnatal week virtually all motor end-plate potentials are from single units (Brown et al., 1976; Rosenthal and Taraskevich, 1977). At the same time the size of motor units begins to decline (Brown et al., 1976). In the experiments presented in this chapter the amplitude of the response evoked from P12 animals compared to P9 animals was significantly smaller whilst the duration of the responses remained unchanged. Falling response amplitudes

would seem to go hand in hand with falling motor unit size, whilst the explanation for the changing duration of responses may lie elsewhere. As muscles grow and motor units remain unchanged in young adults, the recording electrode will record from fewer muscle fibres. It is possible, therefore, that the diminishing reflex amplitude seen from P22 to P41, is in, part attributable to the changing size of muscles.

2.7.2.2 Specificity of noxious input from pinch stimulus in young animals

In the newborn cord A-fibre terminals are found in the superficial DH. In contrast, the adult A-fibre terminals are concentrated in the deeper DH (Beggs et al., 2002). The gradual reshaping of A-fibre connections from superficial to deeper areas of the DH occurs over the first three postnatal weeks and can be prevented by the chronic administration of NMDA receptor antagonists (Beggs et al., 2002). Indeed, A-fibre inputs can be seen in the DH in the immature rodent and when stimulated evoke c-fos expression in LII, something that only occurs following C-fibre strength stimulation in the adult (Coggeshall et al., 1996; Jennings and Fitzgerald, 1998). A-fibre strength stimuli can evoke a unique form of sensitisation in the newborn cord whereby following a sensitising stimulus, direct A-fibre evoked activity does not increase, but induces profound after-discharge in 33% of neurones at P3 which is still present in 6% of neurones recorded at P10 (Jennings and Fitzgerald, 1998). Additionally the architecture of C-fibre inputs to the DH in the first postnatal weeks undergo changes. Post-synaptic activity from C-fibre inputs is not seen until the second postnatal week at P10, 35% of DH neurones show long-latency spiking responses typical of slow conducting C-fibre inputs (Fitzgerald, 1988; Jennings and Fitzgerald, 1998).

Some of the reflex excitability seen at early postnatal ages, particularly the large and prolonged reflex responses, is likely to reflect the large contribution of low threshold mechanoreceptors to DH inputs. Furthermore, the changing architecture of A-fibre inputs into the DH is reflected in the progressive refinement of reflex responses in this study.

2.7.2.3 Receptive fields and reflexes

In this study, the only direct examination of receptive fields was made in P21 animals for the purpose of choosing the most appropriate sites for noxious stimulation. Reflex receptive fields are known to become smaller over the first three postnatal weeks and in the first postnatal week are large and have a variable distribution across the skin of the hind paw (Fitzgerald, 1985; Holmberg and Schouenborg, 1996b). A single DH neurone may have a receptive field covering approximately 50% of the hind paw plantar skin at P3, 36% at P6, 20% at P10 and 15% at P21. Thus, mechanical stimulation of the ankle or the toe in the P9 group is likely to evoke greater activity in the DH than in any other group in this study, and therefore as receptive field size decreases the concomitant DH activity will decrease.

2.7.2.4 Immaturity of local spinal inhibitory and excitatory neurotransmission

The changing architecture of the spinal cord excitatory and inhibitory influences is outlined in Section 1.8.3. The switch of inhibitory control from facilitation in the first postnatal weeks to inhibition in adulthood occurs during the developmental period of interest in this chapter. The intrinsic excitability of spinal DH neurones does not change over this period, although changes do occur in excitatory neurotransmission (Baccei et al., 2003; Pattinson and Fitzgerald, 2004). High levels of expression of excitatory neurotransmitter receptors are seen throughout the cord in immaturity and are increasing restricted to the superficial DH in adulthood (Jakowec et al., 1995; Brown et al., 2002; Pattinson and Fitzgerald, 2004). This may have accounted for, in part, the general level of excitability in younger animals' reflex responses to pinch and the progressive diminution of reflex responses with age. The mature profile of AMPA and NMDA receptor expression seems to be in place at P28 and P21 respectively, although the subunit composition of these receptors continues to develop until at least P35 (Jakowec et al., 1995; Brown et al., 2002). This may underlie the small, but significant differences between the responses of P22 and P41 animals.

The development of fast inhibitory transmission, also summarised in Section 1.8.3, undergoes maturation in the postnatal period. GABAergic inhibition is more mature at birth and GABA acts as an inhibitory neutrotransmitter postsynaptically

in postnatal development. The immaturity of GABAergic transmission may underlie the large and poorly directed reflexes seen at early postnatal ages (Cordero-Erausquin, 2005). GABAergic transmission in the younger DH appears to be of a lower fidelity, rather than absent version of that seen in the adult (Ingram et al., 2008). The most striking deficits in inhibitory signalling are found the immature glycinergic signalling in the DH (Ingram et al., 2008). Lack of glycinergic inhibitory control in the developing spinal cord is apparent (Koch et al., 2012). At P21, glycinergic blockade leads to enhanced behavioural sensitivity and neuronal responses in the DH *in vivo*, whilst in the same paradigm, application of the glycine receptor antagonist strychnine, has no effect on the DH responses in the P3 cord (Koch et al., 2012). The combination of enhanced excitatory neurotransmission and the lack of glycinergic inhibition in the first postnatal weeks seems to play a pivotal role in the 'excitable' state of the spinal cord at this stage. The DH is a site of onward transmission of sensory information both to higher brain structures and through the spinal cord to the motor neurone pools of the ventral horn. It seems likely that gating of sensory inputs that occurs in the adult spinal cord is not present at this early stage and contributes to the large easily evoked reflexes seen in the younger groups of animals in this study.

2.7.2.5 The influence of developing descending control on spinal reflexes

The difference between young and adult reflex responses may be explained by the changing nature of descending influences on the spinal DH. In adult spinalised cats, Sherrington demonstrated that when the spinal cord is cut at the thoracic level that mechanical reflex thresholds fall and responses to noxious stimuli become exaggerated (Sherrington, 1910; Sherrington and Sowton, 1915). This was a clear demonstration of the descending inhibitory control of reflex circuits, though this was not recognised as such until the work of Reynolds in 1969, which showed that electrical stimulation in awake rats was sufficient to perform a laparotomy with no additional analgesia (Reynolds, 1969). The role of descending fibres in the immature spinal cord has been investigated in a similar manner. Electrical stimulation in the dorsolateral funiculus dampen DH responses to stimulation of the hind paw, but only from P9 onwards (Fitzgerald and Koltzenburg, 1986). By P12, stimulation weakly inhibits around 50% of cells and by P18, stimulation causes widespread inhibition in the DH but requires a more

intense stimulation protocol than in adults. Another similar study, employing direct electrical stimulation of the PAG, demonstrated analgesia from P21 onwards with stimulus intensities greater than that required in adults (van Praag and Frenk, 1991). These data would suggest that before P21 descending inhibitory influences are ineffective in modulating the excitability of DH circuits. This may further account for the profile of excitability seen in younger animals that declines with postnatal age.

Important experiments by Hathway et al., (summarised in Section 1.8.4) showed that descending systems in the immature (prior to P21) brainstem and spinal cord are purely facilitatory and that ablation of the RVM in animals up to the age of P21 results in a rise in mechanical thresholds. (Hathway et al., 2009). The same treatment in adulthood results in a fall in mechanical thresholds. Tonic influence from the brainstem is likely to contribute further to enhanced dorsal horn excitability in young animals. Together, these observations suggest that the immature spinal cord is under tonic descending facilitation, whilst after the age of P21, this turns into a tonic inhibition.

2.8 Conclusions

2.8.1 Reflex duration and amplitude is greater in young animals and declines with increasing postnatal age

The major difference in reflex evoked by pinch between young and adult animals is their duration. At the youngest ages studied, P9, reflexes are large and prolonged compared to more mature animals. In adulthood, reflexes are shorter in duration and smaller in amplitude than those from more immature animals.

2.8.2 Postnatal time course of reflex maturation continues beyond the third postnatal week

The time course of reflex development is consistent with previous studies. There were significant differences between P22 animals and P41 animals. Clearly the majority of the postnatal reflex changes are complete by the end of third postnatal week, and there are no further changes in reflex duration beyond this stage. Reflexes continue to change into the forth and fifth postnatal weeks which is likely to be due to the maturing descending control of spinal reflexes and also to the size of the growing muscle.

Chapter 3 Postnatal development of inflammatory pain processing

3.1 Introduction

In the previous chapter the postnatal development of nociceptive reflexes evoked from the hindlimbs of naïve rats was characterised. This chapter will focus on characterising the evoked activity in reflex circuitry in the presence of an inflammatory arthritic injury over the postnatal period. Inflammatory joint disease is a major cause of chronic pain but the mechanisms that initiate and maintain this type of pain are not well understood. This is particularly true of pain in children with rheumatic disease, where treatment of the disease alone is often not sufficient to alleviate the pain (Kimura and Walco, 2007). In addition, the extended postnatal plasticity of pain pathways raises the possibility that joint inflammation in young children may have long-term adverse effects upon developing pain pathways (Fitzgerald, 2005; Walker et al., 2009b). The aim of this chapter is to investigate inflammatory joint pain at different postnatal ages using the reflex model described in Chapter 2. Further detail on the processing of inflammatory pain can be found in Sections 1.7.3 and 1.7.4.

3.1.1 Inflammatory pain processing

Inflammatory pain refers to nociceptive input that is driven by inflammatory processes. The genesis and maintenance of inflammatory pain has been linked to both peripheral and central mechanisms (Fitzgerald, 1995). The following sections will outline the understanding of the drivers, both peripheral and central, of inflammatory pain. Clinically, many autoimmune mediated conditions such as inflammatory arthritis can become chronic and rarely resolve completely. Destruction of articular cartilage, fibrosis of peri-articular structures, proliferation of the synovial membrane (pannus formation), loss of movement and pain are key features of this disease process. The pain associated with arthritis is almost universal and is typified by hyperalgesia - enhanced responses to painful stimulation, and pain at rest - spontaneous pain. Previously innocuous stimulation such as joint pressure or the movement of joints becomes painful (Arendt-Nielsen et al., 2010).

3.1.1.1 Peripheral Processes

Direct trauma, mechanical wear and autoimmune destruction of cartilage are common causes of joint inflammation, and therefore pain. The peripheral

sensitization of nociceptors and other joint nerve terminals is thought to arise from interactions between cells of the immune and nervous systems. Indeed, the extrusion of plasma proteins and fluid associated with acute inflammation increase the pressure within the joint capsule provoking afferent barrages from mechano-sensitive terminals (Andrew and Dodt, 1953). In addition, inflammatory mediators directly sensitise peripheral nerve terminals. This is outlined in greater detail in Section 1.7.3.

Molecules released from damaged cells activate TLRs expressed on the membranes of immune cells (Ren and Dubner, 2010). A plethora of cytokines and small molecules are also released into the joint milieu. These include 5-HT, PGE_2, IL-1β, IL-6 and TNFα. Further detail concerning the mechanisms of peripheral sensitisation can be found in Section 1.6.1.

The 'soup' of inflammatory mediators and cells within an arthritic joint directly affects the sensitivity of primary sensory neurone terminals (Schaible et al., 2002) . These mediators act to sensitise joint afferents in the first instance, but also to maintain hypersensitivity of sensory terminals in the periphery (Grubb et al., 1988; Schaible et al., 1988; Richter and Schaible, 2007; Kawasaki et al., 2008; Richter et al., 2010). In the presence of ongoing joint destruction and inflammation there is also evidence to suggest the sprouting of nerve terminals and formation of neuroma-like nerve tangles that may maintain nociceptive input from damaged joints (Jimenez-Andrade and Mantyh, 2012).

In an effort to elucidate the role of different types of joint fibres in inflammation, Schaible and colleagues followed single joint primary afferents through the induction of arthritis in the cat knee (Schaible and Schmidt, 1988b). Within an hour of inflammation the responsiveness of low threshold myelinated fibres had increased, and continued to do so from 2-4 hours. Movement within the normal working range of the joint, which had previously evoked minimal activity, provoked an afferent barrage. Small, high threshold afferents became more responsive later in the course of inflammation, at about 4 hours, which, the authors argue correlates well with the onset of behavioural signs of hypersensitivity in rodent models of arthritis.

The nerves that supply joints are commonly found in articular capsule, ligaments and the tough fibrous network of connective tissue that surround the articular surfaces, including high and low threshold mechanoreceptors, proprioceptors and sympathetic terminals (Gardner, 1948; Zimny, 1988; Jimenez-Andrade and Mantyh, 2012). Articular cartilage itself is devoid of innervation (Freeman and Wyke, 1967).

3.1.1.2 Central Processes

The barrage of nociceptive afferent activity that follows the initiation of joint inflammation leads to changes in excitability of spinal DH neurones. Using extracellular recordings from electrodes placed in the deep DHs of adult animals, Neugebauer and colleagues investigated the acute development of hyperexcitability following the joint inflammation. Neurones, previously only responsive to stimulation of the knee joint, begin to respond robustly to stimuli across the hind limb, paw and on occasion the contralateral limb (Neugebauer and Schaible, 1990). In spinalised animals the expansion of receptive fields is pronounced, suggesting a role for descending control of deep afferent inputs in arthritic pain processing (Neugebauer and Schaible, 1990). Additionally, the response threshold of these neurones is simultaneously lowered and responses to electrical stimulation of the sural nerve are enhanced (Neugebauer and Schaible, 1990; Neugebauer et al., 1993). The level of spontaneous activity in the deep DH is also increased along with responses to high threshold unmyelinated inputs in those neurones receiving afferent joint input (Martindale et al., 2007). So within the hours following the initiation of inflammation the spinal cord is rendered hyperexcitable.

This DH hyperexcitability, accompanied by enlarged receptive fields and increased afferent inputs, is the neural substrate of hyperalgesia and allodynia or the increased pain and tenderness that are features of inflammatory pain. While the joint pain is initiated by sensitisation of peripheral sensory terminals, the pain rapidly takes on a central mechanism whereby joint and other inputs are amplified and enhanced by DH circuits. It is not known, however, to what extent these changes and processes are present in the immature nervous system.

3.2 Aims of the experiments

The primary aim of this chapter is to investigate the effect of inflammation of the ankle joint upon the pattern and time course of hindlimb nociceptive reflex responses in young rats using EMG from the biceps femoris in response to noxious stimulation of the ankle joint and hind paw. The key objectives were:

I. To establish a reliable and repeatable method of inducing inflammation in the ankle joint in postnatal rats.
II. To provide a quantitative analysis of the postnatal development of ankle joint inflammation using ipsilateral and contralateral nociceptive flexion reflex recording.
III. To assess the immediate, short and medium term consequences of joint inflammation on the pattern of reflex activity at different postnatal ages.
IV. To make a comparative analysis of electrophysiological and behavioural measures of inflammatory hypersensitivity.

3.3 Methods

The author gratefully acknowledges the assistance of Stéphanie Koch (SK) for the completion of dorsal horn recordings in this chapter. SK performed the initial anaesthesia, tracheotomy insertion and laminectomy. The author performed the recordings, pinch stimulation, data collection and analysis.

3.3.1 Animals

Male Sprague-Dawley rats were obtained from UCL Biological Services Unit as described in Chapter 2. A total of 144 animals aged P8, P21 and P40 received intra-articular ankle injections of CFA; see Table 3-1 for details. Data from naive controls are from the same 45 naive animals used in Chapter 2.

Postnatal age at time of injection (days)	Experimental time-point post injection	No. of animals used for EMG	
		Naïve	CFA
8	24 Hours	6	9
	4 Days	12	16
	10 Days	6	6
21	24 Hours	6	5
	4 Days	6	8
	10 Days	6	6
40	24 Hours	6	12
	4 Days	6	16
	10 Days	6	6

Table 3–1 Age, experimental time-points and numbers of naïve and CFA animals used for EMG recording

3.3.2 Inflammation

To our knowledge, there are no studies that have accurately established the volume of the joint space volume within rodents. For the purposes of all studies the hock (referred to as the 'ankle joint' from now on) was injected. This joint was chosen for its proximity to the paw, a common site for behavioural testing, and to allow easy visual monitoring of joint appearance and size. In all behavioural experiments, saline injected and naïve animals were used as controls. Commercially available CFA (Sigma-Aldrich, UK) was used. As joint volume is likely to vary substantially with age, a standardised volume of approximately

0.1µl/gram bodyweight was injected using a 30-gauge needle in conjunction with a 100µl Hamilton Syringe into the joints of animals of different ages. P8, P21 and P40 animals received 2ul, 6ul and 20ul of CFA respectively.

3.3.3 Saline vs. Naïve

All control data for these experiments were gathered from age matched naïve animals. Saline injected animals showed no significant differences in EMG response, morphology, amplitude, or behaviour compared to naïve animals and so this data is not presented here.

3.3.4 Observation of behaviour

Behaviour was quantitatively measured using weight bearing and mechanical thresholds (detailed below). In the course of these experiments notes were made on the way that animals moved around their home cages although these general behaviours were not scored.

3.3.5 Weight bearing testing – incapacitance meter

Weight bearing measurements were made using a Linton Incapacitance Meter (Linton Instruments, UK). One animal at a time was placed into a small perspex container so that the animal's hind paws were placed on two separate microbalances (Figure 3-1b) and the forepaws rested lightly on the sloping front part of the container (Figure 3-1c). During measurement the animal's tail was lightly restrained to prevent excessive movement (Figure 3-1a). A mouse container was used for the smaller animals and the adult rat container was used at P40. The device was zeroed prior to animals being placed on the microbalance. Animals were allowed to acclimatise to the container for at least 5 minutes prior to testing. Three measurements were made for each animal and an average used as a final value.

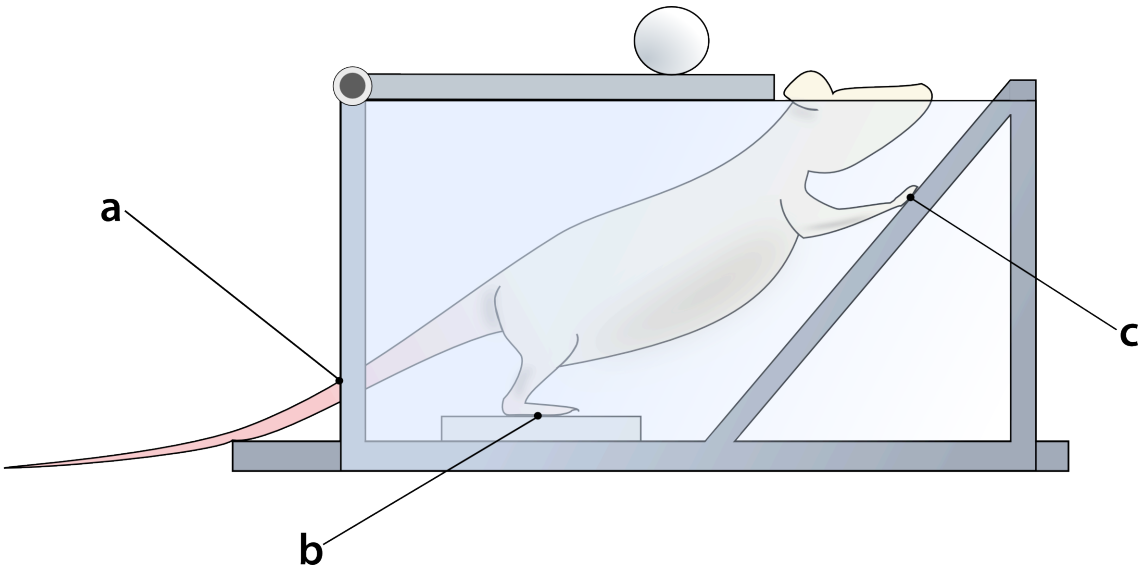
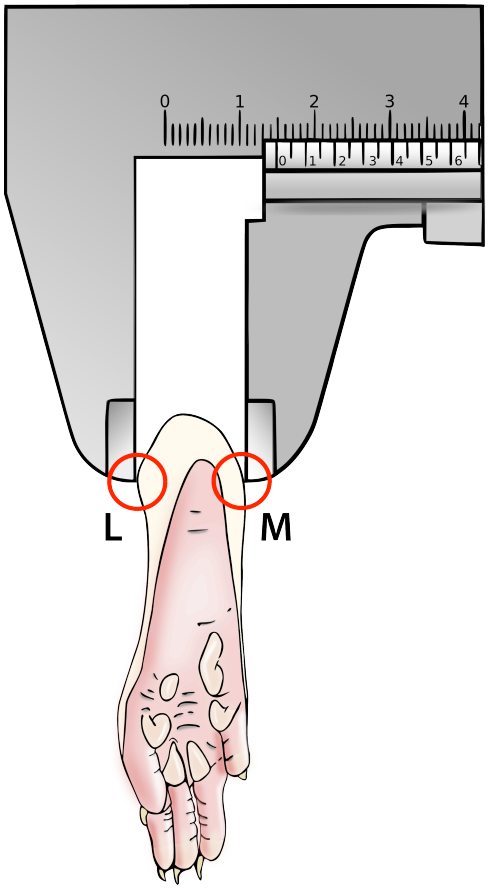


Figure 3-1 Weight Bearing Measurement set-up
Weight bearing measurements were made using a Linton Incapacitance meter. Measurements were made whilst the rat was lightly restrained by the tail **(a)**. The two hind paws rested on two separate microbalances **(b)** and the fore paws rested on the front of the perspex chamber **(c)**.

3.3.6 Mechanical threshold testing - manual von Frey

Mechanical withdrawal thresholds were measured in P8 and P40 rats pups who had received CFA, 0.9% saline or no treatment with von Frey (vF) filaments. P40 animals were placed in boxes with a wire mesh bottom to allow filament application to the plantar surface of the hind paw. Single vF filaments were applied 6 times to one paw. Paw flick, rocking away from the stimulus or withdrawal on 3 or more occasions was taken as threshold. In the P8 group, due to the size of the animals, and the wire mesh available, animals were gently held whilst vF filaments were applied to the the plantar skin on the infero-lateral part of the hind paw. At all time-points the experimenter was blinded to the treatment the animals had received. Prior to injection of CFA or saline, baseline threshold measurements were made. As time of day has been shown to affect nociceptive thresholds in rodents, all measurements were made within a three-hour window between 11am and 2pm (Frederickson et al., 1977; Rosenfeld and Rice, 1979). Threshold measurements were followed for 21 days after injection of CFA (n=4), saline (n=4) or no injection (n=4).

3.3.7 Ankle size measurement



Prior to every EMG recording, when the animals were lying prone on a flat bench fully anaesthetised, the width of the left and right ankles was assessed using Vernier callipers. The measurements were made across the widest part of the ankle – from the most lateral bony landmark (the distal end of the fibula) to the most medial bony outcrop (the distal end of the tibia). Measurements were accurate to 3 significant figures. Data were normalised to the non-injected ankle for the purposes of across-age comparisons.

Figure 3-2 Ankle measurement
This figure illustrates how the width of ankles was measured using Vernier callipers. The most medial (M) and lateral (L) bony outcrops were taken as the anatomical landmarks for measurement. It should be noted that the ankle is fully extended for measurement.

3.3.8 EMG recording

EMG recording was performed as described in Chapter 2.

3.3.9 *In vivo* extracellular recordings

DH recordings were performed as described in (Koch et al., 2012). Rats were anesthetized with isoflurane (induction at 3.5% (vol/vol) in medical O2), tracheotomized, and artificially ventilated under isoflurane anesthesia of 1.8%. The airflow was adjusted according to the animal's size and heart rate monitored via electrocardiogram. A homeothermic blanket and heating lamp were used to maintain body temperature at physiological levels. A laminectomy was performed to expose the lumbar spinal cord L4 and L5 following which the vertebral column was secured using a vertebral clamp and the dura and pia mater were removed. To isolate individual neurons in the spinal cord, a 7-µm tipped glass-coated carbon fibre microelectrode was lowered through the DH in 2- or 10-µm steps by microdrive. Stroking of the skin overlying the ankle and light pinch were used as

search stimuli. When a single unit was isolated, 5 minutes of background activity was recorded followed by 4 pinches of 1s duration – an isolated single pinch, followed by a train of 3 with 5s between each pinch. Neuronal activity recording and analysis was recorded using Chart 7 software (Chart 7 version 7.3.7; AD Instruments). Spike activity was discriminated using the 'Spike Histogram v2' plugin for Chart 7. The number of spikes in a 5s period (starting at the onset of the pinch stimulus) was taken as the evoked activity per cell. The background firing rate of each cell was then subtracted from the total.

3.3.10 Statistical analysis

All statistical analyses were performed using Graph Pad Prism and is described alongside each figure where appropriate. For all tests, $p<0.05$ was considered statistically significant. The following statistical analyses were used:

(1) EMG Intra-age comparisons were made using a 2-way analysis of variance (ANOVA) a Bonferroni post-hoc test to compare differences at individual time-points (250ms time bin). These were performed to establish differences between left and right-sided responses as well as the effect of age on response at different time-points.

(2) Ankle size comparisons were made using an unpaired, two tailed t-test between naive and CFA animals. Comparison of ankle size across ages was performed with a one-way ANOVA with a Bonferroni post-hoc for multiple comparisons.

(3) Dorsal horn extracellular recordings from CFA and naïve animals were compared using a Mann-Whitney test. Comparison between consecutive pinches was made using 2-way ANOVA with Bonferroni post-hoc analysis for comparison between pinches 1, 2 and 3.

3.4 Results

3.4.1 Terms of reference

In the previous chapter, the normal development of the nociceptive flexion hind paw and ankle joint withdrawal reflex was characterised in naive animals across the postnatal period. In this chapter the effect of ankle joint inflammation upon these nociceptive reflex properties was investigated at different postnatal ages and at various times post injury. In all cases ankle inflammation was induced using CFA. Naïve animals of matched postnatal age (reported in Chapter 2) were used as controls.

In the previous chapter the responses that were evoked from the BF on the same side as the pinch were referred to as *ipsilateral* responses and those that were evoked in the biceps femoris on the opposite side to the pinch were referred to as *contralateral* responses. This nomenclature is continued in this chapter and in addition, when referring to effects of pinch on the side of inflammation the term *inflamed* side will be used, while pinching the opposite side will be referred to as the *non-inflamed* side.

For example, if the inflamed ankle joint was pinched and the responses were recorded from the BF on the same side as the pinch then this would be an *ipsilateral inflamed ankle* response.

The postnatal ages of animals are given as the day that they were injected with CFA.

3.4.2 Ankle Inflammation

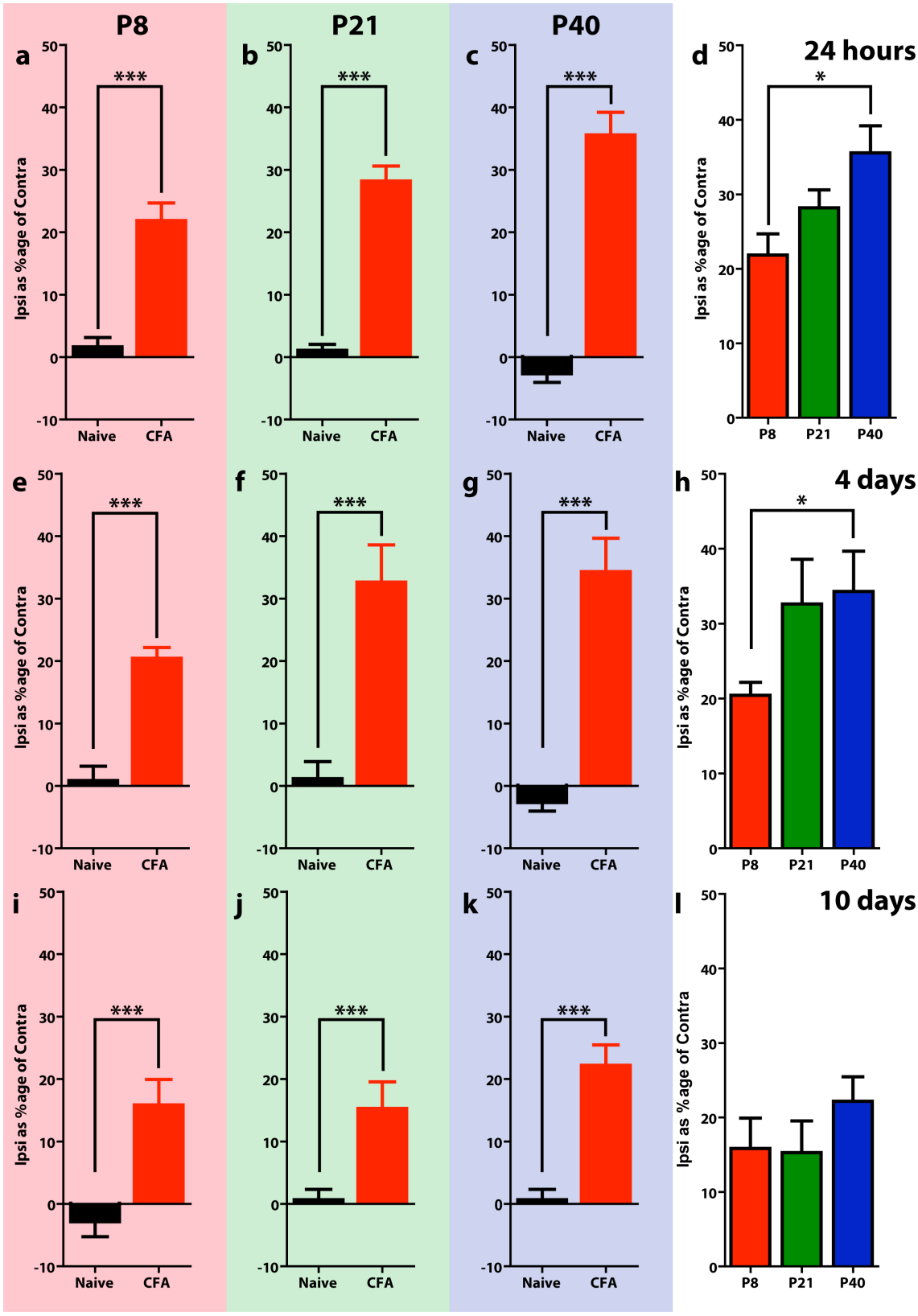


Figure 3-3 Injection of CFA into the ankle leads to swelling of the injected joint
Graphs, on previous page, show the severity of ankle swelling following ankle injection of CFA at different postnatal ages –, P8, P21 and P40 at 24 hours, 4 days and 10 days post injection. All graphs show the right or inflamed ankle as a proportion of the left or uninflamed ankle. There is a significant swelling of the ankle at all ages and all time-points (*** $p<0.001$, two-tailed unpaired t-test). **(d, h, l)** show the severity of swelling at different ages – with increasing postnatal age. Swelling is significantly less pronounced at 24 hours and 4 days in P8 animals, but this age related difference is lost by 10 days post-CFA (One-way ANOVA, * $p<0.05$ Bonferroni post-hoc).

Injection of CFA into the ankle joints of rats caused redness, swelling and loss of movement in all animals, though the latter observation was more variable between animals. Guarding, such that the limb was held clear of the ground, was apparent for 3-4 days following injection. The size of the ankle joint was measured prior to all EMG recordings to assess the severity of swelling in CFA injected and control animals. Figure 3-3 shows the ankle size of the inflamed ankle as a percentage of the uninflamed ankle 24 hours, 4 days and 10 days following injection at different postnatal ages: P8, P21 and P40 compared to naïve animals of the same age. 24 hours post injection of CFA induces significant inflammation at all ages injected (Figure 3-3a, b, c unpaired two tailed t-test, $p<0.001$). There is a significant increase in ankle size at 4 days (Figure 3-3e, f, g) and up to 10 days post inflammation at all ages (Figure 3-3i, j, k). Interestingly, there is a significant trend for the inflammation to be greater with increasing postnatal age, at 24 hours, 4 days but not 10 days post inflammation (Figure 3-3d, h, l – One way ANOVA, Bonferroni post-hoc, $p<0.05$).

3.4.3 Behavioural effects of inflammation

Using manual von Frey and incapacitance (weight bearing) testing, the pattern of behaviour following ankle inflammation has been investigated. Animals aged either P8 (n=12) or P40 (n=12) received either intra-articular injection of CFA (n=4), saline (n=4) or no injection (n=4). Weight bearing and mechanical thresholds were measured longitudinally. Mechanical thresholds are expressed as absolute values or as the right/inflamed side as a proportion of the left/non-inflamed side ± SEM.

3.4.4 Mechanical von Frey hair thresholds

At baseline mechanical paw withdrawal thresholds of P8 animals were 2.1±0.4g (Figure 3-4a,b), substantially lower than the mean baseline of P40 animals, 60±0g (Figure 3-4d,e). There were no significant baseline differences between the animals assigned to the CFA, saline or naïve groups at either age.

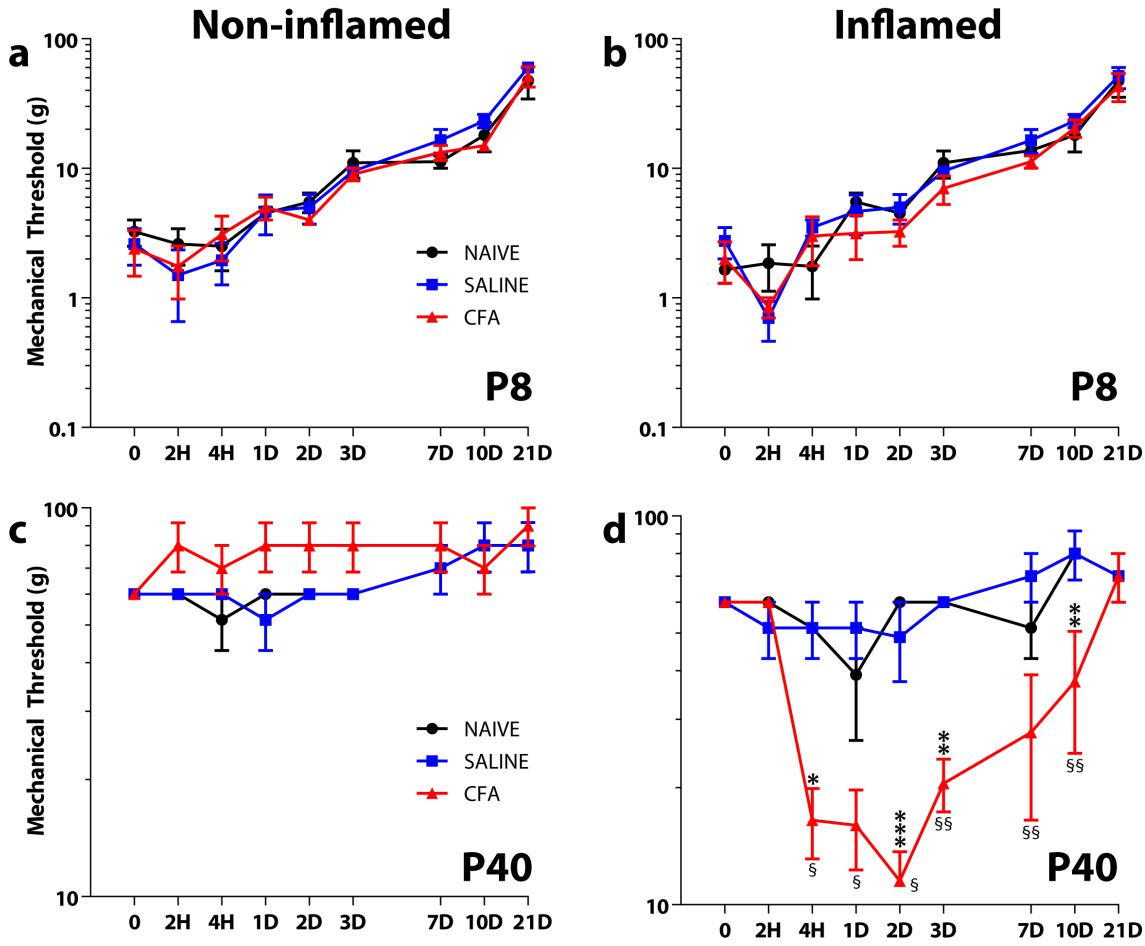


Figure 3-4 Ankle inflammation leads to lowered cutaneous mechanical thresholds at P40 but not P8
Ankle inflammation leads to a detectable fall in mechanical thresholds on the inflamed compared to the uninflamed side in adult (P40) animals (lower half of figure) but not young animals (upper half of figure). **H** refers to hours and **D** refers to days post injection, baseline mechanical thresholds are shown at 0 on the x-axis. The mechanical thresholds of P8 animals (in grams) are shown for the left **(a)** and right **(b)** hind paws. There are no significant differences between P8 naïve (n=4), saline (n=4) or CFA (n=4) treated animals. **(c)** and **(d)** show the mechanical thresholds of left and right hind paws of P40 animals, respectively. Animals that received CFA have significantly lower mechanical thresholds on the right hand paw compared to both naïve and saline treated animals All tested with a 2-way ANOVA, Bonferroni post hoc. CFA vs. Naïve: ***p<0.001, **p<0.01, *p<0.05, CFA vs. Saline §§§p<0.001, §§p<0.01, §p<0.05.

The mean mechanical paw threshold of P40 animals that received ankle CFA injections fell dramatically to 16.5±3.4g by 4 hours post-injection which was significantly lower than saline and naïve animals. By 3 days post-injection the mean mechanical threshold was at its lowest, 11.5±2.2g, but remained significantly different from controls until 10 days post-inflammation (37.5±13g), and returned to baseline by 21 days post-injection (70±10g). The mean threshold of saline injected animals was not significantly different from those of naïve animals at any time post-injection, in addition, the mean mechanical thresholds of P8 animals that received ankle CFA injections did not differ from those of control at any time-point post injection. Consistent with previous literature, there is a steady increase in mechanical thresholds with postnatal age and this occurred

equally on both inflamed and non-inflamed sides. By 21 days post-injection thresholds have reached near adult levels (43±9.8g).

3.4.4.1 Weight bearing

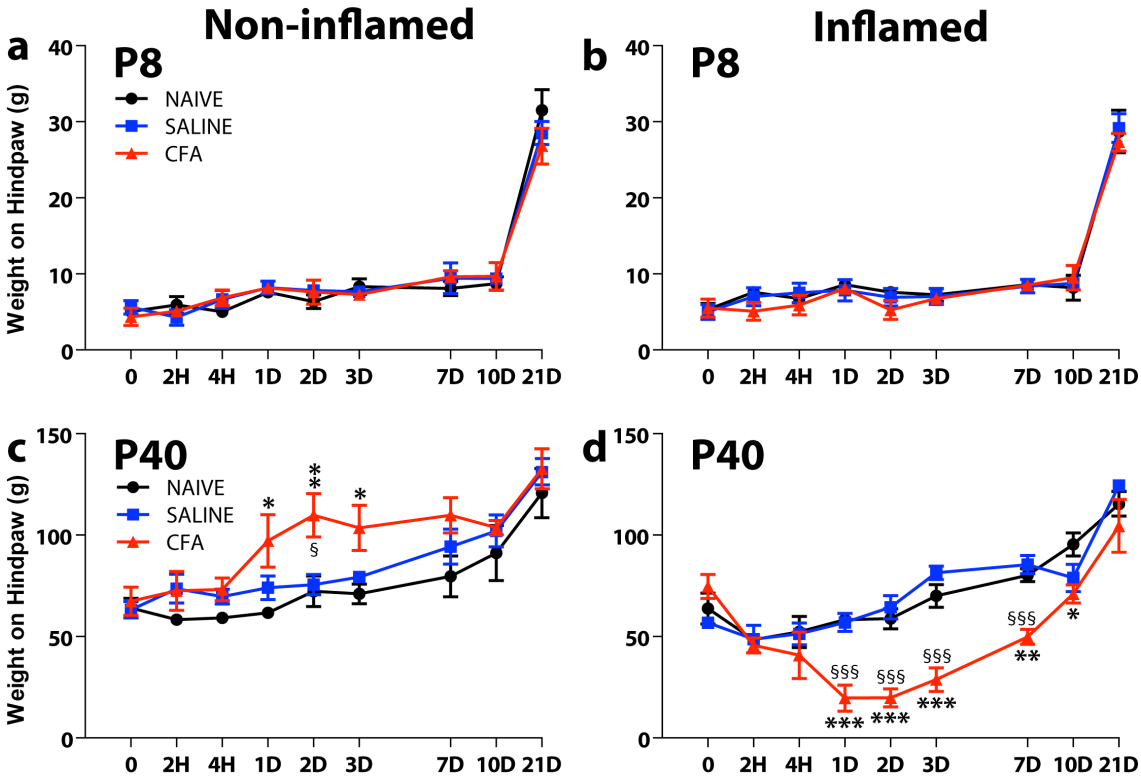


Figure 3-5 Weight bearing is reduced on the inflamed side at P40 but not at P8
Ankle inflammation leads to a detectable shift in weight from the inflamed to the uninflamed side in adult animals (lower half of figure) but not young animals (upper half of figure). In all parts: **H** refers to hours and **D** refers to days post injection, baseline weight bearing is shown at 0 on the x-axis. The weight (g) borne on the left and right hind paws is shown in **(a)** and **(b)** respectively. There are no significant differences at P8 between naïve (n=4), saline (n=4) or CFA (n=4) treated animals on the left or right hind paw. **(c)** and **(d)** show the weight borne on the left and right hind paws of P40 animals. Animals that received CFA bear significantly less weight on the right hand paw and significantly more on the left hind paw compared to both naïve and saline treated animals at the time-points shown. All tested with a 2-way ANOVA, Bonferroni post hoc. CFA vs. Naïve: ***p<0.001, **p<0.01, *p<0.05, CFA vs. Saline §§§p<0.001, §p<0.05.

At baseline, the mean weight borne on the left and right hind paws was 65.1±3.8g at P40 and 5.3±0.5g at P8 group. The marked increase seen in weight bearing between P18 and P29 (10 days to 21 days in Figure 3-5a, b), is a function of the rapid growth that occurs over this age period. Following CFA injection, P40 animals gradually bear less weight on the affected limb and consequently more on the unaffected side, becoming significantly different from control 1 day after injection (Figure 3-5d). P40 CFA-injected animals bore less weight on the affected limb compared to naïve and saline controls at 1, 2, 3 and 7 days post-injection (Figure 3-5d, 2-way ANOVA, Bonferroni post-hoc, $p<0.001$). Differences between naïve and CFA groups remained significant until 10D post-inflammation. CFA injection at P8 had no effect upon weight bearing. At no point in the time course

were there any significant differences between P8 CFA injected animals and the naïve and saline controls.

In summary no significant effect was seen on vF mechanical thresholds or weight bearing at any time-point post P8 CFA ankle injection.

3.4.5 Effect of Ankle Inflammation upon Nociceptive Flexion Reflex EMGs

In this section the data from electromyographic recordings is presented.

3.4.5.2 P8 ankle joint inflammation: 24 hours post injection

Figure 3-6 shows the mean EMG activity in the ipsilateral biceps femoris (Figure 3-6a,b) and the contralateral biceps femoris (Figure 3-6c,d) evoked by pinch of the ankle and toe 24 hours post ankle CFA injection at P8. Responses to pinch on the inflamed side, the opposite, non-inflamed side and naive controls are plotted on the same graph. In the ipsilateral biceps femoris, EMG activity was readily evoked from both the inflamed and non-inflamed sides both at the toe and the ankle. There were no significant differences in the reflex evoked from the inflamed ankle compared to the non-inflamed ankle. Reflexes evoked from the toe, by contrast, were significantly enhanced on the inflamed side. Inflammation did not affect either the rate of onset or rate at which the reflex subsides. Responses were also evoked in the biceps femoris contralateral to the side of the pinch (Figure 3-6c,d). Figure 3-6c,d shows that there is a profound enhancement of the contralateral reflex evoked by stimulating the inflamed ankle and toe. Importantly these responses are not seen in naive animals.

In summary:

At 24 hours post ankle inflammation at P8, there is central sensitization of spinal reflex circuits on the side of the inflammation, as shown by

- Enhanced responses from stimulation of the inflamed toe.
- Bilateral responses to stimulation of the inflamed ankle and toe.
- Responses from the inflamed ankle itself were unaffected.

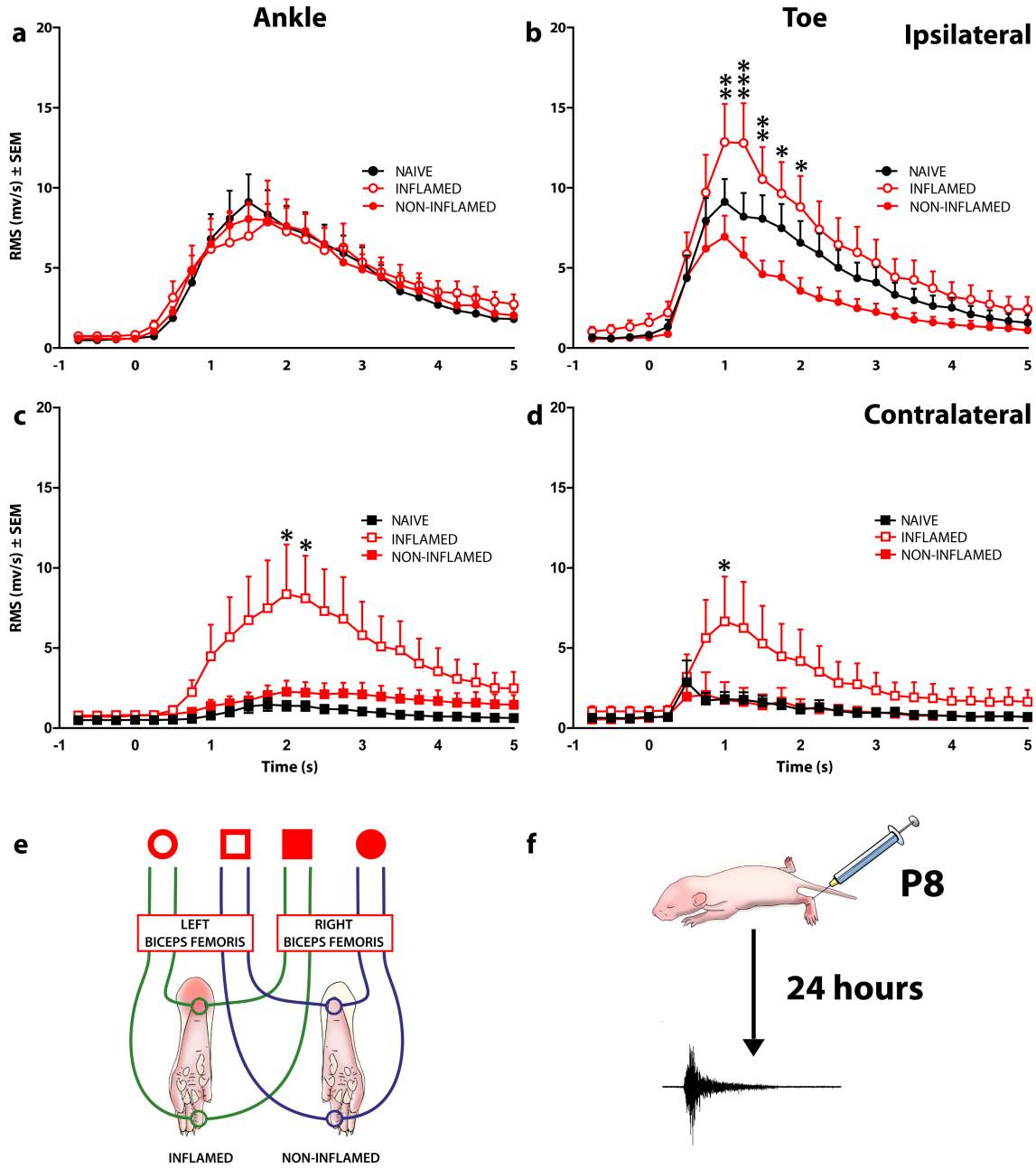


Figure 3-6 P8 EMG responses to pinch following CFA ankle inflammation
Responses in the biceps femoris ipsilateral and contralateral to pinch in CFA injected animals (n=6) at the ankle **(a,c)** and toe **(b,d)** on both the inflamed and non-inflamed sides are shown. Pooled naïve (n=6) responses are shown for reference in black. There is a significant enhancement of responses on the inflamed side compared to the non-inflamed side at the toe, but not the ankle. Inflamed vs. non-inflamed, *** p<0.001, ** p<0.01, *p<0.05). RMS Data is represented in 250ms time bins. Time(s) is shown on the x-axis, from 1s pre-stimulus, to 5s post-stimulus. Stimulation occurred at the '0s'. **(e)** Hollow coloured circles on the hind paw show stimulation sites, whilst the lines connect first to the biceps femoris in which the response was recorded and upward to the symbol that represents these responses on the graphs. **(f)** The age at which an animal was injected and the time after which a recording (represented by the raw trace) was performed are indicated on all graphs in the section that follows.

3.4.5.3 P21 and P40 ankle joint inflammation: 24 hours post injection

Figure 3-7 shows the mean EMG activity in the ipsilateral biceps femoris evoked by pinch of the ankle and toe 24 hours post ankle CFA injection at P21 (Figure 3-7a,b) and at P40 (Figure 3-7c,d). Responses to pinch on the inflamed side, the opposite, non-inflamed side and naive controls are plotted on the same graph. In contrast to the effects at P8, the ipsilateral nociceptive reflexes were attenuated following inflammation of the ankle joint at P21 or at P40. EMG activity evoked in the ipsilateral biceps femoris following both ankle and toe stimulation on the inflamed paw, resulted in a reflex that was substantially attenuated or in many cases, absent. Responses evoked from the inflamed ankle of P21 animals were significantly smaller than those evoked from naive and from the non-inflamed ankle in the first 250-750ms following pinch (Figure 3-7a, 2-way ANOVA, Bonferroni post-hoc). Responses evoked from the toe in CFA injected animals follow a similar pattern. There is an almost complete abolition of the reflex evoked from the inflamed toe in comparison to that evoked in naive control and from the non-inflamed toe (Figure 3-7b).

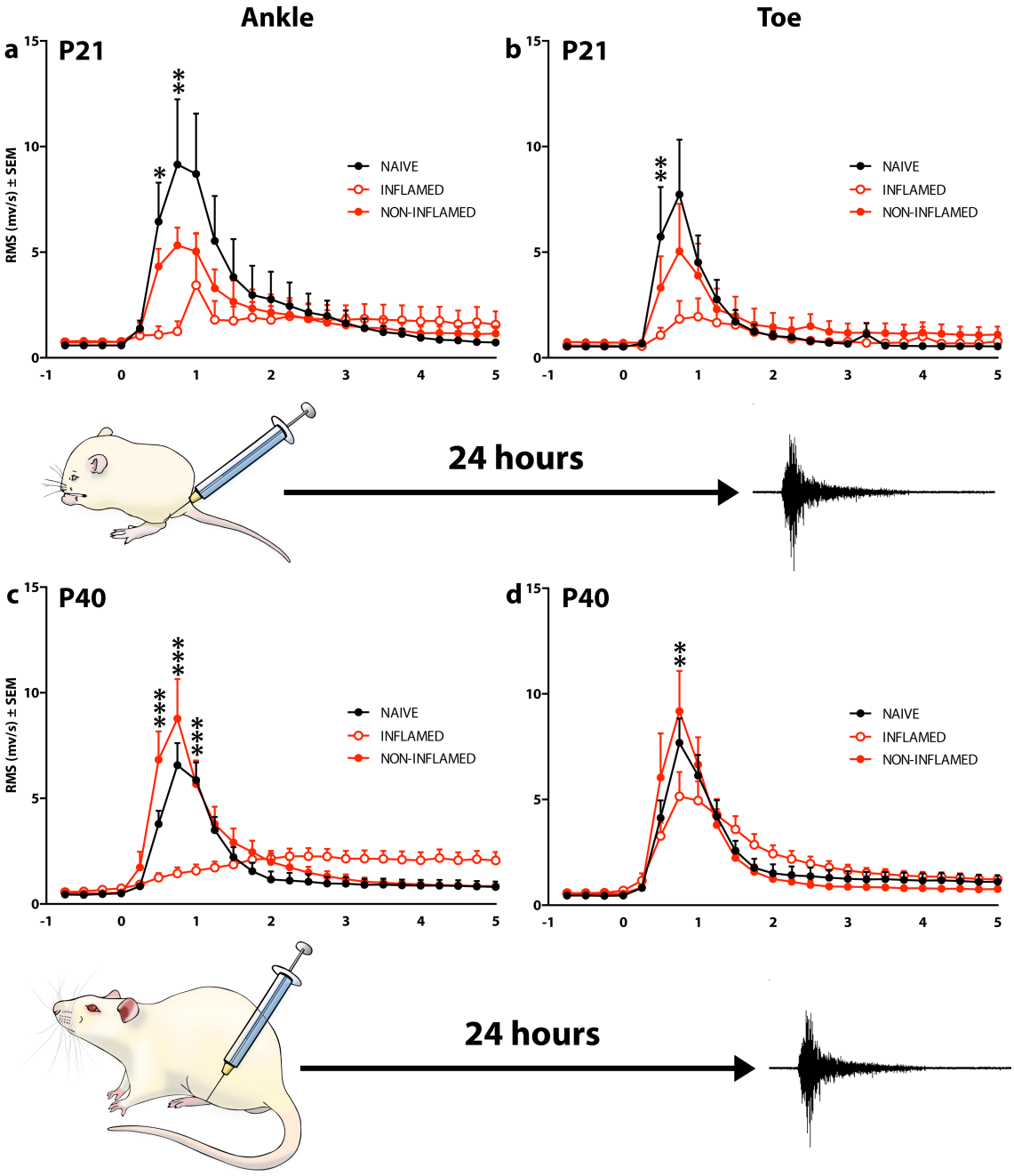


Figure 3-7 EMG responses ipsilateral to pinch 24 hours after CFA injection at P21 and P40
This figure shows the EMG responses from CFA injected animals (P21 n=12, P40 n=12) evoked in the BF on the side ipsilateral to pinch. Solid red points, represent the responses evoked from the BF on the inflamed side following pinch of the non-inflamed side at the ankle **(a)** and **(b)** toe. Hollow red circles represent responses evoked from the BF on the non-inflamed side, by pinch of the inflamed side. The black lines indicated the pooled responses of naïve animals (P21 n=12, P40 n=6) at the same site. Reflex responses to pinch were attenuated in P21 and P40 animals evoked from the ankle and the toe when compared to naïve. ***p<0.001, **p<0.01, *p<0.05, 2-way ANOVA, Bonferroni post-hoc RMS Data are represented in 250ms time bins. Time(s) is shown on the x-axis, from 1s pre-stimulus, to 5s post-stimulus. Stimulation occurred at '0s'.

In P40 animals, inflammation dramatically reduced the peak amplitude of the reflex EMG in the ipsilateral biceps femoris evoked by pinch of the inflamed ankle when compared to the non-inflamed side (Figure 3-7c, 2-way ANOVA, Bonferroni post-hoc). Additionally, responses remain significantly elevated compared to baseline throughout the 5s epoch. Ipsilateral EMG activity evoked by the inflamed toe is also significantly lower that that from the non-inflamed toe in this age group, within the 500-750ms time bin, though this is not as marked as the suppression of the ipsilateral reflex evoked from the inflamed ankle (Figure 3-7d).

The ipsilateral reflex responses evoked from the inflamed side of more mature animals, at both P21 and P40, following pinch of both the ankle and toe were also significantly attenuated when compared to naïve control. In contrast to younger animals there was no significant change in the reflex evoked in the biceps femoris contralateral to the pinch site in either P21 or P40 animals. There was no significant difference between contralateral responses of the P21 and P40 animals and their aged matched controls.

In summary:

- In animals inflamed at P21 reflexes evoked from the ankle and the toe on the inflamed side were significantly less than those of aged matched naïve controls.
- In P40 animals, inflammation resulted in almost complete abolition of the reflex from the inflamed ankle and a significant attenuation of reflexes from the inflamed toe.
- Responses from ankle or toe in P21 or P40 animals the non-inflamed side were not significantly different from control.

3.4.6 P8, P21, P40 ankle joint inflammation: 4 days post injection

At 4 days post injection, the P8 group were aged P12, but shall be referred from now on as P8 animals. Figure 3-8 shows EMG responses from the BFs ipsilateral to the site of pinch was P8, P21 and P40 animals, alongside pooled naïve controls. In contrast to EMG responses evoked at the ankle of inflamed P8 animals after 24 hours (Figure 3-6a) there is a reduction of the reflex on the inflamed side compared to the non-inflamed side. (Figure 3-8a, 2-way ANOVA, Bonferroni post-hoc). Pinch of the toe on the inflamed and non-inflamed sides evoked responses that were not significantly different from each other or pooled age matched naïve responses.

The pattern of reflex activity evoked from stimulation the ankle in P21 animals (Figure 3-8c) at four days was similar to that seen 24 hours following inflammation. Inflammation results in a highly significant attenuation of reflex responses to pinch in the inflamed limb compared to naïve controls, but not compared to the non-inflamed limb (2-way ANOVA, Bonferroni post-hoc). As was the case in the P8 group, responses to toe pinch were not significantly different between the inflamed and non-inflamed limbs or the inflamed limb responses and those of naïve controls (Figure 3-8d)

The reduced reflex response to pinch of the ankle remained at 4 days following CFA injection in P40 animals (Figure 3-8e). There was a highly significant reduction of reflex activity on the inflamed compared to the non-inflamed side in response to pinch of the ankle. There is also a highly significant difference between responses from the inflamed side and those from naïve controls (2-way ANOVA, Bonferroni post-hoc). There was striking similarity between responses evoked from the non-inflamed, inflamed and naïve toe at P40, and there were no significant differences between these groups.

At 4 days there was no effect of inflammation in any of the groups on reflexes evoked on the side contralateral to pinch (data not shown).

In summary:

At 4 days post ankle inflammation at P8, P21 or P40 the effects were as follows:

- In animals inflamed at **P8**, reflexes were not significantly different compared to naïve controls. There was an enhancement of reflexes on the non-inflamed side compared to the inflamed side. Reflexes evoked from the toe on the inflamed, non-inflamed sides and in naïve age matched animals were not significantly different.
- In animals inflamed at **P21** responses from the inflamed ankle were significantly reduced compared to age matched naïve animals, whist reflexes evoked from the toe on the inflamed, non-inflamed sides and in naïve age matched animals were not significantly different.
- In animals inflamed at **P40** responses from the inflamed ankle were significantly reduced compared to age matched naïve animals and when compared to the non-inflamed side. Reflexes evoked from the toe on the inflamed, non-inflamed sides and in naïve age matched animals were not significantly different.

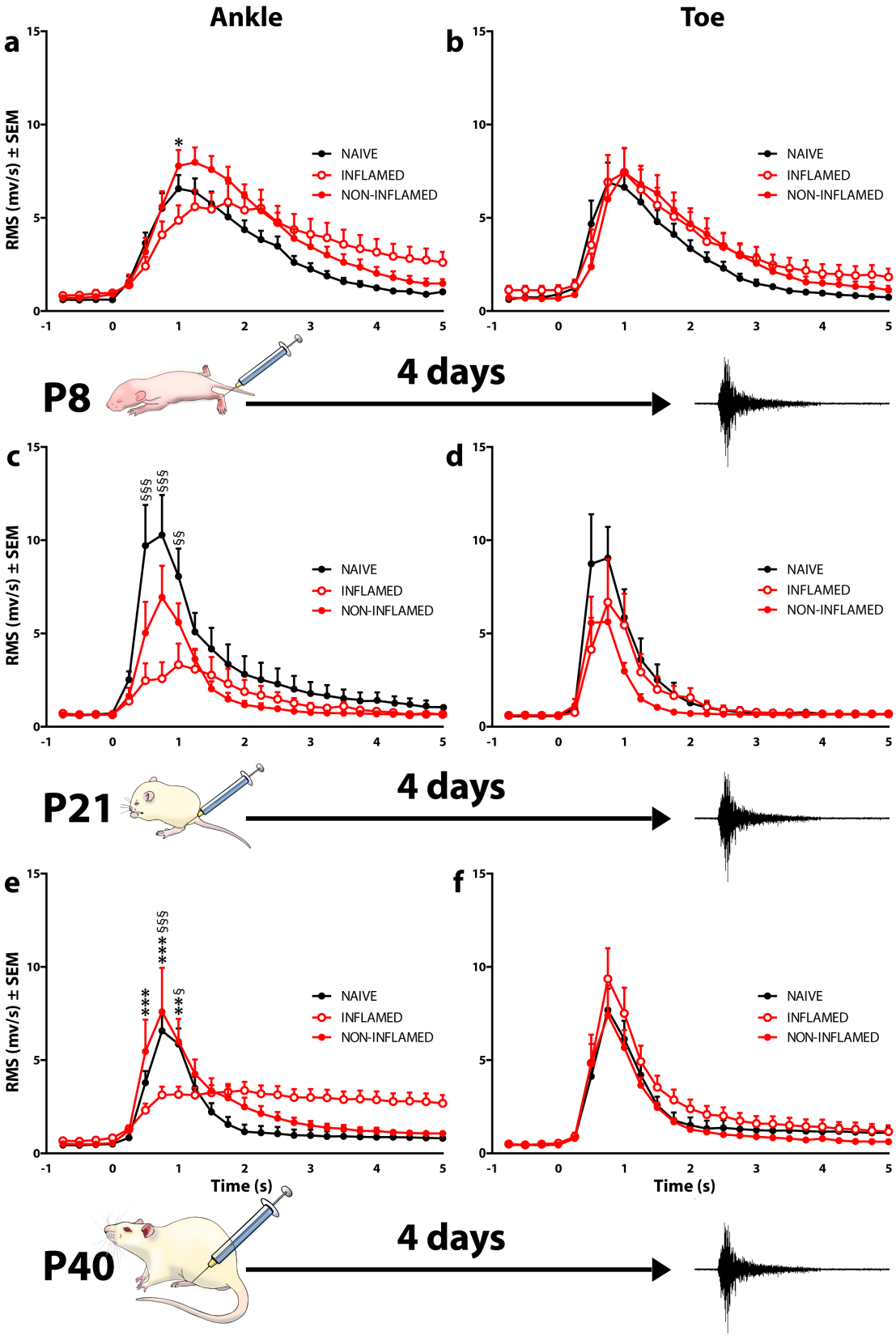


Figure 3-8 Pattern of ipsilateral reflexes 4 days following inflammation in P8, P21 and P40 animals
Responses from the BF ipsilateral to pinch on the inflamed and non-inflamed ankle **(a,c,e)** and toe **(b,d,f)** are plotted alongside pooled age matched naïve controls**.** In P8 animals **(a,b)**, inflammation results in a slight but significant suppression of reflex responses to pinch of the ankle but not the toe on the inflamed side compared to the non-inflamed side. In P21 animals **(c,d)** reflex responses evoked from the inflamed ankle but not the, toe were significantly attenuated compared to the non-inflamed side. In P40 animals **(e,f)** reflex responses from the inflamed side were significantly attenuated when compared to responses from naïve control. **For all graphs**: inflamed vs. non-inflamed (***p<0.001, **p<0.01, *p<0.05) and inflamed vs. naïve control (§§§p<0.001, §§p<0.01, §p<0.05) All tests 2-way ANOVA, Bonferroni post-hoc. RMS Data is represented in 250ms time bins. **Time(s)** on the x-axis, from 1s pre-stimulus, to 5s post-stimulus. Stimulation

3.4.6.1 P8, P21, P40 ankle injection: 10 days post-injection

Ten days post-injection, the pattern of EMG responses changed substantially. Unlike the depression observed at 24 hours and 4 days there was a clear and substantial enhancement of reflex response to pinch of both the inflamed and non-inflamed ankles compared to control. From 1000-2500ms the inflamed and non-inflamed responses are significantly greater than naïve control (Figure 3-9a). In addition, pinch of the toe on the inflamed side resulted in an enhanced response compared to the non-inflamed side (1000-1500ms) and naïve control (1000-2000ms) (Figure 3-9b).

Responses to pinch recorded in the P21 group after 10 days were the largest of any time-points or age groups presented in this chapter. It should be noted that the scale employed in Figure 3-9c,d is 0-40mV rather than the usual 0-15mV or 0-20mV. Responses evoked from the ankle in the inflamed side were significantly enhanced compared to naïve control (1250-3000ms), but not compared to responses from the non-inflamed side (Figure 3-9c). Interestingly there was a significant enhancement of responses on the non-inflamed side (not indicated on graph; 250-500ms, $p<0.05$ and 500-750ms, $p<0.01$) to pinch of the toe (Figure 3-9d) compared to naïve control. In addition, there was a significant enhancement of responses evoked from the inflamed toe compared to both responses from the non-inflamed side (250-1250ms) and naïve control (250-2000ms).

The pattern of reflex responses in the P40 group 10 days post-injection of CFA, closely resembles that seen in the P21 group, though differing in magnitude. A significantly larger response was evoked from the inflamed ankle compared to naïve controls (1250-3000ms), but not compared to the non-inflamed side (Figure 3-9e). There was a significantly larger response from the toe on the inflamed side compared to naïve control (500-750ms), however, there were no significant differences between the responses from the inflamed side and the non-inflamed side (Figure 3-9f).

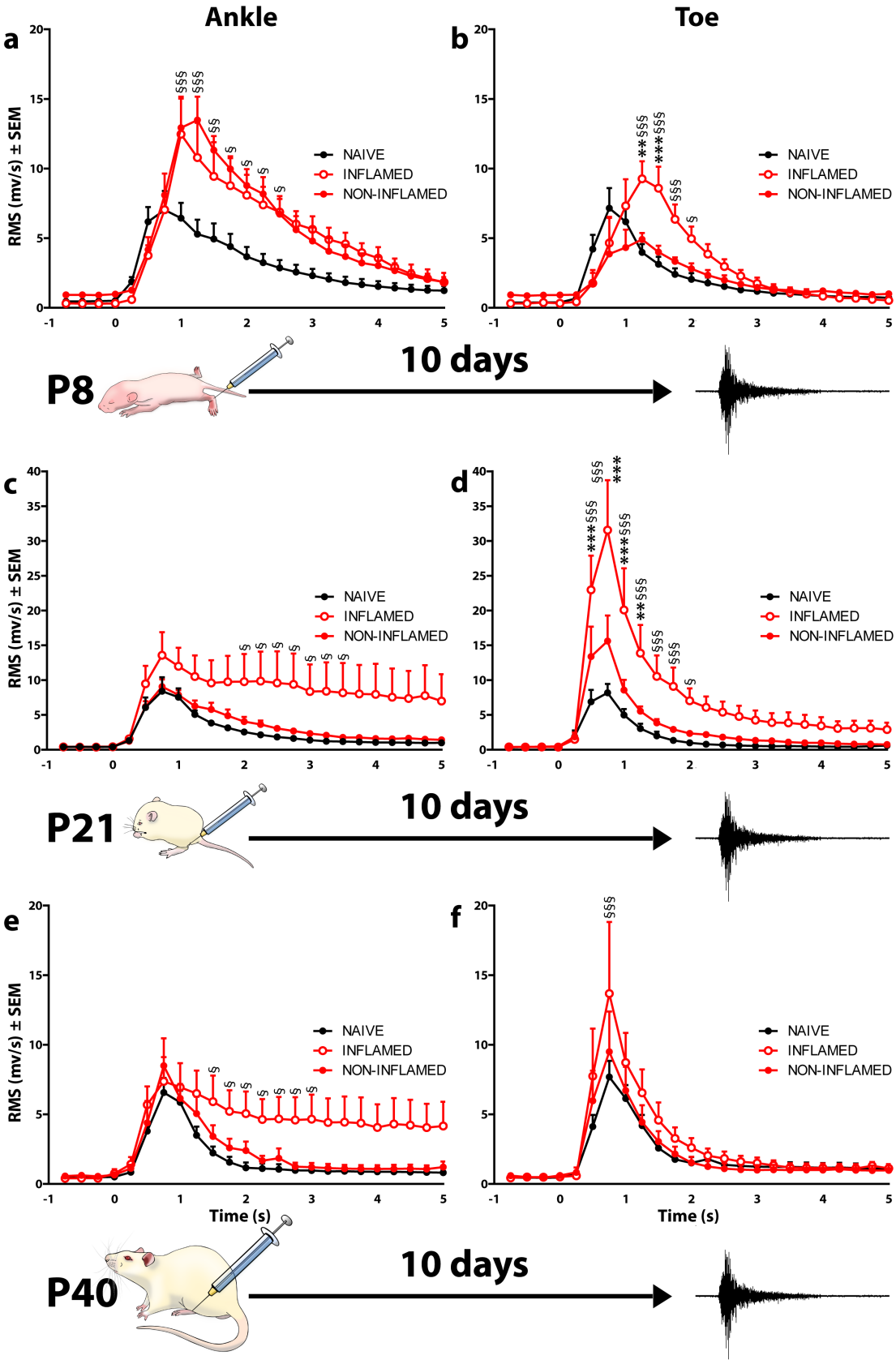

Ankle
Toe
a
b
c
d
e
f
RMS (mv/s) ± SEM
NAIVE
INFLAMED
NON-INFLAMED
Time (s)
P8
P21
P40
10 days
10 days
10 days

Figure 3-9 Pattern of reflexes 10 days following inflammation in P8, P21 and P40 animals
Figure, on previous page, shows responses from the BF ipsilateral to pinch on the inflamed and non-inflamed ankle **(a,c,e)** and toe **(b,d,f)** alongside pooled age matched naïve controls**.** In P8 animals **(a,b)**, inflammation results in a significant enhancement of reflex responses to pinch of the ankle **(a)** on the inflamed side and non-inflamed sides compared to naïve controls. Responses to pinch of the toe were enhanced compared to the non-inflamed side and naive controls. In P21 animals **(c,d)** reflex responses evoked from the inflamed ankle **(c)** are enhanced compared to naïve control. Response to pinch of the inflamed side toe is significantly enhanced compared to the non-inflamed side and naïve control. In P40 animals **(e,f)**, reflex responses from the inflamed side were significantly enhanced compared to naïve control. **For all graphs**: inflamed vs. non-inflamed (***p<0.001, **p<0.01, *p<0.05) and inflamed vs. naïve control (§§§p<0.001, §§p<0.01, §p<0.05) All tests 2-way ANOVA, Bonferroni post-hoc. Data is represented in 250ms time bins. Time(s) is shown on the x-axis, from 1s pre-stimulus, to 5s post-stimulus. Stimulation occurred at '0s'.

In summary:

By 10 days inhibition has turned into excitation:

- In animals inflamed at **P8**, reflexes were bilaterally enhanced at the ankle compared to naïve. Responses from the toe on the inflamed side were significantly larger than those on the non-inflamed side and those of naïve controls.
- In animals inflamed at **P21** responses from the inflamed side were profoundly enhanced compared to control.
- In animals inflamed at **P40** responses from the inflamed ankle and toe were enhanced compared to naïve control but were not significantly different from the non-inflamed side.

3.4.7 Dorsal horn responses to pinch in the presence of inflammation

Figure 3-11 shows data collected from animals four days post-CFA injection at P8 or P40, plus age matched controls. Extracellular recordings of individual DH cells were performed *in vivo* and the total numbers of cells recorded and the numbers of animals from each experimental group were as follows: P8 – Naïve (n=4) 19 cells, CFA (n=4) 20 cells, P40 – naïve (n=4) 23 cells, CFA (n=4) 23 cells.

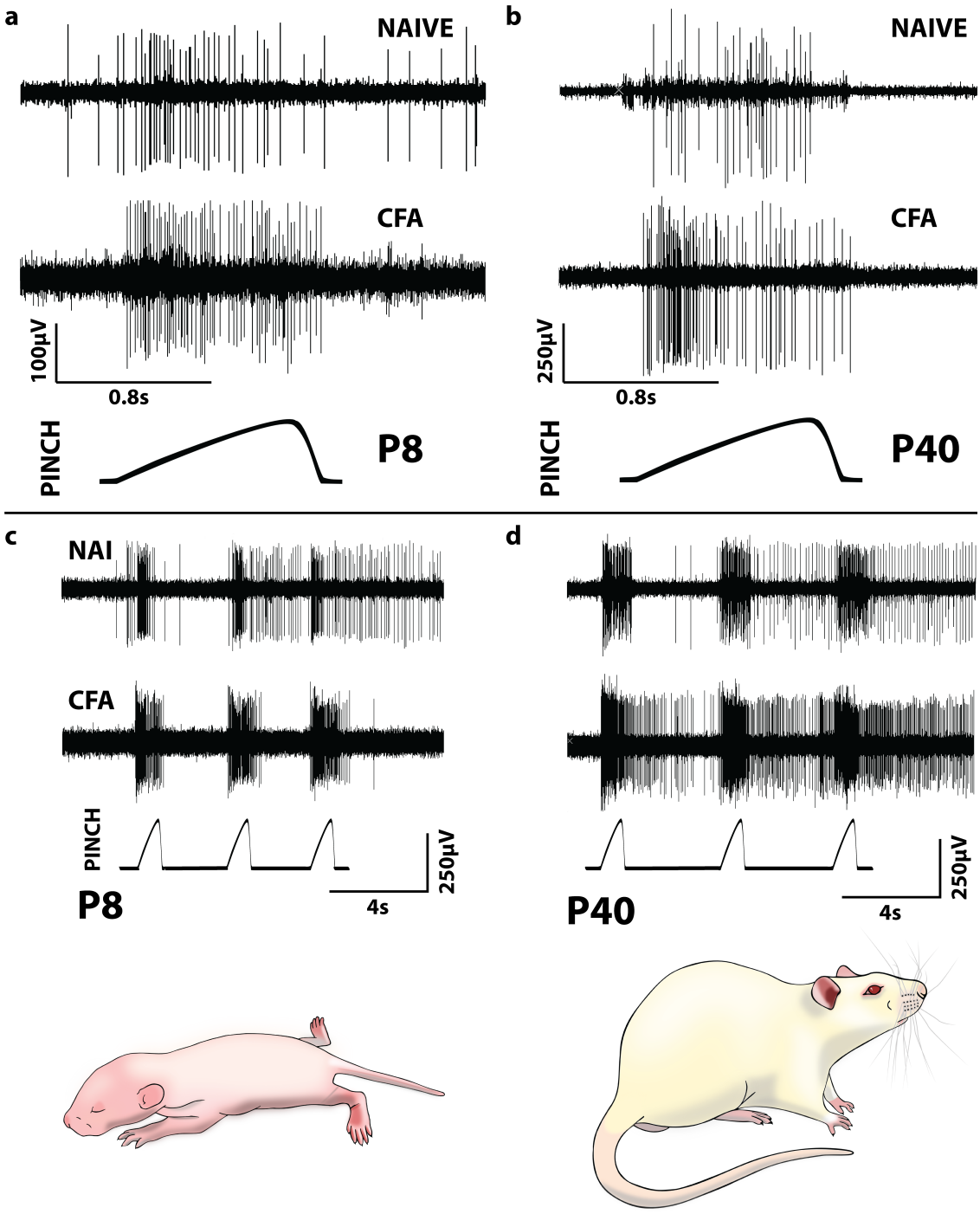


Figure 3-10 Raw recordings from single DH neurones four days post CFA injection
Raw representative recording data is shown from naïve and CFA injected animals. **(a)** and **(b) s**how the response of an individual DH neurone to a single noxious pinch of the ankle 4 days post-CFA injection at P8 or P40 compared to aged matched controls. **(c)** and **(d)** show the response of single neurones to 3 consecutive pinches separated by 3 seconds on naïve or CFA treated ankles.

Once a single unit was isolated a baseline level of 5 minutes was recorded. DH neurones in P8 CFA inflamed animals displayed a significantly lower level of spontaneous activity compared to control. There was no significant reduction in adult animals, but the same trend was evident (Figure 3-11a). After a period of 5 minutes the ankle was pinched once and all ongoing activity was recorded until activity ceased. Pinch evoked activity (in 5s from the beginning of the pinch) was doubled when evoked from an adult inflamed joint, but not in a young inflamed joint (Figure 3-11b). Following the single pinch the response to a train of 3 pinches with 5s between pinches was recorded. The average ankle pinch evoked response in DH neurones in the P8 CFA treated animals from three consecutive pinches was not significantly different from control (Figure 3-11c). The activity evoked by each consecutive pinch was not significantly different in the P8 or P40 groups (Repeated measures 2-way ANOVA, Bonferroni post-hoc). Post-hoc analysis confirmed that P40 animals in the CFA group showed significantly enhanced responses to pinch across all pinches (Figure 3-11c). Mean responses from each pinch were greater in the neurones of animals injected at P8 than naïve controls, however, this was not significant (Figure 3-11c).

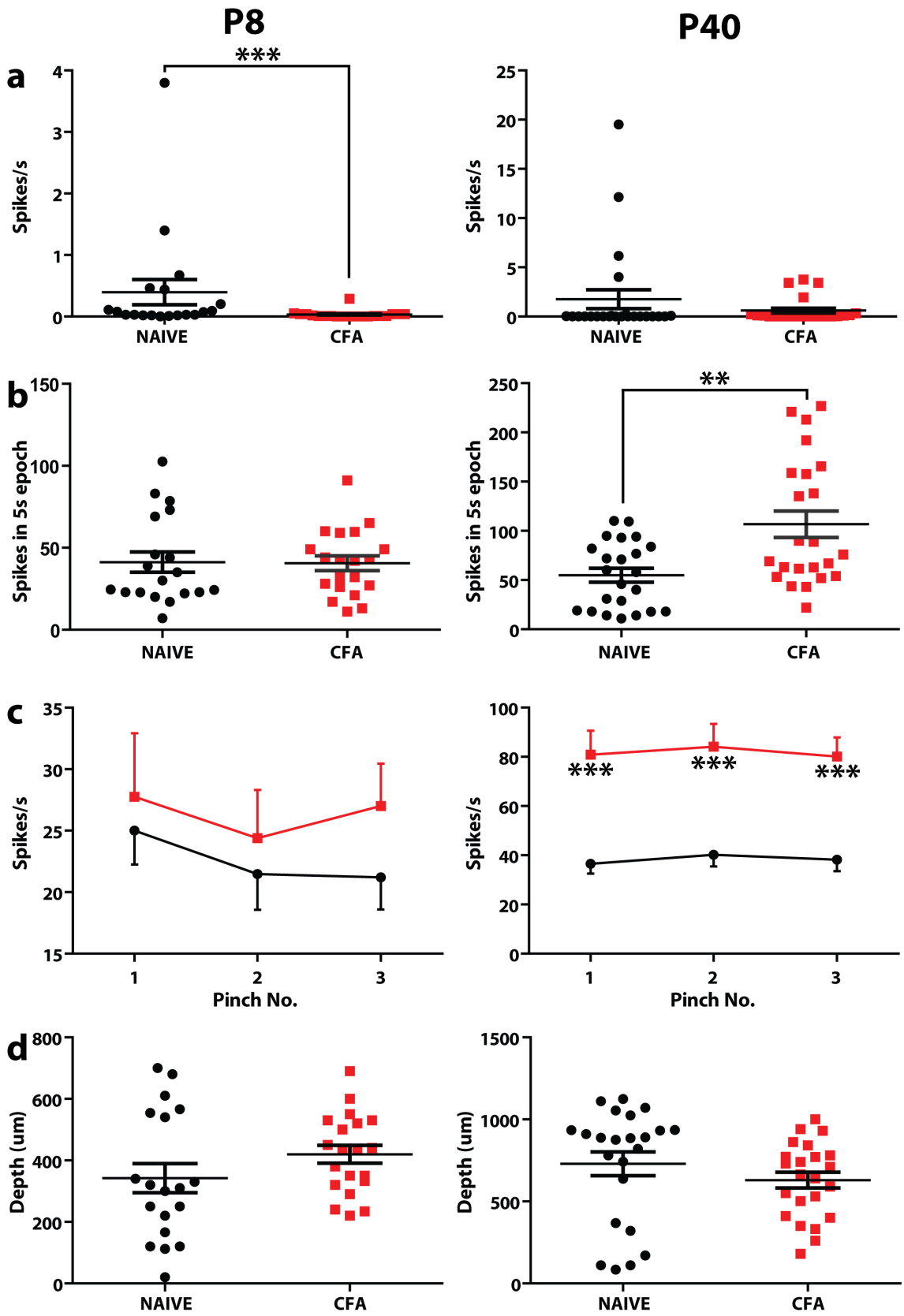


Figure 3-11 DH neuronal responses to noxious pinch of naive and inflamed joints 4 days following CFA
Dorsal horn extracellular recordings were performed on animals that had received CFA 4 days previously P8 – naïve (n=4) 19 cells, CFA (n=4) 20 cells, P40 – naïve (n=4) 23 cells, CFA (n=4) 23 cells. **(a)** Spontaneous activity was significantly reduced in the presence of CFA ($p<0.001$, unpaired two tailed t-test) at P8, but not P40. **(b)** A single noxious ankle pinch evokes significantly more activity in the DH of animals that received a CFA injection at P40 compared to naïve ($p<0.01$, unpaired two tailed t-test) but not in animals injured at P8. **(c)** There was no significant change in the response with each subsequent pinch (naïve – black, red – CFA) at either age, there was a significant effect of treatment and post-hoc analysis revealed significant enhanced responses the CFA group (Repeated measures ANOVA, Bonferroni post-hoc). **(d)** Shows the recording depths for individual cells.

Summary of results

The data presented above can be summarised as follows:

1. Injection of CFA into the ankle reliably induced redness and swelling across all three ages (P8, P21 and P40). CFA injected ankles were significantly larger than naïve controls. Ankle inflammation was significantly greater in P40 animals than P8 animals at one, four but not 10 days post-injection.

2. Inflammation led to significantly increased sensitivity of the hind paw to mechanical stimulation and significantly reduced weight bearing compared to control in adult (P40) animals. Increased mechanical sensitivity was evident from four hours post-injection for up to 10 days, peaking at two days following injection of CFA. Weight bearing was significantly different from controls from days one through to three and returning to baseline more rapidly than mechanical sensitivity.

3. In contrast to adult animals, injection of CFA at P8 had no significant effect on either of these behavioural measures.

4. Nociceptive flexion reflex EMG recording revealed effects at P8, not evident from behavioural analysis. 24 hours following P8 CFA injection reflexes evoked from the inflamed ankle were no different from the non-inflamed ankle but there was an enhancement of reflex responses to pinch of the toe compared to the non-inflamed side and naïve control. Interestingly, pinch of the non-inflamed limb led to exaggerated contralateral reflex responses on the inflamed side, suggesting some secondary hyperalgesia. By 4 days following CFA there is a reduction of the response from inflamed side compared to the non-inflamed side but by 10 days reflexes from both inflamed and non-inflamed limbs in the P8 group are significantly facilitated compared to naïve control suggesting continuing central sensitization.

5. At P21 and P40 animals the pattern of reflex activity to pinch differed strikingly from that of P8, and also revealed changes not observed in behavioural analysis. At both P21 and P40, a profound and significant

attenuation of reflex responses to pinch of the inflamed ankle occurred 24 hours after CFA compared to responses from the non-inflamed ankle and naïve controls. There was smaller but significant attenuation of reflexes evoked from the inflamed toe at these ages. The suppression of reflex activity in response to pinch of the ankle on the inflamed side remained present at four days after CFA but no longer affected the toe at this time-point. At 10 days post CFA, inhibition of reflex activity turns to facilitation of the reflex response to pinch at both the ankle and the toe. The facilitation is particularly marked in the P21 group.

6. CFA ankle injection at P8 had no effect upon the dorsal horn responses to pinch of the inflamed ankle four days later. By contrast, in adult animals ankle inflammation increased pinch evoked DH responses 2-fold.

3.5 Discussion

The results presented in this chapter describe behavioural responses as well as the hindlimb flexion reflex activity following joint inflammation in P8, P21 and P40 at 24 hours, 4 days and 10 days post inflammation. In addition extracellular recordings of DH neurones' responses to pinch of the inflamed ankle 4 days post inflammation were examined at P8 and P40.

3.5.1 Methodological considerations

3.5.1.1 Control Animals

In this section EMG response data from animals that received intra-articular injections of CFA were compared to responses from naïve animals. It was established in pilot experiments that EMG responses from animals that received intra-articular injections of saline were not significantly different from those of naïve animals. It was therefore unnecessary and unjustifiable to use saline-injected animals as controls.

3.5.1.2 Joint volume and injections

Size differs dramatically between animals of different postnatal ages in this study. Of central importance to this study is the size and volume of the ankle joint space at difference postnatal ages. In order to develop a rodent model of juvenile arthritis it has been necessary to critically evaluate the literature and adapt a model of adult inflammation to younger rodents. Concerns arose initially about the volumes of various inflammogens that authors of previous studies had injected into joint cavities. Forty to fifty micro-litres is a volume that is commonly used in both the knee and ankle joints (Danziger et al., 1999; Cruz et al., 2005; Vermeirsch et al., 2007; Angeby-Möller et al., 2008), though some key studies have employed 100µl (Radhakrishnan et al., 2003; Kelly et al., 2007), or even 150µl (Butler et al., 1992; Hanesch et al., 1995). The pressure of large volumes is likely to cause discomfort to the injected animal independent of inflammation. Furthermore, if excessive volumes are forced into a closed space under pressure, it stands to reason that some of that volume is likely to spill out and inflame surrounding tissues. This may be a source of confounding in previous studies. Despite comparatively small volumes of CFA injected into the ankle for these experiments,

measures of joint swelling demonstrate CFA injection in small volumes could reliably and repeatedly induce inflammation across all age groups (see Figure 3-3).

3.5.2 Comparison of behavioural and EMG measures of inflammatory pain

There is an apparent disconnect between the results of EMG studies presented in this study and results from behavioural assays of hypersensitivity. They are as follows:

1) Joint inflammation at P8 results in secondary hypersensitivity which is apparent in EMG studies at 24 hours post-inflammation. By contrast, mechanical thresholds of CFA injected animals and weight bearing in this group are no different from naïve or saline injected controls.
2) At 10 days post-inflammation of P8 animals EMG studies reveal that reflex circuits from the ankle and the toe are sensitised. There are, however, no changes to mechanical thresholds on the hind paw or the weight borne on the inflamed limb.
3) In P8 animals 24 hours post-inflammation contralateral reflex circuits are driven by inputs from the inflamed side in absence of perturbations of mechanical sensitivity on the non-inflamed side.
4) In adults, flexor reflex responses from the ankle and the toe are significantly dampened by the presence of joint inflammation. This is consistent with the reduced weight bearing on the affected limb, but is inconsistent with reduced mechanical thresholds as assessed by von Frey hairs.
5) In adults, at 10 days post-inflammation, while mechanical hypersensitivity as assessed is no different from controls, EMG studies show that flexion withdrawal circuits from the ankle and toe are facilitated compared to naïve animals.

These observations are two sides of the same metaphorical coin, which if considered separately may appear incongruous, but together provide more insight into the central changes associated with joint inflammation in young and adult animals. This is addressed in more detail in the following sections.

3.5.3 Behavioural measures of hypersensitivity associated with joint inflammation

3.5.3.1 Mechanical thresholds

Cutaneous mechanical thresholds of the skin on the hind paw were determined using calibrated vF hairs following the injection of CFA. Close observation of inflamed animals revealed a 'reluctance' to bear weight on the affected limb whilst moving around their home cage, meanwhile the paw, toes curled, was held tight and close to the body elevated away from the bottom of the cage. When vF hairs were applied, the reflex withdrawal and flick well described in this behavioural paradigm (Chaplan et al., 1994), were seldom seen, rather the animal would 'rock away' from the stimulus, transferring the weight onto the non-inflamed side. Nevertheless, inflamed adults would rock away from stimuli to which saline injected and naïve animals did not react. Often, when the vF filament was applied, the paw was pushed out of the way with the animal offering little or no resistance to the stimulus. This appears to be a highly appropriate behaviour to adopt in the presence of a damaged joint. Moving the joint is likely to evoke a substantial afferent barrage, even slight flexion within the normal working range is likely to be unpleasant (Schaible and Schmidt, 1985b). This behaviour appears to be a 'protective' inhibition whilst joint inflammation is most severe and has been observed before (Butler et al., 1985; Coderre and Wall, 1987, 1988a). It is disadvantageous to move the joint at this stage following inflammation when a vF hair is applied in spite the sensitivity of the joint and surrounding skin of the hind paw (Calvino et al., 1987a). Flexor EMG recordings tell a similar story. Reflexes are dampened by inflammation of both the ankle and the toe, as contraction of the biceps femoris, regardless of input, would result in unwarranted movement of the joint. Flexion reflex EMG considers the action of a single muscle in isolation, without knowing how other surrounding muscle groups react. This is in contrast to the observation of classic behavioural hypersensitivity which requires reflex circuits to be intact. Enhancing reflexes in cutaneous inflammation is an appropriate response to remove the affected skin from harm, yet this is not true in joint inflammation. The assessment of pain behaviours in models of joint inflammation clearly requires more integrated measures to accurately characterise hypersensitivity and dysfunction where the joint is compromised. This has, in part, driven investigators to employ new techniques. Videoradiographic analysis,

for instance, can accurately assess range of movement, the swing and stance phases of gait and postural changes in freely moving animals following experimental joint inflammation (Boettger et al., 2011). Other forms of gait analysis are useful dynamic measures of joint dysfunction and hypersensitivity and both measures are sensitive to analgesic and anti-inflammatory treatments (Angeby-Möller et al., 2008).

Despite of the limitations of traditional assessments of behavioural hypersensitivity there appears to be reluctance to use more integrated measures of inflammatory pain (Butler et al., 1992; Danziger et al., 1999; Cruz et al., 2005; Seino et al., 2006; Wilson et al., 2006; Geranton et al., 2007; Vermeirsch et al., 2007; Lu et al., 2008). The discrepancies between EMG studies and behavioural studies presented here reiterate that behavioural assessments of pain must be both appropriate to the model under scrutiny and not reliant on systems that may be compromised by the experimental pain itself.

3.5.3.2 Behavioural hypersensitivity in young rodents following joint inflammation

Strikingly, animals showed no mechanical hypersensitivity at any time-point following CFA injection at P8 (see Figure 3-4b). At 2 hours post-injection there was a non-significant fall in thresholds in the saline and CFA injected groups which was the only perturbation seen in this period; most likely due to some residual sensitivity following the trauma of injection. From 4 hours until 21 days post injection, mechanical thresholds developed normally (Fitzgerald, 2005). Others have established that at this age and even as early as P0 mechanical and thermal thresholds can be measured in rat neonates following peripheral inflammation although the effects observed were not as pronounced as those seen in older animals (Marsh et al., 1999b; Marsh et al., 1999a; Lidow et al., 2001; Walker et al., 2003). Withdrawal reflexes at this age are immature, often inappropriate and may move the stimulated skin toward the stimulus (Waldenström et al., 2003). It is important to consider that disrupting muscle action across a joint, for instance by neonatal tendon transfer, results in a reorganisation of reflex receptive fields on the hind paw (Holmberg et al., 1997). It is possible that the impediment of a swollen and ungainly joint may have the same effect compromising the display of 'classic' behavioural hypersensitivity. Taken together, and in light of the

hypersensitivity seen in reflex EMG studies, it is clear that testing of mechanical thresholds on the hind paw in the presence of joint inflammation in young animals does not reflect the true excitability of spinal cord nociceptive circuits.

These observations suggest that measures of experimental pain must not only be appropriate to the model studied, but also make careful consideration of the development of the system studied.

3.5.3.3 Weight bearing

Weight bearing was assessed using an incapacitance meter. Weight bearing has been a widely used measure of dysfunction and a correlate of hypersensitivity in experimental joint pain (Otsuki et al., 1990; Vermeirsch et al., 2007; Boettger et al., 2010; Boettger et al., 2011). By one day post-injection weight bearing in adult CFA-injected rats is significantly reduced on the inflamed side compared to control (Figure 3-5d). There is a concurrent shift of weight onto the uninflamed paw. This is consistent with many previous studies. By comparison to mechanical thresholds measured by vF, changes in weight bearing appear to be a less variable and more robust measure of hypersensitivity associated with ankle joint inflammation. Both measures showed that at sometime between 10 days and 21 days post injection weight bearing and mechanical thresholds returned to near normal values. By 1 day post–injection weight bearing in adult CFA injected animals was significantly different from control. By comparison, mechanical thresholds in CFA injected animals were significantly different from control by 4 hours post-injection. Clearly, dysfunction and changes to stance and gait are slower to develop than frank hypersensitivity.

In younger animals there was no evidence for shifts in weight bearing between left and right hindlimbs (Figure 3-5d). Surprisingly, the weight borne on both hindlimbs over first 10 days remained unchanged despite substantial growth of the animal. One possibility is that following inflammation, young animals place an increasing proportion of weight on their forelimbs but there is little information on weight bearing in this age group. Locomotor activity in young rats is limited to jerky and poorly directed movements, typified by pivoting and crawling (Altman and Sudarshan, 1975). From observation of this age group, it is clear that a substantial proportion of weight is placed on the belly with iimbs

splayed out laterally. Hindlimb weight bearing in an open field does not appear until P12-13, and walking until P14-15 (Altman and Sudarshan, 1975). What is interesting is that even when these animals begin to weight bear in a more adult-like fashion there is no change in the weight distribution between limbs in spite of the presence of ongoing inflammation (Figure 3-3k).

These results are unlikely to be due to limitations of equipment. Incapacitance meters have been repeatedly and reliably used to assess changes in mice of similar size and weight (Lolignier et al., 2011; McNamee et al., 2011; Jimenez-Andrade and Mantyh, 2012; Knights et al., 2012). although it is important that the mice were adults and therefore weight bearing is likely to have differed.

In light of the EMG data presented here the absence of changes in weight bearing in younger animals is consistent with the lack of with joint freezing and may represent a lack of adaptive joint protection.

3.5.4 Hypersensitivity and freezing: two forms of protective responses to joint inflammation

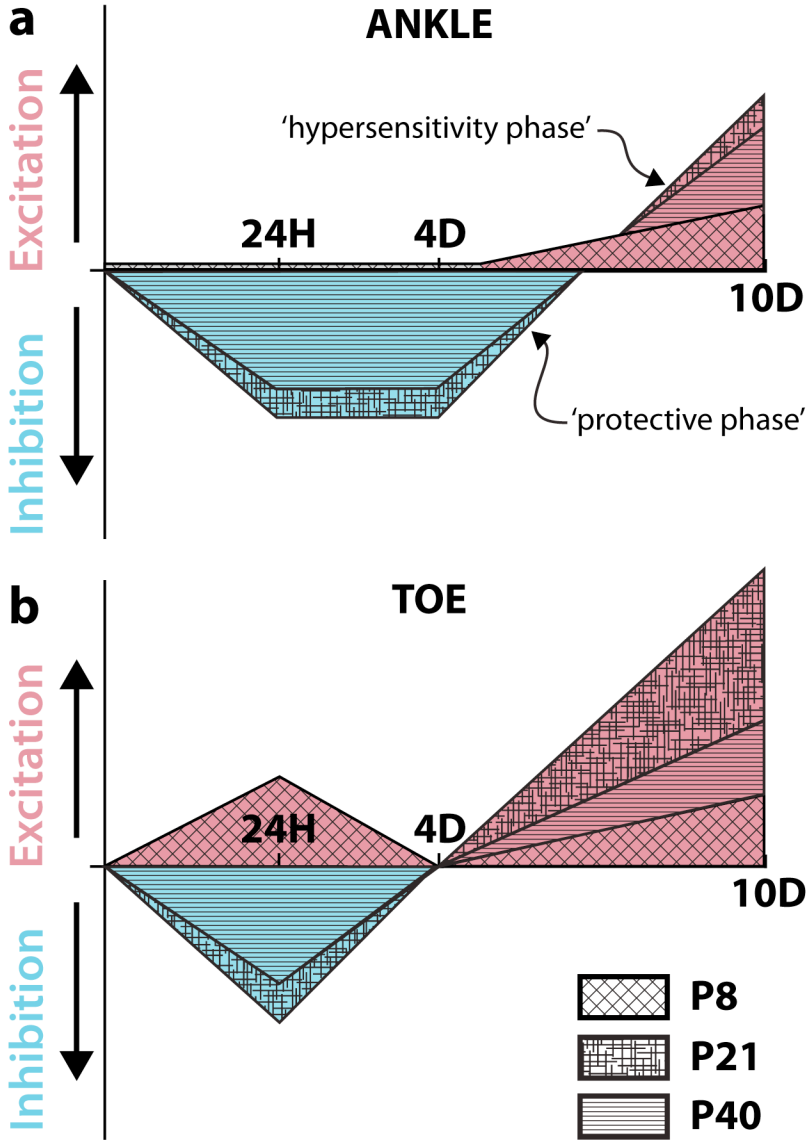


Figure 3-12 Schematic representation of changing reflex excitability following inflammation at different postnatal ages

The figure above is a schematic representation of flexor reflex activity on the **side ipsilateral to pinch only** for each age relative to naïve animals. **(a)** For instance, the flexor reflex activity evoked from the ankle at 24hrs post-inflammation was the no different in P8 CFA treated animals compared to naïve, but toe evoked reflexes **(b)** were enhanced (shown as red) at this time-point. Inhibition (shown as blue) of reflex activity was apparent from the ankle and the toe at 24 hours post-inflammation in animals inflamed at P21 and P40. At 4 days P21 and P40 animals showed reflex inhibition at the ankle but not the toe. By 10 days post-inflammation reflexes evoked from both the ankle and the toe were enhanced in all age groups. Note: in **(a)** there is a grey bar for the P8 group at 24 hours and 4 days post-inflammation as reflexes were not significantly different from naïve at these time-points.

Figure 3-12 is a schematic representation of the flexor reflex data presented in this chapter. Inhibition of reflex activity is represented by blue whilst red represents enhanced reflex activity compared to naïve. The response to joint inflammation is characterised by two discrete phases, an acute protective phase which transforms into a hypersensitivity phase by 10 days. Intriguingly, the hypersensitivity emerges as behavioural measures are beginning to return to baseline. Postural adjustments may account for the apparent reduction in sensitivity. Indeed, regardless of the cause, behavioural measures normalise in a period of growing hypersensitivity in spinal reflex circuits. It would be interesting to establish the full extent of spinal reflex sensitivity following joint inflammation using EMG. In P21 and P40 groups the specific time course is different depending on the site stimulated, but the overall pattern is very similar. In stark contrast to P21 and P40 animals, whose reflex responses to pinch were dampened following joint inflammation, there was a bilateral enhancement of reflex activity in the P8 group 24 hours after injection. These findings are in contrast to, but not incompatible with, the classical enhancement of reflex responses and fall of reflex thresholds following skin inflammation (Woolf and McMahon, 1985; Ma and Woolf, 1996). Animals inflamed at P8 lack the protective phase, but clearly show the hypersensitivity phase.

Recordings from the DHs of arthritic adult rats showed the well-described enhancement of individual neuronal responses to pinch (Grubb et al., 1993) (Schaible and Schmidt, 1985b; Schaible and Schmidt, 1988b). Unexpectedly, there was no difference between the evoked DH responses of young animals that were inflamed compared to control. The following section will address the differences that exist between pain and inflammation originating from the joint and contrast it with that originating from the skin as well as examining the extent to which the immaturity of sensory systems in young animals may contribute to the responses seen in animals of different ages.

3.5.5 Central sensitisation following joint inflammation

Reduced mechanical reflex thresholds and enhanced reflex amplitude and duration are well-described phenomena in both young and adult animals following peripheral cutaneous inflammatory injury (Woolf and Swett, 1984; Woolf and Wall, 1986; Walker et al., 2007). To date, the changing reflex excitability

following joint inflammation over an extended period or in the context of development has not been investigated. In cutaneous inflammation in adult animals, behavioural hypersensitivity distant from the original site of injury is brought about by the changing excitability of central reflex circuits i.e., central sensitisation (Woolf, 1983; Woolf and Wall, 1986). Enhanced neuronal and behavioural responses to noxious stimuli (hyperalgesia) and the perception of previously innocuous stimuli as painful (allodynia) are also features of sensitised central circuits in models of arthritic pain (Neugebauer and Schaible, 1990; Grubb et al., 1993; Martindale et al., 2007).

24 hours following inflammation in the P8 group there was a profound facilitation of the reflex evoked from the toe on the inflamed side. Pinch on the inflamed side also evoked reflexes on the non-inflamed side which were greater than those seen in naïve animals (Figure 3-6d). These findings imply that a profound and widespread sensitisation is induced in young animals following inflammation. Whilst the net effect of joint inflammation on motor output in adults is inhibitory, the widespread excitation in young animals and the DH of adult animals demonstrates the well-described sensitisation present following CFA joint inflammation (Schaible and Schmidt, 1988b; Schaible et al., 1991; Grubb et al., 1993; Neugebauer et al., 1993; Boettger et al., 2008; Telleria-Diaz et al., 2010). Neurones show enhanced responses to noxious stimulation of the inflamed joint, and the normally high threshold nociceptors become responsive to non-noxious stimuli (Schaible et al., 2009). In spinalised animals, where descending influences are absent, responses are enhanced compared to animals with intact descending systems (Neugebauer and Schaible, 1990). As joint inflammation progresses descending controls begin to modulate DH neuronal responses more powerfully (Schaible et al., 1991). This may in part contribute to enhanced pain transmission in young animals where descending control is still immature and facilitatory (Hathway et al., 2009). A state of central sensitisation may be apparent within hours of joint inflammation, but can persist for weeks (Menétrey and Besson, 1982; Grubb et al., 1993; Martindale et al., 2007). Cutaneous receptive fields of arthritic animals are chronically enlarged and show enhanced responses to A- and C-fibre inputs (Martindale et al., 2007). Central sensitisation in arthritic pain is dependent on the glutamatergic NMDA and AMPA receptors (Neugebauer et al., 1993). Blocking the action of excitatory neurotransmission through these

receptors prevents reflex wind-up to mustard oil or electrical stimulation (Woolf and Thompson, 1991). Other factors that contribute to enhanced DH excitability following joint inflammation include the release of excitatory amino acids as well as the neuropeptides SP, neurokinin A and CGRP (Schaible et al., 2009). All of these factors are likely to have contributed to the enhancement of DH neuronal responses to noxious pinch of the ankle which showed a doubling of discharge rate compared to control (Figure 3-11).

Importantly, high levels of expression of AMPA and NMDA receptors are seen in the immature spinal cord and over the first postnatal weeks the expression of these receptors becomes increasingly anatomically restricted to the superficial DH (Jakowec et al., 1995; Brown et al., 2002; Pattinson and Fitzgerald, 2004). The potential for joint inflammation at P8 to induce profound changes in the excitation of the DH is great. It was strange, therefore, to observe little change in the responses of DH neurones 4 days following inflammation at P8.

Four days following joint inflammation, the P8 group did not show significantly enhanced responses to noxious pinch of the ankle compared to naïve animals. Using a similar method, Torsney and Fitzgerald have shown that peripheral inflammation in the hind paw enhances DH neuronal responses to a suprathreshold mechanical stimulus (Torsney and Fitzgerald, 2002). Any activity in a 12s window following the stimulus was included in their analysis, whereas data presented here represents 5s of activity following the beginning of the stimulus. At 4 days post inflammation, there is little change in reflex patterns from animals inflamed at P8. It may be that this time-point is a point of transition between the immediate effects of CFA inflammation and the more long-term changes associated with peripheral inflammation. The DH response to ankle inflammation in the developing nervous system has not previously been examined and it is assumed that joint afferents should have the same ability to initiate a state of central sensitisation as adult and skin afferents from animals of a similar age. It is unclear why this was not seen in the present study.

By 10 days post-inflammation reflex facilitation is seen at all postnatal ages. Both ankle and toe responses are facilitated relative to naïve animals. At this point post-injury, the inhibition of the reflex appears to be minimal. Reflexes are

abnormal and responses from the inflamed ankles of P21 and P40 injured animals are prolonged and remain significantly different from naïve for between 3-4s (Figure 3-9). Responses 10 days post-inflammation are presumably driven by the mechanisms elucidated above, although it would be necessary to examine the responses of DH neurones at this time-point to confirm this supposition.

3.5.6 Reflex inhibition following CFA induced joint inflammation

When joint inflammation was induced with CFA in animals from P21 onwards, the affected joint was rendered immobile. Despite the numerous studies that have injected CFA into joints, few have addressed this issue. Butler and colleagues originally proposed an isolated joint injection of CFA as a model of inflammatory arthritis to avoid the systemic effects of CFA administration (Butler et al., 1992). In this study, the authors note that there was a marked flexion of the hip, knee and ankle which they attribute to shortening of 'soft tissues' around the hip and knee as it was difficult to release post-mortem. Whilst in the long-term this may account for the changes they observed, in this study inhibition of reflex responses to pinch were present 24hrs after the injection of CFA, suggesting an alternative explanation for such behaviour - at least in the period immediately following injection. The relative scarcity of studies that address the issue of frozen joints in the pain literature most likely stems from the tools employed to characterise the neural responses to joint inflammation. For the large part these have been behavioural and electrophysiological with no attention paid to patterns of reflex activity following joint inflammation. A diminishingly small number of studies have examined reflex activity in the presence of an inflamed joint. A group of studies by Coderre and Wall touched on the subject in both awake behaving animals and decerebrate preparations. In a model of urate arthritis, a gouty inflammatory disease, Coderre and Wall describe similar behaviour but make no real attempt to examine the phenomenon they observed (Coderre and Wall, 1987, 1988b; Wall et al., 1988).

Reflexes to pinch were also significantly inhibited at 4 days post-injection on the inflamed side (see Figure 3-8). There was no significant difference in responses evoked from the toe on the inflamed side compared to the non-inflamed side in naïve animals, suggesting that the inhibition of toe evoked reflexes had been lost (Figure 3-8d,f). This speaks against Butler's account of the degenerative changes

underlying loss of function in CFA induced arthritis (Butler et al., 1992). They, however, made this assertion at the end of 6 weeks and used more CFA than was used here, thus it could be that the inflammation and subsequent disability they describe was more severe. There is, however, clear evidence of degenerative change in the muscles surrounding the inflamed knee within a week of CFA injection (Ozawa et al., 2009). Studies that used the range of movement joint and other gait parameters have shown the lack of use and joint freezing to be sensitive to pharmacological agents (Boettger et al., 2009; Boettger et al., 2010; Boettger et al., 2011). Daily administration of intraperitoneal morphine or intrathecal ketamine can return weight bearing and measures of gait disturbance to baseline in 7 days, suggesting that this may in part be a centrally mediated phenomenon (Boettger et al., 2009; Boettger et al., 2010).

Inhibition, present in adult animals, was absent in young animals throughout the time-course of this investigation and therefore suggests that the factors responsible are developmentally regulated. Extracellular recordings of joint responsive neurones here and elsewhere suggest that the excitatory and facilitatory effects of joint inflammation, also present in this study, are due to inhibitory changes occurring in the intermediate zone or ventral horn. That young animals do not develop joint freezing behaviour may mean that they fail to protect their joint from further damage in the presence of ankle inflammation.

3.5.7 Peripheral neuroimmune changes associated with joint inflammation

An important question to address when discussing the profile of inflammatory joint pain during development is to what extent the inflammation in the ankles of P8 animals is equivalent to inflammation in adult joints. Approximately 2µl, 6µl and 20µl of CFA was injected into the joints of animals aged P8, P21 and P40, respectively. At all ages CFA produced swelling, though this was more pronounced in older animals (see Figure 3-3). It was not possible to observe any behavioural sensitivity to mechanical stimuli in animals injected at P8 whilst older animals showed clear changes in weight bearing and mechanical sensitivity on the hind paw (see Figure 3-4 and Figure 3-5). Using manual vF hairs to assess cutaneous sensitivity in young rodents is a well established technique, and can reveal cutaneous hypersensitivity in certain conditions (Walker et al., 2009a;

Walker et al., 2010; Walker et al., 2012). Such differences may be in part explained by differences between the immature and adult immune systems. This difference includes diminished T-cell activation and infiltration as well as macrophage recruitment to the site of inflammation or injury. Both young and adult animals have few CD2-positive T-cells before injury, but while the numbers of these cells increase markedly in adults after nerve injury, this does not occur in young animals (Costigan et al., 2009). This is paralleled by a lack of upregulation of CD2 or CD3 mRNA in young animals (Costigan et al., 2009). T-cell numbers in the neonate are lower relative to the adult in many tissues and while the immune response is Th2-biased in the neonate, it is Th1-biased in the adult (Forsthuber et al., 1996; Ridge et al., 1996; Sarzotti et al., 1996; Adkins et al., 2004; Morein et al., 2007). Th1-biased responses are broadly responsible for the recruitment of macrophages to sites of infection and injury, thus if a Th2 response predominates in the neonatal immune system, macrophage recruitment may also be impaired in younger animals. This difference is likely to allow the development of tolerance to new antigens in young animals, and prevents potentially self-damaging inflammation (Adkins et al., 2004). Numerous experiments have shown, that, if provoked, the young immune system is capable of mounting adult-like responses (Adkins et al., 2004; Morein et al., 2007). Furthermore, peripheral nerve injury results in a differential regulation of immune related genes in sensory ganglia in young and adult rats (Vega-Avelaira et al., 2009). The same study demonstrated that nerve injury at P10 resulted in little recruitment of macrophages within DRGs, whereas in the DRGs of adult nerve injured animals macrophages moved to encircle damaged neurones.

In spite of the evidence suggesting weaker or different immune responses in young animals, it is clear that CFA injection can provoke a response at these ages. Walker and colleagues found that peripheral inflammation (CFA injection into the hind paw) provoked a robust inflammatory response at P3, as measured by paw diameter, although no direct assessment of inflammatory cell infiltration was made at this age (Walker, 2003). In the same study, administration of CFA resulted in a small but significant fall in mechanical thresholds two hours following injection, however no mechanical sensitivity was seen at any other time-point in the eight weeks of behavioural testing (Walker, 2003). In addition, inflammation has been seen following intraplantar CFA injection at P10 (Ren et al., 1997) and

mustard oil can also evoke hyperalgesia in the first postnatal week (Jiang and Gebhart, 1998).

There has long been a suggestion that the symmetry of disease progression seen in adult rheumatoid arthritis patients may be a function of neuroimmune interactions in the periphery (Levine et al., 1985). Indeed, sciatic nerve section in poly-arthritic rats delays the onset and reduces the severity of adjuvant-induced arthritis on the denervated side (Courtright and Kuzell, 1965). If inflammation is severe, dorsal root reflexes are evoked, resulting in antidromic stimulation of joint afferents and release of pro-inflammatory or pro-nociceptive mediators, which is mitigated by severing of the dorsal root (Rees et al., 1994, 1995). Whilst cytokine responses were not directly assessed in these studies it is likely that these phenomena would have contributed to the general level of afferent excitability in arthritic animals.

3.5.8 Central neuroimmune changes associated with joint inflammation

The immature spinal cord is different to that found in adult animals. As described in the discussion of Chapter 2, profound differences are evident in descending control pathways, the maturity of afferent inputs and the properties of individual neurones. As well as these neuronal differences, there are substantial non-neuronal and central neuro-immune features of the immature spinal cord that undergo significant changes in the first postnatal weeks. In the presence of injury and inflammation in the adult, the interaction of the nervous with the immune system plays a substantial role in the sensitisation and maintenance of hypersensitivity in the spinal cord. The extent to which these factors are important in the spread of sensitisation seen in the P8 group shall be explored below.

24 hours following the intra-articular injection of CFA into the joint of a P8 rat, a noxious pinch on the inflamed ankle was sufficient to evoke a reflex of similar size and duration on both the inflamed and non-inflamed side. The same pattern of reflex activity was not seen in naïve controls. Although an unexpected result, it feeds into a wealth of often incidental findings of bilateral effects of unilateral injury (Koltzenburg et al., 1999; Shenker, 2003). Recent investigations have examined central neuroimmunological explanations for the emergence of 'mirror-

image' and spreading hypersensitivity in models of inflammatory and neuropathic pain. Inflammation around the sciatic nerve, termed Sciatic Inflammatory Neuropathy (SIN) is a common model through which spreading sensitisation has been explored. Low-grade inflammation results in hypersensitivity that is restricted to the treated limb, a more substantial inflammatory insult results in spreading sensitisation to other body parts including the contralateral limb (Ledeboer et al., 2005). This effect can be delayed by intrathecal pre-treatment with minocycline or fluorocitrate to reduce glial activation. Treatment with p38 mitogen activated kinase inhibitors prevents the release of pro-inflammatory mediators and signalling of pro-inflammatory cytokines as does the administration of monclonal antibodies blocking the action of IL-6, TNFα and IL-1β in the spinal cord (Milligan et al., 2003; Ledeboer et al., 2005). Additionally, administration of carbenoxolone, a gap junction decoupler results in complete loss of mirrored hypersensitivity in the same model (Spataro et al., 2004). There was however, no evidence from behavioural data in the experiments presented here that hypersensitivity had spread to the non-inflamed side. However, as Figure 3-9a demonstrates, 10 days following CFA injection in P8 animals responses to pinch on the non-inflamed side were significantly enhanced compared to naïve controls. Interestingly, this was not a feature of the responses from either P21 or P40 animals at the same time post-CFA.

The fact that large responses could be elicited on the non-inflamed side by stimulation of the inflamed side in the P8 inflamed animals would suggest contralateral motor pools can be driven by ipsilateral inputs. This was not a feature of naïve animals of the same age or at any other age. It is well established that ipsilateral inputs can modulate contralateral outputs, and vice versa. However, neuroanatomical tracing of dorsal root fibres has provided evidence for direct input from ipsilateral afferents into the contralateral spinal cord, though such connections are thought to occur in low numbers and are concentrated in cervical and thoracic regions of the spinal cord (Culberson et al., 1979; Light and Perl, 1979). The connections more likely to be implicated in contralateral facilitation of hind-limb reflexes are second order interneurones that cross to synapse in the contralateral deep DH (Sotgiu et al., 2004). Furthermore, contralateral inhibition of sensory inputs, whereby ongoing discharges on the stimulated side can be inhibited by inputs from the contralateral side, are well

documented (Fitzgerald, 1982a; Fitzgerald, 1982b; Schaible et al., 1991). In decerebrate and spinalised adult preparations the biceps femoris muscle has excitatory receptive fields on the contralateral limb and tail (Woolf and Swett, 1984; Woolf and Wall, 1986). Furthermore, in the presence of joint inflammation the number of neurones excited by contralateral inputs on the side of the injury is increased (Neugebauer and Schaible, 1990; Grubb et al., 1993). In the naïve adult spinal cord, whilst flexor units are facilitated on the stimulated side, extensors are reflexively facilitated on the contralateral side in order to maintain posture (Sherrington, 1910). This study did not record from extensors, and thus comment on changes to extensor units is unwarranted. However, by virtue of the simultaneous bilateral recordings performed here, a previously undocumented facilitation of flexor unit activity in the presence of inflammation in an intact preparation was seen. Monoarticular inflammation failed to potentiate EMG responses in either P21 or P40 groups. This suggests that the immaturity of descending inhibitory pathways and segmental inhibitory interneurone pools may underlie the presence of contralateral responses to ipsilateral stimulation in the P8 group.

3.5.9 CFA induced joint inflammation as a model of juvenile inflammatory arthritis

CFA induced arthritis is a commonly used model of inflammatory pain in adult rodents. Injection of CFA into the base of the tail results in the development of a poly-articular arthritis which is associated with hypersensitivity to mechanical stimuli in the hind paws (Menétrey and Besson, 1982). This may be a better model of some forms of disease that fall under the category of juvenile inflammatory arthritis in view of poly-articular features, however, it is difficult to disentangle the specific effects of joint inflammation when much of the animal's body is sensitive to mechanical stimuli. This technique also carries the added risk of systemic illness associated with immune complex formation (Pearson et al., 1961). Rather, a single joint injection of CFA was made to induce a limited arthritis that was restricted to a single joint. It is important to note that this is a model of inflammatory pain and as such does not take account of immunological mechanisms of arthritis. As such, if comparisons were made to a human form of JIA, this model closely resembles the phentoype seen in Enthesitis Related Arthritis. Mechanisms of inflammatory joint pain in adults are well described and

are characterised by immune cell infiltration, joint damage and destruction, the release of pro-inflammatory cytokines and the sensitisation of peripheral nociceptors. Thus the method of arthritis induction is independent of the associated pain. Clearly the immune competence of neonatal animals is important in the severity of swelling and associated pain. The presence of CFA induced swelling and sensitivity at the youngest age tested, indicated that immunological machinery necessary to induce an inflammatory response was in place by this age.

Thus, in summary, the advantages of this model are three-fold. Firstly, a single injection results in an isolated arthritis that is limited to the injected joint at all postnatal ages tested. Secondly, following CFA injection in young animals, wide-spread sensitivity is seen in nociceptive circuits, indicating this model's biological validity. Finally, CFA is a widely used model of inflammatory pain and therefore an extensive literature exists with which to contextualise the results of any studies.

In order to further validate this model of inflammatory joint pain in juveniles, it will be important to establish the efficacy of existing clinical therapies for juvenile arthritic pain.

3.6 Conclusions

3.6.1 Young and adult responses to joint inflammation are profoundly different

Young animals that suffer joint inflammation at the beginning of the second postnatal week show enhanced reflex responses for the first 10 days following injury to noxious pinch. Simultaneously, young rats do not develop the protective inhibition of reflexes that was seen in animals of older ages. This difference is most likely due to differences in immune system competence and development of spinal and brainstem nociceptive circuits.

3.6.2 Postnatal age determines the severity and spread of sensitisation following joint inflammation

The short-term impact of inflammation at different ages is profoundly different, as inflammation begins to resolve, reflex inhibition seen in older animals turns into a profound reflex facilitation 10 days after the initial injury. Postnatal age at the time of injury does not appear to be important for the medium term (10 days post-injury) consequences of joint inflammation. It does, however, have an effect on the severity and spread of sensitisation 10 days after injury.

3.6.3 Monoarticular injection of CFA shows promise as a model of juvenile joint inflammation

Injection of CFA into the intra-articular space of the ankle in young rats, provides a potentially extremely useful paradigm within which to explore the long-term pain associated with articular inflammatory disease in early life. CFA injection results in the cardinal signs of inflammation and does not have long-term consequences on the systemic health of the animal.

Chapter 4 Long term alterations in sensory processing following joint inflammation

4.1 Introduction

In recent years a growing body of evidence from human and animal studies suggest prolonged painful experiences in early life are able to alter long-term pain sensitivity. This chapter will examine how joint inflammation in young rats affects the processing of nociceptive inputs in adulthood. As in previous chapters, the FWR is used here as a metric to quantitatively assess spinal sensitivity.

The postnatal development of cutaneous sensory systems is known to be highly learning dependent. 'Normal' sensory inputs including light touch, movement and acute pain are thought to be crucial for the development of spinal networks. Within this context chronic joint pain is regarded as an 'abnormal' sensory input. The consequences of prolonged joint pain in childhood and the extent to which early nociceptive input alters spinal sensitivity to subsequent injury are poorly understood.

4.1.1 The effects of early injury

The normal development of sensory processing in young mammals is highly activity dependent and can be disrupted by altering the inputs to the spinal cord in the first postnatal weeks (Waldenström et al., 2003; Fitzgerald, 2005; Granmo et al., 2008; Koch et al., 2012). Sensory development can also be altered by abnormal patterns of inputs arising from injured or inflamed tissues. It has been suggested that such injuries may lead to long-term changes in somatosensory processing, pain transmission and future analgesic responsiveness (Walker et al., 2009b; Walker et al., 2009a; Beggs et al., 2012a). Hind paw inflammation is known to produce long-term structural changes in nociceptive pathways (Tachibana et al., 2001; Walker, 2003; Ren et al., 2004). Early hind paw inflammation results in a generalised hypoalgesia in adulthood and a larger hyperalgesic response if the previously injured paw is re-inflamed in adulthood (Tachibana et al., 2001; Ren et al., 2004; Ren et al., 2005; Chu et al., 2007). The effects of neonatal injury have also been investigated in the context of a surgical incision model of pain, in which the skin and muscle of the hind paw are incised and then sewn together at P3 (Walker et al., 2009a; Beggs et al., 2012a). Animals injured at P3 and then reinjured at P60 showed enhanced mechanical and thermal sensitivity which was prolonged

compared to rats that only had an injury in adulthood (Walker et al., 2009a; Beggs et al., 2012a).

Non-nociceptive stressors such as early immune stress can also affect future pain processing in rats. When young rats are exposed to intraperitoneal LPS, persistent changes are reported in centrally mediated inflammatory responses and increased sensitivity to mechanical and thermal stimuli as adults (Boissé et al., 2004; Boissé et al., 2005).

The growing awareness of the effects of early life experience and injury on sensory systems has seen the long-term effects of pain in early life explored in the context of many other models. Bladder inflammation, colonic irritation, abdominal wounding, repeated needling, full thickness skin wounding and intramuscular injections of low-pH solutions have all been used to demonstrate long-lasting changes to sensory processing in adulthood.

Female rats treated with intravesical zymosan between P14 and P16 have enhanced responses to bladder re-inflammation in adulthood but no changes to hind paw thermal and mechanical stimulation (Randich et al., 2006). Neonatal laparotomy at P0 or P1 results in decreased sensitivity to thermal stimuli as adults on the tail and hind paws in addition to reduced writhing on injection of intraperotineal acetic acid (Sternberg et al., 2005). Interestingly, and perhaps more clinically relevant, repeated needling in the neonatal period (P0-P7), an attempt to model repeated heel lancing that occurs in neonatal intensive care units, results in reduced c-fos responses in the adult somatosensory cortex whilst enhancing behavioural responses to repeat injury in adulthood (Anand et al., 1999; Hermann et al., 2006; Hohmeister et al., 2009; Knaepen et al., 2012).

Further details of the long-term consequences of early-life injury are outlined in Section 1.9 and in Figure 1-5.

Inflammation, surgical incision and colonic distension models in early life have all shown robust and consistent effects on adult nociceptive processing. These generally result in a baseline hypoalgesia coupled with enhanced responses to supra-threshold stimuli to repeat injury in adulthood. However, no previous research has been undertaken on the effects of early life joint inflammation,

which has potential clinical implications for children with chronically inflamed joints.

Aims of the experiments

The primary aim of this chapter was to study the long-term effects of ankle joint inflammation on the hindlimb flexion reflex in rats using electromyographic EMG from the biceps femoris in response to noxious stimulation of the ankle joint and hind paw.

The key objectives were:

I. To examine the long term effects of joint inflammation in young rodents upon the pattern of adult nociceptive reflex activity.
II. To examine the long-term effects of adult joint inflammation upon the pattern of reflex activity later in life.
III. To investigate the effects of a second joint inflammation on the pattern of reflex activity, following an initial joint inflammation in early life or adulthood.

4.2 Methods

4.2.1 Joint inflammation and re-inflammation

In the re-inflammation groups animals were given two injections separated by 32 days. The volume of CFA was at a dose of 0.1ul/g body weight at the time of injection. Eight P8s and eight P40s received their first injection and then were re-inflamed when they were P40 and P72, respectively. The responses of animals that were re-inflamed were compared to age matched controls that received a single injection of CFA at the time of re-inflammation (P40 and P72).

4.2.2 Animals and experimental groups

In total 78 animals were used to collect data for this chapter. The numbers and treatments of all animals used in this study are detailed below.

4.2.3 Experiment 1: long-term consequences of single inflammation

The first set of experiments was designed to investigate the long-term consequences of joint inflammation at different postnatal ages set out in the aims of this chapter. The animals used and respective treatments are detailed below.

Figure 4-1 Numbers of animals and experimental groups used to explore the long-term effect of joint inflammation
(ai) P8 animals (n=4) received an injection of CFA and their flexor reflex responses to pinch 36 days later was assessed when they were P44. **(aii)** Another group of animals received an injection of CFA at P40 (n=10) and their flexor reflex responses to noxious pinch were assessed 36 days later when they were P76. **(b)** The responses of single inflamed animals were compared to those of naïve aged matched controls **(bi)** P44 (n=6) and **(bii)** P76 (n=10). Coloured boxes indicate how these groups of animals are displayed in this chapter.

4.2.4 Experiment 2: effects of repeat inflammation

The second set of experiments was designed to understand how the effect of single joint inflammation affects the response to a second inflammation, set out in the aim 3 of this chapter. The ages at which inflammation was induced and the age when animals were re-inflamed are detailed below.

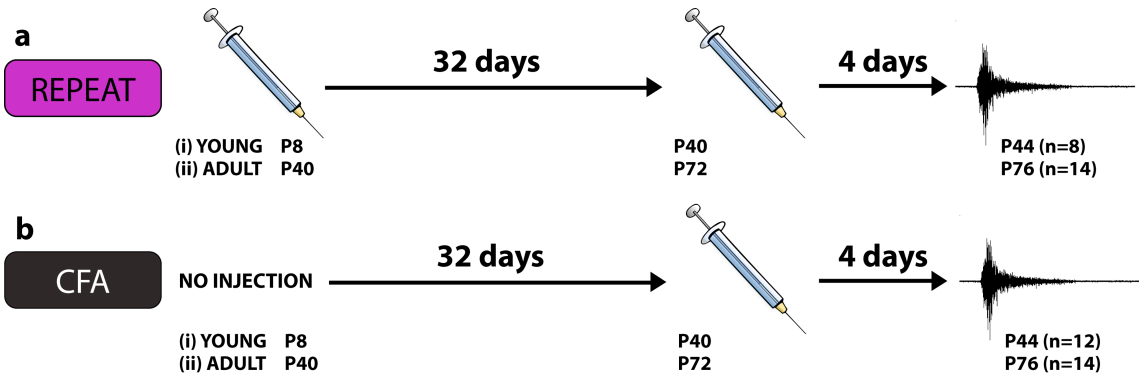


Figure 4-2 Numbers of animals and experimental groups used to explore the repeat inflammation
(ai) P8 animals (n=8) received an injection of CFA followed by a second injection of CFA at P40, four days after which EMG recording was performed. **(aii)** Another group of animals received the first inflammation at P40 (n=14) and the second at P72, four days after which EMG recording was performed. **(b)** Responses of repeat inflamed animals were compared to animals that had only received a single injection of CFA at either **(bi)** P40 (n=12) or **(bii)** P76 (n=14). Coloured boxes indicate how these groups of animals are displayed in this chapter.

4.2.5 Early anaesthesia and maternal separation

In a recent and very similar study of the long term effects of surgical injury, Beggs and colleagues established that the effect of the maternal separation, the stress of anaesthesia and the anaesthetic had no effect on repeat injured animals' nociceptive flexion reflex responses in adulthood (Beggs et al., 2012a). For this reason it was not deemed necessary to sham anaesthetise naïve pups. Handling and time apart from the mother was minimised as much as possible. On average, pups left their mother for no more than 5 minutes at the time of the initial injection.

4.2.6 EMG recording

EMG recording was performed as described in Chapters 2 and 3. Figure 4-1 and Figure 4-2 detail when recording was performed.

4.2.7 Ankle measurements

Ankle measurements were performed as described in Chapter 3.

4.3 Terms of reference

In this chapter animals that received an injection of CFA at P8 or P40 and had their reflex responses to pinch recorded 36 days later are called *single long-term* animals. The responses from these animals are compared to naïve animals that have experienced no joint inflammation, these animals are called *naïve* animals.

Rather than refer to inflamed and non-inflamed sides, the different limbs are referred to as the *treated* and *untreated* sides, respectively.

The data from these experiments are presented in *Experiment 1: Long-term consequences of a single joint inflammation*.

Animals that received an injection of CFA at P8 or P40 followed by a repeat injection of CFA at P40 or P76 and had their reflex responses to pinch recorded four days later are called *repeat inflamed* animals. The responses of these animals are compared to animals that received a single injection of CFA at P40 or P76 and had their reflex responses to pinch recorded four days later, these animals are called *single short-term* animals.

The data from these experiments are presented in *Experiment 2: Long-term Effects of repeat joint inflammation*.

4.4 Results

4.4.1 Experiment 1: long-term consequences of a single joint inflammation

This chapter presents data examining the long-term effect of joint inflammation. Joints were inflamed 36 days prior to EMG recording. Some animals received an injury when young (P8), others as adults (P40). Data in the previous chapter show that by 7-10 days behavioural measures of mechanical hypersensitivity and weight bearing have normalised.

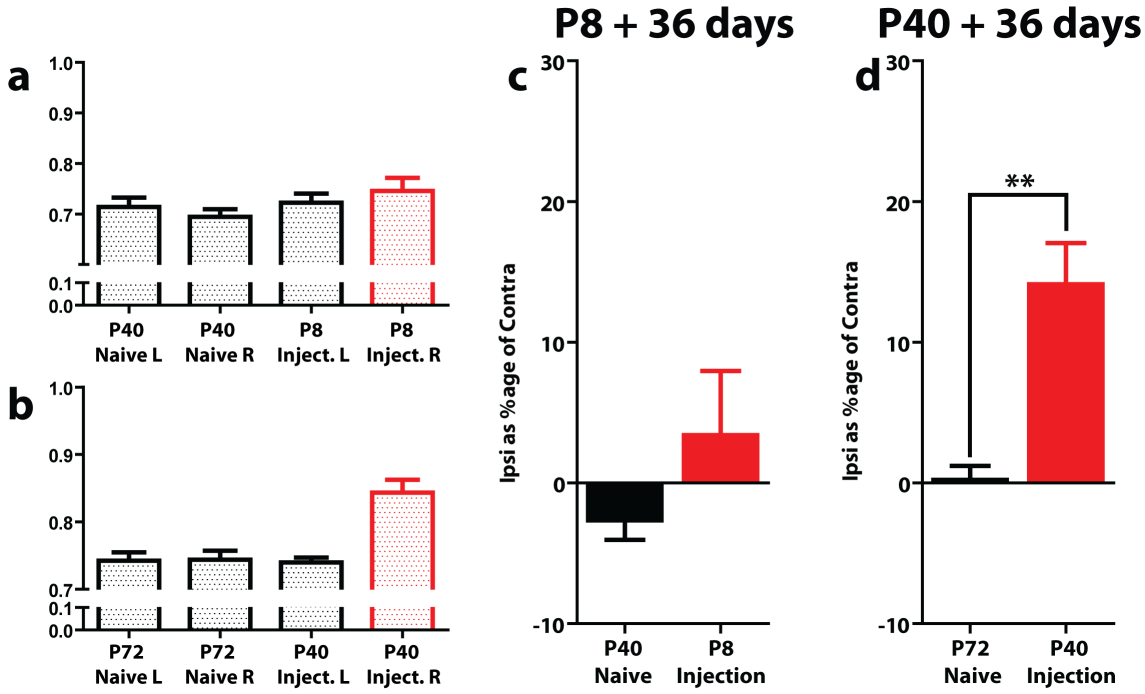


Figure 4-3 Joint swelling is evident 36 days later following CFA injection at P40 but not P8
(a) Shows raw ankle size data from P44 animals that received a single injection 36 days previously compared to age matched naïve animals which is expressed in graph **(c)** as the ipsilateral ankle as a percentage of the contralateral ankle. **(c)** The treated ankle in P8 single long-term animals (n=4) was not significantly different from age matched naïve animals (n=6, unpaired t-test, p=0.1679) **(b)** Shows raw ankle size data from P76 animals that received a single injection 36 days previously compared to age matched naïve animals which is expressed in graph **(c)** as the ipsilateral ankle as a percentage of the contralateral ankle. **(d)** The joints of P40 single long-term animals (n=8) remained significantly enlarged compared to age matched naïve animals (n=10, unpaired t-test, p<0.01).

Four animals received an injection of CFA aged P8 and were then returned to their littermates until they were P40. Animals were checked for any signs of systemic disease which is known to occur in some cases following CFA injection. The EMG responses to pinch of the ankle and toe were made at 36 days post injection. Prior to recording, ankle size was measured. Following a CFA injection at P8, single long-term animals showed no significant swelling of the treated joint compared to naïve P40 animals (Figure 4-3a). By contrast single long-term animals

injured at P40 had joints that had remained significantly enlarged compared to age matched naïve animals (Figure 4-3b, unpaired, t-test, $p<0.01$, n=8).

4.4.2 Experiment 1: long-term effect of a single injury at P8

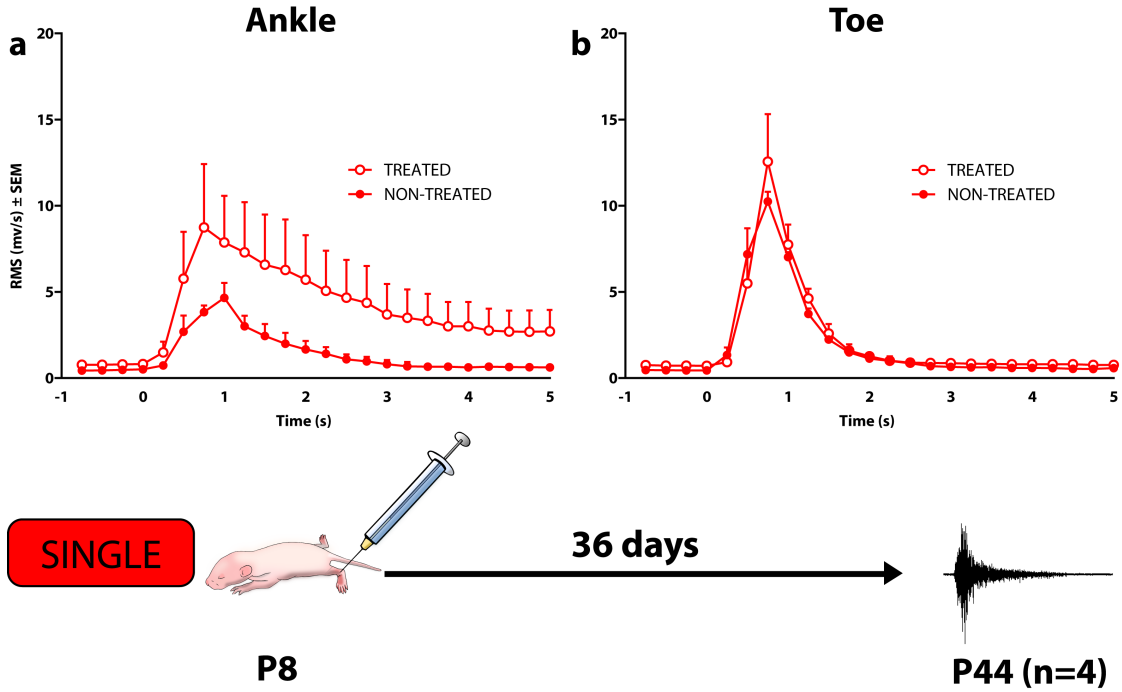


Figure 4-4 EMG Responses of P8 single long-term animals (n=4) recorded at P44
This figures shows the responses of animals in which behavioural signs of inflammation are absent. 'Inflamed' refers to the side that was inflamed aged P8. There is a non-significant enhancement of responses at the ankle whilst at the toe there is little difference in responses evoked on the previously inflamed and non-inflamed sides.

Figure 4-4 shows EMG responses from the biceps femoris muscle in adult animals (P44) that had received a single ankle injection at P8. Inspection of Figure 4-4 would suggest that EMG responses to pinch of the ankle on the treated side were enhanced compared to pinch of the ankle on the previously non-treated side, but this was not significant ($p=0.0762$, Figure 4-4a). There was no significant difference in the responses evoked at the toe on the inflamed and non-inflamed sides.

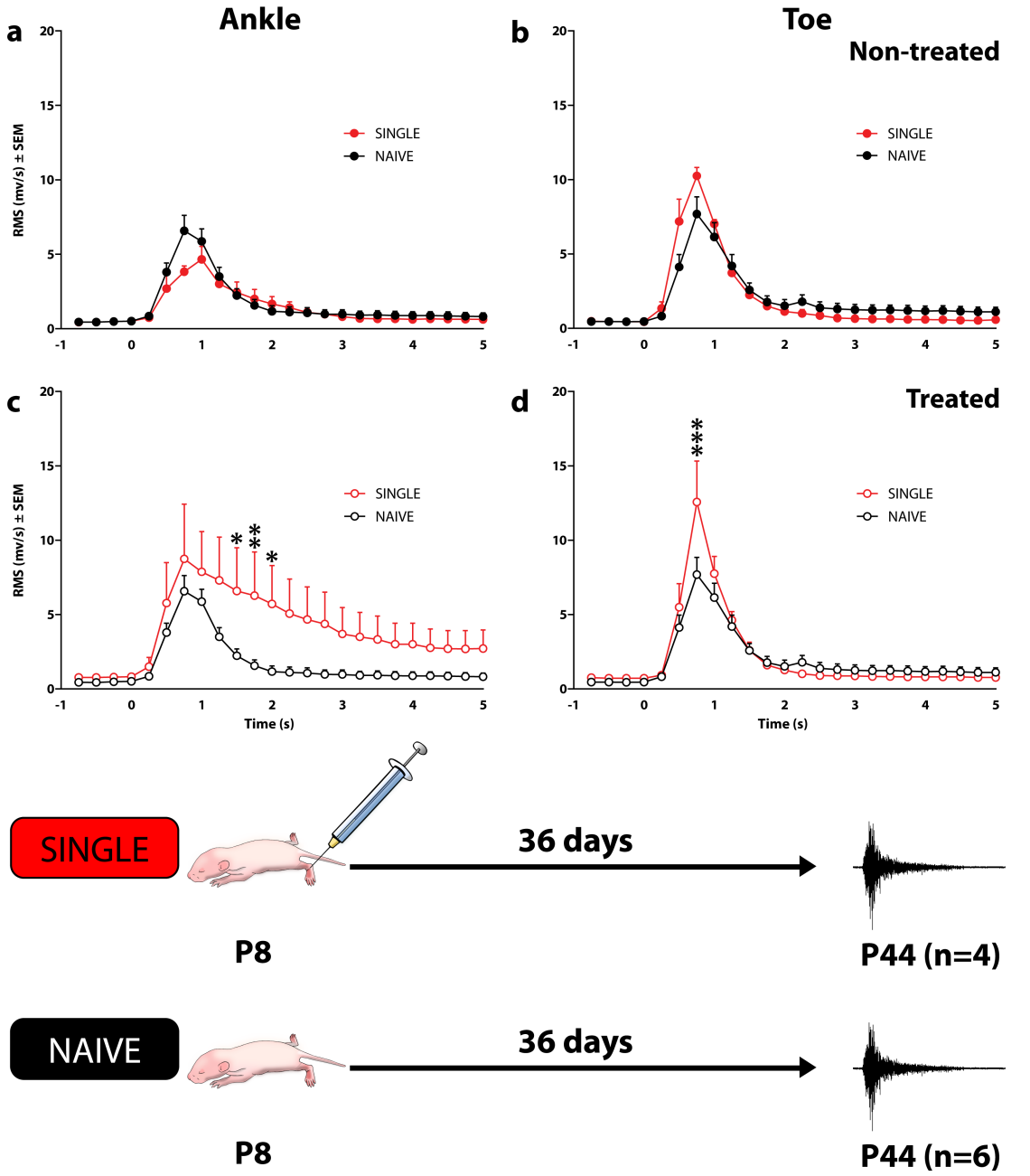


Figure 4-5 Responses of P44 animals inflamed at P8 compared to age matched naïve animals
The upper two graphs of this panel show the responses of animals to pinch on the non-inflamed (early injury) side compared to left side (no injury). Responses from the early injury group are compared to pooled responses from the ankle (a) and toe (b) of naïve P44 animals. The lower two show the responses on the previously inflamed side, again, compared to pooled P44 animals. Responses evoked from the previously inflamed ankle (c) and toe (d) were significantly greater than those in naïve animals. All tested with a 2-way ANOVA, Bonferroni post hoc. Single vs. Naïve: ***p<0.001, **p<0.01, *p<0.05.

When compared to the responses of age matched naïve controls (Figure 4-5) P8 single long-term (shown in red) reflex responses evoked from the treated ankle and toe on the treated side were significantly enhanced. Treatment with CFA had a significant effect on the reflex evoked from the treated ankle compared to naïve ($p=0.043$, Figure 4-5c) and there was a significant interaction between the responses from the naïve ankle and the toe on the treated side ($p=0.0005$). Post-

hoc analysis revealed significant differences between reflex responses to pinch at the ankle (1250-2000ms) and toe (500-750ms).

Thus, in summary, 36 days following inflammation at P8 there was no maintained swelling but there is evidence of maintained hypersensitivity in the nociceptive reflex responses from the ankle and toe on the treated side compared to age matched naïve controls.

4.4.3 Experiment 1: long-term effects of joint inflammation at P40

A group of adult (P40) animals were given a single injection of CFA and reflex responses to pinch were examined 36 days later and compared to age matched naïve controls.

Figure 4-6 shows the responses of P76 animals that received a single injection of CFA aged P40 (n=10). There was no significant difference between the treated and untreated sides (p=0.2587). Likewise, pinch of the toe evoked the same responses on the treated and non-treated sides.

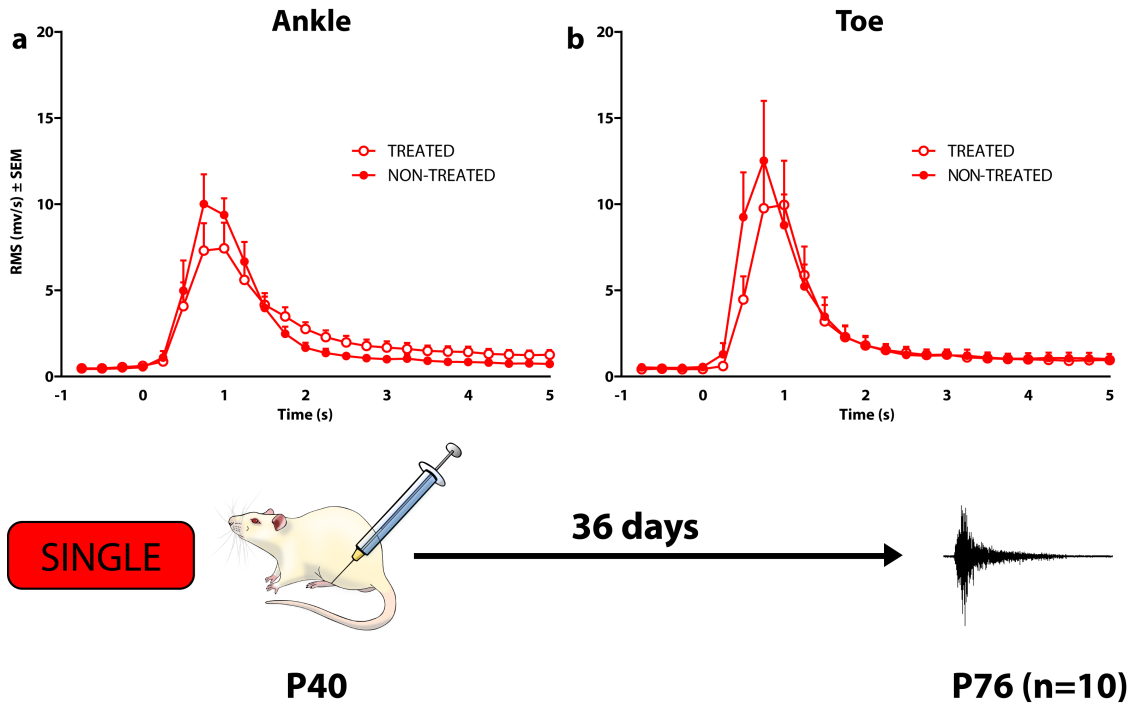


Figure 4-6 Responses of animals (n=10) inflamed when P40 and then recorded at P76
This figure shows the responses of animals in which behavioural signs of inflammation are absent. 'Inflamed' refers to the side that was inflamed aged P40. There is a significant enhancement of responses at the ankle (2-way ANOVA, Bonferroni post-hoc $p<0.05$ at 1000-1250ms) whilst at the toe there is little difference in responses evoked on the previously inflamed and non-inflamed sides.

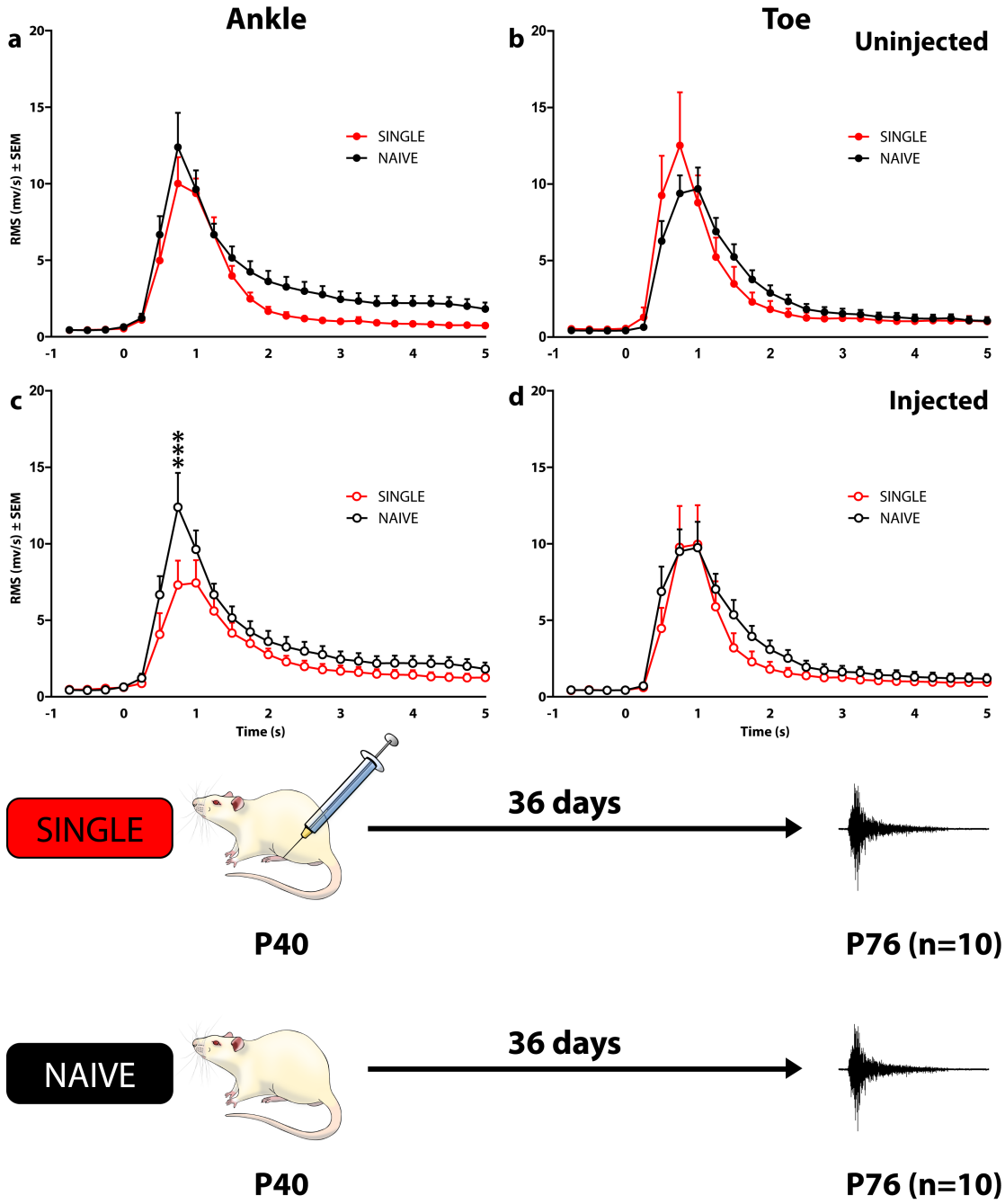


Figure 4-7 Responses of P76 animals inflamed at P40 compared to age matched naïve animals
The upper two graphs of this panel show the responses of animals to pinch on the non-inflamed (adult injury) side and left side (no injury). Responses from the adult injury group are compared to pooled responses from the ankle **(a)** and toe **(b)** of naïve P76 animals. The lower two show the responses on the previously inflamed side, again, compared to pooled P76 animals. Responses evoked from the non-inflamed toe **(b)** were not significantly different from those in naïve animals. Responses from the previously inflamed ankle were suppressed compared to naïve responses. All tested with a 2-way ANOVA, Bonferroni post hoc. CFA vs. Naïve: ***p<0.001.

To establish if baseline sensitivity had changed, responses of P76 animals inflamed at P40 were compared to age matched naïve controls (n=10, Figure 4-7). Animals that were injured in adulthood were largely similar to age matched naïve controls. There were significant changes in reflex sensitivity, but this was restricted to the site of the initial injury (Figure 4-7c). Responses from the previously inflamed ankle were significantly smaller than pooled responses from the ankles of naïve animals.

Thus, in summary, 36 days following joint inflammation at P40 there is still some residual swelling of the treated ankle. There is no maintained hypersensitivity in the reflex from the treated hind paw compared to age matched naïve controls. There is, however, evidence for a residual protective inhibition.

4.4.4 Experiment 2: repeat ankle inflammation

In order to investigate the effects of prior injury at different postnatal ages, animals that were inflamed at either P8 or P40 were re-inflamed 32 days following the initial injury, at P40 and P72 respectively. Reflex responses to pinch were then assessed four days later and compared to animals that only received a single injection of CFA at P40 and P72 respectively.

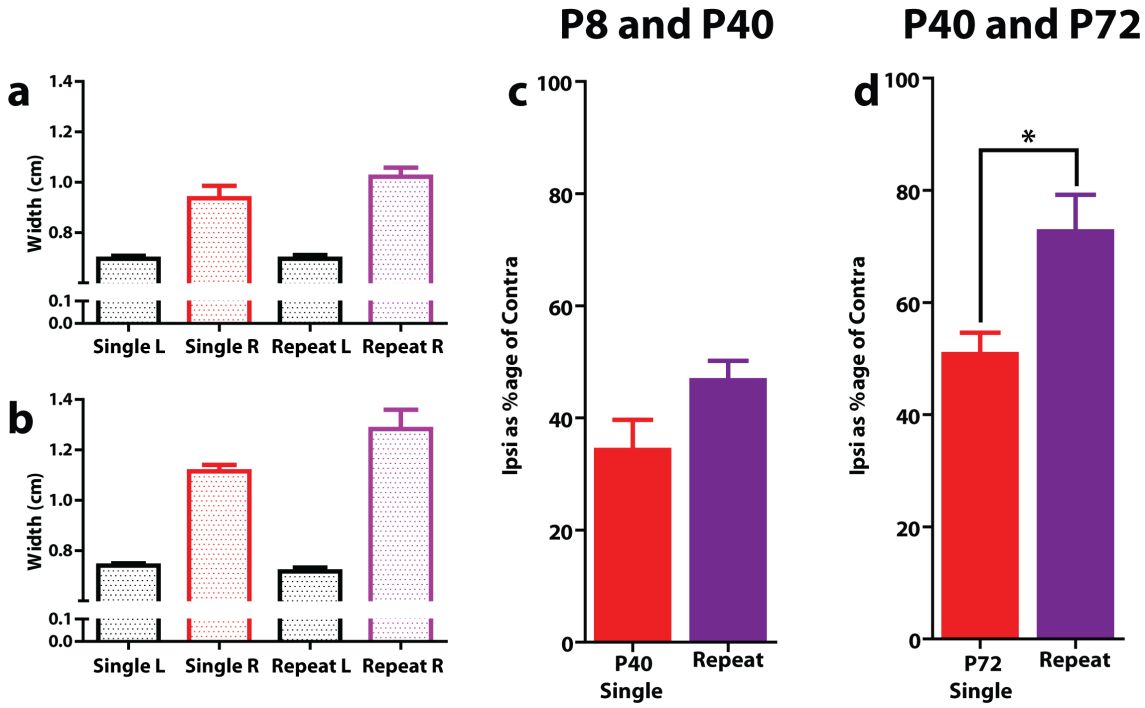


Figure 4-8 Ankle size is increased in adult repeat injured animals
(a) Shows raw ankle size data from single (P40) injection compared to repeat (P8 and P40) injections which is expressed in graph **(c)** as the ipsilateral ankle as a percentage of the contralateral ankle. Swelling following joint injection at P8 and P40 (n=8) was not significantly different from animals inflamed only at P40 (n=12). **(b)** Shows raw ankle size data from single (P72) injection compared to repeat (P40 and P72) injections which is expressed in graph **(d)** as the ipsilateral ankle as a percentage of the contralateral ankle. **(d)** Animals injected at P40 and P72 (n=14) had more swelling than those injected only at P72 (n=14, unpaired t-test, $p<0.05$).

Animals that were P8 at the time of the first injection of CFA were re-inflamed at P40 (n=8) and compared to P40s that had received only received an injection at P40 (n=12). Ankle swelling was not significantly different in animals that received repeat injury at P8 and P40 and those that received a single injury at P40 (Figure 4-8a). Animals that received an injection of CFA at P40 and P72 had significantly greater swelling of injected joint than those that received a single injection at P72 (Figure 4-8b, n=14, unpaired t-test, $p<0.05$).

4.4.5 Experiment 2: effect of repeat inflammation on animals first inflamed when young (P8)

Responses evoked from the inflamed ankle or toe of repeat injured animals (P8 and P40, n=8) were not significantly different from responses evoked on the non-inflamed side (Figure 4-9a,b).

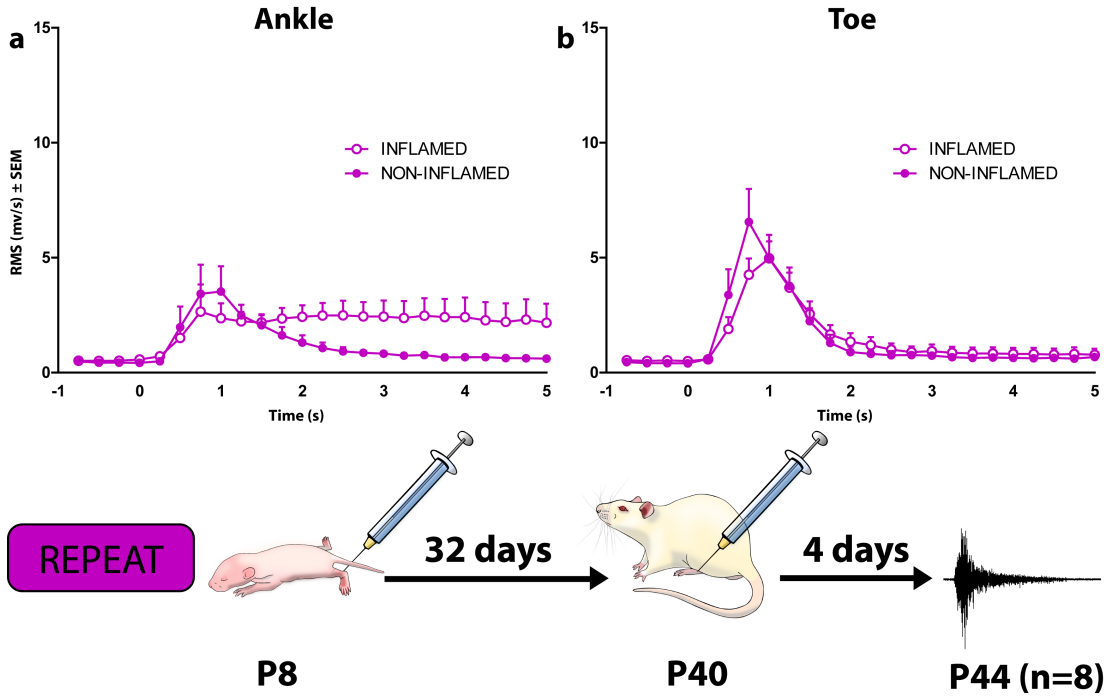


Figure 4-9 Inflamed and non-inflamed responses from repeat injured animals (P8 and P40)
The responses of animals inflamed first at P8 and then P40 (n=8) are shown. The inflamed side is compared to the non-inflamed side. Reflexes following pinch of the toe on the inflamed side compared are significantly different from non-inflamed side (2-way ANOVA, Bonferroni post-hoc, *p<0.05).

Responses from repeat injured animals were compared to animals that had only received an injury 4 days prior to recording (n=12). Repeat injury did not lead to any significant changes in the reflexes evoked from the inflamed ankle (Figure 4-10c). Away from the inflamed ankle there was a widespread inhibition of reflexes in repeat injured animals compared to single injured animals. Reflex responses to pinch of the ankle on the non-inflamed side as well as the toe on the inflamed side were significantly reduced (Figure 4-10a,d,) there was no difference in reflex responses evoked from the non-inflamed toe (Figure 4-10b).

In summary, repeat inflammation in adulthood post early life inflammation does not enhance normal ankle swelling following CFA injection. Flexion reflex EMG responses of repeat inflamed animals showed a more widespread 'protective inhibition' than single injected animals.

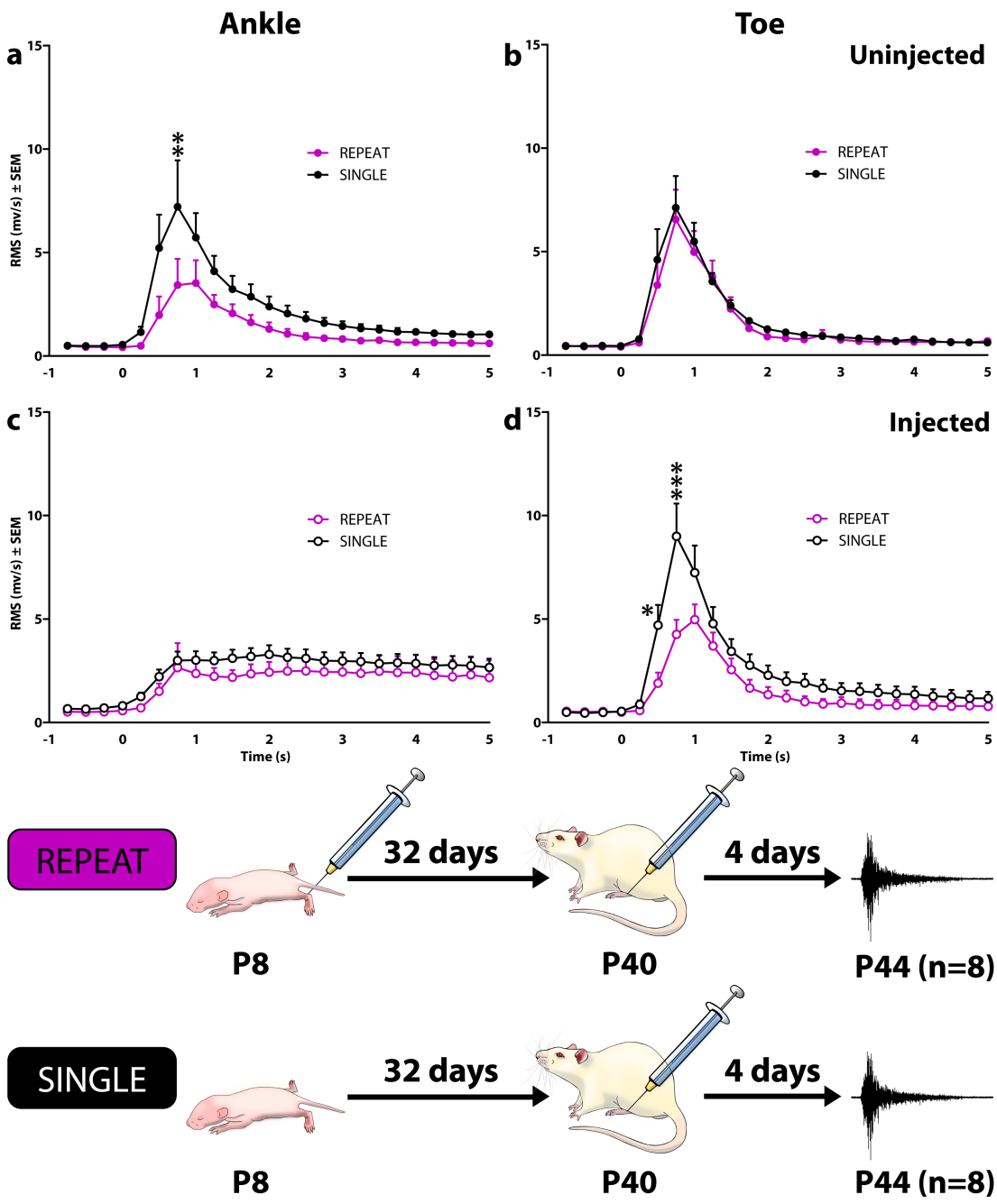


Figure 4-10 Responses of repeat inflamed (P8 and P40) animals compared to P40 CFA animals
The upper two graphs of this panel show the responses of animals to pinch on the non-inflamed side. Responses from the repeat injury group are compared to responses from the ankle **(a)** and toe **(b)** of animals that only received CFA at P40**.** The lower two **(c)** and **(d)** show the responses on the inflamed side, again, compared to P40 CFA animal. Responses evoked from the repeat inflamed toe **(d)** were significantly reduced compared to animals that had only received a single injury (***p<0.001, *p<0.05)

4.4.6 Experiment 2: effect of repeat inflammation on animals first inflamed when adult (P40)

To establish the significance of the time of the first injury a group of P40 animals (n=14) received an injection of CFA and 32 days later received a second injection aged P72. Their EMG responses were compared to animals that had only received an injection at P72 (n=14). Recordings were performed at P76 in both groups.

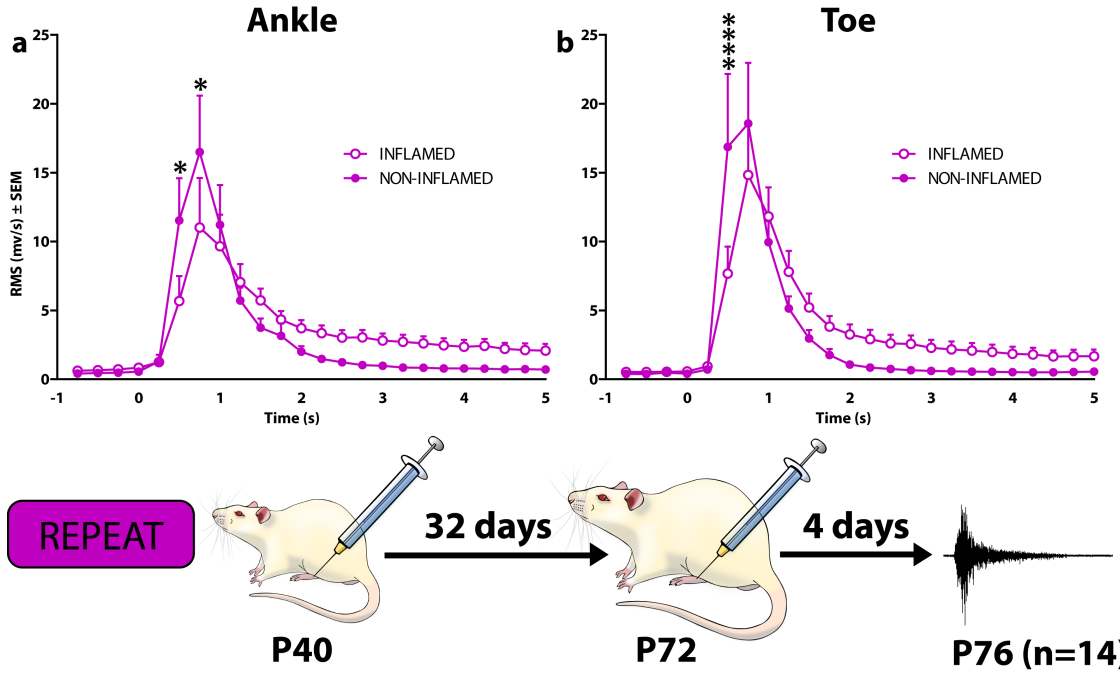


Figure 4-11 Inflamed and non-inflamed responses from repeat injured animals (P40 and P72)
The responses of animals inflamed first at P40 and then P72 (n=8) are shown. The inflamed side is compared to the non-inflamed side. Reflexes are significantly reduced on the inflamed side compared to the non-inflamed side (2-way ANOVA, Bonferroni post-hoc, ****p<0.0001, *p<0.05).

Responses evoked from the ankle and toe on the inflamed side were inhibited compared to the non-inflamed side. There was a significant interaction between responses evoked on the repeat inflamed side compared to the non-inflamed side from the ankle ($p<0.021$) and the toe ($p=0.0007$). Responses to pinch from the inflamed ankle were significantly inhibited between 500-1000ms (Figure 4-11a) and from the toe between 500-750ms.

The responses of repeat injured animals were then compared to the responses of animals that had only received an injury when they were P72. In contrast to animals that received the first injury at P8, the reflex responses to pinch of animals that received a CFA injection aged P40 and again at P72, were facilitated

compared to

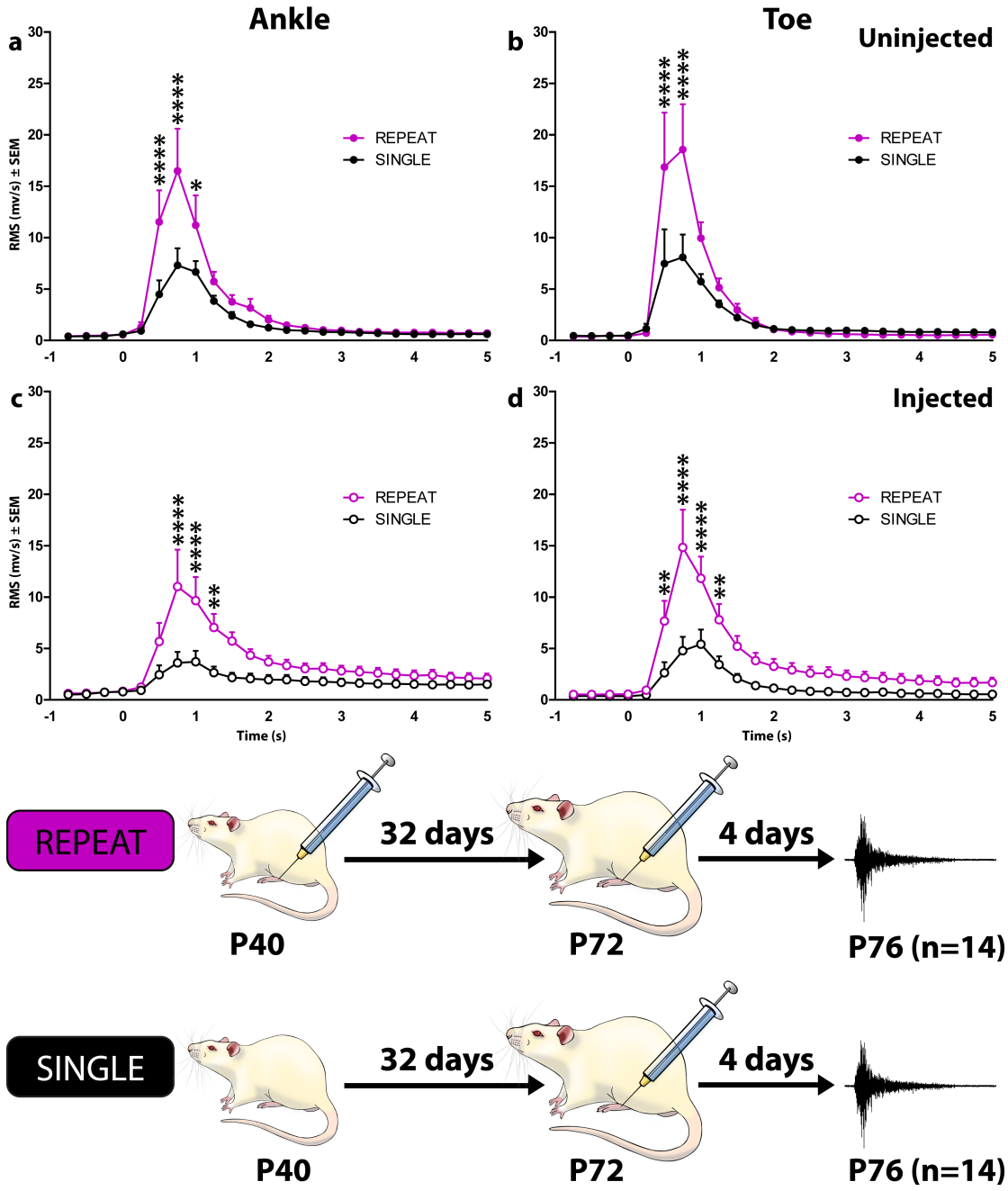


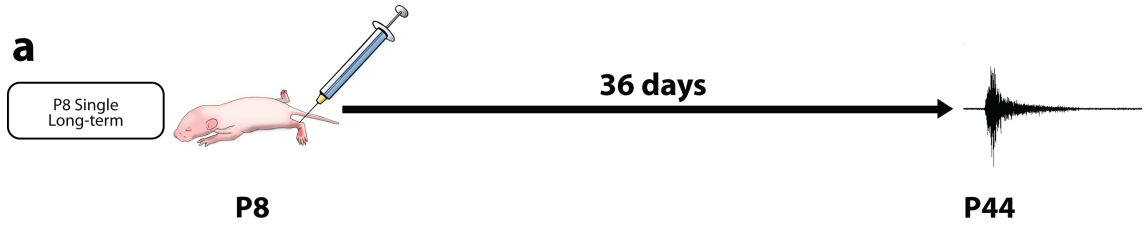
Figure 4-12 Responses of repeat inflamed (P40 and P72) animals compared to P72 CFA single inflamed animals

The upper two graphs show the responses of animals to pinch on the non-inflamed side. Responses from the repeat injury group are compared to responses from the ankle **(a)** and toe **(b)** of animals that only received CFA at P72**.** The lower two **(c)** and **(d)** show the responses on the inflamed side, again, compared to P72 CFA animals. Pinch of the ankle on both the inflamed and non-inflamed sides result in enhanced responses compared to animals that received a single injection of CFA (****p<0.0001, ***p<0.001, **p<0.01). Reflex response to pinch of the toe of repeat inflamed animals is significantly greater than responses from the single injury group.

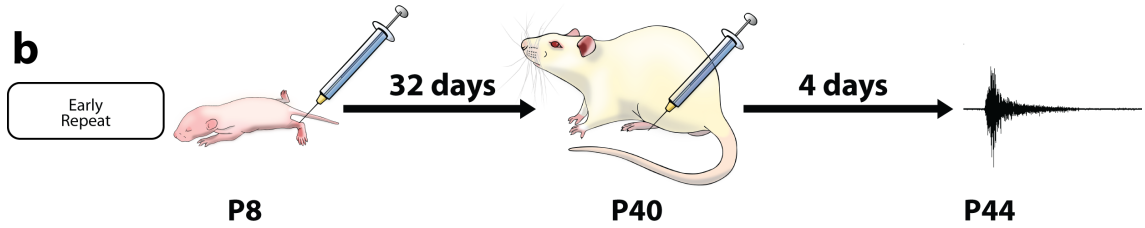
those that had only received an injection at P72 (Figure 4-12). There was a significant interaction between responses from animals that received a single inflammation compared to those that received repeat inflammation at all sites tested ($p<0.0001$ at all sites). Responses were enhanced at the non-inflamed ankle (Figure 4-12a, 250-1250ms), the toe on the non-inflamed side (Figure 4-12b, 500-1000ms), the inflamed ankle (Figure 4-12c, 750-1500ms) and the toe on the inflamed side (Figure 4-12d, 250-1000ms)

Thus, in summary, repeat inflammation following an inflammation at P40 results in greater swelling of the affected ankle. EMG responses to noxious pinch were facilitated on the inflamed and non-inflamed sides and animals showed no protective inhibition in response to joint inflammation.

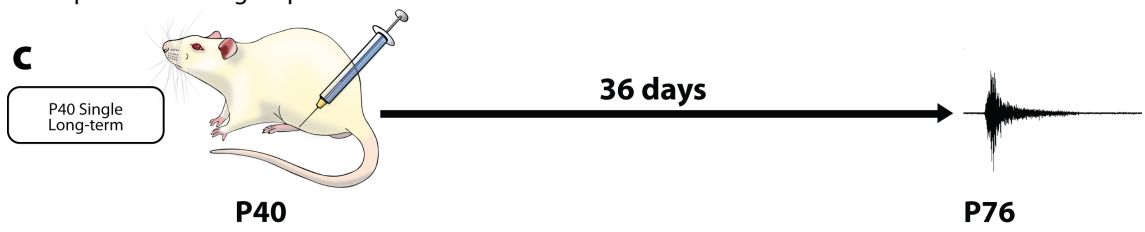
4.5 Summary of results



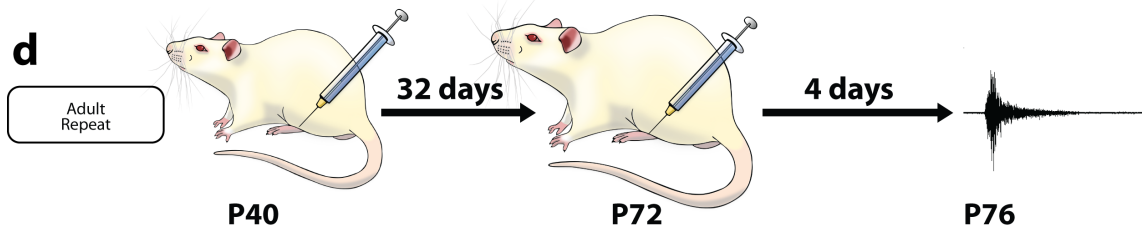
(a) Ankle inflammation at P8 results in a generalised increased flexion reflex sensitivity on the side of the inflammation in adulthood compared to age matched naïve controls, whilst there is no difference in the size of the previously inflamed joint.



(b) Repeat inflammation in adulthood (P40) following an initial injury when young (P8) results in reflex suppression compared to animals that only received a single joint inflammation at P40. Reflex suppression spreads beyond the injured ankle to the ankle on the non-inflamed side and the toe on the inflamed side. Joint size is not significantly different between the single inflamed and repeat inflamed groups.



(c) Ankle inflammation in adulthood, at P40, results in a reduced flexion reflex response to noxious pinch compared to age matched naïve controls which is restricted to the injected joint, at P76. The previously inflamed ankle joints of P76 animals were larger than the ankles of age matched naïve controls.



(d) By contrast, repeat injury in adulthood, at P72, preceded by an injury at P40, results in widespread and profound enhancement of reflex responses to noxious pinch at all sites tested. Joint swelling was significantly enhanced in the repeat injured group when compared to single injured group.

4.6 Discussion

The first experiment was designed to examine whether there was a long-term effect of inflammation on baseline nociceptive processing. The results show that the long-term effect of joint inflammation depends upon the timing of the inflammation. Ankle joint inflammation in young animals (P8) results in enhanced nociceptive reflexes from the ankle later in life (P44) despite apparent full recovery from the inflammation itself. In contrast, ankle joint inflammation when an animal is already an adult (P40) has no such effect. Weeks later in life (P76) the joint is still swollen and the nociceptive reflexes are slightly depressed. This appears to be a residual protective inhibition.

The second experiment was designed to investigate the effects of a history of joint inflammation on a second bout of joint inflammation. Again, the results show that the consequences of such a repeat injury depend upon the timing of the original joint inflammation. If the first bout was when the animal was young, the second bout causes a strong inhibition of nociceptive reflexes; in other words a more profound inhibition than expected. If the first bout was when the animals was already an adult, then the second bout appears to accelerate and exacerbate the reflex excitation; or in other words, a greater hypersensitivity than expected.

Thus, early life and adult inflammation both have long-term consequences on both the baseline nociceptive processing but also on the sensitivity to a fresh bout of inflammation, but the pattern of these consequences is different in young and adult animals.

There is a growing body of preclinical data which suggest that exposing developing sensory circuits to pain leads to long-term changes in the sensitivity of sensory systems in adulthood as well as change in the sensitivity of these circuits in the presence of subsequent injury (Anand et al., 1999; Ruda et al., 2000; Lidow et al., 2001; Boissé et al., 2005; Ren et al., 2005; Sternberg et al., 2005; Chu et al., 2007; Fitzgerald and Walker, 2009; Walker et al., 2009a; Beggs et al., 2012a; Knaepen et al., 2012). Within this context, the data from the experiments above will be discussed.

4.6.1 Methodological considerations

All of the results presented here are premised on a dichotomy of youth and adulthood. This distinction is made on the basis of what is known of developing sensory circuits, and is outlined in detail in Chapter 2. Profound changes to sensory systems occur in the first postnatal weeks and have stabilised by P28 (Fitzgerald, 2005). Studies presented in this thesis and elsewhere have shown that developmental changes in sensory circuits are complete well before the animals reach P40. To all intents and purposes therefore, by P40 the rats in this study can be regarded as adults (Hathway et al., 2012).

4.6.2 Early injury: baseline changes to reflex sensitivity following inflammation

The first set of experiments presented in this chapter examined the long-term effects of a single joint inflammation on the sensitivity of reflex circuits 36 days later. Previous studies have shown that following cutaneous inflammation in early life, behavioural responses to thermal and mechanical stimuli are dampened (Ren et al., 2004; Chu et al., 2007). Here we show, for the first time, that a single bout of joint inflammation results in long-lasting hypersensitivity to mechanical pinch of the ankle many weeks later.

It is evident that the absolute amount of Mycobacterium tuberculosis injected into P8 animals would have been smaller than that injected into P40 animals. It is probable, therefore, that the intensity of inflammation at P8 was less than that at P40. Nevertheless, it cannot be discounted that these animals have a continuing low-grade inflammation in the previously inflamed joint.

36 days after an inflammation at P8 there were no significant effects of joint inflammation on reflex responses to pinch when comparing responses from the inflamed and non-inflamed sides together (Figure 4-5). There is a suggestion that reflex responses were greater from the inflamed ankle than the non-inflamed ankle, but this did not reach significance and may in part be due to the low number of animals in the group (n=4). In comparison to age matched naïve animals, however, reflex responses were significantly enhanced. Thus, a long-lasting sensitivity to noxious pinch was established by a single episode of joint

inflammation and these animals showed enhanced responses to noxious stimuli 32 days later.

The prolonged hypersensitivity after joint inflammation may be due to changes in the peripheral nociceptor terminals within the joint peripheral response to inflammation in early development. Skin wounding in the neonatal period is known to produce robust and long-lasting hyper-innervation of the wound site which is dependent on neurotrophin 3 (NT-3) (Reynolds and Fitzgerald, 1995; Moss et al., 2005; Beggs et al., 2012b). Currently it is unknown whether joint inflammation in early life results long-term changes in the innervation of joints. This would be an interesting question to address.

Another possible explanation of the long-lasting heightened responses seen in P8 single inflamed animals is mediated by the CNS response to injury in early life. There are also developmentally regulated changes apparent in central synapses following inflammation or incision injury (Li and Baccei, 2009; Li et al., 2009). Neonatal surgical injury increases the frequency of glutamatergic miniature excitatory postsynaptic currents (mEPSCs) 2-3 days followed by a reduction 9-10 days post injury, however, the same effect was not seen in older animals (P17) (Li et al., 2009). Inflammation of the hind paw at a similar postnatal age also modulates excitatory input into the spinal DH in a similar fashion to surgical injury and again these effects were not seen in more mature animals (Li et al., 2009). Interestingly, foot injury but not intraplantar injection of carrageenan results in the persistent increase of individual DH neurones' receptive field size that does not occur in more mature animals (Torsney and Fitzgerald, 2003). Carrageenan associated inflammation is less severe and resolves more rapidly than CFA inflammation (Radhakrishnan et al., 2003). Perhaps in cases of more powerful inflammation, receptive fields are altered by early inflammation which may go some way to explaining the presence of enhanced responses from the toe on the previously inflamed side in P8 animals in this study.

4.6.3 Early injury: response to repeat injury

Against this baseline of heightened nociceptive reflexes in adult animals with an early pain history, we asked what the effect was of a second joint inflammation in adulthood. To address this animals received a joint injection of CFA at P8 and

then again at P40 and their reflex responses 4 days post-inflammation were compared to animals that received an injection only 4 days prior (at P40).

There was no significant difference in the severity of joint swelling following injection, however, reflex responses to pinch differed greatly between single short-term inflamed animals and repeat-inflamed animals (Figure 4-10). At the inflamed ankle there was no difference between the repeat inflamed and single inflamed animals, that is, both repeat-inflamed and single short-term inflamed animals developed a protective inhibition. The protective inhibition at the inflamed ankle was no different between single and repeat inflamed animals, although it should be noted that repeat-inflamed inhibition occurred in the context of enhanced responses before the second inflammation.

Both at the toe on the inflamed side and the ankle on the non-inflamed side there was a depression of reflex activity in repeat inflamed animals compared to single short-term animals. If inhibition of reflex activity is taken to be a mechanism of joint protection within spinal circuits then it is clear that the presence of a repeat inflammation results in a more widely spread inhibition.

As was demonstrated in Chapter 3, the increase in reflex inhibition observed in acutely inflamed animals in adulthood was seen in conjunction with increased DH sensitivity to noxious pinch. It may be that there is a greater degree of reflex depression in the repeat injured animals as a function of the increased onward transmission of nociceptive information through the DH although this was not tested here. This would be consistent with other studies that have shown increased behavioural, immunohistochemical and electrophysiological responses to repeat injury in adulthood following an early injury (Ren et al., 2005; Beggs et al., 2012a; Knaepen et al., 2012) (Chu et al., 2007). The data presented here show that a spreading of sensitisation was apparent 24 hours following CFA injection at P8. By 10 days post-inflammation bilateral enhancement of reflexes to noxious pinch was apparent. The immaturity of segmental spinal inhibition as well as immature descending control might have allowed a greater sensitisation of nociceptive circuits that was only revealed by a second inflammation (Hathway et al., 2006b; Hathway et al., 2009; Koch et al., 2012).

4.6.4 Adult injury: response to repeat injury

The long-term effect of early joint injury led us to ask whether the age of the first inflammation was important or simply the time between bouts of inflammation. Therefore, adult rats were inflamed at P40 and re-inflamed at P72, the same time interval between bouts of inflammation as was used to model early inflammation. Reflex responses following a second inflammation were compared to responses from animals that only received an injury at P72. Reflex responses to pinch were recorded 4 days after inflammation at P76. Reflexes evoked from the inflamed side in repeat inflamed P76 animals were smaller in magnitude than those on the non-inflamed side both at the ankle and the toe (Figure 4-11). Rather than becoming inhibited, as the early inflamed repeat group did, the adult repeat injured group were less inhibited or even enhanced relative to the single inflamed animals.

It is difficult to say in absolute terms whether reflexes are inhibited, facilitated or no different following repeat injury. In animals that were injured at P40 and not repeat inflamed at P72, it appears that reflexes normalised over the 36 days following the CFA injection excepting the reflex evoked from the previously inflamed ankle which was diminished compared to the naïve P76 (Figure 4-7). Following repeat injury the reflex was identical to that evoked from age-matched naïve controls. Without further speculation, however, one can confidently conclude that the reflexes evoked from adult repeat injured animals did not display the protective inhibition that was so evident in adult animals presented in the previous chapter (see Chapter 3). It may be that the phase of joint protection seen in acutely inflamed animals was shorter or did not occur at all in repeat injured animals. Rather, repeat inflammation accelerated the transition from joint protection to hypersensitivity. Regardless, the absence of reflex inhibition at the inflamed ankle is evidence that previous joint inflammation in adulthood is able to alter the response to repeat inflammation and is driven by different mechanisms to repeat inflammation in early-inflamed rats.

4.6.5 Early injury: response to repeat injury: the case for microglial involvement

In recent years there has been a growing interest in the role of glia in the maintenance of central sensitisation in multiple chronic pain conditions (Milligan

and Watkins, 2009). Microglia, resident macrophages within the CNS, are long-lived cells that have the ability to retain an innate immune memory (Town et al., 2005). In the brain, primed by ongoing neuropathology such as prion disease, multiple sclerosis or Alzheimer's disease, microglia react more vigorously to the introduction of inflammatory mediators associated with infection releasing pro-inflammatory cytokines and exacerbating the pre-existing pathology (Perry et al., 2007).

CFA monoarthritis results in the robust activation of spinal cord microglia for at least 14 days following injection (Sun et al., 2007; Hernstadt et al., 2009; Xu et al., 2010a; Yang et al., 2012). In this model, microglia are thought to contribute to thermal and mechanical hyperalgesia. Blocking or reversing the activation of microglia in the spinal cord results in the reversal of changes to thermal and mechanical sensitivity associated with CFA inflammation (Raghavendra et al., 2004; Sun et al., 2007; Hernstadt et al., 2009; Yang et al., 2012). Meanwhile, enhanced activation of microglia in the spinal cord has been reported following repeat incision compared to single incised animals (Beggs et al., 2012a). The spread, density and duration of microglial activation in repeat injured animals is enhanced compared to single injured animals (Beggs et al., 2012a). The tetracycline antibiotic minocycline is a non-selective inhibitor of microglial activation. The drug prevents the morphological changes and cytokine release commonly seen following microglial activation (Lai and Todd, 2006). Intrathecal administration of minocycline attenuates flinching in the second phase of formalin-induced behaviour as well as the thermal hypersensitivity following intraplantar injection of carrageenan (Hua et al., 2005). The hypersensitivity-associated sciatic inflammatory neuropathy and intrathecal administration of human immunodeficiency virus-1 (HIV-1) envelope glycoprotein gp120 is attenuated by minocycline administration, as too is the associated release of pro-inflammatory cytokines (Raghavendra et al., 2003; Ledeboer et al., 2005).

Importantly, in a model of repeat injury, low dose intrathecal minocycline attenuated hyperalgesia in repeat injured animals only (Beggs et al., 2012a). Minocycline is thought to act on p38 mitogen-activated protein kinase (p38 MAPK), which is widely implicated in the processing of inflammatory pain and can be responsible for the release of pro-inflammatory cytokines which are known to

sensitise nociceptive circuits (Ji et al., 2002; Svensson et al., 2003; Svensson et al., 2005). Interestingly, intrathecal administration of a low dose of an inhibitor of phosphorylated p38 MAPK results in the reversal of the enhanced hyperalgesia seen in repeat injured animals so that they become indistinguishable from single injured animals (Schwaller & Walker, personal communication).

Evidently microglia are in a powerful position to modulate the excitability of nociceptive circuits, whilst their longevity and immune memory makes them a strong candidate to mediate long-term changes associated with repeated joint inflammation.

4.6.6 Critical periods in the development of nociceptive processing

The early postnatal period is a time of exceptional plasticity during which inputs have a powerful ability to affect the wiring and functioning of neural circuits (Waldenström et al., 2003; Hensch, 2004; Schouenborg, 2004; Koch et al., 2012). These periods in development are known as *critical periods*. Injury within a critical period, resulting in an abnormal sensory input to the spinal cord, may lead to long-lasting alterations in nociceptive circuitry and may underlie the reflex sensitivity that can be seen following joint inflammation at P8 (Walker et al., 2009a). The timing and type of injury is key. Incision between P3 or P6 results in enhanced responses to repeat injury two weeks later that does not occur if the first injury occurs at P10, P21 or P40 (Walker et al., 2009a). Similarly, intraplantar inflammation at P3 results in an expansion of termination fields in the DH that is not seen if the inflammation occurs at P14 (Ruda et al., 2000). Additionally, there is a transient increase in the size of C-fibre terminals fields as well as a denser innervation of $CGRP^+$ fibres in LII of the DH which does not occur in adults (Walker, 2003; Chien et al., 2007). Neonatal inflammation at P1 also accelerates the development of $IB4^+$ nociceptive fibres and increases the proportion of small and large $CGRP^+$ fibres in the DRG, a neuropeptide important for the transmission of nociceptive information (Beland and Fitzgerald, 2001). Furthermore, the release of NGF in the skin is markedly increased following neonatal wounding, not seen if the injury occurs at P22 or older (Constantinou et al., 1994).

Clearly, as evidenced above, there is a period within the first 2-3 postnatal weeks where the central processing of pain associated with inflammation and surgical

injury is highly developmentally regulated and is fundamentally different from that seen in the adult spinal cord. These processes may underlie enhanced responses to nociceptive input following an early injury, whilst renewed afferent input from a re-inflamed joint is likely to pass through circuits altered by the presence of inflammation in a vulnerable period in early life.

4.6.7 Epigenetic regulation of pain

Epigenetic mechanisms of gene regulation have brought a new understanding to the study of chronic pain (Géranton, 2012). Epigenetic mechanisms are important for the regulation of long-term neuronal plasticity, which has become particularly apparent in the fields of learning and memory (Day and Sweatt, 2010). Epigenetic changes in many of the genes that are important in long-term synaptic plasticity in the hippocampus underlie mechanisms in chronic pain states (Kuner, 2010). Epigenetic changes as a result of injury in early life may lead to priming of the nociceptive machinery that is uncovered by subsequent injury or stress in adulthood. Indeed, pups reared by inattentive mothers show epigenetic changes in glucocorticoid-associated genes that result in altered stress responses in adulthood (Weaver, 2009). The exact mechanisms behind the changes are slowly becoming apparent, it is interesting to consider that long-term changes in nociceptive circuits may, in part, be due to epigenetic regulation of important genes in pain processing.

4.6.8 Other potential mechanisms

We may speculate that long-term changes to local and descending inhibitory circuitry also contribute to the changes observed here. To date there is limited information about this. For further information see Baccei and Fitzgerald, 2013.

4.7 Conclusions

4.7.1 Joint inflammation in early life leads to a long lasting sensitivity that spreads beyond the joint that was originally inflamed

A single joint inflammation in P8 animals resulted in sensitivity that was evident just over 4 weeks later. This is not confined to the original inflamed joint but was also evident at the toe. Long lasting changes in the peripheral innervation of joints and changes within the DH may serve to potentiate the transmission of nociceptive information from the previously inflamed joint.

4.7.2 The long term effects of joint inflammation are dependent on the postnatal age of the injury

The postnatal age at which joint inflammation occurs determines the outcome in later life. Early inflammation leads to long-term alterations in the processing of nociceptive input from joints and skin in contrast to animals that received the first injury in adulthood. Single early inflammation results in widespread sensitivity that spreads beyond the affected joint, whilst single adult inflammation results in a restricted change in reflexes at the affected ankle.

4.7.3 Repeat injury results in reflex inhibition if the initial injury occurs early but reflex facilitation if the initial injury occurs in adulthood

Repeat injury following early injury leads to a widespread inhibition of reflex activity that affects the inflamed and non-inflamed sides, this presumably reflects widespread sensitisation of both sides of the DH. If the first injury occurs in adulthood, however, reflexes are bilaterally facilitated. Repeat inflammation in adulthood appears to accelerate the development of hypersensitivity by-passing the protective seen following joint inflammation in Chapter 3.

Chapter 5 General Discussion

5.1 Introduction

Inflammatory pain is a significant clinical problem in juvenile inflammatory joint disease. However, it is yet to be established how inflammatory joint pain is processed in the immature nervous system. In this thesis FWR responses to noxious pinch have been characterised in the presence of joint inflammation and compared to the normal development of nociceptive reflex responses. It has been demonstrated that the profile of FWR responses in the presence of joint inflammation in young animals and adult animals differs greatly. Furthermore, it has been established that suffering joint inflammation early in life results in changes to nociceptive processing in adulthood and that the changes observed are specific to the age at which the injury occurs. This chapter will explore broader questions that data in this thesis have raised, experimental limitations and discuss the clinical implications of the data in the context of human studies of childhood and adult arthritis.

5.2 Summary of findings

5.2.1 Chapter 2

In this chapter the development of joint evoked reflexes was mapped out from the beginning of the second postnatal week to the middle of the fifth postnatal week. Previously well-characterised reflexes evoked from the toe provided a reference point for the analysis of joint evoked reflexes. The duration and amplitude of nociceptive reflexes evoked from the ankle are prolonged and more variable in young animals than older animals. There is a progressive refinement of reflex responses to pinch much of which occurs in the first three postnatal weeks. Interestingly, there was significant change in reflex responses beyond the third postnatal week, but these were not of the same magnitude seen in the preceding weeks.

5.2.2 Chapter 3

This chapter investigated the immediate and medium term effects of joint inflammation on nociceptive reflex circuitry. Inflammation at a young age (P8) results in a bilateral facilitation of EMG reflex responses to noxious pinch after 24 hours. By contrast, joint inflammation at P21 or P40 results in a profound

depression of reflex responses to noxious input 24 hours later that spreads from the originally inflamed joint. The relation between behavioural measures of hypersensitivity and reflex responses to mechanical stimuli were often at odds on first inspection. Under further investigation it is apparent that reflex inhibition and behavioural hypersensitivity are two parts of a biphasic response to joint inflammation. The first phase is typified by a joint protection, in which protection of the inflamed joint is prioritised over protection of the limb. In spite of reflex inhibition DH neuronal responses to pinch of the inflamed ankle were enhanced compared to naïve controls. In the second phase, protecting the limb from further damage is prioritised over specific joint protection. By 10 days following joint inflammation profound hypersensitivity is evident in all age groups. It is interesting to note that the protective phase following joint inflammation was completely absent from P8 animals.

5.2.3 Chapter 4

The long-term effects of early joint inflammation were considered in this chapter. Injury at P8 resulted in sensitivity that was apparent 36 days later and had spread beyond the originally inflamed joint. When a similar injury was performed in adulthood and reflex responses to pinch were assessed after the same period of time, changes to the reflex circuitry was less marked and restricted to the originally inflamed joint. The second section of experiments in this chapter explored the consequences of repeat inflammation following inflammation at different postnatal ages. The prior experience of early injury resulted in a more widespread depression of reflex responses to pinch, which is assumed to be a correlate of hypersensitivity. In rats that had received an injury at P40 and were re-inflamed at P72, there was a widespread facilitation of reflex activity. This result may have been confounded by the leak of CFA into the skin of the hind paw from what was presumably an abnormal joint.

5.3 Experimental considerations

5.3.1 Joint volumes and injections

A detailed discussion of this issue can be found in Chapter 3. The primary concern when performing joint injections in animals of different postnatal age was the variation in the size of joints. There are no studies that have examined the joint

volume of adult rats, let alone young ones. The author considered that a substantial proportion of studies into adult joint pain mechanisms had vastly overloaded the joint by injections of large volumes of inflammogens. Great care was taken to practice joint injection on cadaveric material with the express intention of making a more accurate estimation of joint volume at different ages. Injections of more than 5µl of blue dye were observed to spill into surrounding tissues in P8 animals, thus a volume slightly lower than this was chosen for injections at this age. The same procedure was used at each postnatal age injected to insure a localised inflammation without systemic spread. Animals in long-term studies were monitored daily for signs of systemic illness which can result from administration of CFA. Importantly, at no point in any study presented here was there evidence of systemic illness in any animals.

5.3.2 The use of CFA to induce inflammation

CFA inflammation was used here as model of joint inflammation, not specifically of juvenile idiopathic arthritis. There are many mouse models of inflammatory arthritis (Lindqvist et al., 2002), however the purpose of the studies presented here was to understand the processing of inflammatory pain in an immature spinal system, rather than investigate the underlying immunological mechanisms of the disease. Multiple studies have used CFA injection as a model of cutaneous inflammation in neonatal systems (Ruda et al., 2000; Tachibana et al., 2001; Walker et al., 2003), showing that immune competence required for a robust inflammatory reaction is functional at an early stage. Developmental differences in immune responses to peripheral inflammation are a potential factor in the development of inflammatory pain and thus are explored in Chapter 3.

5.3.3 Electromyography

EMG is a powerful technique with which to investigate the segmental processing of sensory information. Additionally, it is minimally invasive and allows the investigator to probe the sensitivity of nociceptive circuitry in the absence of widespread tissue damage associated with more invasive electrophysiology in an intact animal. This technique is, however, limited to the muscle studied and requires sedation. The FWR is a highly stable and well characterised metric for the excitability of spinal sensory circuits in animals and man (Sandrini et al., 2005), but does not allow more detailed characterisations of specific inputs and may be

affected by changes in the motor output. Inflammatory arthritis in adults is associated with postural and gait adjustments while weight bearing on the limb is reduced (Angeby-Möller et al., 2008; Boettger et al., 2011). During recordings, animals were suspended and did not weight bear, thus minimising the influence of proprioceptive inputs which are known to affect reflex responses to noxious inputs and the influence of anti-gravity muscle activity (Sandrini et al., 2005).

It is likely that the inhibition of flexion reflexes following joint inflammation was mediated at the level of the intermediate ventral horn. This fact and its implications are discussed below. Damage to the studied muscle was unavoidable, incision of the skin overlying the muscle allowed for accurate and less traumatic electrode placement which hopefully minimised this. Incisions were made before all recordings and so this was kept as constant as possible

5.3.4 Anaesthesia

Anaesthesia is known to affect the processing of sensory information in the spinal DH. The level of anaesthesia between experiments was kept constant. Though equilibration times changed depending on the size of the animal the final anaesthetic depth was held at 1-1.1% for all ages and all experiments as has been used elsewhere (Hathway et al., 2006a; Walker et al., 2007; Beggs et al., 2012a). Beggs and colleagues established that prior neonatal anaesthesia does not affect responses to repeat injury; the only instance in this thesis where anaesthesia may confound the experimental observations (Beggs et al., 2012a). Many studies have used decerebration in order to eliminate the potential effects of anaesthesia on reflex systems (Woolf and Swett, 1984; Coderre and Wall, 1987), but the effects of decerebration surgery can be very severe on young animals and the results generally are less easy to translate to clinical conditions.

5.3.5 Pinch stimulation

Pinch is a widely used noxious stimulus in neurophysiology. It is considered to be an intense mechanical stimulus that activates Aδ and C-fibres predominately. Pinch across the ankle and the toe is a commonly means of assessing joint pain clinically and was chosen for this reason. Difference in the intensity of pinch may explain small differences in the amplitude of responses, but would not account for changes of duration.

5.4 Wider discussion of the work presented

5.4.1 Deep versus cutaneous afferent inputs

It was apparent throughout the experiments performed for this thesis that pinch of the ankle and the toe had differing abilities to drive motor responses in the biceps femoris. Whilst afferent input from the adult inflamed ankle would frequently fail to elicit a reflex, it was clear that afferents from the toe were perfectly able to drive normal or slightly reduced reflexes in the same motor pool. This strongly suggests that there are functional and anatomical differences in afferent fibres arising from different hindlimb locations. Enhanced responses following cutaneous inflammation are well described, unlike the inhibition observed here (Woolf and Wall, 1986; Walker et al., 2007). It is reasonable to suggest that joint afferents have access to a population of inhibitory interneurones, unavailable to cutaneous afferents, within the deep dorsal and intermediate zone. Joint inflammation in young animals does not recruit the same inhibition, which is consistent with reports that local inhibitory networks in DH are immature at this stage (Baccei, 2004; Fitzgerald, 2005; Koch et al., 2012). During postnatal development the tuning of sensori-motor circuits is driven by activity dependent learning and can be altered by intervention in early life (Schouenborg, 2004). Blocking excitatory neurotransmission from afferent input at an early stage results in a failure of A-fibre withdrawal from the superficial DH and a failure of the development of appropriate segmental glycinergic inhibition (Beggs et al., 2002; Koch et al., 2012). Furthermore, sensory deprivation of the tail using local anaesthetic, hair removal or covering in a plastic tube results in a perpetuation of abnormal tail flick reflexes in response to noxious laser stimulation (Waldenström et al., 2003). As well as having immature A and C-fibre input in the first 3 postnatal weeks that is not seen in adult circuits, patterns of A and C-fibre terminations within the spinal cord are overlapping and poorly organised (Fitzgerald and Gibson, 1984; Granmo et al., 2008).

The termination patterns of cutaneous afferent in the spinal cord are well described (Molander and Grant, 1985; Todd, 2010). Surprisingly, given the number of studies that have investigated the effect of ankle joint inflammation on sensory systems, this is not true for joint afferents arising from the ankle. From

studies of the knee and muscle in various species it is clear that different terminal patterns exist for deep afferents and cutaneous afferents. Anatomical tracing of the medial and posterior articular input to the spinal cord from the knee reveals terminations in laminae I as well as V-VI, but no terminations in laminae II-IV (Craig et al., 1988). By contrast, cutaneous inputs are largely restricted to laminae I-III (Todd, 2010). Laminae II neurones have been implicated in the mechanical hypersensitivity associated with neuropathic and inflammatory injury, whilst ablation of a population of these neurones which express IB4 results in reduced mechanical hypersensitivity in peripheral cutaneous inflammation (Malmberg et al., 1997; Martin et al., 2001; Cavanaugh et al., 2009). Interestingly $IB4^{+}$ neurones are not found in joint and bone (Ivanavicius et al., 2004; Nakajima et al., 2008; Jimenez-Andrade et al., 2010). Joint and muscle afferents are also neurochemically distinct in other ways to cutaneous afferents. It may be that the higher levels of expression of the powerful neuromodulatory neuropeptides CGRP and SP in joints, which are also upregulated following inflammation, are poised to influence nociceptive circuitry in a unique way (O'Brien et al., 1989; Hanesch et al., 1995; Ling et al., 2003).

In electrophysiological studies, joint responsive neurones are found as far ventral as lamina VIII and generally receive convergent cutaneous and muscle input even at this level (Schaible et al., 1986, 1987). In their functional classification of afferent input to the spinal cord Menétrey and colleagues exclusively found cells responsive to joint stimulation in deep laminae and named them 'Class 4' neurones, differing from classes 1-3 by virtue of their deep location and limited responses to cutaneous stimuli (Menétrey et al., 1977).

The differences in lamina terminations of joint and skin afferents may result in functional differences between pain evoked from deep and cutaneous structures. Injection of the chemical irritant capsaicin into the skin results in a mechanical hyperalgesia that resolves rapidly, generally in less than 3 hours, whilst the same injury in muscle or joint results in a bilateral hyperalgesia that was evident for between 1 and 4 weeks (Sluka, 2002). Similarly, unilateral intradermal injection of carrageenan results in a short lived inflammation and associated hyperalgesia, whereas if injected into muscle or joint has a impact on sensitivity that is evident for up to 8 weeks following the initial injury (Radhakrishnan et al., 2003).

Furthermore, ventral-root reflexes are more substantially enhanced following C-fiber strength stimulation of joint and muscle afferents compared to C-fiber strength stimulation of cutaneous afferents (Wall and Woolf, 1984). Taken together these data suggest that deep afferents have a particularly powerful ability to affect the sensitivity of spinal circuits.

5.4.2 Role of ventral horn interneurone populations in reflex inhibition

Inhibition observed in this thesis may be mediated at the level of the ventral horn. Ib interneurones, so named because these neurones receive monosynaptic input from Ib afferents which carry input from Golgi tendon organs (GTOs), can in turn exert monosynaptic inhibitory input to motor neurone pools. Populations of inhibitory interneurones in the intermediate zone and ventral horns are critical for normal locomotion and coordination of reflexes. Ib afferents are poised to influence the excitability of motor pools locally and even contralaterally (Jankowska and Edgley, 2010). They are found in the intermediate zone of the spinal cord, namely laminae VIII, and generally receive input from muscle spindles as well as GTOs alongside input from multiple descending tracts (Brownstone and Bui, 2010). Little is known of the nociceptive inputs to these inhibitory interneurones (Hultborn et al., 1976; Harrison and Jankowska, 1985). Selective activation of fine unmyelinated muscle afferents can increase motor neurone excitability by disinhibition of Ib interneurones (Rossi et al., 1999). It appears that nociceptive muscle afferents can also modulate the excitability of extensor reflexes in humans (Rossi and Decchi, 1997). To date, the effect of nociceptive input into the intermediate zone has been observed to increase the excitability of motorneurones (Hultborn et al., 1976; Harrison and Jankowska, 1985). The altered input following joint inflammation may result in a change in the balance around Ib interneurones and result on a net inhibition of motorneurone pools, though this is unconfirmed.

5.4.3 Concluding thoughts on spinal cord circuitry

One of the most noteworthy results from the experiments contained within this thesis is the inhibition of evoked reflex activity to pinch following inflammation of the ankle in adult animals. In initial experiments this finding was difficult to interpret given the large body of electrophysiological literature that invariably

described enhanced reflexes following cutaneous inflammation. Little is known of the different nociceptive circuits activated by afferent input from deep tissues and muscles and to what extent they parallel the development of cutaneous inputs. It is assumed here that inhibition of motor unit activity following inflammation results from enhanced inhibition of the onward transmission of nociceptive inputs to motor neurones. It can be proposed, therefore, that reduced responses of adult arthritic rats to stimulation of the toe may be a result of shared articular and cutaneous inputs to deep dorsal and ventral horn neurones. This is summarised in Figure 5-1.

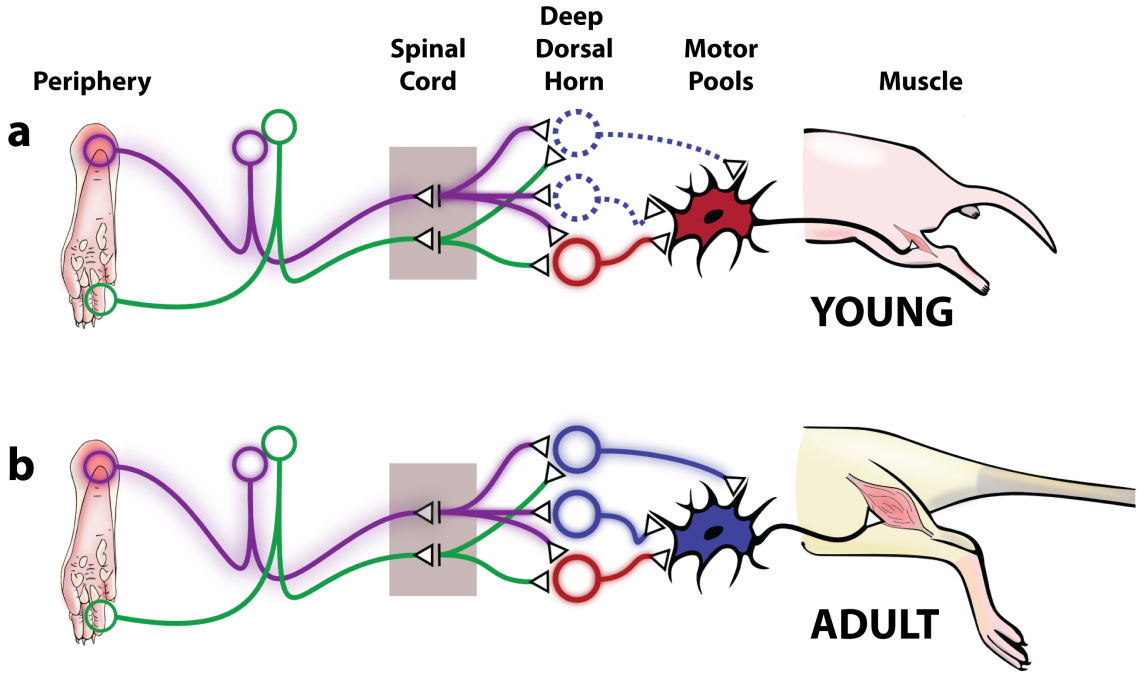


Figure 5-1 Potential mechanism underlying reflex inhibition differences at different ages
(a) In young animals joint inflammation results in sensitisation of joint afferents which terminate in the spinal cord. Joint afferents sensitise dorsal horn circuits whose common output is both excitatory and inhibitory interneurones in the deep dorsal horn. Inhibitory processing, however, is immature in the young spinal cord thus the predominant effect of joint inflammation at this developmental stage results in excitation of motor pools and enhanced reflex responses to noxious stimuli. **(b)** By contrast in the adult spinal cord, inhibitory processing is more appropriately targeted and more powerful. Joint afferent associated circuits have greater access to inhibitory interneurones than cutaneous inputs. Thus, in the presence of joint inflammation enhanced transmission through joint associated circuits results in a net inhibitory influence on motor neurone excitability.

In addition, it is proposed, in light of the evidence above of fundamental differences between afferents carrying information from joints and those from skin, that the profile of long-term changes to nociceptive circuits may differ depending on the site of original injury. Indeed, the consequences of early injury to deep sites result in a widespread sensitisation of nociceptive circuits distant from the site of original injury that is not true of the same injury in a purely cutaneous context (Anand et al., 1999; Al-Chaer et al., 2000; Sternberg et al., 2005; Miranda et al., 2006; Knaepen et al., 2012).

5.4.4 Clinical perspective and implications

Surprisingly, given the incidence and severity of pain in juvenile inflammatory joint disease, little is known of the mechanisms of joint pain in the immature nervous system. Historically, discussions of childhood inflammatory pain have been couched in terms of psychological well being, coping strategies and family interactions. Little attention has been directed to the neurobiological mechanisms of childhood joint pain and the long-term consequences of chronic pain in childhood. It is clear from this thesis that early joint pain experiences in rodents have the ability to shape the sensitivity of nociceptive circuits and inflammatory joint pain processing differs greatly between young and adult animals. To what extent this is true in humans is unknown.

Pain in JIA frequently has profound consequences on the activities of daily living. (Kimura and Walco, 2007). Pain can also often be a more important factor in disability than the progression of the disease itself and its alleviation can lead to appreciable improvements in well-being {Wolfe, 2000 #143}{Dhanani, 2002 #144}.

Despite the importance of pain in paediatric joint disease, it has received very little attention. This partly reflects a general neglect of chronic paediatric pain and a tendency of physicians to underestimate pain in chronically ill children compared to the children themselves and their parents (Howard, 2003; Janse et al., 2008). More recently other types of paediatric pain have received increased research attention, however, the need for proper evaluation and management of pain in children with arthritis continues to be unmet (Kuis et al., 1997; Malleson and Clinch, 2003; Kimura and Walco, 2007; Walker, 2008).

The lack of protective inhibition in young animals with inflamed joints is of clear clinical relevance. There is a disconnect between behavioural signs of pain in animals and the state of reflex excitability and by extension the sensitivity of reflex circuits. Perhaps the underestimation of pain in paediatric joint disease stems in part from the lower levels of behavioural change associated early joint inflammation. Gait disturbance, for instance, is often progressive in juvenile inflammatory joint disease and difficult to assess in a clinical setting due to often rapid growth and other developmental changes (Witemeyer et al., 1981; Fairburn

et al., 2002). The severity of gait disturbance associated with joint inflammation in childhood appears not to be as severe in adults (Lechner et al., 1987). This may be partly explained by the relative lack of inhibition seen in the developing nervous system. Unlike the young rats in this study that experienced only a single bout of inflammation, the relapsing and remitting nature of juvenile arthritis can result in multiple flare-ups throughout childhood. In the context of developing inhibition and the long-term consequences of early injury, flare-ups may compound pre-existing sensitivity in nociceptive circuits established by historic inflammation. The spread of sensitivity following repeat inflammation in adulthood raises the possibility that juvenile joint disease may be associated with increased pain and disability when flare-ups occur.

One may hypothesise, based on the results presented here, that adults who have suffered joint inflammation in childhood may have alterations to pain in adulthood. Due to the chronic nature of the disease in childhood and the potential for childhood disease to persist into adulthood it is difficult to disentangle the effects of early inflammation on adult pain processing in humans. What is evident is that early joint pain may persist into adulthood. As Packham and colleagues showed, adult patients with juvenile onset disease continued to suffer the painful consequences of their condition well into adulthood, often in the absence of ongoing inflammation (Packham et al., 2002). The disconnect between disease activity in adulthood and pain is also reflected in paediatric populations (Ilowite et al., 1992; Schanberg et al., 1997). Yet unexplored long-term mechanisms may govern the pain experience of adults exposed to joint inflammation as children.

Whilst this study was primarily focused on the developmental profile of inflammatory joint pain, there have been intriguing incidental findings in the adult processing of inflammatory joint pain that have not been widely explored. Changes to posture and movement typify responses to both cutaneous and deep painful injuries. This is often short lived and resolves with the injury. In chronic pain, especially arthritis, disturbances of gait and loss of movement of affected areas of the body are serious and poorly understood problems (Hodges and Tucker, 2011; Pietrosimone et al., 2011). Whilst commonly observed in animal studies of pain, gait abnormalities and loss of function have eluded the gaze of

sensory neurophysiologists. The phenomenon of reflex inhibition in the presence of joint damage is documented in the human literature and is recognized as *arthrogenic muscle inhibition* and can be established by knee or ankle effusion and is commonly seen following ankle injury such as sprains (Palmieri et al., 2004b; Palmieri et al., 2004a). The mechanisms underlying this are unclear, however, there is increasing evidence that points to responsible party being interneurones in ventral horn that have a powerful ability to control motorneurone excitability.

5.5 Further work

The experiments presented here have raised numerous questions, which given more time should be addressed to better understand the effect of joint inflammation in early life on sensory systems. These include, but are not limited to, the following:

5.5.1 Immature processing of joint pain

1. Is the innervation of joints in early life different from that of adult animals and to what extent does inflammation at an early age alter the course of primary afferent development in articular tissues?

2. Why does joint inflammation at P8 result in profound changes in reflex excitability from the inflamed ankle at P18, that is to say, why is there a delay in the appearance of sensitivity when there is increased sensitivity at the toe on the inflamed side?

3. What processes underlie the sensitivity in reflex circuits seen in adulthood after early injury and can this be prevented?

5.5.2 Adult processing of joint pain

1. Which inhibitory interneurones are responsible for joint inhibition and how are they related to the excitation of DH circuits?

2. Where is reflex inhibition co-ordinated and to what extent is this dependent on the severity or location of the injury?

3. Why does repeat inflammation in adulthood decrease protective inhibition?

5.6 General Conclusions

The following conclusions have been drawn from the work presented above.

5.6.1 Joint inflammation in early postnatal life is processed differently to joint pain in adults

Inflammation at an early age leads to widespread sensitisation of nociceptive reflexes that is unparalleled in adult animals. Young animals fail to develop protective inhibition of the inflamed joint that may lead to further damage and pain. By contrast in adult animals, in the acute phase of joint inflammation reflexes are inhibited presumably to prevent joint movement that would be painful. Once this inhibition has passed animals begin to show signs of classical reflex hypersensitivity following injury. Behavioural testing did not detect the changing balance of excitation and inhibition in reflex circuits, and should be used with caution in models of joint inflammation. It is concluded that the processing of inflammatory joint pain in early life differs from that in adults, furthermore, young animals are potentially at greater risk of joint damage following inflammation as they fail to develop protective behaviours.

5.6.2 The long-term consequences of a single joint inflammation are different depending on the time of life that the injury occurs

Animals that experienced joint inflammation in early life remained sensitive to noxious mechanical stimulation even after joint inflammation had passed. Joint inflammation in young animals results in long-term increase of reflex sensitivity in adulthood that is not seen if inflammation first occurs in adulthood. Instead animals that are older, have a residual protective inhibition at the previous inflamed joint. It is concluded that the development of hypersensitivity following joint inflammation is developmentally regulated and joint inflammation in early life has long-term potentially detrimental effects on the sensitivity of adult animals.

5.6.3 The effect of repeat inflammation in adulthood is dependent on pain history

If inflammation occurs in adulthood following an initial inflammation in early life the consequences on the excitability of spinal reflex circuits are different to those

if both inflammations occurred in adulthood. Re-inflammation as an adult following an inflammation when young results in widespread inhibition of reflex changes to mechanical stimuli on the inflamed and non-inflamed sides. Re-inflammation as an adult results in a widespread sensitisation of reflex circuits. Animals move more quickly, or bypass altogether the protective phase of joint inflammation and move rapidly to the hypersensitivity phase.

It is hoped that the results described here of previously unknown characteristics and mechanisms of joint pain in early life will contribute to a better understanding and treatment of pain in JIA.

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