Spinal Processing of Somatosensory Information in the Developing Dorsal Horn

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Declaration

I confirm that the work presented in this thesis is my own. Where information has been derived from other sources, or performed in collaboration with others, I confirm that this has been indicated in the thesis.

Emma Battell 25/10/2021

Abstract

Somatosensory processing is immature at birth and significant changes occur during postnatal development throughout the neuroaxis, which produce mature processing of these sensory modalities. There is increasing evidence that disruptions to normal development are associated with an increased risk of developing pain-related conditions in adulthood. As the spinal dorsal horn is a key integrative centre in the somatosensory system, fully understanding how the processing of peripheral stimulation matures at this site is undoubtedly beneficial. Previous research into postnatal maturation of the physiological responses of the dorsal horn predominantly relied upon single-unit electrophysiology and has demonstrated multiple critical periods of development over the first four postnatal weeks in rats. However, as the dorsal horn is very heterogeneous, with distinct roles for each of the laminae, we propose that investigating the development of the whole dorsal horn requires a broader approach which is not achievable from just measuring the activity in single neurons. Therefore, the aim of this thesis was to use spinal multielectrode array (MEA) electrophysiology, developed in our laboratory, in postnatal rats. This would enable the simultaneous study of all laminae of the dorsal horn across postnatal development, but also in rats which are older than those normally used in pre-clinical pain research, allowing changes across the whole life course to be investigated.

Studies were conducted using a range of peripheral stimulation modalities including, thermal, mechanical, and electrical stimulations, applied to the ipsilateral footpad to the MEA recording, as well as protocols designed to study inhibitory modulation and dorsal horn plasticity. Clear differences in evoked activity occurred between dorsal horn regions following peripheral stimulation, predominantly matching the excepted response profiles. Dorsal horn processing of A-fibre stimulation showed maturation over the first three postnatal weeks and increased conduction velocities until adulthood. Although C-fibre processing was present by the second postnatal week dorsal horn responses continued to increase until adulthood. Similarly, wind-up of dorsal horn neurons, a preclinical model of synaptic plasticity, was also significantly increased in postnatal rats (Postnatal day (P)9-30) compared to adults. Interestingly, GABAergic disinhibition produced differential effects throughout the life course suggesting that even in young adults (P42), GABAergic inhibition is not yet mature in the dorsal horn. Ultimately the data presented here suggests a longer duration of development occurs than previously thought in the processing of primary afferent fibre inputs, GABAergic activity, and wind-up in the dorsal horn, with maturation not occurring until adulthood as opposed to by the third or fourth postnatal week.

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Publications and External Conference Presentations

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Abbreviations

5-HT Serotonin

ACC Anterior Cingulate Cortex

aCSF Artificial Cerebrospinal Fluid

AgCl Silver Chloride

ANOVA Analysis of Variance

ASIC2 Acid-Sensing Ion Channel 2

ATP Adenosine Triphosphate

BOLD Blood-Oxygen-Level-Dependent

Ca^{2+} Calcium

$CaCl_2$ Calcium Chloride

CAP Compound Action Potential

CCK Cholecystokinin

CFA Complete Freund's Adjuvant

CGRP Calcitonin Gene-Related Peptide

CNS Central Nervous System

CR Calretinin

DCML Dorsal Column-Medial Lemniscus

dFC dynamic Functional Connectivity

DLF Dorsolateral Funiculus

DNA Deoxynucleic Acid

DNIC Diffuse Noxious Inhibitory Control

DRG Dorsal Root Ganglion

DRT Dorso Reticular Nucleus

DYN Dynorphin

E Embryonic Day

EEG Electroencephalography

EMG Electromyography

fMRI Functional Magnetic Resonance Imagining

GABA Gamma-aminobutyric Acid

GABAR Gamma-aminobutyric Acid Receptor

GAD Glutamate Decarboxylase

GAL Galanin

GlyR Glycine Receptor

GRP Gastrin-releasing Peptide

IASP International Association for the Study of Pain

IB4 Isolectin B4

K^+ Potassium

KCl Potassium Chloride

L(1-6) Lumbar

L(I-X) Lamina

LC Locus Coeruleus

LFP Local Field Potential

LTMR Low-Threshold Mechanoreceptor

MEA Multi-Electrode Array

$MgSO_4$ Magnesium Sulfate

Mrgprd Mas-related G-protein-coupled receptor D

mRNA Messenger Ribonucleic Acid

Na^+ Sodium

NAc Nucleus Accumbens

NaCl Sodium Chloride

$NaHCO_3$ Sodium Hydrogen Carbonate

NaH_2PO_4 Sodium Phosphate

Na_v Voltage Gated Sodium Channel

NK1R Neurokinin 1 Receptor

NKB Neurokinin B

NMDA N-methyl-D-aspartate

nNOS Neuronal Nitric Oxide Synthase

NPFF Neuropeptide FF

NPY Neuropeptide Y

NRM Nucleus Raphe Magnus

NTS Neurotensin

NTS Nucleus Tractus Solitarius

P Postnatal Day

PAF Primary Afferent Fibre

PAG Peri-Aqueductal Gray

PBN Parabrachial Nucleus

PD Post-Discharge

PFA Paraformaldehyde

PFC prefrontal Cortex

PIEZO2 Piezo-type Mechanosensitive ion channel component 2

PKCγ Protein Kinase C gamma

PNS Peripheral Nervous System

PSDC Postsynaptic Dorsal Column

PV Parvalbumin

RA Rapidly Adapting

RORβ Retinoid-related Orphan Receptor β

RVM Rostroventral Medulla

SA Slowly Adapting

SD Sprague-Dawley

SEM standard error of the mean

SOM Somatostatin

SP Substance P

TREK-1 TWIK-related Potassium Channel 1

Trk Tropomyosin Receptor Kinase

TRP Transient Receptor Potential

TRPA1 Transient Receptor Potential cation channel subfamily A member 1

TRPM8 Transient Receptor Potential cation channel subfamily M member 8

TRPV1 Transient Receptor Potential cation channel subfamily V member 1

TRPV2 Transient Receptor Potential cation channel subfamily V member 2

TRPV3 Transient Receptor Potential cation channel subfamily V member 3

TRPV4 Transient Receptor Potential cation channel subfamily V member 4

TTL Transistor-Transistor Logic

UoN University of Nottingham

vFh von Frey Hair

vGLUT2 Vesicular Glutamate Transporter 2

vGLUT3 Vesicular Glutamate Transporter 3

WDR Wide Dynamic Range

Chapter 1

General Introduction

1.1 Defining Pain

Pain is a complex phenomenon encompassing sensory-discriminative, affective-motivational, and cognitive-evaluative aspects (Melzack and Casey, 1968). This means that pain perception and responses can be affected by several factors including anxiety, fear, stress, and memory. The International Association for the Study of Pain (IASP) defines pain as "An unpleasant sensory and emotional experience associated with, or resembling that associated with, actual or potential tissue damage" and emphasise that pain is always subjective (IASP). Thus the studies presented in this thesis are better described as studying nociception "The neural process of encoding noxious stimuli" (IASP) as this removes the affective and cognitive aspects associated with pain.

The exteroceptive somatosensory system allows the external environment to be perceived and appropriate responses to be made, for example the movement away from a painful stimulus. The somatosensory system is incredibly complex, it includes multiple sensory and modulatory steps within the peripheral and central nervous systems. This introduction will give a brief overview of the mature systems involved in nociceptive signalling and the ongoing development occurring from birth to adulthood.

An important consideration in all pre-clinical work, possibly even more so in pain research, is the sex of the animals being used. An increasing focus within the pain research field is being put on the need to study both sexes following a significant number of studies identifying sex-dependent differences in adult rodents (Shansky and Murphy, 2021). Sex-differences have been found in the activity of microglia within the periaqueductal gray (PAG) (Doyle *et al.*, 2017), in migraine models (Avona *et al.*, 2019) and in the development of allodynia following spared nerve injury (Ahlström *et al.*, 2021; Boullon, Finn and Llorente-Berzal, 2021). Importantly this reflects the sex based differences observed in the clinic, for example in chronic pain, and as such policies on using both sexes in research are increasingly being introduced (Mogil, 2020). Additionally, pain-related sex differences have been found following various early life stresses (LaPrairie and Murphy, 2010), including repetitive needle prick (Knaepen *et al.*, 2012), intra-plantar injection of Complete Freund's adjuvant (CFA) (Lima *et al.*, 2014), and plantar hind-paw incision (Beggs *et al.*, 2012). These results suggest a possible sex-dependent maturation of these pathways. However, as historically the majority of research has used males only, it should be assumed that this is the case for the studies referred to in this introduction unless otherwise stated.

1.2 Somatosensory Processing in Adults

1.2.1 Cutaneous Sensory Receptors

The skin functions as a barrier to the outside world and as such accommodates the first stage in peripheral sensory processing. Specialised somatosensory organs are able to detect various aspects of light touch and are termed low-threshold mechanoreceptors (LTMRs) (Abraira and Ginty, 2013). These somatosensory organs were first identified in the 18th and 19th Centuries and include Pacinian corpuscles (Vater, 1741; Pacini, 1840), Meissner corpuscles (Wagner and Meissner, 1852), Merkel discs (Merkel, 1875), and Ruffini corpuscles (Ruffini, 1894). However, new research is still unravelling more details of their functional mechanisms (Cobo *et al.*, 2021). For example, a recent study has demonstrated the role of non-neuronal lamellae cells in touch detection at Meissner and Pacinian corpuscles (Nikolaev *et al.*, 2020) and another showed that two distinct mechanoreceptor subtypes are present within Meissner corpuscles (Neubarth *et al.*, 2020).

Specific specialised receptors are located in different skin types, the main types being glabrous and hairy skin. The studies conducted for this thesis involve the application of stimuli to glabrous skin, so that will be the focus in this introductory section, see Figure 1.1 for a schematic of the receptors discussed below. In glabrous skin Meissner corpuscles and Pacinian corpuscles are rapidly adapting, sensing vibration and light stroking whilst Merkel cells and Ruffini corpuscles are slowly adapting and respond to indentation and stretch (Abraira and Ginty, 2013). Rapidly adapting (RA) responses produce action potentials at only the onset and offset of a mechanical stimulus, and can be divided in to two types RAI and RAII, with type I associated with Meissner corpuscles and type II with Pacinian corpuscles, which have larger receptive fields (Knibestöl, 1973). Slowly adapting (SA) responses maintain firing during sustained indentation by a stimuli and can also be divided into two categories SAI and SAII; SAI are associated with Merkel cell complexes and responses involving indentation and SAII with stretch. SAII show more regular static-phase firing rates and a much larger (5x) receptive field (Johansson and Vallbo, 1980).

The LTMRs involved in innocuous touch are specialised because the perception of the shape, roughness, and vibration are important in discriminating what an object is. Conversely, free nerve endings are linked mostly with the perception of high threshold, nocuous stimuli, where the important information is simply that there is a high intensity of mechanical, heat or cold stimulation that could cause damage. A- and C-primary afferent fibres (PAFs) are present in all layers of the epidermis as free nerve endings (Hilliges, Wang and Johansson, 1995). Interestingly keratinocytes have also been linked

with the generation of action potentials in response to innocuous and noxious stimuli for a variety of primary afferent subtypes (Baumbauer *et al.*, 2015) as well as specifically in the processing of mechanical (Moehring *et al.*, 2018), cold, and heat signalling via ATP pathways (Sadler, Moehring and Stucky, 2020). Supporting the concept that an intact *in vivo* system may be best when researching somatosensation.

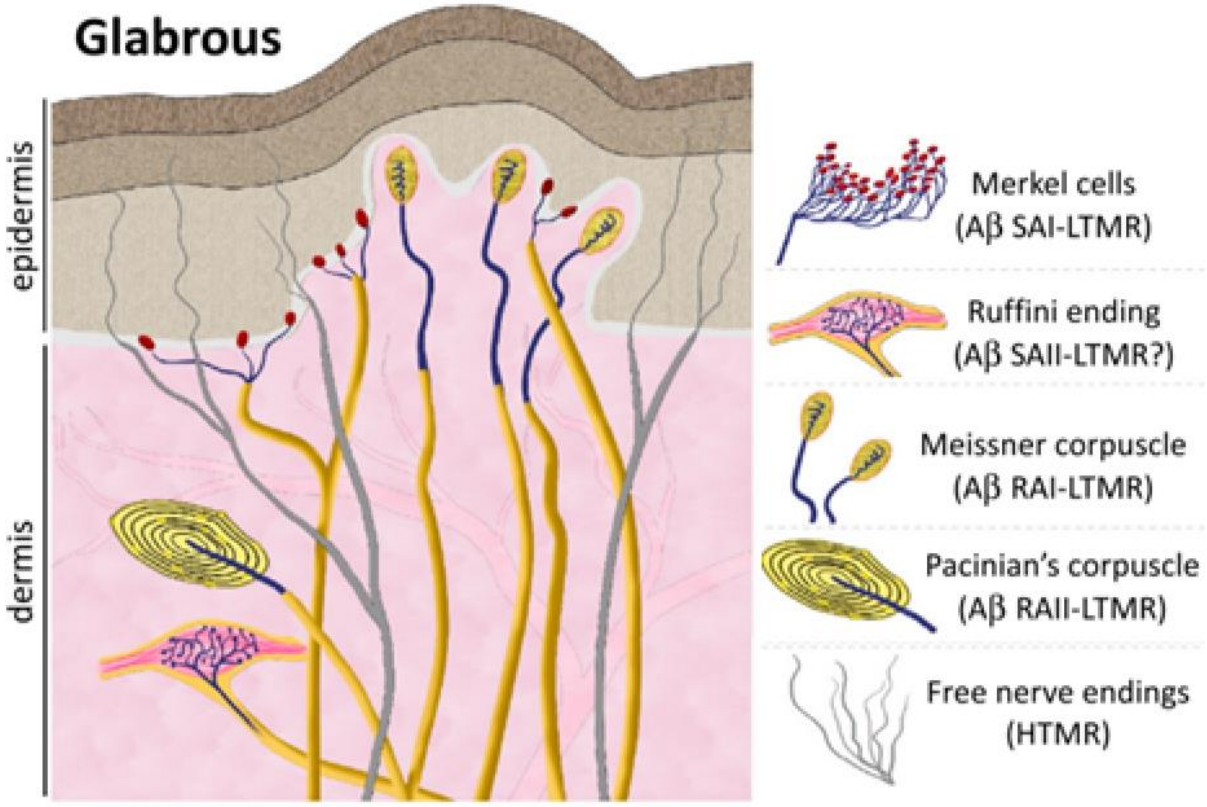


Figure 1.1 Cutaneous sensory receptors. Specialised sensory receptors in glabrous skin are shown at their respective location in the dermis and epidermis. Slow adapting (SA) and rapidly adapting (RA) types are specified as well as low threshold mechanoreceptors (LTMR) and high threshold mechanoreceptor (HTMR) endings. (Modified from Abraira and Ginty, 2013).

1.2.2. Cutaneous Sensory Receptors and Ion Channels

LTMRs and Free nerve endings express membrane bound receptors and ion channels that respond to distinct stimuli, with some possessing multiple channel types resulting in them being polymodal (Bessou and Perl, 1969). A key family of these membrane bound receptors are transient receptor potential (TRP) cation channels which are ionotropic receptors that play a role in sensing stimuli throughout the body (Bennett, A. J. Clark, *et al.*, 2019). Peripheral cutaneous processing of mechanical, thermal, and chemical stimuli encompasses a variety of these channels, shown in Table 1.1. These channels have been identified in mammals so far but many more have been identified in other species, so this most likely does not represent an exhaustive list. Additionally, this list does not

reflect the expression levels of these receptors, which can vary by PAF subtype (Kobayashi *et al.*, 2005). TRPC1, acid-sensing ion channel 2 (ASIC2), and piezo-type mechanosensitive ion channel component 2 (PIEZO2) receptors have also been linked with detection of innocuous mechanical stimulation (Woo *et al.*, 2014; Sexton *et al.*, 2016; Cobo *et al.*, 2021).

Receptor	Sensory Modality	Associated Sensory Fibre
TRPA1	Mechanical, Thermal (<18°c), chemical	C-fibre
TRPM8	Thermal (<28°c), chemical	C-fibre
TRPV1	Thermal (>42°c)	Aδ- and C-fibres
TRPV2	Mechanical, Thermal (>52°c)	Aβ- and Aδ-fibres
TRPV3	Thermal (>34-39°c), chemical	C-fibre
TRPV4	Thermal (>27-34°c), chemical	Aβ-(?), Aδ- and C-fibres
TREK-1	Mechanical, Thermal	Aβ-(?), Aδ- and C-fibres
TRPC1	Mechanical	Aβ-, Aδ- and C-fibres
ASIC2	Mechanical	Aβ- and Aδ-fibres
PIEZO2	Mechanical	Aβ-fibres

Table 1.1 Sensory receptor ion channels. The sensory modality and associated primary afferent fibre types are listed for the key ion channel receptors. (Modified from Lumpkin and Caterina, 2007 and Cobo *et al.*, 2021).

These receptors allow transduction of the original stimulus into electrical activity in the form of an action potential. This transduction is facilitated by a range of ion channels allowing the movement of associated ions across the neuronal cell membrane including calcium ions (Ca^{2+}), potassium ions (K^{+}), and sodium ions (Na^{+}) (Coste *et al.*, 2012; Bennett, A. J. Clark, *et al.*, 2019).

Voltage gated sodium channels (Na_v), play a particularly key role in the initiation and amplification of action potential. Na_v channels are comprised of an α subunit and β subunits; currently 10 different α subtypes and four β subtypes have been identified (Glass, Duncan and Biggin, 2020). Na_v subtypes confer different functional roles in the generation of action potentials and are differentially expressed on primary afferent fibres (Bennett, X. A. J. Clark, *et al.*, 2019). Mature PAFs all express Na_v 1.1, 1.6, 1.7, and 1.8, and non-peptidergic C-fibres also express Na_v 1.9 (Dib-Hajj, Black and Waxman, 2015). The expression level, however, varies between the PAF types; Na_v 1.1 is expressed predominantly on myelinated neurons (Fukuoka *et al.*, 2008), Na_v 1.6 is expressed on non-myelinated neurons (Black, Renganathan and Waxman, 2002) and at the nodes of Ranvier in myelinated neurons (Caldwell *et al.*, 2000), Na_v 1.7 has greater expression on small DRG neurons and Na_v 1.8 on nociceptive neurons (Djouhri, Fang, *et al.*, 2003; Djouhri, Newton, *et al.*, 2003).

Unsurprisingly, these sodium channels have all been linked with the processing of painful stimuli; Na_v 1.1 with mechanical pain (Osteen *et al.*, 2016) and increases in Na_v 1.6 expression in several pain states (Deuis *et al.*, 2013; Xie, Strong and Zhang, 2015). However, being the predominant channels on nociceptive neurons, the main research focus remains on Na_v 1.7, 1.8, and 1.9 (Goodwin and McMahon, 2021). The mechanisms underlying the roles of these channels in pain are still being investigated. For example it has been recently suggested that Na_v 1.7 is involved in mediating analgesia at the level of the spinal cord, but not at the periphery, via an opioid mediated pathway (MacDonald *et al.*, 2021).

1.2.3 Primary Afferent Fibres

As mentioned above there are several primary afferent sensory neuron sub-types which are associated with the detection of different aspects of somatosensation. The three main types considered in cutaneous somatosensory studies are Aβ-, Aδ-, and C-fibres. The primary afferent types are grouped according to several factors including their cell surface markers expressed, cell body size, axon diameter, degree of myelination, and conduction velocity. Despite the anatomical and functional differences between the different primary afferents they all use glutamate as their main neurotransmitter thus having excitatory effects on postsynaptic targets (Todd, 2010). However, recent studies using transcriptional analysis have suggested there are at least 11 subtypes of sensory neurons (Usoskin *et al.*, 2015; Sharma *et al.*, 2020). Although these have not been fully correlated with functional groups (Gatto *et al.*, 2019).

Primary afferents neurons facilitate the passing of sensory information from the periphery to the CNS. When an appropriate stimulus is detected at the skin, an action potential is generated at the axon terminal of the primary afferent fibre, which is then passed to the cell body of the neuron located in the dorsal root ganglia (DRG) (Mesulam and Brushart, 1979). DRGs are pseudo-unipolar neurons with a single bifurcating axon allowing the action potential generated at the periphery to be passed to the spinal cord (Ranson, 1912). It is worth noting that the anatomical and functional characteristics of these different primary afferent fibre types have historically been investigated in a range of species and some interspecies variations have been observed (Djouhri and Lawson, 2004; Abraira and Ginty, 2013).

1.2.3.1 A-Fibres

A-fibres are large, myelinated afferents, and as such are capable of saltatory conduction. A wide range of conduction velocities have been reported for A-fibres, partly due to variations between species (Djouhri and Lawson, 2004)and with age (Fitzgerald, 1985; Vejsada *et al.*, 1985). Aβ-fibres are thickly myelinated, have the largest diameter of the three main fibre types, and the fastest conduction velocity. They are predominantly involved in sensing low threshold mechanical stimuli via the specialised cutaneous sensory receptors (Section 1.2.1) but are also involved in nociceptive signalling (Djouhri and Lawson, 2004). In adults LTMR Aβ-fibres innervate the intermediate and deep dorsal horn (lamina III-V) (Todd, 2010) but nociceptive Aβ-fibres innervate the superficial dorsal horn (lamina I and IIo) as well as lamina V (Djouhri and Lawson, 2004).

Aδ-fibres are thinner than Aβ-fibres and as such have a slower conduction velocity, but as they are myelinated they are still significantly faster than C-fibres. They can be divided into two subgroups; Aδ-HTMR which are peptidergic and express calcitonin gene-related peptide (CGRP) and Aδ-LTMR express the molecular marker tropomyosin receptor kinases B (TrkB) (Gatto *et al.*, 2019). Aδ-HTMR are associated with the processing of higher threshold touch as well as heat and cold (McGlone and Reilly, 2010) and innervate lamina I and IIo (Abraira *et al.*, 2017). Although associated with innocuous mechanosensation in hairy skin, Aδ-LTMR have also been linked with Pacinian corpuscles (Gatto *et al.*, 2019) and innervate the dorsal horn at lamina IIi/III (Abraira *et al.*, 2017).

1.2.3.2 C-Fibres

C-fibres have the smallest diameter but are grouped into Remak bundles (Harty and Monk, 2017). They are unmyelinated and have the slowest conduction velocity of the three fibre types, as they utilise continuous conduction instead of saltatory conduction. C-fibres can be subdivided into peptidergic neurons expressing neuropeptides including CGRP and non-peptidergic neurons expressing receptors including mas-related G-protein coupled receptor member D (MrgprD) and IB4; but these groups can also be further divided. For example, Salio et al. (2020) characterised three non-peptidergic subgroups in mice. C-fibres are involved in processing innocuous and noxious mechanical pain, heat, and cold and have been shown to be polymodal (McGlone and Reilly, 2010). However some studies have suggested specific subgroups are involved in processing distinct stimuli types for example $MrgprD^{+}$ fibres are involved in sensing noxious mechanical stimulation but not thermal (Cavanaugh *et al.*, 2009). Additionally, C-LTMR have been identified which are associated with lanceolate endings in hairy skin (Gatto *et*

al., 2019). C-fibres innervate the dorsal horn predominantly in lamina II but in a subtype specific pattern, peptidergic neurons innervate lamina I-IIo, $IB4^+$ neurons innervate the dorsal horn in lamina IIi and C-LTMR innervate lamina IIiv (Todd, 2010; Abraira *et al.*, 2017).

1.2.4 The Spinal Cord Dorsal Horn

The spinal cord, in particular the dorsal horn, is a key site of somatosensory processing, with the ventral horn primarily involved in movement. Primary afferents innervate the spinal cord in a somatotopically organised pattern relating to their site of origin both rostro-caudally as well as dorsoventrally (Molander, Xu and Grant, 1984). This allows incoming information to be processed within the spinal cord in specific microcircuits involving inhibitory and excitatory interneurons, propriospinal neurons and projection neurons (Todd, 2010). Additionally, whilst out of the scope of this thesis it is important to note that non-neuronal cells, particularly glial cells, have been shown to have roles in somatosensory processing (Gareth J. Hathway *et al.*, 2009; Kohro *et al.*, 2020; Gazerani, 2021).

1.2.4.1 Laminar Structure of the Dorsal Horn

The spinal cord can be divided into different regions across the dorsoventral axis; historically this has been based on factors such as the morphology of the neurons and resulted in 10 distinct regions termed laminae. Laminae I-VI comprise the dorsal horn and VII-X the ventral horn (Figure 1.2) (Rexed, 1954; Molander, Xu and Grant, 1984). The structure of the dorsal horn has been investigated in a range of species, historically in the cat and more recently in rodents, particularly mice, and interestingly a large proportion of the deeper dorsal horn research has been conducted in hamsters. It is worth noting that some inter-species and inter-strain differences exist, such as lumbar location of sciatic nerve origins (Rigaud *et al.*, 2008). The dorsal horn can be considered as three regions: laminae I and II as the superficial dorsal horn, III-IV as the intermediate and V-VI as the deep. However, each lamina has distinct cell populations, PAF inputs, ascending tracts and descending tracts (Figure 1.3), which will be briefly covered below, more details can be found in several recent studies and reviews (Abraira *et al.*, 2017; Todd, 2017; Häring *et al.*, 2018; Peirs, Dallel and Todd, 2020). The vast majority of research has focussed on the superficial dorsal horn, partly due to its key role in the gate control theory of pain (Melzack and Wall, 1965), with the intermediate

and deep regions being relatively poorly characterised (Abraira *et al.*, 2017; Wercberger and Basbaum, 2019).

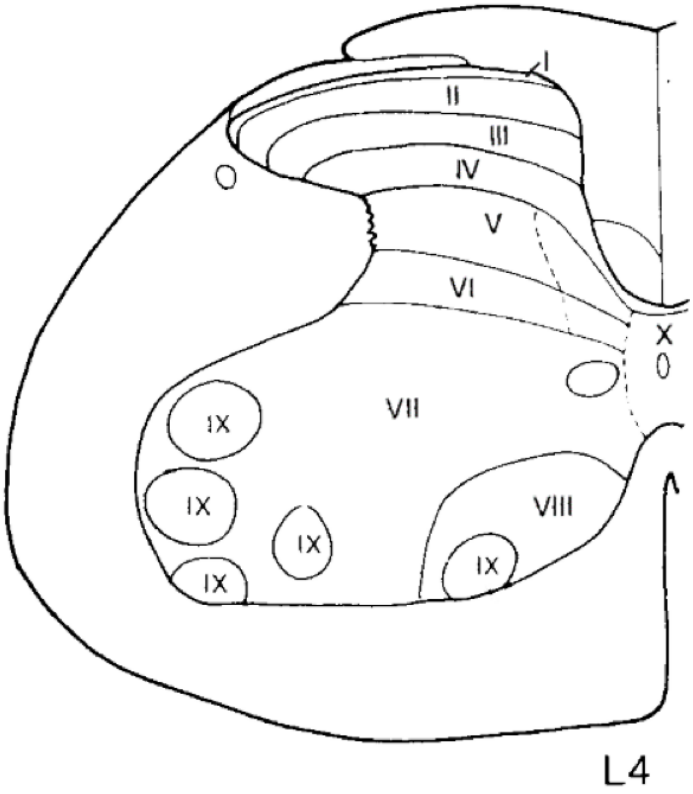


Figure 1.2 Laminae structure of the rat spinal cord (at the lumbar 4 segment). Schematic drawing showing laminae I-X (Modified from Molander, Xu and Grant, 1984).

1.2.4.2 The Superficial Dorsal Horn

Lamina I (also known as the marginal layer) is a key site for relaying peripheral input to supraspinal regions via projection neurons. The majority of lamina I projection neurons receive direct inputs from nociceptive Aδ- and C-fibre PAFs and indirect inputs from Aβ- and non-peptidergic C-fibre PAFs (Torsney and MacDermott, 2006; Cordero-Erausquin *et al.*, 2009). Despite their important role, projection neurons account for only 5% of the neurons in lamina I with the remainder being interneurons (Spike *et al.*, 2003). Inhibitory interneurons ($GABA^+$) account for 25% of lamina I neurons, with 12% of lamina I neurons expressing both GABA and glycine, but none expressing glycine exclusively (Polgár *et al.*, 2003).

Lamina II (also known as the substantia gelatinosa) is often further divided into an outer (IIo) and inner (IIi) regions and more recently into three regions (IIo,IIid, IIiv) due to

different neuronal subtypes present (Duan *et al.*, 2014). Lamina II is densely packed and comprised almost exclusively of interneurons (Spike *et al.*, 2003), with inhibitory interneurons thought to represent 30% of the neurons in lamina II. As with lamina I, approximately 12% of lamina II neurons express both GABA and glycine, but none express glycine exclusively (Polgár *et al.*, 2003).

Somatostatin (SOM^+) excitatory interneurons account for 7% of lamina I neurons and 37% of lamina II neurons (Duan *et al.*, 2014). Lamina II SOM^+ interneurons receive inputs from C-PAFs (89% monosynaptic) as well as inputs from Aδ- and Aβ- fibres, depending on their exact location within lamina II (Duan *et al.*, 2014). (Protein Kinase Cγ (PKCγ) excitatory interneurons are also found in lamina IIi, they receive inputs from Aδ-LTMR and C-LTMR PAFs (Abraira *et al.*, 2017) and activate lamina I projection neurons (Braz *et al.*, 2014). Laminae I and II are the main location of Galanin/Dynorphin (GAL/DYN^+) inhibitory interneurons (Duan *et al.*, 2014) which account for approximately 26% of inhibitory interneurons in lamina I and 10% in lamina II (Tiong *et al.*, 2011). Parvalbumin (PV^+) inhibitory interneurons are also located in the superficial dorsal horn and are predominantly along the laminae IIiv and III border (Petitjean *et al.*, 2015). Neuronal nitric oxide synthase ($nNOS^+$) expressing interneurons are also found in the superficial laminae, however it is less clear if they are all inhibitory interneurons (T. C.P. Sardella *et al.*, 2011). GAL/DYN^+ inhibitory interneurons receive inputs from Aβ-PAFs and have been implicated in gating the output of circuits involving SOM^+ and PKCγ excitatory interneurons (Duan *et al.*, 2014). PV^+ inhibitory interneurons have also been shown to receive Aβ-PAF inputs and tonically inhibit PKCγ excitatory interneurons (Petitjean *et al.*, 2015) potentially via a glycinergic mediated pathway (Miraucourt, Dallel and Voisin, 2007; Takazawa and MacDermott, 2010).

1.2.4.3 The Intermediate Dorsal Horn

Lamina III is comprised of projection neurons and interneurons, with interneurons accounting for approximately 98% of the neurons in this region (Abraira *et al.*, 2017). Inhibitory interneurons represent approximately 40% of neurons in lamina III. Unlike in the superficial lamina 90% of the $GABA^+$ interneurons co-express glycine and approximately 1.5% of lamina III neurons express exclusively glycine (Polgár *et al.*, 2003). NPY^+ inhibitory interneurons account for almost 10% of lamina III neurons, PV^+ inhibitory interneurons account for 8% (Abraira *et al.*, 2017), and GAL^+ inhibitory interneurons make up 2% of lamina III neurons (Tiong *et al.*, 2011). The main excitatory interneurons are SOM^+ accounting for approximately 16% of lamina III neurons, with excitatory interneurons expressing CCK^+, VGLUT3, PKCγ, and other

markers also being identified, but with overlapping populations (Gutierrez-Mecinas *et al.*, 2016).

Lamina IV neurons are heterogeneous in size and are mostly larger than the neurons in lamina III (Rexed, 1954). Lamina IV also contains projection neurons and interneurons, although less is known about the exact populations of interneurons in this lamina than laminae I-III (Al-Khater and Todd, 2009).

Laminae III and IV contain the cell bodies of large neurons, sometimes referred to as antenna cells, which have dendrites that extensively branch in laminae I and II (Ramon y Cajal and Azoulay, 1909; Szentágothai, 1964) and some which branch to laminae III and IV (Fernandes *et al.*, 2018) and potentially to all laminae of the spinal cord (Szentágothai, 1964); although it is worth noting that the study by Fernandes et al. (2018) used postnatal day (P)9-12 rats and the golgi studies conducted by Szentagothai used P7-35 kittens and P35-56 dogs, so these results may not reflect the mature state. There are at least two populations of these large neurons; one expressing Neurokinin 1 receptor (NK1r) which associate with substance P (SP) expressing axons and are projection neurons, and a second which do not express NK1r and are thought to be interneurons (Polgár, Al Ghamdi and Todd, 2010). Collectively these large neurons have multiple roles in the circuitry within the dorsal horn; they are thought to allow the transfer of information from the superficial dorsal horn to the intermediate and deep dorsal horn (Szentágothai, 1964; Polgár, Al Ghamdi and Todd, 2010), as well as from the intermediate dorsal horn to lamina I projection neurons (Todd, 2017), and can themselves be projection neurons (Polgár, Al Ghamdi and Todd, 2010). Laminae III/IV projection neurons receive mono-synaptic Aβ inputs as well as LTMR-mediated polysynaptic inhibitory and excitatory inputs (Abraira *et al.*, 2017).

1.2.4.4 The Deep Dorsal Horn

Lamina V receives direct input from Aβ- and Aδ- PAFs (Harding, Fung and Bonin, 2020) as well as inputs from the laminae III-IV large neurons as mentioned previously. Lamina V represents a key site in dorsal horn circuitry, in part due to the presence of wide dynamic range (WDR) neurons. WDR neurons can respond to a variety of innocuous and noxious mechanical, thermal, and chemical stimuli in an intensity dependent manner (Wall, 1960; Mendell, 1966; Carstens, 1997). WDR neurons receive information from more superficial laminae including lamina II (Braz *et al.*, 2005) and a subset of these WDR neurons which express NK1R are projection neurons (Section 1.2.5.1). Lamina VI is only present in the cervical and lumbosacral enlargements of the spinal cord and contains small, regularly arranged neurons compared to lamina V (Peirs, Dallel and

Todd, 2020). A population of neurons originating within laminae V and VI have been shown to extend GABAergic axons to deeper laminae of the ventral horn where they can inhibit motorneurons (Hughes *et al.*, 2005). More recent studies have found specific subpopulations of laminae V/VI inhibitory interneurons which express RORβ aiding in fluid locomotor activity (Koch *et al.*, 2017).

An interesting additional lamina to consider is Lamina X. While not defined as being in the dorsal horn this lamina surrounds the central canal putting it at a similar depth to the deepest dorsal laminae in the lumbar regions. Lamina X receives direct input from visceral Aδ- and C- PAFs and as such has been suggested to play a role in somatosensory, particularly nociceptive processing (Krotov *et al.*, 2019).

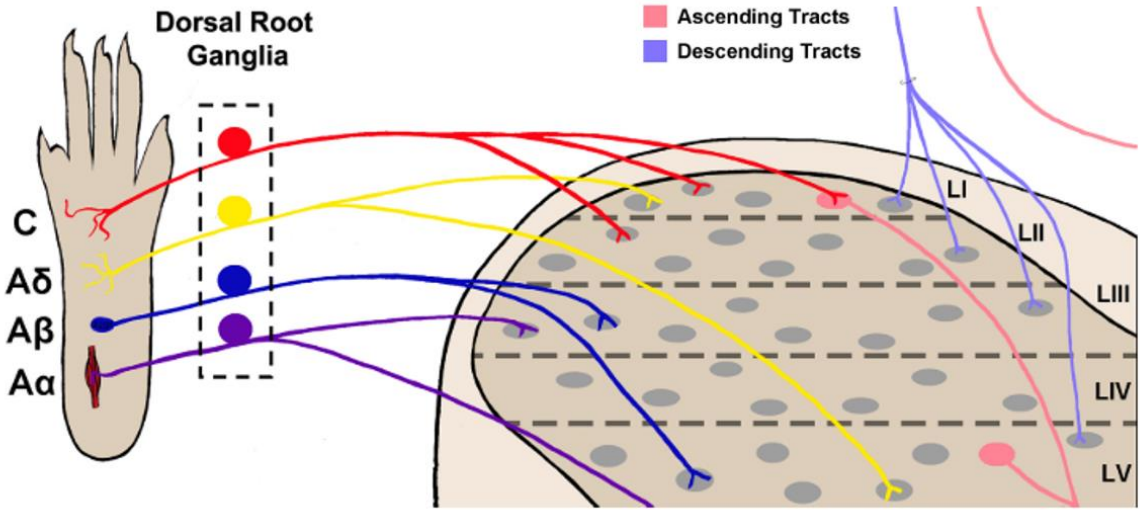


Figure 1.3 Simplified schematic of somatosensory circuitry of dorsal horn inputs and outputs. Primary afferent fibres from the periphery and descending tracts from supraspinal regions innervate the dorsal horn at specific lamina. In general high threshold C-fibres terminate in the superficial laminae (I&II), Aδ-fibres innervate lamina I and V, Aβ-fibres terminate in the intermediate lamina III and V, and Aα-fibres innervate intermediate lamina III and laminae in the ventral horn. Ascending tracts from dorsal horn lamina I and V project to supraspinal regions (modified from Harding, Fung and Bonin, 2020*).*

1.2.4.5 Dorsal Horn Interneurons

Interneurons, also called local circuit neurons, have axonal projections that remain within the spinal cord (Bice and Beal, 1997). However these can extend to adjacent spinal segments. Interneurons can be divided into inhibitory and excitatory groups, with inhibitory interneurons using GABA and/or glycine as their main neurotransmitter and excitatory interneurons using glutamate (Todd, 2017). Interneurons can be further

subdivided in relation to their expression markers, electrophysiological properties, morphology, and involvement in processing specific stimuli (Hughes and Todd, 2020).

At present, five subsets of inhibitory interneurons have been identified in the spinal cord based on their expression markers (Peirs, Dallel and Todd, 2020). (1) Galanin/Dynorphin $(GAL/DYN)^+$ interneurons (Thomas C.P. Sardella *et al.*, 2011) (2) Neuronal nitric oxide synthase (nNOS) expressing interneurons which may have a role in inhibiting noxious mechanical stimuli (Hughes and Todd, 2020). (3) Neuropeptide Y $(NPY)^+$ interneurons may also inhibit noxious stimuli, although possibly only following chronic pain states (Solway *et al.*, 2011). (4) Paravalbumin $(PV)^+$ interneurons have been identified as being involved in inhibition following innocuous sensory stimuli (Petitjean *et al.*, 2015). (5) Calretinin (CR) expressing interneurons are potentially involved in modulating innocuous and noxious input on to spinoparabrachial neurons (Petitjean *et al.*, 2019). However, inter-species differences have been noted between mice and rats, with the CR^+ group only identified in mice so far, as well as changes in overlapping expressing markers in mice compared to rats (Boyle *et al.*, 2017). Additionally, it is important to note that many of these expression markers are also found on excitatory interneurons and primary afferents, and thus additional markers, including Pax2 which is thought to be expressed on all inhibitory interneurons, need to be used in addition (Hughes and Todd, 2020).

Six partially overlapping groups of excitatory interneurons have also been identified in the dorsal horn so far (Peirs, Dallel and Todd, 2020). Although there is evidence that a seventh subgroup may exist (Häring *et al.*, 2018). (1) Neurokinin B (NKB) (Polgár *et al.*, 2006), (2) Neurotensin (NTS) (Todd, Russell and Spike, 1992), (3) Substance P (SP) (Polgár *et al.*, 2020), (4) Cholecystokinin (CCK) which may play roles following innocuous stimulation and in mechanical allodynia in neuropathic pain (Gutierrez-Mecinas, A. M. Bell, *et al.*, 2019), (5) Neuropeptide FF (NPFF) involved in itch and noxious stimuli input (Gutierrez-Mecinas, A. Bell, *et al.*, 2019), (6) Gastrin-releasing peptide (GRP) possibly involved in affective touch and itch (Bell *et al.*, 2020). However, most of the studies mentioned above have been conducted in mice and similar to inhibitory interneurons, some inter-species differences have been found between mice and rats (Peirs, Dallel and Todd, 2020).

1.2.5 Connections with Supraspinal Regions

The spinal dorsal horn is connected to supraspinal structures involved in the processing of somatosensory stimuli via multiple ascending and descending pathways. Several brain regions are involved in different aspects of this processing including the parabrachial nucleus (PBN), the thalamus, the periaqueductal grey (PAG), the rostroventral medulla

(RVM), the somatosensory cortices (S1 and S2), the locus coeruleus (LC), the anterior cingulate cortex (ACC), the insular cortex, the amygdala, the prefrontal cortex (PFC), and the nucleus accumbens (NAc). Of these, the ACC, PFC, amygdala, and NAc are involved in the emotional and contextual aspects of pain (Ossipov, Morimura and Porreca, 2014). The details of these regions and their supraspinal connections is out of the scope of this thesis however regions directly connected with the spinal cord will be highlighted below.

Ascending and descending pathways interact with each other forming modulatory spino-bulbo-spinal loop systems, which can modulate somatosensory processing at the spinal cord. This modulation can be both facilitatory and inhibitory allowing appropriate adaptation to the current environment.

1.2.5.1 Ascending Pathways

Projection neurons in lamina I and III-VI (and X) facilitate the transfer of somatosensory information from the dorsal horn to supraspinal regions via ascending tracts (Al-Khater, Kerr and Todd, 2008; Al-Khater and Todd, 2009). Generally, projection neurons from lamina I are involved in nociceptive stimuli, heat, and cooling sensations (Chisholm *et al.*, 2021) whereas projection neurons in deeper lamina are involved in innocuous and noxious stimulation (Abraira *et al.*, 2017). However, this is a complex topic and research is still ongoing to fully understand the subpopulations of projection neurons and their ascending pathways (Wercberger and Basbaum, 2019).

There are several major ascending tracts, which can be grouped into those ascending via the dorsal column, predominantly involved in transmitting visceral somatosensory information and those ascending via the ventrolateral or anterolateral column which are involved in transmitting cutaneous somatosensory information (Al-Chaer *et al.*, 1996). The dorsal column includes the gracile fasciculus, cuneate fasciculus, and the postsynaptic dorsal column (PSDC). The gracile fasciculus contains axons of primary afferents from the lower trunk and extremities, the cuneate fasciculus contains axons of primary afferents from the upper trunk and extremities, and the PSDC is comprised of projection neurons from lamina III/IV (and X) of the dorsal horn (Watson and Harrison, 2012; Abraira *et al.*, 2017). These dorsal column tracts ascend ipsilaterally in a somatotopically organised pattern to the gracile and cuneate nuclei in the brainstem, where they synapse with neurons with axons forming the medial lemniscus that extends to the contralateral side of the thalamus; forming the dorsal column-medial lemniscus pathway (DCML) pathway (Kayalioglu, 2009).

The anterolateral column includes the spinothalamic tract, which has two components the anterior spinothalamic tract which transmits innocuous somatosensory information and the lateral spinothalamic tract involved in nociceptive and thermal transmission (Almeida, Roizenblatt and Tufik, 2004). It is also important to note that the spinothalamic tract has multiple collateral branches and innervates several brainstem regions as well as the thalamus (Boadas-Vaello *et al.*, 2016). However, only approximately 5% of lamina I projections neurons in the rat lumbar dorsal horn are spinothalamic projection neurons (Polgár, Al Ghamdi and Todd, 2010).

Also predominantly included in the anterolateral column are the spinocerebellar and spinoreticular tracts. Spinocerebellar tracts can be divided into the ventral spinocerebellar tract and dorsal spinocerebellar tract (Xu and Grant, 1994) which follow different routes to the cerebellum with the dorsal tract ascending on the ipsilateral side via the dorsal column and the ventral on the contralateral side within the anterolateral column (Kayalioglu, 2009; Watson and Harrison, 2012). These tracts are involved in proprioception of the lower trunk and hindlimbs (Bosco and Poppele, 2001). However, it has been suggested that spinocerebellar tracts are very infrequent in L4-6 of rats (Molander, Xu and Grant, 1984). Similarly, the spinoreticular tract ascends to the brainstem as two sections, one involved in motor control and the other in nociception (Almeida, Roizenblatt and Tufik, 2004). They project to a number of brain regions including the parabrachial nucleus (Millan, 1999). These tracts predominantly ascend via the anterolateral column but some projections ascend via the dorsal column. Some lamina V projection neurons ascend via the spinoreticular tract (Steeds, 2016).

Additionally, the spinomesencephalic tract innervates multiple regions including the PAG and parabrachial nucleus (Millan, 1999). Approximately 95% of lamina I projection neurons in the rat lumbar dorsal horn are spinoparabrachial projection neurons (Polgár, Al Ghamdi and Todd, 2010), although it has been suggested that these divide into distinct subgroups (Browne *et al.*, 2021). However, due to collateral branches in these tracts it has been shown that some lamina I projection neurons can ascend to at least three supraspinal regions, including the thalamus, lateral PBN, and PAG (Al-Khater and Todd, 2009). In line with this it has been reported that 30% of lamina I projection neurons ascend to the PAG (Spike *et al.*, 2003). A subset of projection neurons from laminae III/IV also ascend to the lateral PBN via spinoparabrachial tracts (Cameron *et al.*, 2015).

1.2.5.2 Descending Pathways

There are multiple descending pathways which form the descending pain modulatory system and enable supraspinal sites to modulate activity at the spinal cord level via facilitatory or inhibitory effects, depending on the specific populations of neurons involved (Harding, Fung and Bonin, 2020). As mentioned previously, several brain regions are involved in different aspects of pain processing. Some regions have direct descending projections to the spinal dorsal horn, including the nucleus tractus solitaries (NTS), PBN, Dorsal reticular Nucleus (DRT) (aka subnucleus reticularis dorsalis (SRD)), and hypothalamus (Millan, 2002). However, the majority of supraspinal regions project to the PAG either directly or indirectly (Quintero, 2013).

The PAG is located in the midbrain and surrounds the midbrain aqueduct. It can be divided into subgroups consisting of four longitudinal columns; the ventrolateral PAG (vlPAG), lateral PAG (lPAG), dorsolateral PAG (dlPAG), and dorsomedial PAG (dmPAG), which have distinct physiologies and functions (Bandler and Shipley, 1994) as well as being distinct along the rostrocaudal axis (Moss, Glazer and Basbaum, 1983; Satpute *et al.*, 2013). The PAG receives inputs from the dorsal horn (Al-Khater and Todd, 2009) as well as multiple supraspinal regions including the hypothalamus, amygdala, and the anterior cingulate cortex (ACC) (Heinricher, Tavares, Leith and B.M. Lumb, 2009). Whilst the cortical input into the PAG is important and alterations in these connections have been linked with the development of chronic pain (Drake *et al.*, 2021), this work will instead focus on descending pathways starting at the level of the PAG or descending directly to the spinal cord, as these are expected to more closely reflect nociception rather than pain.

The PAG functions as an integrative centre for descending modulation and has been the subject of extensive research. Multiple studies have demonstrated the inhibitory effects of stimulating the PAG electrically and pharmacologically with opioids (Reynolds, 1968; Wang and Wessendorf, 2002). As well as the differential descending effects depending on PAF input and lamina depth of the target neurons (Koutsikou *et al.*, 2007; Waters and Lumb, 2008). Additionally, sex differences have been identified in the PAG and have been suggested as a contributing factor to the gender differences observed in opioid pain treatment in humans (Shansky and Murphy, 2021). The main output of the PAG is to the RVM, including direct projections to RVM neurons which project to the spinal cord (Morgan *et al.*, 2008).

The RVM is located in the brainstem and includes the nucleus raphe magnus (NRM) and the adjacent reticular formation, both of which have direct descending inputs to the dorsal horn (Fields and Heinricher, 1985; Heinricher, Tavares, Leith and B. M. Lumb,

2009). The RVM has reciprocal connections to the PAG and receives inputs from other supraspinal regions including the thalamus, locus coeruleus, and parabrachial region (Ossipov, Morimura and Porreca, 2014). The RVM is composed of ON-cells, OFF-cells, and NEUTRAL-cells; which were originally classified by their response to noxious thermal stimulation of the tail with ON-cells increasing in neuronal discharge, OFF-cells decreasing in discharge, and NEUTRAL-cells which showed no response (Morgan and Heinricher, 1992) however this non-responsive group has been suggested to respond to alternative stimuli (Leung and Mason, 1998; Ellrich, Ulucan and Schnell, 2000). Both ON and OFF cells receive inputs from the PAG (Morgan *et al.*, 2008) and project to the spinal cord (Heinricher, Tavares, Leith and B. M. Lumb, 2009). In line with this, ON-cells are involved in facilitation and OFF-cells in inhibition of nociceptive outputs from the RVM (Heinricher, Tavares, Leith and B. M. Lumb, 2009).

Neurons within the RVM can be divided into subpopulations based on their expression of different receptors. As with the spinal cord dorsal horn GABA is inhibitory and glutamate is excitatory (Morgan *et al.*, 2008). Approximately two thirds of PAG-RVM projections are to GABAergic neurons, and approximately two thirds of these PAG projection neurons are also GABAergic (Morgan et al., 2008). The dorsal horn has also been shown to receive GABAergic input from the RVM, including onto PAFs (Zhang *et al.*, 2015). It has recently been suggested that at least two GABAergic descending pathways exist from the RVM to the dorsal horn. An inhibitory pathway involving a subset of GABAergic (GAD2 expressing)/enkephalinergic RVM neurons inhibiting PAF input and a double inhibitory pathway, functioning as an excitatory pathway, involving a different subset of GABAergic (GAD1 expressing)/ non-enkephalinergic RVM neurons projecting to enkephalinergic dorsal horn inhibitory neurons (François *et al.*, 2017).

Approximately 40% of RVM neurons are serotonergic (Marinelli *et al.*, 2002). As with the GABAergic neurons, the downstream effects of serotonergic projections from the RVM are bidirectional with activation resulting in either inhibitory (5-HT7) or excitatory (5-HT2/5-HT3) pain modulation depending on the receptors expressed by the spinal neurons (Dogrul, Ossipov and Porreca, 2009; Bannister and Dickenson, 2017) (Figure 1.4). Additionally, a subset of descending serotonergic projections from the RVM directly input with $TRPV1^+$ spinal cord neurons (Zhao *et al.*, 2014) although it is not clear if this is a completely distinct subset.

Noradrenergic neurons are located in the brainstem in clusters referred to as A1 to A7 (Pertovaara and Almeida, 2006) with A5 – A7 having projections to the spinal cord (Westlund *et al.*, 1983). Specifically, the LC and pontine (subcoeruleus) regions contain noradrenergic nuclei ($A_{5\&6}$ and A_7 respectively) with the A_7 input to lamina I/II, IV, and V being particularly key in descending inhibition (Millan, 2002). The LC can project to the

spinal cord directly or indirectly via the RVM and is thought to function in pain states (Llorca-Torralba *et al.*, 2016). Descending noradrenergic projections to the spinal cord cause noradrenaline release, resulting in the activation of spinal α2 adrenergic receptors, which inhibit pre- and post-synaptic transmission of nociceptive signals (Budai, Harasawa and Fields, 1998; Pertovaara and Almeida, 2006).

These descending projections enter the spinal cord predominantly via the dorsolateral funiculus, which projects to the superficial (laminae I and II) and the deep (lamina V) dorsal horn (Basbaum and Fields, 1979), and to a lesser extent via the ventrolateral funiculus (Fields and Basbaum, 1978). Descending input can modulate activity within the spinal cord at the terminals of PAFs, at interneurons, at projection neurons, and at sites of other descending pathways (Millan, 2002).

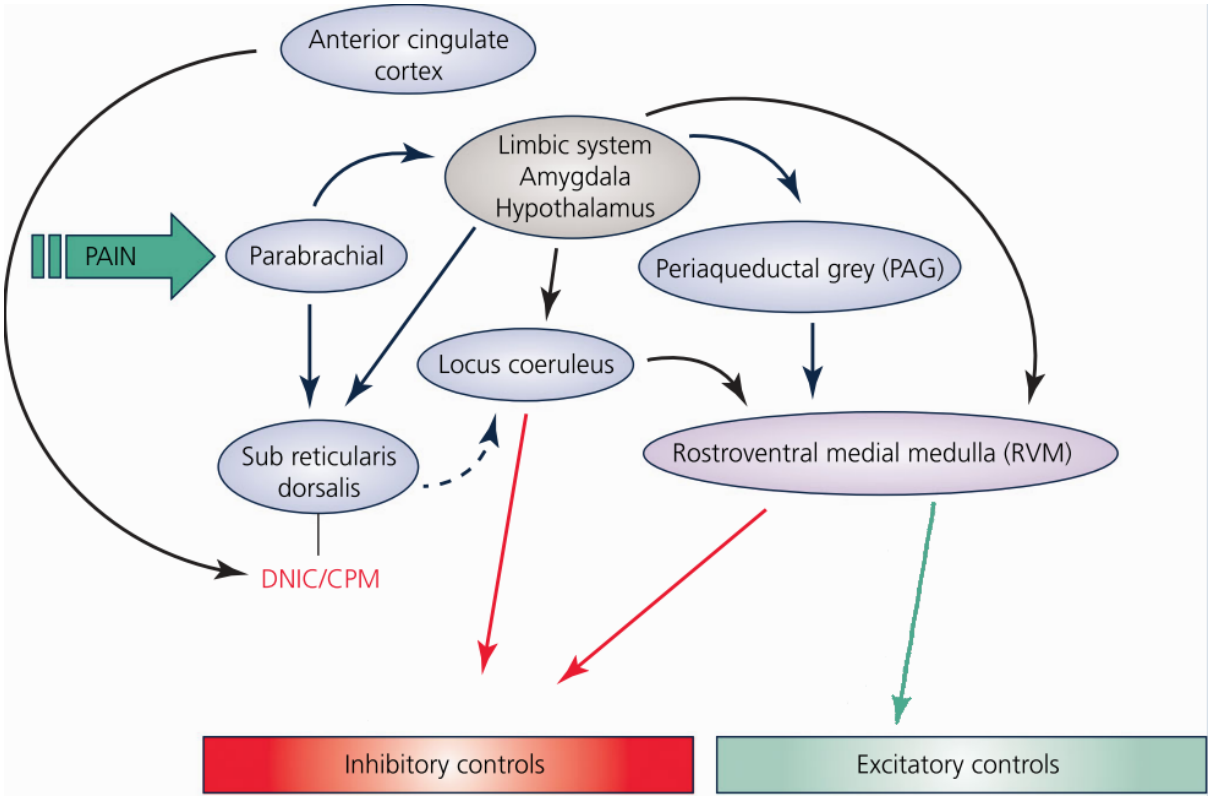


Figure 1.4 Descending control from supraspinal regions. Processing following the detection of pain involves several supraspinal regions culminating in either inhibitory or excitatory modulation of the spinal dorsal horn. (Modified from Bannister and Dickenson, 2017).

1.2.6 Pain Theories

Over the last two and a half millennia multiple theories, including the specificity, intensity, pattern, and gate control theories have attempted to explain how somatosensory information, particularly pain, is perceived (Reviewed by Perl, 2007; Moayedi and Davis, 2013). The most widely recognised is the gate control theory, first

proposed in 1965 by Melzack and Wall (Melzack and Wall, 1965), which combined aspects of the specificity and pattern theories. The gate control theory of pain suggests that cutaneous stimulation evokes impulses that travel to the spinal cord, specifically neurons in the substantia gelatinosa (lamina II) and central transmission cells. The substantia gelatinosa cells act as a 'gate' to the incoming information, modulating the response before it is transmitted to central transmission cells. The theory also considers three key aspects of pain perception; previous ongoing activity, the stimulus-evoking activity, and the balance of activity detected by A- vs C-fibres. However, this theory is still not adequately complex to explain the processing of pain and work is ongoing to fully understand the mechanisms involved (Moayedi and Davis, 2013).

1.3 Postnatal Development of the Somatosensory System

Pain is detectable at all postnatal ages, however as altricial species humans and rats are born in an immature state and the pathways involved in processing innocuous and nocuous stimuli from the periphery are still maturing over the postnatal period (Fitzgerald, 2005). Whilst the processes involved in postnatal development are comparable between rats and humans, the ages as which developmental progress occurs in not directly comparable. Complications occur in comparing ages between the two species as life-period e.g. weaning, adolescence, adulthood, and old age, affects the comparative rate of aging (Sengupta, 2013) as well as the specific tissues being studied (Ohmura and Kuniyoshi, 2017). Rough estimates of comparative ages suggest the first postnatal week in rats is comparable to premature new-born humans, the following two weeks in rats, before weaning occurs, compares to the end of the gestational period and the first two years of development in humans, and from postnatal day (P)21 until adulthood (approximately P60) correlates with childhood and adolescence in humans (Figure 1.5) (Kort, 2021).



Figure 1.5 Comparative postnatal ages between rodents and humans. GW: gestational weeks, P: postnatal day, y:years. (Kort, 2021).

The immaturity of these systems results in multiple quantifiable changes during postnatal development. In both rats and humans, neonates display lower thresholds to nociceptive stimuli, have larger cutaneous receptive fields, and respond with exaggerated movements to painful stimulation compared to adults (Fitzgerald, 2005). However, it was not until the late 1980s that the occurrence of neonatal pain was becoming more widely acknowledged (Hall and Anand, 2014). As such, research into the maturation of these pathways is still ongoing and whilst substantial progress has been made into unravelling the complex systems involved, it is far from completely understood.

Gaining a better knowledge of the postnatal maturation of the somatosensory system is required for several reasons. Primarily that babies, in particular pre-term babies, undergo multiple painful procedures in early life including injections and heel lancing, appropriate analgesic treatments for which are still being trialled (Slater *et al.*, 2017). Associated with this is the growing amount of evidence that disruption to the maturation of these pain processing pathways through early life pain, or following treatment with chemotherapeutics such as vincristine and cisplatin (Hathway *et al.*, 2018; Schappacher *et al.*, 2019), can increase the risk of developing chronic pain states as an adult (Williams and Lascelles, 2020).

Secondly, the age of animals used in *in-vivo* and *in-vitro* work appears to be overlooked in many studies even though it has large implications on the results (Michel *et al.*, 2020). This is in part due to technical limitations with certain techniques. For example neonatal preparations are preferentially used for *in-vitro* studies because it is harder to maintain adequate oxygenation to an adult section and it is easier to visually identify cells as there is less myelination in younger samples (McCutcheon and Marinelli, 2009).

1.3.1 Postnatal Development of Cutaneous Somatosensory Receptors

Free nerve endings of both A- and C-fibres begin to innervate the hindpaw epidermis at embryonic day (E)16, innervate the whole paw by E18, and then withdraw to the dermal/epidermal border by E20 (Jackman and Fitzgerald, 2000). The majority of specialised LTMR (Section 1.2.1) develop following the presence of the primary afferent nerve fibres and as such, do not begin their maturation until the end of the gestational period (Zelena, 1976; Zelená *et al.*, 1990). The exception to this are Merkel cells, as they are derived from epithelial cell lines and are almost mature at P7 (English, Burgess and Norman, 1980) thus allowing the (slow adapting) detection of indentation by the first postnatal week.

Meissner corpuscles begin to be recognisable from P8 in the mouse toe pad and their maturation is complete at approximately P20-25 (Idé, 1977). Similarly, the Pacinian corpuscle inner core is morphologically mature by P7 and by P20 the corpuscle resembles the mature form, although outer core layers continue to be added and their mean size continues to increase until P60 (Zelená, 1978; Fleming and Luo, 2013). These developments result in the ability to discretely detect (rapidly adapting) vibration and light stroking via Meissner and Pacinian corpuscles being almost mature by the third postnatal week. However, the developmental process of Ruffini endings and Lanceolate endings is still not clear (Fleming and Luo, 2013).

1.3.2 Postnatal Development of Cutaneous Sensory Receptors and Ion Channels

Postnatal changes have been identified in key sensory receptors in the skin. Expression of TRPV1, which is expressed on Aδ- and C-fibres and involved in thermal sensation, is detectable at E12.5 in the mouse and appears to reach adult levels of mRNA expression at P0 (Hjerling-leffler *et al.*, 2007). However, there is a postnatal decrease in mRNA expression at P7 and P14, where the expression is almost half of the adult levels (Hjerling-leffler *et al.*, 2007). In line with this, another study found that TRPV1 expression in the skin more than doubles between P10 and adults (Guo *et al.*, 2001). Similarly, TRPM8, which is present on C-fibres and involved in thermal and chemical sensation, is present at E16.5 and has comparable mRNA expression levels at P0 and in adults but again shows a decrease during postnatal development (Hjerling-leffler *et al.*, 2007). Interestingly TRPA1, which is expressed on C-fibres and involved in mechanical, thermal, and chemical responses was not detectable until P0 in peptidergic neurons and not until P14 in non-peptidergic neurons, and did not reach mature levels of mRNA expression before adulthood (although P14 was the oldest developmental age used in this study) (Hjerling-leffler *et al.*, 2007). In line with this, there have been reports of a postnatal switch around P10 during which a subset of mechanically sensitive C-fibres become polymodal, responding to thermal stimulation as well as mechanical (Jankowski *et al.*, 2014).

Sodium channel expression on PAFs is also developmentally regulated; almost all of the sodium channels expressed in embryonic DRGs undergo changes before reaching the adult state. Na_v 1.2, 1.3, and 1.5 all decrease in expression with increasing postnatal age (Bennett, A. J. Clark, *et al.*, 2019). Conversely, there is an increase in expression of Na_v 1.1, 1.8, and 1.9 from late embryological development to adult levels by P7, although the distribution pattern of Na_v 1.8 and 1.9 doesn't reach the adult state until P21 (Benn *et al.*, 2001). In addition to overall expression level changes, some Na_v

channels undergo developmental regulation including Na_v 1.7 and 1.9 which are both involved in nociceptive signalling (Bennett, A. J. Clark, *et al.*, 2019). Na_v 1.7 undergoes alternative splicing at two sites (coding exons 5 and 11), producing functionally different isoforms, the ratios of which alter with postnatal development; the ratio of 5N:5A isoforms is 62:38 in neonates (P2-4) and levels out to 50:50 in adults whereas the 11S:11L isoforms are found at a 56:44 ratio in neonates and at 31:69 in adults (Raymond *et al.*, 2004; Choi *et al.*, 2010). The 5N form is more prevalent in neonates and has faster inactivation at negative potentials and smaller current amplitudes with faster depolarisations. The 11S form, more highly expressed in neonates, is sensitive to cAMP regulation resulting in a negative activation shift which is linked with hyperexcitability, whereas the 11L isoform is not (Chatelier *et al.*, 2008). Na_v 1.9 undergoes developmental changes in glycosylation state between P7 and P21 with an additional form present in neonatal DRGs, which has a smaller depolarizing influence on resting potential and a smaller amplification of depolarizing inputs (Tyrrell *et al.*, 2001).

Within the dorsal horn Na_v 1.1 mRNA expression is low in the neonatal dorsal horn and increased during early life, between P10 and P21, to adult levels (Beckh *et al.*, 1989; Blankenship, Coyle and Baccei, 2013). Conversely, Na_v 1.2 and 1.3 have been shown to be highly expressed throughout the first two to three postnatal weeks, although there is disagreement on the exact age their expression decreases (Beckh *et al.*, 1989; Hildebrand *et al.*, 2011; Blankenship, Coyle and Baccei, 2013). The amplitude of Na_v currents in dorsal horn neurons increases during postnatal development (Safronov, Wolff and Vogel, 1999) as well as the current density and decay time of sodium channels in the dorsal horn which have also been shown to increase over the first three postnatal weeks (Tadros *et al.*, 2015). Lamina specific differences in expression levels have also been shown previously in the adult cord but have not yet been investigated throughout development. Additionally, the Na_v 1.7 isoforms can determine the binding of B1 subunits altering the activation with the neonatal form producing a hyperpolarizing shift in activation 5 times larger than the adult form (Farmer *et al.*, 2012). One thing to note, however, is that the older/adult ages used in some of these studies are still very young; P25-30 (Hildebrand et al., 2011) and >P21 (Tadros *et al.*, 2015).

1.3.3 Postnatal Changes in Primary Afferents

Primary afferent fibres are still undergoing myelination following birth (Vejsada *et al.*, 1985). Schwann cells are responsible for the myelination of primary afferent fibres; immature Schwann cells surround large groups of axons at E18 with myelination starting at birth (Jessen and Mirsky, 2005). Initially, fibres are grouped into bundles containing

all fibre types and surrounded by these immature Schwann cells. Postnatal radial sorting occurs until approximately P10 and involves the segregation of larger fibres to the edge of the bundles to be myelinated. This results in the removal of A-fibre afferents from the bundles, leaving behind only smaller C-fibres which become Remak bundles (Feltri, Poitelon and Previtali, 2016), see Figure 1.6.

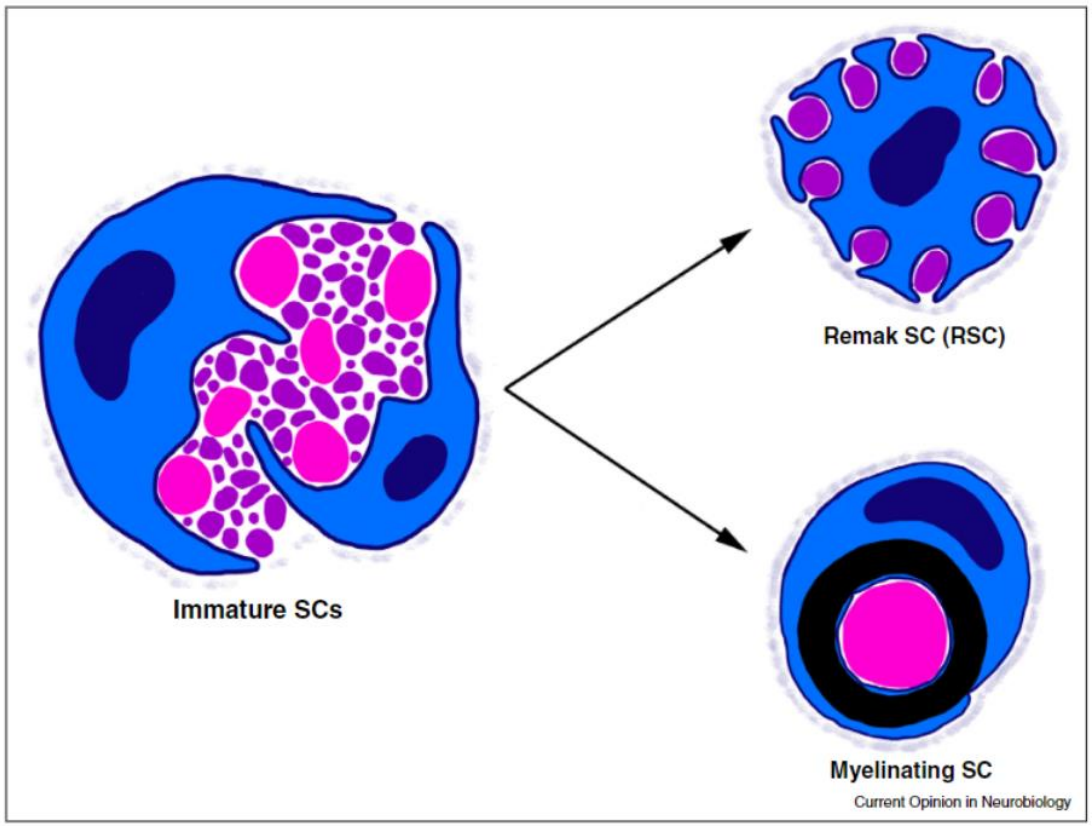


Figure 1.6 Postnatal maturation of Schwann cells (SCs). Immature Schwann cells are sorted during development by radial sorting. Large diameter axons (>1mm, pink) are individually myelinated by myelinating Schwann cells and small axons (<1mm, purple) are myelinated by Remak Schwann cells becoming Remak bundles. (Modified from Harty and Monk, 2017*).*

As C-fibres are not individually myelinated there is not a significant change in nerve diameter between P0 and adults and accordingly no significant change in the conduction velocity of these fibres (Lizarraga, Chambers and Johnson, 2007). However, some studies have suggested that C-fibres are not functionally mature until P14 (Fitzgerald and Gibson, 1984). Conversely, peripheral A-fibres undergo considerable postnatal myelination and as such their conduction velocities increase significantly during early life and are reported to approach adult levels at approximately P21 (Vejsada *et al.*, 1985).

1.3.4 Postnatal Changes in the Dorsal Horn

At birth the laminar structure of the spinal cord is present and recognisable, however the dorsal horn is still growing in size during postnatal development, including in the dorsoventral axis, and does so in a lamina specific manner (Lorenzo *et al.*, 2008). The superficial lamina does not change dramatically and has reached the adult size by P20, with most of the growth occurring in lamina IIi. Whereas the intermediate and deep dorsal horn lamina show a progressive increase in thickness with postnatal age; with this growth likely reflecting the increase in myelination of the innervating PAFs and the increase in dendrites of neurons in this region (Lorenzo *et al.*, 2008). The dorsal horn also shows a postnatal decrease in neuronal density, even in laminae where there is little growth. For example in lamina I there is approximately a 25% decrease in neuronal density between P10 and adults (S. H. W. Man, Géranton and Hunt, 2012).

Both A-fibres and C-fibres are present in the spinal cord at birth, being detectable in the rat lumbar dorsal horn at E15-17 and 18-20, respectively (Mirnics and Koerber, 1995; Jackman and Fitzgerald, 2000). However, their termination pattern is not mature; A-fibre afferents extend throughout lamina I and II at birth but during the first three postnatal weeks, the majority withdraw to terminate in lamina III and IV as seen in the adult dorsal horn (M. Fitzgerald, Butcher and Shortland, 1994) or by P28 (Nakatsuka and Kumamoto, 2000; Beggs *et al.*, 2002). The withdrawal of superficial A-fibre projections is predominantly a withdrawal of Aβ- and not Aδ-fibres (Park *et al.*, 1999) with changes in Aβ-, Aδ-, and C-fibre afferent inputs to lamina II neurons at P21-23 going from 51%/46%/36% to 9%/86%/84% in adults (Nakatsuka and Kumamoto, 2000). The withdrawal of A-fibres is thought to be NMDA receptor dependent (Beggs *et al.*, 2002) and involved in the maturation of dorsal horn inhibition and associated postnatal changes in receptive fields (Bremner and Fitzgerald, 2008). Additionally, although C-fibres are present in the early postnatal dorsal horn their processing is not functionally mature until the second postnatal week (Fitzgerald and Gibson, 1984). The activity of these C-fibres is required for the development of mature glycinergic inhibition and possibly NMDA receptor maturation (Hori and Kanda, 1994; Koch *et al.*, 2012).

Dorsal horn projection neurons and interneurons also undergo postnatal changes, while the majority of previous work has focused on lamina I neurons, it could be expected that postnatal changes occur throughout the dorsal horn. Lamina I spinoparabrachial projection neurons undergo developmentally regulated changes in their gene expression over the postnatal period (Serafin *et al.*, 2020), and, at least the $NK1^+$ spinoparabrachial neurons, are capable of responding to peripheral stimuli at P3 (S. H. W. Man, Géranton and Hunt, 2012). Additionally, a subset of lamina I spinoparabrachial neurons (approximately 10% (Li *et al.*, 2015)) display intrinsic burst firing/pacemaker activity

during early life, although this is still present at P38 (Li and Baccei, 2021) so may persist into adulthood. Interestingly, lamina I inhibitory DYN$^+$ interneurons, which can directly inhibit spinoparabrachial projection neurons, exclusively receive C-fibre input at P6-7 with Aβ-fibre inputs joining at P21-22; resulting in the PAF evoked EPSC amplitude increasing significantly after P21 as the overall glutamatergic drive increases (Brewer *et al.*, 2020). Additionally, they display an increase in their inhibitory synapse efficacy onto lamina I spinoparabrachial neurons over the postnatal period (Brewer *et al.*, 2020). Inhibitory nNOS interneurons in the superficial dorsal horn also may not reach a mature state until the third postnatal week (Liuzzi *et al.*, 1993). Furthermore, the expression of excitatory PKCγ-immunoreactive interneurons in lamina II of the rat lumbar spinal cord increases significantly over the first three postnatal weeks (Sweitzer *et al.*, 2004).

Changes in GABAergic and glycinergic mechanisms occur during postnatal development with a switch from predominantly GABAergic signalling to a balance between the two as seen in the adult dorsal horn (Baccei and Fitzgerald, 2004). In the adult dorsal horn Aβ-fibre evoked responses are normally under tonic glycinergic inhibition which is developed between P14 and P21, although exogenous glycine can lead to inhibition at P3 (Koch *et al.*, 2012). The development of glycine inhibition is associated with the maturation of C-fibre afferents as well as changes in glycine receptor location and isoforms (Koch *et al.*, 2012). The pattern of GABA/Glycine miniature postsynaptic currents also doesn't mature until three postnatal weeks when only individually mediated currents occur (Keller *et al.*, 2001).

1.3.5 Postnatal Changes in Connections with Supraspinal Regions

1.3.5.1 Postnatal Changes in Ascending Pathways

Dorsal horn projection neurons to multiple supraspinal sites are developed prenatally, but are not mature (Li and Baccei, 2012). As mentioned above, at P3 lamina I NK1$^+$ spinoparabrachial projection neurons are able to receive peripheral input and output to the PB, however their projection efficiency is still immature. Following peripheral stimulation with formalin superficial dorsal horn neurons showed no age-related changes in activation (c-fos expression) but the activity in the PB increased significantly with postnatal age, suggesting maturation of the ascending pathway (S. H. W. Man, Géranton and Hunt, 2012). However different ascending pathways appear to mature at different rates (Li and Baccei, 2012). Peripheral stimulation with noxious hind paw pinch does not result in activity (c-fos expression) in the RVM or PAG until P12 (Schwaller, Kwok and Fitzgerald, 2016) even though the ascending pathways are present at birth (Li and Baccei, 2012). Similarly, spared nerve injury in P10 rats does not evoke an increase in

ERK activation in the RVM as in adults (Géranton *et al.*, 2010). These studies suggest ascending pathways to the PAG and RVM, and potentially the integration of other ascending systems in the PAG, is not mature until after P10. Additionally, although immature until after the third postnatal week, peripheral A- and C-fibre stimulation results in activity in the somatosensory cortex at P7 (Chang, Fabrizi and Fitzgerald, 2020). Overall, these studies suggest that ascending pathways develop at different rates over the postnatal period, although how this reflects the development of the supraspinal regions involved compared to the ascending pathways directly is unclear.

1.3.5.2 Postnatal Changes in Descending Pathways

Some descending projections are present at birth (Leong *et al.*, 1984) including serotonergic descending fibres (Rajaofetra *et al.*, 1989) however others, such as noradrenergic descending fibres, are not present in the dorsal horn until P7 (Rajaofetra *et al.*, 1992). These descending fibres are all initially in immature states and mature over the three weeks following their innervation of the dorsal horn (Leong *et al.*, 1984; Rajaofetra *et al.*, 1989, 1992). Similarly, descending DLF fibres are not functional until P10 and do not mature until the third postnatal week, also coinciding with the myelination of the DLF, which is not mature until P24 (Fitzgerald and Koltzenburg, 1986). However, recent studies suggest the presence of tonic descending facilitatory activity at P8, and potentially earlier (Schwaller, Kwok and Fitzgerald, 2016a). Descending input continues to be exclusively facilitatory until approximately P21 when a critical period of development occurs, resulting in both facilitatory and inhibitory modulation (G. J. Hathway *et al.*, 2009a; Hathway, Vega-Avelaira and Fitzgerald, 2012; Kwok *et al.*, 2014a). Supporting this, P21 is also the age at which descending noxious inhibitory control (DNIC) can first be identified in the postnatal rat (Boucher, Jennings and Fitzgerald, 1998). Both the PAG and RVM undergo substantial postnatal changes which mediate the changes in descending modulation.

Electrical stimulation of the PAG in adults results in analgesia (SPA), however, this does not occur until P21 and requires a higher stimulation intensity than in adults (van Praag and Frenk, 1991), suggesting that although an adult-like response is beginning to occur the pathways involved are still immature. However, endogenous signalling does not appear to be reaching a mature state at P21, as microinjection of the mu opioid receptor agonist DAMGO into the PAG has inhibitory effects in adults, but facilitatory effects at P21 and younger (Kwok *et al.*, 2014a).

As with the PAG, administration of DAMGO into the adult RVM results in the inhibition of nociceptive reflexes, but in P21 and younger rats results in facilitation due to a tonic

endogenous mu opioid facilitatory system (Hathway, Vega-Avelaira and Fitzgerald, 2012). Further supporting this critical period of development excitation of RVM neurons with DL-homocysteic acid at P10 has no descending effect on dorsal horn neurons, however, by P21 this results in facilitatory effects (Schwaller, Kwok and Fitzgerald, 2016a). Additionally, the modulatory targets of RVM pathways changes with A-fibre inputs to the dorsal horn being targeted in young rats but by P28 C-fibre inputs are modulated (G. J. Hathway *et al.*, 2009a; Koch *et al.*, 2014). Descending serotonergic fibres from the RVM also show a significant change at P21, from an immature facilitatory state, to a system resulting in facilitation following innocuous peripheral inputs and inhibition following noxious stimulation (Schwaller, Kanellopoulos and Fitzgerald, 2017a). For a recent review of developmental changes of serotonergic descending modulation see de Kort et al. (2021).

1.4 Somatosensory Changes in Middle Age and Old Age

Reaching adulthood, however, does not result in a fixed state and changes occur to most sensory systems, including the somatosensory system, as middle age and old age are reached. Interestingly the changes occurring at these life stages have only been appreciated since the early 1990s (Gagliese, 2009). Findings in this area are inconsistent in the literature in both clinical and preclinical studies; in part due to a wide variety of factors including the animal being used, exact age groups selected, and testing protocols (Yezierski, 2012). As in early life, matching rodent ages to an equivalent human age in later years is not simple. A widely accepted comparison would make an 18month old rat, as used in this thesis, comparable to about 50 years of age in humans (Sengupta, 2013).

Clinical studies suggest a decrease in the number of Meissner corpuscles, Merkel cells, and to some extent Pacinian corpuscles in the skin with increasing age (García-Piqueras *et al.*, 2019). There are also changes in PAFs in old age, in A-fibres these include a thinning of myelin, reductions in conduction velocity, as well as a reduction in the number of large myelinated fibres, although these peak at around 15months of age in mice and then decline (Verdú *et al.*, 2000). There has been evidence that aged animals (18months+) do not show the same C-fibre response that younger adults do in response to acute or chronic peripheral inflammation (Weyer *et al.*, 2016). Similarly, human studies have demonstrated altered C-fibre function with age (Namer, 2010). Additionally, fewer $TRPV1^{+}$ afferents are found in hindpaw skin in aged mice (24months)

as well as reductions in $Na_v1.8$ channels and TRPV1 protein expression in lumbar DRGs, with the decline starting at 15months (Wang *et al.*, 2006).

Patch-clamp recordings in the dorsal horn of older adult mice (28-30 months) compared to younger adult (3-4 months) found some excitable neurons (initial bursting neurons) displayed an increased action potential number and increased response duration in older animals, suggesting a more excitable phenotype. However, the same study showed a decrease in sEPSC amplitude, and an increase in GABAergic signalling with advanced age, suggesting a more inhibitory phenotype (Mayhew *et al.*, 2019). Similarly, aged rats (24-39months) had an increased level of spontaneous activity, increased noxious evoked responses, longer response latencies, and an increase in the receptive field of WDR neurons, although the low threshold area was smaller (Iwata *et al.*, 2002; David-Jürgens *et al.*, 2008). Non-neuronal cells in the dorsal horn also undergo age-related changes in middle age and old age, and show increases in proinflammatory cytokines (Galbavy *et al.*, 2015; Mayhew *et al.*, 2020).

Age-related changes in descending modulation also occur, serotonergic and noradrenergic expression decreases in the dorsal horn in aged rats (29-34months) compared to younger adults, additionally application of lidocaine to descending pathways resulted in increased neuronal responses to thermal stimulation in young adults but not aged rats (Iwata *et al.,* 2002). Decreases in DNIC have also been identified in aged rats (20-24months) which are suggested to be due to alterations in the functional connectivity of the PAG with other supraspinal regions (Da Silva *et al.*, 2020; Da Silva *et al.*, 2021). Decreased functional connectivity between supraspinal regions involved in descending modulation have also been demonstrated in humans in older age (González-Roldán *et al.*, 2020). Interestingly DNIC, and its age-related alterations, have been shown to be sex-dependent in rats; males showed stronger functional connectivity between supraspinal regions involved in the endogenous pain inhibitory system and stronger DNIC than females (Da Silva *et al*., 2018). Additionally, alterations in brain connectivity between these regions was found in older male and female rats (20-24months), resulting in a decrease in the DNIC response, however the alterations in functional connectivity were sex-dependent (Da Silva *et al*., 2020).

1.5 Multi-Electrode Array Electrophysiology

Electrophysiology allows the study of electrical activity within cells and tissues of the body, including the nervous system. Electrophysiology techniques can be broadly categorised as *in vivo*, *ex vivo,* and *in vitro* as well as extracellular or intracellular.

Extracellular recordings detect action potentials and as such suprathreshold activity whereas intracellular recordings can detect subthreshold activity and synaptic potentials.

In vivo electrophysiology techniques allow the recording of neuronal activity as single-unit action potentials from individual cells, multi-unit activity from multiple cells, or as local field potentials from living subjects. This is predominantly extracellular recordings as although *in vivo* intracellular recordings can take place they require a very high level of technical skill and equipment (Kodandaramaiah *et al.*, 2018). *In vivo* intracellular recordings have, however, been successfully conducted, particularly by the Yoshimura group, in the superficial and deep dorsal horn (Sonohata *et al.,* 2019; Uta *et al*., 2021). *Ex vivo* electrophysiological techniques allow the recording of neuronal activity from tissues explanted from the body into an appropriate medium. These are mostly conducted on spinal or brain slices allowing some microcircuits to be retained but removing the tissue from the larger somatosensory network (Lossi and Merighi, 2018). They do however offer a more stable format for intracellular recordings, primarily via patch-clamp recordings, which have been, and still are, extensively used in somatosensory research to study membrane properties, ion channels, and subthreshold currents (Li and Baccei, 2012; Schaefer *et al.*, 2018; Tadros *et al.*, 2018). *In vitro* studies involve recording from cultured cells, which allows a specific cell type to be studied,. Although cultured cells can only provide limited information about the intact *in vivo* system, this technique can be invaluable in other aspects, including exploring the function of transducer receptors. Of note, historically the term *in vitro* has also been used to describe *ex vivo* preparations (Fitzgerald *et al.*, 1987).

One of the recent advances in electrophysiological techniques has been the creation of multi-electrode arrays (MEAs). The majority of MEAs record extracellular, population data simultaneously recorded from multiple locations allowing the spatial-temporal aspects of neuronal activity to be analysed. MEAs range drastically in their number of electrodes with the latest arrays reaching over 1000 recording channels (Miccoli *et al.*, 2019) however the focus of these in *in vivo* studies has been on brain recordings. Recently, MEA electrophysiology has been used in the brain to record from multiple regions simultaneously at high temporal resolutions (Jun *et al.*, 2017) as well as in the spinal cord to investigate sensory and motor networks (Inácio *et al.*, 2016a). Additionally, MEAs are becoming increasingly used in *in vitro* studies as they can be incorporated into the base of a well on which cells can be cultured (Lam *et al.*, 2019). The experiments described in this thesis use a linear MEA for multi-spike *in vivo* spinal recordings, allowing population recordings to be obtained throughout the depth of the dorsal horn in anaesthetised rats allowing spatial-temporal analyses to take place (Greenspon *et al.*, 2019).

1.5.1 MEA Electrophysiology Compared to Alternative Electrophysiology Techniques

The most common *in vivo* electrophysiology technique used in preclinical pain studies has been single-unit electrophysiology, following protocols described initially in 1960 (Wall, 1960) and more recently in 2003 (Urch and Dickenson, 2003). Similar to MEA electrophysiology, these utilise extracellular recordings detecting action potential/spike generation. However, in single-unit recordings only one cell is located and identified, often a WDR neuron in the deep dorsal horn or to a lesser extent the superficial dorsal horn. As such in comparison to MEA population recordings there are a range of advantages and disadvantages between the techniques. As MEA recordings are from multiple cells, the analysis of specific waveforms is harder and as cutaneous receptive fields of neurons overlap they cannot be mapped. Additionally, the arachnoid membrane must be removed in addition to the dura, as the array tip is not as sharp as those used in single-unit recordings, which may lead to increased damage of cells at the recording site. However, the MEA setup is more forgiving of slight movements in the set-up or of a single cell's death. Population recordings allow the heterogeneous neuronal populations in the dorsal horn to be better represented and importantly the MEA allows the analysis of the spatial-temporal aspects of baseline and evoked activity to be investigated following peripheral stimulation.

Non-penetrating arrays have also been developed to allow extracellular recordings from the surface of neuronal structures such as the DRGs. These offer the ability to record from more intact systems but would not be suitable for measuring deeper structures and suffer from worse signal to noise ratios compared to the above techniques (Kashkoush *et al.*, 2019).

The most clinically relevant electrophysiological technique for studying the somatosensory system is electroencephalography (EEG), as it is widely used in human patients due to its non-invasive nature. EEG allows temporally sensitive measurements to be recorded at rest or during application of a variety of sensory stimuli (Tiemann *et al.*, 2018; Tayeb *et al.*, 2020). Whilst EEG recordings have been conducted in rodents there are several issues, a key one being that the depth of the majority of pain-related brain regions limits its use in preclinical pain research; with studies primarily involving cortical regions such as the somatosensory cortex (Devonshire, Greenspon and Hathway, 2015a).

1.5.2 MEA Electrophysiology Compared to Imaging Techniques

Functional magnetic resonance imaging (fMRI) is another technique that can be used to study pain at both supraspinal and spinal levels, usually by indirectly measuring neuronal activity using blood-oxygen-level-dependent (BOLD) signal analysis (Logothetis and Wandell, 2004). The main advantages in fMRI compared to MEA electrophysiology are the non-invasive nature of the technique and the more direct comparisons to clinical data. However, there are several drawbacks (Cohen-adad, 2017). One of the biggest issues with functional imaging in the spinal cord is a poor signal-to-noise ratio due to physiological noise from cardiac and respiratory sources (Paquette *et al.*, 2018). Temporal limitations are also a problem in fMRI with improvements in this often coming at a compromise with spatial resolution (Yacoub and Wald, 2018). Additionally, the small size of the spinal cord and pain related regions of the brain makes generating quality data difficult, even in humans, due to the low resolution of the data (Satpute *et al.*, 2013). Thus, although fMRI can be used in rodents (Mandino *et al.*, 2020), due to the limitations mentioned above this has currently only involved imaging the brain, and would be unlikely to be usable in postnatal animals.

Several other techniques have been developed recently to study functional connectivity via the hemodynamic responses in the brain, focusing on dynamic functional connectivity (dFC), in comparison to the averaged data often used (Preti, Bolton and Van De Ville, 2017). These include, using ultrafast ultrasound imaging which can detect a spatiotemporal resolution of 10-100μm and 2ms, (Rahal *et al.*, 2020). Functional ultrasound has also been used to image the rodent spinal cord following spinal cord stimulation; this, however, was a feasibility study and several issues with this technique, including motion correction analysis and resolution, need to be developed further (Song *et al.*, 2019). More recently ultrasound imaging in the rodent spinal cord was used to detect inflammation-dependent alterations in responses following specific PAF stimulation (Claron *et al.*, 2021). 2-photon calcium imaging can also be used to investigate the *in vivo* spinal cord, however, due to its microscopy depth limits this technique cannot be used in the intermediate and deep dorsal horn (Xu and Dong, 2019), although 3-photon imaging may offer improved resolution and depth (Ouzounov *et al.*, 2017).

1.6 Hypotheses and Aims

1.6.1 Hypotheses

Previous studies have demonstrated that the postnatal somatosensory system undergoes substantial development before reaching maturity. A-fibres are dominant in the neonatal dorsal horn with C-fibre functional maturity not being reached until the second postnatal week (Fitzgerald and Gibson, 1984). The innervation of these primary afferent fibres changes location during the first three to four postnatal week with a retraction of A-fibre inputs from the superficial laminae to the intermediate regions (M. Fitzgerald, Butcher and Shortland, 1994; Nakatsuka and Kumamoto, 2000; Beggs *et al.*, 2002). The postnatal dorsal horn is also considered to be excitatory in younger life with local glycinergic inhibition not developing until the second to third postnatal week (Koch *et al.*, 2012) and descending inhibition to the dorsal horn absent until the third to fourth postnatal week (G. J. Hathway *et al.*, 2009a; Hathway, Vega-Avelaira and Fitzgerald, 2012). As multi-electrode array electrophysiology has been successfully used in adult rats supporting and building upon many previous single-unit findings (Greenspon *et al.*, 2019) we hypothesise this technique will be able to record dorsal horn population activity at a range of postnatal ages and be able to detect changes occurring during the development of dorsal horn processing. As such we hypothesise our findings will build upon previous studies, but as using a population recording approach allows temporal-spatial aspects of processing to be investigated in more detail than previously achievable, we may identify developmental changes which occur outside of the WDR neurons generally used in single-unit recordings.

Specific hypotheses

- Chapter 3:

- The MEA setup used will be capable of measuring dorsal horn activity in postnatal rats.
- Higher levels of spontaneous and evoked activity will be detected in the younger dorsal horn decreasing over the postnatal period.
- Dorsal horn responses will show a retraction of Aβ-fibre innervation over the first 4 postnatal weeks.

- Chapter 4:

- A-fibre conduction velocity will increase with increasing postnatal age until adulthood but no changes in C-fibre conduction velocity will occur.
- Dorsal horn responses to both A- and C-fibre input will mature until adulthood.

- Regional difference in dorsal horn responses will be observed following different peripheral stimulation intensities.

-Chapter 5:

- $GABA_A$ disinhibition in the dorsal horn will result in an increase in the excitability at baseline and following peripheral stimulation, with effects increasing with increasing age.

-Chapter 6:

- DNIC will only be observable at P28 and above.
- Wind-up will be detectable in the deep dorsal horn in all age groups and responses will increase with increasing postnatal age.

1.6.2 Aims

Overall Aims:

- To confirm the usability of the MEA electrophysiology technique in postnatal rats.
- To investigate somatosensory processing across the dorsal horn throughout the life course.
- To analyse age-related differences in dorsal horn processing of peripheral stimulation throughout postnatal development and in adults.

Specific aims

- Chapter 3: To determine whether the MEA can be used to measure dorsal horn activity in postnatal rats and to compare spontaneous and evoked activity throughout the life course

- Chapter 4: To calculate A- and C-fibre input latency periods in postnatal rats and to compare A- and C- fibre evoked dorsal horn responses throughout the life course

-Chapter 5: To analyse dorsal horn activity following local $GABA_A$ disinhibition and to compare this throughout the life course

-Chapter 6: To investigate neuronal plasticity in the dorsal horn using DNIC and wind-up protocols and to compare dorsal horn plasticity throughout the life course

Specific hypotheses and aims are discussed in more detail within each results chapter.

Chapter 2

General Methods

2.1 Spinal MEA Electrophysiology

2.1.1 Animals

All procedures were performed in accordance with the Animals (Scientific Procedures) Act 1986/2012 and were licensed by the UK Home Office under the project licence PB3DA999F. Sprague-Dawley rats between postnatal day (P) 9 and 18 months, which unless stated otherwise, were used in the age groups P9-11, P14-16, P21-23, P28-30, P42-60 which are considered as young adults, and 18months old which are considered as aged adults in this thesis.

For rats used at P9-P60: Both male and female rats were used in these age groups. Time-mated females were purchased from Charles River (arrived E18), all neonatal and young adult rats used in experiments were born in-house and were weaned at P22. Rats were group-housed as a litter until weaning and then group housed in individually sexed cages of 2-4; rats were never individually housed for more than two days. Cages were individually ventilated cages located in a room maintained at 21°c, 55% humidity, and on a 12 hour dark/light cycle. Food and water was provided ad libitum. All experiments took place in the same room and during the animals' light cycle. Details of age groups, sex, and the number of animals used in each study are specified in the methods section of the relevant results chapters.

For rats used at 18months: Aged Sprague Dawley rats were all male and on average 18months old (17-20months). Rats were obtained from Eli Lilly in partnership with a different PhD studentship. Rats were raised in-house at an Eli Lily site and underwent no previous procedures before being transported to the University of Nottingham Bio-Support Unit. Due to arrival from an alternative housing location these rats were caged in either an isolator or in a quarantined room. Rats were group housed when possible but due to size restrictions of the isolator cages some rats were individually caged for up to 2 weeks. Aged rat weights were monitored and rats were placed on a restrictive diet if needed due to housing size limits.

Aged rat electrophysiology was performed in collaboration with postdoc Steve Woodhams who performed the tracheal cannulation and laminectomy for all aged rats as well as electrophysiology recordings for approximately half of the group. The remaining recordings and all subsequent analysis was performed by myself.

Reporting of the studies completed for this thesis are based on the ARRIVE Guidelines for reporting animal research (Kilkenny *et al.*, 2010; Percie du Sert *et al.*, 2020). Details are provided in each results chapter on the study specific design, experimental

procedures used, sample size, any reasons that data was not included in the analysis, randomization and blinding, outcome measures, statistical methods used, and results.

2.1.2 Anaesthesia and Monitoring

2.1.2.1 Anaesthesia Induction and Maintenance

All animals used in these studies were anaesthetised using isoflurane delivered in 100% medical oxygen (BOC) at a flow rate of 1L/min. For P9-60 rats, the rat was placed into an induction box and 3% isoflurane was delivered until loss of the righting reflex occurred. Following this the isoflurane level was reduced to 2.75% and the rat was transferred to a stereotaxic frame (UoN) fitted with a nose cone (UoN). After 10 minutes areflexia was confirmed using a toe-pinch and the initial rostro-caudal incision for the laminectomy occurred (Section 2.1.4) following this the isoflurane level was reduced to 2.5%. After completion of the laminectomy and meningostomy the isoflurane level was reduced to 2%. 15 minutes later the isoflurane level was decreased to 1.75%, and a further 15 minutes later reduced to 1.6% for initial testing. Recordings did not take place until at least 15 minutes following this final reduction.

Aged rats had their anaesthetic delivered via a tracheal cannula. The tracheal cannulation occurred after induction at 3% isoflurane and confirmation that the rat was areflexic. The tracheal cannulation was done by Steve Woodhams but briefly involved placing the animal on to its back on an insulating mat and making an incision to the skin over the throat. The fat and muscle covering the trachea was blunt-dissected using iris scissors and the smooth muscle covering the trachea cut away using vannas scissors. Two sutures were placed under the trachea, at the top and bottom of the exposed section. An incision was then made approximately in the middle of the exposed trachea parallel to the cartilage rings and a cannula inserted (2.1mm diameter polythene tubing). The two sutures were then tied to secure the cannula in place and the respiratory apparatus was connected. Following this the rat was placed on the stereotaxic frame which had the nose cone removed. The anaesthetic protocol was the same as for the younger animals once placed on the frame.

2.1.2.2 Justification of Chosen Anaesthetic, Gas, and Delivery Routes

The anaesthetic, carrier gas, and delivery method to be used are important considerations in all electrophysiology experiments but an additional complication with these studies was anaesthetising animals at a wide range of age points. As blood loss, especially in the neonates would be detrimental to the study, an inhaled anaesthetic

delivered via a nose cone (UoN) was chosen to avoid any additional surgical procedures. An exception to this was the aged rats who had the anaesthetic delivered via a cannula following a tracheotomy as, possibly due to their size, they did not free breathe well on the nose cone. The nose cone was specially designed to fit over the tooth bar used on this rig, be shaped inside to fit the snout of the rats at all ages, and has an inlet for the anaesthetic/ gas mixture and an outlet to a scavenger system to remove excess gas.

Isoflurane, a halogenated ether widely used in veterinary surgery, was chosen as the inhalational anaesthetic for all of the experiments in this thesis for several reasons. An issue with all inhalational anaesthetics is that they cause a reduction in neuronal activity, including in the dorsal horn, however, isoflurane has been found to cause less of a reduction than other inhaled anaesthetics such as halothane (Jinks *et al.*, 2003). In rats (and humans) lung development mostly occurs postnatally (Figure 2.1), this results in significant changes in the minimum alveolar concentration (MAC); the concentration of inhaled anaesthetic at which 50% of the subjects do not respond to a surgical stimulus (Eger, Saidman and Brandstater, 1965). Interestingly the pattern of MAC change during early life is the same in both humans and rats with an increase following birth reaching a maximum at P9, or 1-6months in humans, and then decreasing with age until adulthood (Orliaguet *et al.*, 2001). Specifically for isoflurane delivered in 100% oxygen in free breathing rats the lowest MAC level required is in adults, with increases of 66% at P2, 108% at P9 and 49% at P30 compared to the adult levels (Orliaguet *et al.*, 2001). Considering the expected variation in the concentration of anaesthetic required at the different ages used one that allowed quick adjustment of anaesthetic depth, such as isoflurane, was ideal. Additionally, isoflurane has been shown to be functional at all postnatal ages (Sanders *et al.*, 2005).

The carrier gas used in these experiments was 100% medical oxygen (BOC) this is considered best practice in non-recovery experiments lasting less than 12 hours (Flecknell, 2015) and matches with previous work from the group (Greenspon *et al.*, 2019). An alternative option was a mixture of oxygen and nitrous oxide (N_2O), as often used in electrophysiology studies, however N_2O functions via descending control pathways and as such does not work effectively in neonatal rats (Ohashi *et al.*, 2002) making it inappropriate for these studies.

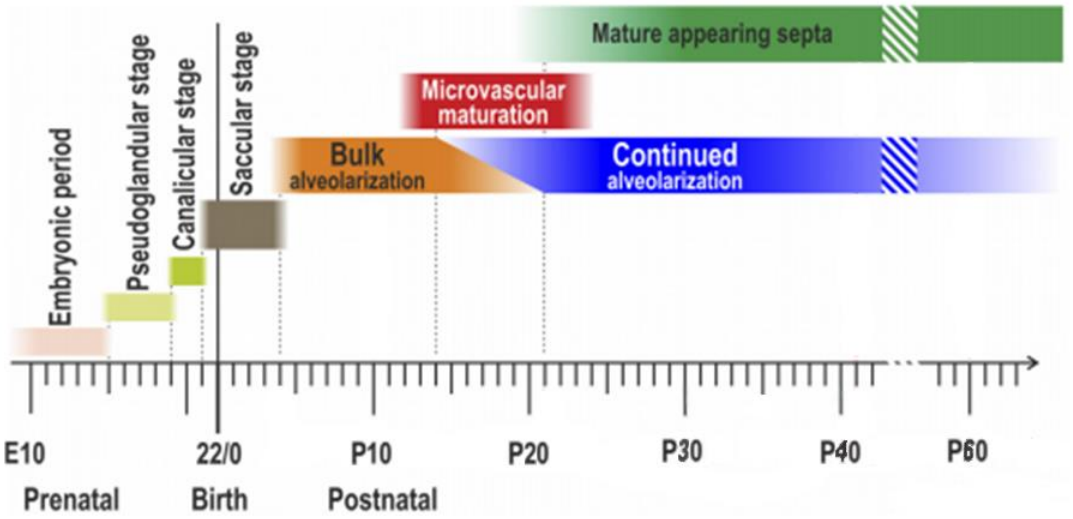


Figure 2.1. Timeline of lung development in rats. Highlights the main stages of lung development, although these are often overlapping it demonstrates that a mature state is not reached until after P21. This is linked with the different isoflurane levels and breathing rates used at the different age groups (modified from Tschanz *et al.*, 2014*).*

2.1.2.3 Anaesthetic Monitoring

The breathing rate was monitored by counting the respirations per minute (rpm) every 15-30 minutes. Effort of breathing was also monitored and if this looked shallow or laboured adjustments were made to the isoflurane level or the positioning of the rat on the stereotaxic frame (Section 2.1.3). Toe-pinch tests on the contralateral paw to experimental testing were repeated throughout the experiment to confirm areflexia. Isoflurane levels were generally adjusted if the breathing rate fell below 40rpm or increased beyond 80rpm, the time and amount of any isoflurane changes were recorded.

2.1.2.4 Temperature

Body temperature was monitored and maintained at approximately 37°c using a rectal thermometer attached to a heated blanket with feedback control (Harvard Apparatus). In P9-P30 rats the thermometer was placed directly under the rat (Urch and Dickenson, 2003). Additionally the temperature of the room in which the recordings took place was set to 26-28°c. This higher than normal room temperature was set for two reasons; to assist the younger rats in maintaining an appropriate body temperature and to increase the temperature of the exposed spinal cord, as the temperature of nervous tissue has been associated with its level of activity (Kalmbach and Waters, 2012).

2.1.2.5 Saline

For all experiments physiological saline was applied topically to the spinal cord following the laminectomy and meningostomy to hydrate the spinal cord. This was reapplied throughout the experiment if required; this was frequently the case in the younger animals where less of a reservoir is made due to the lack of tissue over the spinal column. In some studies the experiment was much longer in duration, approximately 10 hours, in these physiological saline was administered subcutaneously approximately every 2 hours at 0.1ml for the youngest age groups and up to 1ml for the aged rats to keep the rats hydrated. Subcutaneous saline was also given following the surgery if the rat had lost a substantial amount of blood. To avoid disruption to the data saline was never applied either topically or subcutaneously within 2 minutes of a stimuli being delivered and if possible was not given during trains of stimuli. No topical saline was applied in the experiments in Chapter 5.

2.1.3 Stereotaxic Placement

The same stereotaxic frame was used for all experiments, and was designed for adult rats, as opposed to neonatal or juvenile. P21 rats and older had their heads secured in place on the frame using a tooth bar and traditional ear bars inserted into the ear canals so that the head was secure. However, as the ear canals had not fully developed in the youngest rats those used at P9-P16 had their head secured using the ear bars with white tack on the tips. Similarly, the bite bar was not used in the youngest groups and instead the chin was placed on the bar to provide the correct positioning for the nose cone. All rats were placed on to a heat blanket which was above blocks to raise the rat to an appropriate height for the frame. If the rats are not in the correct position the thoracic cavity can become stretched causing breathing difficulties.

2.1.4 Laminectomy and Meningostomy

The skin on the back of the rat was cleaned using a 10% hydrex solution. A laminectomy was then performed to expose the L3-5 region of the spinal cord, this was done under a bench top dissecting microscope (Olympus Tokyo 272235). A rostro-caudal incision was made above the spinal column using a scalpel (#3 handle with #11 blade) and the 13th thoracic (T13) and 1st lumbar 1 (L1) vertebrae were located using the lowest ribs, located on T13, as an anatomical marker.

Two longitudinal incisions, of an appropriate length to accommodate a spinal clamp, were made to the tissue immediately lateral to the vertebral bodies of the spinal column above the T13 vertebra and a spinal clamp of an appropriate size was placed. This clamp was used to raise the animal's chest allowing for easier breathing, and helped stabilize the spinal column during the laminectomy. Two sizes of spinal clamps were used; for

rats aged P28 and above a larger more traditional set were used and for younger ages modified small rat-toothed forceps were used as clamps.

Small incisions were made perpendicular to the spinal column above the T13 vertebrae and below the L1 vertebrae, two more incisions were then made to connect these two forming a rectangle. This allowed the tissue above the T13 and L1 vertebrae to be easily removed using rongeurs (straight 1 mm Friedman-Pearson; FST) in the P28+ rats and using forceps (Dumont #2; FST) in the younger rats. The clamp was rotated or raised to allow easier removal of the L1 and T13 vertebrae with the rongeurs or forceps used previously, exposing the L3-5 region of the spinal cord. The placement of the laminectomy was the same in animals, of all ages, used in this study.

As the electrode array is relatively large and blunt, a meningostomy was performed to remove the dura mater and arachnoid membranes to make insertion of the electrode easier and less damaging to the spinal cord. This was done under the dissecting microscope used for the laminectomy, with fine forceps (Dumont #5; FST) and a 25 gauge needle (BD). The pia membrane was left in place. A second spinal clamp was then placed caudally to the laminectomy, both clamps were moved caudally to help elevate the rat and allow for easier breathing. Loose skin was pinned caudally to the second clamp using a crocodile clip or straight forceps to help reduce heat and water loss during the experiment.

2.1.5 Electrode Placement

A 16-channel linear multielectrode array with 50 µm inter-electrode spacing (A1x16-5mm-50-177; Neuronexus) (Figure 2.2) was positioned using a stereotaxic arm (UoN) to a location half way along the exposed region of spinal cord (L4), and one third of the distance from the central vessel to the lateral margin of the exposed spinal cord. This location was based on historical somatotopic mapping studies (Swett and Woolf, 1985) and previous work done by the group (Greenspon *et al.,* 2019). The electrode was manually inserted into the cord using a stereotaxic arm to an approximate depth of 950 µm by visibly confirming all electrodes in the array were lowered into the spinal cord, using the dissecting microscope.

To determine if the correct location along the dorsal-ventral axis had been achieved a train of 5 electrical stimuli (3mA, 50ms, 0.1Hz) was delivered to the hindpaw (Section 2.1.6) and the subsequent local field potential (LFP) profile inspected (Figure 2.3). The electrode placement was adjusted if necessary using a micro-manipulator (IVM Single; Scientifica) and the electrical train repeated. The correct placement was determined from several criteria, initially, the overall shape of the profile was considered as the depolarization pattern generated is subtly different if the array is placed into the spinal cord in an incorrect location – sometimes due to avoiding blood vessels present at the dorsal surface of the cord. Another measure for determining the correct

depth is the change from negative to positive deflection at the border to the ventral horn. As the length of the dorso-ventral axis of the dorsal horn increases during postnatal development (Section 1.3.4) the array is longer than required in the youngest groups and the LFP profile reflected this (Figure 2.3). Positioning in the younger rats was aligned so that the dorsal horn was covered by the central electrodes in the array, resulting in the deepest electrodes being potentially placed within the ventral horn and occasionally the most dorsal electrode being in the white matter of the spinal cord. The electrodes included in subsequent data analysis reflected this (Section 2.2.1).

After it was determined that the correct location and depth had been found (Section 2.2.1), the array was kept in place for 10min and the electrical train run again to confirm no movement of the spinal cord tissue in relation to the array had occurred (electrode drift). In some studies it was possible to rerun the electrical train between different stimuli protocols to confirm the placement was correct before starting the new protocol, and at the end of each experiment to check the correct placement had remained throughout the testing period. This movement is multifactorial and could relate to the clamps slipping slightly, the spinal cord tissue adjusting to the insertion of the array, or mild swelling of the spinal cord.

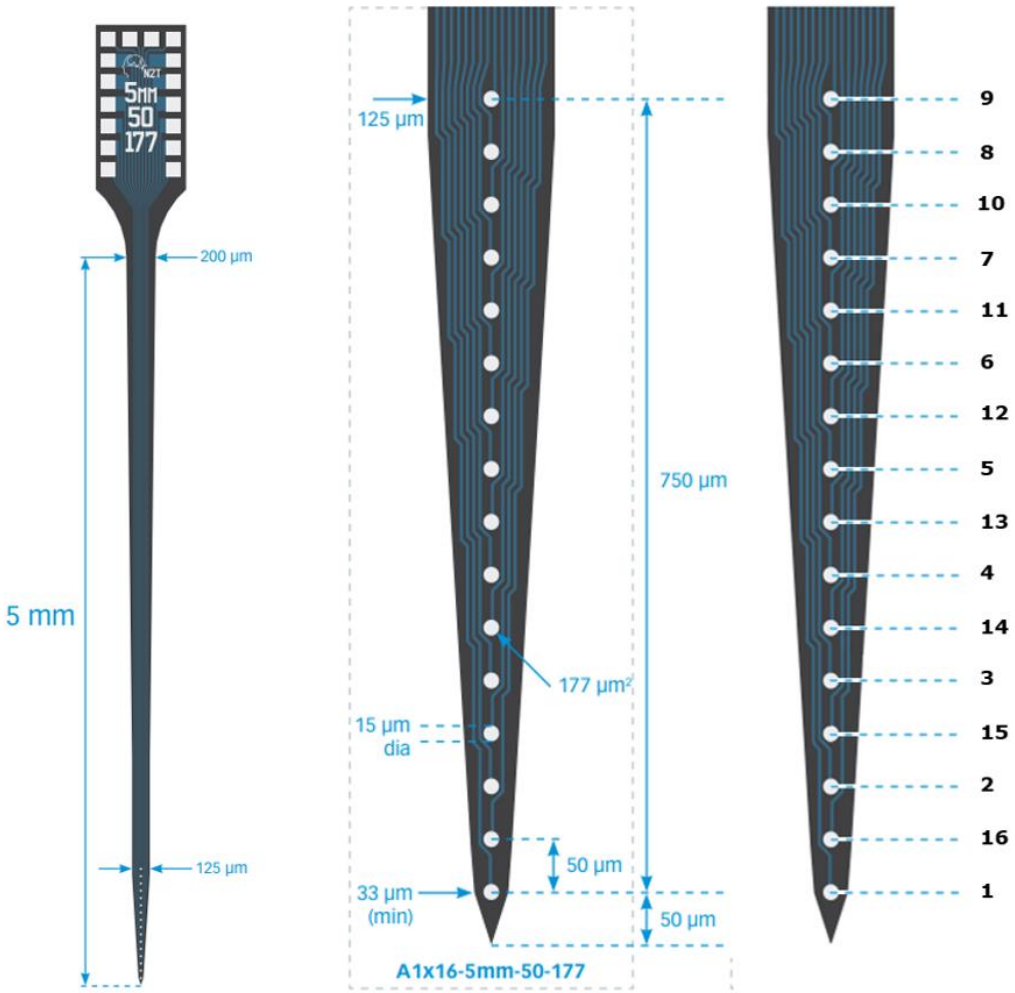


Figure 2.2. Schematic of the Multi-Electrode Array used. Left: The whole of the A1x16-5mm-50-177 electrode array is shown with dimensions. Not noted is the thickness of the array (15µm). Middle: A detailed schematic of the tip to show the dimensions of the 16 electrodes. Right: Channel numbers are shown, these are renumbered during analysis to represent the spatial location, with 1 being the most dorsal and 16 the most ventral (Section 2.1.7). (Modified from Neuronexus documentation).

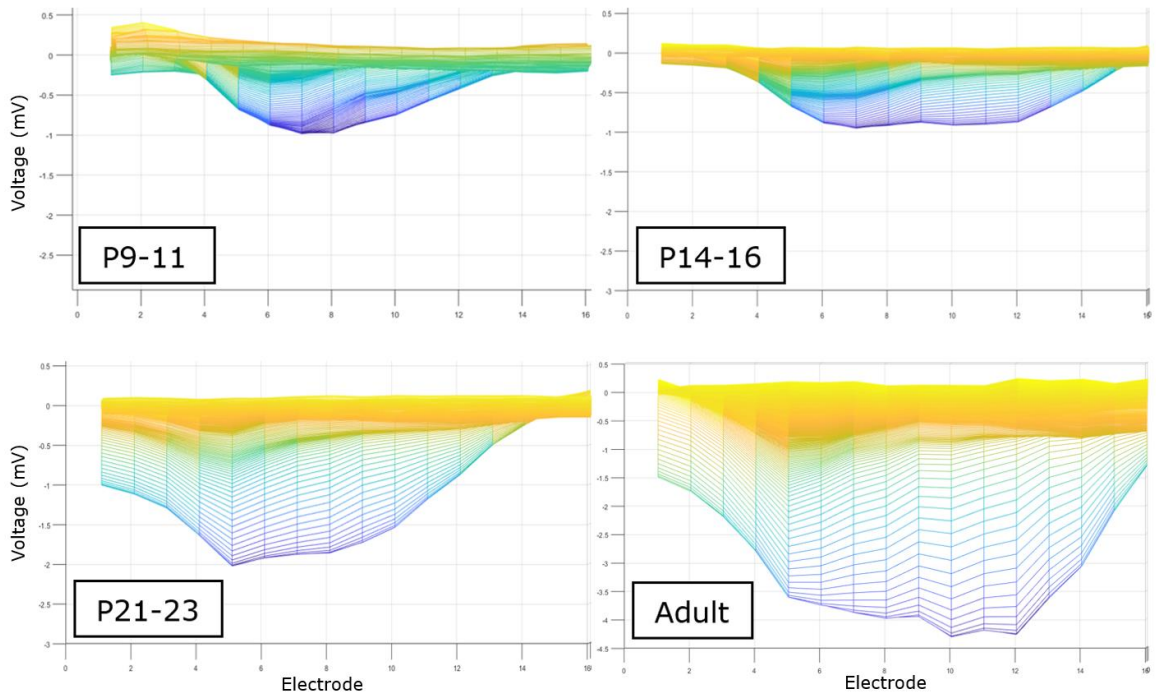


Figure 2.3. Example LFP profiles used for alignment. LFP responses following electrical stimulation of the hindpaw (3mA, 50ms, 0.1Hz; x5). Electrodes are shown on the x-axis in order with 1 being the most dorsal and 16 the most ventral. The response at each electrode is shown on the y axis as the evoked voltage, and time is shown coming towards the viewer. Time shown is the response over the first 180ms following stimulation. The width of the depolarisation wave increases along the electrode array with increasing postnatal age.

2.1.6 Hindpaw Stimulation

A range of stimuli were applied to the hindpaw in different experiments. These were time-locked as an event channel by the recording software by transistor-transistor logic (TTL) pulses. A frequency stimulator (D330, Digitimer) was used to generate these TTL pulses. For electrical stimulation the frequency stimulator controlled a stimulus generator (DS3, Digitimer) which delivered the stimulus via two needle electrodes inserted into the proximal metatarsal pads of the paw. Stimuli varied in amplitude, duration, and frequency; specific protocols are detailed in the relevant results chapters. For mechanical and thermal stimulation, the D330 frequency stimulator instead triggered a pneumatic picopump (World Precision Instruments) attached to a spring loaded pneumatic pin cylinder (CJ1B4-10SU4, SMC) attached to either a 3D-printed von Frey hair holder (UoN) or aluminium heating block (UoN). These were positioned approximately 0.5 cm above the centre of the hindpaw and the stimulus applied for 5 seconds. The pneumatic pump was set to a pressure of 35psi for all testing. Von Frey hairs (vFhs) with calibrated forces between 1.4g and 26g were used to test spinal cord responses to mechanical stimulation of the plantar surface of the hindpaw, specific vFhs used are detailed in the relevant results chapters. For testing spinal responses to thermal stimulation the aluminium block was

heated to 30°c or 55°c (Devonshire, Greenspon, & Hathway, 2015). The applied surface of the block is circular with a diameter of 3.14cm, as such the same block was used at all ages tested, as the entire plantar surface of the hindpaw was always covered.

2.1.7 Data Collection and Analysis

Recording took place from all electrodes (channels) in the array simultaneously. The electrode array was connected via a custom flexible connector (Omnetics) to a headstage (HSt/16o25-18p-xR; 1× gain; Plexon). The headstage was also connected to a PBX preamplifier (1000x gain; Plexon), which split and filtered the raw data by frequency into LFP (0.7-300Hz, 5kHz sampling frequency) and spike data (150Hz-8kHz, 40kHz sampling frequency). Spike data was then sent via a Multichannel Acquisition Processor (Plexon) and LFP data sent directly to a computer. The Plexon Sort Client program was used during experiments to set sorting parameters and visualise waveform activity at each electrode. LFP data was continuously sampled but spike waveforms were captured when a depolarisation of 10% or greater from baseline was detected. Individual spikes were not sorted as population (unsorted spike) data was used throughout for analysis. The depolarisation threshold and the refractory period selected (1200µs) were recommended by Plexon.

Unsorted spike and LFP data was exported from NeuroExplorer 4 (Plexon) to MATLAB 2017b (MathWorks) for processing. LFP data was used for alignment purposes only and all results presented here use spike activity. Custom MATLAB scripts (Written by Charles Greenspon) were used and subsequent statistical analysis was done using Graphpad Prism 7. The main script used is an 'MSP_Pipeline' (all scripts are included in the appendices). Briefly, several variables are declared so that appropriate data points can be extracted; resolution in milliseconds, time pre-stimulation, duration of stimulation, and time following stimulation. The number of stimuli given per group and the number of stimuli to calculate a baseline can also be declared. This script then initialises additional relevant scripts (see Appendices).

Using 'MSP_Core' the epoch length is calculated based on these declared parameters and transformed from a continuous time vector to a binned vector with the values as the centre point of the bin. 'MSP_Arrange' sorts the channels and allocates them to the correct spatial location. A depth vector is created and resultant data and the stimulus event vector are moved to a structure called 'data'. Following on from this, an empty matrix is created based on the sizes of the matrices already created. A triple 'for' loop then moves along each dimension of the 'data.raw' matrix; for each stimulus the relative

epoch limits are calculated. As the channels are of uneven lengths as they contain time points and not continuous variables, it is necessary to calculate the index of the stimulus specific start and stop points. The 'find_ind' function finds the closest value to each start stop point for each channel. The unbinned vector is then excised using the index values obtained. The absolute time is then converted to stimulus relative time. For each bin the total number of waveforms that exists in the range is determined from the cumulative spike count. The unbinned vector is then allocated to a new fixed length vector.

A baseline subtraction is also performed to normalize evoked activity. This is done on an individual channel basis. The average number of threshold crossings, in each bin, during the pre-stimulus time is calculated and subtracted from the number of threshold crossings recorded in each bin during the declared stimulus duration and post-stimulus period. Adjusted values are allocated to a new matrix so uncorrected data can be assessed too.

The 'MSP_Pipeline' script then allows selection from subsequent scripts to correctly analyse the data collected e.g. electrical or physiological (mechanical and thermal). For electrical analysis the latency names and values are declared and the matrix is arranged so that the first element of every row is the start of the latency range and the second the end. A 'for' loop using 'find_ind' finds the values in the 'data.time_ms' vector. Values are allocated to empty matrices. The sum and mean of the number of spikes in the range is created for each stimulus, channel, and latency. The baseline firing rate (as calculated previously) can be subtracted. The 'MSP_Elec_Sorting' script is then used and responds to the declared experiment type. Several output indices are created to allow easy use of the data. For physiological data the order of stimuli application (alternate or continuous) needs to be declared and the 'Etho_MSP_SecSub' script is then run. This sorts data into second and subsecond bin sizes, creates separate files and groups the stimuli.

To combine data from different animals the 'Elec_Combine' function is used which loads the declared variable from each file (animal) and adds it to a matrix one dimension larger than the input variable. For grouped analysis the mean response to each stimulus is found for each individual animal and then the mean response is calculated for all animals in the age group. Details of exact data and statistical analysis is included in the results chapters.

2.2 Correlating Electrode Number with Dorsal Horn Regions

2.2.1 Spinal Cord Lesioning and Staining

2.2.1.1 Electrolytic Lesioning

As discussed in (Section 1.3.4) the dorsal horn increases in size during postnatal development from approximately 700µm in P9 rats to 1000µm in adult rats (Lorenzo *et al.*, 2008). This was apparent in the LFP and placement of the multielectrode array was adjusted accordingly to best cover the dorsal horn region. We attempted to confirm the location histologically in the different age groups by lesioning the spinal cord following recordings in several studies. Lesioning was attempted by applying an electrical stimulus through the electrode array at electrodes 1 and 16, to identify the exact region from which the recording had occurred. This involved unplugging the headstage and instead attaching a lesion breakout box (Trent Thermal Technology) via custom flexible connectors (Omnetics). The DS3 stimulator was used to deliver the stimulus via the lesion breakout box. Multiple lesioning protocols were attempted ranging in intensity between 5µA-50µA for 2s, termination occurred between 30min-4h following this to allow glial scarring to occur.

2.2.1.2 Transcardial Perfusion and Tissue Collection

Rats were given an intra-peritoneal (i.p.) injection of sodium pentobarbital (P9-11 1ml, P14-16 1ml, P21-23 2ml, P28-30 2ml, young adults 2ml, aged rats 3ml) and remained on maintenance isoflurane until breathing ceased. Rats were then quickly moved to the laboratory sink with a custom down-flow set-up. The rat was placed on its back and the chest cavity quickly opened so the heart could be accessed. A 25G needle connected to an appropriately sized syringe (5ml P9-16, 50ml P21+) was inserted into the left ventricle and the right atrium was cut using a pair of vannas scissors. The rat was then perfused with physiological saline (15ml P9-16, 100ml P21-30, and 150ml adults) and the same volume of 4% paraformaldehyde (PFA) solution (Sigma Aldrich). The spinal cord, and in some cases additional tissue, was collected.

2.2.1.3 Lesion Staining

Following tissue collection the spinal cord segment was post-fixed in 4% paraformaldehyde (PFA) for at least 48 hours and then transferred to sucrose azide (0.04%) for at least 48 hours to cryoprotect the tissue before slicing. Cords were cut to the lumbar region and then sliced

into 40μM thick sections using a freezing microtome (Leica, SM2010R) and stored in sucrose azide (0.04%) at 4°c.

Cresyl violet acetate was used to stain the spinal cord to identify the depth of the electrodes in the array. As this is a basic dye it stains the DNA and RNA in the tissue, helping to identify the laminae of the spinal cord as well as the lesion. Sections where the lesioning was expected to be found were placed on glass slides. The sections were then dehydrated in increasing dilutions of methanol, submerged in xylene, and then rehydrated before being stained with 5% cresyl violet acetate, then dehydrated again. More specifically they were submerged in the following for 2 minutes each unless otherwise stated 50% methanol, 75% methanol, 90% methanol, 100% methanol, xylene, 100% methanol, 90% methanol, 75% methanol, 50% methanol, distilled water, cresyl violet acetate (4 minutes) distilled water, distilled water, acidic 50% methanol (4 seconds), 50% methanol, 75% methanol, 90% methanol, 100% methanol, xylene, xylene. Slides were then covered in DPX mounting medium and cover slipped.

2.2.1 Previously Reported Dorsal Horn Laminae Depths

Unfortunately no lesions were identified using this method. As an alternative method for assessing the likely recording site at each electrode in each age group, dorsal horn laminae depths described in previous studies were reviewed (Table 2.1). From these studies the electrodes expected to be in the superficial, intermediate, and deep dorsal horn regions were determined for each age group used in this thesis (Table 2.2). For the younger age groups this resulted in some electrodes being removed from the analysis; electrodes 1 and 16 are excluded during data analysis of P9-11 recordings and electrode 1 is excluded from P14-16 recordings. Additionally, we expect some of the deeper dorsal horn to be missing in the adult recordings. However, as the superficial region is a key area in the somatosensory circuits being studied in this thesis it was decided to prioritise including this region.

Table 2.1. Dorsal horn laminae depths during postnatal development previously reported in the literature. Depths from three previous studies are aligned to show depths of the whole dorsal horn and superficial (Lamina (L)I-IIi) and intermediate/deep (LIII-VI) dorsal horn depths between birth (day 0) and adulthood (day 60).

	Depth of specified region (µm)								
Age (days)	Whole dorsal horn	Whole dorsal horn (Mean ± SEM)	LI	LIIo	LIIi	Sup. dorsal horn	Int. and deep dorsal horn	Sup. dorsal horn	Int. and deep dorsal horn
0	500	525 ± 7	43	39	65	147	380		
3								200	450/500
5		623 ± 4	45	42	68	155	470		
7	700								
10		692 ± 19	43	39	73	155	537	250	650/700
15	800	776 ± 17.5	41	44	84	169	525		
20		789 ± 13	42	42	90	174	620		
21								300	750/800
60	1000	1086 ± 5.5	42	47	93	182	905		
Reference	(Fitzgerald, 1985) (Wistar rats. Spinal cord surface to ventral lamina VI)	(Lorenzo *et al.*, 2008)(SD rats. Measured from surface of grey matter to central canal)						(Torsney and Fitzgerald, 2002)(SD rats. From surface of spinal cord)	

Table 2.2. The approximate depth (µm) of each electrode at all age groups. The value in the table represents the lower depth of the region in which the electrode is expected to record (e.g. 50 represents 0-50µm). Dorsal horn regions are colour coordinated, yellow representing the superficial laminae (LI-II), green representing the intermediate laminae (LIII-IV), and blue representing the deep laminae (LV-VI). Electrodes 1 and 16 are excluded during data analysis of P9-11 recordings and electrode 1 is excluded from P14-16 recordings. P=postnatal day, YA=young adults, and AA= aged adult.

Electrode	P9-11	P14-16	P21-23	P28-30	YA	AA
1			50	50	50	50
2	50	50	100	100	100	100
3	100	100	150	150	150	150
4	150	150	200	200	200	200
5	200	200	250	250	250	250
6	250	250	300	300	300	300
7	300	300	350	350	350	350
8	350	350	400	400	400	400
9	400	400	450	450	450	450
10	450	450	500	500	500	500
11	500	500	550	550	550	550
12	550	550	600	600	600	600
13	600	600	650	650	650	650
14	650	650	700	700	700	700
15	700	700	750	750	750	750
16		750	800	800	800	800
--				850	850	850
--					900	900
--					950	950

2.3 Peripheral Nerve Recordings

Peripheral nerve recordings were conducted to calculate the conduction velocity of the primary afferent fibres at different postnatal ages. Male and female SD rats aged P9-11, P15-17, P23-24, P31-32, and P49-51 were used. Both sciatic nerves (left and right) were used from each rat resulting in n=6 for all groups, except P15-17 where n=8. Previous work has suggested that the use of both sciatic nerves from one animal can be considered as two units (Rich *et al.*, 2020). Briefly, rats underwent a cervical dislocation followed by decapitation. An incision was made along the hind leg and spinal column to expose the sciatic nerve. Sciatic nerves were carefully removed ensuring the largest length of nerve possible was removed. Each nerve was placed in a separate superfusion chamber (Harvard Apparatus) maintained at 37°C and pH 7.45. Chambers were superfused with 10mM glucose artificial cerebrospinal fluid (aCSF) solution (126mM NaCl, 3mM KCl, 2mM $MgSO_4$, 26mM $NaHCO_3$, 1.25mM NaH_2PO_4 and 2mM $CaCl_2$) and continuously aerated with a 95% O_2 and 5% CO_2 mixture. The length of each dissected nerve was then measured using digital calipers. Both ends of the nerve were attached to suction electrodes containing silver chloride (AgCl) wires, which were filled with substrate free aCSF, with the hindpaw end of the nerve inserted into the stimulating electrode and the spinal cord end inserted into the recording electrode. Nerves were then allowed to equilibrate for at least half an hour before starting the experiment.

The nerves were stimulated with a double pulse stimulator (Grass S88, A-M Systems, Washington, USA) with a second stimulus applied 20ms after the first to enable simultaneously recording of the A- and C-fibre compound action potentials (CAPs). These pulses were applied at a control frequency of 1Hz with the voltage selected following a stimulus response protocol to ensure selective recruitment of the fibre types. CAPs were amplified 1000x, filtered at 30kHz (Stanford Research Systems preamplifier, SR560), and acquired at 50kHz using PClamp 10.7 (Molecular Devices software) and a Digidata 1440A analogue to digital converter. Data was analysed using Clampfit 10.7 (Molecular Devices) to determine CAP amplitude (A-fibres) and area (C-fibres). The latency to peak was calculated and divided by the nerve length to calculate conduction velocities for each nerve. These recordings were conducted in collaboration with another group at the University of Nottingham (PhD student Laura Rich), who also performed the subsequent conduction velocity analysis. Statistical analysis was performed on GraphPad Prism.

Chapter 3

Multi-Electrode Array Electrophysiology in the Developing Dorsal Horn

3.1 Introduction

The dorsal horn of the spinal cord is a key site in the processing of touch and nociceptive stimuli, not only relaying information between the periphery and brain supraspinal sites but containing its own endogenous processing microcircuits (Todd, 2010; Abraira and Ginty, 2013). Historically the dorsal horn has been divided into six distinct laminae (Rexed, 1954; Molander, Xu and Grant, 1984) but over the years the complex heterogeneity of the dorsal horn in regards to both the laminae and the cells located within each lamina has become increasingly apparent (Häring *et al.*, 2018; Gatto *et al.*, 2019; Peirs, Dallel and Todd, 2020). The integration and processing of incoming somatosensory information is modality specific with distinct response patterns observed following different peripheral stimulation types including innocuous touch, noxious mechanical, heat, and cold (Abraira and Ginty, 2013; Ran, Hoon and Chen, 2016). Although an oversimplification it can be generally considered that innocuous touch involves neurons in the intermediate laminae (lamina III and IV), innocuous heat to the deeper dorsal horn laminae (lamina V and VI), and noxious touch and heat to the superficial laminae (lamina I and II) and to a lesser extent the deep laminae (Basbaum *et al.*, 2009; Abraira and Ginty, 2013).

Multi-electrode array electrophysiology enables the *in vivo* population recording of numerous neurons throughout the dorsal horn. Thus offering an alternative insight to the somatosensory circuits within the intact dorsal horn compared to other commonly used techniques including single-unit electrophysiology, in-vitro patch clamp recordings, and optical imaging techniques (Section 1.5). The benefits of MEA electrophysiology have led to an increase in their use, initially in the brain (Liu *et al.*, 2012; Obien *et al.*, 2015) and more recently in the spinal cord including in neonatal rats, however at a relatively low spatial resolution focusing on circuits between sensory (dorsal) and motor (ventral) zones (Inácio *et al.*, 2016b).

Previous work by our group using linear MEA electrophysiology in the dorsal horn of male adult SD rats demonstrated their ability to detect region-specific activity showing differential response patterns to distinct stimuli types and intensities (Greenspon *et al.,* 2019). However, in this chapter for the first time MEA electrophysiology has been used to look at the maturation of somatosensory circuits in the dorsal horn during postnatal development and in later life. As such the main aim of the work presented in this chapter is to confirm that the technique is able to detect regional and modality specific responses in the dorsal horn following peripheral stimulation in neonatal, juvenile, and aged rats.

3.1.2 Postnatal Maturation of the Somatosensory Processing Pathways

It is well known that the spinal cord, including the dorsal horn, undergoes substantial maturation during postnatal development (Section 1.3.4). However it is important, particularly in *in vivo* studies, to consider changes occurring at all levels of the somatosensory processing system including the peripheral sensory organs and free nerve endings, the primary afferent fibres relaying these signals to the dorsal horn, and the descending modulation from supraspinal sites. At all of the age groups used in this thesis the free nerve endings responsible for detecting noxious stimuli in the skin are present (Jackman and Fitzgerald, 2000), as well as the cutaneous sensory structures, although not all are in their mature form (English, Burgess and Norman, 1980; Zelená *et al.*, 1990). Primary afferent fibres are present, although immature in the younger age groups (Fitzgerald and Gibson, 1984; Vejsada *et al.*, 1985; Lizarraga, Chambers and Johnson, 2007), however, due to the duration of mechanical and thermal stimulation used in this chapter the individual PAFs cannot be distinguished. Ascending projections to multiple supraspinal regions are present from birth (Li and Baccei, 2012) and although there is variation in the age at which functional maturity is reached this is established in the PB nucleus, RVM, and vlPAG by P12 (Schwaller, Kwok and Fitzgerald, 2016b). Descending serotonergic and noradrenergic projections mature later in postnatal life (Rajaofetra *et al.*, 1989, 1992; Boucher, Jennings and Fitzgerald, 1998) with descending modulation transitioning from a purely facilitatory effect to a balance between facilitatory and inhibitory effects by P28 (G. J. Hathway *et al.*, 2009b; Hathway, Vega-Avelaira and Fitzgerald, 2012; Kwok *et al.*, 2014b).

At P9 the dorsal horn is approximately 700μm deep (in the dorsal-ventral axis) and grows in size until at least P60 when it measures ~1000μm (Fitzgerald, 1985; Torsney and Fitzgerald, 2002; Lorenzo *et al.*, 2008). Interestingly the rate of growth is not consistent between the different laminae; during this time there is no change in the size of lamina I and lamina II increases by only 30μm, thus the majority of the increase is seen in the intermediate and deep regions possibly reflecting the increase in myelination of the inputting Aβ-fibres (Lorenzo *et al.,* 2008). However, the superficial laminae still undergo postnatal changes including reductions in neuronal density (S. H. Man, Géranton and Hunt, 2012) and changes in PAF innervation patterns with some Aβ-fibres terminating throughout lamina I and II at birth, withdrawing to lamina III and IV over the first 3 or 4 postnatal weeks (M Fitzgerald, Butcher and Shortland, 1994; Park *et al.*, 1999; Beggs *et al.*, 2002; Granmo, Petersson and Schouenborg, 2008). Although some Aβ afferents do remain in the superficial laminae (Nakatsuka and Kumamoto, 2000).

The immaturity of the somatosensory system is reflected in the behavioural responses of neonates who have lower thresholds to nociceptive stimuli, larger receptive fields, and

exaggerated responses (Fitzgerald, 2005). Responses to mechanical stimulation have been shown to be longer in duration until P16 (Fitzgerald and Gibson, 1984) with mechanical and thermal thresholds increasing with postnatal age reaching a mature level at approximately P28 (Fitzgerald, M, Shaw, A, MacIntosh, 1988) and P12 (Falcon *et al.*, 1996) respectively.

The majority of electrophysiological studies involving the neonatal dorsal horn have investigated WDR neurons located in the deep dorsal horn, and to a lesser extent the superficial dorsal horn regions (Section 1.5.1). These have mostly measured changes in receptive field size and evoked spike number following mechanical stimulation. Receptive field size of WDR neurons consistently decreases with increasing postnatal age, however, comparing spike numbers these have mostly shown a trend of increasing evoked activity with increasing postnatal age (Fitzgerald and Jennings, 1999; Koch *et al.*, 2012; Schwaller, Kanellopoulos and Fitzgerald, 2017b) although some do not show any age-related trends (Torsney and Fitzgerald, 2002; Schwaller, Kwok and Fitzgerald, 2016b). Whilst there is no doubt that these neurons play important roles in somatosensory processing they are only one population of neurons in the dorsal horn that are involved in these processing circuits. As such there is a huge amount of research still required to fully understand the postnatal changes in the dorsal horn, particularly in less studied regions such as the intermediate laminae, and in regards to the spatial-temporal nature of responses throughout the dorsal horn.

As discussed in Section 1.4 aged adult animals also undergo multiple changes in the somatosensory system including reductions in cutaneous LTMR (García-Piqueras *et al.*, 2019), increases in receptive fields (Iwata et al. 2002), changes in PAFs (Verdú *et al.*, 2000; Weyer *et al.*, 2016) and reduced tonic descending inhibition (Iwata *et al.*, 2002; Da Silva *et al.*, 2021). As such a more excitatory dorsal horn would be expected compared to younger adults and has been demonstrated in patch clamp recordings of WDR neurons (Iwata *et al.*, 2002; David-Jürgens *et al.*, 2008; Mayhew *et al.*, 2019).

3.2 Hypotheses and Aims

3.2.1 Hypotheses

The MEA electrophysiology protocol used in these experiments has previously been shown to be capable of detecting region-specific and modality-specific response patterns in the dorsal horn of adult rats following stimulation of the hindpaw. As the depth of the dorsal horn, in particular the superficial dorsal horn, changes only slightly during postnatal development we predict that the same MEA setup will also be capable of

detecting the region-specific and modality-specific response patterns, throughout the dorsal horn, in younger rats.

Although previous electrophysiology experiments have shown lower amplitudes of evoked responses in neonates these have been primarily from WDR neurons and do not necessarily reflect the responses we would see with population recordings. As population recordings will record from multiple neurons we would expect more receptive fields to be activated following stimulus application and as younger animals have larger receptive fields, the number of receptive fields activated may be higher than in adults. Additionally, as inhibitory signalling is immature in the dorsal horn with glycinergic signalling only developing at P14 and tonic descending facilitation being present until the third to fourth postnatal week, when descending inhibition can be detected, it seems logical that the neonatal dorsal horn would be more excitatory. Thus considering the developmental changes during the postnatal period being studied (P9+), the behavioural changes during development, and the differences in the data that can be acquired by single-unit and MEA electrophysiological recordings, we predicted the population recordings would demonstrate higher levels of spontaneous and evoked activity in the younger dorsal horn decreasing over the postnatal period. Additionally, as the somatosensory changes that occur in aged animals generally appear to be a reversal of the maturation occurring during postnatal development, although most likely via alternative mechanisms, we would expect the aged adults to show dorsal horn responses more similar to the juvenile rats than the aged adults.

We also predict that region-specific response will change over the postnatal period, particularly following innocuous stimulation of the hindpaw, as Aβ-fibre innervation retracts from the superficial laminae to the intermediate region of the dorsal horn.

3.2.2 Aims

- To determine whether the MEA can be used to measure evoked activity across the dorsal horn in postnatal rats following mechanical and thermal stimuli
- To detect expected region-specific and stimuli-specific response patterns at the different postnatal ages
- To compare spontaneous and evoked dorsal horn activity at different ages throughout the life-course

3.3 Methods

3.3.1 Animals

Male and female Sprague Dawley rats at postnatal day (P)9-11, P14-16, P21-23, P28-30, P42-60 (considered as young adults), and males at 18months old (considered as aged adults) were used for data presented in this chapter. Details of animal origin and housing can be found in Section 2.1.1. Group sizes, sex, weights, and specific groupings are detailed in Section 3.3.4.

3.3.2 Surgery and Electrode Placement

Rats were anaesthetised with isoflurane (Section 2.1.2) and placed on to a stereotaxic frame (Section 2.1.3). A laminectomy and meningostomy took place to expose the L4 and L5 spinal cord regions and the dura matter and arachnoid membranes carefully removed (Section 2.1.4). The electrode array was then placed as described in Section 2.1.5.

3.3.3 Hindpaw Stimulation

The data presented in this chapter involves the application of mechanical and thermal stimuli to the hindpaw; von Frey hairs corresponding to 2g, 6g, 15g, and 26g were used for mechanical testing and an aluminium heating block set to a temperature of 30°c or 55°c was used for thermal testing, as described in Section 2.1.6. All stimuli were applied with a 10 minute inter-stimulus duration.

3.3.4 Specific Study Design

Data presented in this chapter was obtained over multiple groups, some of which received slightly different stimulation protocols (Tables 3.1 and 3.2).

*Table 3.1. Mechanical stimulation protocols in specific groups. Animals shown here were those included in data analysis, excluded data is stated below. *In study 4 26g was not given to rats younger than P21.*

N numbers	Total			Cohort 2: 2, 6, 15 g	Cohort 3: 2, 6, 15 g	Cohort 4: 2, 6, 15, 26g*	Cohort 5: 6, 15, 26g	Cohort 6: 2, 6, 15, 26g
	n	Sex (m/f)	Weight (g) mean ± SD					
P9-11	7	4/3	26 ± 6	2	-	5	-	-
P14-16	10	5/5	41 ± 6	2	-	8	-	-
P21-23	10	5/5	63 ± 5	3	-	7	-	-
P28-30	5	3/2	112 ± 15	3	2	-	-	-
Young Adult	7	6/1	252 ± 40	3	1	3	-	-
Aged Adult	10	10/0	521 ± 29	-	-	-	5	5

Table 3.2. Thermal stimulation in specific groups. Animals shown here were those included in data analysis, excluded data is stated below.

	Total			Number from each Cohort	
	n	Sex (m/f)	Weight (g) mean+/- SD	Cohort 2: 30 & 55°c	Cohort 3: 30 & 55°c
P9-11	5	2/3	27 ± 8	2	3
P14-16	9	4/5	42 ± 10	3	6
P21-23	8	4/4	60 ± 5	3	5
P28-30	6	4/2	87 ± 34	3	3
Young Adult	6	4/2	203 ± 12	3	3

Following these studies, data from 4 rats was excluded from final analysis. For mechanical testing one was from the P14-16 group collected in cohort 2 and one from the aged adult group in cohort 6 due to the recordings being too noisy. One was also removed from mechanical analysis from the young adult group in cohort 3 due to drift of the electrode during recording. For thermal testing, data was removed for one P9-11 rat as the stimuli protocol was not completed due to breathing difficulties.

3.3.5 Data Analysis

Details of data collection and initial sorting are described in 2.1.7. Data analysis and statistical analysis were performed using Matlab 2017b and GraphPad Prism 7.05.

Initial sample sizes were based on previous MEA electrophysiology data collected by the group in male adult SD rats (Greenspon *et al.,* 2019). However, subsequent collection showed an increase in variability possibly due to the use of different postnatal ages as well as both sexes. A power calculation was conducted using the initial data collected; specifically the mean spike activity, and standard deviation measures, across the whole array between the P9-11 and young adult groups following the 15g vFh hindpaw stimulations was used. From this a group size of 10 was recommended to give a statistical power of 80% or higher to detect an effect size of Cohen's d = 1.3 for differences between age groups with a significance level of $p<0.05$ using a two-tailed test. As such additional rats were used to increase the group sizes. Additionally, a power calculation for the thermal study was conducted using the initial data collected, specifically using the mean difference in spike activity across the whole array following 30°c and 55°c, and the corresponding standard deviation, between the P9-11 and young adult groups was used. From this a group size of 10 was also recommended to give a statistical power of 80% or higher to detect an effect size of Cohen's d = 1.2 for differences between age groups with a significance level of $p<0.05$ using a two-tailed test. As such additional rats were used to increase the group sizes. However, this was not designed to be sufficiently powered to compare differences between sex, and as an n of 10 was not reached in every group the study may be underpowered.

To sort the mechanical and thermal stimuli looked at in this chapter the 'Etho_MSP_Pipeline' script was used with the bin size set as 100ms, pre-stimulus time set as 1 second, the duration as 5seconds, and the post stimulation as 60seconds. The data was baseline corrected and sorted into 'subsecond' and 'second' files to allow the duration of the stimulus application to be investigated or the whole 70second time frame set. Data was combined between animals using the 'Elec_Combine' script creating a 4D matrix. In some cases the mechanical data had to be manually aligned to the onset before being combined. This was the case across several ages and stimuli intensity and is most likely due to a slight delay in the picopump.

Statistical comparisons were made using the mean response of three stimulus applications per stimuli group from each animal. The mean response and SEM was then found for all animals within an age group. Comparing regional and stimulation responses at individual ages two-way RM ANOVAS were used followed by Tukey's post hoc tests to reveal significant changes between groups. Changes in the stimuli given were

predominantly age dependent and study dependent. This resulted in mixed numbers of animals in the P21-23, young adult, and aged adult groups having received some stimuli, in most cases, only a subset had received the 26g stimulation. In these cases 2-way ANOVAs were run followed by Tukey's post hoc tests. Comparing between age groups, one-way ANOVAs were used followed by Dunnett's post hoc tests to determine changes between postnatal age groups and aged adults compared to the young adult responses.

3.4 Results

3.4.1 Baseline Dorsal Horn Activity Significantly Increases with Postnatal Age

The baseline level of spike activity was found to significantly increase with increasing postnatal age until young adulthood ($F(4, 121)=12.38$, $P<0.0001$, Two-way ANOVA, Figure 3.1). However, no sex differences ($F(1, 121) <1$) or interaction ($F(4, 121) = 1.602$, $P=0.18$) were found using a two-way ANOVA (Figure 3.1). A Tukey's multiple comparisons test between the age groups showed statistically significant differences between the young adult group and all four postnatal age groups (P9-11, P14-16, P21-23 $P<0.0001$ and P28-30 $P<0.001$). Although it is worth noting that as only males were used in the aged adult group, this age group was not included in the analysis, however it appears that the baseline activity in the aged adults is similar to that in the young adults (Figure 3.1). As significant age-dependent differences were found in the baseline recordings the decision was made to use baseline corrected data for the rest of this chapter. This removes the baseline level of activity independently at each electrode in the array, allowing the evoked responses to be investigated without being affected by the age-related changes in spontaneous baseline activity.

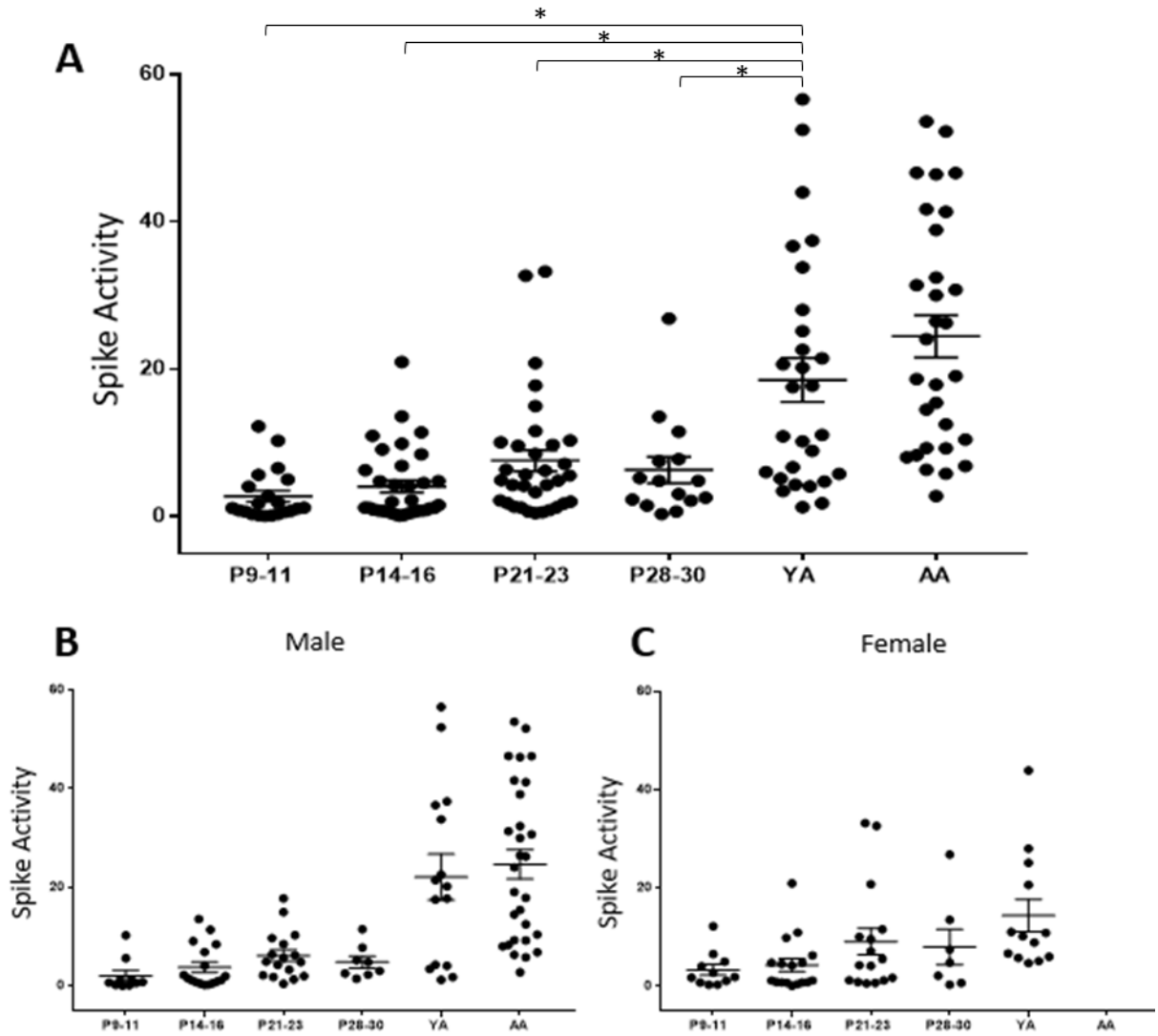


*Figure 3.1. Baseline activity increases during the life-course and is not sex-specific. Spike activity over the 60second period before the first test stimuli was delivered using the mean activity recorded across the whole array (adjusted for age, see Section 2.2.1). Individual points represent an individual rat with lines representing mean +/- SEM. A) shows difference between all rats grouped by age P9-11 n=21, P14-16 n= 34, P21-23 n=33, P28-30 n=15, YA n=28, AA n=30 (only males used in the aged adult group). B) Shows baseline activity of the male rats C) shows baseline activity of the female rats used. YA = young adults, AA = aged adults. A two-way ANOVA comparing baseline activity by sex and age (not including AA) found significant age-related differences (P<0.0001) but no sex-dependent differences or interaction (P>0.05). Asterisks * represent a significant differences in baseline activity between age groups (P<0.001).*

3.4.2 MEA Detection of Mechanical and Thermal Responses throughout the Life Course

To determine whether the multi-electrode array (MEA) could be used to measure evoked activity across the dorsal horn in neonatal and juvenile rats, as well as detect regional and stimulus dependent responses following mechanical and thermal stimulation, each age group was analysed individually.

To examine the spatial-temporal activity, heatmaps were generated representing the duration of the response on the x-axis, the electrode number in the array and as such the depth of the dorsal horn on the y-axis, and the spike number by the colour gradient. These allow the subtle changes to be observed that are lost when grouping the data by region or latencies, however, they do not allow the associated error to be displayed and as such no statistical analyses have been performed with them. Instead the data is sorted and presented as bar charts with the relevant statistical analysis.

Postnatal Days 9-11

The application of a mechanical stimulus to the hindpaw produces a clear onset and offset which can be observed on the heatmaps (Figure 3.2A) in response to the 2g, 6g, and 15g vFhs, as well as an ongoing level of activity during the application, all of which appear to increase with the higher intensity hairs. In the P9-11 age group regional and stimulus intensity (Figure 3.2C) dependent changes were able to be detected in response to mechanical stimulation of the hindpaw. When considering the regional aspect of the dorsal horn response, although each electrode is unique in the recorded response we grouped the electrodes into the approximate regions of the superficial, intermediate, and deep dorsal horn and calculated the mean spike number in that region (Section 2.1.7). Following the mechanical stimuli there were region dependent differences ($F(2, 12)=3.975$, $P<0.05$, Two-way RM ANOVA) and stimulus intensity dependent differences ($F(2, 12)=10.16$, $P<0.01$, Two-way RM ANOVA) but no interaction ($F(4, 24) = 1.326$, $P=0.29$). A Tukey's post hoc test showed a significant difference between the superficial and deep regions ($P<0.05$). Additionally, a Tukey's post hoc test showed in all regions there was an increase in spike activity in response to 15g compared to 2g ($P<0.01$) and 6g ($P<0.05$).

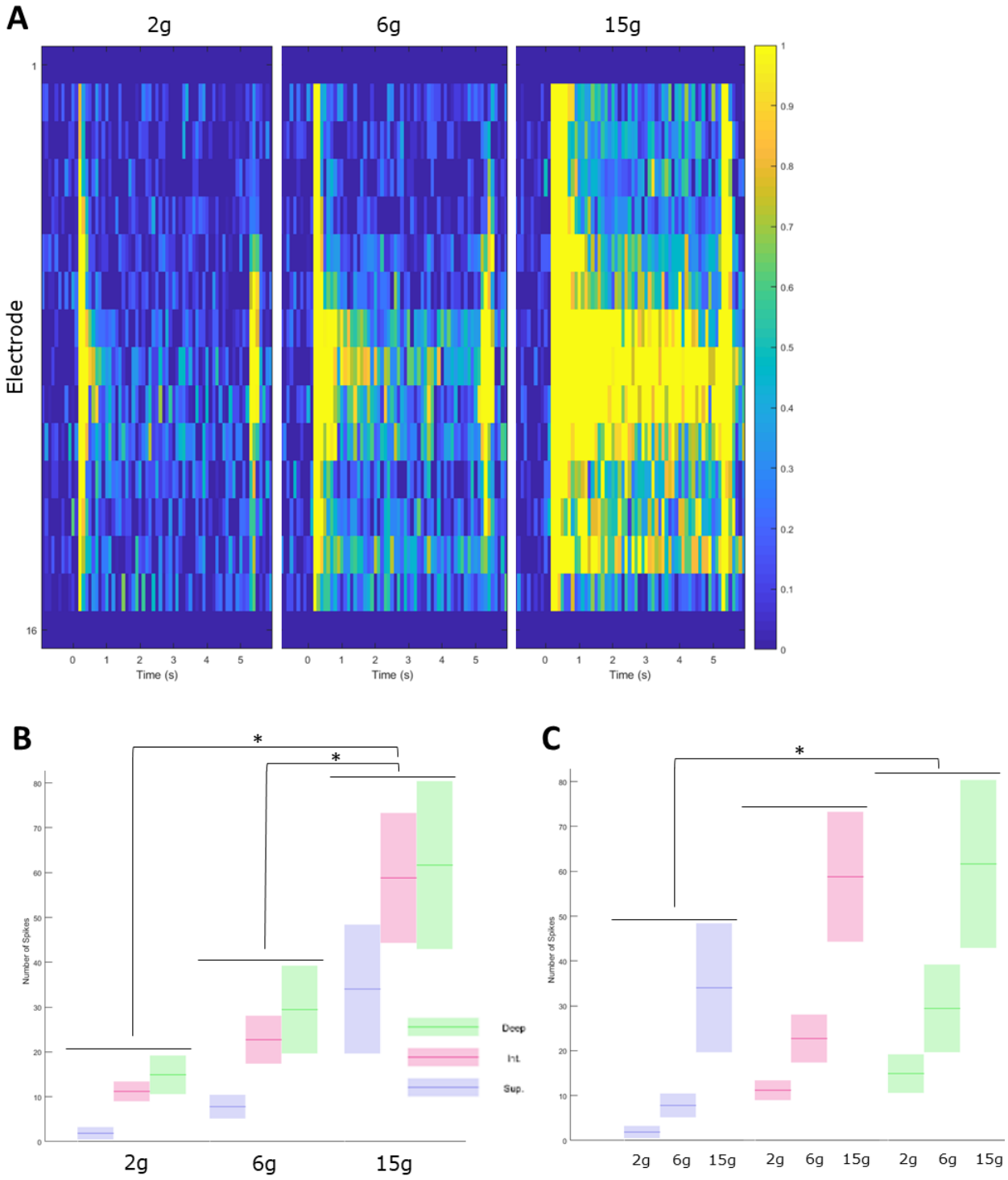


Figure 3.2. Region and stimulus dependent changes occur in the P9-11 dorsal horn following mechanical stimulation of the hindpaw and these changes can be analysed with the MEA. A) Heatmaps of activity at each electrode following stimulation with 2g, 6g, or 15g vFhs. Responses shown are averaged responses from all animals in the age group, n=7. Time period shown is 1second before the stimulus is applied, the 5 seconds of application, and 1second post application. Electrodes are in order; 1 is the most dorsal and 16 the most ventral, electrodes 1 and 16 are removed to better represent the size of the P9-11 dorsal horn. Colours represent mean number of spikes respective to the colour bar shown on the right. B) Regional responses to each stimulus over the time period shown in (A), lines represent mean of electrodes in each region and shaded area represents SEM. C) Stimulus responses within each region, data shown in B is reorganised to allow easier representation of comparisons. A two-way RM ANOVA found significant region-dependent and stimulus intensity-dependent differences but no interaction. Asterisks indicate significant differences between groups with a Tukey's post hoc test (P<0.05).

Thermal stimulation was applied using a heated block (Section 2.1.6), which also results in mechanical stimulation when applied to the hindpaw, the onset and offset of this can be seen on the thermal heatmaps (Figure 3.3A). To attempt to separate the mechanical and thermal responses the 30°c response was subtracted from the 55°c response and the remaining activity considered to represent the evoked thermal response (difference). However, as can be seen in the heatmaps, there is still a visible onset remaining. This may be due to a compounding effect of the thermal and mechanical stimulation resulting in a higher number of spikes at the on-set of the 55°c than the 30°c stimulation. To avoid comparing any mechanical component still contributing at the stimulus on-set, only the response between 0.5seconds post stimuli to 5seconds was used. The heatmaps demonstrate that the thermal response has a delayed onset, possibly representing the time taken for the skin the increase in temperature. Additionally, the highest response appears to be in the intermediate regions, however, a two-way RM ANOVA found no statistical significance between the regions ($F(2, 8) = 1.406$, $P=0.30$) or the different temperature stimuli ($F(2, 8) = 3.645$, $P=0.07$), and no interaction ($F(4, 16) = 1.537$, $P=0.24$).

Comparing the evoked responses of the P9-11 age group also demonstrates different response patterns following mechanical compared to thermal stimulation (Figure 3.2 vs Figure 3.3). Although these differences have not been statistically compared it is clear that the MEA protocol used can record modality-specific responses in the neonatal dorsal horn.

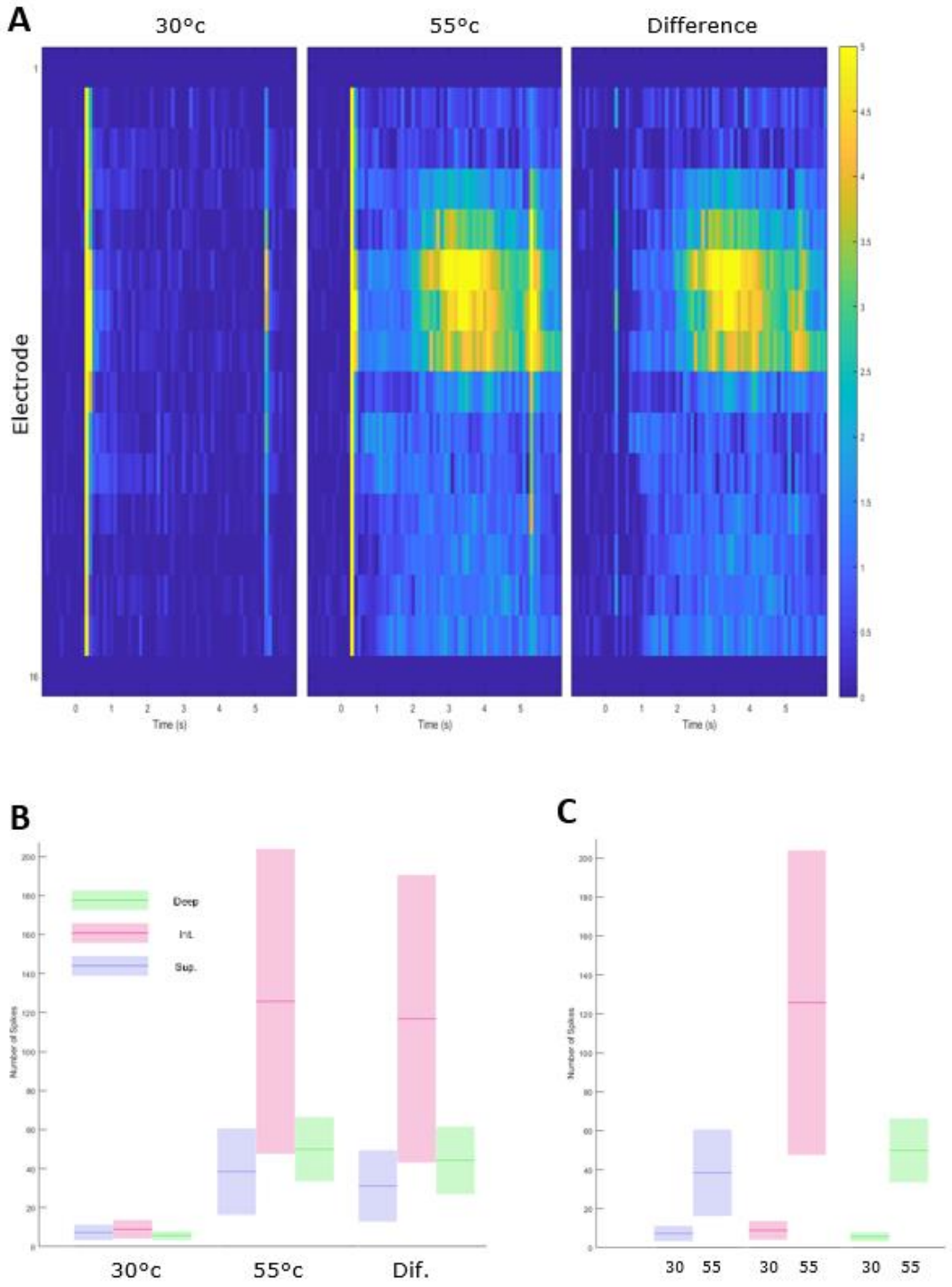


Figure 3.3. Spike activity in the P9-11 dorsal horn following thermal stimulation of the hindpaw and these changes can be analysed with the MEA. A) Heatmaps of activity at each electrode following stimulation with 30°c and 55°c is shown, and the difference in response between the two groups. Responses shown are averaged responses from all animals in the age group, n=5. Time period shown is 1second before the stimulus is applied, the 5 seconds of application, and 1second post application. Electrodes are in order; 1 is the most dorsal and 16 the most ventral, electrodes 1 and 16 are removed to better represent the size of the P9-11 dorsal horn. Colours represent mean number of spikes respective to the colour bar shown on the right. B) Regional responses to each stimulus between 0.5seconds and 5seconds of stimulus application. Lines represent mean spike number of electrodes in the region and shaded area represents SEM. C) Stimulus responses within each region, data shown in B is reorganised to allow easier representation of comparisons. No statistical significance was found following a 2-way RM ANOVA.

Postnatal Days 14-16

The heatmaps (Figure 3.4A) show the mean stimulus onset, ongoing activity, and offset in response to 2g, 6g, and 15g vFhs, all of which appear to increase with the higher intensity hairs. No regional difference ($F(2, 18)<1$), or interaction ($F(4, 36)<1$), were detected using a two-way RM ANOVA, however, the heatmaps do show that each electrode is recording different responses so although the regions may not be significantly different, the MEA is detecting spatial differences in activity throughout the dorsal horn. However, there are stimulus intensity (Figure 3.4) dependent changes with a significant increase in spike activity occurring with the increasing stimulus intensity ($F(2, 18) = 24.29$, $P<0.0001$, two-way RM ANOVA). Additionally a Tukey's post hoc test showed an increase in spike activity in response to 15g compared to 2g ($P<0.0001$) and 6g ($P<0.001$).

Following thermal stimulation of the hindpaw, the heatmaps (Figure 3.5A) suggest an increase in activity occurred at all electrodes during the application of the 55°c stimulus with a particular increase in the deep region. Again the mechanical element was minimised by looking at the difference in response between 30°c and 55°c as well as using the response between 0.5seconds post stimuli to 5seconds. There was a stimulus-dependent change in spike activity ($F(2, 16)=30.5$, $P<0.0001$, two-way RM ANOVA) (Figure 3.5) but not a regional change ($F(2, 16) = 2.593$, $P=0.11$; Figure 3.5). However, an interaction between stimulus intensity and region was also identified ($F(4, 32)=3.178$, $P<0.05$). Tukey's post hoc tests showed a significant increase in the 55°c stimulation response, and in the difference group, compared to the 30°c response ($P<0.0001$, Tukey's post hoc test).

Comparing the evoked mechanical and thermal responses (Figure 3.4 vs Figure 3.5) it is clear that the spike activity in response to these different stimulation types are distinct in the P14-16 age group, as with the younger age group.

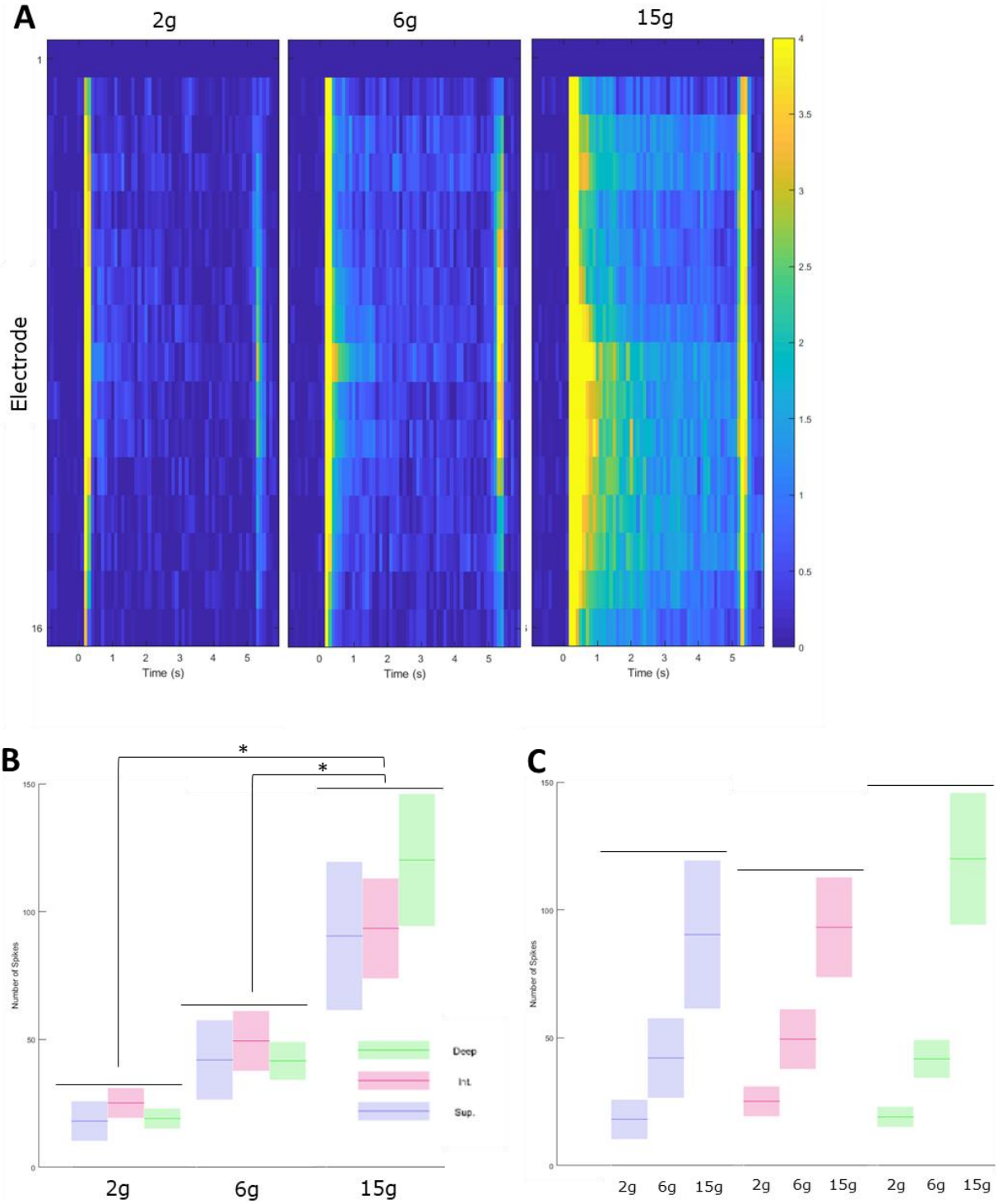


Figure 3.4. Stimulus dependent changes occur in the P14-16 dorsal horn following mechanical stimulation of the hindpaw and these changes can be analysed with the MEA. A) Heatmaps of activity at each electrode following stimulation with 2g, 6g, or 15g vFhs. Responses shown are averaged responses from all animals in the age group, n=10. Time period shown is 1second before the stimulus is applied, the 5 seconds of application, and 1second post application. Electrodes are in order; 1 is the most dorsal and 16 the most ventral, electrode 1 is removed to better represent the size of the P14-16 dorsal horn. Colours represent mean number of spikes respective to the colour bar shown on the right. B) Regional responses to each stimulus over the time period shown in (A), lines represent mean of electrodes in each region and shaded area represents SEM. C) Stimulus responses within each region, data shown in B is reorganised to allow easier representation of comparisons. A two-way RM ANOVA showed significant stimulus intensity dependent differences but not regional differences or interaction. Asterisks indicate significant differences between groups with a Tukey's post hoc test (P<0.001).

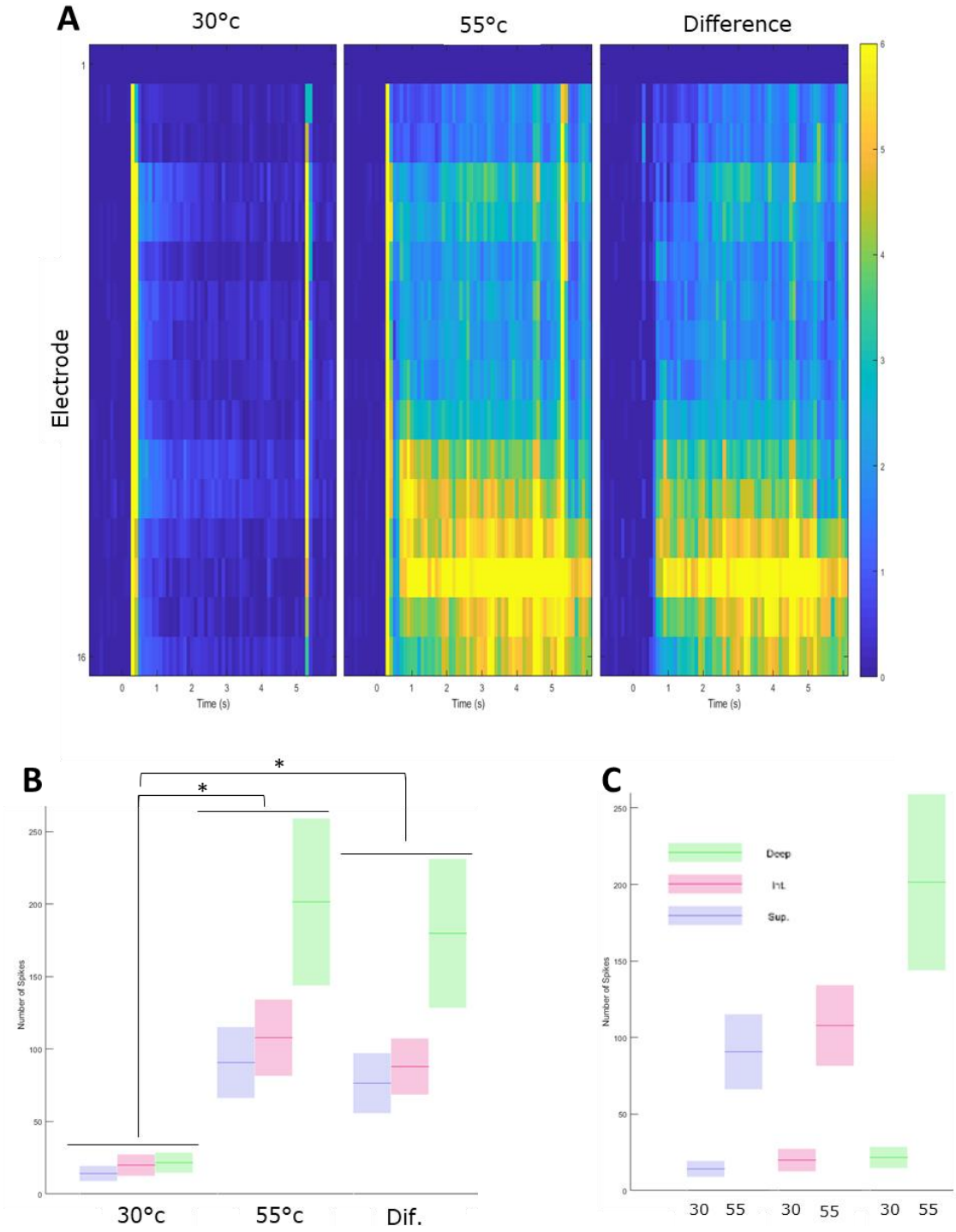


Figure 3.5. Stimulus dependent changes occur in the P14-16 dorsal horn following thermal stimulation of the hindpaw and these changes can be analysed with the MEA. A) Heatmaps of activity at each electrode following stimulation with 30°c and 55°c is shown, and the difference in response between the two groups. Responses shown are averaged responses from all animals in the age group, n=9. Time period shown is 1second before the stimulus is applied, the 5 seconds of application, and 1second post application. Electrodes are in order; 1 is the most dorsal and 16 the most ventral, electrode 1 is removed to better represent the size of the P14-16 dorsal horn. Colours represent mean number of spikes respective to the colour bar shown on the right. B) Regional responses to each stimulus between 0.5seconds and 5seconds of stimulus application. Lines represent mean spike number of electrodes in the region and shaded area represents SEM. C) Stimulus responses within each region, data shown in B is reorganised to allow easier representation of comparisons. A two-way ANOVA showed stimulus-dependent differences and an interaction between region and stimulus intensity but no difference between regions. Asterisks indicate significant differences between groups with a Tukey's post hoc test (P<0.0001).

Postnatal Days 21-23

In the P21-23 age group some rats also received a 26g vFH stimulation (Section 3.3.4). The heatmaps (Figure 3.6A) show the stimulus onset and offset, as well as the increased duration of the evoked response with increasing mechanical stimulation intensity. Whilst the dorsal horn activity increases in all regions of the dorsal horn this is predominantly in the intermediate and deep regions where the longer evoked activity is observed. In the superficial region although there appears to be a trend in increased spike number with increasing stimulus intensity this is mostly at the onset of the stimulus. Possibly suggesting different mechanoreceptors are terminating in different regions. However, a two-way ANOVA showed no statistically significant regional changes ($F(2, 111) = 2.247$, $P=0.11$; Figure 3.6) or interaction ($F(6, 111)<1$). However, stimulus intensity dependent changes were detected ($F(3, 111)=11.27$, $P<0.0001$; Figure 3.6C). Tukey's post hoc tests showed spike activity following 26g was significantly higher than the 2g ($P<0.0001$), 6g ($P<0.0001$), and 15g ($P<0.001$) responses.

Following thermal stimulation of the hindpaw the P21-23 age group the heatmaps (3.7A) suggest a whole dorsal horn increase in response with a particular increase in the deep region. A two-way RM ANOVA showed stimulus dependent differences ($F(2, 14)=5.595$, $P<0.05$) but interestingly did not identify any regional differences ($F(2, 14) = 5.595$, $P=0.02$), or interaction ($F(4, 28) = 2.662$, $P=0.05$) although the deep region appears to show the greatest evoked response activity on the heatmaps. Additionally, Tukey's post hoc tests showed a significant increase within the deep dorsal horn region following the 55°c stimulation compared to the 30°c stimulation ($P<0.0001$).

Again clear differences in the dorsal horn response can be seen following mechanical or thermal stimulation of the hindpaw (Figure 3.6 vs. Figure 3.7).

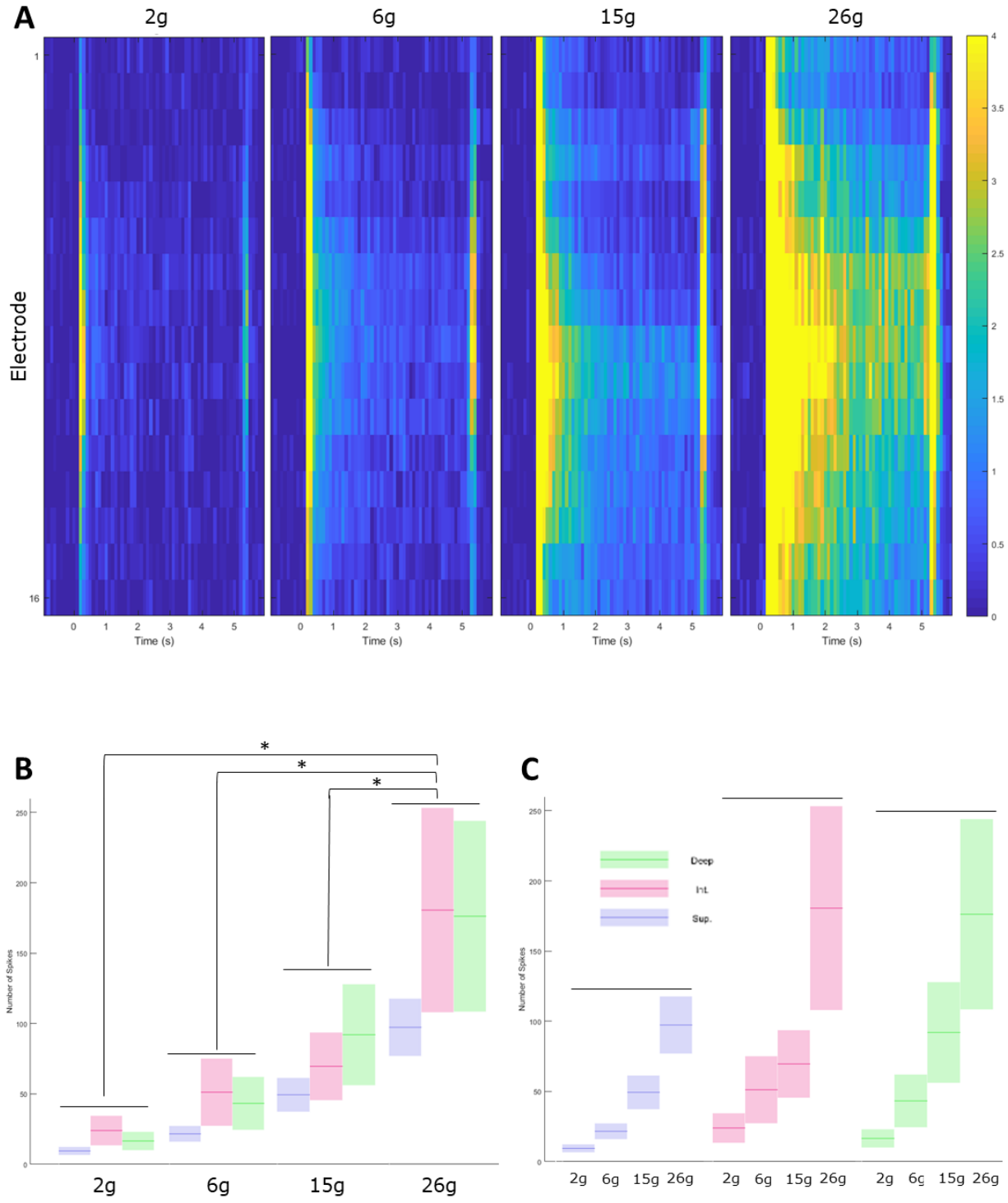


Figure 3.6. Stimulus dependent changes occur in the P21-23 dorsal horn following mechanical stimulation of the hindpaw and these changes can be analysed with the MEA A) Heatmaps of activity at each electrode following stimulation with 2g, 6g, 15g, or 26g vFhs. Responses shown are averaged responses from all animals in the age group, n=10 for 2g, 6g, and 15g responses, n=7 for 26g responses. Time period shown is 1second before the stimulus is applied, the 5 seconds of application, and 1second post application. Electrodes are in order; 1 is the most dorsal and 16 the most ventral. Colours represent mean number of spikes respective to the colour bar shown on the right. B) Regional responses to each stimulus over the time period shown in (A), lines represent mean of electrodes in each region and shaded area represents SEM. C) Stimulus responses within each region, data shown in B is reorganised to allow easier representation of comparisons. A two-way ANOVA showed significant stimulus intensity dependent changes but no regional differences or interaction. Asterisks indicate significant differences between groups with a Tukey's post hoc test (P<0.01).

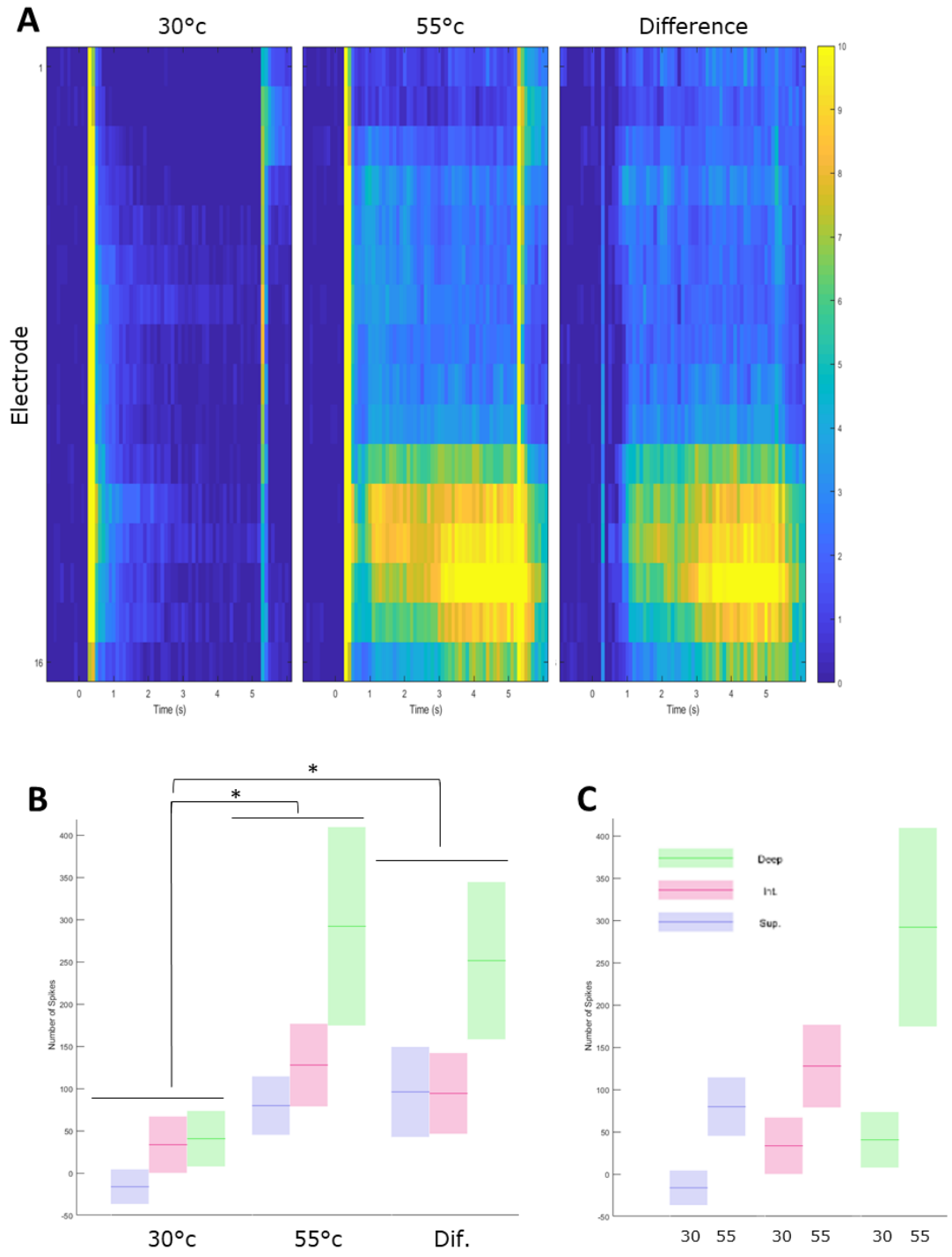


Figure 3.7. Stimulus dependent changes occur in the P21-23 dorsal horn following thermal stimulation of the hindpaw and these changes can be analysed with the MEA. A) Heatmaps of activity at each electrode following stimulation with 30°c and 55°c is shown, and the difference in response between the two groups. Responses shown are averaged responses from all animals in the age group, n=8. Time period shown is 1second before the stimulus is applied, the 5 seconds of application, and 1second post application. Electrodes are in order; 1 is the most dorsal and 16 the most ventral. Colours represent mean number of spikes respective to the colour bar shown on the right. B) Regional responses to each stimulus between 0.5seconds and 5seconds of stimulus application. Lines represent mean spike number of electrodes in the region and shaded area represents SEM. C) Stimulus responses within each region, data shown in B is reorganised to allow easier representation of comparisons. Two-way ANOVA showed stimulus intensity differences but no regional difference or interaction. Asterisks indicate significant differences between groups with a Tukey's post hoc test (P<0.05).

Postnatal Days 28-30

The P28-30 age group was unexpectedly low in spike activity (Figure 3.8). It is worth noting that with a group size of n=4 this age group is most likely underpowered for mechanical analysis, however, P28-30 responses to thermal and electrical (Chapter 4) stimulation were also unexpectedly low so the decision was made to include it. There were no significant regional ($F(2, 8) < 1$), stimulus dependent changes ($F(2, 8) = 1.753$, $P=0.03$), or interaction ($F(4, 16) < 1$), following a two-way RM ANOVA.

The thermal response in this age group was also low and no regionally dependent ($F(2, 10) = 1.945$, $P=0.19$) or stimuli dependent changes ($F(2, 10) = 1.568$, $P=0.26$), or interaction ($F(4, 20) = 2.014$, $P=0.13$), were found following a two-way RM ANOVA. The heatmaps (Figure 3.9A) show that the thermal response was delayed in the whole dorsal horn not visibly appearing until approximately 2seconds post stimulus application. As with the P14-16 and P21-23 age groups the evoked thermal response appears to be predominantly in the deep dorsal horn.

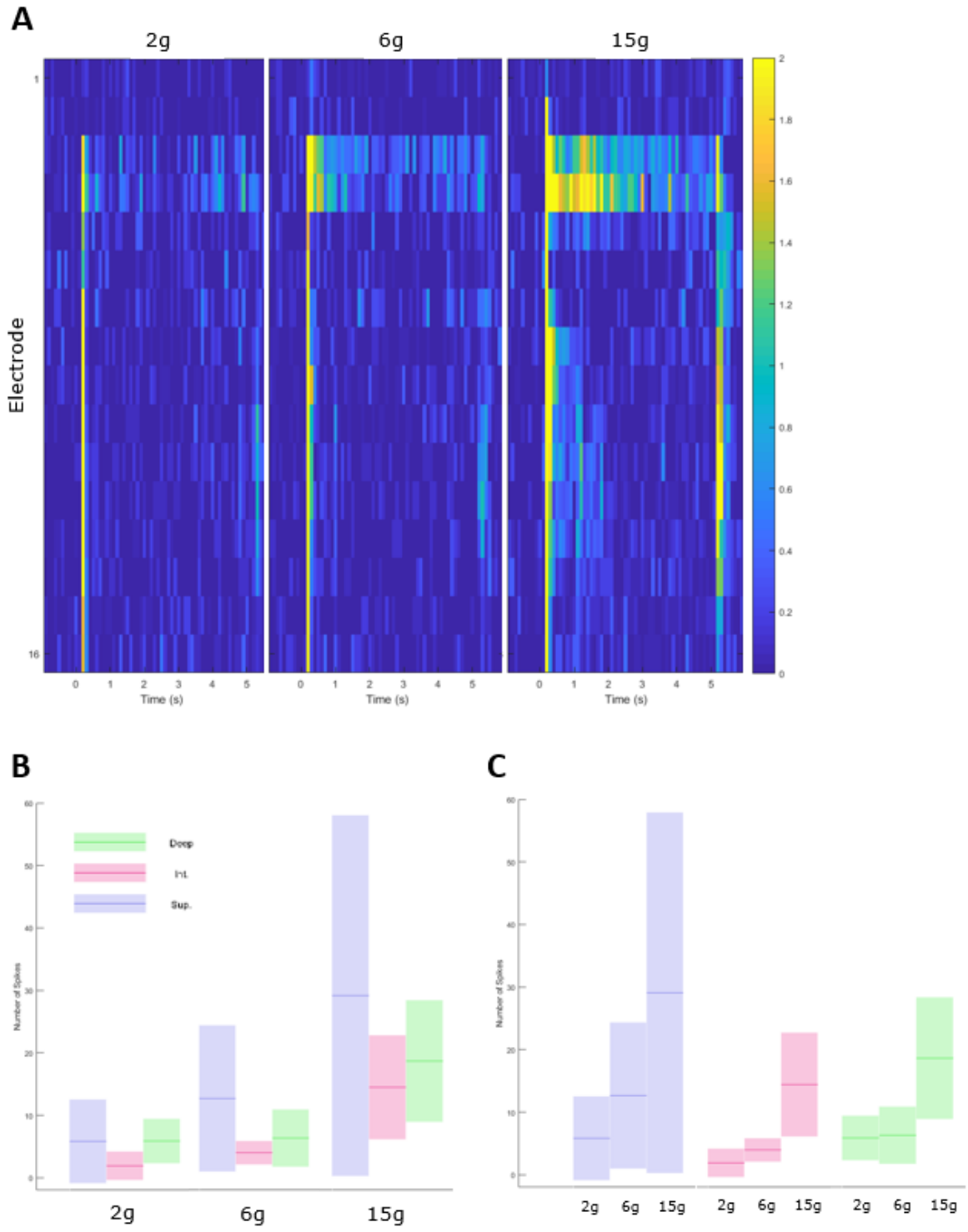


Figure 3.8. Spike activity in the P28-30 dorsal horn following mechanical stimulation of the hindpaw and these changes can be analysed with the MEA A) Heatmaps of activity at each electrode following stimulation with 2g, 6g, or 15g vFhs. Responses shown are averaged responses from all animals in the age group, n=5. Time period shown is 1second before the stimulus is applied, the 5 seconds of application, and 1second post application. Electrodes are in order; 1 is the most dorsal and 16 the most ventral. Colours represent mean number of spikes respective to the colour bar shown on the right. B) Regional responses to each stimulus over the time period shown in (A), lines represent mean of electrodes in each region and shaded area represents SEM. C) Stimulus responses within each region, data shown in B is reorganised to allow easier representation of comparisons. No statistically significant difference were found using a two-way RM ANOVA.

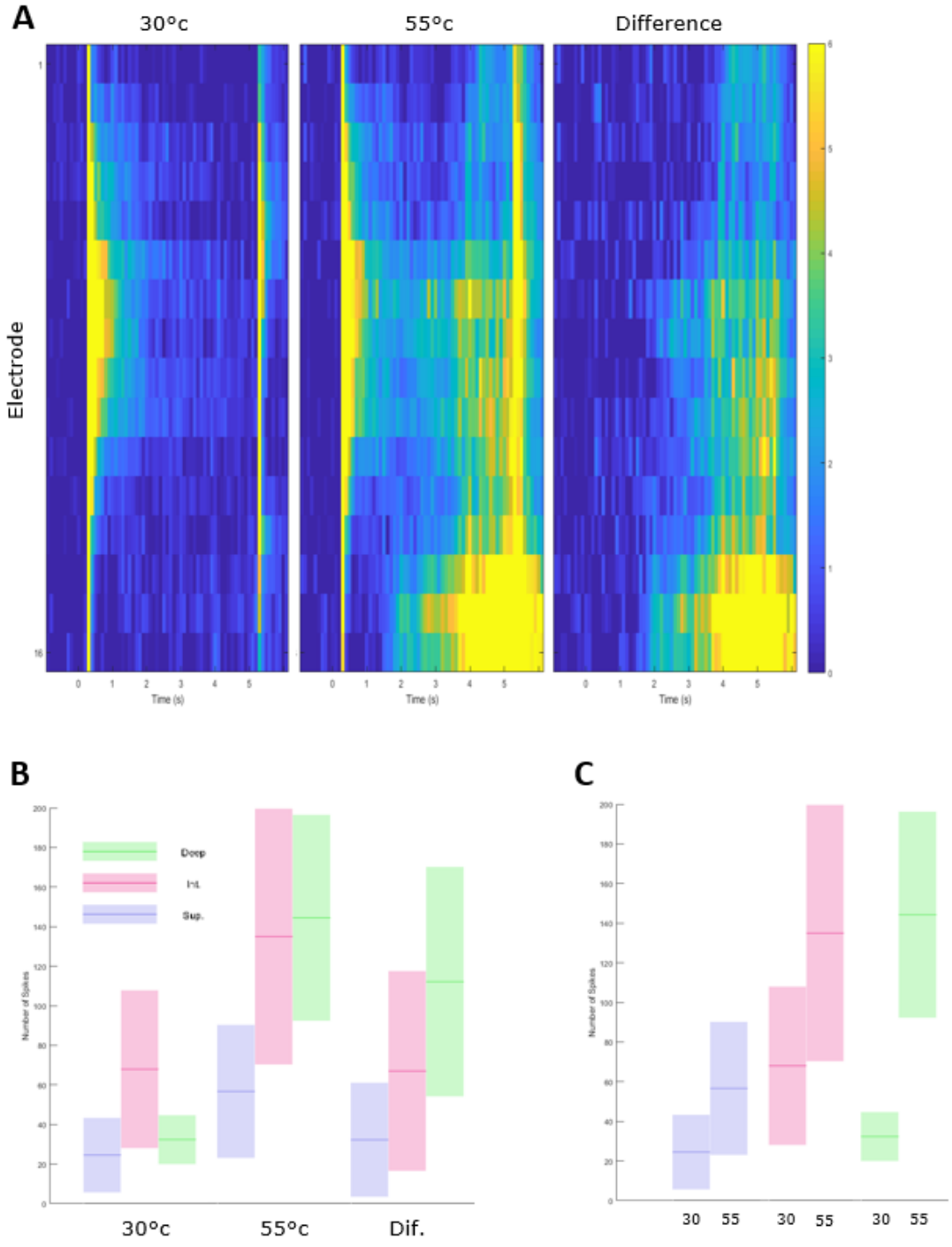


Figure 3.9. Spike activity in the P28-30 dorsal horn following thermal stimulation of the hindpaw and these changes can be analysed with the MEA. A) Heatmaps of activity at each electrode following stimulation with 30°c and 55°c is shown, and the difference in response between the two groups. Responses shown are averaged responses from all animals in the age group, n=6. Time period shown is 1second before the stimulus is applied, the 5 seconds of application, and 1second post application. Electrodes are in order; 1 is the most dorsal and 16 the most ventral. Colours represent mean number of spikes respective to the colour bar shown on the right. B) Regional responses to each stimulus between 0.5seconds and 5seconds of stimulus application. Lines represent mean spike number of electrodes in the region and shaded area represents SEM. C) Stimulus responses within each region, data shown in B is reorganised to allow easier representation of comparisons. No statistically significant difference were found using a two-way RM ANOVA.

Young Adults

In the young adult group only some rats received 26g vFh stimulation (Section 3.3.4). The heatmaps (Figure 3.10A) show an increase in spike activity with increasing stimulus intensity, particularly increasing in the 15g and 26g groups. The response is relatively consistent across the dorsal horn although the very superficial and deep electrodes are detecting less spike activity than the more intermediate electrodes. Additionally following the 26g vFh stimulation there is also an increased activity in the superficial region although no regional differences ($F(2, 96) = 2.866$, $P=0.06$), or interaction ($F(6, 96)<1$), were identified following a two-way ANOVA. However stimulus intensity dependent changes were shown ($F(3, 96)=32.55$, $P<0.0001$). A Tukey's post hoc test showed substantial stimuli dependent changes, the 26g response was higher than the 2g, 6g, and 15g responses ($P<0.0001$) and the 15g response was significantly higher than the 2g response ($P<0.001$) and the 6g response ($P<0.01$).

Following thermal stimulation of the hindpaw in the young adult rats, the heatmaps (Figure 3.11A) indicate there is an increase in spike activity throughout the dorsal horn but particularly in the very deep region. Whilst no significant regional changes ($F(2, 10) = 3.836$, $P=0.06$) or stimuli related changes ($F(2, 10) = 3.93$, $P=0.06$) were found using a two-way RM ANOVA, a significant interaction between the two factors was identified ($F(4, 20)=5.562$, $P<0.01$). Additionally, a Tukey's post hoc test revealed a significant difference between the 55°c and 30°c response ($P<0.05$).

As expected following previous work in the group (Greenspon *et al.,* 2019) in the young adult age group the MEA can show clearly different dorsal horn response profiles following mechanical and thermal hindpaw stimulation (Figures 3.10 vs. Figure 3.11).

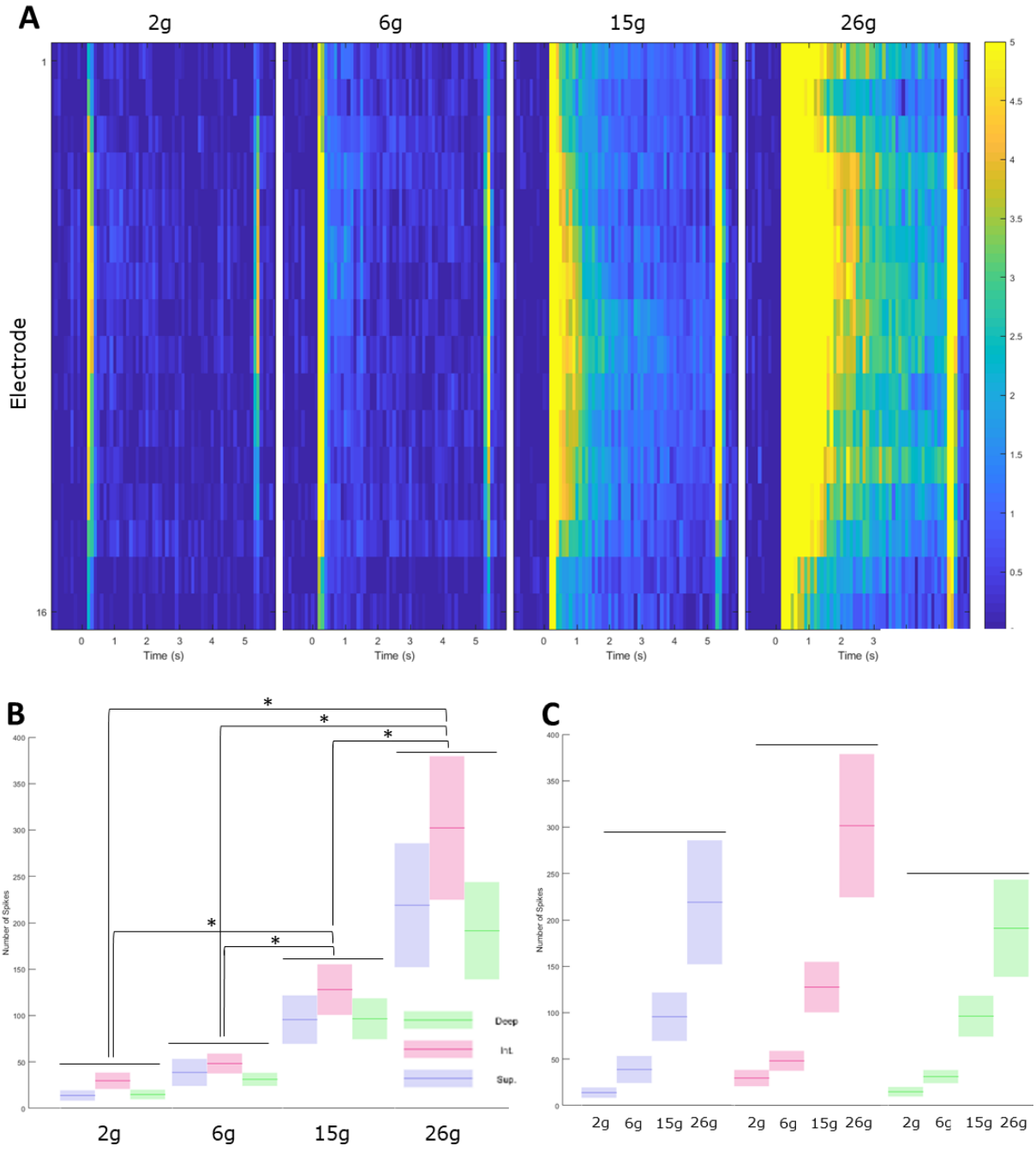


Figure 3.10. Stimulus dependent changes occur in the young adult dorsal horn following mechanical stimulation of the hindpaw A) Heatmaps of activity at each electrode following stimulation with 2g, 6g, 15g, or 26g vFhs. Responses shown are averaged responses from all animals in the age group, n=7 for 2g, 6g, and 15g responses, n=3 for 26g responses. Time period shown is 1second before the stimulus is applied, the 5 seconds of application, and 1second post application. Electrodes are in order; 1 is the most dorsal and 16 the most ventral. Colours represent mean number of spikes respective to the colour bar shown on the right. B) Regional responses to each stimulus over the time period shown in (A), lines represent mean of electrodes in each region and shaded area represents SEM. C) Stimulus responses within each region, data shown in B is reorganised to allow easier representation of comparisons. A two-way ANOVA showed significant stimulus intensity differences but no regional differences or interaction. Asterisks indicate significant differences between groups with a Tukey's post hoc test (P<0.01).

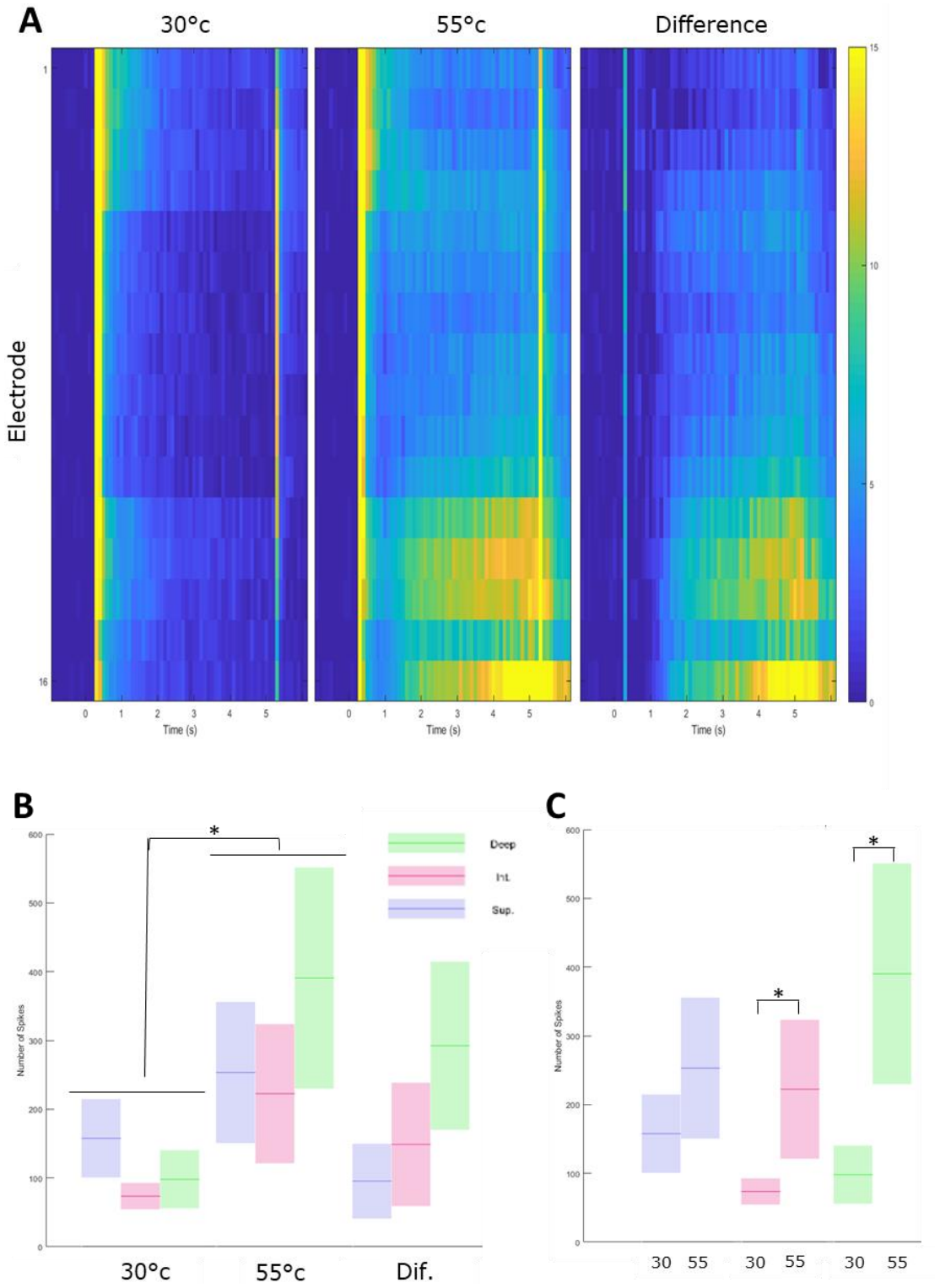


Figure 3.11. Stimulus dependent changes occur in the young adult dorsal horn following thermal stimulation of the hindpaw. A) Heatmaps of activity at each electrode following stimulation with 30°c and 55°c is shown, and the difference in response between the two groups. Responses shown are averaged responses from all animals in the age group, n=6. Time period shown is 1second before the stimulus is applied, the 5 seconds of application, and 1second post application. Electrodes are in order; 1 is the most dorsal and 16 the most ventral. Colours represent mean number of spikes respective to the colour bar shown on the right. B) Regional responses to each stimulus between 0.5seconds and 5seconds of stimulus application. Lines represent mean spike number of electrodes in the region and shaded area represents SEM. C) Stimulus responses within each region, data shown in B is reorganised to allow easier representation of comparisons. Asterisks indicate significant differences between groups with a Tukey's post hoc test.

Aged Adults

In the aged adult group all rats received a 26g vFh stimulation however only 5 rats received 2g vFh stimulation (Section 3.3.4). The heatmaps (Figure 3.12A) show an increase in spike activity and a two-way ANOVA identified a significant stimulus intensity dependent change ($F(3, 93)=16$, $P<0.0001$) but not a regional change ($F(2, 93) = 2.222$, $P=0.11$), or interaction ($F(6, 93)<1$). A Tukey's post hoc test showed multiple significant differences between stimulus intensity responses with an increase in activity to higher intensity stimulation. Following 26g stimulation there was an increase in activity compared to the 2g, 6g, and 15g responses (2g and 6g $P<0.0001$, 15g $P<0.01$). Additionally, the 15g response was significantly higher than the 2g and 6g, responses ($P<0.05$) (Figure 3.12).

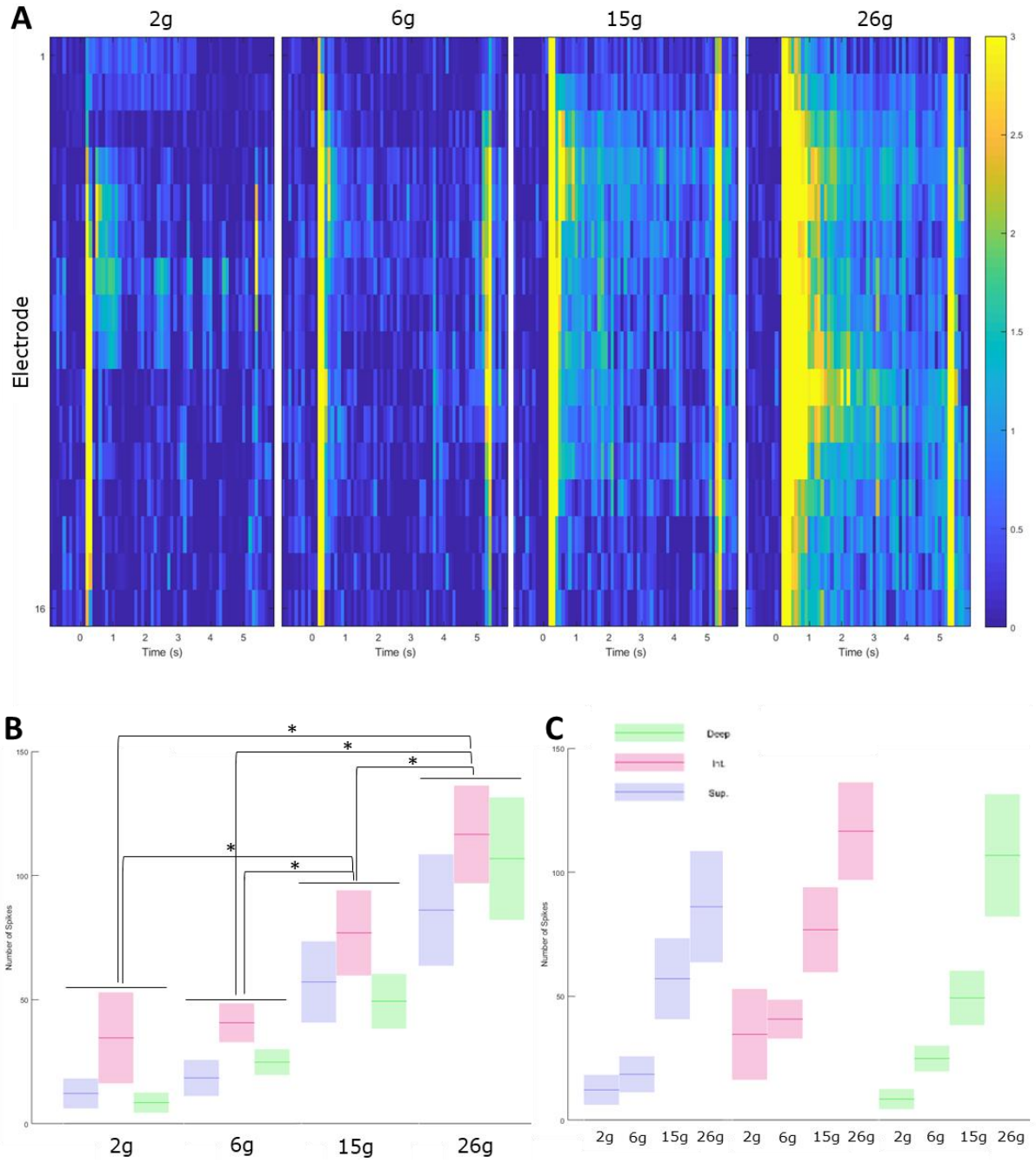


Figure 3.12. Stimulus dependent changes occur in the aged adult dorsal horn following mechanical stimulation of the hindpaw and these changes can be analysed with the MEA. A) Heatmaps of activity at each electrode following stimulation with 2g, 6g, 15g, or 26g vFhs. Responses shown are averaged responses from all animals in the age group, n=5 for 2g responses, n= 10 for 6g, 15g, and 26g responses. Time period shown is 1second before the stimulus is applied, the 5 seconds of application, and 1second post application. Electrodes are in order; 1 is the most dorsal and 16 the most ventral. Colours represent mean number of spikes respective to the colour bar shown on the right. B) Regional responses to each stimulus over the time period shown in (A), lines represent mean of electrodes in each region and shaded area represents SEM. C) Stimulus responses within each region, data shown in B is reorganised to allow easier representation of comparisons. A two-way ANOVA showed significant different in activity following different stimulus-intensity but not regional differences or interaction. Asterisks indicate significant differences between groups with a Tukey's post hoc test (P<0.05).

3.4.3 Mechanical Responses Undergo Region-Specific Changes during the Life Course

Having confirmed that the array is suitable for measuring regional and stimulus specific responses in the dorsal horn at each age group used, comparisons between the ages can now be made. The heatmaps allow the visualisation of the mean data from each age group to be compared on the same scale (Figure 3.13). As stated previously the increasing stimuli results in an increase in spike activity in all ages, this is easiest to see on at the onset. Interestingly there is not a clear increase in response to each stimulus with increasing age.

In the superficial region the spike activity in response to the different mechanical stimuli was compared between ages (Figure 3.14). One-way ANOVAs showed age-dependent differences in the superficial regions following 26g vFh stimulations ($F(2, 16)=4.288$, $P<0.05$) a Dunnett's post hoc test, comparing postnatal and aged adult responses to young adult responses, showed significantly lower responses were detected in the aged adults compared to the young adults ($P<0.05$). Statistically significant changes with age were not identified in the intermediate or deep dorsal horn region following any stimulation intensity ($P>0.05$ for all). However, there does appear to be a trend of young adults having a greater evoked response than any of the postnatal, or aged adults, following the higher stimulus intensities in the intermediate region (Figure 3.15).

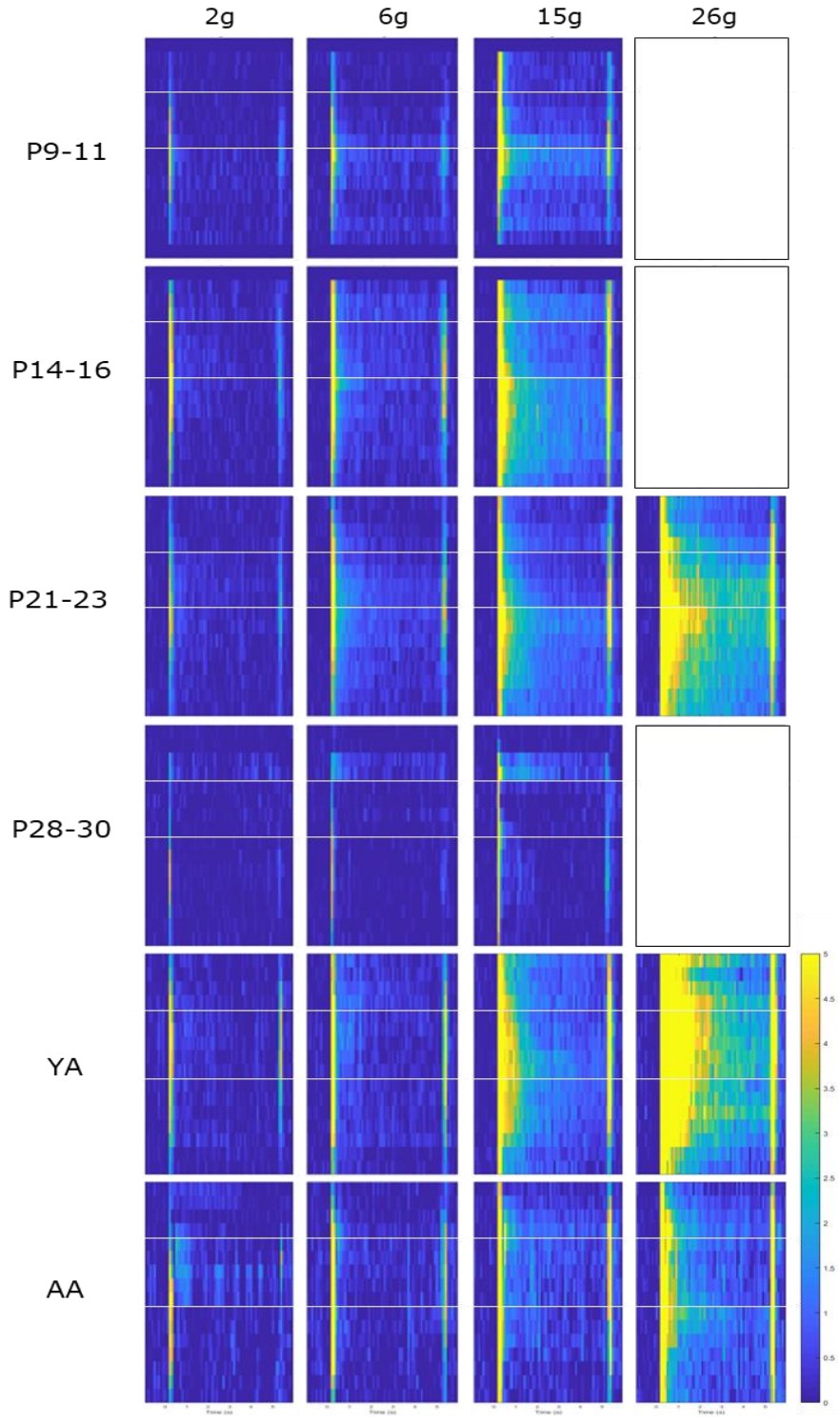


Figure 3.13. Heatmaps of spike activity in response to mechanical stimulation of the hindpaw throughout the life course. Heatmaps of activity at each electrode following stimulation with 2g, 6g, 15g, or 26g vFhs. Responses shown are averaged responses from all animals in the age group, P9-11 n=7, P14-16 n=10, P21-23 n=10 (n=7 for 26g vFh), P28-30 n=5, YA n=7 (n=3 for 26g vFh), AA n=10 (n=5 for 2g vFh). Time period shown is 1second before the stimulus is applied, the 5 seconds of application, and 1second post application. Electrodes are in order; 1 is the most dorsal and 16 the most ventral. Data from electrodes 1 and 16 has been removed from the P9-11 age group and from electrode 1 in the P14-16 age group, to represent the size of the dorsal horn. Colours represent mean number of spikes respective to the colour bar shown on the right. Empty rectangles were used where the stimuli was not given to that age group. Horizontal white lines indicate the divide between the superficial, intermediate, and deep regions electrodes are grouped into for subsequent analysis. YA = young adults, AA = aged adults.

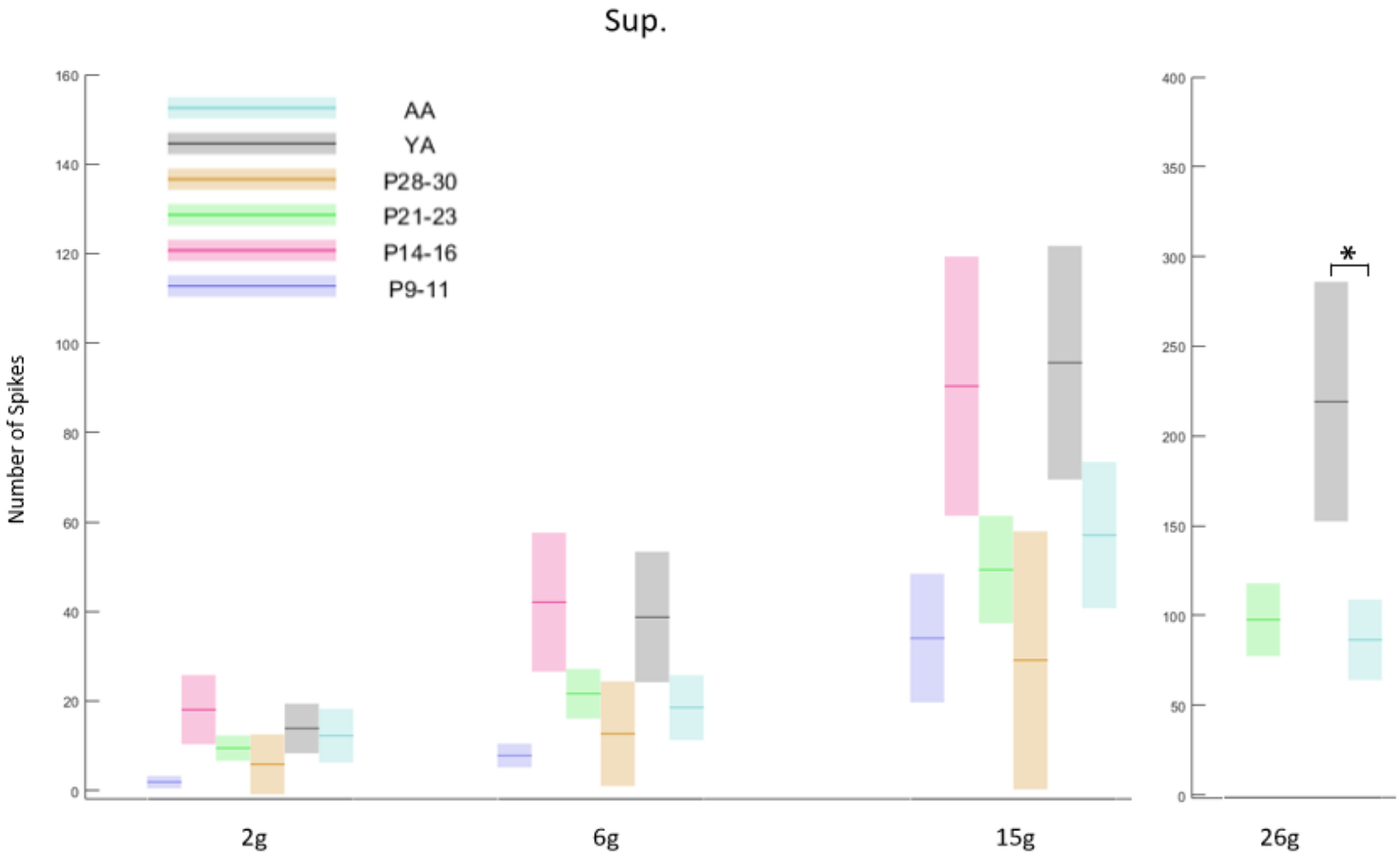


Figure 3.14. Age dependent changes occur in the superficial (Sup.) dorsal horn following mechanical stimulation of the hindpaw. Spike activity in the superficial dorsal horn in response to 2g, 6g, or 15g vFh stimulation are shown for each age group, P9-11 n=7, P14-16 n=10, P21-23 n=10 (n=7 for 26g vFh), P28-30 n=5, YA n=7 (n=3 for 26g vFh), AA n=10 (n=5 for 2g vFh). The time period used is represented by the associated heatmaps (Figure 3.13) 1second before the stimulus is applied, the 5 seconds of application, and 1second post application. Lines represent mean of electrodes in the region and shaded area represents SEM. 26g responses are on a separate scale. Asterisks indicate significant differences compared to the young adult age group determined by Dunnett's post hoc tests (P<0.05). YA = young adults, AA = aged adults.

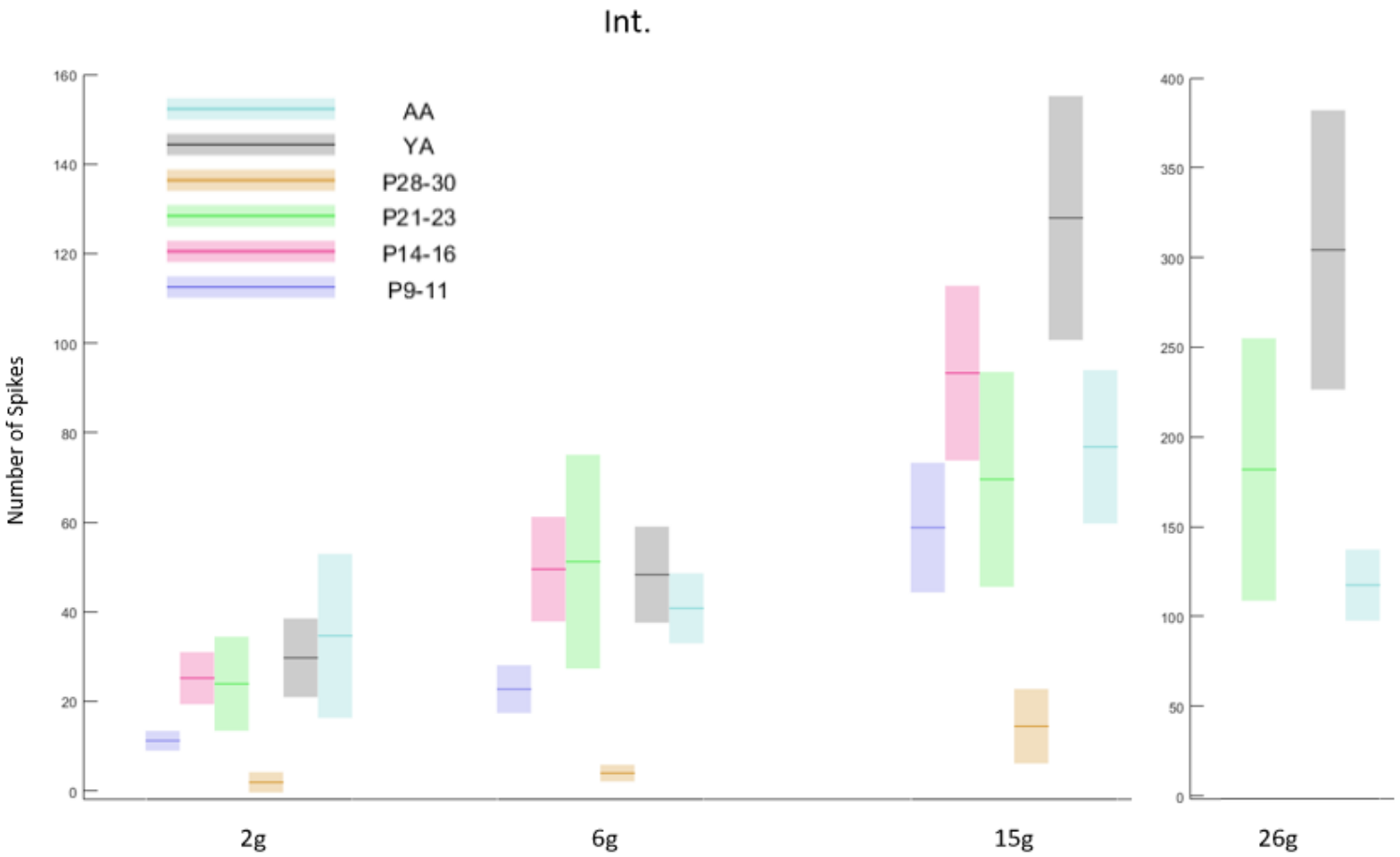


Figure 3.15. Spike activity in the intermediate (Int.) dorsal horn following mechanical stimulation of the hindpaw. Spike activity in the intermediate dorsal horn in response to 2g, 6g, or 15g vFh stimulation are shown for each age group, P9-11 n=7, P14-16 n=10, P21-23 n=10 (n=7 for 26g vFh), P28-30 n=5, YA n=7 (n=3 for 26g vFh), AA n=10 (n=5 for 2g vFh). The time period used is represented by the associated heatmaps (Figure 3.13) 1second before the stimulus is applied, the 5 seconds of application, and 1second post application. Lines represent mean of electrodes in the region and shaded area represents SEM. 26g responses are on a separate scale. No statistical differences were detected between age groups using one-way ANOVAs. YA = young adults, AA = aged adults.

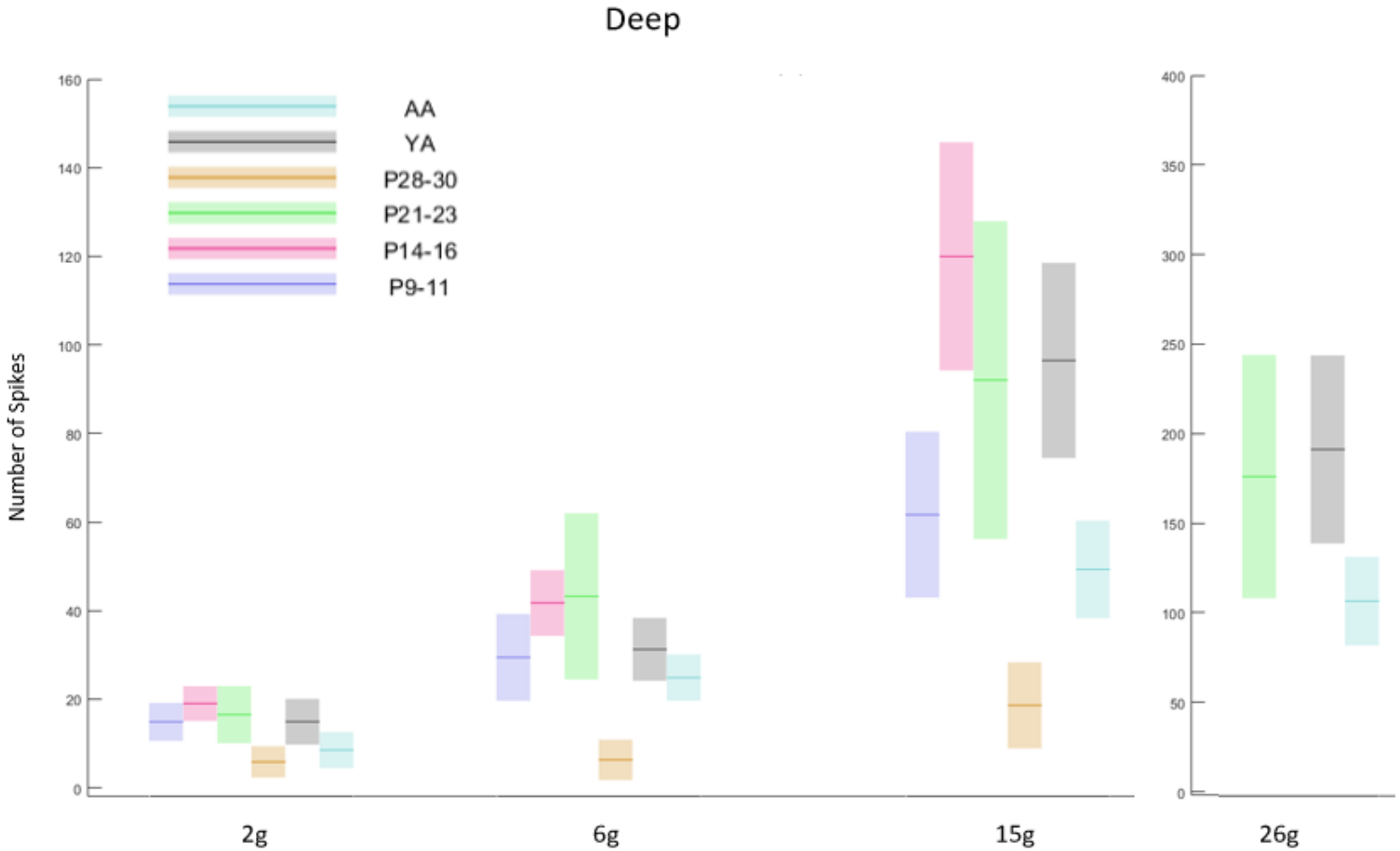


Figure 3.16. Spike activity in the deep dorsal horn following mechanical stimulation of the hindpaw. Spike activity in the deep dorsal horn in response to 2g, 6g, or 15g vFh stimulation are shown for each age group, P9-11 n=7, P14-16 n=10, P21-23 n=10 (n=7 for 26g vFh), P28-30 n=5, YA n=7 (n=3 for 26g vFh), AA n=10 (n=5 for 2g vFh). The time period used is represented by the associated heatmaps (Figure 3.13) 1second before the stimulus is applied, the 5 seconds of application, and 1second post application. Lines represent mean of electrodes in the region and shaded area represents SEM. 26g responses are on a separate scale. No statistical differences were detected between age groups using one-way ANOVAs. YA = young adults, AA = aged adults.

3.4.4 Thermal Responses May Undergo Region-Specific Changes during Postnatal Development

The thermal heatmaps (Figure 3.17) when all set to the same scale show an interesting pattern during development. As discussed earlier the intermediate region in the P9-11 age group shows the greatest response, however from P14-16 onwards this moves to the deep dorsal horn. Additionally, this response appears to increase with increasing age, apart from in the P28-30 group. In the adult group it appears that the most ventral region of the dorsal horn is missing from the recordings, as the array is positioned to prioritise recordings from the superficial region (Section 2.2.1). When presented as line graphs the changes in thermal response patterns can be seen more easily (Figure 3.18), in particularly the thermal 'difference' group in the deep region shows different speeds of response, with the P28-30 group having a much slower onset than the other age groups as well as changes during the response. For example, the P14-16 response stays relatively steady but the P21-23 and young adult group continue to increase in the latter half of the stimulus application. When the mean spike activity is examined for the 'difference' between age groups in each region using a two-way ANOVA no age dependent differences are found, however a region related change is ($F(2, 87)=4.157$, $P<0.05$) and using a Dunnett's post hoc test a significant difference is revealed between the P9-11 and young adult groups in the deep region ($P<0.05$) (Figure 3.19).

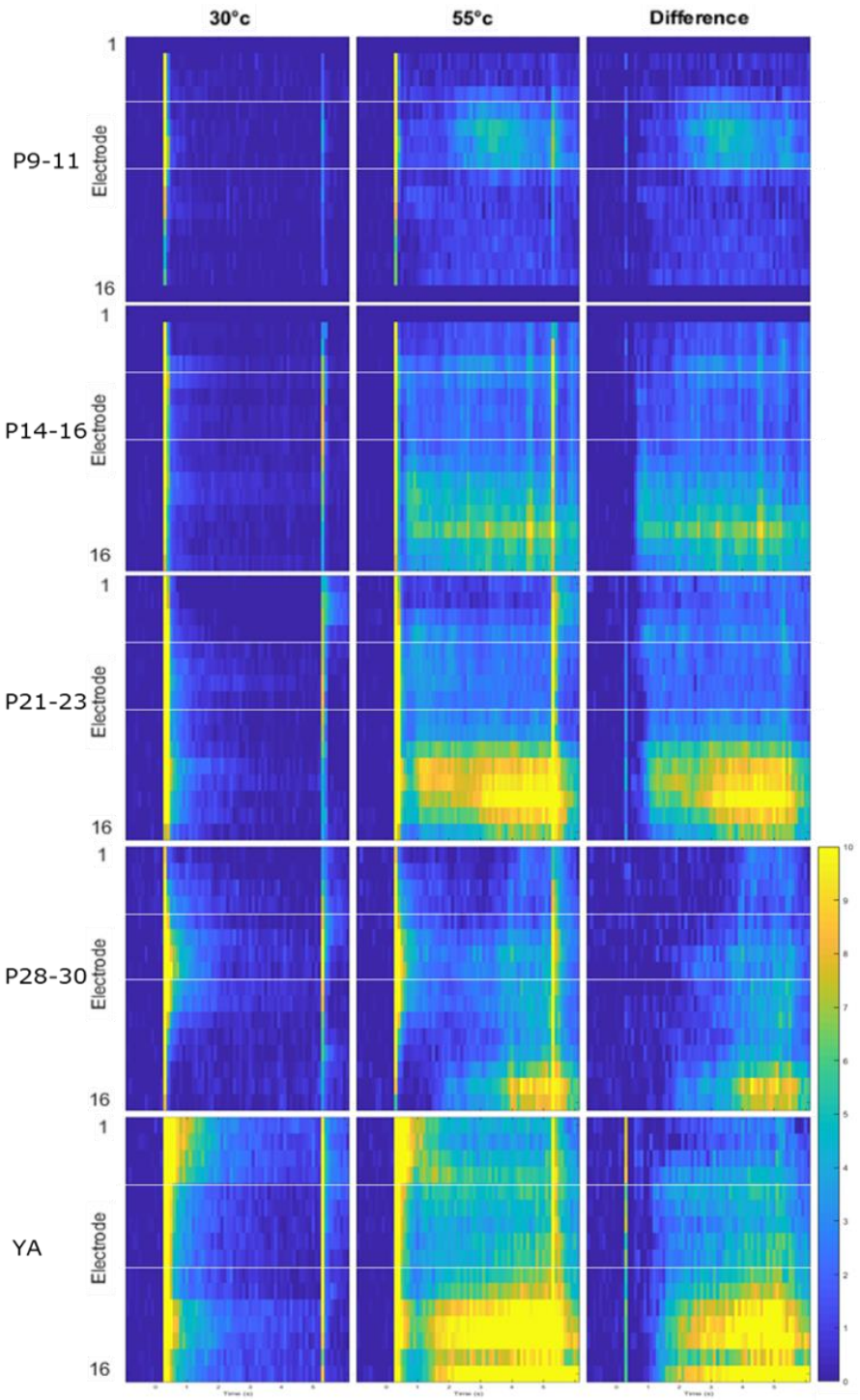


Figure 3.17. Heatmaps of spike activity in response to thermal stimulation of the hindpaw throughout early life. Heatmaps of activity at each electrode following stimulation with 30°c, 55°c, or the difference between the two responses. Responses shown are averaged responses from all animals in the age group, P9-11 n=5, P14-16 n=9, P21-23 n=8, P28-30 n=6, YA n=6. Time period shown is 1second before the stimulus is applied, the 5 seconds of application, and 1second post application. Electrodes are in order; 1 is the most dorsal and 16 the most ventral. Data from electrodes 1 and 16 has been removed from the P9-11 age group and from electrode 1 in the P14-16 age group, to represent the size of the dorsal horn. Colours represent mean number of spikes respective to the colour bar shown on the right. Horizontal white lines indicate the divide between the superficial, intermediate, and deep regions electrodes are grouped into for subsequent analysis. YA = young adults.

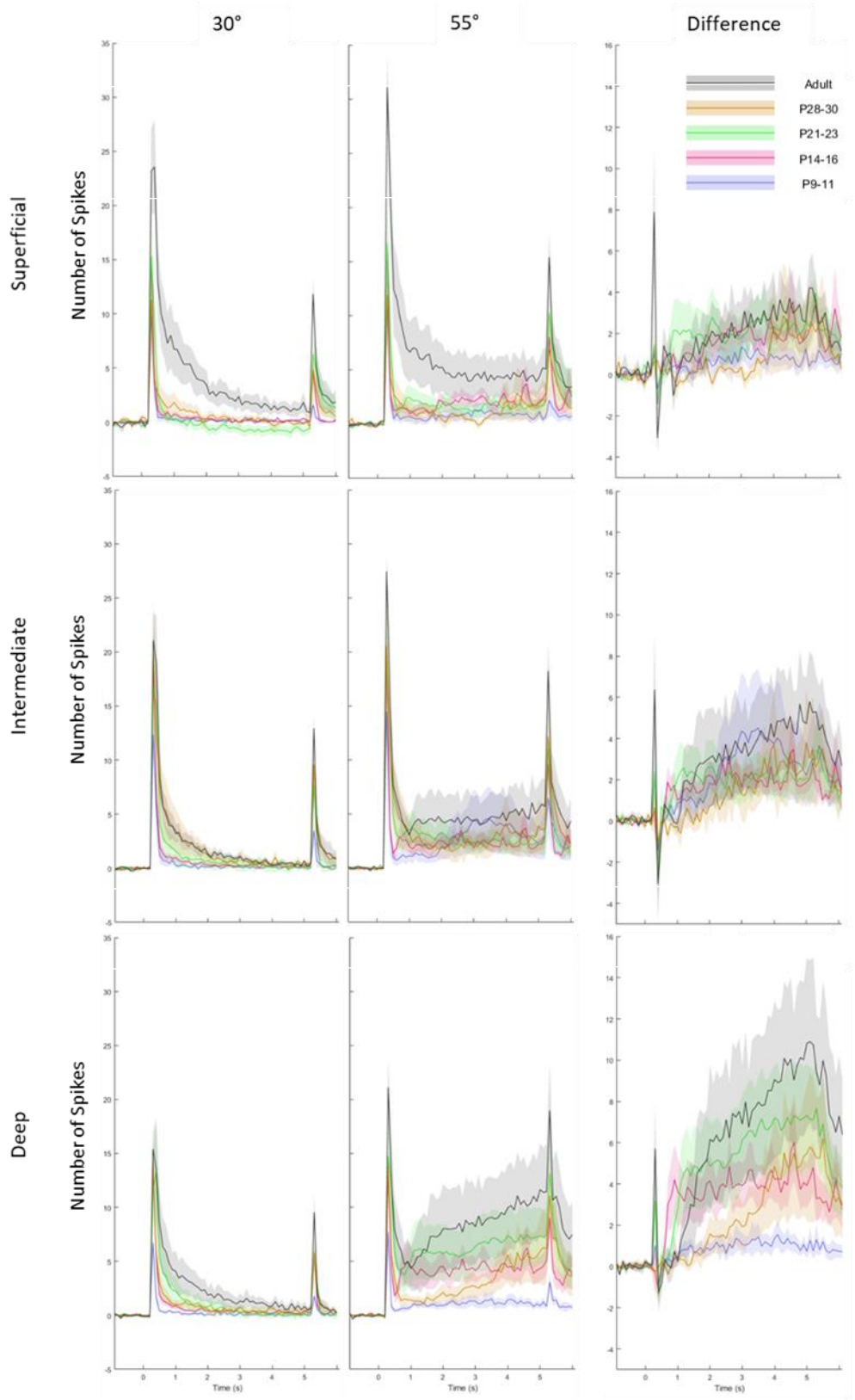


Figure 3.18. Spike activity in response to thermal stimulation of the hindpaw throughout early life. Mean activity of the electrodes in each region is shown following stimulation with 30°c, 55°c, or the difference between the two responses for each age group, P9-11 n=5, P14-16 n=9, P21-23 n=8, P28-30 n=6, YA n=6. The time period used is represented in the thermal heatmaps (Figure 3.18); 1second before the stimulus is applied, the 5 seconds of application, and 1second post application. Lines represent mean data and shaded area represents SEM. The difference between 30°c and 55°c responses are on a separate scale. YA = young adults.

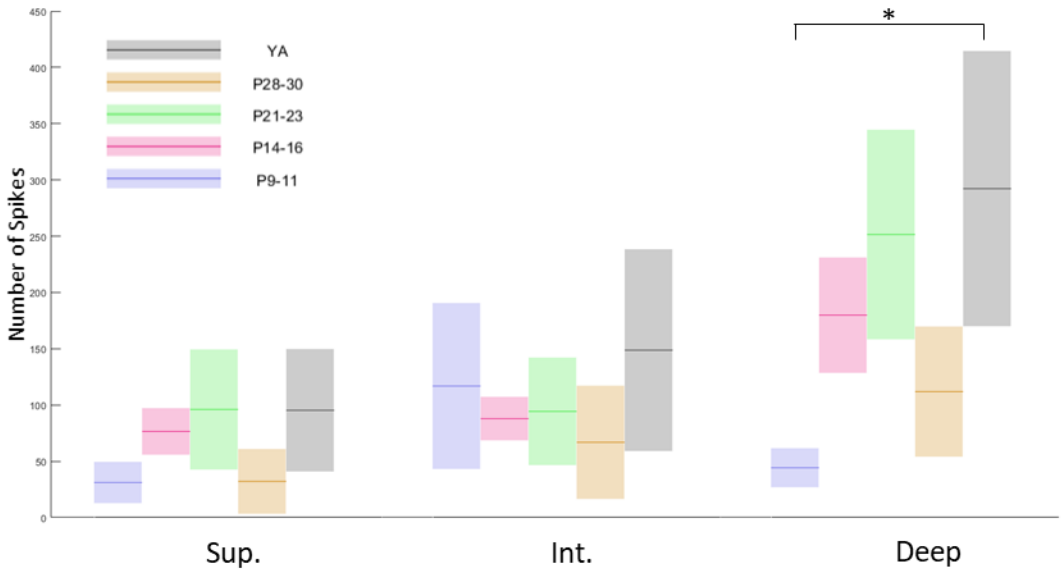


Figure 3.19. Age dependent changes occur in the dorsal horn during thermal stimulation of the hindpaw. The difference in spike activity following 30°c and 55°c hindpaw stimulation in the superficial, intermediate, and deep dorsal horn are shown for each age group, P9-11 n=5, P14-16 n=9, P21-23 n=8, P28-30 n=6, YA n=6. The time period used is 0.5seconds to 5 seconds during application of the stimuli. Lines represent mean of electrodes in the region and shaded area represents SEM. A two-way ANOVA showed regional and age related differences but no interaction. Asterisks indicate significant differences compared to the young adult age group determined by Dunnett's post hoc tests (P<0.05). YA = young adults.

Unlike the mechanical response the spike activity to the thermal response continued to increase in the P21-23, P28-30, and young adult groups during the stimulus application. To determine if this was immediately resolved following removal of the thermal stimulus the spike activity examined before and the activity over the following minute was plotted on line graphs (Figure 3.20). These show, particularly in the deep dorsal horn, a trend of increasing evoked response with increasing postnatal age, apart from the P21-23 and P28-30 groups. Comparing the mean spike activity over this time period at the different ages in each region age-related changes ($F(4, 87)=3.101$, $P<0.05$) and region dependent changes ($F(2, 87)=3.776$, $P<0.05$) were found, but no interaction ($F(8, 87) <1$; Figure 3.21). Additionally, a Dunnett's post hoc test showed a significant decrease in the P9-11 spike activity compared to the young adult response ($P<0.05$). Interestingly the general response pattern between the ages in the same when looking at the response during or during with the 60seconds following apart from in the P14-16 age group which decreases with the longer time frame, suggesting less ongoing activity after the thermal stimulus is removed.

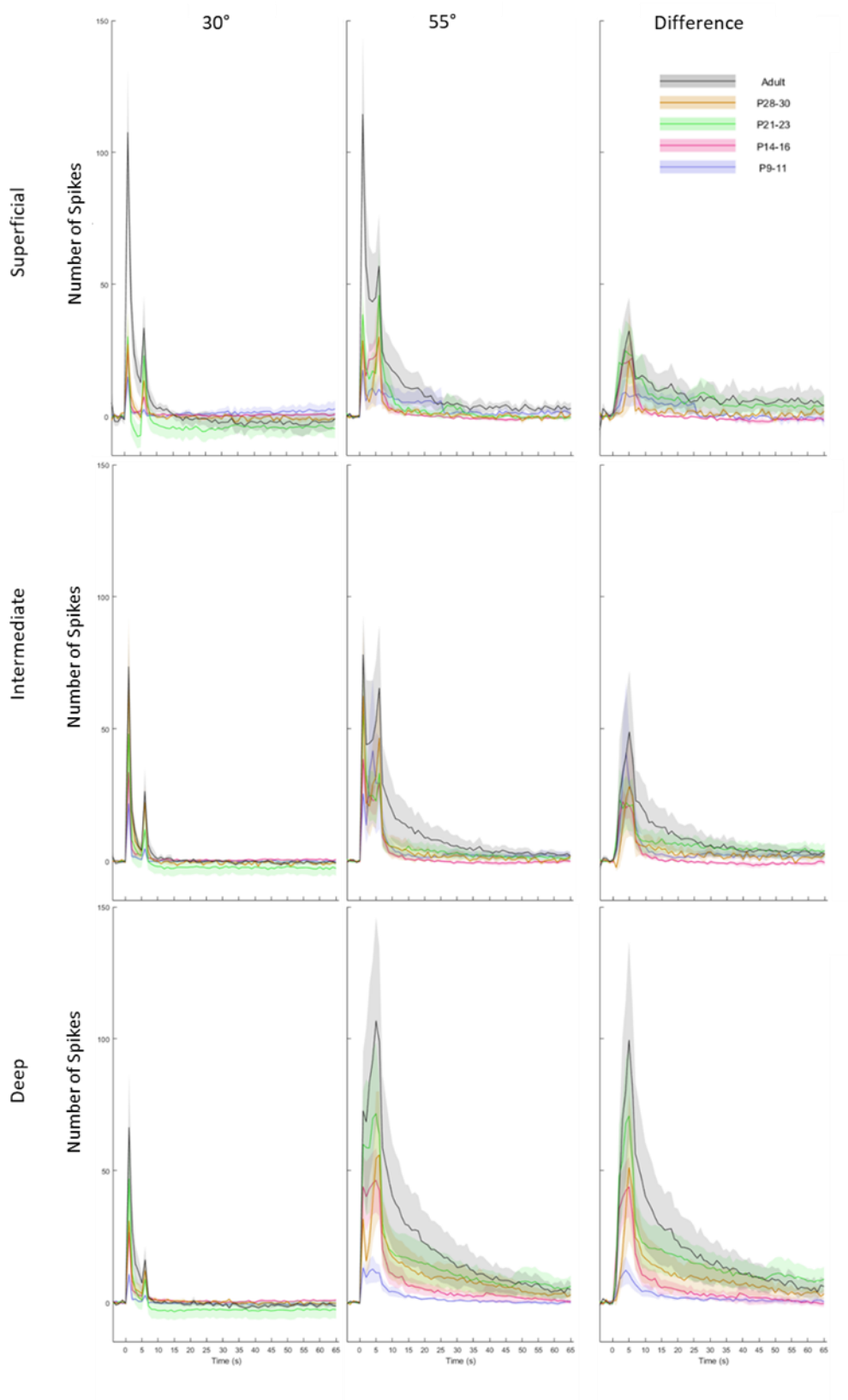


Figure 3.20. Spike activity 1 minute following thermal stimulation of the hindpaw throughout early life. Mean activity of the electrodes in each region is shown following stimulation with 30°c, 55°c, or the difference between the two responses for each age group, P9-11 n=5, P14-16 n=9, P21-23 n=8, P28-30 n=6, YA n=6. The time period used is 1 seconds pre-stimulus, 5 seconds of stimulus application, and 60 seconds post application. Lines represent mean data and shaded area represents SEM. YA = young adults.

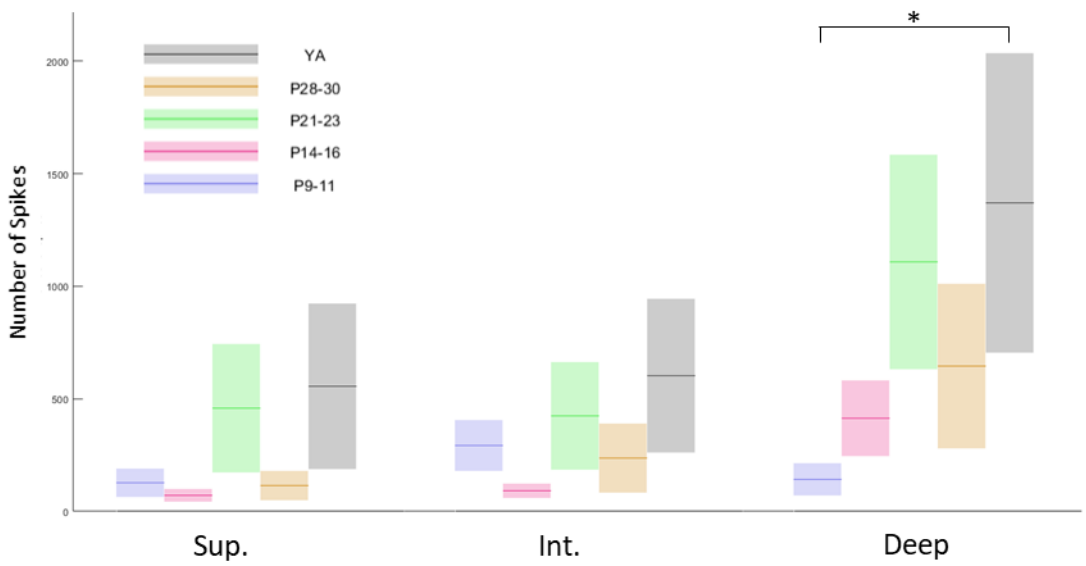


Figure 3.21. Age and region dependent changes occur in the dorsal horn 1 minute following thermal stimulation of the hindpaw. The difference in spike activity following 30°c and 55°c hindpaw stimulation in the superficial, intermediate, and deep dorsal horn are shown for each age group, P9-11 n=5, P14-16 n=9, P21-23 n=8, P28-30 n=6, YA n=6. The time period used is represented on the corresponding line graphs (Figure 3.21), 1 second before stimulus application to 60 seconds post application. Lines represent mean of electrodes in the region and shaded area represents SEM. Asterisks indicate significant differences compared to the young adult age group determined by Dunnett's post hoc tests. YA = young adults.

3.5 Discussion

The work presented in this chapter demonstrates that the MEA electrophysiology set-up can be used to measure evoked activity across the dorsal horn in postnatal rats from at least P9. We found an increase in spontaneous activity with increasing postnatal age as well as a general trend of increased spike activity following mechanical and thermal stimulation of the hindpaw during the postnatal period. Interestingly, evoked mechanical responses in aged adults appear to be generally lower than young adults.

3.5.1. Technique Development

An initial pilot study was conducted to confirm that the electrode array could be used in the neonatal rats used in this study. An initial concern was that although single-unit electrophysiology is frequently performed in neonatal rats the increased size of the MEA could cause damage to the neonatal spinal cord. However, we've shown that the array used was able to record spontaneous and evoked dorsal horn activity at P9 and above.

This would also suggest that the array may be suitable for dorsal horn recordings in mice as the P9-11 rats are a similar weight and size, if not smaller, than an adult mouse.

Although a substantial amount of spinal electrophysiology work has been undertaken in neonatal rats to help elucidate the mechanisms taking place during the processing of touch and pain (Section 1.2.3) this is the first time population data using multi-unit spike recordings has been used. Whilst this technique has been used previously in the group (Greenspon *et al.,* 2019) this has been exclusively in adult rats. As such, a level of method development was required with adjustments to the surgical tools use (Section 2.1.4) and the analysis performed (Section 2.1.7). Additionally, previous work using the MEAs in adult male rats (Greenspon *et al.,* 2019) suggested a group size of 3 to 4 was required to be statistically powered. However, during this work it became clear that there was more variation in the neonatal groups. This increased variation could be due to neonates having larger hindpaw receptive fields than adults (Fitzgerald, 1985). This is in part why the data presented in this chapter, particularly the mechanical stimulation data, has been collected over several groups of animals.

3.5.2 Increases in Baseline Dorsal Horn Activity with Increasing Age

Baseline activity, representing spontaneous activity of the dorsal horn, significantly increased with age. Most previous studies have employed single-unit electrophysiology, which predominantly uses wide dynamic range neurons, however, these cells have relatively little spontaneous activity (Jennings and Fitzgerald, 1998). Patch-clamp recordings of lamina I projection neurons during postnatal development suggest there is little change in spontaneous activity (Li and Baccei, 2012). This supports the hypothesis that population recordings can add new contributions to the understanding of dorsal horn development.

3.5.2 Dorsal Horn Processing of Hindpaw Mechanical Stimulation

As innocuous mechanical stimulation of the hindpaw should evoke primarily Aβ primary afferent fibres we expected to see the majority of the evoked dorsal horn activity occurring in the intermediate and deep regions in adulthood, as this is where Aβ-LTMR fibres are thought to innervate (Todd, 2010). Interestingly, we observed relatively consistent responses across the dorsal horn from P14. This may suggest other primary afferent fibres are also be being activated, possibly C-LTMRs which terminated in lamina IIiv or Aδ-LTMRs which innervate lamina IIi/III (Abraira *et al.*, 2017). Alternatively the widespread mechanical responses could reflect a wider innervation profile for Aβ-PAFs

than previously thought. These data do however show similar responses to those obtained previously by the group using MEA electrophysiology in adult rats (Greenspon *et al.,* 2019). In line with this A-fibre innervation into the dorsal horn has been shown by multiple groups to change during postnatal development with a retraction from laminae I and II to more intermediate laminae occurring (M Fitzgerald, Butcher and Shortland, 1994; Nakatsuka and Kumamoto, 2000; Beggs *et al.*, 2002). We do not see this with our data, however it may be due to not being able to isolate the A-fibre response following the 5 second mechanical stimulation.

Higher mechanical stimulations resulted in an increased number of spikes throughout the dorsal horn at all ages. However it is interesting to note the large increase in activity following the 26g vFh stimulus in the superficial dorsal horn possibly demonstrating the activation of high threshold A- and C-fibres, which heavily innervate the superficial laminae (Todd, 2010). Additionally, as the superficial dorsal horn, in particular lamina II, is a key site in mechanical pain processing microcircuits which involve excitatory somatostatin interneurons (Duan *et al.,* 2014) we may be observing an increase in spike activity from these neurons following the higher intensity mechanical stimulations.

Although age-related trends of increased dorsal horn activity can be seen, we detected very few statistically significant differences between ages following mechanical stimulation of the hindpaw. What we tend to see over postnatal development are low evoked responses in P9-11 and P28-30 rats, increased activity at P14-16 particularly in the superficial regions, and lower or similar responses at P21-23 compared to P14-16. Young adult evoked responses tended to be relatively similar in spike number to P14-16 responses, and aged adult responses normally declined to below young adult responses.

We also potentially observe a change in the regional response pattern. Although not statistically tested a change can be seen with increasing age; at P9-11 all stimuli resulted in the highest response occurring in the deep dorsal horn, then the intermediate, and least in the superficial. However, at P14-16 and P21-23 that pattern only occurs in response to the higher stimuli (15g and 26g) and the lower stimuli (2g and 6g) have the greatest response in the intermediate region. Both adult groups then produce the highest response to each stimuli (2g, 6g, 15g, and 26g) in the intermediate region. This may also link with an increase in behavioural thresholds as withdrawal thresholds to mechanical stimuli are lower in neonates and increase with postnatal age until reaching a mature level at approximately P28 (Fitzgerald, M, Shaw, A, MacIntosh, 1988).

This suggests that ongoing changes in dorsal horn processing occur throughout the life course. However, it is important to note that the behavioural thresholds following

mechanical stimulation increase over the postnatal period (Fitzgerald, 2005) and may affect the comparisons between ages following specific vFh stimulation intensities.

3.5.2 Dorsal Horn Processing of Hindpaw Thermal Stimulation

Dorsal horn responses following thermal stimulation of the hindpaw showed a trend of increasing with postnatal age, specifically in the deep dorsal horn. This may be due to activity from the wide dynamic range neurons in the deep dorsal horn which respond to a range of temperatures (Menetrey, Chaouch and Besson, 1979); this would suggest that the temperatures used here were not noxious enough to evoke increased activity in the superficial region possibly suggesting that the 5 seconds is not sufficient to heat the skin to 55°c, which may explain the still increasing response towards the end of the stimulus application seen particularly in the older age groups. However, it is also possible that the heatblock used was not reaching the temperature stated on the stimulator and this should be tested or an alternative method of application used for future studies.

Additionally, there is a regional change in the evoked thermal activity between P9-11 and P14-16 with the majority of the response moving from the intermediate to deep region. This may suggest that the processing of thermal stimulations is mature by P14-16. Interestingly, these findings are in line with previous behavioural findings which have suggested that thermal thresholds are mature at approximately P12 (Falcon *et al.,* 1996). $TRPV1^+$ receptors are known to detect noxious heat (Caterina *et al.*, 2000) and are found predominantly on peptidergic DRGs in adults. In the first two postnatal weeks a higher percentage of DRGs are TRPV1 positive, being found throughout the dorsal horn, and the mature state, in which they are in the most superficial laminae is reached by P15 (Cavanaugh *et al.*, 2011). As such this previous study suggested that with increasing postnatal age there is a refinement of $TRPV1^+$ DRG neurons to the superficial dorsal horn, not matching our findings that from P14-16 thermal processing moves to the deep dorsal horn. However, this previous data used immunohistochemistry to determine these postnatal changes. As such the discrepancy may be due to the difference of techniques employed. However, as C- and Aδ-PAFs are associated with noxious heat processing, we would expect to see a large response in the superficial region (Abraira *et al.,* 2017). Previous studies using patch-clamp recordings have shown that superficial dorsal horn neurons have functional TRPV1 receptors, which respond to capsaicin, from P0 and mature between P5 and P10 (Baccei *et al.,* 2003). As such we would expect to see a response in the superficial region at all ages used in this study. Additionally, at approximately P10 there are reports that a subset of mechanically

sensitive C-fibres become polymodal and respond to thermal stimulation as well as mechanical (Jankowski *et al.*, 2014), this could contribute to the increased response seen at P14-16 compared to the young age group.

3.5.3 Postnatal days 28-30

Interestingly the P28-30 age group response activity was comparatively low following mechanical and thermal stimulation. For the mechanical analysis the group size is only 4 rats due to this age group not being included in one of the studies used to obtain this data, which almost certainly means the group is underpowered. However, the group used for the thermal stimulation analysis should be sufficiently powered and again showed a different response to the surrounding age groups, with a slower response time and lower spike activity. It is currently unclear why this age group would show delayed and lower evoked activity.

3.5.4 Technical Considerations

It is unfortunate that the data presented in this chapter was obtained over multiple studies, however, it was unclear from the previous work that a group size of 6-10 would be suggested. This has however resulted in animals within the same age group receiving different vFh stimulations, this will have reduced the power of analysing the 26g responses, and the 2g responses for the aged adults. As mentioned before, as mechanical and thermal thresholds increase during the postnatal period comparing responses following a set stimulus may not be ideal.

For some animals the mechanical responses had to be manually aligned to the onset before analysis could take place. No trend amongst ages or stimuli intensity was found and so this may reflect a slight delay in the picopump occurring sporadically. This required a relatively long amount of time to manually sort and although care was taken with delivering the mechanical stimulations it may suggest that extra care need to be taken to ensure the picopump is primed with the correct pressure and delivered from a consistent height above the paw.

Additionally, the thermal stimulation involving a mechanical element is less than ideal. The heating block used in these experiments was not capable of changing its temperature quickly, so instead had to be applied to the paw for the stimulation. The

group recently purchased a peltier device which should be capable of rapid heating and cooling allowing thermal processing to be investigated more accurately in the future.

A limitation with attempting to understand the data is the inability to specifically look at different primary afferent fibre responses. This could be addressed by using electrical stimulation of the hindpaw instead of mechanical or thermal.

3.5.4 Conclusion

In conclusion the data presented in this chapter has demonstrated the multi-electrode electrophysiology protocol used in these experiments is capable of measuring spontaneous and evoked activity throughout the rat dorsal horn from postnatal day 9. Mechanical stimulation of the hindpaw showed intensity dependent changes in the dorsal horn processing throughout the life course. Thermal stimulation appeared to be regionally mature by the second postnatal week, however trends of increasing evoked spike number with increasing age were seen. This data supports further work using MEA electrophysiology to investigate postnatal development of somatosensory processing.

Chapter 4

Changes in Dorsal Horn Processing of Primary Afferent Fibre Inputs through the Life Course

4.1 Introduction

The previous chapter identified age-dependent changes in the dorsal horn processing of peripheral mechanical stimulation. However, although we expect this is in part due to age-related changes associated with the different primary afferent fibres (PAFs) this could not be determined with the relatively long duration of stimulus application used. Electrical stimulation can be delivered with a much shorter duration and allows the dorsal horn processing of different PAF subsets to be investigated due to the differences in their thresholds and conduction velocities.

4.1.1 Primary Afferent Fibres

PAFs are responsible for the transmission of action potentials generated at the periphery to the central nervous system. As discussed in Section 1.2.3, PAFs are modality specific, with subsets responding to different stimuli intensities and modalities including; mechanical, heat, cold, and chemical, although some fibres are polymodal and can respond to multiple stimulation types (Todd, 2010). As with all neurones, and other electrically excitable cells, PAFs can also be activated by direct electrical stimulation. Again, with different subsets having different activation thresholds, with larger diameter fibres responding to shorter and lower intensities than thinner ones (Gasser and Grundfest, 1939).

A-fibres are large, thickly myelinated afferents capable of saltatory conduction and as such have the fastest conduction velocities of the PAFs. They are further divided into two subgroups Aβ- and Aδ; Aβ-fibres are larger in diameter, have thicker myelination, faster conduction velocities and are associated with the cutaneous specialised LTMRs, although they are also present as free nerve endings. Due to their association with specialised cutaneous sensory receptors, Aβ-fibres are predominantly involved in sensing low threshold mechanical stimuli but are also involved in nociceptive signalling (Djouhri and Lawson, 2004). Generally LTMR Aβ-fibres innervate the intermediate and deep dorsal horn (laminae III-V) (Todd, 2010) and nociceptive Aβ-fibres innervate the superficial dorsal horn (lamina I and IIo) as well as lamina V (Djouhri and Lawson, 2004). Aδ-fibres can again be divided into two subgroups; Aδ-HTMR and Aδ-LTMR (Gatto *et al.*, 2019). Aδ-HTMR are polymodal and involved in processing high threshold mechanical, heat, and cold stimuli (McGlone and Reilly, 2010); they innervate lamina I and IIo (Abraira *et al.*, 2017). Aδ-LTMR are primarily associated with innocuous mechanosensation in hairy skin, however they are also associated with Pacinian corpuscles which are found in glabrous skin (Gatto *et al.*, 2019) and innervate the dorsal horn at laminae IIi/III (Abraira *et al.*, 2017).

C-fibres have the smallest diameter, are unmyelinated, have the slowest conduction velocities, and are grouped into Remak bundles (Harty and Monk, 2017). C-fibres are involved in processing innocuous and noxious mechanical, heat, and cold stimulation and can be polymodal (Cavanaugh *et al.*, 2009; McGlone and Reilly, 2010). They innervate the dorsal horn predominantly in the superficial laminae (Todd, 2010; Abraira *et al.*, 2017).

The differing conduction velocities of the PAF subgroups results in their input arriving at the dorsal horn at different times following electrical stimulation; the greater the distance from the stimulation site to the recording site then the greater the separation. As such, the dorsal horn response can be divided into distinct latency periods which correlate with the specific afferent subtypes. In adult rats following hindpaw stimulation these are traditionally considered to be 3-20ms, 20-90ms, and 90-300ms for Aβ-, Aδ-, and C-fibres respectively (Urch and Dickenson, 2003). However, previous work from our group has suggested alternative latencies of 3-11ms, 11-90, and 90-300ms for Aβ-, Aδ-, and C-fibres respectively (Greenspon *et al.,* 2019).

4.1.2 Age-Related Changes of Primary Afferent Fibres

PAFs are present in the hindpaw at the dermal/epidermal border, as in adults, as well as terminating in the dorsal horn by E20 (Mirnics and Koerber, 1995; Jackman and Fitzgerald, 2000). A-fibres undergo considerable postnatal development, myelination begins at birth and takes at least three postnatal weeks to reach adult levels, increases in diameter and changes in activation thresholds also occur over the first postnatal weeks with lower intensity stimuli activating A-fibres with increased age (Vejsada *et al.*, 1985; Lizarraga, Chambers and Johnson, 2007). As C-fibres are not myelinated there is not a significant change in nerve diameter in early life and they are mature in the first postnatal days (Lizarraga, Chambers and Johnson, 2007). However, other studies have suggested that despite the lack of anatomical change during early life C-fibres are not functionally mature until the second postnatal week (Fitzgerald and Gibson, 1984; Urch and Dickenson, 2003).

As fibre myelination and diameter affect the conduction velocity of the PAFs several studies have investigated changes in conduction velocity during postnatal development in rats. These have shown very little change in C-fibres, with any such changes occurring in the first postnatal week, but significant increases in A-fibre conduction velocities; these studies have, however, mostly focused on the first two postnatal weeks (Fitzgerald, 1985, 1987; Fulton, 1987; Lizarraga, Chambers and Johnson, 2007). Studies that have looked at changes in conduction velocities in later postnatal development have

used nerves other than the sciatic nerve but suggest the increase in conduction velocity continues until adulthood (Sato, Sato and Suzuki, 1985; Vejsada *et al.*, 1985).

Aged animals can have reduced myelination of A-fibres and an associated decrease in conduction velocity, as well as a decline in the number of large myelinated fibres following 15months of age (Verdú *et al.*, 2000). C-fibres have also been shown to decrease in number in aged mice (27-33months) with studies also showing altered C-fibre function with age in humans (Namer, 2010) and in rodents following inflammation (18months+) (Weyer *et al.*, 2016).

4.2 Hypotheses and Aims

4.2.1 Hypotheses

As previous studies have shown changes in C-fibre conduction velocity are small and occur within the first postnatal week we expect to see no changes in the conduction velocity of C-fibres in our study as we are investigating changes following P9. However as these fibres may not be functionally mature yet in the youngest group (P9-11) we expect to see much lower levels of activity compared to at P14 and above. Similarly as aged animals can have altered C-fibre responses we expect to see an altered response pattern in the aged animals too. In regards to the A-fibres we would expect to see an increase in the conduction velocity in the first two postnatal weeks as this has been well documented, we would however, expect this to continue until adulthood.

We expect to see regional difference following higher intensity stimulation, with the superficial and deep regions showing an increased response as they are the main innervation sites of nociceptive Aδ and C-fibres. Additionally, we hypothesise that the A-fibre response following low intensity stimulation will show regional changes with age as the superficial Aβ-fibres retract from the superficial region over the postnatal period.

4.2.2 Aims

- To calculate the appropriate latency periods of A- and C-fibre inputs in postnatal rats
- To analyse A- and C-fibre responses throughout the dorsal horn at different ages throughout the life course
- To compare A- and C-fibre responses between different ages throughout the life course

4.3 Methods

4.3.1 MEA Electrophysiology

4.3.1.1 Animals

Male and female Sprague Dawley rats at postnatal day (P)9-11, P14-16, P21-23, P28-30, P42-49 (considered as young adults), and male rats at 18months old (considered as aged adults) were used for data presented in this chapter. Details of animal origin and housing can be found in Section 2.1.1. Group sizes, sex, weights, and specific groupings are detailed in Section 4.3.1.4.

4.3.1.2 Surgery and Electrode Placement

Rats were anaesthetised with isoflurane (Section 2.1.2) and placed on to a stereotaxic frame (Section 2.1.3). A laminectomy and meningostomy took place to expose the L4 and L5 spinal cord and the dura matter and arachnoid membranes carefully removed (Section 2.1.4). The electrode array was then placed as described in Section 2.1.5.

4.3.1.3 Hindpaw Stimulation

The data presented in this chapter involves the application of electrical stimuli to the hindpaw using two subdermal needle electrodes as described in Section 2.1.6. Stimuli of 0.01, 0.1, 0.5, 1, 1.75, 2.5, and 5mA were given for a 2ms duration at 0.016Hz (1 per minute). Stimuli were delivered 5 times per intensity group with an inter-group duration of 10 minutes. The electrical protocol used was based on previous work by the group (Greenspon et al., 2019).

4.3.1.4 Specific Study Design

Electrophysiology data presented in this chapter was obtained from two cohorts, one involving all P9 to young adult rats and a second involving the aged adults approximately a year later. All animals received the same stimulation protocol.

Age	n	M/F	Weight (g) (Mean±SD)
P9-11	4	2/2	21 ± 3
P14-16	6	3/3	36 ± 5
P21-23	5	3/2	65 ± 10
P28-30	6	3/3	114 ± 11
Young Adult	5	4/1	208 ± 25
Aged Adult	5	5/0	535 ± 26

Table 4.1. Details of rats included in electrophysiological analysis in this chapter. The number of animals combined in each age group (n), the split of males and females (m/f), and the average weight are included. Details of excluded data are stated below and not included in the table.

Data is not included for one P10 rat (female) as incomplete recordings were obtained during the experiment. Subsequent processing also found noise in recordings from one P11 rat (male) and one P21 rat (female) which were removed from the analysis, and drift occurred in one young adult rat (female) resulting in that data also not being included.

4.3.1.5 Data Analysis

Details of data collection and initial sorting are described in 2.1.7. Data analysis and statistical analysis were performed using Matlab 2017b and GraphPad Prism 7.05.

Data collected from a pilot study, specifically the mean evoked spike activity across the whole array, and standard deviations, during the A fibre latency period following 5mA stimulation from the P9-11 and young adult age groups was used to calculate the recommended group size of 6 to give a statistical power of 80% or higher to detect an effect size of Cohen's d = 1.7 for differences between age groups with a significance level of $p<0.05$ using a two-tailed test. However, this study was not designed to be sufficiently powered to compare differences between sexes. To sort the electrical stimuli looked at in this chapter the 'Etho_MSP_Pipeline' script was used with the bin size set as 1ms, pre-stimulus time set as 0.1 second, the duration as 0seconds, and the post stimulation as 1second. The data was baseline corrected and sorted into fibre latency periods, regions, and stimulation groups for analysis. The only exception to this was when analysing the initial A fibre onset, in this case the bin size was set to 0.1ms. Data was combined between animals using the 'Elec_Combine' script creating a 4D matrix.

Statistical comparisons were made using the mean response of five stimulus applications per stimuli group from each animal. The mean response and SEM was then found for all animals within an age group. Comparing within ages one-way RM ANOVAS were used to determine stimulus intensity changes followed by Tukey's post hoc tests to reveal significant changes between groups. Comparing between age groups two-way ANOVAs were used followed by Tukey's post hoc tests to determine changes between all age groups.

4.3.2 Conduction Velocity Analysis

4.3.2.1 Animals

Male and female Sprague Dawley rats at postnatal day (P)9-11, P15-17, P23-24, P31-32, and P49-51 (considered as young adults) were used for peripheral nerve recordings Details of animal origin and housing can be found in Section 2.1.1. Group sizes, sex, and weights are detailed in Section 4.3.2.4.

4.3.2.2 Peripheral Nerve Recordings

Rats underwent cervical dislocation followed by decapitation. Both sciatic nerves were dissected out and placed in separate superfusion chambers for recordings to occur (Section 2.3). Simultaneous recordings of A- and C-fibre compound action potentials were collecting following electrical stimulation at 1Hz and a voltage selected following a stimulus response protocol. These recordings were conducted in collaboration with another group at the University of Nottingham (PhD student Laura Rich), who also performed the subsequent conduction velocity analysis.

4.3.2.3 Data Analysis

Data was analysed using Clampfit 9.2 (Section 2.3). The latency to peak was calculated and divided by the nerve length to calculate A-fibre and C-fibre conduction velocities for each nerve. Following terminal cardiac perfusion (Sections 2.2.1.2) of animals used in the electrophysiology recordings the skin over the hind leg was removed and the distance between the hindpaw stimulation site and the spinal recording site were measured following the natural path of the nerves.

4.3.2.4 Specific Study Design

A separate set of animals were used to obtain conduction velocity data; in this study both (left and right) sciatic nerves were used from each animal and considered as separate experimental units.

Age	N of animals	M/F	Weight (g) (Mean±SD)
P9-11	3	1/2	25 ± 2
P15-17	4	1/3	45 ± 3
P23-24	3	2/1	84 ± 6
P31-32	3	0/3	95 ± 2
P49-51	3	0/3	170 ± 10

Table 4.2. Details of rats included in conduction velocity analysis. The number of animals combined in each age group (n), the split of males and females (m/f), and the average weight are included. Recordings were obtained from both sciatic nerves from each animal.

C-fibre recordings were not able to be calculated in 1 P15-17 sciatic nerve and 2 P49-51 (young adult) nerves.

4.3.5 Statistical Analysis

Statistical analysis was performed using GraphPad Prism 7.05. One-way ANOVAs followed by post hoc Tukey's tests were used to test for age differences in the conduction velocity of each primary afferent fibre type.

4.4 Results

4.4.1 Postnatal Changes in Primary Afferent Fibre Conduction Velocity and Latency Periods

PAF conduction velocity (Figure 4.1) was measured in collaboration with another group as describe in Section 2.3. These conduction velocities were then used to determine the expected fibre latency periods for A- and C-fibres at each postnatal age used in subsequent MEA studies. Aged rat conduction velocity could not be measured due to the unavailability of rats at this time. However, as previous studies have found no change in C-fibre conduction velocity and very small decreases in A-fibre conduction velocity in aged animals (Sato, Sato and Suzuki, 1985; Verdú *et al.*, 2000) young adult conduction velocity values were used to estimate the fibre latencies in the aged adult group (Table 4.3).

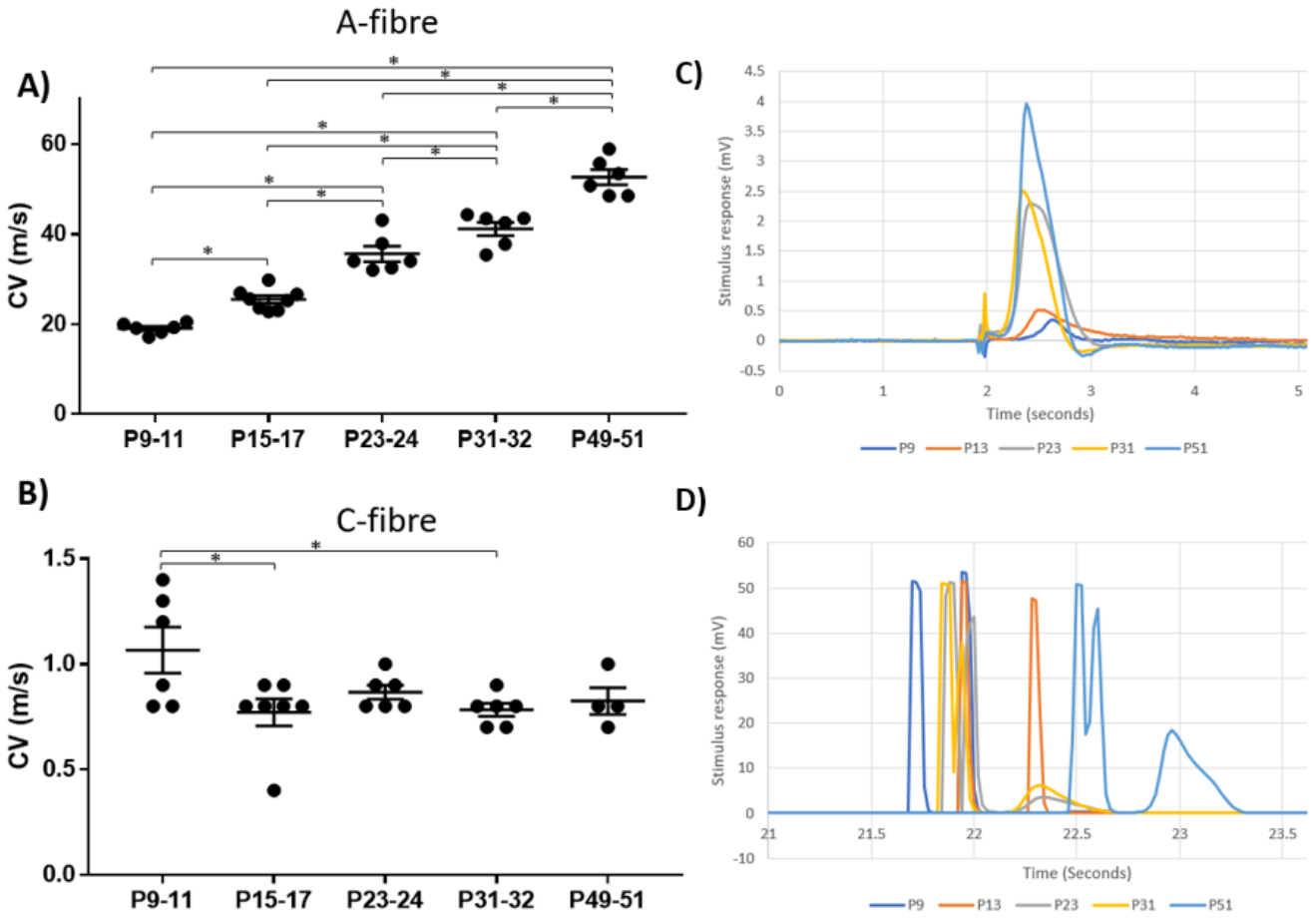


Figure 4.1. Conduction velocity of A-fibres but not C-fibres increases with postnatal age. Conduction velocity (CV) is shown as m/s values A) shows A-fibre CVs and B) shows C-fibre CVs at the postnatal days displayed on the x-axis. Each point represents recordings from 1 sciatic nerve and bars represent the mean value +/- SEM for each age group. C) and D) show example traces of the recordings used to calculate the conduction velocities from a single nerve at each age group; C) shows A-fibre example traces following 40V stimulation and D) shows C-fibre example traces following 120V stimulation. Asterisks represent statistically significant responses between ages following a one-way ANOVA and post-hoc Tukey's test.

A- and C-fibre waveforms were detected at all ages used. The A-fibre conduction velocities increased with postnatal age between P9 and young adults ($F(4, 27)=101.2$, $P<0.0001$, one-way ANOVA), supporting previously reported findings (Lizarraga, Chambers and Johnson, 2007). A-fibre conduction velocities increased from 19m/s at P9-11 to 53m/s in young adults in a linear manner (Figure 4.1). A post hoc Tukey's test showed significant increases in conduction velocity occurred between every age group tested (P9-11 vs. P15-17 $P<0.01$, P23-24 vs. P31-32 $P<0.05$, all other comparisons $P<0.0001$).

C-fibre conduction velocity also showed age dependent differences ($F(4,24)=3.312$, $P<0.05$) however from P15-17 the values calculated are very consistent. In line with this a post hoc Tukey's test showed significant differences only between the P9-11 group and both the P15-17 and P31-32 age groups ($P<0.05$). Conduction velocity values were more varied in the youngest P9-11 age group with three values again being similar to the P15-17 and above velocities. It appears that the C-fibre conduction velocity is mature by P15 at approximately 0.8m/s.

Age	Approximate distance: hindpaw to recording site (cm)	Latency onset calculated from CV (ms)		Latency range used (ms)	
		A-fibre	C-fibre	A-fibre	C-fibre
P9-11	5.5	2.9	51	3-50	50-250
P14-16	6.6	2.6	77	3-50	75-275
P21-23	8.8	2.4	97	3-50	95-295
P28-30	9	2.0	106	3-50	105-305
YA	11	2.1	133	3-50	120-320
AA	12	2.3	145	3-50	130-330

Table 4.3. PAF latency periods throughout the life course. The approximate distance between the hindpaw where stimulus application occurred to the spinal recordings site increases with age. Ages used here are matched to the ages used for electrophysiological recordings. The calculated onset for the A- and C-fibres latencies and the latency periods used for subsequent analysis in this chapter are stated in ms.

The distance between the stimulus application site of the hindpaw and the spinal recording site was measured and used to calculate the latency onsets. Whist classically the A-fibre latency period is divided into Aβ- and Aδ-fibre latencies the conduction velocity measurements we collected were unable to clearly distinguish between these, however previous reports have suggest grouping Aβ- and Aδ-fibre recordings in neonates anyway (Urch and Dickenson, 2003). This resulted in a very consistent

calculated A-fibre onset time varying by a maximum of 1ms between all of the ages tested but increasingly delayed C-fibre latency periods with increased age (Table 4.3). The C-fibre latency period onset occurred later with increasing postnatal age as the distance between the hindpaw and recording site increased. These latencies match previous findings which suggest single-unit electrophysiology recordings should use latency periods in pups (35-100g; approximately P14-28) of 0-50ms for A-fibre recordings and 50-250ms for C-fibre recordings (Urch and Dickenson, 2003). The calculated A-fibre latencies suggest a very consistent onset of between 2-3ms for all ages however, the MEA recordings of A-fibre onset appear to vary slightly with the onset occurring quicker with increased postnatal age (Figure 4.2) however, as the onset variation is small compared to the total latency period used for the A-fibres 3-50ms was chosen for all ages. The calculated C-fibre latency period reflects the onset observed by the MEA recordings at all of the postnatal ages, the adult conduction velocities however appears to suggest a slightly later onset than observed using the MEA recordings.

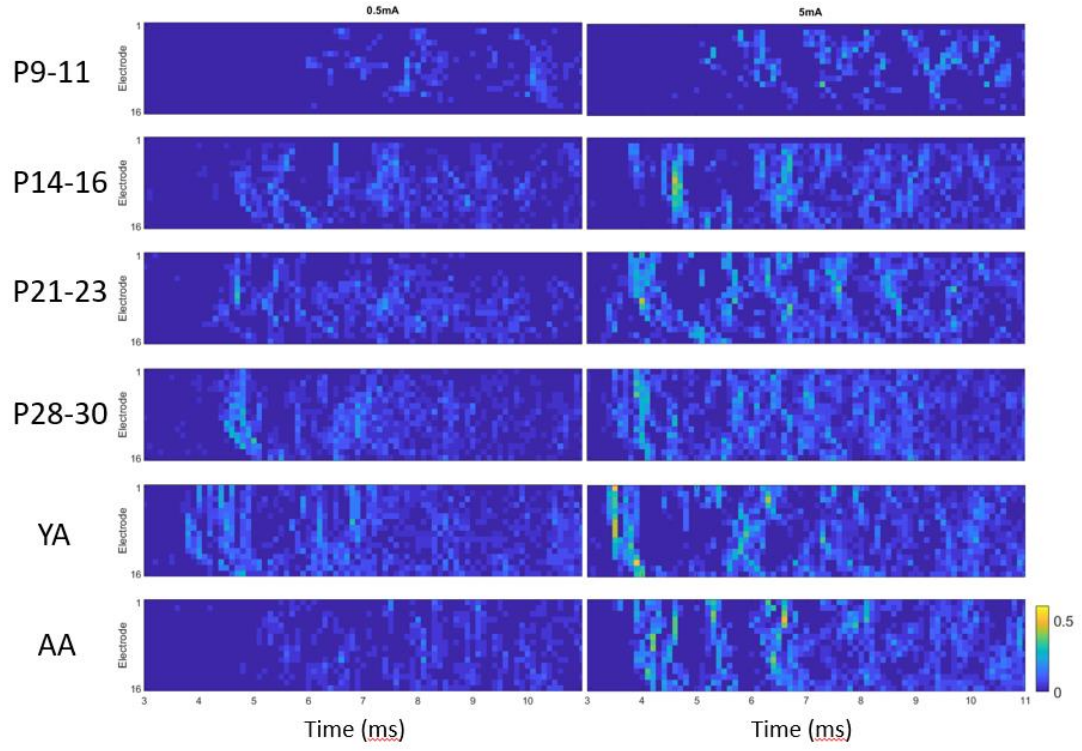


Figure 4.2. Onset of the A-fibre response in the dorsal horn throughout the life course following low and high intensity stimulation of the hindpaw. Heatmaps represent the spike activity at each electrode following electrical stimulation at 0.5mA and 5mA at P9-11, P14-16, P21-23, P28-30, Young Adults (YA), and Aged Adults (AA). Responses shown are averaged responses from all animals in the age group, P9-11 n=4, P14-16 n=6, P21-23 n=5, P28-30 n=6, YA n=5, AA n=5. Electrodes are in order; 1 is the most dorsal and 16 the most ventral, for P9-11 electrodes 1 and 16 are removed and at P14-16 electrode 1 is removed, to better represent the size of the dorsal horn at these ages. Data shown is 3-11ms post stimulus with a bin size of 0.1ms. Colours represent mean number of spikes respective to the colour bar shown on the right. YA = young adults, AA = aged adults.

The onset of the A-fibre period, which most likely represents the Aβ-fibres, occurs earlier following 5mA stimulation compared to 0.5mA stimulation of the hindpaw. This may represent the difference in activating the HTMR population of Aβ-fibres, additionally the 5mA response appears to have increased activation in the superficial region compared to the 0.5mA response. However as accurate Aβ- vs Aδ-fibre conduction velocities were not obtained specific Aβ responses were not analysed.

4.4.2 Dorsal Horn Responses to PAF Stimulation throughout the Life Course

In the previous chapter we demonstrated that modality, intensity, and regional differences could be detected in the dorsal horn following peripheral stimulation. We were unable however to directly link these with PAF input due to the nature of the stimulation used. The A-fibre and C-fibre responses were analysed at each age group before being compared between the ages. Low intensity stimulation is expected to recruit A-fibres and higher intensity stimuli to evoke C-fibres.

Postnatal Days 9-11

At postnatal days 9-11, as in the previous chapter, the most dorsal and most ventral electrodes were removed from the analysis to better reflect the size of the dorsal horn at this age. The heatmaps (Figure 4.3A&B) represent the mean response following the increasing stimulus intensities tested in the A-fibre latency period and C-fibre latency period. The heatmaps are all on the same colour bar scale however the A-fibre response has been grouped into bin sizes of 1ms and the C-fibre into bins of 5ms. The 0.1mA stimulus produced almost no response in the P9-11 dorsal horn but the 0.5mA and above did, suggesting the thresholds at this age lie between 0.1 and 0.5mA (at 2ms and 0.016Hz). The summed activity over the fibre latencies is shown in the bar charts below (Figure 4.3 C-E). Whilst there is a trend of increased spike number as the intensity increases in both the A- and C-fibre latency periods, this is only statistically significant for the A-fibre across the whole array ($F(1.809, 5.426) = 10.05$, $P<0.05$, one-way ANOVA). Using a two-way RM ANOVA to compare the stimulus intensity and regional response during the A fibre latency period showed significant differences in dorsal horn activity were identified between stimulus intensities ($F(5, 15) = 9.165$, $P<0.001$), but not between regions ($F(2, 6) = 1.319$, $P=0.33$), similarly no interaction was detected ($F(10, 30) = 1.859$, $P=0.09$). Additionally, using a Tukey's post hoc test in the A-fibre latency period there were significant differences in evoked spike activity between 0.1mA and 1.75, 2.5, and 5mA ($P<0.01$, $P<0.01$, and $P<0.001$ respectively) as well as between 0.5mA and 1mA compared to 5mA ($P<0.05$). There appears to be a larger response in the intermediate region during the A-fibre latency period which is consistent with the

majority of A-fibre inputs however, no significant differences were found between the dorsal horn regions in either latency period. A significant difference was also found using a two-way RM ANOVA between the dorsal horn response to different stimulus intensities during the C-fibre latency period at P9-11 ($F(5, 15) = 4.549$, $P<0.05$) but a difference between the regions ($F(2, 6) <1$), or interaction between factors ($F(10, 30) <1$) was not found. A Tukey's post hoc test in the C-fibre latency period showed there were significant differences in evoked spike activity between 0.1mA and 5mA ($P<0.01$). The responses are much lower than during the A-fibre latency period, possibly representing the immaturity of the C-fibres at this age.

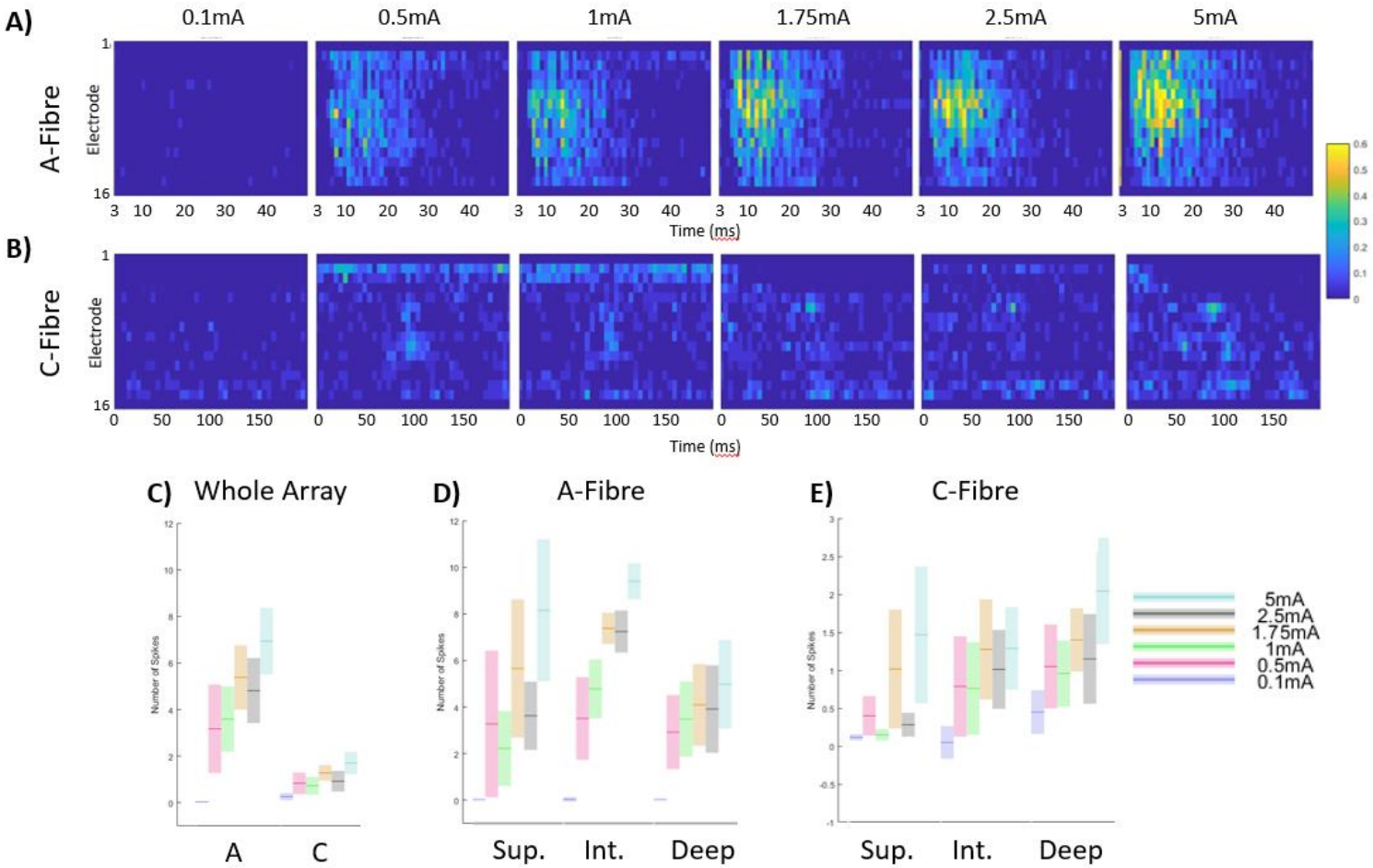


Figure 4.3. Primary afferent fibre responses in the P9-11 dorsal horn. A & B) Heatmaps show spike activity at each electrode following electrical stimulation at 0.1, 0.5, 1, 1.75, 2.5, and 5mA. Responses shown are averaged responses from all animals in the age group, n=4. A) Shows the A-fibre latency period (3-50ms post stimulation) with bin sizes of 1ms, B) shows the 200ms C-fibre latency period (50-250ms post stimulation) with bin sizes of 5ms. Electrodes are in order; 1 is the most dorsal and 16 the most ventral, electrodes 1 and 16 are removed to better represent the size of the P9-11 dorsal horn. Colours represent mean number of spikes respective to the colour bar shown on the right. C, D, & E) Summed spike activity across A- and C-fibre latency periods in the whole array(C) or superficial, intermediate, and deep regions of the dorsal horn (D & E) (mean activity across the electrodes) is shown in the bar charts, lines represent mean and shaded area represents SEM. Two-way RM ANOVAs found stimulus dependent changes but no regional changes or interaction during both the A-Fibre and C-fibre response periods.

Postnatal Days 14-16

At P14-16 there is again no response in either latency period to 0.1mA stimulation but a clear response in both following 0.5mA (Figure 4.4). The most dorsal electrode has been removed from analysis to better reflect the size of the dorsal horn in this age group. At this age the C-fibre response is clear on the heatmaps but very short, with the response period lasting approximately 50ms and abruptly stopping (Figure 4.4B). Both the A- and C-fibre latencies appear to have an increased response with increasing stimulus intensity and this is significant throughout the dorsal horn in the A-fibre latency period (Whole array: $F(1.478, 7.391) = 28.43$, $P<0.001$, 1-way RM ANOVA). A two-way RM ANOVA comparing the stimulus intensity and regional response during the A fibre latency period showed stimulus dependent changes ($F(5, 25) = 20.16$, $P<0.0001$) but no regional changes ($F(2, 10) <1$) or interaction ($F(10, 50) <1$). A Tukey's post hoc test also identified significant difference between stimulus intensity, however, as so many were detected these have been displayed in Table 4.4.

The increase in intensity produced significantly different responses in the C-fibre period across the whole array ($F(1.437, 7.183) = 5.097$, $P<0.05$, 1-way RM ANOVA). Significant differences were again found between responses to the different stimulus intensities ($F(5, 25) = 3.423$, $P<0.05$) but as with the A fibre latency period no regional differences ($F(2, 10) <1$), or interaction ($F(10, 50) <1$), were found using a two-way RM ANOVA. A Tukey's post hoc test also identified significant difference between evoked stimulus intensity responses between 0.1mA and 1.75mA and 5mA ($P<0.05$). As seen in the C-fibre heatmaps and bar charts (Figure 4.4B&E) there is an increased activity with increased stimulus intensity, which appears to be particularly clear in the deep dorsal horn. Overall the clear presence of the C-fibre response would suggest that by P14-16 the C-fibre response has matured compared to the P9-11, which supports the previous findings suggesting maturation of C-fibres occurs at P14 (Fitzgerald and Gibson, 1984). Similar to the P9-11 age group, no regional differences were found in either latency period at P14-16.

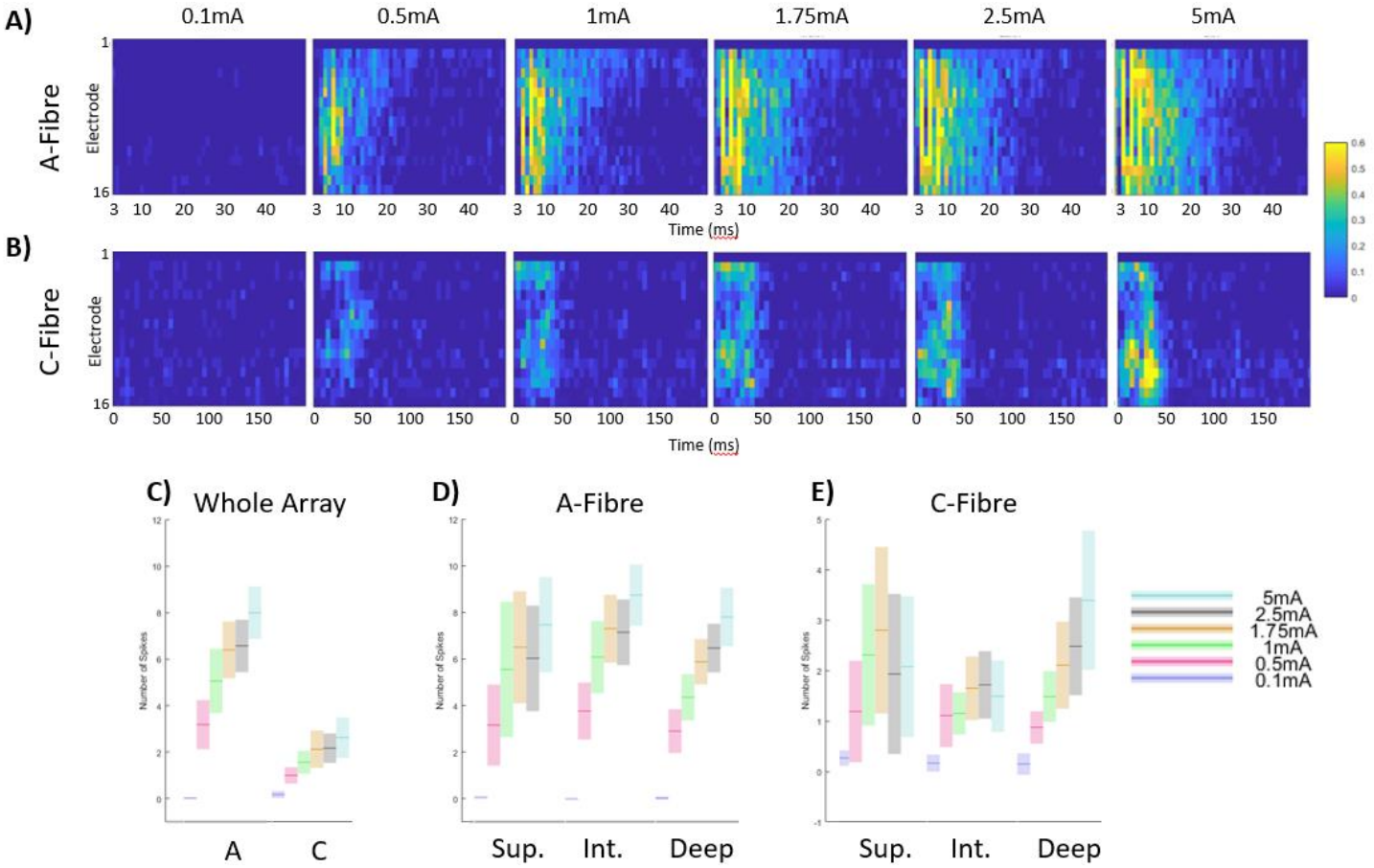


Figure 4.4. Primary afferent fibre responses in the P14-16 dorsal horn. A & B) Heatmaps show spike activity at each electrode following electrical stimulation at 0.1, 0.5, 1, 1.75, 2.5, and 5mA. Responses shown are averaged responses from all animals in the age group, n=6. A) Shows the A-fibre latency period (3-50ms post stimulation) with bin sizes of 1ms, B) shows the 200ms C-fibre latency period (75-275ms post stimulation) with bin sizes of 5ms. Electrodes are in order; 1 is the most dorsal and 16 the most ventral, electrode 1 is removed to better represent the size of the P14-16 dorsal horn. Colours represent mean number of spikes respective to the colour bar shown on the right. C, D, & E) Summed spike activity across A- and C-fibre latency periods in the whole array(C) or superficial, intermediate, and deep regions of the dorsal horn (D & E) (mean activity across the electrodes) is shown in the bar charts, lines represent mean and shaded area represents SEM. Two-way RM ANOVAs found stimulus dependent changes but no regional changes or interaction during both the A-Fibre and C-fibre response periods, significant differences following a Tukey's test are shown in table 4.4.

Between stimuli intensities (mA)	P value
0.1 vs. 0.5	<0.05
0.1 vs. 1	<0.0001
0.1 vs. 1.75	<0.0001
0.1 vs. 2.5	<0.0001
0.1 vs. 5	<0.0001
0.5 vs. 1.75	<0.05
0.5 vs. 2.5	<0.05
0.5 vs. 5	<0.001

Table 4.4 Tukey's post hoc test significance between stimulus intensities in the A-fibre latency period at P14-16. Following a two-way RM ANOVA corresponding data is in Figure 4.4.

Postnatal Days 21-23

Interestingly at P21-23 the A-fibre response appears again not following 0.1mA but following 0.5mA stimulation (Figure 4.5A) however the C-fibre response is not visible in the heatmaps until following 1.75mA stimulation (Figure 4.5B). The A-fibre heatmaps also now appear to display two distinct periods of activity particularly following the higher intensity stimulations, the first occurring between 3 and 10ms and the second between 10 and 20ms. This may represent the Aβ- and Aδ- fibre subgroups as these fit the expected adult latency periods (Greenspon *et al.*, 2019) or nociceptive and low threshold A-fibres (Djouhri and Lawson, 2004). Summed activity during both the A- and C-fibre latencies shows a trend of increasing activity with increasing intensity in all regions with the A-fibre having significant increases when summed across the whole array (Whole array: $F(1.134, 4.538) = 42.24$, $P<0.001$ 1-way RM ANOVA). A two-way RM ANOVA comparing the stimulus intensity and regional response during the A fibre latency period showed stimulus dependent changes ($F(5, 20) = 34.03$, $P<0.0001$) but no regional changes ($F(2, 8) <1$) or interaction ($F(10, 40) <1$). As with the previous age group the Tukey's post hoc results for the A-fibre latency period have been displayed in table 4.5.

The C-fibre latency period showed no statistically significant differences using a two-way RM ANOVA between the stimulus intensities ($F(5, 20) = 2.69$, $P=0.05$) or regions ($F(2, 8)<1$), there was also no interaction ($F(10, 40) = 1.125$, $P=0.37$). As such no differences between regions were found in either latency period.

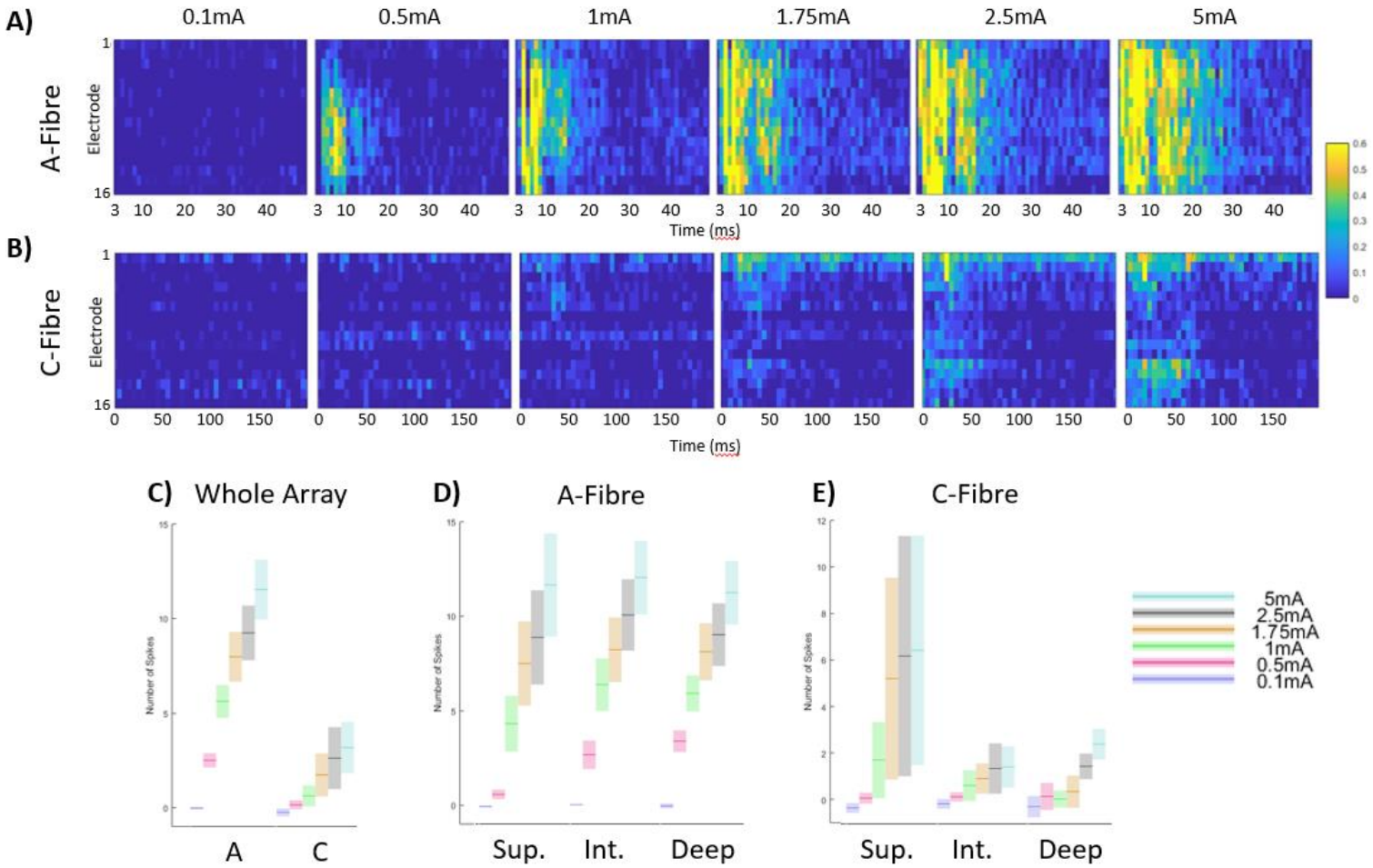


Figure 4.5. Primary afferent fibre responses in the P21-23 dorsal horn. A & B) Heatmaps show spike activity at each electrode following electrical stimulation at 0.1, 0.5, 1, 1.75, 2.5, and 5mA. Responses shown are averaged responses from all animals in the age group, n=5. A) Shows the A-fibre latency period (3-50ms post stimulation) with bin sizes of 1ms, B) shows the 200ms C-fibre latency period (95-295ms post stimulation) with bin sizes of 5ms. Electrodes are in order; 1 is the most dorsal and 16 the most ventral. Colours represent mean number of spikes respective to the colour bar shown on the right. C, D, & E Summed spike activity across A- and C-fibre latency periods in the whole array(C) or superficial, intermediate, and deep regions of the dorsal horn (D & E) (mean activity across the electrodes) is shown in the bar charts, lines represent mean and shaded area represents SEM. Two-way RM ANOVAs found stimulus dependent changes but no regional changes or interaction during the A-Fibre response period and no statistically significant differences during the C-fibre response period, significant differences following a Tukey's test are shown in table 4.5.

Between stimuli intensities (mA)	P value
0.1 vs. 1	<0.001
0.1 vs. 1.75	<0.0001
0.1 vs. 2.5	<0.0001
0.1 vs. 5	<0.0001
0.5 vs. 1.75	<0.001
0.5 vs. 2.5	<0.0001
0.5 vs. 5	<0.0001
1 vs. 2.5	<0.05
1 vs. 5	<0.001
1.75 vs 5	<0.05

Table 4.5. Tukey's post hoc test significance between stimulus intensities in the A-fibre latency period at P21-23. Following a two-way RM ANOVA, corresponding data is in Figure 4.5.

Postnatal Days 28-30

At P28-30 there is a clear A-fibre response following stimulation at 0.5mA and above (Figure 4.6). There is also a visible C-fibre response on the heatmaps at 1.75mA stimulation and higher, although the summed activity suggests a trend of increased activity in all regions following the 0.1mA stimulation. When summing activity across the whole array during the A-fibre latency period, but not the C-fibre latency period, there is a significant increase in the evoked response with increasing stimulus intensity ($F(1.269, 6.344) = 72.24$, $P<0.0001$, one-way RM ANOVA). A two-way RM ANOVA comparing the stimulus intensity and regional response during the A fibre latency period showed stimulus dependent changes ($F(5, 25) = 68.93$, $P<0.0001$) as well as regional changes ($F(2, 10) = 9.363$, $P<0.01$) and an interaction between stimulus intensity and region ($F(10, 50) = 4.673$, $P=0.0001$). A Tukey's post hoc analysis found multiple significant differences between stimulus intensity groups as shown in table 4.6. The A-fibre response was also significantly lower in the superficial region compared to the intermediate ($P<0.01$) and deep ($P<0.05$) in the P28-30 age group.

The activity during the C-fibre response latency shows significantly increased spike activity with increasing stimulus intensity ($F(5, 25) = 3.503$, $P<0.05$, two-way RM ANOVA) but no regional differences ($F(2, 10) = 1.513$, $P=0.27$) or interaction ($F(10, 50) <1$) were found. Additionally a post hoc Tukey's test only showed significant increases specifically between the 0.1mA and 5mA responses ($P<0.01$).

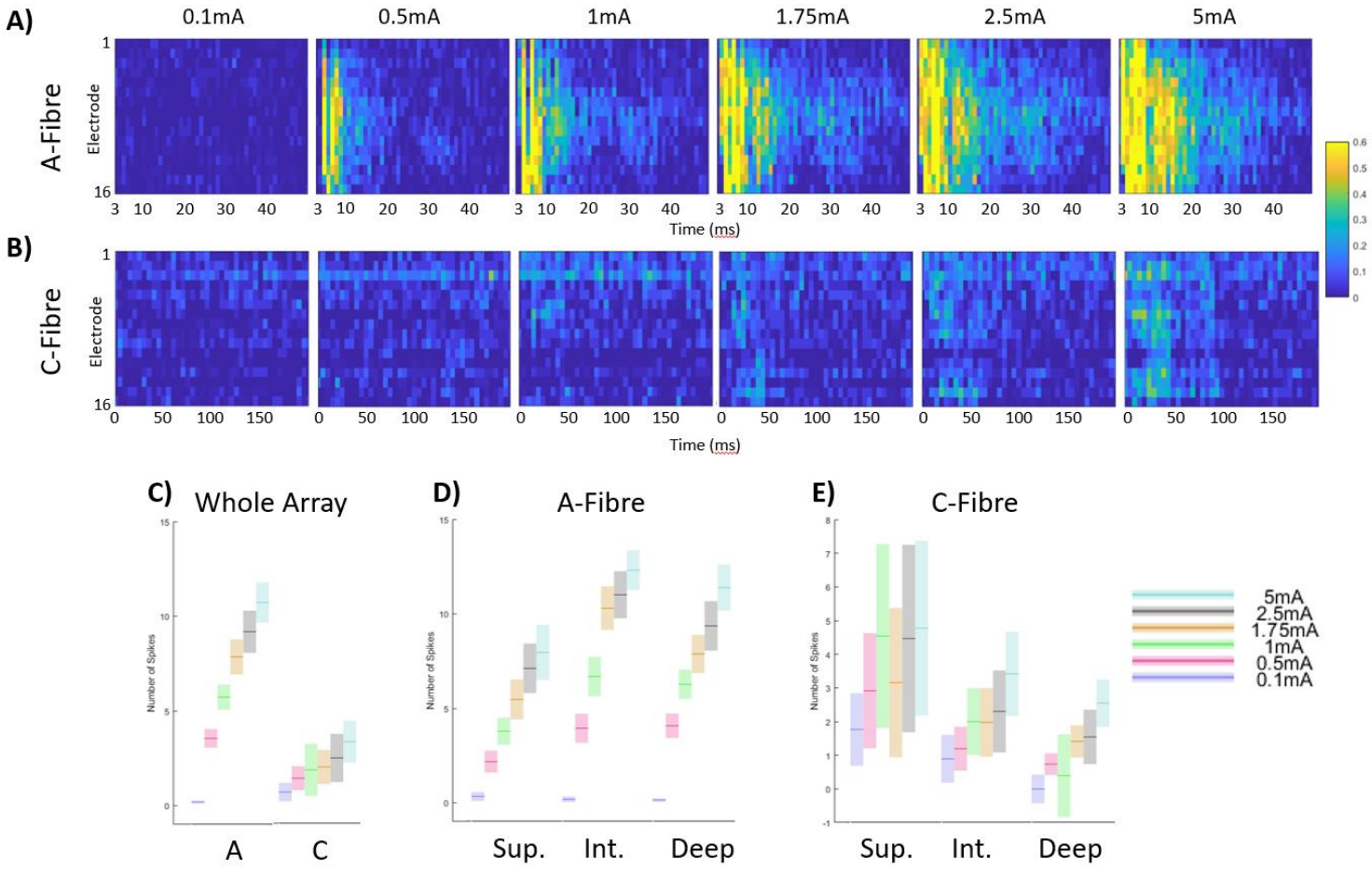


Figure 4.6. Primary afferent fibre responses in the P28-30 dorsal horn. A & B) Heatmaps show spike activity at each electrode following electrical stimulation at 0.1, 0.5, 1, 1.75, 2.5, and 5mA. Responses shown are averaged responses from all animals in the age group, n=6. A) Shows the A-fibre latency period (3-50ms post stimulation) with bin sizes of 1ms, B) shows the 200ms C-fibre latency period (105-305ms post stimulation) with bin sizes of 5ms. Electrodes are in order; 1 is the most dorsal and 16 the most ventral. Colours represent mean number of spikes respective to the colour bar shown on the right. C, D, & E) Summed spike activity across A- and C-fibre latency periods in the whole array(C) or superficial, intermediate, and deep regions of the dorsal horn (D & E) (mean activity across the electrodes) is shown in the bar charts, lines represent mean and shaded area represents SEM. Two-way RM ANOVAs found stimulus dependent changes, regional change and an interaction during the A-fibre response period but only stimulus dependent differences during the C-fibre response period, significant differences following a Tukey's test are shown in table 4.6.

Between stimuli intensities (mA)	P value
0.1 vs. 0.5	<0.001
0.1 vs. 1	<0.0001
0.1 vs. 1.75	<0.0001
0.1 vs. 2.5	<0.0001
0.1 vs. 5	<0.0001
0.5 vs 1	<0.05
0.5 vs. 1.75	<0.0001
0.5 vs. 2.5	<0.0001
0.5 vs. 5	<0.0001
1 vs 1.75	<0.05
1 vs. 2.5	<0.001
1 vs. 5	<0.0001
1.75 vs 5	<0.01

Table 4.6. Tukey's post hoc test significance between stimulus intensities by region in the A-fibre latency period at P28-30. Following a two-way RM ANOVA, corresponding data is in Figure 4.6.

Young Adults

In the young adult group there is again a significant increase in the A-fibre response latency period with increasing stimulus intensity when summed across the whole dorsal horn ($F(1.301, 5.202) = 30.8$, $P<0.01$, One-way RM ANOVA, Figure 4.7). This shows a similar initial response during the first 10ms which appears to be in the superficial and deep dorsal horn. As well as a second burst of activity at approximately 10-20ms in the intermediate and deep regions which shows the majority of the A-fibre related increase to stimulus intensity (Figure 4.7A). A two-way RM ANOVA comparing the stimulus intensity and regional response during the A fibre latency period showed stimulus dependent changes ($F(5, 20) = 28.75$, $P<0.0001$) but no regional changes ($F(2, 8) = 3.035$, $P=0.10$) or interaction ($F(10, 40) = 1.839$, $P=0.08$) were detected. Tukey's post hoc differences are displayed in table 4.7.

The C-fibre latency period has ongoing activity throughout the intensities investigated, although the data presented here is baseline subtracted this may represent a build up over the stimulus protocol as each stimulus is given 5 times with 1 min in between (0.016Hz). A more distinct response is seen following the 1mA stimulation and above predominantly in the deeper dorsal horn but also in the superficial region (Figure 4.7B&E), this represents the expected innervation sites of C-fibres. Interestingly the 5mA stimulation results in two peaks of activity although there is also a relatively large amount of variation. A two-way ANOVA found no stimulus intensity ($F(5, 20) = 1.771$, $P=0.16$) or regional differences ($F(2, 8) = 1.815$, $P=0.22$), but did detect an interaction between stimulus intensity and region ($F(10, 40) = 2.211$, $P<0.05$). However, no differences were detected using a Tukey's post hoc analysis.

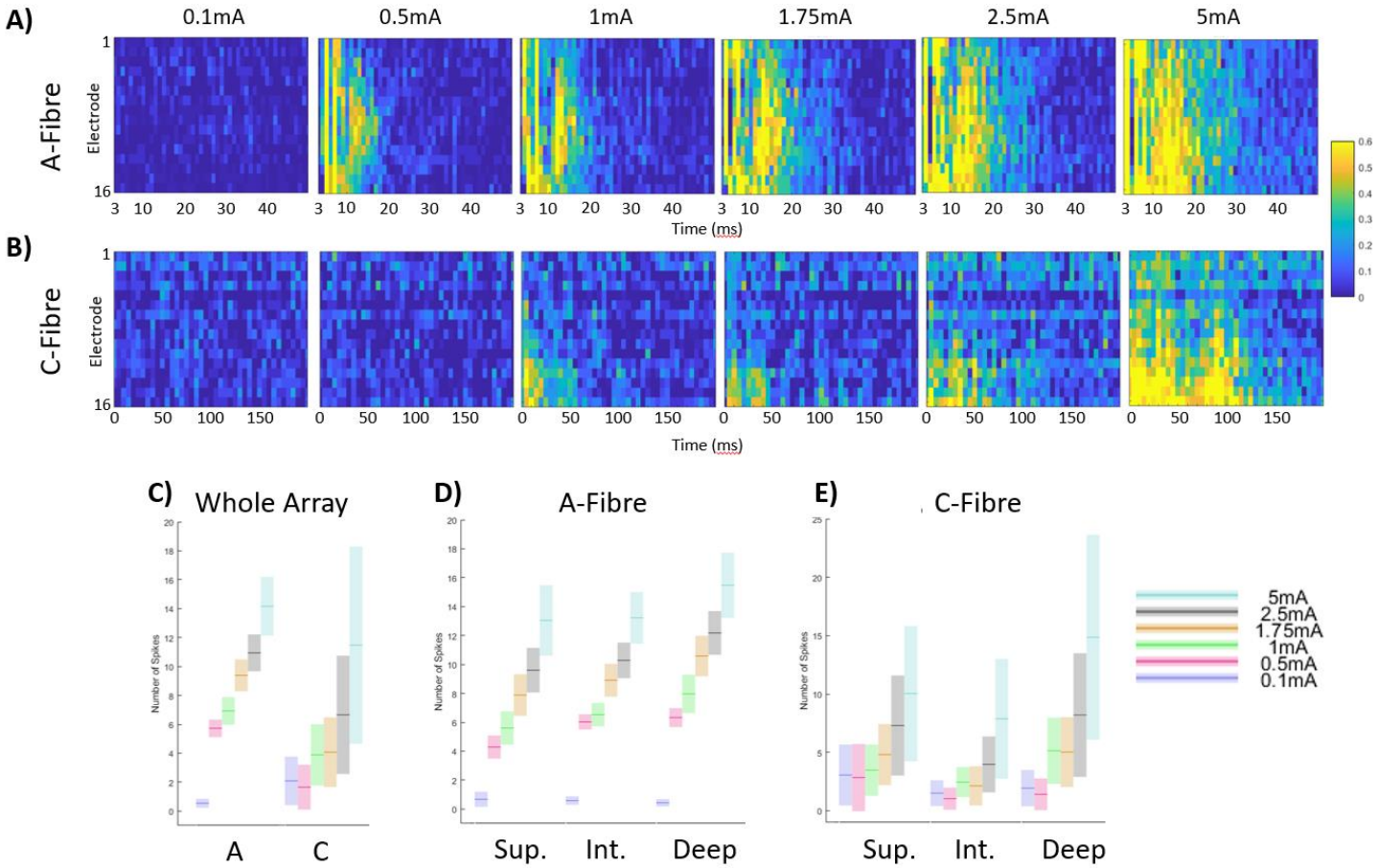


Figure 4.7. Primary afferent fibre responses in the young adult dorsal horn. A & B) Heatmaps show spike activity at each electrode following electrical stimulation at 0.1, 0.5, 1, 1.75, 2.5, and 5mA. Responses shown are averaged responses from all animals in the age group, n=5. A) Shows the A-fibre latency period (3-50ms post stimulation) with bin sizes of 1ms, B) shows the 200ms C-fibre latency period (120-320ms post stimulation) with bin sizes of 5ms. Electrodes are in order; 1 is the most dorsal and 16 the most ventral. Colours represent mean number of spikes respective to the colour bar shown on the right. C, D, & E) Summed spike activity across A- and C-fibre latency periods in the whole array(C) or superficial, intermediate, and deep regions of the dorsal horn (D & E) (mean activity across the electrodes) is shown in the bar charts, lines represent mean and shaded area represents SEM. Two-way RM ANOVAs found stimulus dependent changes but no regional changes or interaction during the A-Fibre response period, during the C-fibre response period no stimulus dependent or regional differences were identified but there was an interaction. Significant differences following a Tukey's test are shown in table 4.7.

Between stimuli intensities (mA)	P value
0.1 vs. 0.5	<0.01
0.1 vs. 1	<0.001
0.1 vs. 1.75	<0.0001
0.1 vs. 2.5	<0.0001
0.1 vs. 5	<0.0001
0.5 vs. 2.5	<0.01
0.5 vs. 5	<0.0001
1 vs. 2.5	<0.05
1 vs. 5	=0.0001
1.75 vs 5	<0.01

Table 4.7. Tukey's post hoc test significance between stimulus intensities by region in the A-fibre latency period in young adults. Following a two-way RM ANOVA, corresponding data is in Figure 4.7.

Aged Adults

Aged adults showed A-fibre responses at stimulation intensities of 0.5mA or above and response activity increased with increased stimulus intensity when summed across the whole array ($F(1.504, 6.017) = 28.32$, $P<0.01$, One-way RM ANOVA, Figure 4.8). Whilst the duration of the response increased with increased intensity there is a less clear divide into two response bursts than seen in the younger adults. A two-way RM ANOVA comparing the stimulus intensity and regional response during the A fibre latency period showed stimulus dependent changes ($F(5, 20) = 27.14$, $P<0.0001$) but no regional changes ($F(2, 8) <1$) or interaction ($F(10, 40) = 1.136$, $P=0.36$) were detected. Post hoc Tukey's tests showed significant differences in evoked dorsal horn activity (Table 4.8).

C-fibre responses are visible on the heatmaps following 1mA stimulation in the very deep dorsal horn and following 1.75mA and above in the deep and superficial dorsal horn. A two-way RM ANOVA showed significant differences in responses between stimulus intensities in the C-fibre latency period ($F(5, 20) = 2.92$, $P<0.05$), with a Tukey's post hoc test showing a difference between 0.1mA and 5mA evoked responses ($P<0.05$). However, no region dependent changes were found ($F(2, 8) <1$) or interaction ($F(10, 40) <1$). As with the postnatal age groups there appears to be more A-fibre activity than C-fibre activity across the whole array.

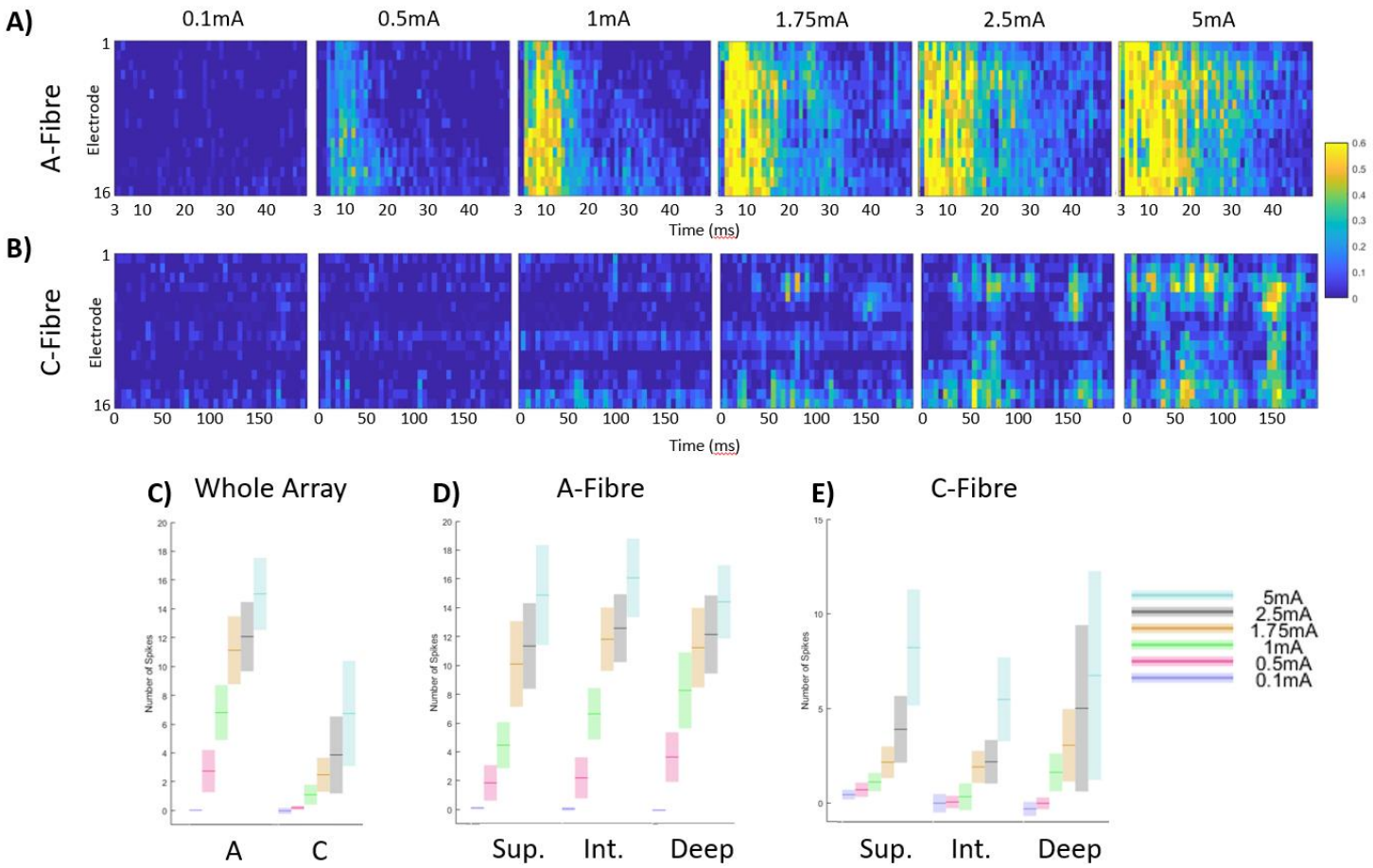


Figure 4.8. Primary afferent fibre responses in the aged adult dorsal horn. A & B) Heatmaps show spike activity at each electrode following electrical stimulation at 0.1, 0.5, 1, 1.75, 2.5, and 5mA. Responses shown are averaged responses from all animals in the age group, n=5. A) Shows the A-fibre latency period (3-50ms post stimulation) with bin sizes of 1ms, B) shows the 200ms C-fibre latency period (130-330ms post stimulation) with bin sizes of 5ms. Electrodes are in order; 1 is the most dorsal and 16 the most ventral. Colours represent mean number of spikes respective to the colour bar shown on the right. C, D, & E) Summed spike activity across A- and C-fibre latency periods in the whole array(C) or superficial, intermediate, and deep regions of the dorsal horn (D & E) (mean activity across the electrodes) is shown in the bar charts, lines represent mean and shaded area represents SEM. Two-way RM ANOVAs found stimulus dependent changes but no regional changes or interaction during both the A-fibre and C-fibre response periods, significant differences following a Tukey's test are shown in table 4.8.

Between stimuli intensities (mA)	P value
0.1 vs. 1	<0.01
0.1 vs. 1.75	<0.0001
0.1 vs. 2.5	<0.0001
0.1 vs. 5	<0.0001
0.5 vs. 1.75	<0.001
0.5 vs. 2.5	=0.0001
0.5 vs. 5	<0.0001
1 vs. 2.5	<0.05
1 vs. 5	<0.001

Table 4.8. Tukey's post hoc test significance between stimulus intensities in the A-fibre latency period in aged adults. Following a two-way RM ANOVA, corresponding data is in Figure 4.8.

4.4.3 A-fibre Responses Undergo Changes throughout the Life Course

At every age used in this study the 0.1mA stimulation did not explicitly evoke an A-fibre response but the next lowest stimulation of 0.5mA did. The intensity of this response however varied with age; the P9-11 group and 18month old rats both appeared to have a reduced response following 0.5mA compared to the other age groups, although this change was not significant (Figure 4.9). Additionally, lower stimulation intensities (Figure 4.9) produced similar responses in the heatmaps at P14-16, P21-23, and P28-30, but at the higher intensities (Figure 4.10) responses were similar from P21-23 where a double burst of activity occurred. Although this double burst activity occurs at all ages from P21-23 the young adult group showed regional differences in the heatmaps with higher responses specifically in the superficial and deep regions in the first 10ms compared to across the array as seen in the younger ages.

The duration of the evoked response appears to increase with age following higher intensity stimulations (Figure 4.10) particularly between P14-16 and P21-23. This may suggest an immaturity in the A-fibre evoked responses, particularly to higher intensity stimulations before P21. The age-related variations seen in the heatmaps are also demonstrated when comparing the sum of the activity within the A-fibre latency period (Figure 4.11 & 4.12). Dorsal horn activity across the whole array (Figure 4.11) showed stimulations dependent and age dependent changes, as well as an interaction between stimulus intensity and age, following a two-way RM ANOVA (Stimulation, $F(5, 130)=172.2$, $P<0.0001$; Age, $F(5,26)=2.715$, $P<0.05$; Interaction, $F(25, 130) = 4.02$, $P<0.0001$). Post hoc Tukey's tests showed no age-related differences following 0.1 or 0.5mA stimulations, but a trend of increased evoked A-fibre activity is seen following 1mA stimulations and higher, although statistically significant differences only occurred

at 1.75mA and above, predominantly between the P9-11 and P14-16 responses compared to the older ages (Figure 4.11).

Additionally, following 2.5mA and 5mA stimulations there appears to be a divide in the response between the neonatal (P9-11 and P14-16), juvenile (P21-23 and P28-30) and adult (YA and AA) groups, most likely reflecting the maturation of the second burst of A-fibre response. It is however interesting to note that the P9-11 and P14-16 groups show a very similar summed activity level following the electrical stimulations but markedly different response patterns on the heatmaps, highlighting the importance of considering the spatial and temporal aspects of the responses and not just the total activity levels.

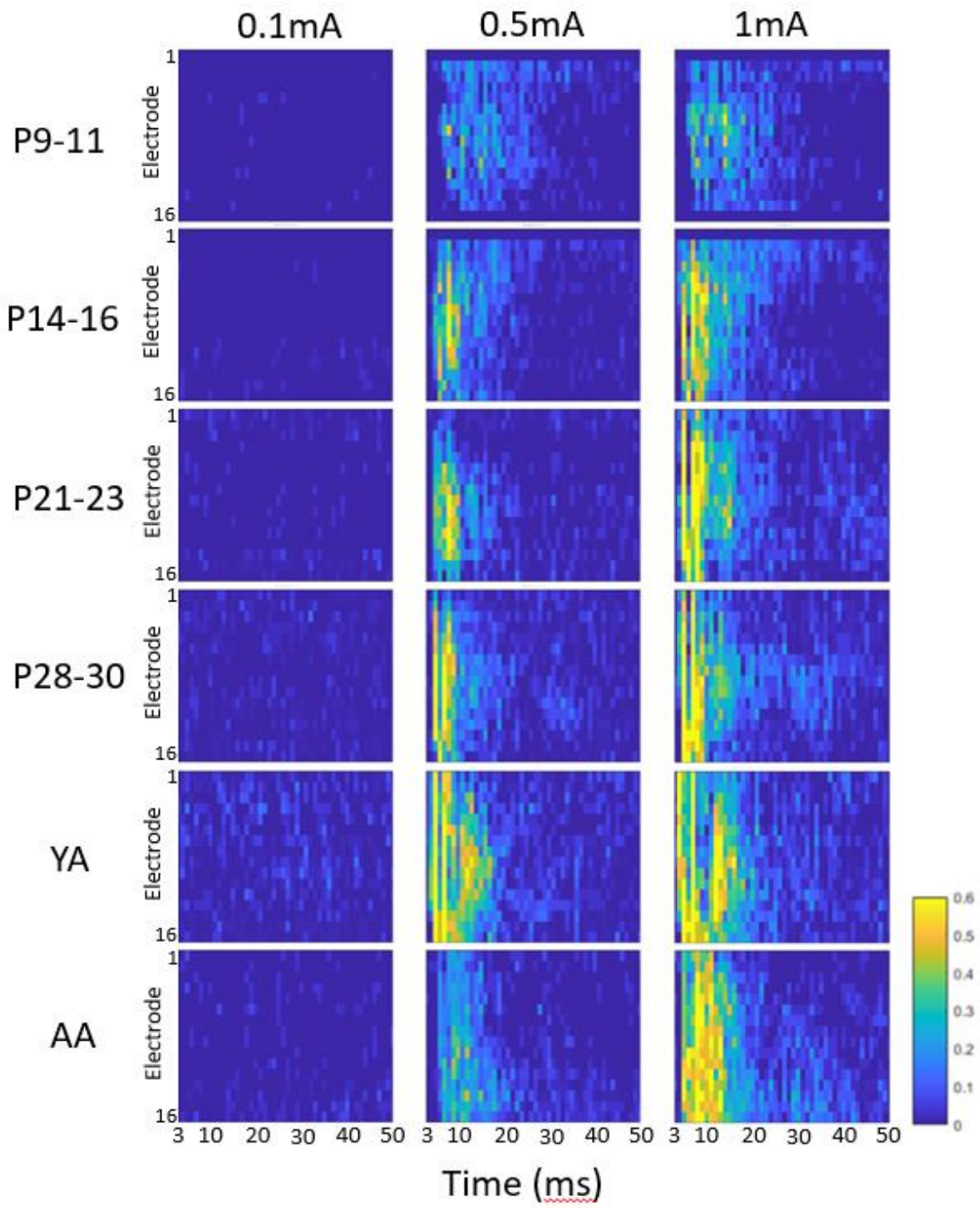


Figure 4.9. Low intensity A-fibre stimulation produces different response profiles throughout the life course. Heatmaps show spike activity at each electrode at P9-11 n=4, P14-16 n=6, P21-23 n=5, P28-30 n=6, young adults (YA) n=5, and aged adults (AA) n=5, following electrical stimulation at 0.1, 0.5, and 1mA. Responses shown are averaged responses from all animals in the age group. The A-fibre latency period is 3-50ms post stimulation for all ages with bin sizes of 1ms. Electrodes are in order; 1 is the most dorsal and 16 the most ventral, for P9-11 electrodes 1 and 16 are removed and at P14-16 electrode 1 is removed, to better represent the size of the dorsal horn at these ages. Colours represent mean number of spikes respective to the colour bar shown on the right.

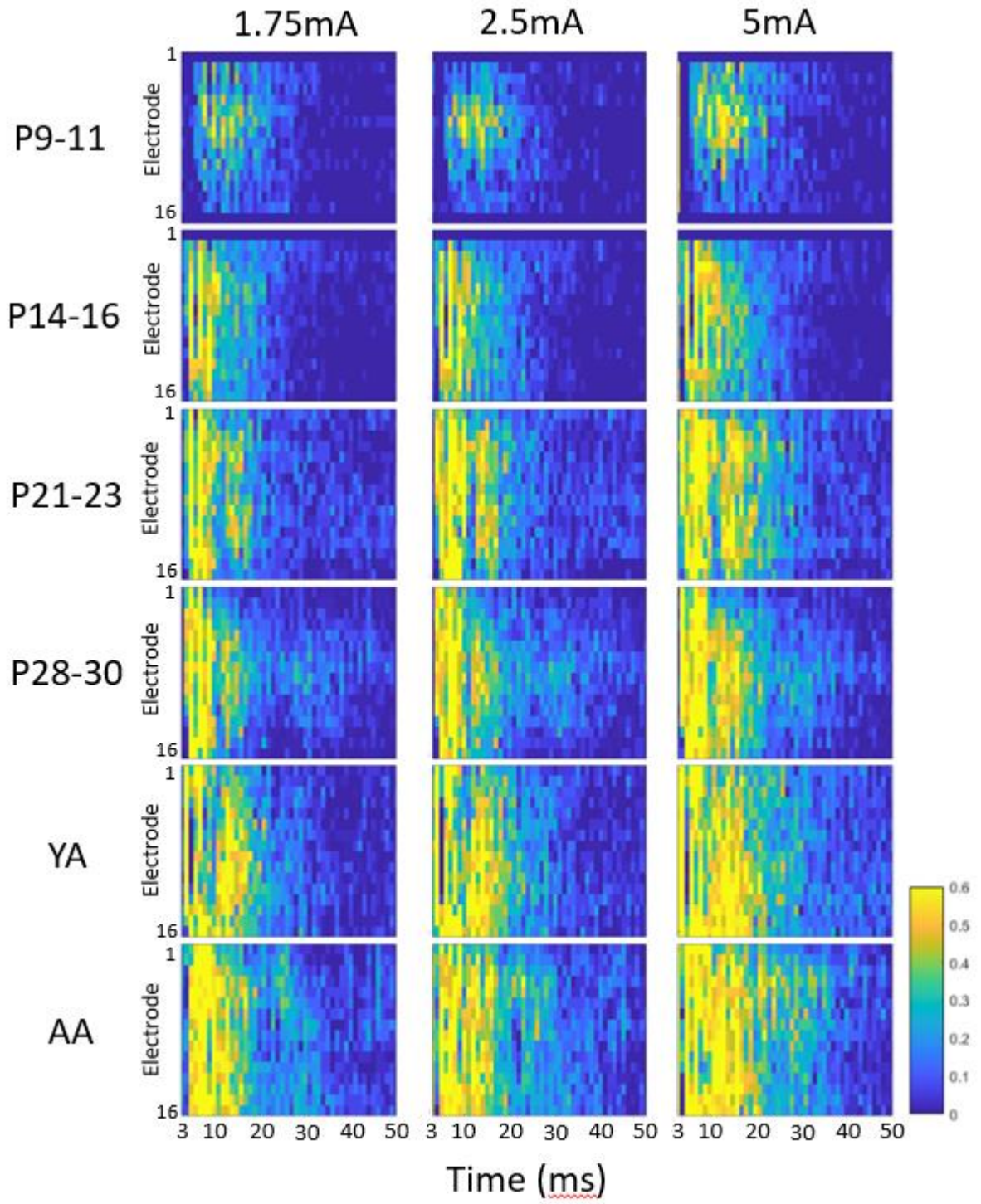


Figure 4.10. High intensity A-fibre stimulation produces different response profiles throughout the life course. Heatmaps show spike activity at each electrode at P9-11 n=4, P14-16 n=6, P21-23 n=5, P28-30 n=6, young adults (YA) n=5, and aged adults (AA) n=5, following electrical stimulation at 1.75, 2.5, and 5mA. Responses shown are averaged responses from all animals in the age group. The A-fibre latency period is 3-50ms post stimulation for all ages with bin sizes of 1ms. Electrodes are in order; 1 is the most dorsal and 16 the most ventral, for P9-11 electrodes 1 and 16 are removed and at P14-16 electrode 1 is removed, to better represent the size of the dorsal horn at these ages. Colours represent mean number of spikes respective to the colour bar shown on the right.

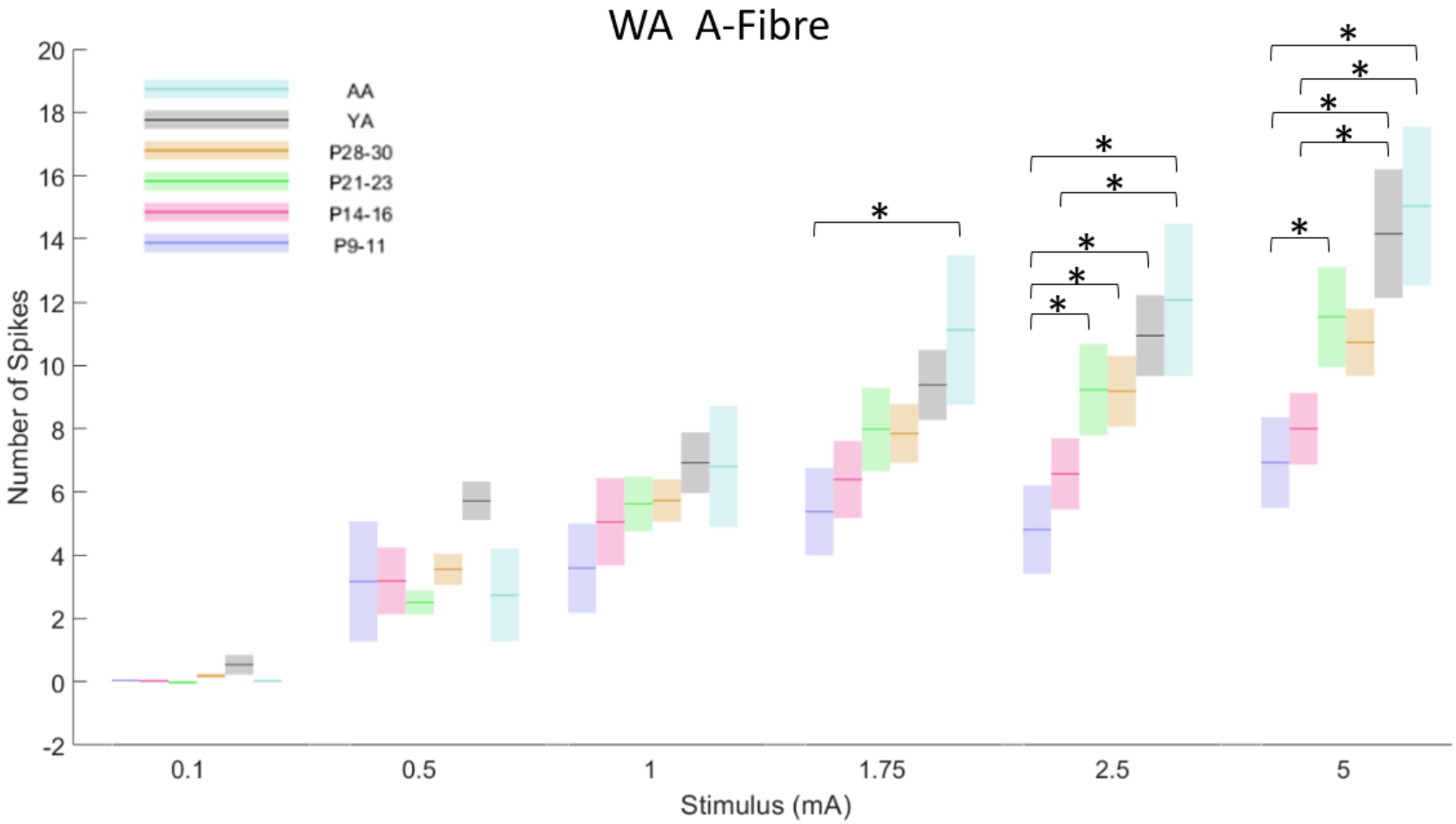


Figure 4.11. Age-dependent changes in A-fibre response occur in the dorsal horn following electrical stimulation of the hindpaw. Spike activity in the dorsal horn is shown for each age group, P9-11 n=4, P14-16 n=6, P21-23 n=5, P28-30 n=6, YA n=5, AA n=5, as the summed activity in the A-fibre latency period (3-50ms post-stimulation for all ages) across the whole array (mean activity across the electrodes). Data was not included from electrodes 1 and 16 for the P9-11 group or from electrode 1 for the P14-16 group to better represent the size of the dorsal horn at these ages. Lines represent mean spike number and shaded area represents SEM. A two-way RM ANOVA showed stimulation dependent and age dependent changes, as well as an interaction between stimulus intensity and age. Asterisks indicate significant differences between age groups determined by a Tukey's post hoc test.

The A-fibre response in the superficial, intermediate, and deep dorsal horn follows a similar pattern of increased activity with increased stimulus intensity and increased age, although less so following 0.5mA stimulation (Figure 4.12). The total activity recorded over the latency period in each region is also very similar at all ages, supporting the previous adult MEA results which suggested a broad innervation pattern of A-fibres throughout the dorsal horn (Greenspon *et al.,* 2019). In the superficial and intermediate regions a 2-way RM ANOVA shows stimulus intensity changes and an interaction between the stimulus intensity and age, but not specifically differences between ages (Superficial: stimulus intensity, $F(5, 125) = 61.28$, $P<0.0001$; interaction: $F(25, 125) = 1.841$, $P<0.05$; age: $F(5, 25) <1$ and Intermediate: stimulus intensity, $F(5, 130) = 153.4$, $P<0.0001$; interaction $F(25, 130) = 2.948$, $P<0.0001$; age: $F(5, 26) = 1.549$, $P=0.21$), the deep region however demonstrates stimulation intensity dependent changes, and age dependent changes as well an interaction (Stimulation intensity, $F(5, 130) = 152.9$, $P<0.0001$; Age, $F(5, 26) = 3.5$, $P<0.05$; Interaction, $F(25, 130) = 4.236$, $P<0.0001$) these changes are particularly evident in response to the higher stimuli intensities as shown by the post hoc differences. Additionally, as mentioned above the summed activity following the 0.5mA stimulation is very similar across the lifespan as well as following the 1mA stimulation in the superficial and intermediate regions. As the intermediate region is the main site of LTMR A-fibre innervation this may suggest that this is relatively mature by P9-11. The age-related variation in the deep dorsal horn following the higher intensity stimulations may suggest a postnatal maturation in A-fibre innervation or processing in this region. Interestingly the P28-30 response in the superficial region is lower than would be expected following the general increase in response with increased age as seen in the intermediate and deep regions, this is similar to the results in the previous chapter and may suggest an altered response at this age group. As P28 is the age at which the critical period of descending inhibition is developed it may be linked with this. Additionally, postnatal retraction of some A-fibres from the superficial laminae of the dorsal horn is completed by the third to fourth postnatal week (M Fitzgerald, Butcher and Shortland, 1994; Nakatsuka and Kumamoto, 2000; Beggs *et al.*, 2002) which could result in the decrease A-fibre response activity in this region, however this is not seen in the adults.

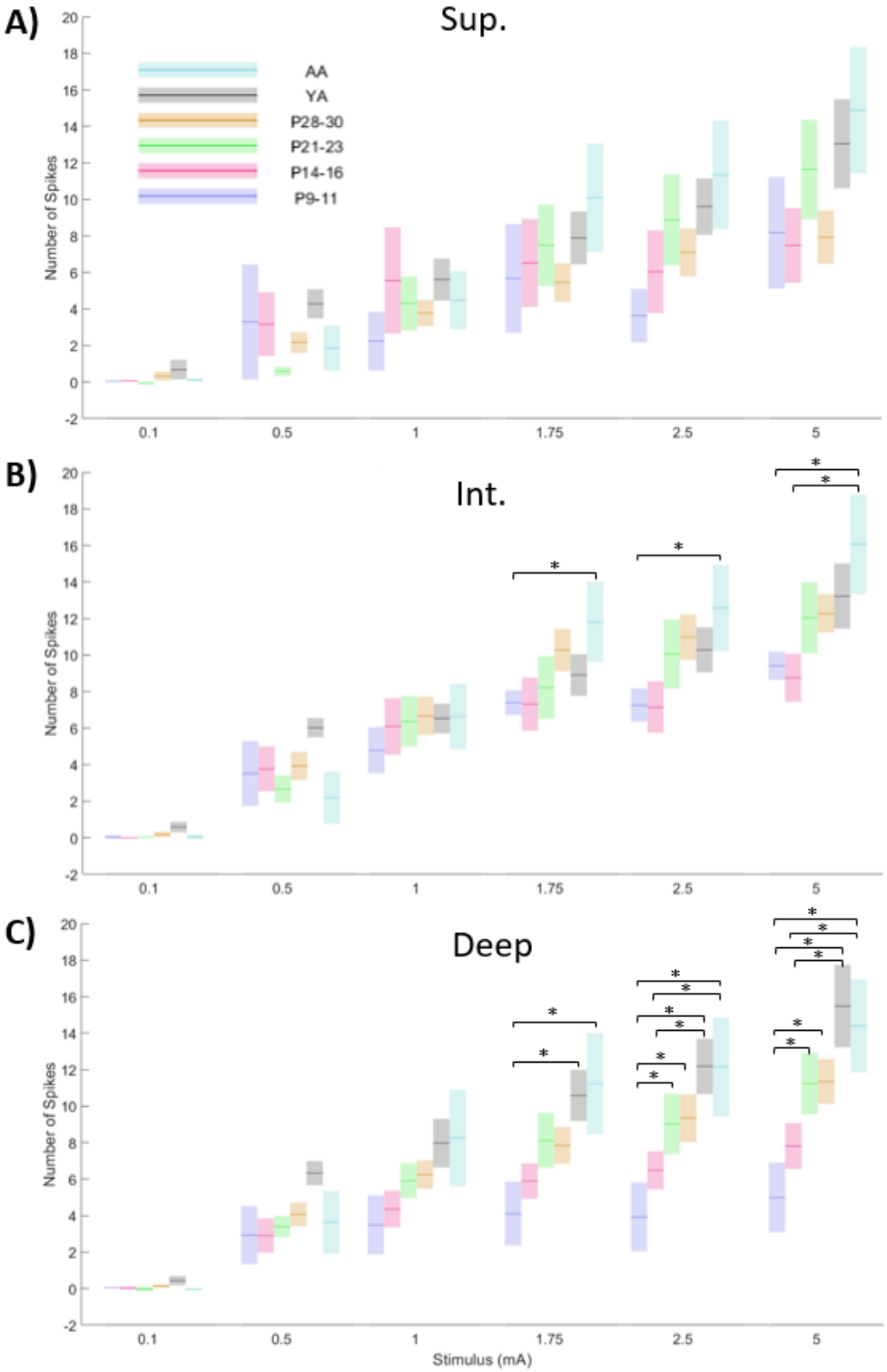


Figure 4.12. Age-dependent changes in A-fibre response occur in the intermediate and deep dorsal horn following electrical stimulation of the hindpaw. Spike activity in the dorsal horn is shown for each age group, P9-11 n=4, P14-16 n=6, P21-23 n=5, P28-30 n=6, YA n=5, AA n=5, as the summed activity in the A-fibre latency period (3-50ms post-stimulation for all ages) across the A) superficial, B) intermediate, and C) deep regions (mean activity across the electrodes). Data was not included from electrodes 1 and 16 for the P9-11 group or from electrode 1 for the P14-16 group to better represent the size of the dorsal horn at these ages. Lines represent mean spike number and shaded area represents SEM. Two-way RM ANOVAs show stimulus dependent changes and an interaction between stimulus intensity and age, in all regions as well as differences between ages in the deep region. Asterisks indicate significant differences between age groups determined by a Tukey's post hoc test.

4.4.4 C-fibre Responses Undergo Changes throughout the Life Course

The C-fibre response is hard to distinguish in the heatmaps following lower intensity stimulations (Figure 4.13), as C-fibres are generally associated with higher threshold stimulation this is not too surprising. It does appear however that following 0.5mA stimulation C-fibre responses are observed in the P14-16 group in the superficial and deep regions, as well as potentially in the P9-11 group, but not at any other age. This could reflect an age-related change in C-fibre thresholds following the second postnatal week. Following 1mA stimulation there is a clear increase in the deep region in the young adult group lasting approximately 50ms, interestingly this is not observed in the aged adult group possibly reflecting a change in thresholds between the two adult groups. Following higher intensity stimulations (Figure 4.14) there is little change in the P9-11 group but an increase in activity with increased intensity stimulation at every other age group, supporting previous studies that C-fibre responses mature at P14 (Fitzgerald and Gibson, 1984). The duration of the C-fibre response appears to increase with increased age, this is particularly clear following 5mA stimulation. Additionally, the young adult group displays a much greater response than any other age tested here, which appears to be predominantly in the deep dorsal horn, although there is a relatively large amount of variation within the young adult group in all but especially the deep dorsal horn. The aged adult group also shows a decline in summed activity during the C-fibre response latency period compared to the young adults, especially in the superficial region of the dorsal horn, although this was not statistically significant. This may reflect the altered C-fibre response in old age reported in previous studies (Weyer *et al.*, 2016). Two-way RM ANOVAs across the whole array and at each region identified only stimulation intensity differences (Whole array, $F(5, 125) = 10.88$, $P<0.0001$; Superficial, $F(5, 125) = 7.362$, $P<0.0001$; Intermediate, $F(5, 130) = 8.455$, $P<0.0001$; Deep, $F(5, 130) = 7.035$, $P<0.0001$) but not significant age- dependent changes (Whole array, $F(5, 25) <1$; Superficial, $F(5, 25) <1$; Intermediate, $F(5, 26) = 1.005$, $P=0.43$; Deep, $F(5, 26) = 1.694$, $P=0.17$) or interactions (Whole array, $F(25, 125) = 1.217$, $P=0.24$; Superficial, $F(25, 125)<1$; Intermediate, $F(25, 130) = 1.185$, $P=0.27$; Deep, $F(25, 130) = 1.044$, $P=0.42$) in the C-fibre response latency period (Figure 4.15 & 4.16). Although not statistically significant it appears that age-related differences may occur earlier in the superficial region and latest in the deep region (Figure 4.16).

Additionally, as highlighted in the A-fibre section there is a disconnect between the response patterns observed in the heatmaps and the summed activity over the C-fibre latency period. In the bar charts of summed activity (Figure 4.15) the response following the 1.75mA stimulation looks very consistent between the P14-16, P21-23, P28-30, and aged adult group however the heatmaps show very different responses occurring

throughout the dorsal horn during this time, as such it is important to consider the temporal and spatial aspects when investigating these responses.

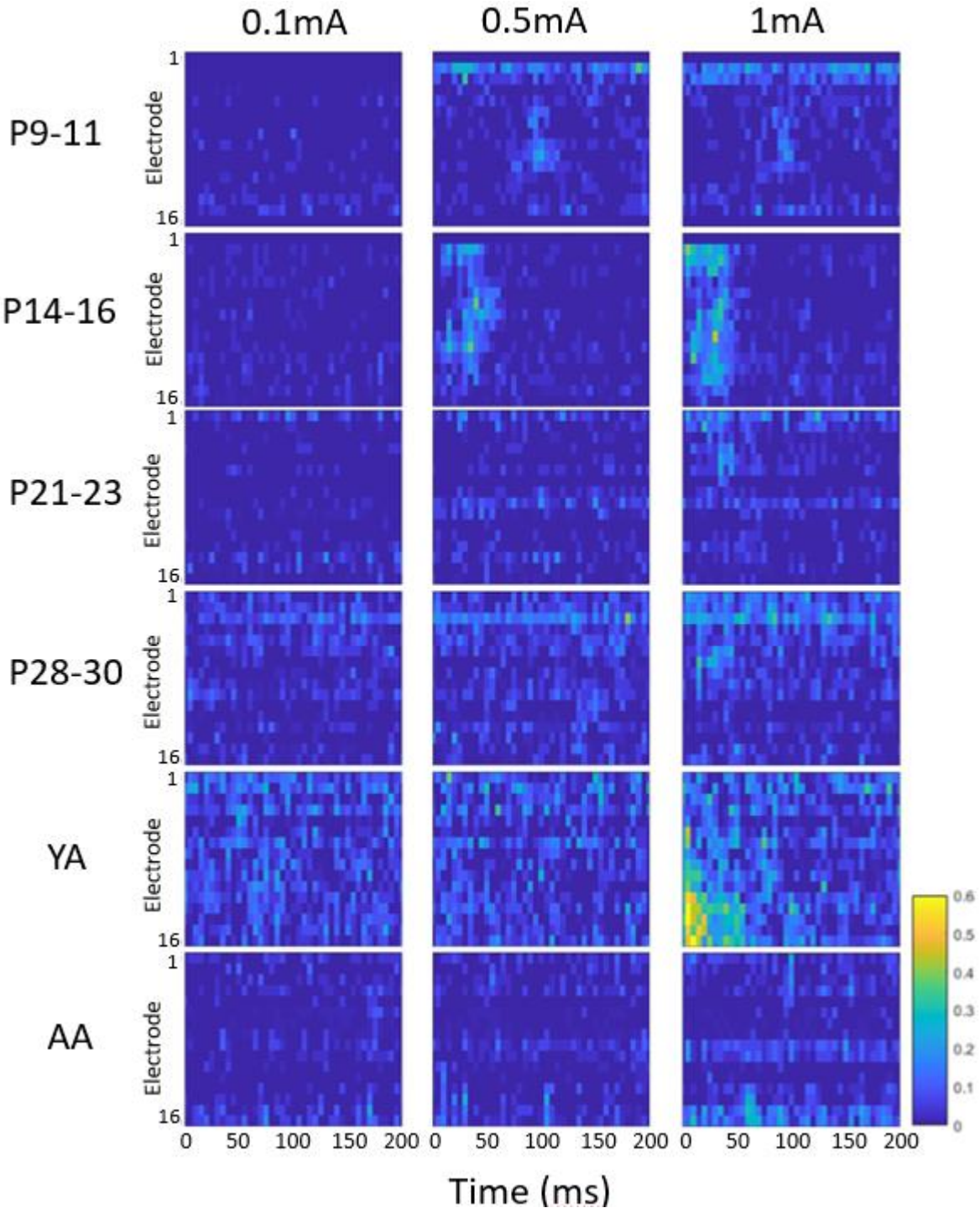


Figure 4.13. Low intensity C-fibre stimulation produces different response profiles throughout the life course. Heatmaps show spike activity at each electrode at P9-11 n=4, P14-16 n=6, P21-23 n=5, P28-30 n=6, young adults (YA) n=5, and aged adults (AA) n=5, following electrical stimulation at 0.1, 0.5, and 1mA. Responses shown are averaged responses from all animals in the age group. The C-fibre latency period is 200ms for all ages with the onset post-stimulation dependent on age (Table 4.3) bin sizes are 5ms. Electrodes are in order; 1 is the most dorsal and 16 the most ventral, for P9-11 electrodes 1 and 16 are removed and at P14-16 electrode 1 is removed, to better represent the size of the dorsal horn at these ages. Colours represent mean number of spikes respective to the colour bar shown on the right.

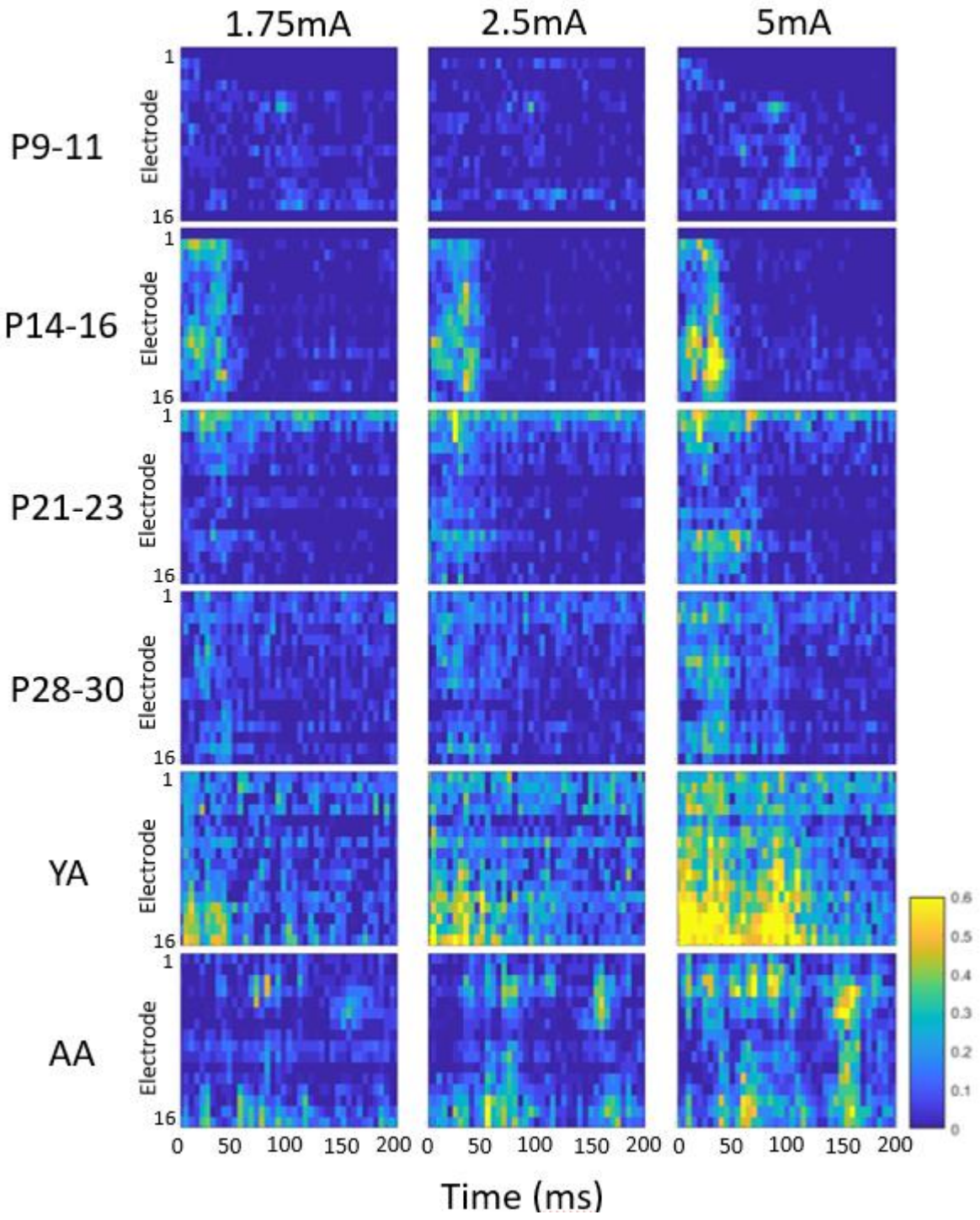


Figure 4.14. High intensity C-fibre stimulation produces different response profiles throughout the life course. Heatmaps show spike activity at each electrode at P9-11 n=4, P14-16 n=6, P21-23 n=5, P28-30 n=6, young adults (YA) n=5, and aged adults (AA) n=5, following electrical stimulation at 1.75, 2.5, and 5mA. Responses shown are averaged responses from all animals in the age group. The C-fibre latency period is 200ms for all ages with the post-stimulation onset dependent on age (Table 4.3) bin sizes are 5ms. Electrodes are in order; 1 is the most dorsal and 16 the most ventral, for P9-11 electrodes 1 and 16 are removed and at P14-16 electrode 1 is removed, to better represent the size of the dorsal horn at these ages. Colours represent mean number of spikes respective to the colour bar shown on the right.

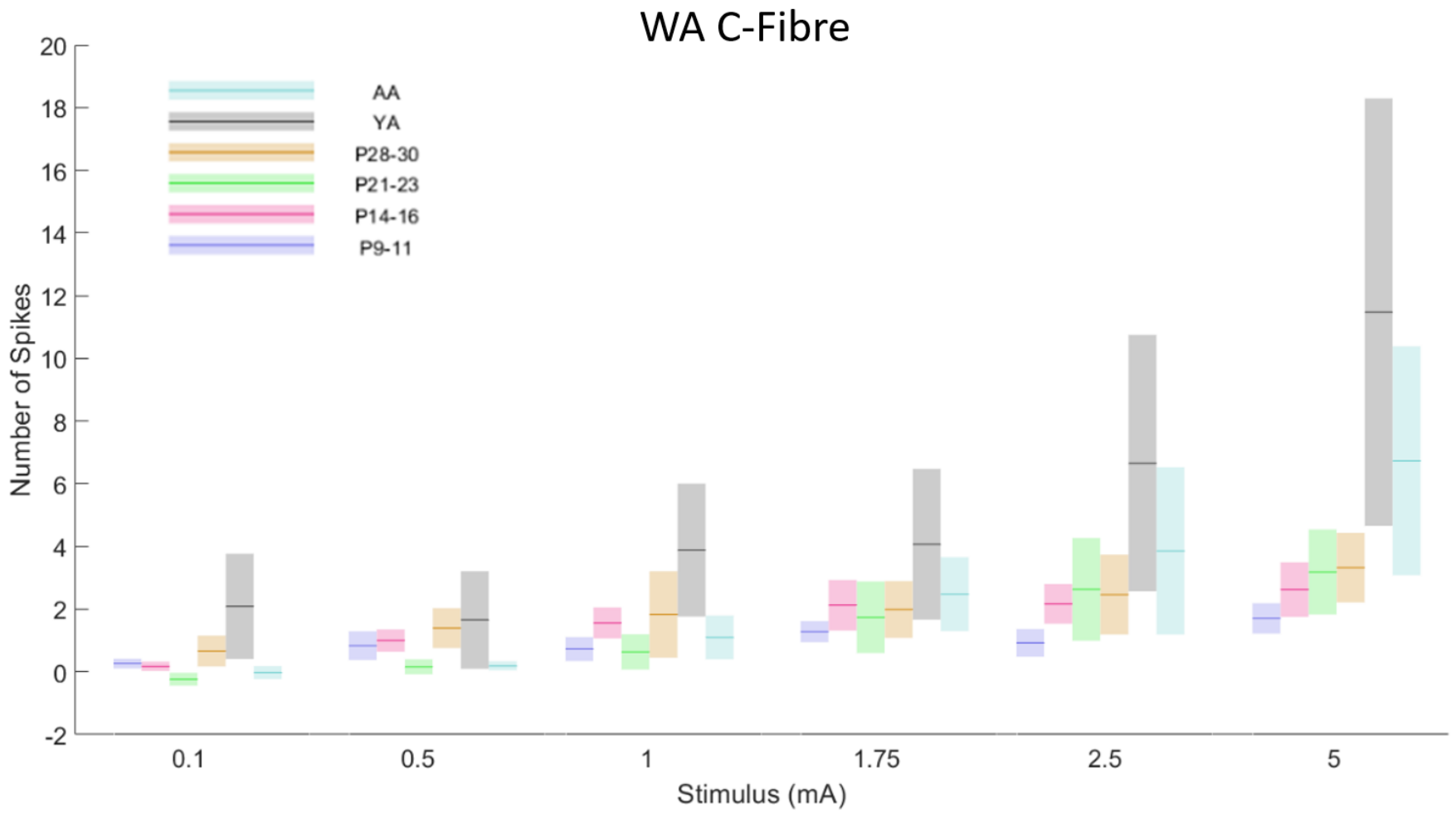


Figure 4.15. Age-dependent changes in C-fibre response occur in the dorsal horn following 5mA electrical stimulation of the hindpaw. Spike activity in the dorsal horn is shown for each age group, P9-11 n=4, P14-16 n=6, P21-23 n=5, P28-30 n=6, YA n=5, AA n=5, as the summed activity in the C-fibre latency period (200ms post-stimulation for all ages with the post-stimulation onset dependent on age (Table 4.3)) across the whole array (mean activity across the electrodes). Data was not included from electrodes 1 and 16 for the P9-11 group or from electrode 1 for the P14-16 group to better represent the size of the dorsal horn at these ages. Lines represent mean spike number and shaded area represents SEM. A two-way RM ANOVA found stimulation dependent differences but no age dependent changes or interaction.

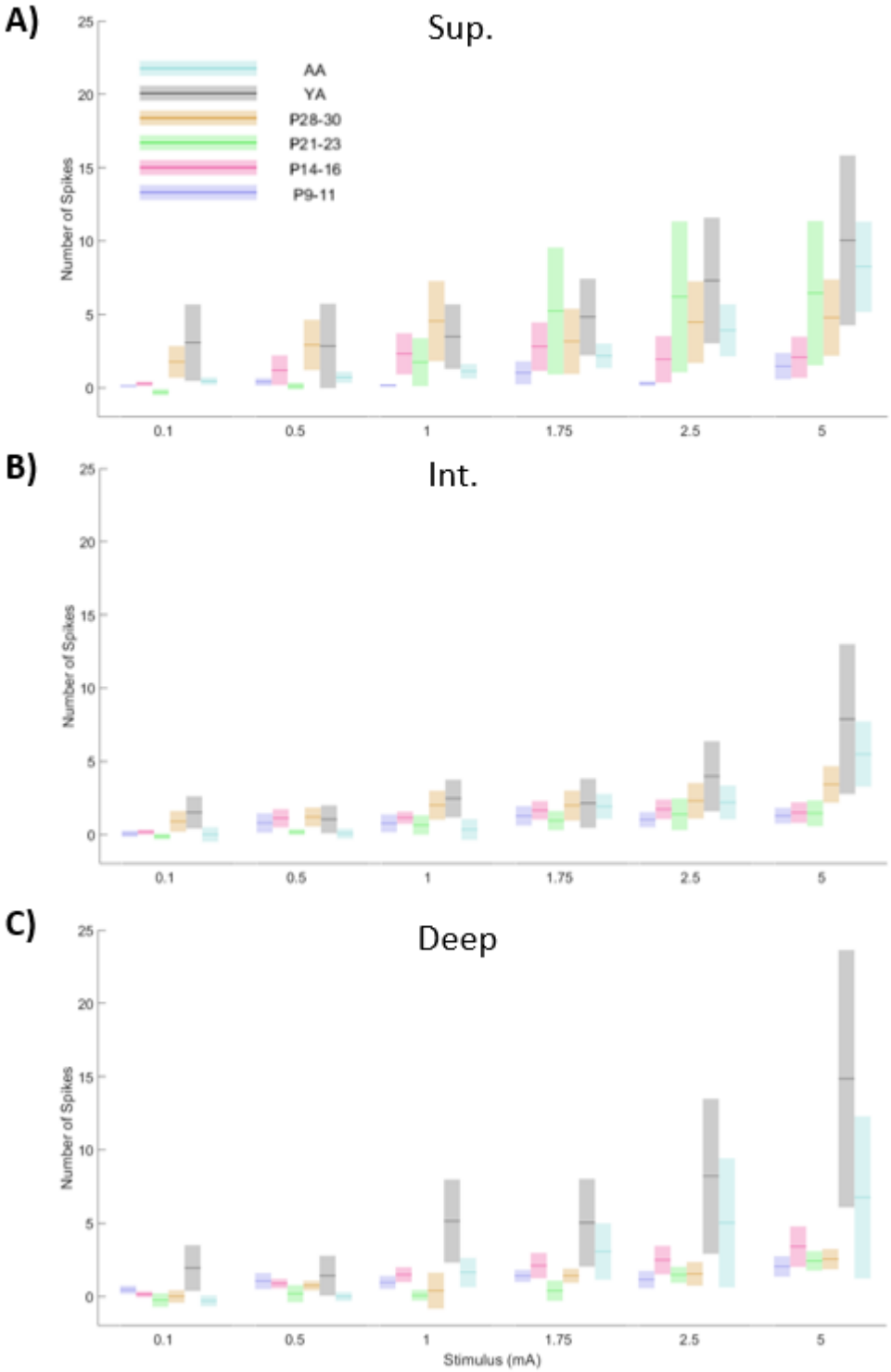


Figure 4.16. Changes in C-fibre response in the superficial, intermediate, and deep dorsal horn across the life course following electrical stimulation of the hindpaw. Spike activity in the dorsal horn is shown for each age group, P9-11 n=4, P14-16 n=6, P21-23 n=5, P28-30 n=6, YA n=5, AA n=5, as the summed activity in the C-fibre latency period (200ms post-stimulation for all ages with the post-stimulation onset dependent on age (Table 4.3)) across the A) superficial, B) intermediate, and C) deep regions (mean activity across the electrodes). Data was not included from electrodes 1 and 16 for the P9-11 group or from electrode 1 for the P14-16 group to better represent the size of the dorsal horn at these ages. Lines represent mean spike number and shaded area represents SEM. Two-way RM ANOVAs found stimulation dependent differences but no age dependent changes or interaction in any region.

4.5 Discussion

The work presented in this chapter resulted in more precise primary afferent fibre conduction velocities and latency periods for the postnatal development than have been previously reported. Age-related increases in evoked spike activity with increasing age were found in the dorsal horn responses to A- and C-fibre stimulation during postnatal development. However this increase was not found to continue throughout the whole life course, as in older age there was a decrease in evoked spike activity compared to the young adults. As such the aims set out at the beginning of this chapter were achieved.

4.5.1 Changes in PAF Conduction Velocity and Latency Periods Occur Throughout Postnatal Development

A- and C-fibre waveforms were detected at all ages used, as these can be detected from birth, although in an immature form (Fitzgerald, 1985) this was unsurprising. The A-fibre conduction velocities increased with postnatal age between P9 and young adults, supporting previously reported findings (Lizarraga, Chambers and Johnson, 2007). Importantly this demonstrated ongoing development of the sciatic nerve in the later postnatal period which has been previously demonstrated in the tibial (Vejsada *et al.*, 1985) and sural (Sato, Sato and Suzuki, 1985) nerves but not the sciatic nerve. A-fibre conduction velocities increased from 19m/s at P9-11 to 53m/s in young adults in a linear manner (Figure 4.1) these values are slightly faster than those previously reported from the tibial nerve at similar ages (Vejsada *et al.*, 1985), and much faster than those reported by some other studies, although this may be a reflection of experimental set up as our conduction velocity measurements took place at 37°c whereas others were conducted at 22-25°c (Lizarraga, Chambers and Johnson, 2007). C-fibre conduction velocity was more varied in the youngest P9-11 age group but is possibly faster than at older ages which were very consistent from P15 at approximately 0.8m/s. Half of the P9-11 values were consistent with the P15-17 and above velocities and so this may reflect a discrepancy between the two superfusion chamber setups used at this age group, repeating the peripheral recordings at this age could determine if this is a set-up factor or not. C-fibre recordings were unable to be obtained from two sciatic nerves in the young adult group, possibly due to limitations in the setup which is predominantly used for recordings of smaller mouse sciatic nerves.

We were however unable to determine exact Aβ- and Aδ-fibre conduction velocities limiting the analysis that could be done into the A-fibre responses within the dorsal horn. However, previous reports have suggest grouping Aβ- and Aδ-fibre responses in neonates (Urch and Dickenson, 2003). Interestingly this resulted in a very consistent calculated A-fibre onset time varying by a maximum of 1ms between all of the ages

tested but increasingly delayed C-fibre latency periods with increased age. The C-fibre latency period onset occurred later with increasing postnatal age as the distance between the hindpaw and recording site increased. These latencies support and build upon previous findings which suggest pups (35-100g; approximately P14-28) use 0-50ms for A-fibre recordings and 50-250ms for C-fibre recordings (Urch and Dickenson, 2003). The calculated A-fibre latencies suggest a very consistent onset of between 2-3ms for all ages however, the MEA recordings of A-fibre onset appear to vary slightly with the onset occurring quicker with increased postnatal age however, as the onset variation is small compared to the total latency period used for the A-fibres 3-50ms was chosen for all ages. The calculated C-fibre latency period reflects the onset observed by the MEA recordings at all of the postnatal ages, the adult conduction velocities however appear to suggest a slightly later onset than observed using the MEA recordings. This could be linked with the increased size of the sciatic nerve of adults resulting in a more challenging placement of the nerves into a setup originally designed for recordings of mouse sciatic nerve. Alternatively it is possible that the C-fibre processing is being affected by the preceding A-fibre inputs, possibly through circuits involving inhibitory control (Torsney and MacDermott, 2006). A possible way to test this would be to optogenetically activate the peripheral C-fibres only.

4.5.2 Dorsal Horn Processing of A-fibre Stimulation Changes throughout the Life Course

A-fibre Onset

The A-fibre activity shown on the heatmaps shows a trend of starting earlier as postnatal age increased, this most likely reflects Aβ-fibre onset, however as Aβ- and Aδ-fibre latencies were not analysed separately these slight latency period changes in A-fibre onset should have minimal impacts on the analysis. Interestingly the A-fibre activity was seen earlier following higher intensity peripheral stimulation which may show the activation/recruitment of high threshold Aβ-fibres which have been shown to have a faster conduction velocity than low threshold Aβ-fibres (Djouhri and Lawson, 2004). However, this also may reflect limitations with the electrical stimulation as to reach the target amplitudes in the same duration the rate of current increase may be increased for the higher amplitude stimuli resulting in Aβ-fibre thresholds being reached earlier.

A-fibre Evoked Thresholds in Dorsal Horn Networks

It is unclear if threshold changes in the A-fibre response were detected in this study. No age group tested here had a clear response to 0.1mA stimulation and all displayed a response at 0.5mA. Although comparing heatmaps suggests that the P9-11 and aged

adults may have had a reduced response intensity compared to the other ages at 0.5mA it is not statistically significant, and it is unclear if this is a result of threshold changes. Previous studies have shown changes in behavioural thresholds mature over the first three postnatal weeks (Fitzgerald and Jennings, 1999), a study using stimuli of between 0.1 and 0.5mA may be able to uncover the thresholds at which a response is obtained at each age and study this further.

A-fibre Double Activity Period Response

From P21 until the young adult group two distinct bursts of activity are seen within the A-fibre latency period, one at 3-10ms and the second at approximately 10-20ms. A similar observation was made in previous adult work by our group and it was suggested that this most likely represented the activity of the Aβ- and Aδ-fibre subgroups (Greenspon *et al.,* 2019). However in the young adults the first burst appears to have higher activity in the superficial and deep regions and the second burst in the intermediate and deep regions and as low threshold A-fibres project to the intermediate laminae (IIi-V) and nociceptive A-fibres project to the superficial laminae (I and IIo) and deep lamina V (Andrew J Todd, 2010) this instead suggests that the initial burst may be nociceptive and the second burst associated with low threshold A-fibre activity. It has been previously suggested that approximately 50% of nociceptive A-fibres in rodents conduct in the Aβ range (Djouhri and Lawson, 2004) supporting both possible theories. Either way, the two burst split appears to be developed between the second and third postnatal weeks suggesting a key developmental step in A-fibre processing within the dorsal horn.

This second burst appears to be where the majority of increases observed following higher stimulus application is observed, as well as increases with increased age. This is illustrated in the associated bar charts as the variation between age groups increases with increased stimulus intensity. This produces very interesting results in the deep dorsal horn in particular where there is a neonatal/juvenile/adult divide in the response to higher stimulus intensities (Figure 4.12). This may suggest that dorsal horn processing of high intensity A-fibre stimulation undergoes more postnatal development than responses to low intensity stimulation.

A-fibre Response across Doral Horn Regions

Responses in A-fibre latency period are relatively consistent across the superficial, intermediate, and deep dorsal horn at all ages although there were generally fewer differences between the intensity groups and ages in the superficial compared to the

intermediate and deep regions. This may suggest that the intermediate and particularly the deep dorsal horn are involved in discriminating stimulus intensity in the range used in this study and that these regions are where the majority of postnatal development occurs. However, looking at the heatmaps of the evoked responses shows how the response pattern changed during the life course and further studies using more complex analysis analysing the response from each electrode individually may be able to delineate regional development in these patterns.

Interestingly during the A-fibre latency period the P28-30 group had lower responses in the superficial region following high intensity stimulation than would be expected with the pattern of age-related increases observed in the other ages and regions. It is unclear why this may be the case, but as lower responses were seen following mechanical stimulation in this age group in the previous chapter it is unlikely that this is an error in this study. It is unclear if this is an alteration in initial A-fibre response or a change in processing within the dorsal horn. P28 is a key age in the postnatal maturation of descending inhibition (G. J. Hathway *et al.*, 2009b; Hathway, Vega-Avelaira and Fitzgerald, 2012; Kwok *et al.*, 2014b) and these results may reflect an adjustment in the processing of high threshold stimulation during this critical period.

4.5.3 Dorsal Horn Processing of C-fibre Stimulation Changes throughout the Life Course

C-fibre Onset

As the onset of the C-fibre latency period differed during postnatal development the decision was made to analyse the activity for a consistent duration of 200ms at each age and adjust the post-stimulation times used. This was based on previous studies that use a 200ms duration to measure C-fibre activity in adults and pups (Urch and Dickenson, 2003).

C-fibre Response across the Life Course

There was very little response during the C-fibre latency period in the P9-11 group which matches previous studies suggesting that C-fibres are immature until the second postnatal week (Fitzgerald and Gibson, 1984). The P14-16 age group showed a remarkable response in the C-fibre latency period at 0.5mA and above, however this was very different than the C-fibre response patterns seen in the older ages. The P14-16 C-fibre response had a much shorter duration and was more intense, suggesting an immature C-fibre response is still occurring in the dorsal horn at this age. This may reflect the difference in inhibitory activity occurring in the spinal cord at this age, or the lack of descending inhibition which is not developed until P21-28 (G. J. Hathway *et al.*,

2009b; Hathway, Vega-Avelaira and Fitzgerald, 2012; Kwok *et al.*, 2014b). In line with this lower response activity was seen in the P21-23 and P28-30 groups compared to P14-16 and young adults, possibly further supporting that descending inhibition may be having an effect at these ages during the C-fibre latency period. Alternatively, as the P14-16 group had a much clearer C-fibre response at stimuli intensities of 0.5mA and above, the decrease in the older ages may reflect an alteration in C-fibre thresholds. As mentioned above behavioural responses mature over the first three postnatal weeks (Fitzgerald and Jennings, 1999), potentially matching the changes seen in this study.

There also appears to be differences in the C-fibre response pattern between young and aged adults as well as a reduction in the summed response in all regions of the dorsal horn in the aged animals. These however require further study as the large amount of variation observed in the young adult group, as well as only males being included in the aged adult group reduces the ability to make any definitive conclusions from this study. It does however support previous findings of altered C-fibre responses in older age (Namer, 2010; Weyer *et al.*, 2016). Fewer significant differences between stimulation intensities and between ages were detected in the C-fibre latency period than the A-fibre latency period. It is possible that more differences would be uncovered if a protocol involving higher stimulus intensity had been used. C-fibre activity again matches the expected innervation pattern with responses detected in all regions but more intensely in the superficial and deep regions.

4.5.4 Experimental Considerations

The electrical stimulation used in this study was applied to the hindpaw via two subdermal needle electrodes. The same needle electrodes were used at every age to reduce variation in the application of the electrical stimuli between ages. However, as these are then larger in proportion to the neonatal hind paws it is possible that they will have resulted in increased tissue damage and inflammation. To reduce the effects of this on the data the needle electrodes were left in the paw for the whole protocol.

The stimulation duration, frequency, and amplitude range were chosen based on previous work by the group. The aims of this study included being able to analyse the population response following A- and C-fibre stimulation at the different stages of the life course and compare these between ages. While we were able to do this the stimulation protocol used did not allow the fibre thresholds to be examined in detail or the full extent of the C-fibre range to be investigated. Further studies would be able to adapt the protocol used here to focus on their specific aims.

It is, however, important to remember that whilst the individual fibres can be investigated as we are recording from the dorsal horn there are still other processing circuits occurring within the dorsal horn and from supraspinal sites. This is particularly important when analysing high intensity and C-fibre responses as previous studies have shown that Aβ-PAF inputs on to different subpopulations of inhibitory interneurons reduces excitatory interneurons in the superficial lamina (Duan *et al.*, 2014; Petitjean *et al.*, 2015). Similarly stimulation of Aβ-fibres can reduce synaptic transmission of nociceptive inputs in lamina II of the dorsal horn (Sdrulla *et al.*, 2015). As A-fibre stimulation is unavoidable when using stimulus intensities high enough to activate C-fibres this may be having an effect on the responses detected. A possible way to selectively test different PAF subtypes is by utilising optogenetic techniques which have become increasingly used in somatosensory research (Harding, Fung and Bonin, 2020). Optogenetic techniques could be used to activate selected PAF subtypes and be done without additional mechanical touch interference (Schorscher-Petcu, Takács and Browne, 2021). Although this could potentially be used in conjunction with the MEA electrophysiology technique to uncover, for example, specific C-fibre processing circuits in the dorsal horn, Hachisuka et al. (2018), suggest an important consideration that activating specific cells using optogenetic techniques may bypass endogenous pathways resulting in physiologically inaccurate responses.

Arguably the main limitation in this study is the lack of expertise in the analysis of the response patterns observed. The MEA electrophysiology setup used here appears to be capable of uncovering incredibly detailed population recordings, for example the A-fibre onset heatmaps (Figure 4.2) show very interesting response patterns as well as the changing response patterns seen on the A- and C-fibre heatmaps that is lost when comparing by summed activity. These temporal-spatial aspects of the dorsal horn response may help in understanding the development of spinal processing throughout the life course and an imporved bioinformatics or machine-learning approach may allow these aspects to be studied fully.

4.5.5 Conclusions

In conclusion primary afferent fibre latency periods were identified for ages throughout postnatal development and we demonstrated that population recordings using the multielectrode array were able to identify distinct A-fibre and C-fibre responses following a range of electrical stimulations of the hindpaw. Although regional differences in PAF responses were not as clear as expected this could be a limitation of the inaccurate activation of specific fibre types and optogenetically activating peripheral primary afferent fibres may make these innervation profiles clearer.

We have suggested the postnatal development of low intensity and high intensity A-fibre responses may be distinct and occur over the first three postnatal weeks. Additionally, our findings support previous work suggesting that C-fibre responses are not functional until the second postnatal week but we have also identified a very different response pattern throughout the second postnatal week to young adults. Overall these findings suggest that postnatal development of PAF responses occurs throughout the postnatal period. These findings also support the theory that aged animals show altered primary afferent responses and warrant further work.

Chapter 5

Changes in GABAergic Inhibition in the Dorsal Horn throughout the Life Course

5.1 Introduction

One of the most important modulatory features of the dorsal horn is the ability to inhibit excitatory activity from the primary afferent fibres (PAD) and regulating the activity of projection neurons (Lucas-Osma *et al.*, 2018; Delgado-Lezama *et al.*, 2021). In the mature dorsal horn γ-aminobutyric acid (GABA) and glycine are the main fast-acting endogenous inhibitory neurotransmitters and their corresponding receptors $GABA_AR$, $GABA_BR$, and GlyR are widely expressed throughout the spinal cord (Rahman *et al.*, 2007). As discussed in Section 1.2.4, there is however a spatial disparity between the receptors in the dorsal horn, with cells in laminae I-IIo mainly expressing GABA receptors and cells in laminae IIi-IV mainly expressing glycinergic receptors (Inquimbert and Rodeau, 2007; Bardoni *et al.*, 2013). Although, subgroups of inhibitory interneurons do express both GABA and glycine (Polgár *et al.*, 2003). Additionally, a population of neurons originating within laminae V and VI have been shown to extend GABAergic axons to deeper laminae of the ventral horn where they can inhibit motorneurons (Hughes *et al.*, 2005).

The adult dorsal horn is under both tonic and phasic inhibition, with extrasynaptic receptors thought to be involved in tonic inhibition and synaptic receptors with phasic inhibition (Takazawa and MacDermott, 2010; Lucas-Osma *et al.*, 2018). Activation of synaptic $GABA_A$ receptors within the CNS causes an influx of Cl^- ions into the cell resulting in hyperpolarisation of the membrane which inhibits the production of an action potential. Whereas, activation of extrasynaptic GABA receptors mediate neuronal excitability through alternative mechanisms including by increasing conductance (Lucas-Osma *et al.*, 2018; Delgado-Lezama *et al.*, 2021). Disinhibition of GABAergic and glycinergic signalling in the dorsal horn results in activation of pathways normally associated with nociceptive input following low-threshold input, predominantly from Aβ-PAFs, in the superficial dorsal horn including in $NK1^+$ neurons (Baba *et al.*, 2003; Torsney and MacDermott, 2006).

5.1.1 Changes in Dorsal Horn Inhibition during the Life Course

Tonic GABAergic inhibition has been reported at P8 and shown to increase with postnatal age and reaching adult levels by P21 (Zell *et al.*, 2014). Tonic glycinergic inhibition is developed between P14 and P21, although exogenous glycine can lead to inhibition at P3 (Koch *et al.*, 2012). The development of glycine inhibition is associated with the maturation of C-fibre afferents as well as changes in glycine receptor location and isoforms (Koch *et al.*, 2012).

Changes in GABAergic and glycinergic signalling occur during postnatal development with a switch from predominantly GABAergic signalling to a balance between the two, as seen in the adult dorsal horn (Baccei and Fitzgerald, 2004). Patch-clamp studies have demonstrated changes in miniature inhibitory postsynaptic currents (mIPSCs) with predominantly GABA mIPSCs present during the first postnatal week and a mature pattern of GABA/Glycine mIPSCs detected by the third postnatal week (Keller *et al.*, 2001; Baccei and Fitzgerald, 2004). Additionally, GABAergic mIPSCs also increase in amplitude, become more consistent, and have a quicker recovery time over the first three postnatal weeks (Ingram, Fitzgerald and Baccei, 2008).

Interestingly, the effect of activating $GABA_AR$s and GlyRs has been shown to change during development in the CNS with embryonic and early neonatal responses resulting in a depolarization. This is proposed to be due to a lack of KCC2 ion transporters, which are developmentally upregulated, producing altered chloride gradients across the cell membrane (Avila, Nguyen and Rigo, 2013). Patch-clamp studies identified only hyperpolarization following GABA receptor activation in P6/7 rats, suggesting this switch from excitatory to inhibitory activity has occurred during the first postnatal week (Baccei and Fitzgerald, 2004), although mature responses are not seen until the third postnatal week (Cordero-Erausquin *et al.*, 2005). These postnatal changes in $GABA_AR$ but not GlyR, may be associated with the development of descending pathways, such as the serotonergic projections, as spinal transection prevents the shift to hyperpolarisation of motor neurons (Sadlaoud *et al.*, 2010). This may be linked to the finding that intrathecal $GABA_AR$ antagonists cause a dose-dependent increase in withdrawal thresholds in P3 rats but following spinalisation there is instead a decrease in withdrawal thresholds, as seen in P21 rats (Hathway *et al.*, 2006).

Patch-clamp recordings in the dorsal horn of older adult mice (28-30 months) compared to younger adult (3-4 months) found some excitable neurons (initial bursting neurons) displayed an increased action potential number and increased response duration in older animals, suggesting a more excitable phenotype. However, the same study showed a decrease in sEPSC amplitude, and an increase in GABAergic signalling, with advanced age suggesting a more inhibitory phenotype (Mayhew *et al.*, 2019). Similarly, aged rats (24-39months) had an increased level of spontaneous activity, increased noxious evoked responses, longer response latencies, and an increase in the receptive field of WDR neurons, although the low threshold area was smaller (Iwata *et al.*, 2002; David-Jürgens *et al.*, 2008) ultimately suggesting changes in inhibitory modulation within the dorsal horn with increased age.

5.2 Hypotheses and Aims

5.2.1 Hypotheses

As inhibitory processing within the dorsal horn has been shown to mature over the first three postnatal weeks and be altered in aged animals, we expect to see changes in the effect of $GABA_A$ disinhibition throughout the life course. With an increase in the excitability at baseline and following peripheral stimulation with increasing age. Previous work suggests the superficial dorsal horn is a key site in $GABA_AR$ mediated inhibition, so we expect to see the majority of increases in this region at all ages.

Aims:

- To analyse neuronal activity across the in vivo dorsal horn following local $GABA_A$ disinhibition and see how this compares to previous patch clamp findings
- To investigate changes in $GABA_A$ inhibition in the dorsal horn throughout the life course

5.3 Methods

5.3.1 Animals

Male and female Sprague Dawley rats at postnatal day (P)14-16, P21-23, P43-56 (considered as young adults), and males at 18months old (considered as aged adults) were used for data presented in this chapter. Details of animal origin and housing can be found in Section 2.1.1. Group sizes, sex, weights, and specific groupings are detailed in Section 4.3.4.

5.3.2 Surgery and Electrode Placement

Rats were anaesthetised with isoflurane (Section 2.1.2) and placed on to a stereotaxic frame (Section 2.1.3). A laminectomy and meningostomy took place to expose the L4 and L5 spinal cord and the dura matter and arachnoid membranes carefully removed (Section 2.1.4). The electrode array was then placed as described in Section 2.1.5.

5.3.3 Drug Preparation and Application

Bicuculline methiodide was dissolved in saline to a stock concentration and placed into 15µl or 50µl aliquots then stored at -20°c until the experimental day. This was then made into serial dilutions of 0.12µg/µl, 0.06µg/µl, and 0.02µg/µl with saline for P14-16, P21-23, and young adult groups and to dilutions of 1.2µg/µl, 0.6µg/µl, and 0.2µg/µl with saline for the aged adult group. Aged adult doses were based on previous work conducted by the group (unpublished) and were the first experiments undertaken for this study. The same doses were attempted for the younger ages, however following a

reflex to any hindpaw stimulation in a neonate following the first dose the dose range was reduced by a factor of 10.

Bicuculline or saline was applied topically to the exposed spinal cord using a 50µl Hamilton syringe. Cumulative dosing was used with up to 3 doses being applied with a 50 minute gap between doses. The volume applied was 12µl for P14-16, 20µl for P21-23, and 50µl for both adult groups. As the P14-16 group often didn't have sufficient tissue around the laminectomy to hold liquid for a prolonged period of time a small amount of Vaseline to applied using a 1ml syringe and 25g needle around the outer/upper boarder of the laminectomy to allow the fluid to stay on the spinal cord.

5.3.4 Hindpaw Stimulation

The data presented in this chapter involves the application of mechanical and electrical stimuli to the hindpaw as described in Section 2.1.6. Stimulation trains were given before any drug doses were administered and 20 minutes after each dose. Trains consisted of 1 x 6g vFh, 1x 15g vFh, and 5x 1mA, 2ms, 0.016Hz electrical stimulation with a 5 minute interval between mechanical stimulations and a 10 minute interval between the mechanical and electrical stimulation. The next dose was applied 10 minutes after completion of the stimulation train to allow 50 minute intervals between drug applications. Timings were based on previous work by the group (unpublished).

5.3.5 Specific Study Design

Electrophysiological data presented in this chapter was obtained from two studies, one involving all P14 to young adult rats and a second involving the aged adults. Although both studies were paused for approximately 1 year during university COVID-19 lockdowns.

Table 5.1. Details of rats included in electrophysiological analysis in this chapter. Animals receiving saline or bicuculline doses are displayed separately. The number of animals combined in each age group (n), the split of males and females (m/f), and the average weight are included. Details of excluded data are stated below and not included in the table.

	Saline			Bicuculline		
Age	n	M/F	Weight (g) (Mean±SD)	n	M/F	Weight (g) (Mean±SD)
P14-16	6	3/3	36±4	6	3/3	38±3
P21-23	6	3/3	64±6	6	3/3	63±10
Young Adult	6	3/3	237±52	6	3/3	224±57
Aged Adult	6	6/0	500±36	11	11/0	535±36

Table 5.2. The number of animals included in analysis following each bicuculline dose. Some animals had reflexes to hindpaw stimulation following bicuculline application to the spinal cord at which point the study was terminated.

	Number of rats still included after each bicuculline dose		
Age	Dose 1	Dose 2	Dose 3
P14-16	6	6	6
P21-23	6	5	4
Young Adult	6	5	4
Aged Adult	11	11	9

Data was collected from 4 P9-11 rats (1 Saline, 3 Bicuculline) but not included; One rat received the original dose plan as given to the aged adults and had a reflex to hindpaw stimulation immediately prompting the decrease in the doses applied. Two other P9-11 rats received the lower doses of bicuculline, but one experiment had to be terminated early due to breathing issues and the other had a reflex to hindpaw stimulation following the second dose. We decided not to continue with testing at this age group. Data was incompletely collected from one young adult male due to breathing issues, and not included due to electrode drift from two P21-23 rats (both female) and two young adults (both male).

5.3.6 Data Analysis

Details of data collection and initial sorting are described in Section 2.1.7. Data analysis and statistical analysis were performed using Matlab 2017b and GraphPad Prism 7.05.

Data collected from studies discussed in Chapter 3 and 4 was used in power calculations to calculate the recommended group sizes of 6 for electrical responses and 10 for mechanical responses, when using both sexes and postnatal rats. These group sizes were calculated to give a power of 80% or higher to detect effect sizes of Cohen's d = 1.7 (electrical data) and 1.3 (mechanical data) between age groups using mean data with a with a significance level of $p<0.05$ using a two-tailed test. However, this was not designed to be sufficiently powered to compare differences between sexes. To sort the stimuli responses looked at in this chapter the 'Etho_MSP_Pipeline' script was used.

For baseline data the bin size was set as 1000ms, pre-stimulus time set as 60 seconds, the duration as 0seconds, and the post stimulation as 0seconds. Data was combined between animals using the 'Elec_Combine' script creating a 4D matrix. The activity was then summed over this 1 minute duration and mean responses and SEM were calculated for all animals within an age group following each bicuculline dose. Statistical comparisons between activity at each dose were made using a one-way ANOVA for each

age group followed by a Dunnett's post hoc test to compare differences to the pre-drug response.

For electrical data the bin size was set as 1ms, pre-stimulus time set as 0.1 second, the duration as 0seconds, and the post stimulation as 1second. For mechanical data the bin size was set as 100ms, pre-stimulus time set as 5 seconds, the duration as 5 seconds, and the post stimulation as 60 seconds. The data was baseline corrected and sorted into 'subsecond' and 'second' files. Data was sorted into regions, stimulation, and dose groups for analysis. Data was combined between animals using the 'Elec_Combine' script creating a 4D matrix. Statistical comparisons were made using the percentage change between the responses to a stimulus before any drug was applied and following each drug dose. The mean response and SEM was then found for all animals within an age group following each dose. These were then compared using one-way ANOVAs for each latency period or stimulus duration across the whole array and within specific regions followed by Tukey's post hoc tests. After confirming no statistically significant changes occurred following saline applications comparisons were not made between the saline and bicuculline groups. Although saline data is presented following electrical stimulations.

5.4 Results

5.4.1 Changes in Spontaneous Activity Following $GABA_A$ Disinhibition throughout the Life Course

As tonic GABAergic inhibition occurs in the dorsal horn (Takazawa and MacDermott, 2010; Lucas-Osma *et al.*, 2018) we investigated the effect of cumulative doses of the $GABA_AR$ antagonist bicuculline to the exposed dorsal horn on spontaneous dorsal horn activity. This resulted in a trend of increased spontaneous activity across the dorsal horn at every age but significantly increased spontaneous activity in the aged adult group (Figure 5.1, ($F(1.389, 13.89)=4.688$, $P<0.05$, RM one-way ANOVA). This difference was specifically significant between pre-drug activity and following dose 3 ($P<0.001$, Dunnett's post hoc). These findings support previous work which has shown an increase in tonic GABAergic inhibition over the postnatal period, however these studies have suggested mature levels are reached by P21 (Zell *et al.*, 2014) whereas our data suggests this occurs later.

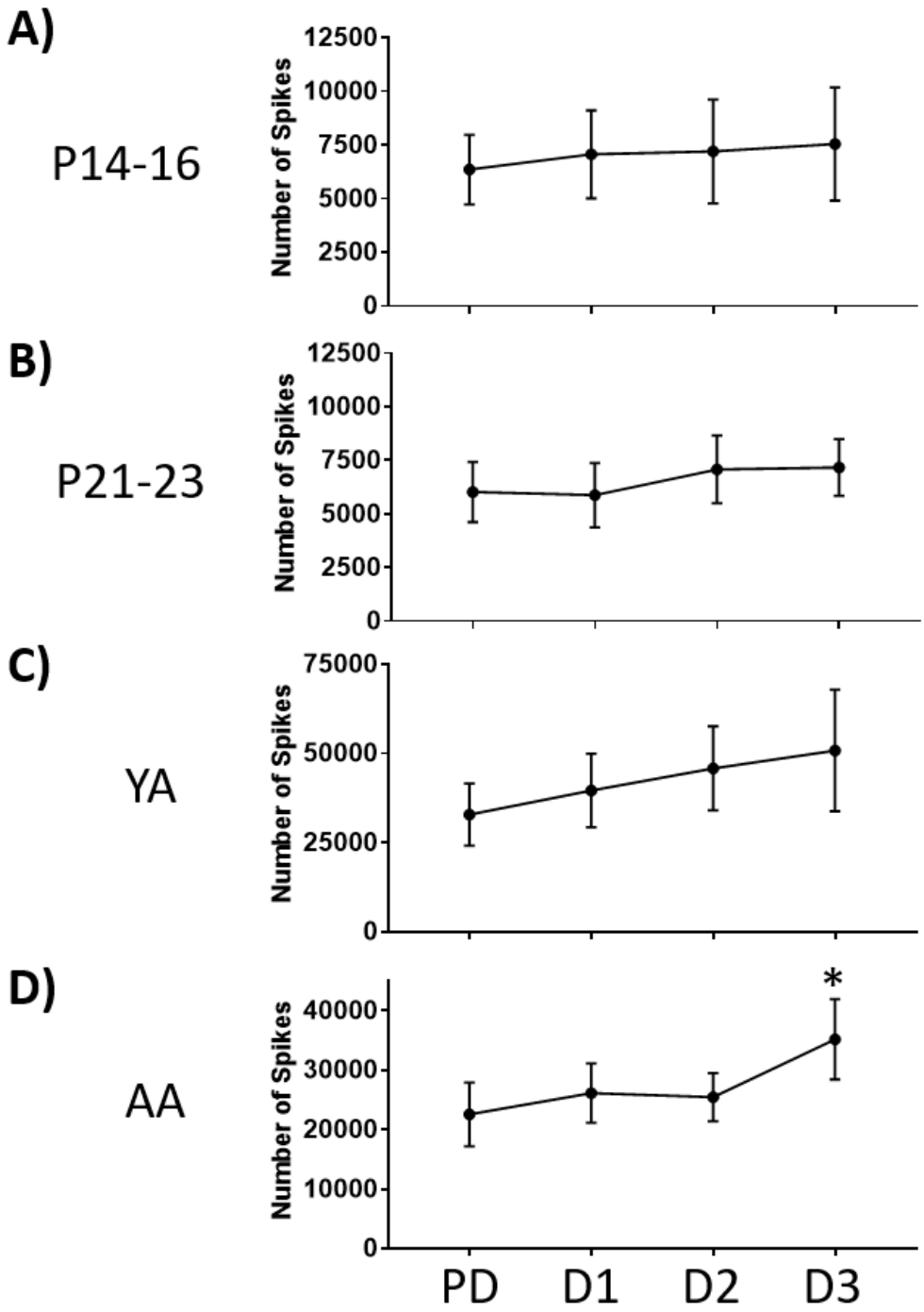


*Figure 5.1 Spontaneous activity in the dorsal horn following cumulative bicuculline doses. The sum of spike activity over 60 seconds before the test stimulation train began was compared at pre-drug (PD), and following bicuculline doses (D) 1-3 at A) P14-16 n=6, B) P21-23 n=6 for PD and D1, n=5 for D2, n=4 for D3, C)young adult (YA) n=6 for PD and D1, n=5 for D2, n=4 for D3, and D) aged adult (AA) n=11 for PD, D1 and D2, n=9 for D3. Data is combined across the whole array (mean response across the array), excluding electrode 1 in the P14-16 age group to better represent the size of the dorsal horn. Dose concentrations are 0.02µg/µl, 0.06µg/µl, and 0.12µg/µl apart from the aged adult group who received 0.2µg/µl, 0.6µg/µl, and 1.2µg/µl. Data is represented as the mean response of the age group ± SEM. * asterisk represents significant increases in spontaneous activity compared to pre-drug determined by RM one-way ANOVAs and post-hoc Dunnetts test (P<0.05).*

5.4.2 Changes in Evoked Activity Following $GABA_A$ Disinhibition throughout the Life Course

Postnatal Days 14-16

At P14-16, cumulative bicuculline doses resulted in a trend of increased dorsal horn activity following electrical stimulation of the hindpaw at 1mA (2ms, 0.016Hz). This increase is spike activity occurred in all regions of the dorsal horn during the A-fibre latency period (Figure 5.2C) although these increases are not statistically significant across the whole array (One-way ANOVA). A two-way RM ANOVA also found no dose dependent ($F(3, 15) = 2.217$, $P=0.13$) or region dependent changes ($F(2, 10) <1$), or interaction ($F(6, 30) <1$) following bicuculline dosing. Interestingly there is very little change following the first dose of bicuculline at this age. It is apparent on the heatmaps (Figure 5.2B) that there is an increase in the duration of evoked A-fibre activity throughout the dorsal horn particularly following the second dose of bicuculline suggesting $GABA_AR$ activity is involved in the shortened duration seen following electrical stimulation in the A-fibre latency period normally.

During the C-fibre latency period a two-way RM ANOVA also found no dose dependent ($F(3, 12) <1$) or region dependent ($F(2, 8) <1$) changes, or interaction ($F(6, 24) <1$) following bicuculline dosing. However, there does appear to be a trend of a general increase in dorsal horn activity across the whole array however there appears to be a slight decrease in the superficial and intermediate dorsal horn following the two lower doses of bicuculline (Figure 5.3C). During the C-fibre latency period the intensity of the response appears to increase within the first 50ms followed by a prolonged activity period in the deep dorsal horn (Figure 5.3B). Interestingly the short and intense C-fibre response observed at P14-16 is not under a significant amount of GABAergic modulation.

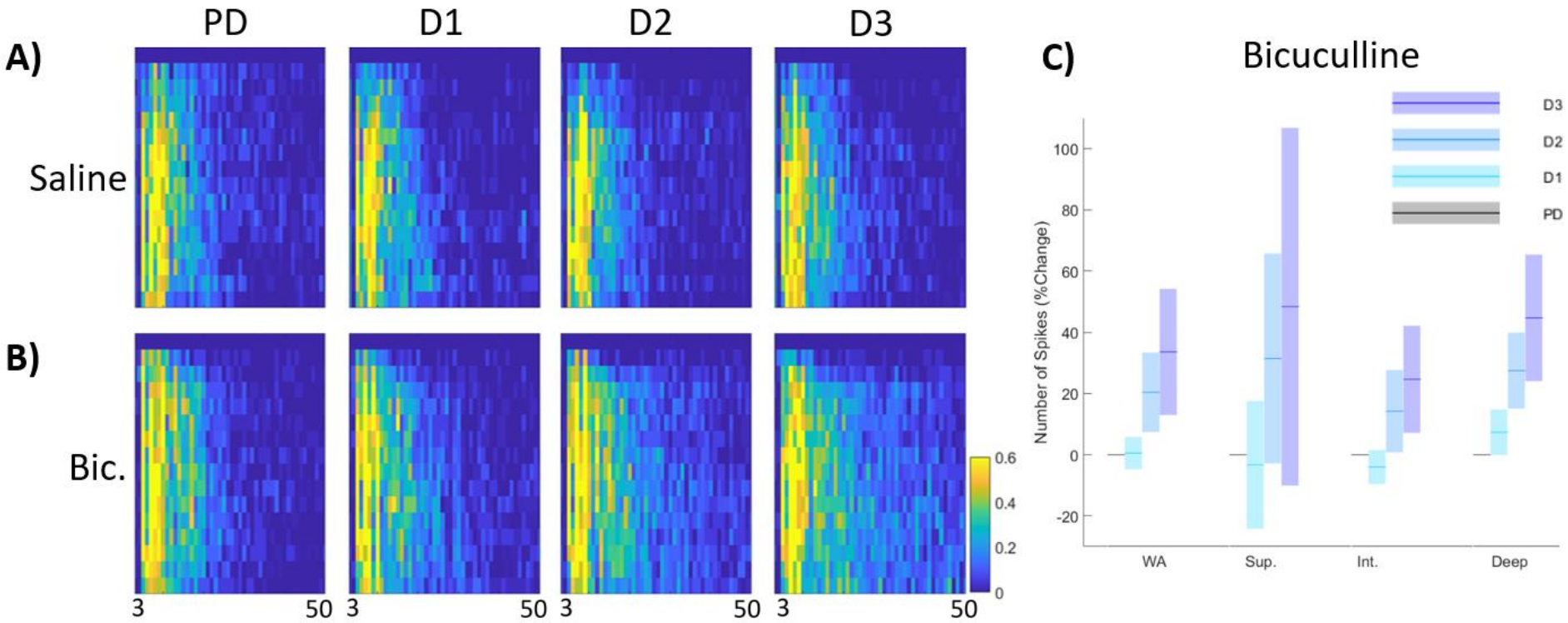


Figure 5.2. Dorsal horn responses during the A-fibre latency period following cumulative bicuculline doses at P14-16. Dorsal horn responses following electrical stimulation of the hindpaw at 1mA (2ms 0.016Hz) are shown before any drug or saline was applied (Pre-drug (PD)), and following three doses of saline (A) n=6, or three cumulative doses of bicuculline (B&C), n=6 for all doses. A&B) Heatmaps show spike activity at each electrode following electrical stimulation during the A-fibre latency period (3-50ms post stimulation) with bin sizes of 1ms. Responses shown are averaged responses from all animals in the age group. Electrodes are in order; 1 is the most dorsal and 16 the most ventral, electrode 1 is removed to better represent the size of the P14-16 dorsal horn. Colours represent mean number of spikes respective to the colour bar shown on the right. C) Summed spike activity during the A-fibre latency period across the whole array (WA), superficial (Sup.), intermediate (Int.) and deep dorsal horn regions (mean activity across the electrodes) is shown for animals receiving cumulative bicuculline doses as a percentage change from the pre-drug response, lines represent mean and shaded area represents SEM. One-way ANOVAs found no significant differences between the response (%change) following bicuculline doses when summed across the WA. A two-way RM ANOVA found no dose dependent or region dependent changes, or interaction.

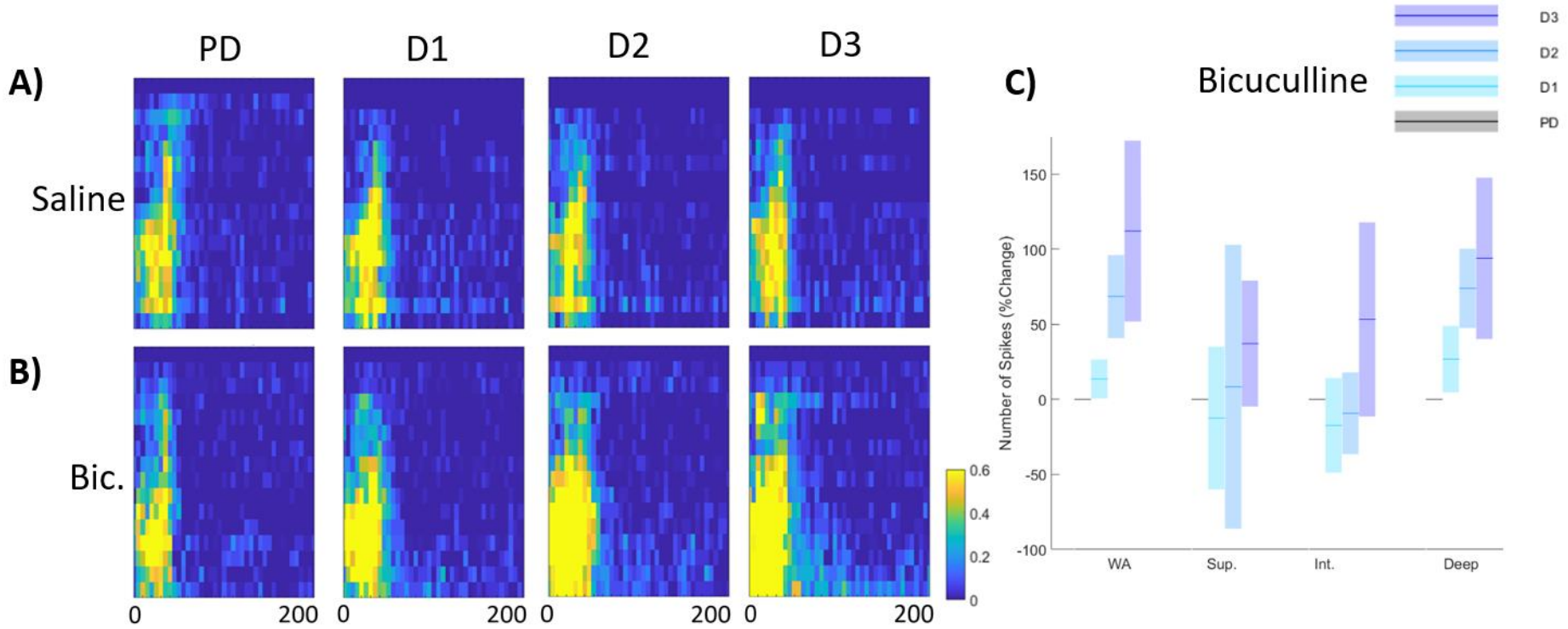


Figure 5.3. Dorsal horn responses during the C-fibre latency period following cumulative bicuculline doses at P14-16. Dorsal horn responses following electrical stimulation of the hindpaw at 1mA (2ms 0.016Hz) are shown before any drug or saline was applied (Pre-drug (PD)), and following three doses of saline (A) n=6, or three cumulative doses of bicuculline (B&C), n=6 for all doses. A&B) Heatmaps show spike activity at each electrode following electrical stimulation during the 200ms C-fibre latency period (75-275ms post stimulation) with bin sizes of 5ms. Responses shown are averaged responses from all animals in the age group. Electrodes are in order; 1 is the most dorsal and 16 the most ventral, electrode 1 is removed to better represent the size of the P14-16 dorsal horn. Colours represent mean number of spikes respective to the colour bar shown on the right. C) Summed spike activity during the A-fibre latency period across the whole array (WA), superficial (Sup.), intermediate (Int.) and deep dorsal horn regions (mean activity across the electrodes) is shown for animals receiving cumulative bicuculline doses as a percentage change from the pre-drug response, lines represent mean and shaded area represents SEM. One-way ANOVAs found no significant differences between the response (%change) following bicuculline doses when summed across the WA. A two-way RM ANOVA found no dose dependent or region dependent changes, or interaction..

Evoked dorsal horn responses to mechanical stimulation of the hindpaw tended to increase with increasing doses of bicuculline at P14-16 (Figure 5.4) with a two-way ANOVA showing statistically significant dose dependent changes ($F(3, 56) = 5.473$, $P<0.01$) but no regional changes ($F(2, 56) <1$) or interaction ($F(6, 56) <1$) following 6g vFh stimulation. However, there were no statistically significant dose-dependent changes ($F(3, 56) = 2.279$, $P=0.09$), regional changes ($F(2, 56) <1$), or interaction ($F(6, 56) <1$) following 15g stimulation. Interestingly, following the third dose there was a decline in the 6g response in the intermediate region (Figure 5.4E) and in the 15g response throughout the dorsal horn (Figure 5.4J). Line graphs showing the mechanical responses allow the maximum response recorded to be visualised. The maximum number of spikes during the onset and offset of the mechanical stimulus appears to increase in a dose-dependent manner as well the activity during the first 2 seconds of stimulus application following 6g stimulation but has a less clear dose dependent change following the 15g stimulation. Previous work using single-unit in-vivo recordings in adult WDR neurons have seen similar dose-dependent increases in neuronal activity to mechanical vFh stimulation of the hindpaw with cumulative spinal bicuculline doses (Rahman *et al.,* 2007). Suggesting that the $GABA_AR$ inhibition following peripheral mechanical stimulation is similar at the second postnatal week to in adults.

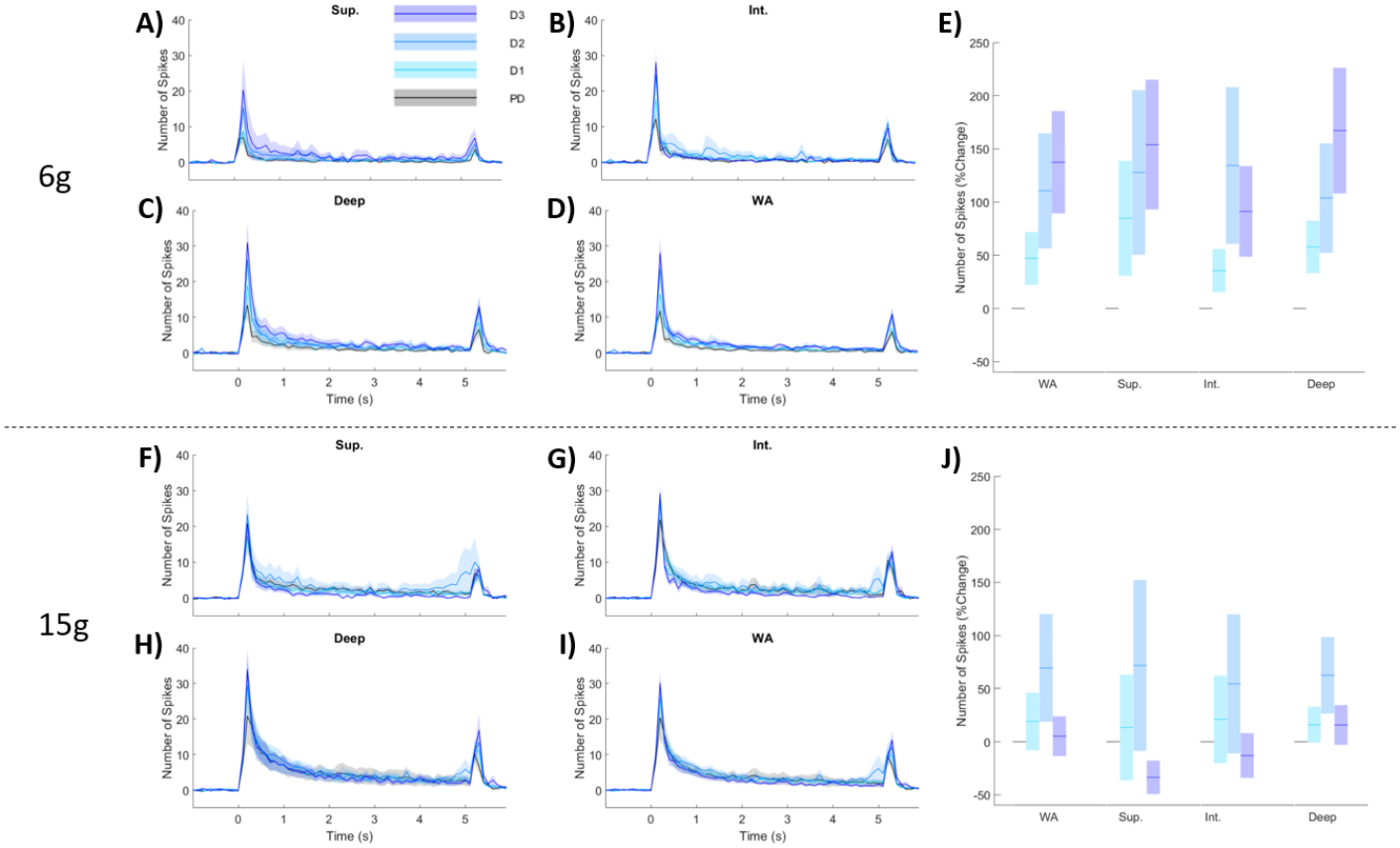


Figure 5.4. Dorsal horn responses to hindpaw mechanical stimulation following cumulative bicuculline doses at P14-16. Dorsal horn responses following mechanical stimulation of the hindpaw with 6g (A-E) and 15g (F-J) vFhs are shown before any drug was applied (Pre-drug (PD)), and following three cumulative doses of bicuculline, n=6 for all doses. Line graphs show spike activity during the 1second before stimulus application, the 5 seconds of application, and 1second post application in the A&F) superficial (Sup.), B&G) intermediate (Int.) and C&H) deep dorsal horn regions, and across the D&I) whole array (WA) (mean activity across the electrodes). E&J) Summed spike activity during the 5 seconds of mechanical stimulation is shown for animals receiving cumulative bicuculline doses as a percentage change from the pre-drug response, lines represent mean and shaded area represents SEM for line graphs and bar charts. No significant differences found. Two-way ANOVAs found significant dose-dependent differences but no regional differences or interaction following bicuculline doses in response to 6g but not 15g vFh hindpaw stimulation.

Postnatal Days 21-23

At the start of the third postnatal week there was a less pronounced response to bicuculline application in the A-fibre latency period (Figure 5.5) following electrical hindpaw stimulation than seen in the P14-16 age group. A one-way ANOVA comparing responses in the A-fibre latency period between doses found no statistically significant differences. Similarly, a two-way RM ANOVA also found no dose dependent ($F(3, 51) = 2.42$, $P=0.077$) or region dependent changes ($F(2, 51) <1$), or interaction ($F(6, 51) <1$) following bicuculline dosing in the A-fibre latency period. Although there was a general increase in the level of dorsal horn activity throughout the regions this was predominantly in the superficial region, however there was also a relatively large amount of variation between individuals in this age group.

Cumulative bicuculline doses also had little effect on the response during the C-fibre latency period with a one-way ANOVA comparing responses between doses showing no statistically significant differences and a two-way RM ANOVA showing no dose dependent ($F(3, 51) = 2.057$, $P=0.12$) or region dependent ($F(2, 51) <1$) changes, or interaction ($F(6, 51) <1$) following bicuculline dosing. However, there does appear to be a trend showing a decline in the number of evoked spikes in the C-fibre latency period following cumulative bicuculline doses (Figure 5.6). Although there may have been an increase following the first bicuculline dose the second and third dose resulted in less activity than recorded during the pre-drug train. Although it is worth noting that at P21-23 there was very little C-fibre response detected following 1mA hindpaw stimulation so any difference in this latency period may be harder to detect. Previous studies have found increased activity during the C-fibre latency period following bicuculline administration (Reeve, Dickenson and Kerr, 1998; Rahman *et al.*, 2007) suggesting that we may not be sufficiently activating C-fibres to see this response.

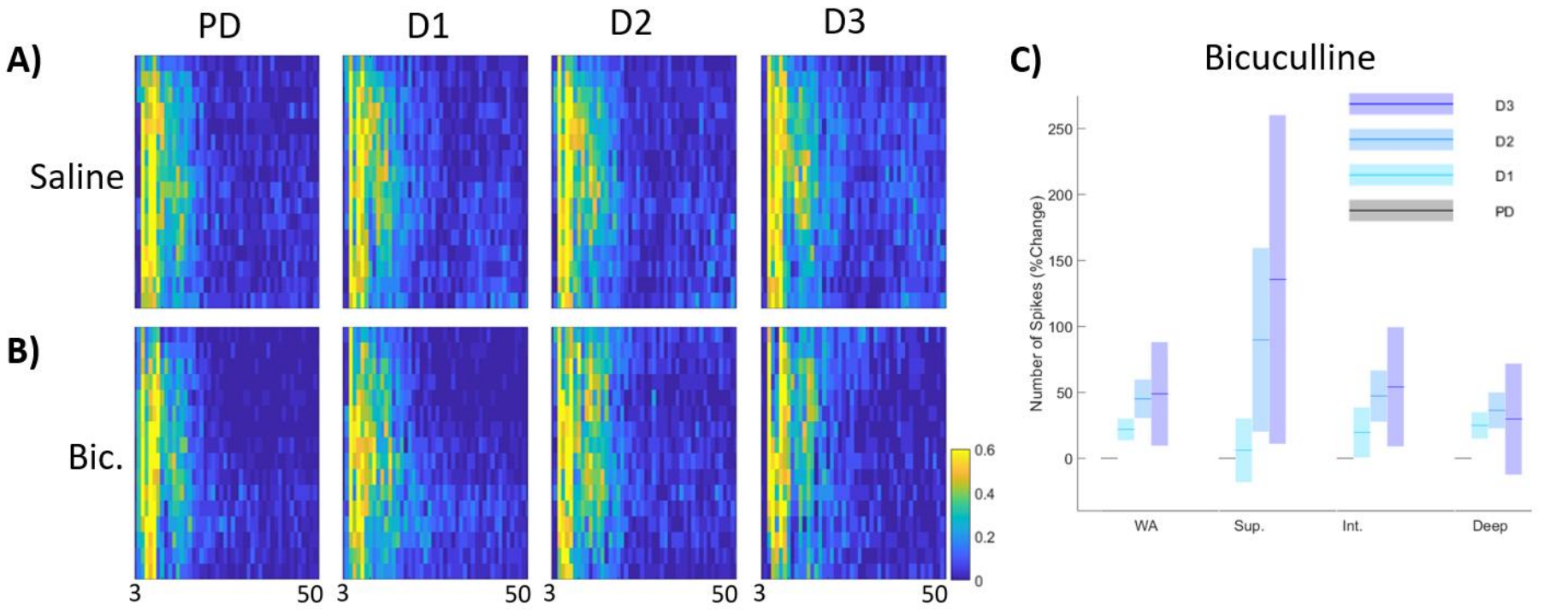


Figure 5.5. Dorsal horn responses during the A-fibre latency period following cumulative bicuculline doses at P21-23. Dorsal horn responses following electrical stimulation of the hindpaw at 1mA (2ms 0.016Hz) are shown before any drug or saline was applied (Pre-drug (PD)), and following three doses of saline (A) n=6, or three cumulative doses of bicuculline (B&C) n=6 for PD and D1, n=5 for D2, n=4 for D3. A&B) Heatmaps show spike activity at each electrode following electrical stimulation during the A-fibre latency period (3-50ms post stimulation) with bin sizes of 1ms. Responses shown are averaged responses from all animals in the age group. Electrodes are in order; 1 is the most dorsal and 16 the most ventral. Colours represent mean number of spikes respective to the colour bar shown on the right. C) Summed spike activity during the A-fibre latency period across the whole array (WA), superficial (Sup.), intermediate (Int.) and deep dorsal horn regions (mean activity across the electrodes) is shown for animals receiving cumulative bicuculline doses as a percentage change from the pre-drug response, lines represent mean and shaded area represents SEM. One-way ANOVAs found no significant differences between the response (%change) following bicuculline doses when summed across the WA. A two-way RM ANOVA found no dose dependent or region dependent changes, or interaction.

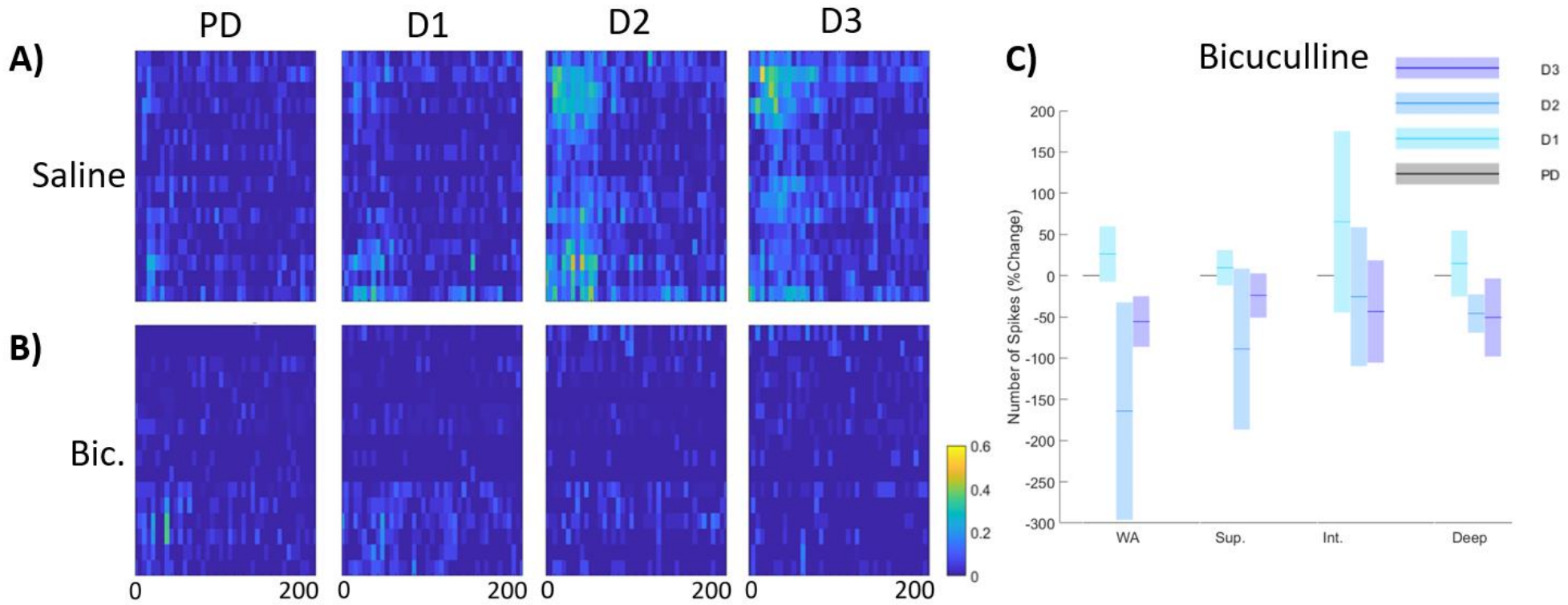


Figure 5.6. Dorsal horn responses during the C-fibre latency period following cumulative bicuculline doses at P21-23. Dorsal horn responses following electrical stimulation of the hindpaw at 1mA (2ms 0.016Hz) are shown before any drug or saline was applied (Pre-drug (PD)), and following three doses of saline (A) n=6, or three cumulative doses of bicuculline (B&C) n=6 for PD and D1, n=5 for D2, n=4 for D3. A&B) Heatmaps show spike activity at each electrode following electrical stimulation during the 200ms C-fibre latency period (95-295ms post stimulation) with bin sizes of 5ms. Responses shown are averaged responses from all animals in the age group. Electrodes are in order; 1 is the most dorsal and 16 the most ventral. Colours represent mean number of spikes respective to the colour bar shown on the right. C) Summed spike activity during the A-fibre latency period across the whole array (WA), superficial (Sup.), intermediate (Int.) and deep dorsal horn regions (mean activity across the electrodes) is shown for animals receiving cumulative bicuculline doses as a percentage change from the pre-drug response, lines represent mean and shaded area represents SEM. One-way ANOVAs found no significant differences between the response (%change) following bicuculline doses when summed across the WA. A two-way RM ANOVA found no dose dependent or region dependent changes.

Although there was a lack of change to the bicuculline doses in the evoked activity of the dorsal horn following electrical hindpaw stimulation at P21-23 there was a clearer increase in the responses following mechanical hindpaw stimulation, particularly 15g vFh stimulation, (Figure 5.7). Two-way ANOVAs found no dose-dependent differences ($F(3, 45) = 1.671$, $P=0.19$), region-dependent differences ($F(2, 45) <1$) or interaction ($F(6, 45) <1$) following 6g vFh stimulation. However, dose-dependent changes were found following 15g stimulation ($F(3, 55) = 5.72$, $P<0.01$), but not regional changes ($F(2, 55) = 1.854$, $P=0.17$) or an interaction ($F(6, 55) <1$). A post hoc Tukey's test showed a difference between the pre-drug response and the response following the third bicuculline dose ($P<0.01$). From the graphs there appears to be a dose-dependent increase in the number of spikes recorded during the onset and offset of the stimuli. In the superficial region following 15g stimulation there may also be an increase in the ongoing activity during the 5 seconds of stimulus application (Figure 5.7F). Interestingly, there also appears to be a second peak/shoulder following the initial onset after the highest bicuculline dose in the 6g and even more so in the 15g responses. To ensure this was not an error in the onset time of the vFh between animals (as discussed in Section 3.3.5) we confirmed that this second peak is occurring within the same animals, however, due to the nature of the population recordings we are unable to determine if this double activity peak is from the same cells or not. As a double peak has not, as far as we can tell, been identified before it may be that this second peak is from different but nearby cells that would normally be under GABAergic inhibition.

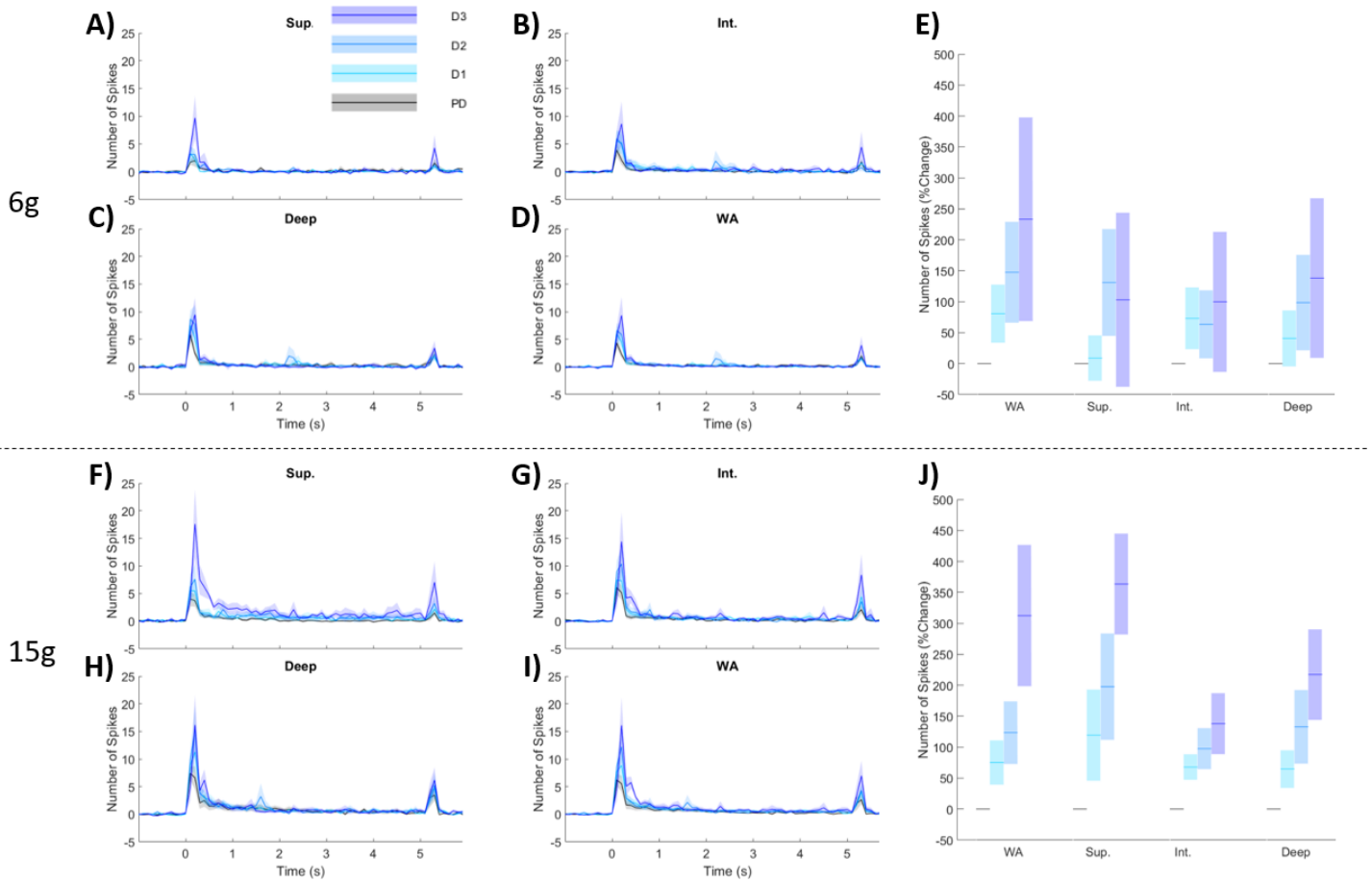


Figure 5.2. Dorsal horn responses to hindpaw mechanical stimulation following cumulative bicuculline doses at P21-23. Dorsal horn responses following mechanical stimulation of the hindpaw with 6g (A-E) and 15g (F-J) vFhs are shown before any drug was applied (Pre-drug (PD)), and following three cumulative doses of bicuculline, n=6 for PD and D1, n=5 for D2, n=4 for D3. Line graphs show spike activity during the 1second before stimulus application, the 5 seconds of application, and 1second post application in the A&F) superficial (Sup.), B&G) intermediate (Int.) and C&H) deep dorsal horn regions, and across the D&I) whole array (WA) (mean activity across the electrodes). E&J) Summed spike activity during the 5 seconds of mechanical stimulation is shown for animals receiving cumulative bicuculline doses as a percentage change from the pre-drug response, lines represent mean and shaded area represents SEM for line graphs and bar charts. No significant differences found. Two-way ANOVAs found no significant dose dependent or regional differences, or an interaction following 6g vFh stimulation but did show a dose-dependent difference in responses to 15g vFh hindpaw stimulation.

Young adult

Young adult responses to electrical hindpaw stimulation increased significantly with cumulative bicuculline doses. During the A-fibre latency period (Figure 5.8) a one-way ANOVA revealed there were dose dependent increases in the number of evoked spikes across the whole array ($F(3, 17)=6.124$, $P<0.01$). Similarly, a two-way RM ANOVA also found a dose dependent change ($F(3, 51) = 14.3$, $P<0.0001$) but no region dependent changes ($F(2, 51) <1$), or interaction ($F(6, 51) = 0.09623$, $P=0.99$) following bicuculline dosing in the A-fibre latency period. A Tukey's post hoc test showed differences between the pre-drug and dose 2 ($P<0.01$) and dose 3 ($P<0.0001$) responses, as well as between the dose 1 and dose 3 responses ($P<0.01$). As seen in the P14-16 age group the duration of the evoked response appears to increase with each bicuculline dose (Figure 5.8B) suggesting the response during the A-fibre latency period is normally under GABAergic inhibition.

During the C-fibre latency period (Figure 5.9) there were also increases in evoked activity following the cumulative bicuculline doses across the whole array ($F(3, 17)=4.391$, $P<0.05$, one-way ANOVA). A two-way RM ANOVA also found a dose dependent change ($F(3, 51) = 5.688$, $P<0.01$) but no region dependent changes ($F(2, 51) = 1.165$, $P=0.32$), or interaction ($F(6, 51) <1$) following bicuculline dosing in the C-fibre latency period. A Tukey's post hoc test showed differences between the pre-drug and dose 3 responses, ($P<0.01$), as well as between the dose 1 and dose 3 responses ($P<0.01$). Following the first bicuculline dose there was a decrease in the C-fibre latency period response compared to the pre-drug response in the intermediate and deep regions, although not statistically significant, as well as following the second dose in the deep region of the dorsal horn (Figure 5.9C), suggesting the dose dependent changes may be region specific.

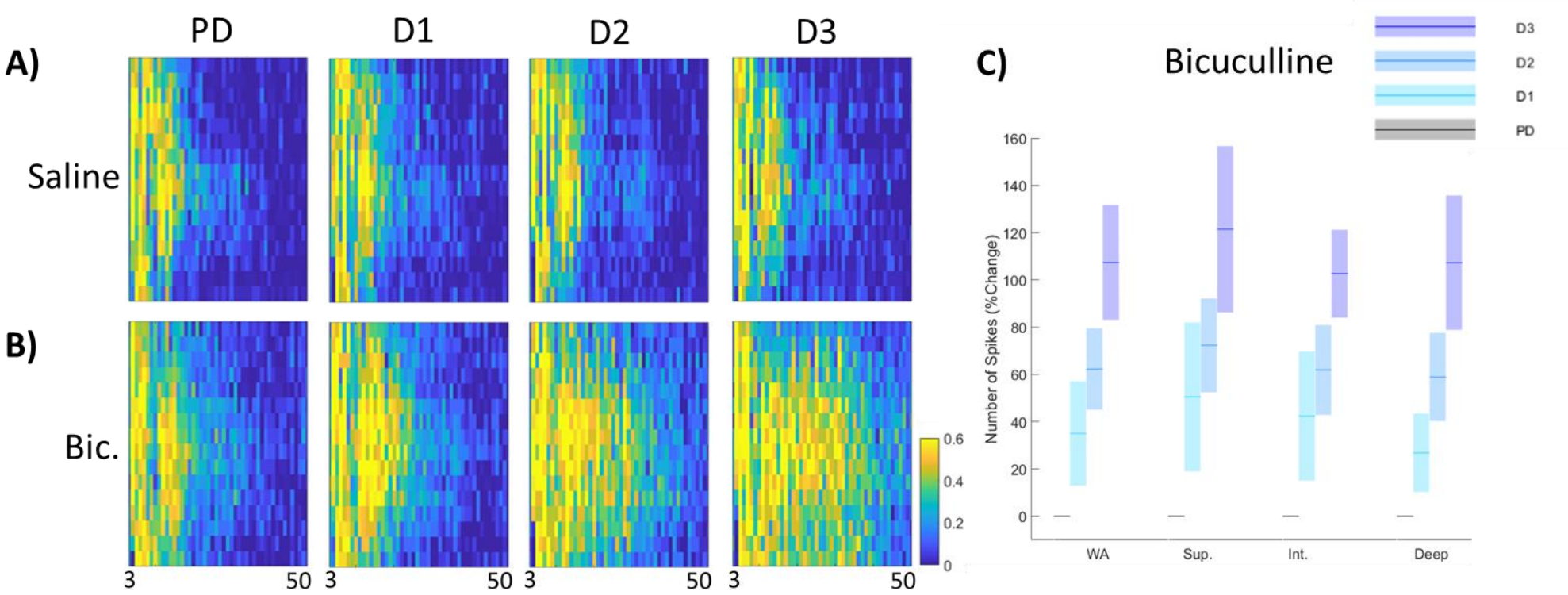


Figure 5.3. Dorsal horn responses during the A-fibre latency period following cumulative bicuculline doses in young adult rats. Dorsal horn responses following electrical stimulation of the hindpaw at 1mA (2ms 0.016Hz) are shown before any drug or saline was applied (Pre-drug (PD)), and following three doses of saline (A) n=6, or three cumulative doses of bicuculline (B&C), n=6 for PD and D1, n=5 for D2, n=4 for D3. A&B) Heatmaps show spike activity at each electrode following electrical stimulation during the A-fibre latency period (3-50ms post stimulation) with bin sizes of 1ms. Responses shown are averaged responses from all animals in the age group. Electrodes are in order; 1 is the most dorsal and 16 the most ventral. Colours represent mean number of spikes respective to the colour bar shown on the right. C) Summed spike activity during the A-fibre latency period across the whole array (WA), superficial (Sup.), intermediate (Int.) and deep dorsal horn regions (mean activity across the electrodes) is shown for animals receiving cumulative bicuculline doses as a percentage change from the pre-drug response, lines represent mean and shaded area represents SEM. One-way ANOVAs found a significant difference between the response (%change) following bicuculline doses when summed across the WA. A two-way RM ANOVA found significant dose dependent changes but not region dependent changes or interaction.

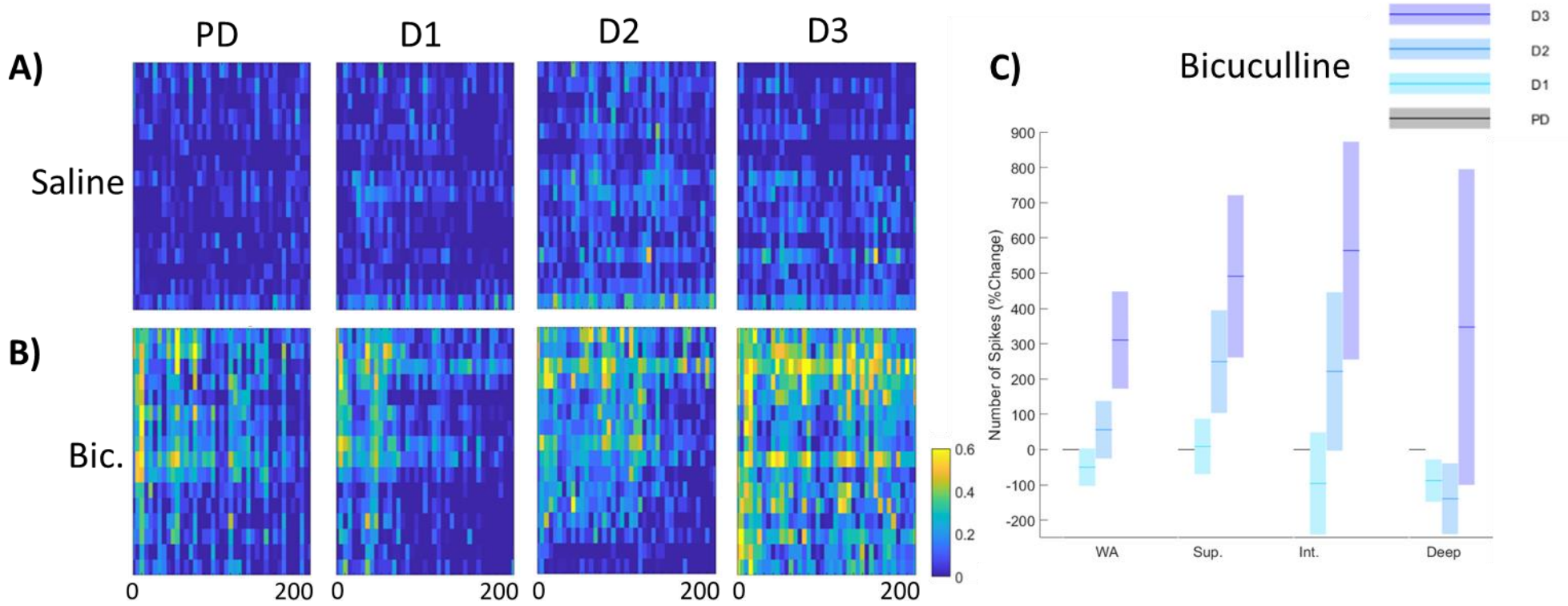


Figure 5.9. Dorsal horn responses during the C-fibre latency period following cumulative bicuculline doses in young adult rats. Dorsal horn responses following electrical stimulation of the hindpaw at 1mA (2ms 0.016Hz) are shown before any drug or saline was applied (Pre-drug (PD)), and following three doses of saline (A) n=6, or three cumulative doses of bicuculline (B&C), n=6 for PD and D1, n=5 for D2, n=4 for D3. A&B) Heatmaps show spike activity at each electrode following electrical stimulation during the 200ms C-fibre latency period (120-320ms post stimulation) with bin sizes of 5ms. Responses shown are averaged responses from all animals in the age group. Electrodes are in order; 1 is the most dorsal and 16 the most ventral. Colours represent mean number of spikes respective to the colour bar shown on the right. C) Summed spike activity during the A-fibre latency period across the whole array (WA), superficial (Sup.), intermediate (Int.) and deep dorsal horn regions (mean activity across the electrodes) is shown for animals receiving cumulative bicuculline doses as a percentage change from the pre-drug response, lines represent mean and shaded area represents SEM. One-way ANOVAs found significant differences between the response (%change) following bicuculline doses when summed across the WA. A two-way RM ANOVA found significant dose dependent changes but not region dependent changes or interaction.

Young adult dorsal horn responses to mechanical stimulation of the hindpaw showed region-dependent changes with cumulative bicuculline doses (6g: $F(2, 53) = 4.355$, $P<0.05$), 15g: $F(2, 53) = 3.841$, $P<0.05$, two-way ANOVAs, Figure 5.10). Post hoc Tukey's tests showed a significant difference in the superficial and deep dorsal horn responses following 6g and 15g vFh hindpaw stimulation ($P<0.05$). No dose-dependent changes ($F(3, 53) <1$) or interaction between dose and region were found ($F(6, 53) <1$) following the 6g stimulation. No interaction was found following 15g vFh stimulations ($F(6, 53) = 1.035$, $P=0.41$) but dose-dependent changes were identified ($F(3, 53) = 3.191$, $P<0.05$, two-way ANOVA) although a Tukey's test found no significant differences between specific dose responses. There was a general trend of an increased response in all regions of the dorsal horn following 15g vFh stimulation (Figure 5.10J) and in the superficial region following 6g vFh application (Figure 5.10E). However, in the intermediate and deep dorsal horn regions the 6g vFh responses were lower following all bicuculline doses compared to the pre-drug response but all were increased in the 15g vFh response. This may suggest a variation in the bicuculline effect depending on stimulus intensity linking with the previously identified difference in GABAergic disinhibition between Aβ- and Aδ-fibres with Aδ-fibres significantly increasing in their response (Rahman *et al.,* 2007). However, as an identified effect of disinhibition is the appearance of Aβ inputs which are normally inhibited this was unexpected (Torsney and MacDermott, 2006). Additionally although there is a slight dose-dependent increase in the peak response activity following mechanical stimulus onset it is much less defined than at P21-23 and the second peak at onset is only visible in the intermediate and deep regions following 6g vFh stimulation.

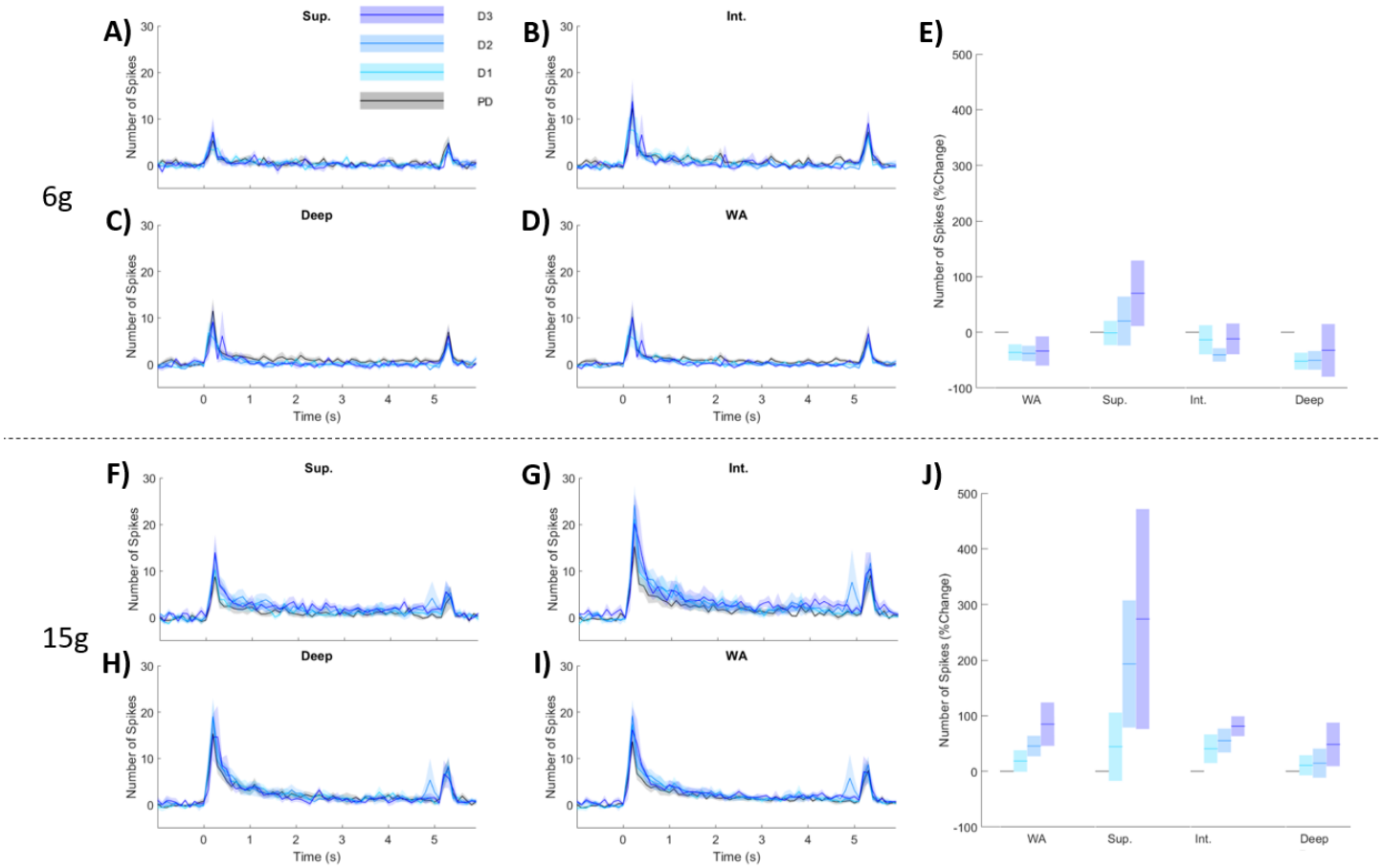


Figure 5.10. Dorsal horn responses to hindpaw mechanical stimulation following cumulative bicuculline doses in young adult rats. Dorsal horn responses following mechanical stimulation of the hindpaw with 6g (A-E) and 15g (F-J) vFhs are shown before any drug was applied (Pre-drug (PD)), and following three cumulative doses of bicuculline, n=6 for PD and D1, n=5 for D2, n=4 for D3. Line graphs show spike activity during the 1second before stimulus application, the 5 seconds of application, and 1second post application in the A&F) superficial (Sup.), B&G) intermediate (Int.) and C&H) deep dorsal horn regions, and across the D&I) whole array (WA) (mean activity across the electrodes). E&J) Summed spike activity during the 5 seconds of mechanical stimulation is shown for animals receiving cumulative bicuculline doses as a percentage change from the pre-drug response, lines represent mean and shaded area represents SEM for line graphs and bar charts. No significant differences found. Two-way ANOVAs found region-dependent differences following 6g and 15g vFh hindpaw stimulation, as well as dose-dependent changes following 15g vFh stimulation.

Aged Adult

It is important to note that the aged rats received a 10x higher concentration of bicuculline at each dose than the younger ages, however we believe this is comparable as reflexes to hindpaw stimulation were produced at the highest dose given at all ages (see Section 5.3.3). Following local bicuculline application the dorsal horn responses to electrical stimulation during the A-fibre latency period (Figure 5.11) were significantly increased across the dorsal horn ($F(3, 38)=20.02$, $P<0.0001$, One-way ANOVA). A two-way ANOVA found dose dependent ($F(3, 114) = 43.31$, $P<0.0001$) and region dependent differences ($F(2, 114) = 8.065$, $P<0.001$) in the A-fibre latency period response but no interaction ($F(6, 114) = 1.031$, $P=0.41$). Differences in dorsal horn responses were identified by a Tukey's post hoc test between the pre-drug dose and following all three bicuculline doses ($P<0.0001$), and between the dose 1 response compared to the second and third dose responses ($P<0.01$). There were also significant differences following a Tukey's test between the responses in the superficial region compared to the responses in the intermediate regions ($P<0.05$) and the deep region ($P<0.001$). There was however very little difference in the evoked response in any region between the second and third doses of bicuculline possibly suggesting that the maximum A-fibre latency period response was reached following the second dose.

During the C-fibre latency period no statistically significant changes were detected following cumulative bicuculline doses across the whole array (one-way ANOVA, Figure 5.12). A two-way ANOVA found region dependent differences ($F(2, 102) = 7.996$, $P<0.001$) no dose-dependent changes ($F(3, 102) <1$) or interaction ($F(6, 102) = 1.796$, $P=0.11$). Additionally a post hoc Tukey's test showed significant differences between the responses in the superficial region compared to the responses in the intermediate regions and the deep region ($P<0.01$). Although no statistically significant there was a trend of increased spike number in the superficial region with increasing bicuculline doses. Conversely, in the intermediate region, apart from an increase following the first dose there was a decline in dorsal horn activity with increased $GABA_A$ disinhibition and very little change in the deep region. Again this may in part be linked with a lack of C-fibre response to 1mA hindpaw stimulation.

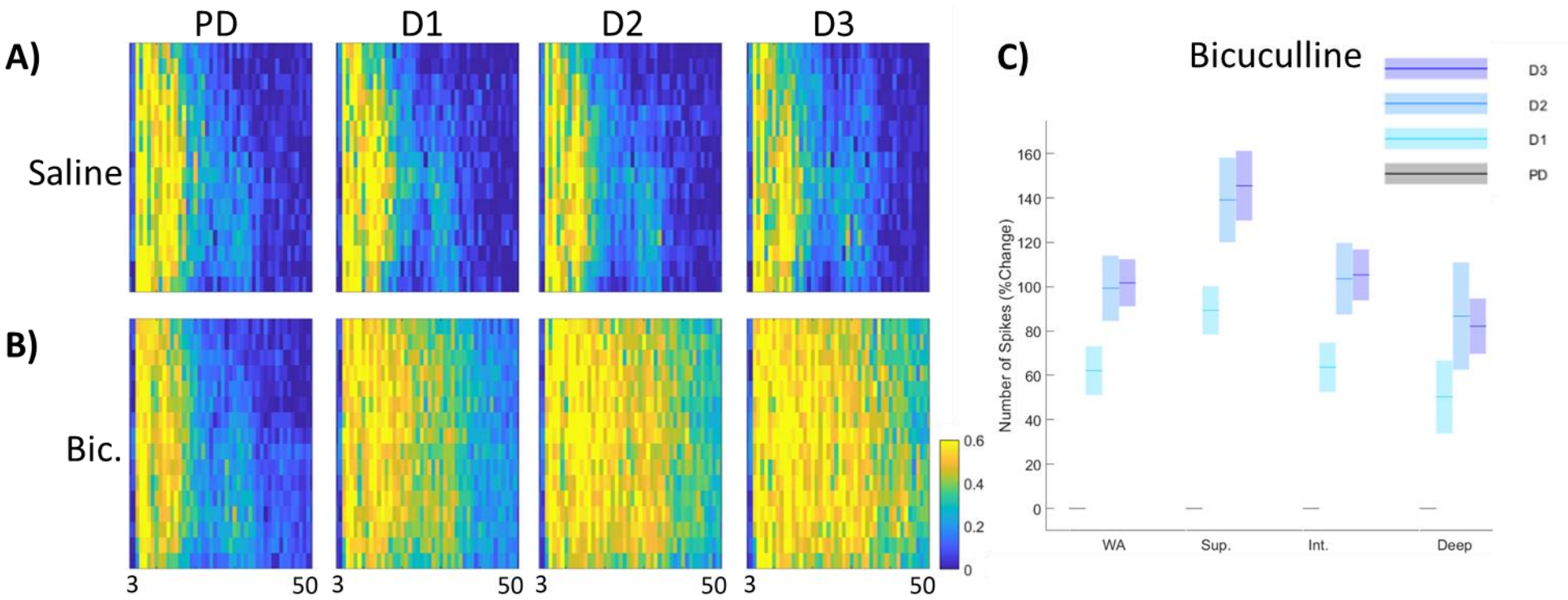


Figure 5.4. Dorsal horn responses during the A-fibre latency period following cumulative bicuculline doses in aged adult rats. Dorsal horn responses following electrical stimulation of the hindpaw at 1mA (2ms 0.016Hz) are shown before any drug or saline was applied (Pre-drug (PD)), and following three doses of saline (A) n=6, or three cumulative doses of bicuculline (B&C), n=11 for PD, D1 and D2, n=9 for D3. A&B) Heatmaps show spike activity at each electrode following electrical stimulation during the A-fibre latency period (3-50ms post stimulation) with bin sizes of 1ms. Responses shown are averaged responses from all animals in the age group. Electrodes are in order; 1 is the most dorsal and 16 the most ventral. Colours represent mean number of spikes respective to the colour bar shown on the right. C) Summed spike activity during the A-fibre latency period across the whole array (WA), superficial (Sup.), intermediate (Int.) and deep dorsal horn regions (mean activity across the electrodes) is shown for animals receiving cumulative bicuculline doses as a percentage change from the pre-drug response, lines represent mean and shaded area represents SEM. One-way ANOVAs found significant differences between the response (%change) following bicuculline doses when summed across the WA. A two-way RM ANOVA found significant dose dependent changes and region dependent changes but no interaction.

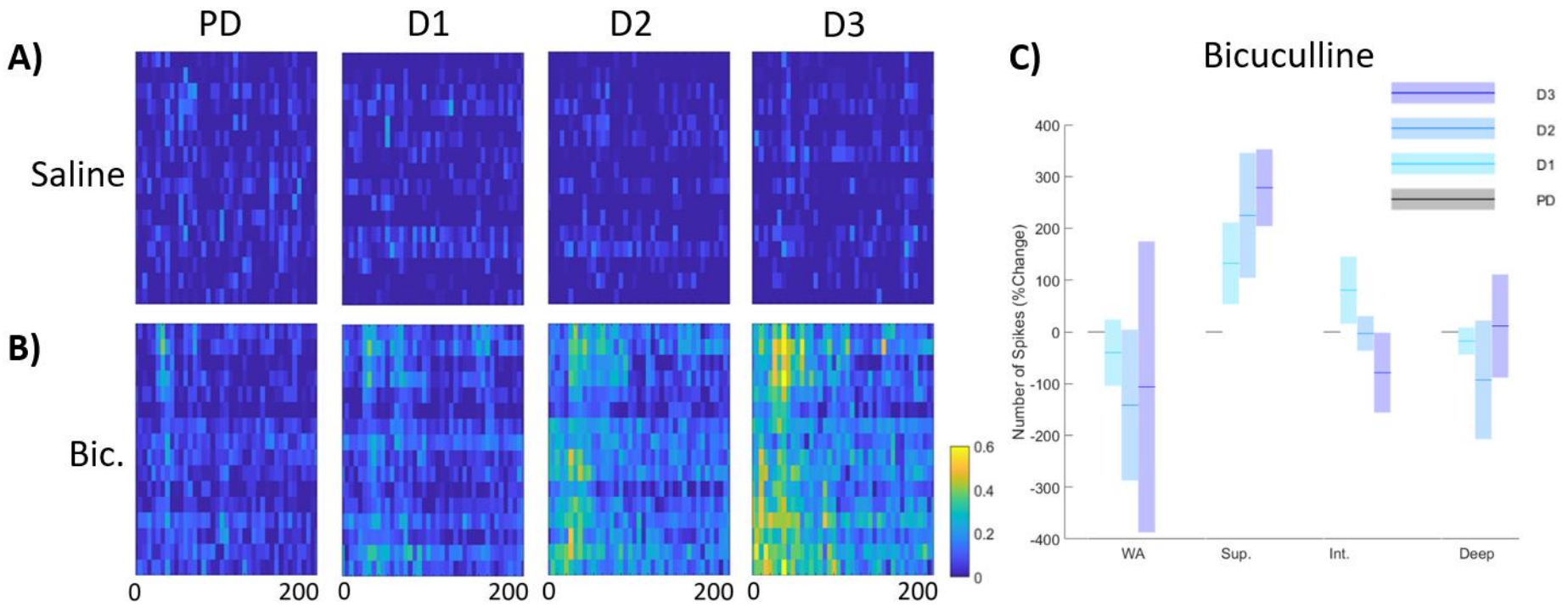


Figure 5.12. Dorsal horn responses during the C-fibre latency period following cumulative bicuculline doses in aged adults. Dorsal horn responses following electrical stimulation of the hindpaw at 1mA (2ms 0.016Hz) are shown before any drug or saline was applied (Pre-drug (PD)), and following three doses of saline (A) n=6, or three cumulative doses of bicuculline (B&C), n=11 for PD, D1 and D2, n=9 for D3. A&B) Heatmaps show spike activity at each electrode following electrical stimulation during the 200ms C-fibre latency period (130-330ms post stimulation) with bin sizes of 5ms. Responses shown are averaged responses from all animals in the age group. Electrodes are in order; 1 is the most dorsal and 16 the most ventral. Colours represent mean number of spikes respective to the colour bar shown on the right. C) Summed spike activity during the A-fibre latency period across the whole array (WA), superficial (Sup.), intermediate (Int.) and deep dorsal horn regions (mean activity across the electrodes) is shown for animals receiving cumulative bicuculline doses as a percentage change from the pre-drug response, lines represent mean and shaded area represents SEM. One-way ANOVAs found no significant differences between the response (%change) following bicuculline doses when summed across the WA. A two-way RM ANOVA found significant region dependent changes but not dose dependent changes or interaction.

Dorsal horn responses to mechanical hindpaw stimulation were interesting in the aged adult group (Figure 5.13). Following 6g vFh stimulation there was a dose dependent increase ($F(3, 115)=3.276$, $P<0.05$, two-way ANOVA), specifically between the pre-drug and dose 2 and 3 responses ($P<0.05$, Tukey's post hoc test). No regional differences ($F(2, 115) = 1.704$, $P=0.19$) or interaction ($F(6, 115) <1$) were found following 6g stimulations. Following the 15g vFh stimulations there appeared to be a trend of increased activity with each dose across the whole array and deep region, but very little change between doses in the superficial and intermediate regions. A two-way ANOVA found dose-dependent ($F(3, 103) = 3.546$, $P<0.05$) and region dependent ($F(2, 103) = 3.511$, $P<0.05$) differences, but no interaction ($F(6, 103) <1$), following cumulative bicuculline doses. Post hoc Tukey's test showed a significant difference between the pre-drug and dose 3 response ($P<0.05$), and between the superficial and intermediate regions ($P<0.05$). Additionally, we again see a higher maximum response and second peak or shoulder, following stimulus onset in the aged adults (Figure 5.13), but this appears to be occurring following all bicuculline doses.

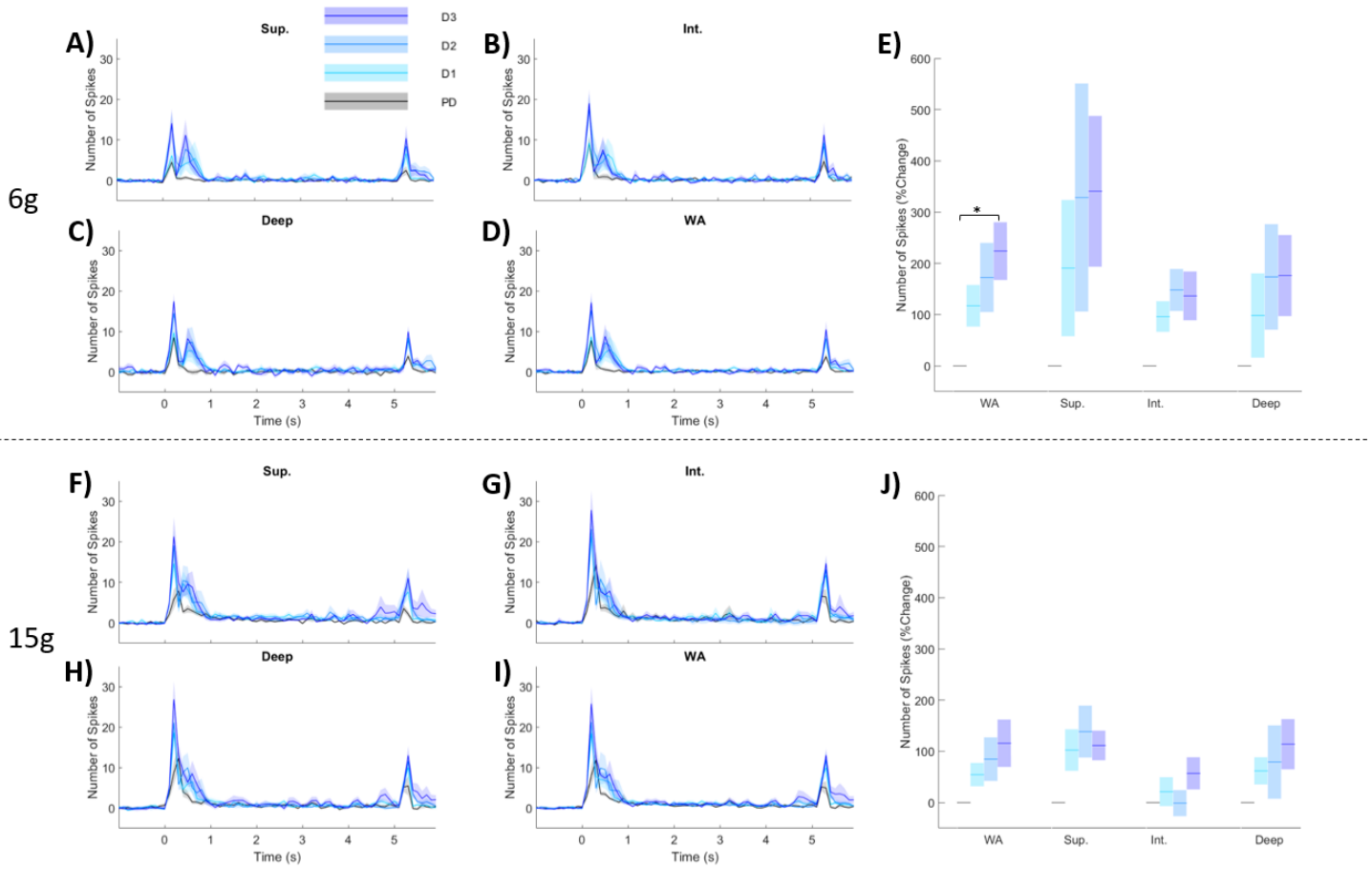


Figure 5.13. Dorsal horn responses to hindpaw mechanical stimulation following cumulative bicuculline doses in aged adult rats. Dorsal horn responses following mechanical stimulation of the hindpaw with 6g (A-E) and 15g (F-J) vFhs are shown before any drug was applied (Pre-drug (PD)), and following three cumulative doses of bicuculline, n=11 for PD, D1 and D2, n=9 for D3. Line graphs show spike activity during the 1second before stimulus application, the 5 seconds of application, and 1second post application in the A&F) superficial (Sup.), B&G) intermediate (Int.) and C&H) deep dorsal horn regions, and across the D&I) whole array (WA) (mean activity across the electrodes). E&J) Summed spike activity during the 5 seconds of mechanical stimulation is shown for animals receiving cumulative bicuculline doses as a percentage change from the pre-drug response, lines represent mean and shaded area represents SEM for line graphs and bar charts. No significant differences found. Two-way ANOVAs foundsignificant dose dependent differences between the response (%change) following bicuculline doses after 6g and 15g vFh hindpaw stimulation, as well as regional difference following 15g stimulation.

5.4.3 Differences across the Life Course following $GABA_AR$ Disinhibition

As mentioned previously the aged adult group received a 10x higher concentration of bicuculline at each dose than the younger ages. Although this means we should take caution in directly comparing between aged we believe these doses are comparable as some rats in each age group had reflexes to hindpaw stimulation following application of the highest dose they received (see Section 5.3.3). As such the aged adult data has been included in comparisons between the other ages (Figures 5.14-5.18) which use the percentage change in the response compared to the pre-drug values at each age, as the effect of bicuculline is being compared not the response to the specific stimuli used.

Dorsal Horn Processing of Peripheral Electrical Stimulation

Dorsal horn responses to electrical stimulation within the A-fibre latency period showed significant dose and age dependent differences, but no interaction, following two-way ANOVAs in the superficial (dose: $F(3, 92)=9.587$, $P<0.0001$, age: $F(3, 92)=4.562$, $P<0.01$, interaction: $F(9, 92) <1$) intermediate (dose: $F(3, 92)=15.46$, $P<0.0001$, age: $F(3, 92)=12.63$, $P<0.0001$, interaction: $F(9, 92) = 1.67$, $P=0.11$), and deep (dose: $F(3, 92)=10.69$, $P<0.0001$, age: $F(3, 92)=4.547$, $P<0.01$, interaction: $F(9, 92) = 1.011$, $P=0.44$) dorsal horn. Although the majority of age-related differences were identified in the intermediate region (Figure 5.14B) with differences between the P14-16 and aged adult groups found following all bicuculline doses (D1: $P<0.01$, D2: $P=0.0001$, D3: $P<0.05$) as well as significant increases in the response between P21-23 and aged adults following the second bicuculline doses ($P<0.05$) and between P14-16 and the young adults after the third dose ($P<0.05$). Additionally, statistically significant increase in dorsal horn activity were seen between the P14-16 and aged adult groups following the second bicuculline dose in the superficial and deep dorsal horn regions ($P<0.05$). Following the first and second bicuculline doses the effect of GABAergic disinhibition in the dorsal horn increases with increasing age however following the third dose the two adult groups display a similar response in all regions and the deep dorsal horn responses possibly suggest a age-related change between P21-23 and the young adults with similar responses seen in the two youngest and two oldest age groups (Figure 5.14C).

In the C-fibre latency period (Figure 5.15) statistically significant dose-related changes in the dorsal horn response were found in the superficial region only (Superficial: $F(3, 80)=3.533$, $P<0.05$; Intermediate: $F(3, 84) = 1.334$, $P=0.27$; Deep: $F(3, 84) = 1.558$, $P=0.21$, two-way ANOVA) and age-related differences found only in the superficial ($F(3, 80)=5.142$, $P<0.01$) and intermediate ($F(3, 84)=5.142$, $P<0.01$) but not the deep ($F(3, 84) <1$) regions of the dorsal horn. The intermediate region also showed an interaction in the response between the age and dose ($F(9, 84)=2.794$, $P<0.01$) but no interaction was found in the superficial ($F(9, 80) = 1.512$, $P=0.16$) or deep regions ($F(9, 84) <1$).

Specifically age-related differences were found using a Tukey's post hoc test between the P21-23 and aged adult responses following the second bicuculline dose in the superficial horn ($P<0.05$) and following the third bicuculline dose between the young adult responses compared to the P14-16 responses ($P<0.05$) and the P21-23 responses ($P<0.01$). In the intermediate region the young adult response following the third bicuculline dose was significantly higher compared to the three other ages (P14-16 and P21-23 $P<0.01$, aged adults $P<0.0001$). This is because the other three age groups had either a very small increase in activity following the maximal $GABA_aR$ disinhibition (P14-16) or a decrease in activity (P21-23 and aged adults). No statistically significant age-dependent or dose -dependent differences were found in the deep dorsal horn using a two-way ANOVA. It is also interesting to note that many of the C-fibre latency period responses were below the pre-drug activity levels particularly following the higher bicuculline doses.

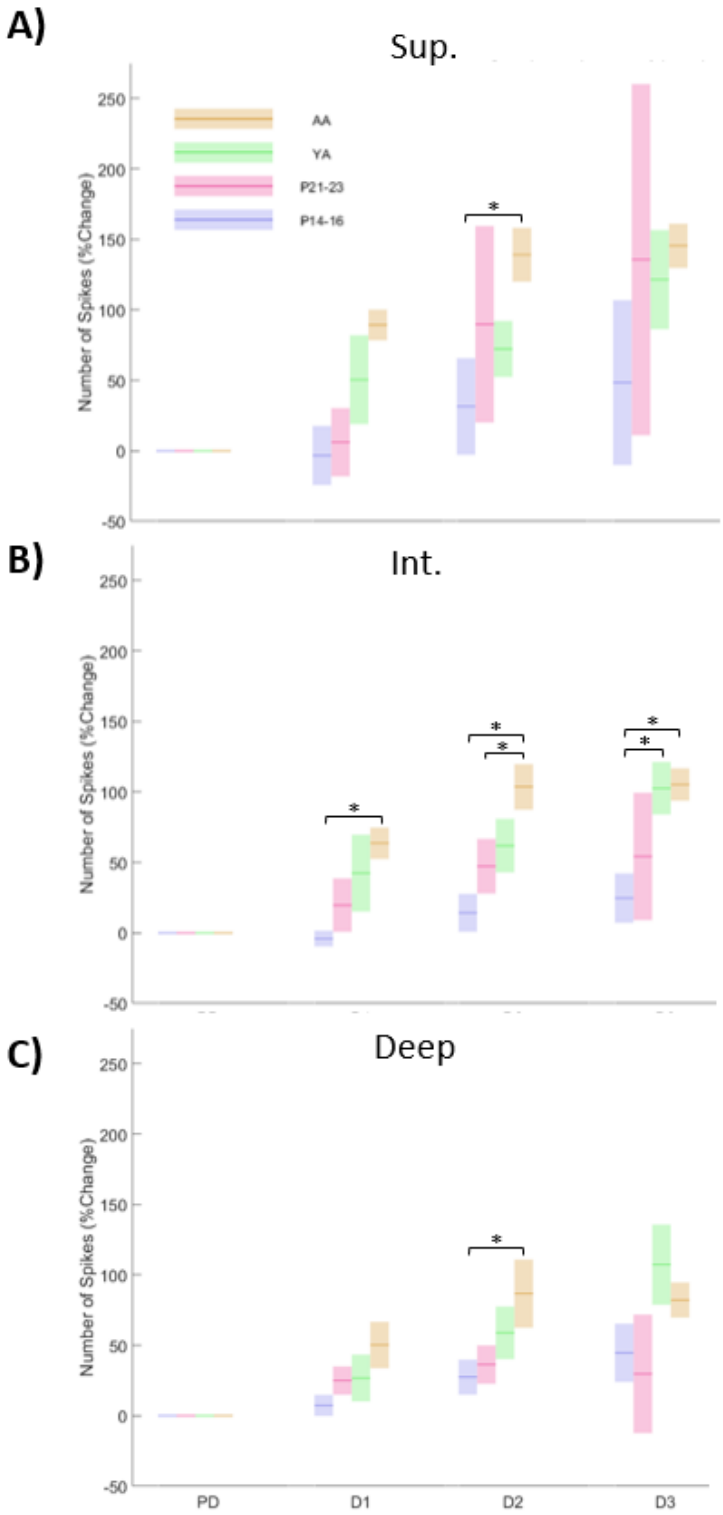


*Figure 5.14. Age-dependent changes in dorsal horn responses occur during the A-fibre latency period following cumulative bicuculline doses in all regions of the dorsal horn. Summed spike activity during the A-fibre latency period (3-50ms post stimulation) across the A) superficial (Sup.), B) intermediate (Int.) and C) deep dorsal horn regions (mean activity across the electrodes) following electrical stimulation of the hindpaw at 1mA (2ms 0.016Hz) is shown for animals receiving 3 cumulative bicuculline doses (D1-3) as a percentage change from the pre-drug (PD) response. P14-16 n=6 for all doses, P21-23 n=6 for PD and D1, n=5 for D2, n=4 for D3, young adult (YA) n=6 for PD and D1, n=5 for D2, n=4 for D3, and aged adult (AA) n=11 for PD, D1 and D2, n=9 for D3. Lines represent mean and shaded area represents SEM. Two-way ANOVAs found significant differences between the response (%change) following bicuculline doses in all regions * asterisk represent significant differences following Tukey's post hoc tests (P<0.05).*

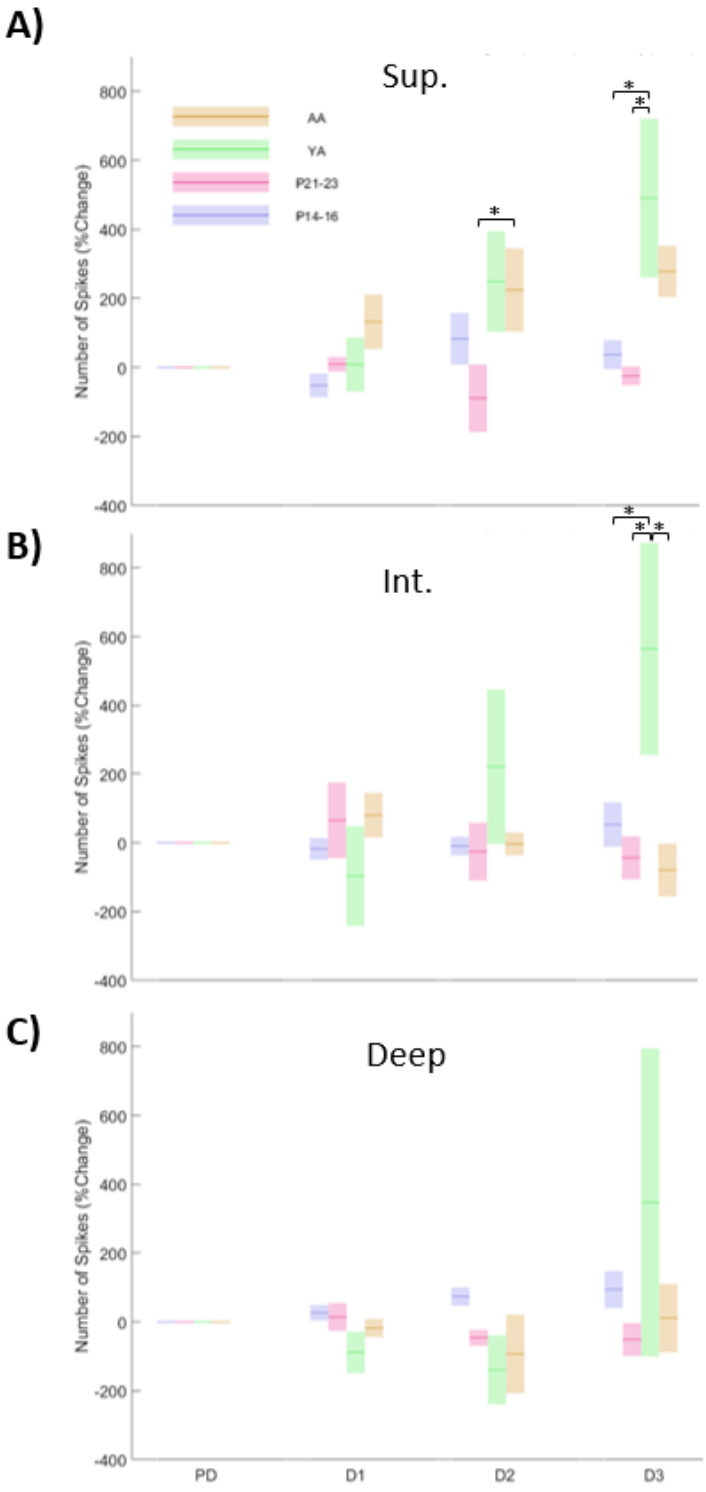


*Figure 5.15. Age-dependent changes in dorsal horn responses occur during the C-fibre latency period following cumulative bicuculline doses in the superficial and intermediate regions of the dorsal horn. Summed spike activity during the C-fibre latency period (200ms post stimulation for all ages with the post-stimulation onset dependent on age (Table 4.3)) across the A) superficial (Sup.), B) intermediate (Int.) and C) deep dorsal horn regions (mean activity across the electrodes) following electrical stimulation of the hindpaw at 1mA (2ms 0.016Hz) is shown for animals receiving 3 cumulative bicuculline doses (D1-3) as a percentage change from the pre-drug (PD) response. P14-16 n=6 for all doses, P21-23 n=6 for PD and D1, n=5 for D2, n=4 for D3, young adult (YA) n=6 for PD and D1, n=5 for D2, n=4 for D3, and aged adult (AA) n=11 for PD, D1 and D2, n=9 for D3. Lines represent mean and shaded area represents SEM. Two-way ANOVAs found significant differences between the response (%change) following bicuculline doses in all regions * asterisk represent significant differences following Tukey's post hoc tests (P<0.05).*

Dorsal Horn Processing of Peripheral Mechanical Stimulation

When comparing the response across the whole array dose-dependent and age-dependent differences, but no interaction, were found following the 6g vFh hindpaw stimulations (dose: $F(3, 93)=4.782$, $P<0.01$, age: $F(3, 93)=6.601$, $P<0.001$, interaction: $F(9, 93) <1$, Figure 5.16A) and the 15g vFh stimulations (dose: $F(3, 96)=5.714$, $P<0.01$, age: $F(3, 96)=3.66$, $P<0.05$, interaction: $F(9, 96) = 1.405$, $P=0.20$, Figure 5.16B). Following the 6g vFh stimulations there was very little increase in the young adult age group following any bicuculline dose compared to the pre-drug response. As such this age group is significantly different to all of the other ages in the effect of $GABA_AR$ disinhibition following innocuous stimulation and statistically significant compared to the aged adult group following the second bicuculline doses ($P<0.05$) and compared to the P21-23 ($P<0.05$) and aged adults ($P<0.01$) following the third dose. Apart from the young adult group there is in general a trend of increased dorsal horn activity following each cumulative dose and increasing with age reflecting the general trend seen in the A-fibre electrical responses.

Looking at the 6g responses in each region of the dorsal horn (Figure 5.17) the young adult response is low across all three regions significantly but to a lesser extent in the superficial region where it is more closely matched to the P21-23 age group following the first and third bicuculline doses. Significant dose and age differences, but no interaction, were found using two-way ANOVAs in the intermediate region (dose: $F(3, 88)=2.892$, $P<0.05$, age: $F(3, 88)=4.931$, $P<0.01$, interaction: $F(9, 88) <1$) and only age-dependent differences were found in the deep region (age: $F(3,93)=6.601$, $P<0.05$, dose: $F(3, 93) = 1.781$, $P=0.16$, interaction: $F(9, 93) <1$). No dose dependent ($F(3, 88) = 1.268$, $P=0.29$) or age dependent ($F(3, 88) = 2.033$, $P=0.12$) changes, or interaction ($F(9, 88) <1$) were detected in the superficial region. A post hoc Tukey's test also found significant differences in the intermediate region following the second bicuculline dose between the young adults and the P14-16 group ($P<0.05$) and the aged adults ($P<0.01$). Apart from the young adult group there were relatively consistent increases in dorsal horn activity in the intermediate and deep regions in between the other ages, particularly following the highest bicuculline dose.

Following the 15g vFh stimulation of the hindpaw the young adult responses were consistently lower than the P21-23 and aged adult groups but not as dramatically as following the 6g vFh stimulations. The largest increase in activity following bicuculline application compared to the pre-drug responses appears to be in the P21-23 age group, especially at the highest dose where the response was statistically significant compared to the other three ages (P14-16 $P<0.001$, both adult groups $P<0.05$). This may suggest that GABAergic inhibition is not mature at this age contrary to previous studies (Ingram,

Fitzgerald and Baccei, 2008; Zell *et al.*, 2014). When comparing within the different dorsal horn regions significant dose and age effects, but not an interaction, were found in the superficial (dose: $F(3, 87)=5.424$, $P<0.001$, age: $F(3, 87)=3.353$, $P<0.05$, interaction: $F(9, 87) = 1.15$, $P=0.34$) and intermediate region (dose: $F(3, 88)=3.212$, $P<0.05$, age: $F(3, 88)=2.998$, $P<0.05$, interaction: $F(9, 88) <1$) but no significant differences in response were found in the deep dorsal horn (dose: $F(2, 68) = 1.264$, $P=0.29$, age: $F(3, 68) = 2.679$, $P=0.05$, interaction: $F(6, 68) <1$). Age-related differences were only found following the third bicuculline dose between the P14-16 group and the P21-23 group (superficial $P<0.01$, intermediate $P<0.05$) and young adults (superficial $P<0.05$). Increases in dorsal horn activity following each bicuculline dose was not seen with increasing age suggesting differences in the effect of GABAergic disinhibition based on stimulus intensity. This supports previous findings that the effect of $GABA_AR$ disinhibition varies between Aβ-, Aδ-, and C-fibre activation as well as between brush and more intense mechanical stimulations (Rahman *et al.,* 2007).

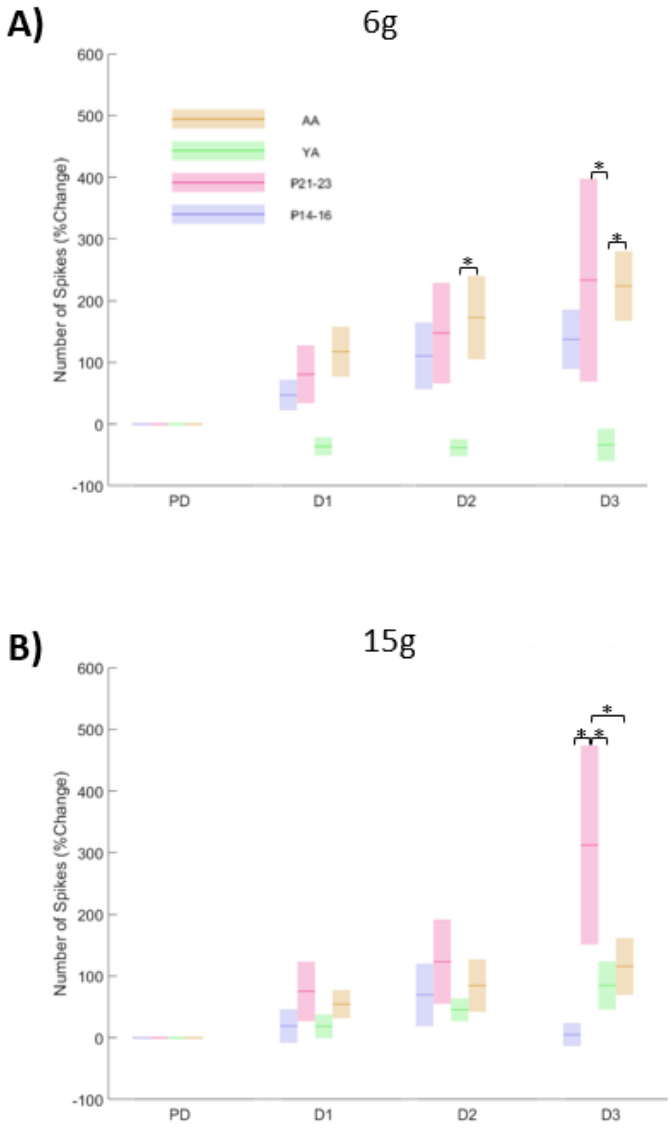


*Figure 5.16. Age-dependent changes in dorsal horn responses to mechanical hindpaw stimulation occur following cumulative bicuculline doses. Summed spike activity during the 5seconds of stimulus application across the whole array (mean activity across the electrodes) following A) 6g and B)15g vFh stimulation of the hindpaw is shown for animals receiving 3 cumulative bicuculline doses (D1-3) as a percentage change from the pre-drug (PD) response. P14-16 n=6 for all doses, P21-23 n=6 for PD and D1, n=5 for D2, n=4 for D3, young adult (YA) n=6 for PD and D1, n=5 for D2, n=4 for D3, and aged adult (AA) n=11 for PD, D1 and D2, n=9 for D3. Lines represent mean and shaded area represents SEM. Two-way ANOVAs found significant differences between the response (%change) following bicuculline doses * asterisk represent significant differences following Tukey's post hoc tests (P<0.05).*

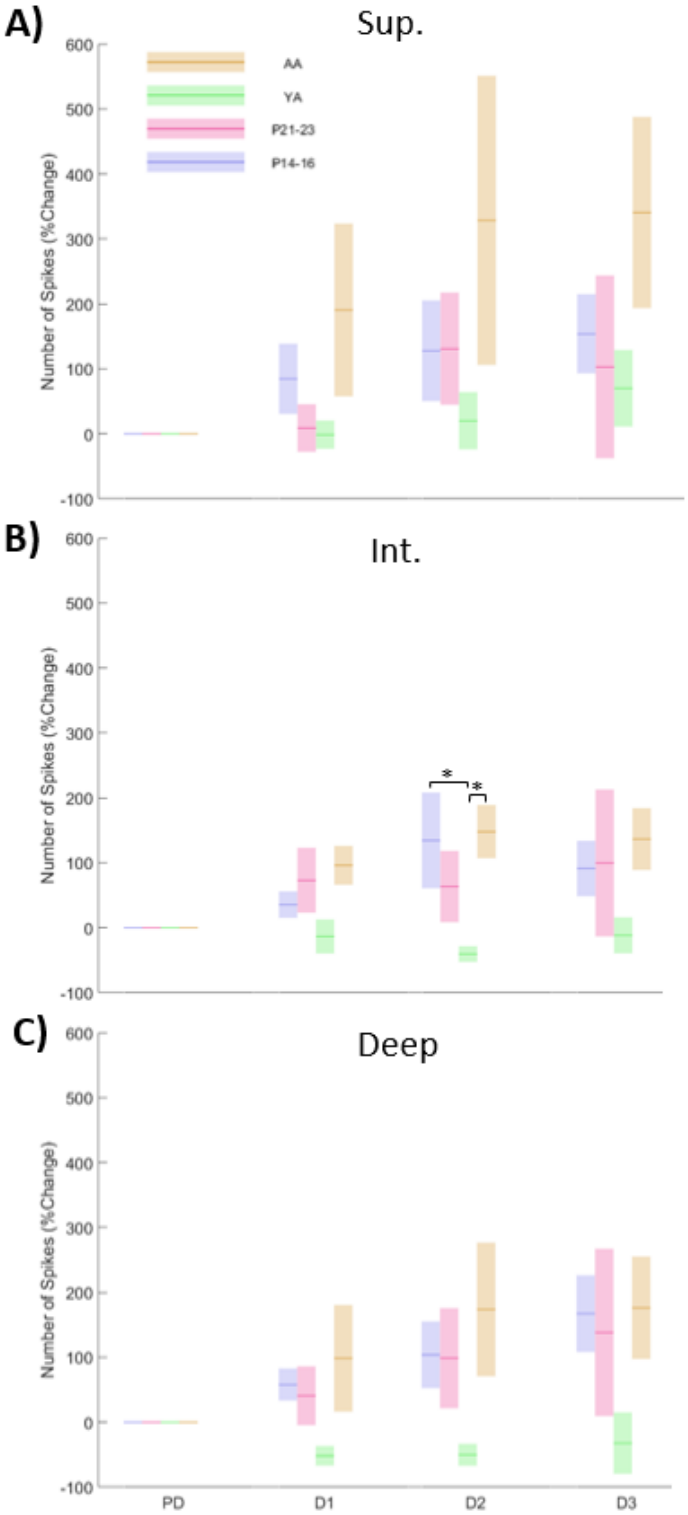


*Figure 5.17. Age-dependent changes in dorsal horn responses to 6g mechanical hindpaw stimulation occur following cumulative bicuculline doses. Summed spike activity during the 5seconds of stimulus application across the A) superficial (Sup.), B) intermediate (Int.) and C) deep dorsal horn regions (mean activity across the electrodes) following 6g vFh stimulation of the hindpaw is shown for animals receiving 3 cumulative bicuculline doses (D1-3) as a percentage change from the pre-drug (PD) response. P14-16 n=6 for all doses, P21-23 n=6 for PD and D1, n=5 for D2, n=4 for D3, young adult (YA) n=6 for PD and D1, n=5 for D2, n=4 for D3, and aged adult (AA) n=11 for PD, D1 and D2, n=9 for D3. Lines represent mean and shaded area represents SEM. Two-way ANOVAs found significant differences between the response (%change) following bicuculline doses in the intermediate and deep regions * asterisk represent significant differences following Tukey's post hoc tests (P<0.05).*

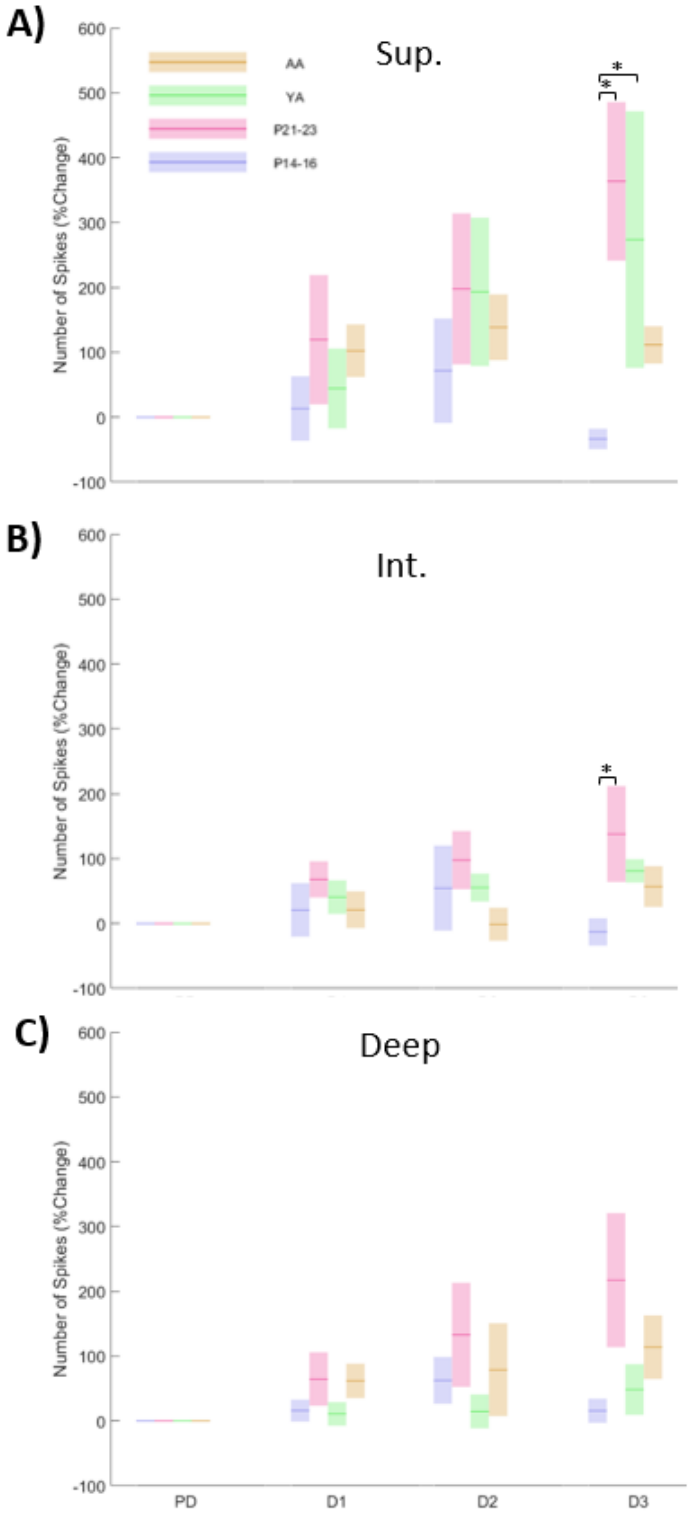


*Figure 5.18. Age-dependent changes in dorsal horn responses to 15g mechanical hindpaw stimulation occur following cumulative bicuculline doses. Summed spike activity during the 5seconds of stimulus application across the A) superficial (Sup.), B) intermediate (Int.) and C) deep dorsal horn regions (mean activity across the electrodes) following 15g vFh stimulation of the hindpaw is shown for animals receiving 3 cumulative bicuculline doses (D1-3) as a percentage change from the pre-drug (PD) response. P14-16 n=6 for all doses, P21-23 n=6 for PD and D1, n=5 for D2, n=4 for D3, young adult (YA) n=6 for PD and D1, n=5 for D2, n=4 for D3, and aged adult (AA) n=11 for PD, D1 and D2, n=9 for D3. Lines represent mean and shaded area represents SEM. Two-way ANOVAs found significant differences between the response (%change) following bicuculline doses in the intermediate and deep regions * asterisk represent significant differences following Tukey's post hoc tests (P<0.05).*

5.5 Discussion

The work presented in this chapter identified age-related changes in GABAergic signalling in the dorsal horn following electrical and mechanical stimulation of the hindpaw which appear to be region specific. Following electrical stimulation this was observed as an increase in the evoked spike activity with increasing age in the A-fibre response period, however, in the C-fibre response period an age-related trend was less clear and the young adults generally showed the highest spike activity. Following mechanical hindpaw stimulations the young adult group showed significantly reduced spike activity compared to the other ages tested. Whilst this work supports previous findings using ex-vivo patch-clamp electrophysiology they ultimately add to the theory of needing to study these complex systems in vivo.

5.5.1 Tonic $GABA_AR$ Activity throughout the Life Course

Spontaneous activity in the dorsal horn following cumulative bicuculline applications was compared within each age group to determine the effect of tonic GABAergic activity. Tonic GABAergic inhibition involves signalling at extrasynaptic receptors and has been reported at P8 and shown to increase with postnatal age, reaching mature levels by the third postnatal week (Zell *et al.*, 2014). Here we see trends of increased spontaneous activity with increased $GABA_AR$ disinhibition supporting the idea that a tonic GABAergic effect is occurring in the dorsal horn at least between P14 and 18 months of age. We do not however see a mature level reached by P21 and instead see differences in the spontaneous response at all ages used in this study. This may be in part due to the previous study suggesting maturation by P21 using patch-clamp recordings compared to in-vivo recordings used here, which have been shown to effect the observed results following spinal application of inhibitory antagonists (Hathway *et al.*, 2006). Additionally, glycinergic tonic inhibition also occurs in the dorsal horn and develops between P14 and P21 (Koch *et al.*, 2012), possibly impacting the GABAergic effects studied here.

5.5.2 Phasic $GABA_AR$ Activity throughout the Life Course

As evoked responses were baseline corrected the effects observed should reflect synaptic/phasic $GABA_AR$ disinhibition. Responses to electrical stimulation during the A-fibre latency period, C-fibre latency period and following mechanical stimulation will be discussed separately.

5.5.2.1 A-fibre Responses

Dorsal horn responses to electrical stimulation during the A-fibre latency period at P14-16 showed dose dependent increases to cumulative bicuculline doses, although these were not statistically significant. However, there was very little difference following the first bicuculline dose throughout the dorsal horn, interestingly, this was the only age at which the first dose had such a small effect. As GABAergic inhibition is the main form of inhibitory activity at this age, with glycinergic inhibition developing between P14 and P21 (Koch *et al.*, 2012) this was surprising. As bicuculline is a competitive $GABA_A$ receptor antagonist this may suggest a higher level of endogenous GABA in the P14-16 dorsal horn.

By P21-23 we saw a less pronounced effect of bicuculline application at any dose on the A-fibre responses to electrical stimulation of the hindpaw. With response difference mostly occurring in the superficial region. While this fits with the expected location of GABAergic activity (Inquimbert and Rodeau, 2007; Takazawa and MacDermott, 2010; Bardoni *et al.*, 2013) there was a large amount of variation within the age group in this region, possibly as the drug is applied to the dorsal surface of the cord reducing its statistical significance. Additionally a slightly altered response to the cumulative doses was observed at this age where the first dose resulted in an increase unlike at P14-16 but there was generally very little difference in activity between the second and third doses.

Dose dependent increases in activity were seen again in the young adults but were now statistically significant in all dorsal horn regions. In the adult dorsal horn GABAergic signalling is associated with the superficial laminae and glycinergic in the deeper laminae (Takazawa and MacDermott, 2010) as such it was unexpected that we would observe relatively comparable increases across the whole dorsal horn. However, in the aged adult, although dose dependent increases are again observed throughout the dorsal horn this is particularly true for the superficial region. Although this was an altered concentration of bicuculline the difference between regions should be comparable and may suggest that even in young adults the GABAergic inhibitory system is not fully mature.

All bicuculline induced increases in dorsal horn activity during the A-fibre latency period involved an increase in the duration of the evoked activity. This suggests that at all ages A-fibre stimulation produces $GABA_AR$ mediated inhibition, although this was less clear in the P21-23 age group. As the bicuculline dependent differences in dorsal horn activity generally increased with age this suggests phasic GABAergic inhibition strengthens with age. Interestingly, age-related differences were mostly found in the intermediate region,

possibly reflecting the development of A-fibre circuits in this region during the life course (Chapter 4).

5.5.2.2 Responses during the C-fibre Latency Period

In the P14-16 dorsal horn we see a trend of increased spike activity with cumulative bicuculline doses in a dose dependent manner, although statistically significant differences were not detected at this age. The increase in activity is predominantly in the deep region of the dorsal horn where increases in activity are observed following all three bicuculline doses as opposed to the superficial and intermediate regions which show increased activity compared to the pre-drug response only after the highest bicuculline dose. This may reflect the location of the C-fibre associated activity which is highest in the deep regions at this age. However, as an increase does occur in the other regions but requires a higher dose of bicuculline, this suggests regional differences in GABAergic signalling in the second postnatal week.

By P21-23 the C-fibre response following cumulative bicuculline doses was completely altered. Instead of dose dependent increases in activity there were instead dose dependent decreases in dorsal horn activity during this latency period, although not statistically significant. Although the first dose did result in an increase in activity compared to the pre-drug response the dorsal horn activity to the following doses progressively declined below the pre-drug response level. It is possible that this may reflect a difference in the C-fibre threshold between these ages (Chapter 4) however as percentage difference is being compared it is more likely to reflect an alteration in $GABA_AR$ signalling. Previous studies have identified increased C-fibre activity following bicuculline administration (Reeve, Dickenson and Kerr, 1998; Rahman *et al.*, 2007) however as 1mA is not a maximal C-fibre stimulus intensity C-fibre effects may be reduced.

Interestingly the C-fibre response in the young adults showed dose dependent increases in dorsal horn activity. Following the second and third dose in the superficial and intermediate region and following the third dose in the deep dorsal horn, although these differences were not statistically significant. The young adult responses during the C-fibre latency period were significantly higher than the other ages in the intermediate dorsal horn and compared to the postnatal ages in the superficial dorsal horn. However by the aged adult group, C-fibre latency period responses again showed a decrease compared to pre-drug responses in the intermediate and deep dorsal horn, but, dose dependent increases in the superficial region. Although the bicuculline associated changes in dorsal horn activity were rarely statistically significant, the data presented

here suggests regional differences in GABAergic signalling occurring throughout the life course.

It is possible that the C-fibre data would be more significant if a higher intensity stimulation had been used and this may be worth repeating in the future.

5.5.2.3 Dorsal Horn Responses to Mechanical Stimulation of the Hindpaw

Few studies have used mechanical stimulation when investigating the effects of GABAergic signalling in the dorsal horn, most likely due to most studies using ex-vivo patch clamp recordings. Adult WDR neuronal responses to peripheral mechanical stimulation have been shown to increase with cumulative spinal bicuculline doses (Rahman *et al.,* 2007) although a higher concentration of bicuculline was used possibly reflecting the increased statistical significance observed in their study. Mechanical stimulation of the hindpaw generally resulted in a dose-dependent increase in dorsal horn activity in all ages although not statistically significant apart from the 6g response in the aged adults.

Interestingly, intrathecal administration of bicuculline to P21 rats results in a decreased mechanical threshold, which would match the data obtained in this study, however, at P3 this increased mechanical thresholds (Hathway *et al.*, 2006). This may suggest that postnatal development of $GABA_AR$ signalling, during which a switch from facilitatory to inhibitory effects is thought to occur, happens between P3 and P14, supporting previous work suggesting this switch occurs in the first postnatal week (Baccei and Fitzgerald, 2004).

Responses following 6g vFh stimulation at P14-16 and 15g vFh stimulation at P21-23 were particularly increased in the superficial and deep dorsal horn regions. In aged adults responses were also highest in the superficial and to a lesser extent the deep region, however in the young adults the superficial regions had the greatest bicuculline induced increase in activity, corresponding with the expected location of GABAergic signalling (Inquimbert and Rodeau, 2007; Takazawa and MacDermott, 2010; Bardoni *et al.*, 2013). Again suggesting postnatal maturation of GABAergic signalling is region specific.

Interestingly from P21-23 a second peak following stimulus onset is observed, occurring only after the highest bicuculline dose. This may reflect polysynaptic Aβ mediated circuits normally under GABAergic inhibition (Baba *et al.,* 2003; Torsney and MacDermott, 2006). This second peak is seen following 6g vFh stimulation in the young adults but again only following the highest bicuculline dose. Aged adults, however, show clear

second peaks at all bicuculline doses, although these concentrations were all higher than those applied to the younger age groups. Whilst the second peak can be seen in the same animals, we are unable with the population recordings to determine if this is activity within the same cells or not. However, this is to our knowledge the first time this has been reported following bicuculline application so the second peak is most likely a result of activity in different cells, as this would not have been detected in patch-clamp or single unit recordings obtained previously.

5.5.3 Technical Considerations

5.5.3.1 Stimulation Protocol

Due to a limited time period in which test stimulations could be administered following each dose, each mechanical stimulus was only given 1x following each dose. As such, it is possible that some of the changes observed are associated with a variation in stimulus application. Although great care was taken to apply the vFh to the same point, at the same angle, and from the same distance this will have slightly changed with each application. Although the time-locked nature of the application will have reduced some user error. Additionally only one electrical stimulus intensity was chosen, 1mA. This stimulation should evoke C-fibre responses (Chapter 4) however a higher intensity stimulus may have allowed C-fibre responses to be investigated further and produced more statistically significant responses.

There is also the possibility that the effect time of the topical bicuculline is different at the different ages, this was not tested in this study and the timescale used was based on previous work by the group (not published) but closely follows timings used in other studies using bicuculline (Hathway *et al.*, 2006; Rahman *et al.*, 2007). This could offer a possible explanation to the relatively low mechanical responses in the young adults compared to the electrical stimulations, as the mechanical preceded the electrical stimulations by 10-15minutes.

5.5.3.2 Bicuculline Application

As the bicuculline solution was applied to the dorsal surface of the spinal cord it is possible that inconsistent concentrations of the drug reached the intermediate and deep dorsal horn compared to the superficial laminae. However, we see changes in the deep and intermediate regions throughout the study suggesting the drug is able to reach

these regions. Additionally, no testing took place for 20 minutes following drug application so there was time for the bicuculline to penetrate the spinal cord.

In the P21-23 and adult rats there was substantial tissue over the spinal column to make a well in which the bicuculline solution could sit. However, in the P14-16 age group there was often not enough tissue to reliably produce the well. In these cases small amounts of Vaseline were used to keep the solution over the exposed spinal cord

Interestingly the aged animals appeared to have similar responses to a x10 higher dosing protocol compared to the young adults. In the A-fibre response latency period a similar change from pre-drug responses was observed at the highest dose in both adult groups in the intermediate and deep regions. As well as similar responses across the whole array following 15g vFh stimulation. There were however differences between the adult groups in the C-fibre response in the intermediate region, particularly following the highest doses. And following the 6g vFh stimulation although this is in part due to the unexpectedly low young adult response to this stimulation.

5.5.3.3. Bicuculline

Bicuculline is a competitive $GABA_A$ receptor antagonist, which has been widely used in research for over 40 years (Johnston, 2013). It binds to the $GABA_A$ receptor at both orthosteric sites; stabilizing it in a closed/resting state (Ueno *et al.*, 1997; Masiulis *et al.*, 2019) and thus reducing the action of the inhibitory neurotransmitter GABA at these receptors. Bicuculline, however, is not very stable at a physiological pH so quaternary salts of bicuculline are often used instead, as with this study where bicuculline methiodide was used. Additionally, the quaternary salts benefit from being more water soluble and having a potency similar to bicuculline, however, they do not cross the blood brain barrier and are less selective than bicuculline (Seutin and Johnson, 1999; Johnston, 2013). Bicuculline, and to a greater extent bicuculline quaternary salts, have been shown to also inhibit cholinergic receptors (Breuker and Johnston, 1975; Demuro *et al.*, 2001) and small conductance calcium-activated potassium channels in neurons (Seutin and Johnson, 1999; Johnston, 2013).

There are several alternative $GABA_A$ receptor antagonists that have been identified and synthesised, including picrotoxin and gabazine. Picrotoxin shows channel blocker and allosteric antagonist activities by binding to the open channel pore and stabilizing the receptor in a closed/resting state (Masiulis *et al.*, 2019). Similarly to bicuculline picrotoxin has been shown to act upon other receptors including $GABA_C$ receptors, glycine receptors, and 5HT3 receptors (Johnston, 2013). Gabazine is also an allosteric

competitive antagonist but has slightly different effects due to interactions with different residues on the $GABA_A$ receptor (Ueno *et al.*, 1997). Although it has been shown that bicuculline, picrotoxin, and gabazine can also block glycine receptors (Wang and Slaughter, 2005).

5.6 Conclusions

Although the disinhibition of $GABA_A$ receptors in the dorsal horn has been extensively studied, including in the neonatal spinal cord, the majority of this work has used ex-vivo patch clamp recordings. The findings presented here suggest that age-related changes occur in GABAergic signalling but not always in line with the expected ages from previous studies. As such the importance of studying these inhibitory circuits in vivo is supported by this work and further in-vivo studies using MEA electrophysiology may uncover the development of inhibitory circuits in the developing dorsal horn.

Chapter 6

Changes in Dorsal Horn Plasticity throughout the Life Course

6.1 Introduction

The term neuronal plasticity is broadly used to describe short- or long-term changes within the nervous system (Buchtel, 2009). The mature somatosensory system exhibits neuronal plasticity in both normal sensory processing and in pathological states such as chronic pain (Gold and Gebhart, 2010). These changes can vary in their temporal extent, can be structural or functional, and can occur throughout the somatosensory pathway, but often involve changes in synaptic strength or changes in descending modulation (Citri and Malenka, 2008; Jamann, Jordan and Engelhardt, 2018). In normal sensory processing plasticity can be observed in multiple forms but mostly occurs as a response to changes in the cellular environment, such as over-stimulation, and can often be considered as activity-dependent (Jamann, Jordan and Engelhardt, 2018). Additionally, in pathological states such as following injury, in chronic pain states, and in inflammatory pain, changes throughout all levels of the somatosensory system have been identified (Waxman, Kocsis and Black, 1994; Bliss *et al.*, 2016; Chen, Li and Zhuo, 2021; Torsney, 2019). Changes in neuronal properties and synaptic strength, therefore, are important aspects of somatosensory and pain processing, with multiple sites at which they can occur.

As alluded to throughout this thesis, the postnatal somatosensory system shows remarkable amounts of neuronal plasticity as it undergoes multiple changes during development (Fitzgerald, 2005). Interestingly, an increasing number of studies are identifying the importance of correct somatosensory development during this time and how an insult to the system, such as through early life pain or stress, can lead to disrupted sensory processing in adulthood (Beggs *et al.*, 2012; Victoria *et al.*, 2014; Hoogen *et al.*, 2018; van den Hoogen *et al.*, 2018; Walker *et al.*, 2018). Although the impact of early life insults is outside the scope of this thesis, it highlights the importance of understanding the neuronal plasticity that takes place in the postnatal somatosensory system, as further understanding in this area may offer new insights in treating pain in early life.

6.1.1 Endogenous Pain Modulation and Diffuse Noxious Inhibitory Control

One method for evoking descending inhibition of dorsal horn processing is via diffuse noxious inhibitory control (DNIC), which is similar to conditioned pain modulation (CPM) in humans (Yarnitsky, 2010). This is thought to trigger endogenous descending inhibitory pathway via a distant noxious stimulus to the control response (Le Bars, Dickenson and Besson, 1979). The inhibitory action requires the conditioning stimulus to be noxious and the presence of functional descending pathways (Dickenson and Le Bars,

1983). DNIC inhibits WDR neuron activity in response to innocuous and noxious stimulation from a range of modalities (Villanueva, Cadden and Le Bars, 1984; Bannister and Dickenson, 2017). Serotonergic, noradrenergic, and opioidergic pathways are thought to be involved, however, the exact mechanisms involved are not fully understood (Kucharczyk, Valiente and Bannister, 2021). A recent study using fMRI has suggested a sex-dependent difference in DNIC efficiency, with female rats having a less efficient analgesic effect than males (Da Silva *et al.*, 2018; Da Silva *et al.*, 2020). Changes in DNIC have also been shown in older adults, in both clinical and pre-clinical studies, with reduced inhibition observed compared to younger adults (Edwards, Fillingim and Ness, 2003; Da Silva *et al.*, 2020).

6.1.1.1 Postnatal Development of Diffuse Noxious Inhibitory Control

Although several studies have looked into the development of descending modulation there is a dearth of research specifically into the postnatal development of DNIC. However, as it is widely agreed that descending inhibition is developed between P21-28 (G. J. Hathway *et al.*, 2009b; Hathway, Vega-Avelaira and Fitzgerald, 2012; Kwok *et al.*, 2014b) and as DNIC is a form of descending inhibition, it is likely this is the age at which DNIC would be first observed. A study by Boucher et al. used dorsal horn c-fos expression as a measure of DNIC, in line with the development of descending inhibition this study found significant decreases in c-fos expression following conditioned stimulus application at P21 and in young adults (P42). Interestingly, although not significant it also appears that this decrease may be beginning at P12 (Boucher, Jennings and Fitzgerald, 1998).

6.1.2 Wind-up of Dorsal Horn Neurons

Wind-up is a phenomenon where a noxious stimulus results in a higher evoked activity following repeated high frequency application (Mendell and Wall, 1965; Mendell, 1966). This type of facilitation has been compared to temporal summation, and linked with central sensitisation, hyperalgesia and allodynia (D'Mello and Dickenson, 2008; Hachisuka *et al.*, 2018) and is a commonly studied form of short term synaptic plasticity (D'Mello and Dickenson, 2008). Wind-up has predominantly been investigated in WDR neurons in the deep dorsal horn but some neurons in the superficial region also demonstrate wind-up (Seagrove, Suzuki and Dickenson, 2004). It has been shown that approximately 18% of spinoparabrachial neurons in lamina I show wind-up, which are thought to contribute to the unpleasant affective component of this phenomenon (Hachisuka *et al.,* 2018).

The mechanisms behind wind-up are not fully understood but it is thought to be a combination of pre- and post-synaptic mechanisms (Herrero, Laird and Lopez-Garcia, 2000). It is however known that NK1 (Suzuki *et al.*, 2002) and NMDA receptors (Davies and Lodge, 1987; Dickenson and Sullivan, 1987) are required for the generation of wind-up, as well as their associated neurotransmitters substance P and glutamate, and ion channels such as $Na_v1.7$ (Bennett, A. J. Clark, *et al.*, 2019). There is also evidence that endogenous inhibitory opioidergic mechanisms may be involved in regulating wind-up and neuronal sensitisation, by reducing the increase in dorsal horn responses (Guan *et al.*, 2006).

6.1.2.1 Changes in Dorsal Horn Wind-up throughout the Life Course

Classical wind-up as described above can be produced in the dorsal horn from P10, however, in fewer cells than in the adult dorsal horn (Fitzgerald and Jennings, 1999). The percentage of cells showing classical wind-up increases with postnatal age from 18% at P10, to 40% at P21 (Fitzgerald and Jennings, 1999). Before P10, stimulating at 2x the A-fibre threshold at 0.5Hz can generate a build-up of prolonged background activity in the neonatal dorsal horn with 33% of P6 cells displaying this, which reduces to 6% at P10 and none by P21 (Jennings and Fitzgerald, 1998).

Interestingly, a subset of lamina I projection neurons which express NK1R and project to the PB nucleus are detectable at P3, and respond to noxious peripheral stimulation (S. H. W. Man, Géranton and Hunt, 2012) suggesting that at least some of the neurons involved in wind-up are present and functional from very early life. However, as mentioned above, wind-up is also known to involve NMDA receptors, which undergo postnatal development in the dorsal horn (Baba *et al.*, 2000; de Geus *et al.,* 2020). The NMDA receptor antagonist AP5, which reduces wind-up in WDR neurons, has a significantly higher potency in postnatal rats (P14-P28) than in adults (Urch, Rahman and Dickenson, 2001).

6.2 Hypotheses and Aims

6.2.1 Hypotheses

Our group has previously shown a predomination of descending facilitation from the RVM in early life and that, whilst present, descending inhibition is not significant until the fourth postnatal week (G. J. Hathway *et al.*, 2009b; Hathway, Vega-Avelaira and Fitzgerald, 2012). We therefore hypothesise that DNIC would fail to evoke significant changes in MEA activity throughout the dorsal horn in the early postnatal period, but

would be observable by P28. Wind-up however should be detectable in the deep dorsal horn in all the ages used in this study but we would expect to see an increase in the response with postnatal age. Additionally we may see wind-up responses in the A-fibre latency period in the P9-11 age group. We also hypothesise that sex-differences will be found or suggested in the DNIC study.

6.2.2 Aims

- Investigate changes in wind-up responses in the dorsal horn throughout the life course
- Determine the age at which DNIC can be observed in the dorsal horn using MEA electrophysiology
- Check for sex-differences in the DNIC study

6.3 Methods

6.3.1 Animals

Male and female Sprague Dawley rats at postnatal day (P)9-11, P14-16, P21-23, P28-30, P42-49 (considered as young adults), and males at 18months old (considered as aged adults) were used for data presented in this chapter. Details of animal origin and housing can be found in Section 2.1.1. Group sizes, sex, weights, and specific groupings are detailed in Section 4.3.4.

6.3.2 Surgery and Electrode Placement

Rats were anaesthetised with isoflurane (Section 2.1.2) and placed on to a stereotaxic frame (Section 2.1.3). A laminectomy and meningostomy took place to expose the L4 and L5 spinal cord and the dura matter and arachnoid membranes carefully removed (Section 2.1.4). The electrode array was then placed as described in Section 2.1.5.

6.3.3 Hindpaw Stimulation

The data presented in this chapter involves the application of mechanical stimuli and electrical stimuli to the hindpaw as described in Section 2.1.6. Two stimulation protocols were used to study somatosensory plasticity, the DNIC protocol utilised mechanical stimulation to the hindpaw and the wind-up protocol used electrical stimulation to the hindpaw as described in Section 2.1.6. Specifically these protocols involved:

DNIC: A control train consisting of 2g, 6g, and 15g von Frey hair applications was applied twice. DNIC trains consisted of the same test stimuli applied to the hindpaw but with a noxious conditioning stimulus application to the ipsilateral ear using a micro bulldog clamp. An inter-stimulus and inter-train duration of 10 minutes was used throughout, in DNIC trains the noxious ear clamp was applied 10seconds before each vFh application and removed immediately after. (Based on Bannister *et al.*, 2015).

Wind-up: A control train of 20 5mA stimuli with a 2ms duration at 0.1Hz (1 every 10 seconds) was given followed by three wind-up trains of 20 5mA stimuli with a 2ms duration at 0.5Hz (1 every 2 seconds). Each train was delivered with an inter-train duration of 15 minutes (As in Greenspon *et al.,* 2019).

6.3.4 Specific Study Design

Electrophysiological data presented in this chapter was obtained from two cohorts, one involving all P9 to young adult rats which received both DNIC and wind-up protocols and a second involving the aged adults who receive the wind-up protocol. Rats receiving both protocols received the DNIC protocol first, following which the alignment protocol was rerun and the depth of the electrode adjusted if required (Section 2.1.5). No stimulation was given for at least 20 minutes before the wind-up protocol was then completed.

Table 6.1 Details of rats included in electrophysiological analysis in this chapter. The number of animals combined in each age group (n), the split of males and females (m/f), and the average weight are included. Details of excluded data are stated below and not included in the table.

	Plasticity study		
Age	n	M/F	Weight (g) (Mean±SD)
P9-11	6	3/3	24±4
P14-16	6	3/3	37±4
P21-23	6	3/3	63±11
P28-30	6	3/3	106±6
Young Adult	6	3/3	207±27
Aged Adult	5	5/0	542±34

Data was incompletely collected from one P10 (female) due to breathing issues during the wind-up protocol, from one P11 (male) rat as there was a reflex to the ear clamp during the DNIC protocol and from one P22 (female) as there was too much noise during the DNIC recording to proceed with data collection. Subsequent data processing revealed noise in one P23 (female) wind-up recordings which was removed from analysis.

6.3.5 Data Analysis

Details of data collection and initial sorting are described in Section 2.1.7. Data analysis and statistical analysis were performed using Matlab 2017b and GraphPad Prism 7.05.

Data collected from studies discussed in Chapter 3 and 4 was used in power calculations to calculate the recommended group sizes of 6 for electrical responses and 10 for mechanical responses, when using both sexes and postnatal rats. These group sizes were calculated to give a power of 80% or higher to detect effect sizes of Cohen's d = 1.7 (electrical data) and 1.3 (mechanical data) between age groups using mean data with a with a significance level of $p<0.05$ using a two-tailed test. These group sizes were not calculated to be adequately powered to address a sex-difference. To sort the stimuli responses looked at in this chapter the 'Etho_MSP_Pipeline' script was used. For wind-up data the bin size was set as 1ms, pre-stimulus time set as 0.1 second, the duration as 0seconds, and the post stimulation as 1second. 'Windup' was selected as the experiment type which results in baseline correction for each stimuli train rather than each stimulus. The data was sorted into fibre latency periods, regions, and stimulation groups for analysis. For DNIC data the bin size was set as 100ms, pre-stimulus time set as 5 seconds, the duration as 5 seconds, and the post stimulation as 60 seconds. The data was baseline corrected and sorted into 'subsecond' and 'second' files.

Data was combined between animals using the 'Elec_Combine' script creating a 4D matrix. Statistical comparisons were made in the wind-up study using the response to each control stimulation compared to the average equivalent response from three wind-up stimulation trains. For example stimulus 1 on the control train was compared to the average response recorded from stimulus 1 in the three wind-up trains. The mean response and SEM was then found for all animals within an age group. Control and wind-up responses were compared in each latency period and region using two-tailed paired t-tests for each age group. Statistical comparisons between ages used the difference in the number of spikes following the control train responses and the averaged wind-up train responses at each equivalent stimulus in the train. These were then compared using one-way ANOVAs for each latency period within specific regions followed by Tukey's post hoc tests.

Statistical comparisons in the DNIC study were made between the average responses obtained from two control trains and three DNIC test trains in each animal. The mean response and SEM was then found for all animals within an age group. Differences between control and DNIC responses following each mechanical test stimulus and sex differences were compared using two-tailed paired t-tests. The difference in activity between the control and DNIC trains was calculated and compared across regions and

stimulus intensity at each age group using one-way ANOVAs. These differences were then used for statistical comparisons between different ages following each stimulus intensity, within each region, using one-way ANOVAs.

6.4 Results

6.4.1 Dorsal Horn Responses to Wind-Up Stimulation throughout the Life Course

At each age the sum of activity in multiple latency periods has been compared following control and wind-up stimulation protocols. These include the pre-stimulus, A-fibre, C-fibre, post-discharge and the combined C-fibre and post-discharge duration. As the C-fibre and post-discharge latencies are the time periods in which we expect to see the largest wind-up effects (Herrero, Laird and Lopez-Garcia, 2000) these were focused on. Additionally, the combined C-fibre and post-discharge latency period was included as this has been previously used when investigating wind-up (Urch and Dickenson, 2003). The difference between control and wind-up responses is then compared between ages.

Postnatal Days 9-11

At P9-11 no significant differences were found in the dorsal horn responses to wind-up vs control stimulation using paired two-tailed t-tests ($P>0.05$) in any latency period compared or between regions during the C-fibre and post discharge latencies (Figure 6.1). Responses can be seen in the heatmaps (Figure 6.1A) in the A-fibre latency period throughout the dorsal horn, matching previous response locations shown in Chapter 4. Although we thought we may see an increase in A-fibre responses at this age we did not, instead there is no change although possibly a trend of decreased activity across the whole array (Figure 6.1B). In the C-fibre latency period and to a lesser extent the post-discharge latency period there are similar responses in the superficial and deep regions following control and wind-up stimulation with a trend of increased activity following wind-up stimulation in these latency periods in all regions (Figure 6.1 C&D) however, these were not significant. The response in the C-fibre latency period is similar following the first wind-up stimulus as it is following the subsequent 19 (Figure 6.1A) unlike what would be expected in classical wind-up during which there is an increased facilitation with each stimulus (Mendell and Wall, 1965; Mendell, 1966). Interestingly the sum of spike activity during the post-discharge period is below 0 in all but the superficial wind-up groups, suggesting that at this age there is a decline below baseline activity levels during this period.

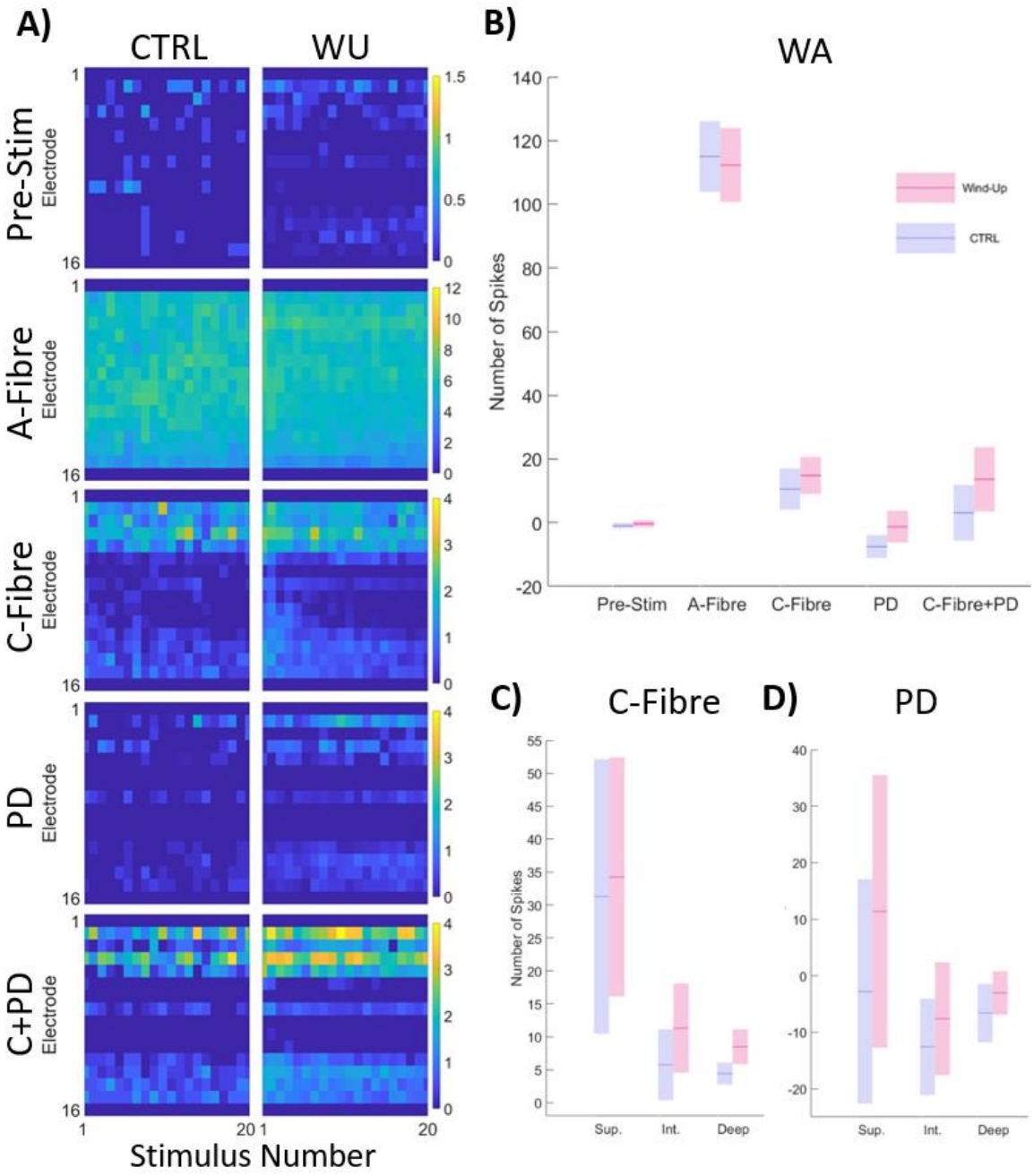


Figure 6.1. Dorsal horn responses to wind-up stimulation at P9-11. A) Heatmaps show spike activity at each electrode following electrical stimulation (20x 5mA at 2ms) of the hindpaw at low (0.5Hz) and high (0.1Hz) frequencies. The first column shows the control (CTRL) responses (low frequency) and the second column shows the responses during the wind-up (WU) protocol (high-frequency).The sum of activity in each latency period is shown as one bin, with the 20 consecutive stimuli displayed along the x-axis. Latency periods correspond to activity at -100-0ms pre-stimulus (Pre-Stim), 3-50ms post-stimulus (A-fibre), 50-250ms post-stimulus (C-fibre), 250-750ms post-stimulus (Post-Discharge (PD)), as well as the C-fibre and Post-Discharge period combined. Responses shown are averaged responses from all animals in the age group, n=5. Electrodes are in order; 1 is the most dorsal and 16 the most ventral, electrodes 1 and 16 are removed to better represent the size of the P9-11 dorsal horn. Colours represent mean number of spikes respective to the colour bar shown on the right. B) Summed spike activity across the latency periods shown in (A) across the whole array following control and wind-up stimulation protocols. C&D) Summed spike activity in the superficial, intermediate, and deep regions of the dorsal horn during the C-fibre (C) and Post-Discharge (D) latency periods. For all bar charts lines represent mean and shaded area represents SEM. Two-tailed paired t-tests found no significant differences between control and wind-up responses in any latency period across the whole array or in the C and PD latency periods in any region of the dorsal horn.

Postnatal Days 14-16

By P14-16 we see significant increases in the post-discharge latency period following wind-up stimulation compared to control ($P<0.05$, two-tailed t-test, Figure 6.2B). Specifically within the intermediate and deep dorsal horn (both $P<0.05$, two-tailed t-test, Figure 6.2D). There is also a trend of increased activity following wind-up stimulation during the C-fibre latency period (Figure 6.2C) and interestingly the pre-stimulus latency period in the deep region (Figure 6.2A), although not significantly different, possibly due to the increased post-discharge activity extending into the following stimulus' pre-stimulation time. Prolonged post-discharge has been previously observed in neonates, although this has been following repetitive A-fibre stimulation and is associated with slightly younger neonates (P6-10) (Fitzgerald and Jennings, 1999). Additionally, as in the P9-11 age groups there is a non-significant decrease in activity during the A-fibre latency period (Figure 6.2B) which appears on the corresponding heatmaps to be due to a reduction in the deep dorsal horn (Figure 6.2A) although this would require further investigation to confirm.

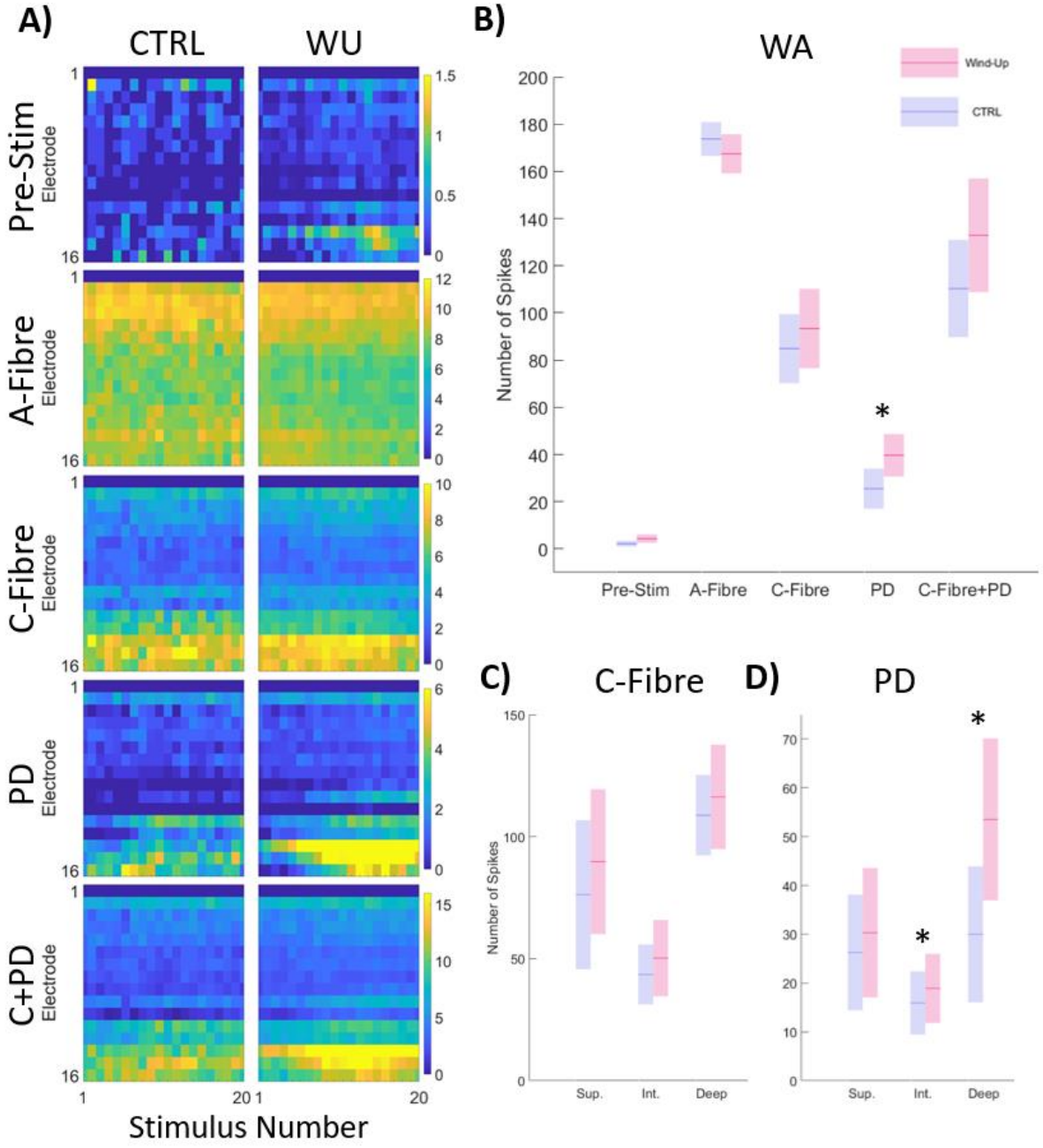


Figure 6.2. Dorsal horn responses to wind-up stimulation at P14-16. A) Heatmaps show spike activity at each electrode following electrical stimulation (20x 5mA at 2ms) of the hindpaw at low (0.5Hz) and high (0.1Hz) frequencies. The first column shows the control (CTRL) responses (low frequency) and the second column shows the responses during the wind-up (WU) protocol (high-frequency).The sum of activity in each latency period is shown as one bin, with the 20 consecutive stimuli displayed along the x-axis. Latency periods correspond to activity at -100-0ms pre-stimulus (Pre-Stim), 3-50ms post-stimulus (A-fibre), 75-275ms post-stimulus (C-fibre), 275-775ms post-stimulus (Post-Discharge (PD)), as well as the C-fibre and Post-Discharge period combined. Responses shown are averaged responses from all animals in the age group, n=6. Electrodes are in order; 1 is the most dorsal and 16 the most ventral, electrode 1 was removed to better represent the size of the P14-16 dorsal horn. Colours represent mean number of spikes respective to the colour bar shown on the right. B) Summed spike activity across the latency periods shown in (A) across the whole array following control and wind-up stimulation protocols. C&D) Summed spike activity in the superficial, intermediate, and deep regions of the dorsal horn during the C-fibre (C) and Post-Discharge (D) latency periods. For all bar charts lines represent mean and shaded area represents SEM. Asterisks indicate significant differences between control and wind-up responses determined by two-tailed paired t-tests (P<0.5).

Postnatal Days 21-23

At P21-23 the dorsal horn response to wind-up compared to control stimulation is similar to the second postnatal week (Figure 6.3 vs. Figure 6.2). There are trends of increased activity in the pre-stimulus and C-fibre latency period in the whole array (Figure 6.3A&B) as well as significant differences in the post-discharge latency period when comparing activity across the whole array ($P<0.05$, two-tailed paired t-test, Figure 6.3B). As with both younger age groups the A-fibre latency period activity may decrease, particularly in the deep dorsal horn, although this was again not a statistically significant difference. As with the P9-11 group, but not the P14-16 age group, a decrease compared to baseline in the post-discharge period is observed with the majority of control responses falling below 0 as well as wind-up responses in the intermediate region.

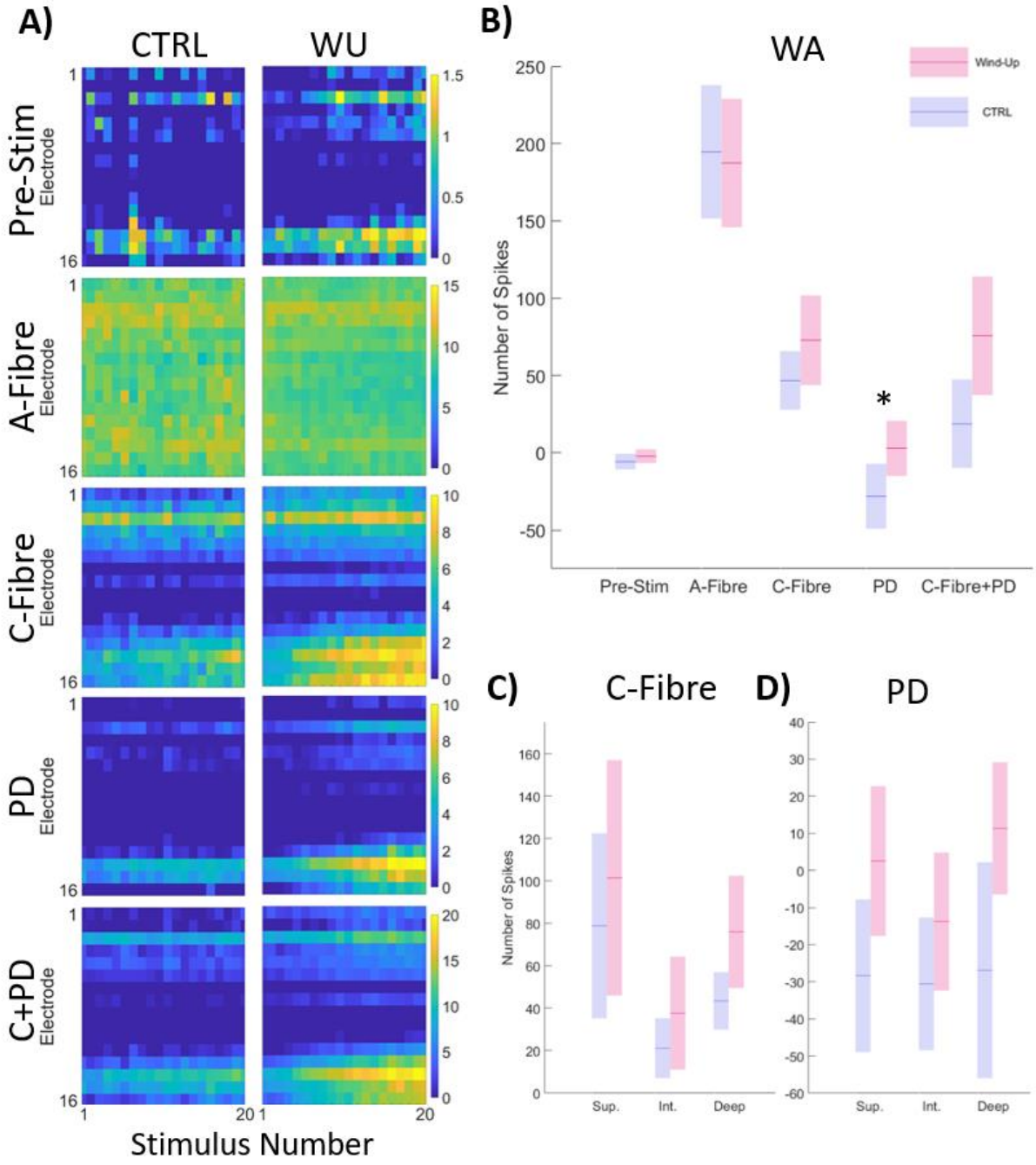


Figure 6.3. Dorsal horn responses to wind-up stimulation at P21-23. A) Heatmaps show spike activity at each electrode following electrical stimulation (20x 5mA at 2ms) of the hindpaw at low (0.5Hz) and high (0.1Hz) frequencies. The first column shows the control (CTRL) responses (low frequency) and the second column shows the responses during the wind-up (WU) protocol (high-frequency).The sum of activity in each latency period is shown as one bin, with the 20 consecutive stimuli displayed along the x-axis. Latency periods correspond to activity at -100-0ms pre-stimulus (Pre-Stim), 3-50ms post-stimulus (A-fibre), 95-295ms post-stimulus (C-fibre), 295-795ms post-stimulus (Post-Discharge (PD)), as well as the C-fibre and Post-Discharge period combined. Responses shown are averaged responses from all animals in the age group, n=5. Electrodes are in order; 1 is the most dorsal and 16 the most ventral. Colours represent mean number of spikes respective to the colour bar shown on the right. B) Summed spike activity across the latency periods shown in (A) across the whole array following control and wind-up stimulation protocols. C&D) Summed spike activity in the superficial, intermediate, and deep regions of the dorsal horn during the C-fibre (C) and Post-Discharge (D) latency periods. For all bar charts lines represent mean and shaded area represents SEM. Asterisks indicate significant differences between control and wind-up responses determined by two-tailed paired t-tests (P<0.5).

Postnatal Days 28-30

By the fourth postnatal week similar trends are observed to the younger ages with a slight decrease in A-fibre latency period responses following wind-up compared to control stimulation and an increase in the pre-stimulus, C-fibre and post-discharge latency periods (Figure 6.4B). These changes were not significantly significant in any latency period across the whole array or in any region during the C-fibre or post-discharge latency periods (Figure 6.4 C&D). This lack of change is visible on the heatmaps which show wind-up-like response profiles following the control stimulation, although these are occurring slightly earlier in the wind-up protocol (Figure 6.4A). There is an apparent lack of activity recorded in the superficial region in all latency periods and in the dorsal half of the array in the C-fibre and post-discharge latencies at this age group (Figure 6.4A,C&D), whilst unclear why this is occurring it supports previous findings in Chapters 3 and 4.

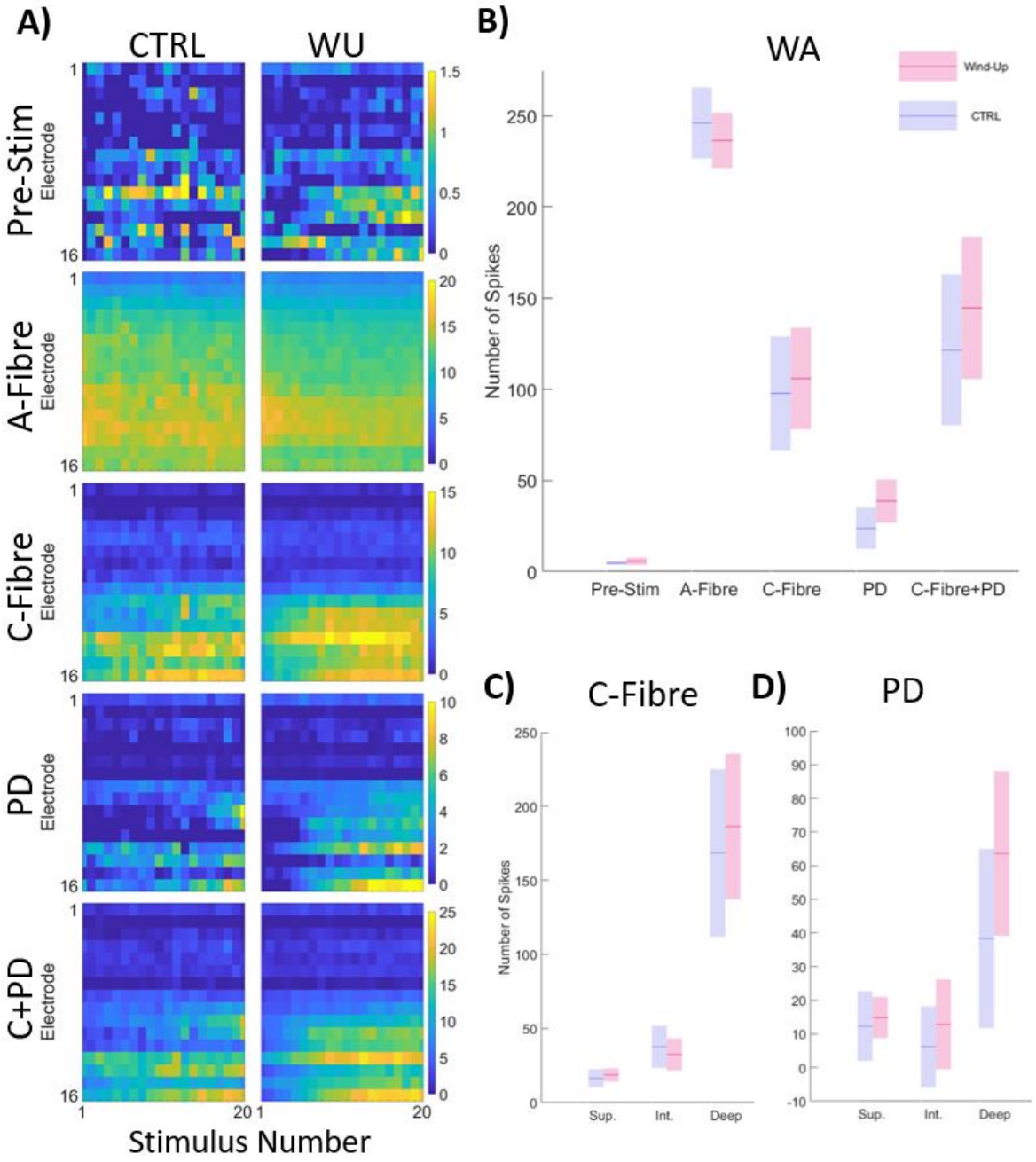


Figure 6.4. Dorsal horn responses to wind-up stimulation at P28-30. A) Heatmaps show spike activity at each electrode following electrical stimulation (20x 5mA at 2ms) of the hindpaw at low (0.5Hz) and high (0.1Hz) frequencies. The first column shows the control (CTRL) responses (low frequency) and the second column shows the responses during the wind-up (WU) protocol (high-frequency).The sum of activity in each latency period is shown as one bin, with the 20 consecutive stimuli displayed along the x-axis. Latency periods correspond to activity at -100-0ms pre-stimulus (Pre-Stim), 3-50ms post-stimulus (A-fibre), 105-305ms post-stimulus (C-fibre), 305-805ms post-stimulus (Post-Discharge (PD)), as well as the C-fibre and Post-Discharge period combined. Responses shown are averaged responses from all animals in the age group, n=6. Electrodes are in order; 1 is the most dorsal and 16 the most ventral. Colours represent mean number of spikes respective to the colour bar shown on the right. B) Summed spike activity across the latency periods shown in (A) across the whole array following control and wind-up stimulation protocols. C&D) Summed spike activity in the superficial, intermediate, and deep regions of the dorsal horn during the C-fibre (C) and Post-Discharge (D) latency periods. For all bar charts lines represent mean and shaded area represents SEM. Two-tailed paired t-tests found no significant differences between control and wind-up responses in any latency period across the whole array or in the C and PD latency period in any region of the dorsal horn.

Young Adults

Young adults show significant increases in dorsal horn activity following wind-up stimulation compared to control using two-tailed paired t-tests in the pre-stimulus ($P<0.05$), C-fibre ($P<0.01$), post-discharge ($P<0.05$), and C-fibre+ post-discharge ($P<0.05$) latency periods across the whole array (Figure 6.5A&B). With the deep dorsal horn specifically showing significant changes in the C-fibre ($P<0.01$) and post-discharge ($P<0.05$) latencies. This was expected as previous studies have shown wind-up to be predominantly associated with WDR neurons in the deep dorsal horn and in the superficial region to a lesser extent (Seagrove, Suzuki and Dickenson, 2004). Interestingly the increase in the pre-stimulus latency period is now significant at this age, this matched previous adult wind-up data collected by the group using MEA electrophysiology (Greenspon *et al.,* 2019).

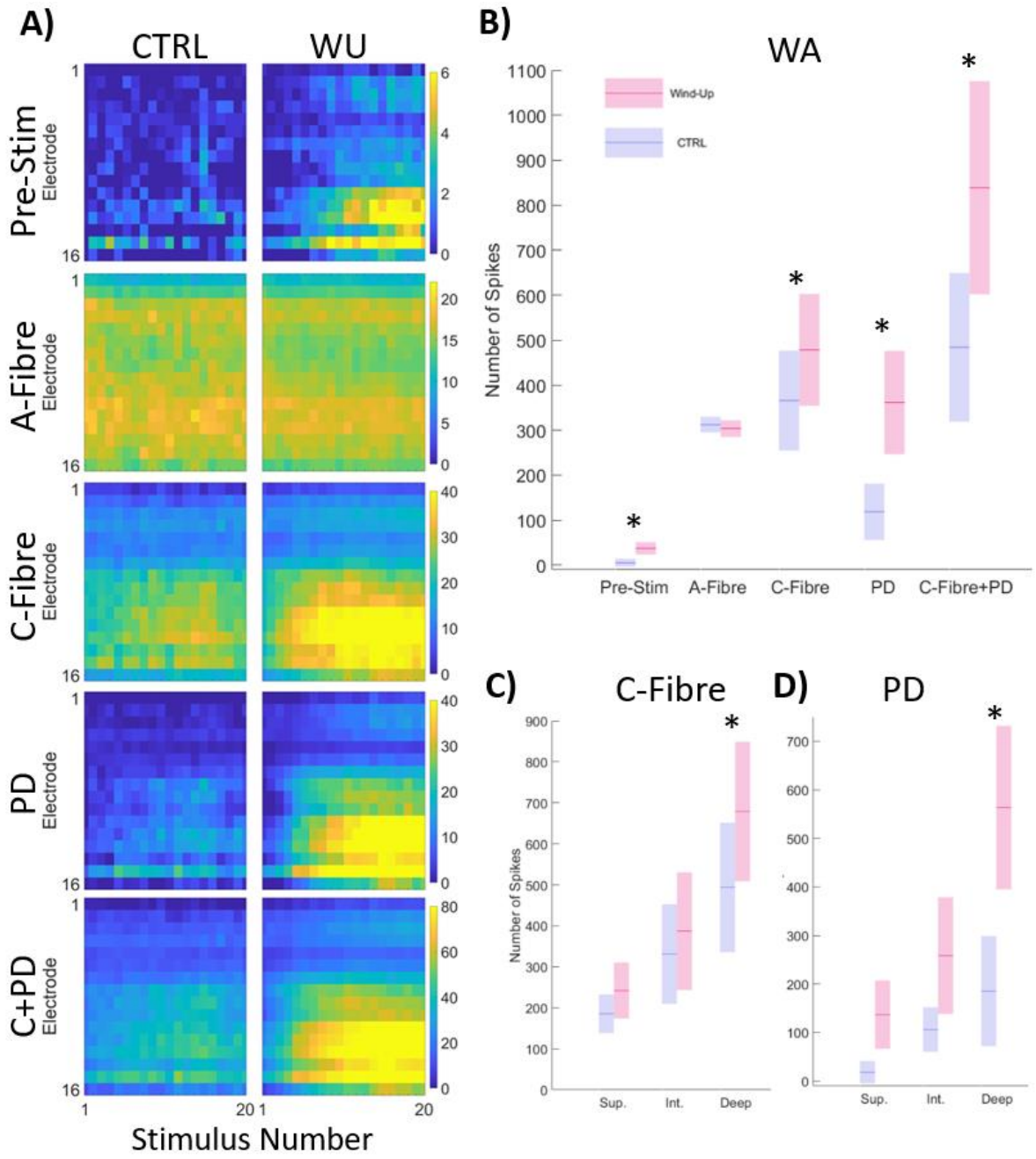


Figure 6.5. Dorsal horn responses to wind-up stimulation in young adults. A) Heatmaps show spike activity at each electrode following electrical stimulation (20x 5mA at 2ms) of the hindpaw at low (0.5Hz) and high (0.1Hz) frequencies. The first column shows the control (CTRL) responses (low frequency) and the second column shows the responses during the wind-up (WU) protocol (high-frequency).The sum of activity in each latency period is shown as one bin, with the 20 consecutive stimuli displayed along the x-axis. Latency periods correspond to activity at -100-0ms pre-stimulus (Pre-Stim), 3-50ms post-stimulus (A-fibre), 120-320ms post-stimulus (C-fibre), 320-820ms post-stimulus (Post-Discharge (PD)), as well as the C-fibre and Post-Discharge period combined. Responses shown are averaged responses from all animals in the age group, n=6. Electrodes are in order; 1 is the most dorsal and 16 the most ventral. Colours represent mean number of spikes respective to the colour bar shown on the right. B) Summed spike activity across the latency periods shown in (A) across the whole array following control and wind-up stimulation protocols. C&D) Summed spike activity in the superficial, intermediate, and deep regions of the dorsal horn during the C-fibre (C) and Post-Discharge (D) latency periods. For all bar charts lines represent mean and shaded area represents SEM. Asterisks indicate significant differences between control and wind-up responses determined by two-tailed paired t-tests (P<0.5).

Aged Adults

In the aged adult dorsal horn no significant differences were found following wind-up stimulation compared to control stimulation. As with the previous ages there is still a trend of increased activity in the dorsal horn in the pre-stimulus, C-fibre, and post-discharge latency periods (Figure 6.6B). Again we observed an increase in the pre-stimulus latency period following wind-up stimulation, although not significant compared to the control responses, as this appears to be in the same region as the post-discharge activity this is most likely still a prolonged after discharge (Figure 6.6A). The aged adults are the first age group to show no trend in decreased responses during the A-fibre latency period (Figure 6.6B). The C-fibre response is clear in both the superficial and deep dorsal horn regions, however the post-discharge activity is much higher in the deep region only, this may suggest that changes are occurring in the PAF processing following repeated stimulations between these two adult age groups.

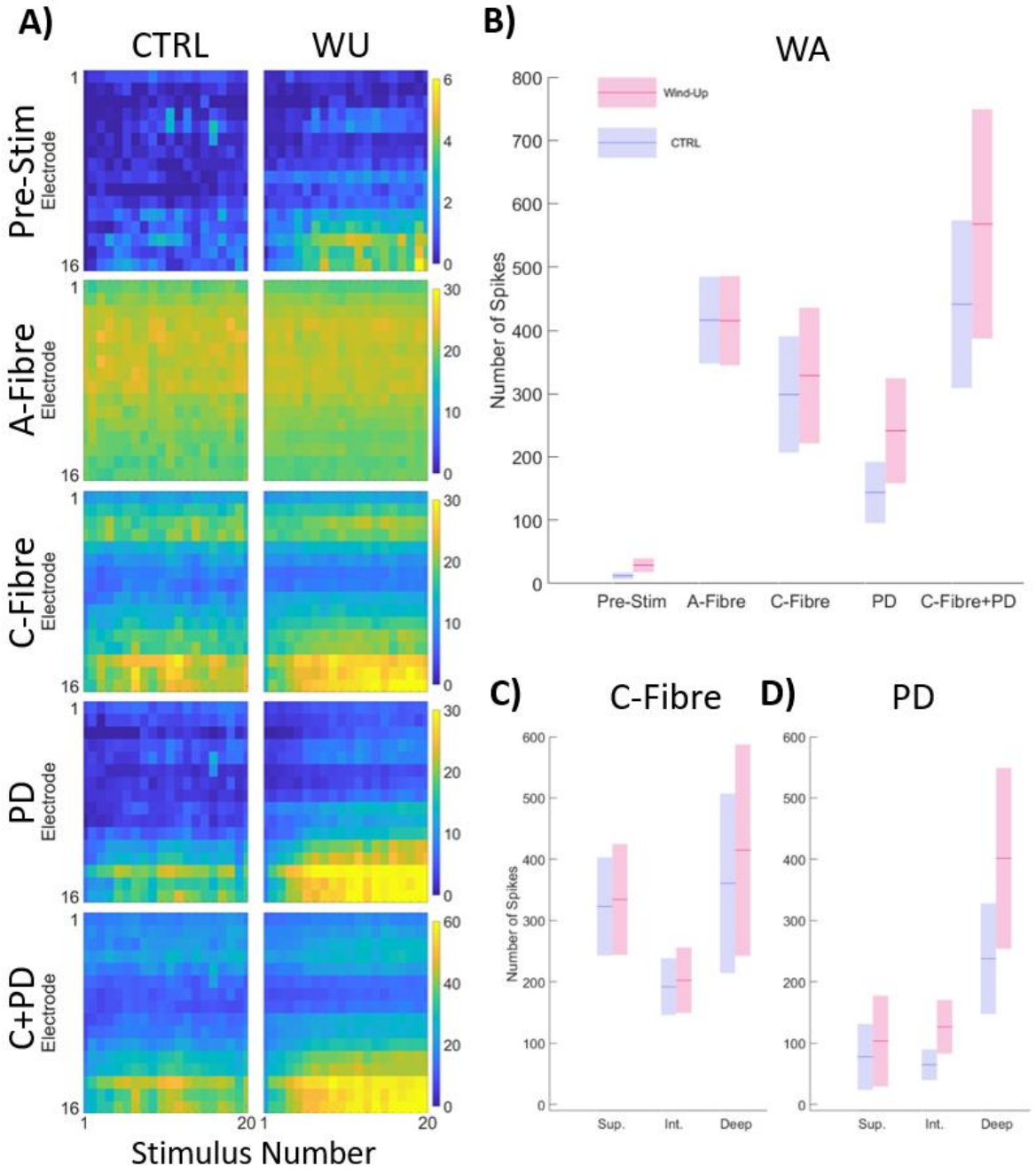


Figure 6.6. Dorsal horn responses to wind-up stimulation in aged adults. A) Heatmaps show spike activity at each electrode following electrical stimulation (20x 5mA at 2ms) of the hindpaw at low (0.5Hz) and high (0.1Hz) frequencies. The first column shows the control (CTRL) responses (low frequency) and the second column shows the responses during the wind-up (WU) protocol (high-frequency).The sum of activity in each latency period is shown as one bin, with the 20 consecutive stimuli displayed along the x-axis. Latency periods correspond to activity at -100-0ms pre-stimulus (Pre-Stim), 3-50ms post-stimulus (A-fibre), 130-330ms post-stimulus (C-fibre), 330-830ms post-stimulus (Post-Discharge (PD)), as well as the C-fibre and Post-Discharge period combined. Responses shown are averaged responses from all animals in the age group, n=5. Electrodes are in order; 1 is the most dorsal and 16 the most ventral. Colours represent mean number of spikes respective to the colour bar shown on the right. B) Summed spike activity across the latency periods shown in (A) across the whole array following control and wind-up stimulation protocols. C&D) Summed spike activity in the superficial, intermediate, and deep regions of the dorsal horn during the C-fibre (C) and Post-Discharge (D) latency periods. For all bar charts lines represent mean and shaded area represents SEM. Two-tailed paired t-tests found no significant differences between control and wind-up responses in any latency period across the whole array or in the C and PD latency periods in any region of the dorsal horn.

6.4.2 Wind-up Responses Undergo Changes throughout the Life Course

To compare wind-up responses between ages the difference between the wind-up and control responses were used to account for the difference in evoked intensity seen between ages in Chapter 4. When comparing these differences in the C-fibre and post-discharge latencies similar response patterns are seen in the dorsal horn from P21 (Figures 6.7 and 6.8). Before this in the P9-11 age group the response is more dispersed throughout the dorsal horn at both latencies but appears to be greatest in the superficial region during the post-discharge period, although this was not statistically tested (Figure 6.8). This most likely reflects the immature C-fibre processing observed previously at this age (Fitzgerald and Gibson, 1984). At P14-16 there is a dispersed C-fibre response to the wind-up stimulation and the post-discharge activity is now most pronounced in the deep dorsal horn on the heatmaps (Figure 6.8). Interestingly the P21-23, young adult, and aged adult wind-up response profiles are very similar in both latencies shown on the heatmaps in Figure 6.8, with activity in the superficial and deep regions of the dorsal horn, although the amount of spike activity varies with age (Figure 6.9 and 6.10). However, the P28-30 group has very little activity in either the C-fibre or post-discharge latency periods in the dorsal half on the array or in the very deepest electrode in the C-fibre latency period response.

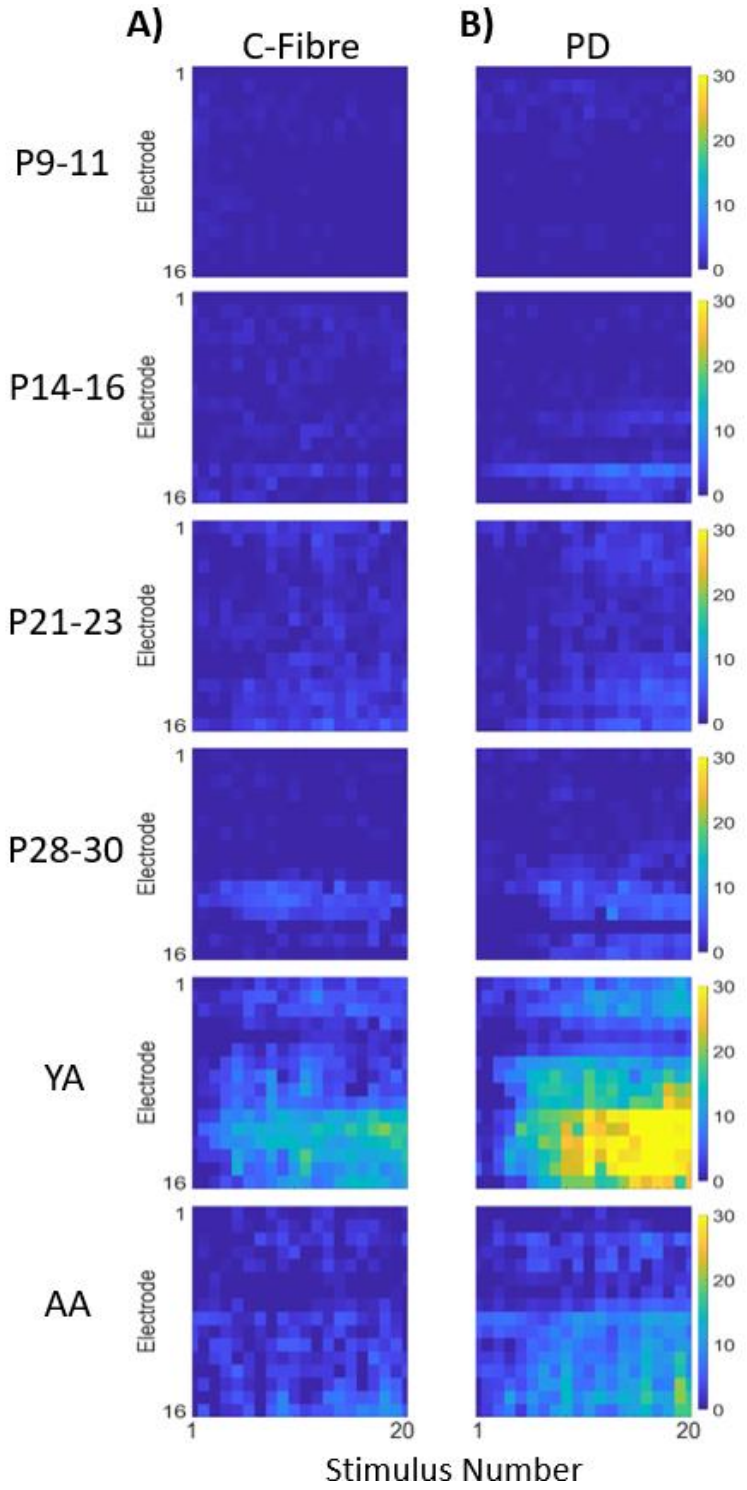


Figure 6.7. Wind-up stimulation produces different response profiles throughout the life course. Heatmaps show the difference in spike activity at each electrode at P9-11 n=5, P14-16 n=6, P21-23 n=5, P28-30 n=6, young adults (YA) n=6, and aged adults (AA) n=5, following wind-up stimulation compared to control stimulation in the C-Fibre (A) and post-discharge (B) latency periods. The C-fibre latency period is 200ms for all ages and the post-discharge period is 500ms after the C-fibre period for each age, the onset post-stimulation is dependent on age (Table 4.3). The sum of activity in each latency period is shown as one bin, with the 20 consecutive stimuli displayed along the x-axis. Responses shown are averaged responses from all animals in the age group. Electrodes are in order; 1 is the most dorsal and 16 the most ventral, for P9-11 electrodes 1 and 16 are removed and at P14-16 electrode 1 is removed, to better represent the size of the dorsal horn at these ages. Colours represent mean number of spikes respective to the colour bar shown on the right. All heatmaps are set to the same colour axis.

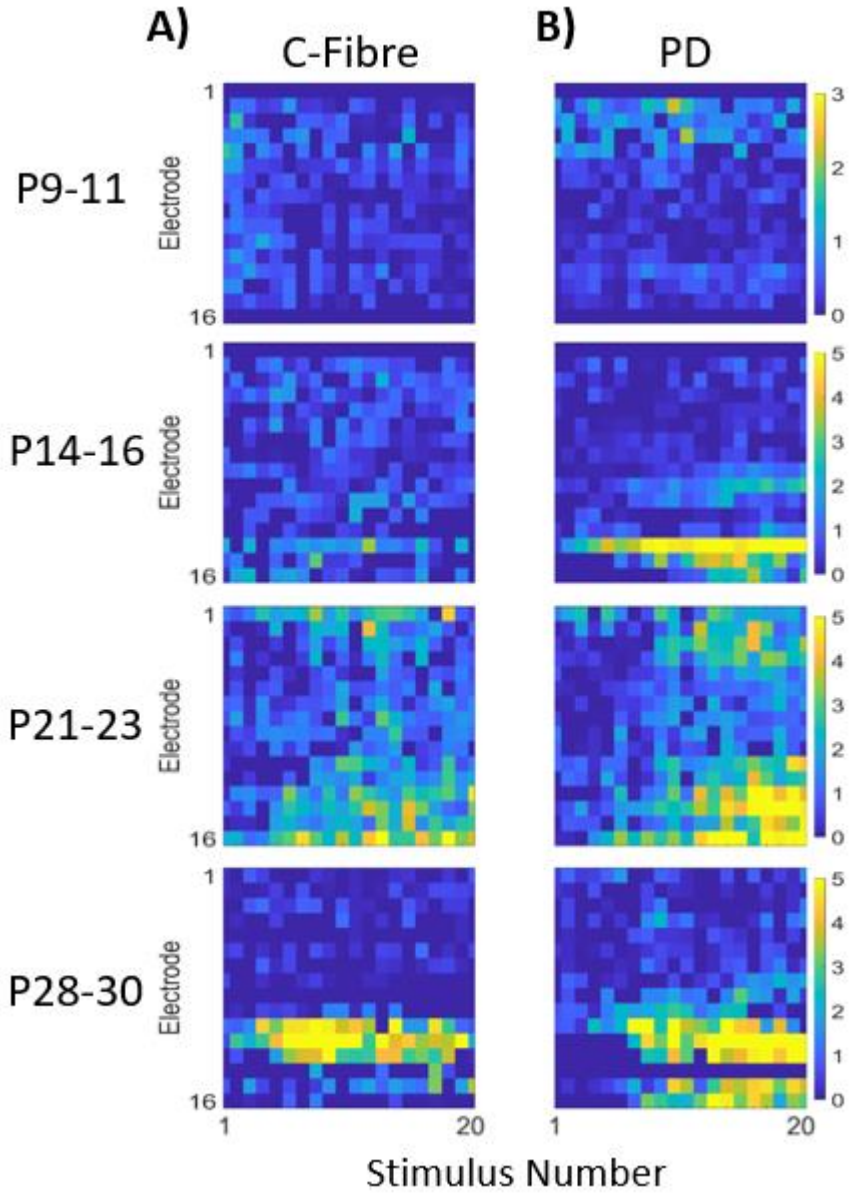


Figure 6.8. Wind-up stimulation produces different response profiles during postnatal development. Heatmaps show the same data as in Figure 6.7 however the caxis has been adjusted to better show the responses at the postnatal ages used. Difference in spike activity at each electrode at P9-11 n=5, P14-16 n=6, P21-23 n=5, and P28-30 n=6, following wind-up stimulation compared to control stimulation in the C-Fibre (A) and post-discharge (B) latency periods. The C-fibre latency period is 200ms for all ages and the post-discharge period is 500ms after the C-fibre period for each age, the onset post-stimulation is dependent on age (Table 4.3). The sum of activity in each latency period is shown as one bin, with the 20 consecutive stimuli displayed along the x-axis. Responses shown are averaged responses from all animals in the age group. Electrodes are in order; 1 is the most dorsal and 16 the most ventral, for P9-11 electrodes 1 and 16 are removed and at P14-16 electrode 1 is removed, to better represent the size of the dorsal horn at these ages. Colours represent mean number of spikes respective to the colour bar shown on the right.

To compare wind-up responses across the whole array at the different ages one-way ANOVAs were used to compare the wind-up response within each latency period. Significant age-related changes were found using Tukey's post hoc tests in the pre-stimulus ($F(5, 27) = 4.832$, $P<0.01$), C-fibre ($F(5, 27) = 8.346$, $P<0.0001$), post-discharge ($F(5, 27) = 6.102$, $P<0.001$), and C-fibre +post-discharge ($F(5, 27) = 7.308$, $P<0.001$) latencies (Figure 6.9). In other words, all fibre latencies investigated showed significant age-related differences in activity apart from the A-fibre latency period. However, this is unsurprising as no A-fibre latency period responses to wind-up stimulation were detected at any age group used in the study.

Both adult groups had more spike activity than the postnatal groups in the pre-stimulus and post-discharge latency periods however only the young adults had statistically significant differences compared to the younger ages (pre-stimulus: P9-11, P14-16 and P21-23 $P<0.05$, P28-30 $P<0.01$; post-discharge $P<0.01$ for all postnatal ages, Figure 6.9). The young adults also showed increased wind-up responses in the C-fibre latency period compared to every other age group tested (P9-11, P14-16, and P28-30 $P<0.001$, P21-23 and aged adults $P<0.01$, Tukey's post hoc test) and the C-fibre + post-discharge latency period (P9-11, P14-16, and P28-30 $P<0.001$, P21-23 $P<0.01$, and aged adults $P<0.05$, Tukey's post hoc test). Interestingly, the P28-30 age group had lower responses than the adult and P21-23 groups, more closely matching the P14-16 age group response level in all latency periods (Figure 6.9).

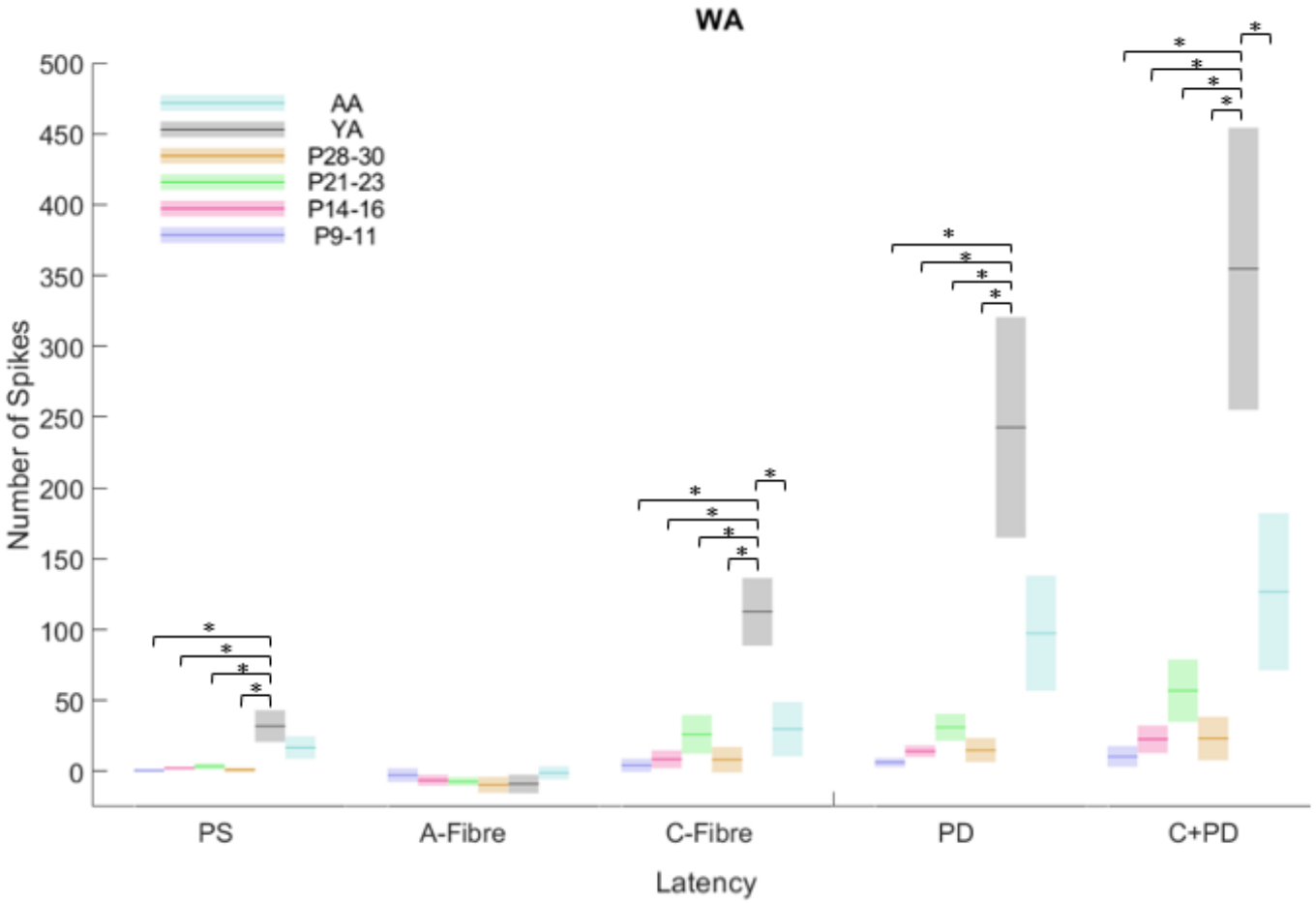


Figure 6.9. Age-dependent changes occur in the dorsal horn response following wind-up stimulation of the hindpaw. Spike activity in the dorsal horn is shown for each age group, P9-11 n=5, P14-16 n=6, P21-23 n=5, P28-30 n=6, YA n=6, AA n=5, as the difference between wind-up and control stimulation summed activity in the pre-stimulus (PS, -100-0ms), A-fibre (3-50ms), C-fibre (200ms post-stimulation for all ages with the post-stimulation onset dependent on age (Table 4.3)), post-discharge (PD, 500ms post-stimulation following the C-fibre latency period for all ages), as well as the C-fibre and post-discharge periods combined. Activity across the whole array is shown but data was not included from electrodes 1 and 16 for the P9-11 group or from electrode 1 for the P14-16 group to better represent the size of the dorsal horn at these ages. Lines represent mean spike number (mean activity across the electrodes) and shaded area represents SEM. Asterisks indicate significant differences between age groups determined by a Tukey's post hoc test.

When comparing between ages within the superficial, intermediate and deep regions of the dorsal horn using one way ANOVAs to compare age difference within each latency period significant differences were only identified in the deep region (Figure 6.10). These significant changes mirror those seen when comparing across the whole array and were again observed in the pre-stimulus ($F(5, 27) = 7.961$, $P=0.0001$), C-fibre ($F(5, 27) = 10.02$, $P<0.0001$), post-discharge ($F(5, 27) = 8.169$, $P<0.0001$), and C-fibre +post-discharge ($F(5, 27) = 9.999$, $P<0.0001$) latencies. Post hoc Tukey's tests also revealed significant differences between wind-up responses in the young adults compared to the postnatal ages (pre-stimulus: P9-11 $P=0.001$, P14-16 and P21-23 $P<0.01$, P28-30 $P<0.001$; post-discharge P9-11, P14-16, and P28-30 $P<0.001$, and P21-23 $P<0.01$, Figure 6.10). The young adults also showed increased wind-up responses in the C-fibre latency period compared to all other age groups tested (P9-11 and P14-16 $P<0.0001$, P21-23 $P<0.001$, P28-30 $P=0.0001$, and aged adults $P<0.01$, Tukey's post hoc test) and the C-fibre + post-discharge latency period (P9-11 and P14-16 $P<0.0001$, P21-23 $P<0.001$, P28-30 $P=0.0001$, and aged adults $P<0.05$, Tukey's post hoc test).

Although significant age-differences were not found in the superficial and intermediate dorsal horn regions the young adult group showed high responses then every other age group in both regions at all latency periods apart from the A-fibre latency period (Figure 6.10A&B).

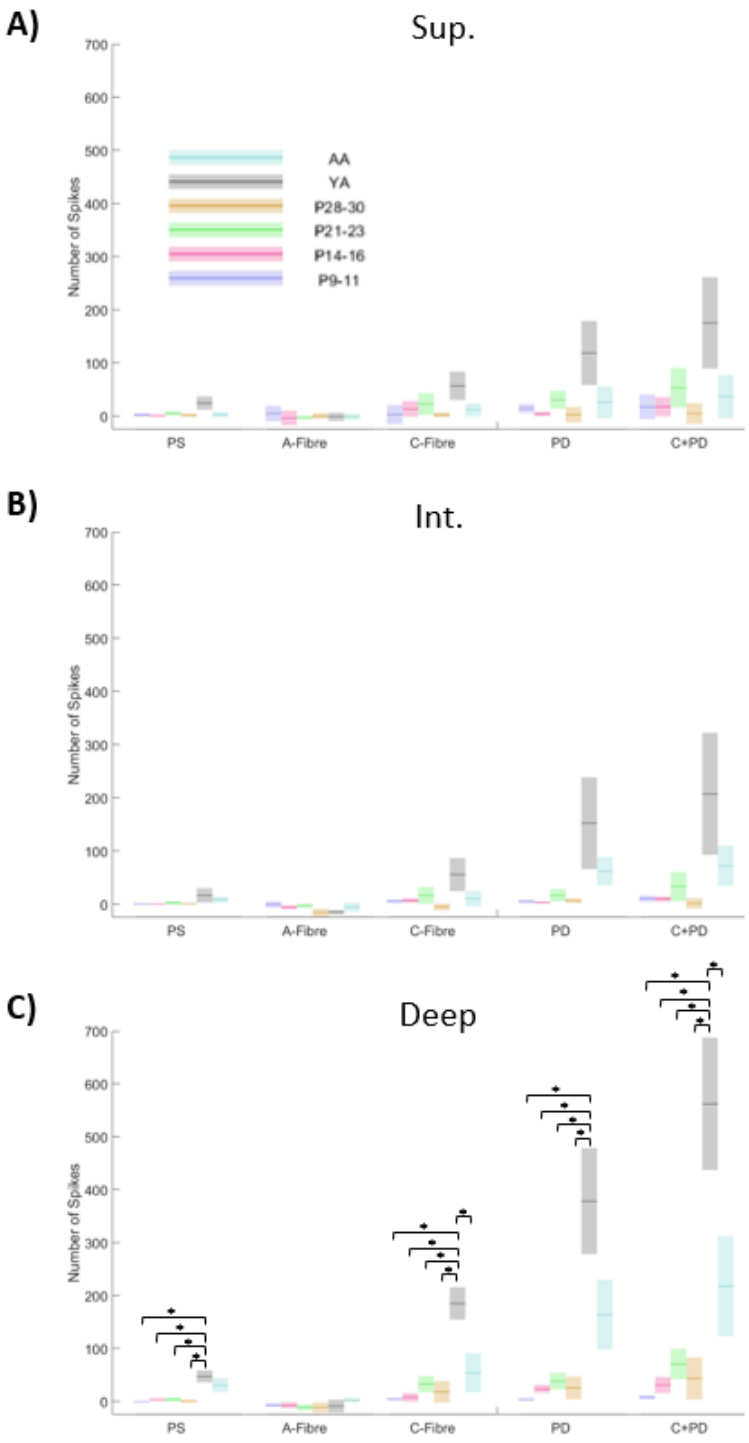


Figure 6.10. Age-dependent changes occur in the deep dorsal horn response following wind-up stimulation of the hindpaw. Spike activity in the dorsal horn is shown for each age group, P9-11 n=5, P14-16 n=6, P21-23 n=5, P28-30 n=6, YA n=6, AA n=5, as the difference between wind-up and control stimulation summed activity in the pre-stimulus (PS, -100-0ms), A-fibre (3-50ms), C-fibre (200ms post-stimulation for all ages with the post-stimulation onset dependent on age (Table 4.3)), post-discharge (PD, 500ms post-stimulation following the C-fibre latency period for all ages), as well as the C-fibre and post-discharge periods combined. Activity across the A) superficial, B) intermediate, and C) deep regions of the dorsal horn is shown but data was not included from electrodes 1 and 16 for the P9-11 group or from electrode 1 for the P14-16 group to better represent the size of the dorsal horn at these ages. Lines represent mean spike number (mean activity across the electrodes) and shaded area represents SEM. Asterisks indicate significant differences between age groups determined by a Tukey's post hoc test.

6.4.3. Diffuse Noxious Inhibitory Control Could Not Be Detected At Any Age Group Tested

A decrease in dorsal horn activity could not be detected at any postnatal age or in young adults using this study protocol. Differences in the number of spikes during the 5 seconds of mechanical stimulation were compared between control applications with no noxious conditioning stimulus (noxious ear clip) and with conditioning stimulus (Figure 6.11-6.15(A)) using two-tailed paired t-tests. The differences between these responses at different mechanical vFh stimulation was also compared using one-way RM ANOVAs within the superficial (Figure 6.11-6.15(B)), intermediate (Figure 6.11-6.15(C)), and deep (Figure 6.11-6.15(D)) dorsal horn regions and well as between regions (not shown). When plotting the difference between DNIC trial responses and control responses values lower than 0 represent a decrease in dorsal horn activity in DNIC trials and as such may reflect decreases due to descending inhibition. Interestingly, when looking at responses from individual rats there are frequently responses below 0, however never for the entire age group. As sex-differences in adults have been shown in DNIC studies (Da Silva *et al.*, 2018; Da Silva *et al.*, 2020) we thought this may explain the variation within the age groups, however these were tested in whole array responses to each test stimulus group and no differences were found at any age (two-way t-tests).

Postnatal Day 9-11

The majority of animals showed a trend of higher dorsal horn responses to hindpaw 2g and 6g mechanical stimulation following application of the conditioning stimulus (Figure 6.11A) at P9-11, although not statistically significant. Interestingly, some decreases in activity were detected when using the 15g stimulation, although again not statistically significant. Changes in the 15g response were spread throughout the whole array with values below 0 being seen in the superficial (Figure 6.11B), intermediate (Figure 6.11C), and deep (Figure 6.11D) regions. 2g and 6g responses showed the lowest values in the deep region although these were showing very little change between control and DNIC trials rather than decreases. A two-way RM ANOVA comparing the difference in spike number between control and DNIC trials across the different regions and following the different stimulus intensities found no significant changes or interaction (regions: $F(2, 8) < 1$, intensities: $F(2, 8) < 1$, interaction: $F(4, 16) = 1.11$, $P=0.3858$). DNIC has previously been suggested to not occur until P21 so these findings are expected (Boucher, Jennings and Fitzgerald, 1998). Additionally no sex differences were found at P9-11.

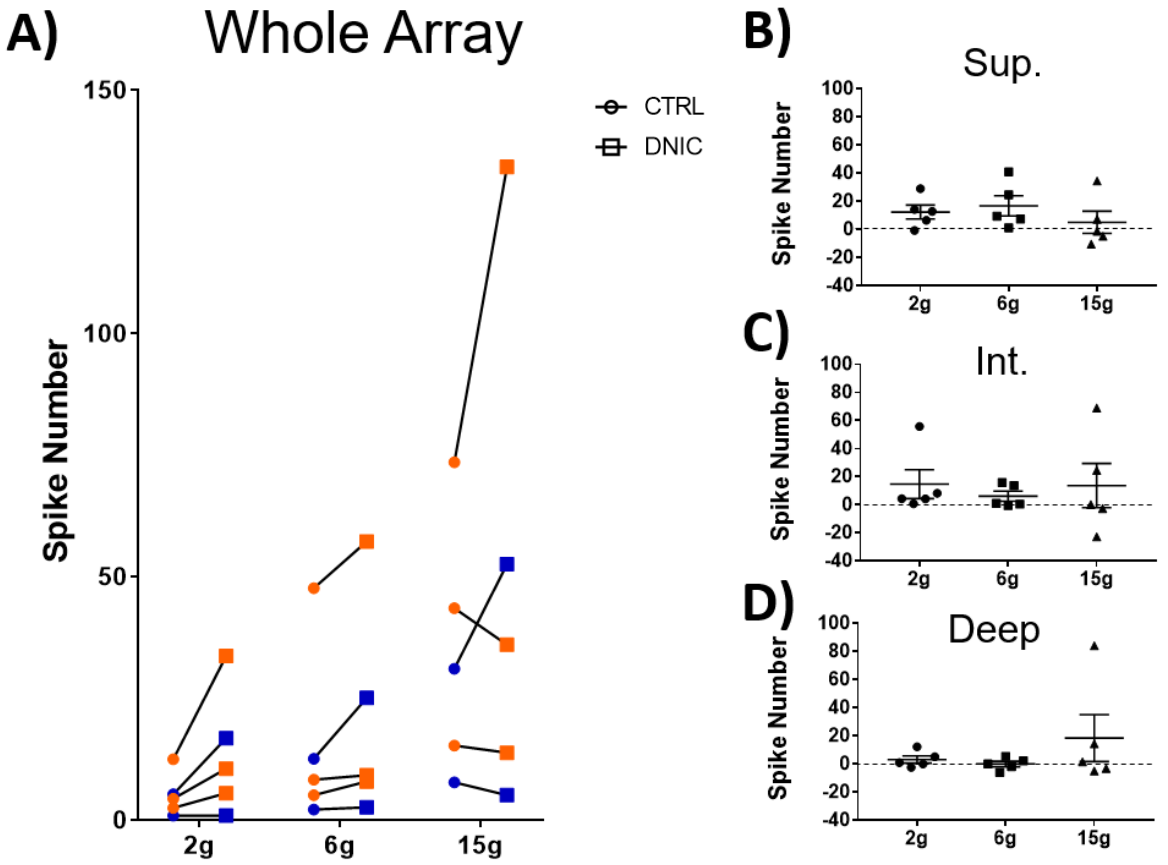


Figure 6.11. Dorsal horn responses to mechanical hindpaw stimulation following a DNIC protocol in P9-11 rats. A) The sum of activity during the 5seconds of 2g, 6g, and 15g vFh hindpaw mechanical stimulation during the control (CTRL) trial is shown as circles and during the DNIC trial is shown as squares. Each circle or square represents an individual, n=5. Data represents the mean activity of the two control trials or three DNIC trials and the mean activity across the whole array, excluding data collected from electrodes 1 and 16 to better represent the size of the P9-11 dorsal horn. Orange is used to detonate female individuals and blue male individuals. B,C,D) the difference in spike number following control and DNIC trials is shown for each individual in the superficial (B), intermediate (C), and deep (D) regions of the dorsal horn. Lines represent mean +/- SEM. No significant differences were found between CTRL and DNIC responses across the WA, or between sexes, following two-tailed paired t-tests. A Two-way ANOVA revealed no significant difference between stimulation intensities or dorsal horn regions, or an interaction.

Postnatal Day 14-16

By P14-16 there appear to be mostly increases in activity following 2g, 6g, and 15g mechanical stimulation when in the presence of the conditioning stimulus (Figure 6.12). However, whilst these increases in evoked activity are similar in all regions following 2g and 6g stimulation there is a shift, although not significant, following 15g stimulation with increased activity being detected for almost all individuals in the intermediate and deep dorsal horn, but decreases observed in over half of individuals in the superficial dorsal horn region. A two-way RM ANOVA comparing the difference in spike number between control and DNIC trials found no region dependent ($F(2, 10) < 1$) or stimulus dependent ($F(2, 10) = 1.062$, $P=0.38$) changes, but did show an interaction between the two factors ($F(4, 20) = 2.954$, $P<0.05$). Although at P14-16 descending inhibitory modulation is not mature previous work has suggested DNIC may be becoming detectable, from P12 (Boucher, Jennings and Fitzgerald, 1998).

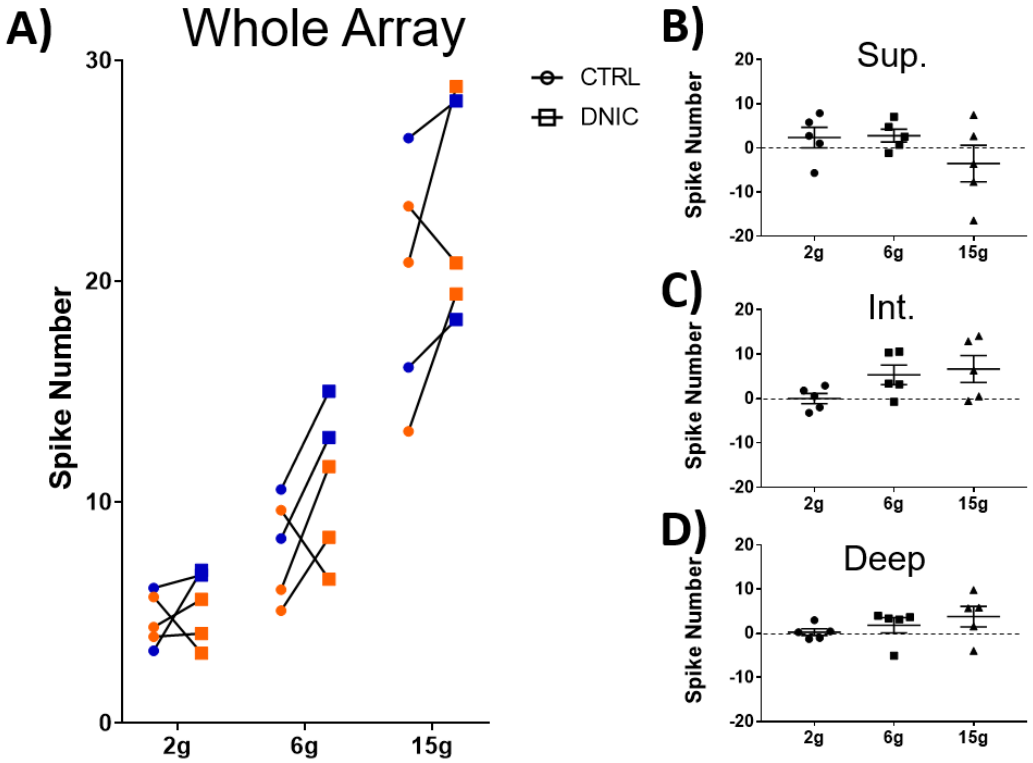


Figure 6.12. Dorsal horn responses to mechanical hindpaw stimulation following a DNIC protocol in P14-16 rats. A) The sum of activity during the 5seconds of 2g, 6g, and 15g vFh hindpaw mechanical stimulation during the control (CTRL) trial is shown as circles and during the DNIC trial is shown as squares. Each circle or square represents an individual, n=6. Data represents the mean activity of the two control trials or three DNIC trials and the mean activity across the whole array, excluding data collected from electrode 1 to better represent the size of the P14-16 dorsal horn. Orange is used to detonate female individuals and blue male individuals. B,C,D) the difference in spike number following control and DNIC trials is shown for each individual in the superficial (B), intermediate (C), and deep (D) regions of the dorsal horn. Lines represent mean +/- SEM. No significant differences were found between CTRL and DNIC responses across the WA, or between sexes, following two-tailed paired t-tests. A two-way ANOVA revealed no significant difference between stimulation intensities or dorsal horn regions, but did show an interaction between the two factors.

Postnatal Day 21-23

By the third postnatal week the evoked dorsal horn response across the whole array following hindpaw mechanical stimulation is predominantly having no change or increasing in DNIC test trials when the conditioning stimulus is applied (Figure 6.13). A two-way RM ANOVA comparing the difference in spike number between control and DNIC trials across the different regions and following the different stimulus intensities found no significant changes, or interaction (regions: $F(2, 8) < 1$, intensities: $F(2, 8) = < 1$, interaction: $F(4, 16) < 1$). DNIC-induced response differences at P21-23 were variable in all regions of the dorsal horn but very few decreases were observed in the deep dorsal horn (Figure 6.13D) as seen in the younger age groups.

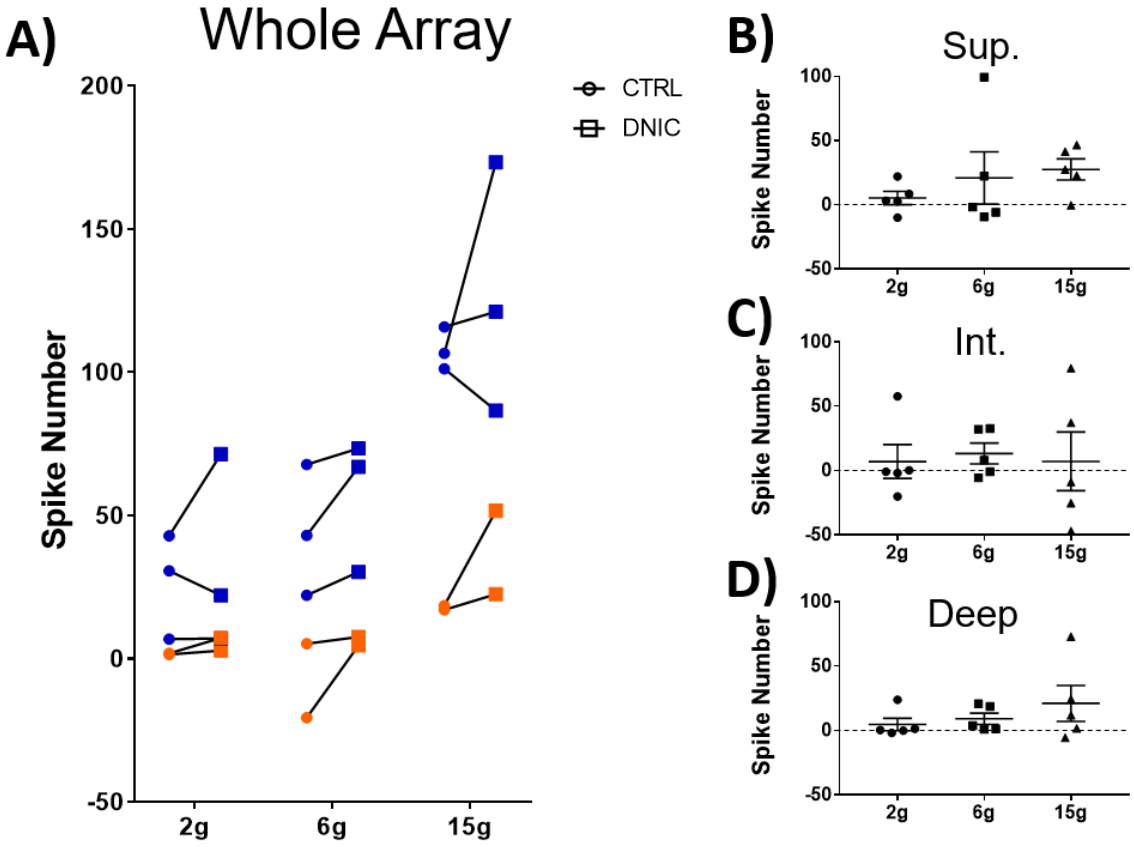


Figure 6.13. Dorsal horn responses to mechanical hindpaw stimulation following a DNIC protocol in P21-23 rats. A) The sum of activity during the 5seconds of 2g, 6g, and 15g vFh hindpaw mechanical stimulation during the control (CTRL) trial is shown as circles and during the DNIC trial is shown as squares. Each circle or square represents an individual, n=5. Data represents the mean activity of the two control trials or three DNIC trials and the mean activity across the whole array. Orange is used to detonate female individuals and blue male individuals. B,C,D) the difference in spike number following control and DNIC trials is shown for each individual in the superficial (B), intermediate (C), and deep (D) regions of the dorsal horn. Lines represent mean +/- SEM. No significant differences were found between CTRL and DNIC responses across the WA, or between sexes, following two-tailed paired t-tests. A two-way ANOVA revealed no significant difference between stimulation intensities or dorsal horn regions, or an interaction.

Postnatal Day 28-30

At P28-30 there is in general little change in evoked dorsal horn activity following hindpaw mechanical stimulation at 2g in DNIC compared to control trials. A mixture of increase and decreases are then observed following 6g and 15g stimulation, although not in a sex dependent manner (Figure 6.14A). Looking at differences within specific regions there are no decreases within the intermediate region of the dorsal horn, few in the superficial region, and several following the higher stimulus intensities in the deep dorsal horn (Figure 6.14B-D). A two-way RM ANOVA comparing the difference in spike number between control and DNIC trials showed region-dependent changes ($F(2, 10) = 4.45$, $P<0.05$) but no stimulus dependent changes ($F(2, 10) <1$) or interaction ($F(4, 20) = 1.109$, $P=0.38$). As DNIC has been shown to function via inhibiting WDR neurons (Le Bars, Dickenson and Besson, 1979), which are located in the deep dorsal horn, this region is where we would expect to see decreases in neuronal activity if DNIC is occurring.

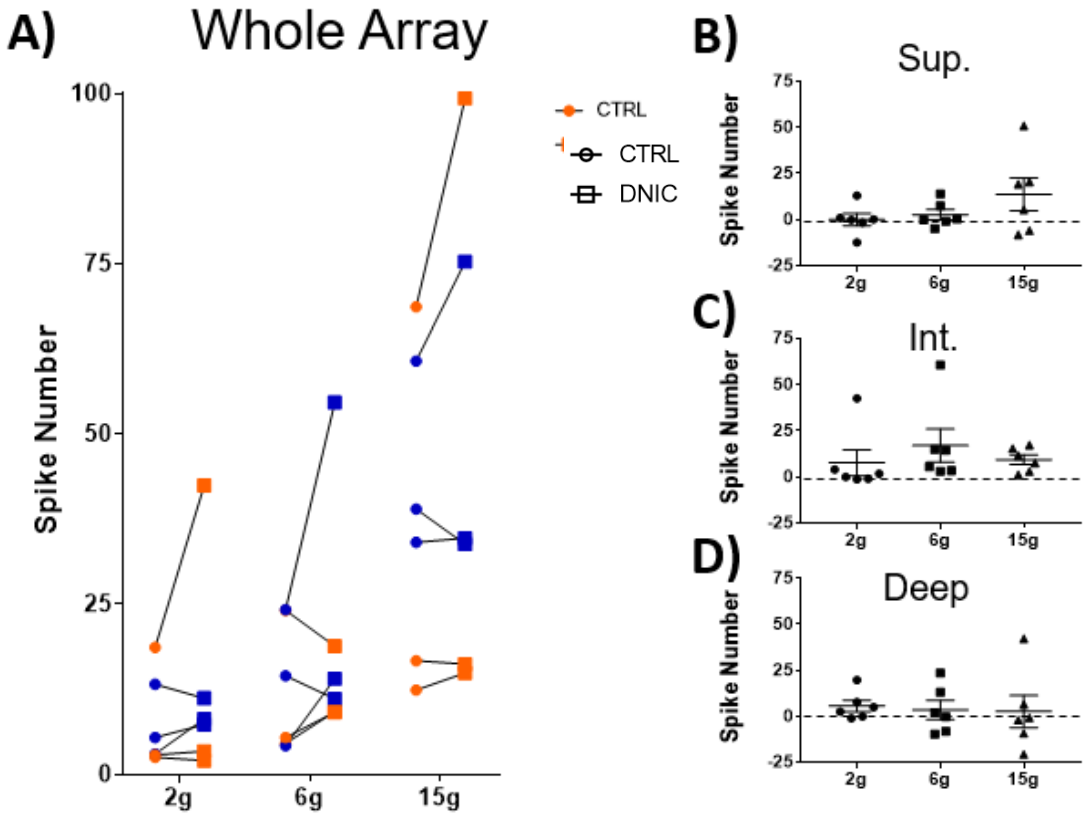


Figure 6.14. Dorsal horn responses to mechanical hindpaw stimulation following a DNIC protocol in P28-30 rats. A) The sum of activity during the 5seconds of 2g, 6g, and 15g vFh hindpaw mechanical stimulation during the control (CTRL) trial is shown as circles and during the DNIC trial is shown as squares. Each circle or square represents an individual, n=6. Data represents the mean activity of the two control trials or three DNIC trials and the mean activity across the whole array. Orange is used to detonate female individuals and blue male individuals. B,C,D) the difference in spike number following control and DNIC trials is shown for each individual in the superficial (B), intermediate (C), and deep (D) regions of the dorsal horn. Lines represent mean +/- SEM. No significant differences were found between CTRL and DNIC responses across the WA, or between sexes, following two-tailed paired t-tests. A two-way ANOVA revealed a significant difference between dorsal horn regions but no difference between stimulation intensities or an interaction between the two.

Young Adults

In the young adults there were a variety of response changes to mechanical stimulation of the hindpaw following application of the conditioning stimulus (Figure 6.15). Looking at the DNIC-induced differences within specific dorsal horn regions there a few decreases occurring within all regions, however slightly more are seen in the deep dorsal horn compared to the other regions. No significant regional ($F(2, 10) < 1$) or stimulus intensity ($F(2, 10) < 1$) changes, or interaction ($F(4, 20) = < 1$) were found using a two-way RM ANOVA.

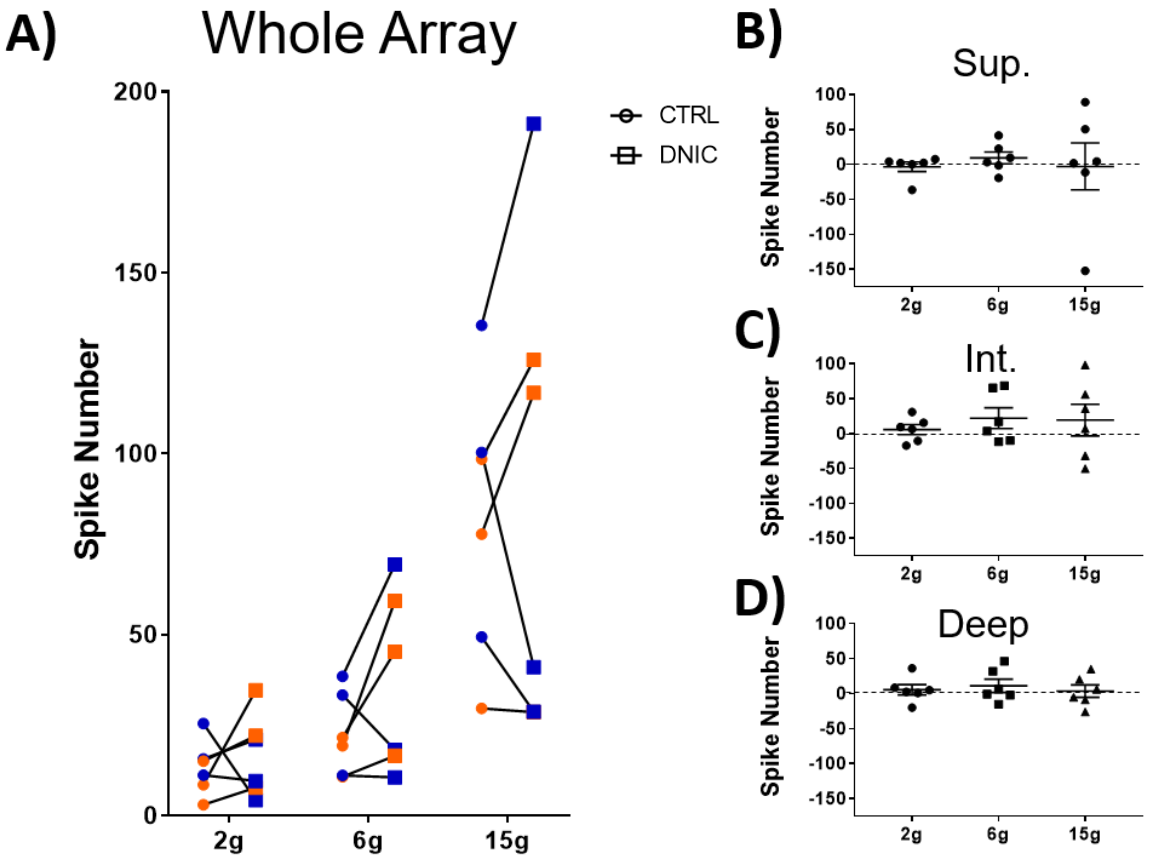


Figure 6.15. Dorsal horn responses to mechanical hindpaw stimulation following a DNIC protocol in young adult rats. A) The sum of activity during the 5seconds of 2g, 6g, and 15g vFh hindpaw mechanical stimulation during the control (CTRL) trial is shown as circles and during the DNIC trial is shown as squares. Each circle or square represents an individual, n=6. Data represents the mean activity of the two control trials or three DNIC trials and the mean activity across the whole array. Orange is used to detonate female individuals and blue male individuals. B,C,D) the difference in spike number following control and DNIC trials is shown for each individual in the superficial (B), intermediate (C), and deep (D) regions of the dorsal horn. Lines represent mean +/- SEM. No significant differences were found between CTRL and DNIC responses across the WA, or between sexes, following two-tailed paired t-tests. A two-way ANOVA revealed no significant difference between stimulation intensities or dorsal horn regions, or an interaction between the two factors.

6.4.4. No Age-Related Difference Were Found Following a DNIC Protocol

No significant differences were found between ages when comparing the difference in responses between control and DNIC trials (one-way ANOVAs, $P>0.05$). This was not surprising as few significant differences were identified at any age. There do however appear to be trends towards reduced activity following the application of the conditioning stimulus (responses lower than 0) possibly reflecting DNIC in the superficial region following low intensity mechanical stimulation and in the deep region following higher mechanical stimulus intensities to the hindpaw, particularly in P28-30 and young adult groups when descending inhibition has matured (G. J. Hathway *et al.*, 2009b; Hathway, Vega-Avelaira and Fitzgerald, 2012; Kwok *et al.*, 2014b).

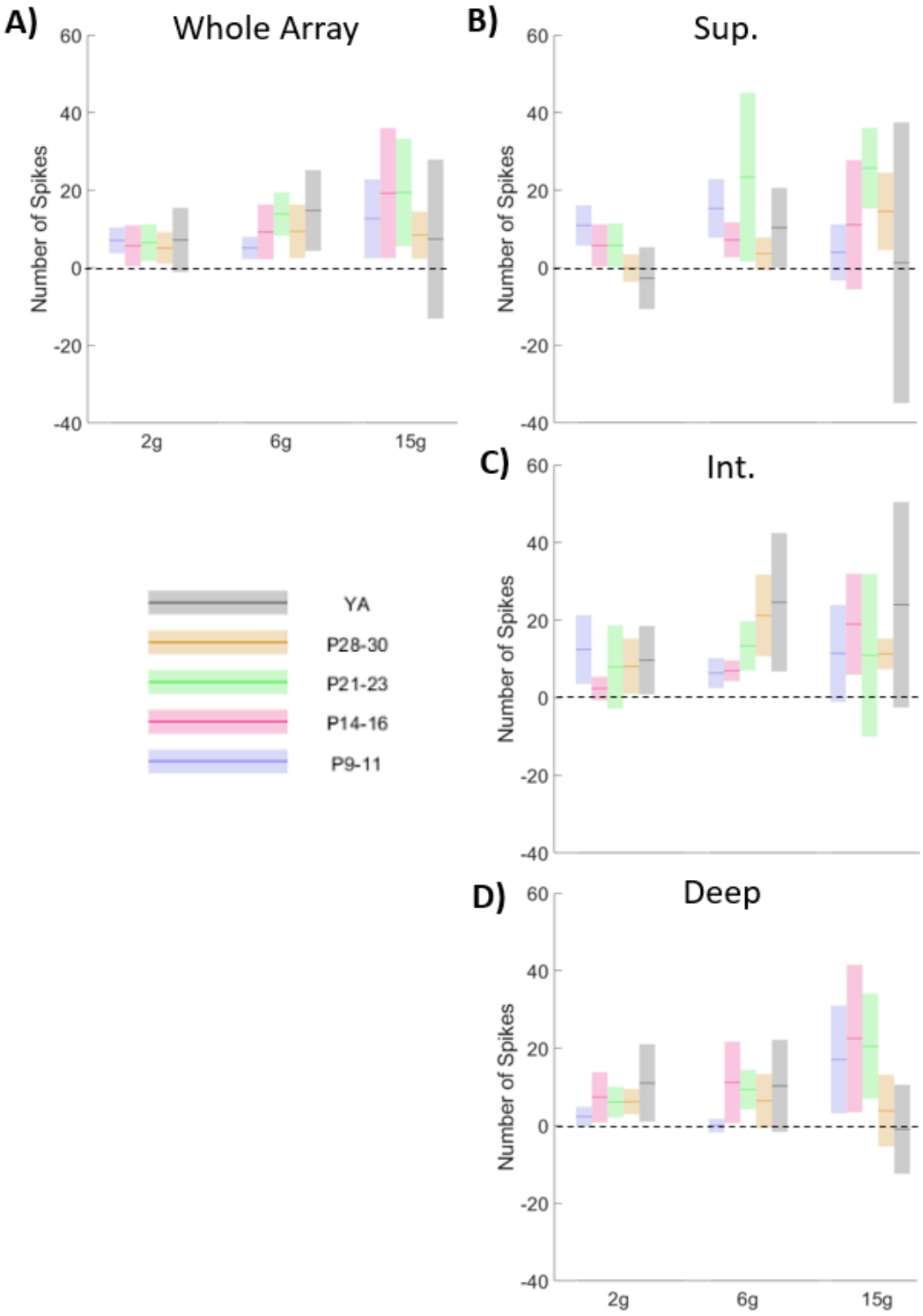


Figure 6.16. Dorsal horn responses to mechanical hindpaw stimulation following a DNIC protocol do not change with age. The difference in the number of spikes recorded over 5seconds of 2g, 6g, and 15g vFh hindpaw mechanical stimulation following control and DNIC trials is shown for each age group, P9-11 n=5, P14-16 n=6, P21-23 n=5, P28-30 n=6, YA n=6, across the whole array (A), superficial (B), intermediate (C), and deep (D) regions of the dorsal horn. Lines represent mean +/- SEM. Dashed line shows y-axis 0 as responses below 0 represent a lower response in DNIC trials compared to control, possibly reflecting DNIC responses. No significant difference were found between age groups following any stimulus intensity or in any region of the dorsal horn using one-way ANOVAs.

6.5 Discussion

The work presented in this chapter showed increased spike activity in the deep dorsal horn of young adults compared to all other ages tested following wind-up stimulation of the hindpaw in the C-fibre latency period and the combined C-fibre and post-discharge period. In addition, we found increased spike activity in the young adults during the pre-stimulus and post-discharge periods compared to all of the postnatal groups.. However, diffuse noxious inhibitory control was not detected at any age, using the current protocol. As such the aims set out at the beginning of this chapter were not all achieved and further work would be required to reach these.

6.5.1 Dorsal Horn Processing Following Wind-Up Stimulation Changes throughout the Life Course

6.5.1.1 Postnatal Changes in Dorsal Horn Wind-Up

Increases in spike activity could be detect in the C-fibre and post-discharge latency periods in every region of the dorsal horn at P9-11, however no statistically significant wind-up responses were detected. As wind-up has been previously shown to occur in the P10 dorsal horn using single-unit electrophysiology (Fitzgerald and Jennings, 1999) we expected to find significant differences following wind-up stimulation at this age group. However, this previous study also suggested that the percentage of cells showing wind-up at this age is less than half of the number showing wind-up responses at P21 (Fitzgerald and Jennings, 1999). Therefore, it is possible that as we are using population recordings, the number of cells showing wind-up at this age is too low to have a significant effect. It is however worth noting that there is also a large amount of variation within the P9-11 age group, possibly reflect the ongoing development of C-fibre processing within the dorsal horn which is immature until the second postnatal week (Fitzgerald and Gibson, 1984), discussed further in Chapter 4. The post-discharge period in the P9-11 rats was below baseline in all regions of the dorsal horn, this may reflect a hyperpolarised state following high intensity stimulation at this age. Interestingly, this is also seen in the P21-23 dorsal horn but not at P14-16.

By P14-16 increases in dorsal horn activity following wind-up stimulation were significant in the post-discharge latency period but not the C-fibre latency period. Although previous studies have found C-fibres to be mature by the second postnatal week (Fitzgerald and Gibson, 1984), we previously suggested that although present, C-fibre processing in the dorsal horn is not mature at this age (See Chapter 4). This immaturity may be why wind-up in the C-fibre latency period is not statistically

significant. As $NK1^+$ spinoparabrachial neurons have been shown to be capable of responding to peripheral stimuli at P3 (S. H. W. Man, Géranton and Hunt, 2012) and NMDA receptors in the dorsal horn, although not fully mature, are functional from at least P14 (Urch, Rahman and Dickenson, 2001) the results may suggest this immaturity represents the C-fibre input more than the wind-up dorsal horn processing. Alternatively, this could be a result of the increase duration over which the activity was summed as the C-fibre latency period was 200ms and the post-discharge latency period 500ms, however if this was the case the C-fibre + post-discharge period would also show significant differences, which it does not. Suggesting the post-discharge period specifically is increased following wind-up stimulation at P14-16. Indeed this has been suggested previously in adult studies where post-discharge activity was produced in the absence of C-fibre activity (Greenspon *et al.,* 2019). The deep dorsal horn in particular showed increases in the post-discharge latency period as well as the intermediate region. Matching the expected location of wind-up effect which is associated predominantly with WDR neurons in the deep dorsal horn laminae (Seagrove, Suzuki and Dickenson, 2004).

Although there was always a trend of increased neuronal activity following wind-up stimulation compared to control stimulation in the C-fibre and post-discharge latency periods fewer significant wind-up responses were observed in the P21-23 and P28-30 dorsal horns. The only significant increase in neuronal activity was in the P21-23 age group across the whole array but not in any individual region of the dorsal horn. By the third postnatal week wind-up responses during the C-fibre latency period were now visible in the heatmaps in the deep and superficial dorsal horn, matching expected wind-up locations, as some wind-up activity has been shown to occur in the superficial lamina (Seagrove, Suzuki and Dickenson, 2004). However, by P28-30 there is almost no activity during the C-fibre or post-discharge latency periods in the dorsal half of the array. A phenomenon observed and discussed in previous studies presented in this thesis.

6.5.1.2 Wind-Up in the Adult Dorsal Horn

Between P28-30 and the young adult group, which in this study were an average age of P41, there is a very significant increase in dorsal horn activity in control and wind-up responses. As the generation of wind-up requires NMDA receptors (Herrero *et al.,* 2000) this could possibly be related to the NMDA receptors in the dorsal horn having a greater effect on wind-up in postnatal rats (P14-28) than in adults (Urch, Rahman and Dickenson, 2001). Significant wind-up responses are observed in the pre-stimulus, C-

fibre, post-discharge, and C-fibre + post-discharge latency periods. This is the only age group in which significant wind-up changes were found in the C-fibre latency period, possibly suggesting maturation of C-fibre processing or the circuits involved in wind-up only occur by this age. The young adult group had significantly higher levels of wind-up activity compared to the younger ages in every fibre latency period investigated, apart from the A-fibre latency period, and compared to the aged adults in the C-fibre and C-fibre + post-discharge periods. The dramatic increase in wind-up responses over the space of approximately 10 days may suggest a critical period in dorsal horn processing occurs at this age point. As expected the wind-up responses in the young adults were mostly in the deep dorsal horn with significance in the C-fibre and post-discharge latency period being found in these regions. Additionally, although it was not significant there were increases in the superficial laminae in these latencies as well, supporting previous findings of wind-up location in the adult dorsal horn (Seagrove, Suzuki and Dickenson, 2004).

Compared to the young adults the aged adult group had smaller increases in neuronal activity following wind-up stimulation. This was significantly different in the C-fibre and C + post-discharge latencies but not the post-discharge or pre-stimulus latencies. This may suggest that the C-fibre processing has declined in the aged adults, something that has been suggested in previous studies (Namer, 2010; Weyer *et al.*, 2016).

6.5.1.3 A-fibre

The lack of wind-up activity during the A-fibre latency period supported previous findings that suggest wind-up is C-fibre and post-discharge specific (Herrero, Laird and Lopez-Garcia, 2000). Interestingly, whilst not significant at any age there was always a trend of reduction in activity following wind-up stimulation compared to control stimulation during the A-fibre latency period. Although previous studies have reported an A-fibre related form of wind-up in the neonatal dorsal horn (Jennings and Fitzgerald, 1998) this used electrical stimuli 2x the A-fibre threshold, and as such the stimulation used in this study may have been too intense at 5mA. Additionally, by P10 this was observed in only 6% of WDR cells (Jennings and Fitzgerald, 1998) and so may not be detectable using the multielectrode array electrophysiology.

6.5.1.4 Wind-up Observed in the Pre-Stimulation Period

Increased activity was observed during the wind-up stimulation protocol in the pre-stimulus time latency period in every age group, and was significant in the young adult group. This is most likely an extension of the increases observed in the post-stimulus

latency period. As the stimuli are delivered at 0.5Hz this would mean there is a prolonged after discharge reaching at least 2 seconds to be detected in the pre-stimulus latency period of the subsequent stimulus. Previous papers have shown prolonged post-discharges of approximately 63 seconds in P10 rats following high frequency A-fibre stimulation, however this was absent by P21 (Jennings and Fitzgerald, 1998). As wind-up is thought to involve multiple pre- and post-synaptic mechanisms (Herrero, Laird and Lopez-Garcia, 2000) this may reflect ongoing activity within these circuits.

6.5.1.5 Increased Responses Following Low Frequency Stimulation

An interesting observation, although not statistically tested, made in this study was that the lower frequency stimulation (0.1Hz) also appeared to produce an increased level of neuronal activity in the C-fibre and post-discharge latencies particularly in P21-23, P28-30, and the young adult groups. Wind-up is usually tested above 0.3Hz, and most commonly at 0.5Hz, as done in this study, so it was unexpected that any build-up in activity would be observed at 0.1Hz. It is possible that the repeated high intensity stimulation even at a low-frequency is activating alternative microcircuits within the dorsal horn which may not involve the WDR neurons to the same extent as classical wind-up and as such may have been missed with previous single-unit studies. Alternatively this may reflect a difference in testing and analysis (see Section 6.5.2).

6.5.2 Technical Considerations in the Wind-Up Study

Usually in wind-up studies using single-unit electrophysiological experiments an individual WDR cell is identified and tested, and the stimulation intensity is then set at 3x the C-fibre threshold for that cell (Urch and Dickenson, 2003). As individual cells are not identified with the population recordings obtained using the MEA this could not be done. In our lab single unit recordings of WDR neurons usually find C-fibre thresholds in adult male SD rats to be between 1.5 and 2.5mA (using a 2ms pulse width in a bipolar setup), resulting in three times the threshold being between 4.5 and 7.5mA. Although 2x C-fibre thresholds have been shown to be sufficient (Smith *et al.*, 2020) thus requiring 3 to 5mA stimulation. 5mA has also been used in the previous adult studies by the group using MEA electrophysiology (Greenspon *et al.,* 2019) and so was chosen as the amplitude in this study.

When analysing the wind-up responses classically the sum activity over the wind-up stimulations is compared to the 1st stimulus multiplied by the number of wind-up stimuli (Urch and Dickenson, 2003). However we compared the sum number of spikes over the 20 stimuli in the control train with the sum number of spikes over the 20 stimuli of the

wind-up train, as this is similar to the analysis used previously by the group (Greenspon *et al.,* 2019). However, we may be seeing reduced effects as analysing the wind-up responses this way as we see small increases in activity over the control stimulation train as discussed above. This results in the difference between the control and wind-up responses being reduced as we appear to be comparing low-frequency 'wind-up' to high frequency wind-up.

6.5.3 Dorsal Horn Processing Following a Diffuse Noxious Inhibitory Control Protocol

Few significant differences were found in the DNIC study at any age group and none were identified between age groups. Although trends were observed in the results obtained suggesting that from P28 there may be a decline in dorsal horn response to peripheral mechanical stimulation in combination with a noxious conditioning stimulus. This trend was observed in the superficial dorsal horn following 2g vFh stimulation and in the deep dorsal horn following 15g vFh stimulation. Although DNIC has been previously suggested to occur by P21 (Boucher, Jennings and Fitzgerald, 1998) as P28 is the age at which descending inhibition has matured (G. J. Hathway *et al.*, 2009b; Hathway, Vega-Avelaira and Fitzgerald, 2012; Kwok *et al.*, 2014b) it seems logical that DNIC would be observed at this age and above. Additionally, no sex differences were found, although other studies have identified sex differences in DNIC (Da Silva *et al.*, 2018; Da Silva *et al.*, 2020) these have related to functional connectivity in supraspinal sites rather than neuronal responses in the dorsal horn.

DNIC should be detectable using innocuous or noxious test stimuli (Villanueva, Cadden and Le Bars, 1984; Bannister and Dickenson, 2017) so the vFhs chosen, although lower in intensity than those used in other groups who used 8, 26, and 60g vFhs (Bannister *et al.*, 2015) they should have been able to demonstrate DNIC if it was occurring. In line with this the 8g vFh in the above mentioned study showed significant decreases in DNIC trials. Similarly, a noxious ear clamp was used as the conditioning stimulus, which is widely used for this purpose (Bannister *et al.*, 2015; Patel and Dickenson, 2020). Additionally, the noxious nature of the ear clamp was unintentionally confirmed following a reflex to its application in a P11 rat, resulting in the termination of recordings for that animal and extra caution in following experiments.

It is possible that the population recordings are not well designed to study inhibitory effects such as DNIC. However, the most likely explanation is that the study was underpowered. Power calculations suggested group sizes of 10 for studies involving mechanical stimulation of the hindpaw, however as the main aim of the study was to investigate wind-up responses which required a group size of 6 this was the group size

used. This decision was in combination with limitations on remaining time and funding. Overall, trends which are in line with expected observations in DNIC were found and this study could act as a pilot for a follow-up using larger group sizes and potentially higher intensity test stimuli.

6.5.4 Conclusions

In conclusion dorsal horn wind-up in the first four weeks of life in rats is related to increased activity in the post-discharge latency period only and not in the C-fibre latency period as seen in adults. As such this study suggests that C-fibre related wind-up is not mature until approximately P41 and a critical period in C-fibre and wind-up processing may occur between P30 and P41. Declines in C-fibre related wind-up responses were also observed in aged adults, possibly supporting previous findings of altered C-fibre processing in old age. Unfortunately, whilst no significant results were obtained in the DNIC study, the trends observed suggest region specific DNIC responses may occur from P28 matching the age of mature descending inhibition and warrant further study.

Chapter 7

General Discussion and Conclusions

The primary aim of this thesis was to employ the multielectrode array electrophysiology technique used previously in adults only, in postnatal rats, enabling the study of dorsal horn development to be investigated with an alternative technique. Studies were conducted using a range of peripheral stimulation modalities involving, thermal, mechanical, and electrical stimulations as well as protocols designed to study inhibitory modulation and dorsal horn plasticity. Our results were often able to support previous findings from single-unit electrophysiology studies, additionally finding results that were contrary or novel, as discussed below.

Chapter 3

Findings presented in Chapter 3 confirmed that the MEA electrophysiology technique used was able to detect dorsal horn responses to hindpaw stimulations in rats as young as P9 and as old as 18months. Initially we compared spontaneous activity levels at different ages. Single unit electrophysiology commonly uses WDR neurons which have a general lack of spontaneous background activity at all postnatal ages (Jennings and Fitzgerald, 1998) so shed little light on this aspect of dorsal horn development. However, patch-clamp recordings of lamina I projection neurons have measured and compared spontaneous activity between postnatal ages but found little change during postnatal development (Li and Baccei, 2012). Conversely, we showed increases in spontaneous activity levels in the dorsal horn which increased with age. This suggests spontaneous activity is produced by cells other than WDR and projection neurons, or that the intact connections to the brain have an effect on spontaneous dorsal horn activity.

Behavioural changes have suggested that mechanical thresholds mature by P28 (Fitzgerald, M, Shaw, A, MacIntosh, 1988) and thermal by P12 (Falcon *et al.*, 1996). Although we can not directly compare spinal activity to behavioural responses mechanical evoked responses showed little regional change between ages but increased in the amount of evoked activity until adulthood. Thermal stimulation resulted in changes in response location between P9-11 and P14-16 moving from a more intermediate to deeper location, potentially fitting with the suggested thermal response maturation, although again the response intensity increased with increasing age until adulthood. Previous work has shown a withdrawal of Aβ-fibre input from superficial laminae I and II down to III and below over the first three or four postnatal weeks (M Fitzgerald, Butcher and Shortland, 1994; Nakatsuka and Kumamoto, 2000; Beggs *et al.*, 2002) but we did not observe that in response to mechanical stimulation which resulted in responses throughout the dorsal horn at all ages. Although more statistically

significant differences were found in the intermediate and deep regions, supporting the findings that these regions have a higher proportion of A-fibre inputs. The data collected here matches previous findings from our group which showed significant increases in mechanical evoked activity in all regions of the dorsal horn in young adults but more so in the intermediate and deep regions (Greenspon *et al.,* 2019).

Chapter 4

In the fourth chapter electrical stimulation of the hindpaw was used to allow the dorsal horn processing of A- and C-fibre stimulation to be investigated. The conduction velocity of PAFs has been measured in neonates previously finding increased A-fibre conduction velocities with postnatal age (Vejsada *et al.*, 1985; Lizarraga, Chambers and Johnson, 2007). As most studies focus on the first two postnatal weeks our results complimented and built upon these findings to show significant increases in A- but not C-fibre conduction velocity with increasing postnatal age until adulthood. This resulted in similar A-fibre onset times but delayed C-fibre input with increasing age, matching suggested latency periods for general neonatal electrophysiology studies reported previously (Urch and Dickenson, 2003).

The neonatal dorsal horn has been previously described as being A-fibre dominant with C-fibre maturation not occurring until the second postnatal week (Fitzgerald and Gibson, 1984, Koch *et al.,* 2012). In line with this we found A-fibre responses were present in all ages, whereas, C-fibre responses were incredibly low at P9-11 but clearly present by P14. However, the A-fibre response did not appear to have the same dorsal horn response profile at P9-11 or P14-16 as it did in P21 and above, where a clear 'double burst' of activity was seen which split around 10/11ms. This was previously observed in adults using this technique (Greenspon *et al.,* 2019) suggesting that A-fibre activity, although present, is not mature until P21. Even then, the intensity increased with increasing age until adulthood resulting in bigger jumps in spike number between stimuli especially in the intermediate region. This possibly reflects an increased ability to distinguish subtle differences in input intensity with increasing age.

Similarly, although present at P14, C-fibre responses were unusually short and intense. In the following ages the evoked activity in the C-fibre latency periods (respective for each age group) appear to increase in duration until adulthood where a double burst is seen, again seen previously in MEA recordings (Greenspon *et al.,* 2019). Further suggesting that C-fibre activity processing in the dorsal horn is maturing until adulthood, with a possible key increase in maturation between P30 and P41.

Interestingly, age-related differences in both A- and C-fibre latency periods occurred only following stimulations at 1.75mA or above suggesting that perhaps the majority of postnatal maturation involves responses to nociceptive stimulations.

PAF innervations as mentioned above are regionally distinct but are thought to change during postnatal development. As with the mechanical data in Chapter 3, we see widespread A-fibre responses in the laminae of the dorsal horn throughout the life course as was observed previously by our group (Greenspon *et al.,* 2019). C-fibre activity, however, matched the expected regions of input with higher responses in the superficial and deep dorsal horn (Andrew J Todd, 2010).

Chapter 5

In the fifth chapter, disinhibition of GABAergic signalling was investigated using the $GABA_AR$ antagonist bicuculline. Previous studies have shown that the dorsal horn is under tonic inhibition from P8 which increases until P21 when adult levels are reached (Zell *et al.*, 2014). Similarly, we see a trend of increased spontaneous dorsal horn activity following cumulative bicuculline application to the dorsal horn at all ages tested, suggesting tonic GABAergic inhibition occurs in all ages tested in this study. However, this is only statistically significant in the aged adult group, potentially supporting the suggestion that tonic inhibition increases with age.

Previous studies have shown A-fibre responses, in particular Aβ responses, to be under inhibitory control, with disinhibition revealing normally silenced polysynaptic A-fibre pathways (Baba *et al.*, 2003; Torsney and MacDermott, 2006). GABA is the predominant inhibitory neurotransmitter in the postnatal dorsal horn (Baccei and Fitzgerald, 2004) but remains as a key neurotransmitter throughout the life course and has been shown to again play a more dominant role than glycinergic inhibition in the dorsal horn of aged animals (Mayhew *et al.*, 2019). In this study local cumulative application of bicuculline resulted in dose dependent increases in A-fibre responses in all ages. Supporting the role of $GABA_AR$ mediated inhibition of A-fibre inputs throughout the life course. Additionally, GABAergic inhibition has been previously shown to be particularly dominant in the superficial dorsal horn (Polgár *et al.*, 2003; Inquimbert and Rodeau, 2007; Bardoni *et al.*, 2013) however the effects seen on A-fibres in this study was similar across all dorsal horn regions, until the aged adults where the superficial region did show an increased effect. Suggesting that even in young adults dorsal horn inhibition may still be developing.

Dorsal horn responses in the C-fibre latency period showed significantly different responses between ages, with increased activity compared to pre-drug levels observed

in P14-16 and young adult rats but decreased activity in P21-23 and aged adults. This is potentially due to the 1mA stimulation not being supramaximal for C-fibre stimulations and as such, we suggest repeating this study with a higher stimulus intensity to more accurately determine C-fibre responses. P21 has been previously identified as the age at which dorsal horn inhibitory signalling is mature (Keller *et al.*, 2001; Baccei and Fitzgerald, 2004; Cordero-Erausquin *et al.*, 2005; Ingram, Fitzgerald and Baccei, 2008), however in this study P21-23 animals were particularly unresponsive to the bicuculline applications following electrical stimulation, suggesting maturation has not been reached at this age.

Changes in dorsal horn activity following mechanical hindpaw stimulation were in general not statistically significant but showed an increased onset response at all ages with increased bicuculline doses, supporting previous findings (Rahman *et al.,* 2007). Interestingly we noticed a second peak at mechanical stimulus onset following the highest bicuculline dose in P21 and above, possibly reflecting Aβ mediated circuits normally under GABAergic inhibition (Torsney and MacDermott, 2006).

Chapter 6

In the sixth chapter dorsal horn plasticity was tested using wind-up and diffuse noxious inhibitory control protocols. Classical dorsal horn wind-up has previously been shown in P10 rats, although in fewer cells than in the adult dorsal horn (Fitzgerald and Jennings, 1999). We detected an increase in dorsal horn activity in P9-11 rats but this was not statistically significant until P14-16, supporting the onset of wind-up occurring at approximately P10.

Classically, wind-up is measured using the combined C-fibre and post discharge latency periods, whilst we did measure this we also looked at the C-fibre and post discharge periods separately. Doing this we found that wind-up evoked increases in dorsal horn activity in the postnatal ages was statistically significant in the post-discharge latency period only, and significant increases in the C-fibre period did not occur until adulthood. It is worth noting that the method of analysis used in this chapter was different to classical analysis and may be linked with a decreased amount of statistical significance, however it takes into account the difference between high-frequency and low frequency evoked increases in dorsal horn activity observed in this study. Additionally, increases were seen in the pre-stimulus period although this is most likely reflective of a prolonged post discharge activity. Previous work by the group showed similar wind-up responses when using male adult rats (Greenspon *et al.,* 2019).

DNIC has been observed using single-unit electrophysiology in many studies (Bannister *et al.*, 2015; Patel and Dickenson, 2020). As it is associated with descending inhibitory pathways (Le Bars, Dickenson and Besson, 1979) we decided to investigate the effect on dorsal horn activity throughout the life course to see if we could observed the development of DNIC. A previous study using c-fos expression in the dorsal horn suggested DNIC occurred from P21 (Boucher, Jennings and Fitzgerald, 1998). However, investigating DNIC effects on dorsal horn responses in this study was not very effective. Although some expected trends were observed with declines in dorsal horn response activity in the superficial and deep regions occurring at P28 and in young adults, no statistically significant results were obtained. We believe this most likely reflects that the study was underpowered for mechanical testing.

7. 1 Dorsal Horn Processing of Somatosensory Input throughout Postnatal Development

In general, previous single-unit electrophysiological studies have shown a trend of increasing evoked activity with increasing postnatal age (Fitzgerald and Jennings, 1999; Koch *et al.*, 2012; Schwaller, Kanellopoulos and Fitzgerald, 2017b) although some do not show any age-related trends (Torsney and Fitzgerald, 2002; Schwaller, Kwok and Fitzgerald, 2016b). Throughout this thesis we have shown an increase in the baseline and evoked dorsal horn activity with increasing postnatal age. Thus it is likely that this increase in dorsal horn activity is not directly indicative of an overall excitable phenotype as behaviourally neonates show lower thresholds to nociceptive stimuli and respond with exaggerated movements to painful stimulation compared to adults (Fitzgerald, 2005). Further studies are needed to fully understand the postnatal maturation of dorsal horn somatosensory processing. Additionally, in patch clamp recordings of WDR neurons previous studies have shown aged adults have higher spike activity compared to younger adults (Iwata *et al.*, 2002; David-Jürgens *et al.*, 2008). We do not see this and instead often see a decline in evoked spike number compared to young adults. This may be a difference of in vivo compared to ex vivo recordings, or possibly a difference in the exact ages used between studies.

In addition to the findings discussed above some interesting observations were made across studies throughout postnatal development. The short and intense C-fibre activity detected at P14-16 was consistent between the studies presented in Chapters 4 and 6. The response increased in spike number following bicuculline applications but did not increase in its duration suggesting that the immature duration length is not linked with GABAergic inhibition. As glycinergic inhibitory activity matures between P14 and P21

(Koch *et al.,* 2012) it is unlikely to be associated with glycinergic inhibition either. Similarly descending inhibition is not developed at this age (G. J. Hathway *et al.*, 2009b; Hathway, Vega-Avelaira and Fitzgerald, 2012; Kwok *et al.*, 2014b). This may suggest a difference in C-fibre input into the dorsal horn which matures until young adulthood, disagreeing with previous suggestions of C-fibre functional maturation by the second postnatal week (Fitzgerald and Gibson, 1984).

During the third postnatal week, dorsal horn activity was frequently lower than baseline levels following evoked responses. This may reflect hyperpolarisation and primarily involved studies involving C-fibre activation; thermal stimulation, wind-up, C-fibre activity, and activity in the C-fibre latency period following bicuculline applications. This did not occur in the age groups immediately below or above the P21-23 group and so it marks a potentially interesting stage in postnatal development. This age group also had very little change in A-fibre activity following bicuculline applications, possibly suggesting an altered level of GABAergic signalling at this age point. As far as we are aware, hyperpolarisation at this age in particular has not been previously reported but as this is the age at which glycinergic inhibition has matured these may be associated. It would be interesting to study the effects of glycinergic disinhibition at this age to investigate whether it is responsible for these findings.

In the fourth postnatal week we observed a decrease in evoked activity recorded within the dorsal horn in Chapters 3, 4, and 6. Following electrical hindpaw stimulation in Chapters 4 and 6 there is a particular decrease in the superficial region. This decline is not present in the first A-fibre burst of activity, where activity throughout the array is detected. As this represents data collected from a separate group of animals, from different litters and collected approximately two years apart it is unlikely to be a result of an unusual set of animals or recording noise. It is possible that the positioning of the electrode has been done incorrectly, however the response profile in the intermediate and deep dorsal horn appears as expected; similar to the shape of the adult response activity on the heatmaps. Additionally, there is very little variation in the superficial responses, suggesting that if this was an error in electrode placement it has been made in every animal in this age range, but not in animals younger or older, which again seems unlikely. It is in retrospect unfortunate that this age group was not included in the bicuculline study as that may have revealed some GABA-related inhibitory changes occurring at this postnatal time point, as GABAergic inhibition is dominant in the superficial dorsal horn and this is the region we are seeing reduced activity. However this could potentially be done later along with an increased pharmacological study to examine the mechanistic changes underlying this decline in superficial dorsal horn activity. As this is a key age in the development of descending inhibition (G. J. Hathway

et al., 2009; Hathway, Vega-Avelaira and Fitzgerald, 2012; Kwok *et al.*, 2014) it may reflect an increase in this, which is balanced fully by the young adult, again this warrants further study. Although this does not fully support previous findings in the literature this is the first time population recordings of this type have been conducted in postnatal rats and may reflect changes outside of the individual WDR and nociceptive neurons investigated previously with single-unit techniques.

Previous studies have suggested alterations in sensory processing in the aged adult dorsal horn (Iwata *et al.*, 2002; David-Jürgens *et al.*, 2008; Mayhew *et al.*, 2019). In the data presented in this thesis aged adults frequently showed responses lower than the young adult group with evoked spike activity more similar to the postnatal age groups, although the mechanisms involved are likely different. As the aged animals used here are arguably late-middle age rather than old (Sengupta, 2013) it would be interesting to study the dorsal horn processing in older animals, although this would be an expensive endeavour.

Additionally, it is worth noting that as the young adult group was repeatedly different in dorsal horn responses compared to every other age group it brings into question if this is the best model to use. Although the postnatal age groups were expected to show alterations in dorsal horn processing compared to the young adult group we frequently saw a large increase in dorsal horn spike numbers between the P28-30 and young adult group. This led to multiple conclusions that processing within the dorsal horn is not mature until adulthood. However, as a plateau in responses was never reached it would be interesting to do a more thorough age-related study from the young adult ages of approximately P42 (used here) compared to at least P60, as this is often considered 'adult' in other studies, to determine how consistent responses are within the adult age range.

7.2 Limitations in the Presented Work

One of the main limitations with this work is that population recordings do not allow us to distinguish which cells are being recorded. A possible solution to this problem would be the combined use of optogenetic techniques to target specific cell types. Most likely we are getting a large amount of recording from interneurons, as these cells represent the greatest portion of the neurons in the dorsal horn (Todd, 2010) and PAF input. Whilst it was relatively straightforward to investigate PAF input activity, it would be much more complex to investigate the full extent of subsequent processing circuits. Additionally, as the population recordings are interpreted in regard to overall excitability it is hard to delineate the contribution of excitatory and inhibitory neurons to this

balance of activity. Again the addition of optogenetic techniques may help in determining dorsal horn circuits.

Overall, whilst multielectrode array electrophysiology offers a methodological advance on single-unit recordings, which is capable of being used throughout the life course to study developmental changes, we have not been able to use the data recorded to its full potential. The extent of the data obtained with this technique is vast and new data analysis techniques are needed to process the complex temporal-spatial activity detected. This may be possible using more complex bioinformatics approaches.

Additionally we had more variation within groups than expected following previous work by the group (Greenspon *et al.,* 2019). This may be due to wider age ranges being used in adult groups as well as the three-day age range used in the postnatal groups considering the relatively fast postnatal maturation occurring in the dorsal horn. For example the difference between C-fibre activity recorded in the P9-11 and P14-16 groups was very large considering there are only two days between these groups. Future studies may be benefit from selecting single postnatal days to compare. Similarly, the mechanical data showed much larger variation within each age group than expected and most likely reflects the activation of multiple cells' receptive fields as opposed to when recording from a single cell.

7.3 Future Work

In addition to combining MEA recordings with optogenetic techniques and more complex data analysis, as mentioned above, there are multiple future studies that would be interesting to pursue. This work has identified age-related changes in somatosensory processing throughout the dorsal horn during postnatal maturation, this could be done following early life pain models to explore the effect of these insults on somatosensory processing within the dorsal horn. Additionally, combining these recordings with electrical or pharmacological manipulation of supraspinal sites, particularly the RVM and PAG to study the effect of descending modulation on dorsal horn processing would be interesting in adults and particularly during postnatal development. Alternatively as recordings successfully took place in P9 rats, the technique could most likely be used in adult mice, which may increase feasibility of studies involving genetically altered aspects.

7.4 General Conclusions

In summary the work presented in this thesis has validated the use of multielectrode array electrophysiology as a technique to study postnatal development in the dorsal horn. Whilst it offers simultaneous recordings throughout the dorsal horn laminae and significantly greater amounts of data than single-unit electrophysiology, it does lack the ability to distinguish which cells are being recorded. We investigated dorsal horn responses to a range of peripheral stimulations throughout the life course and were able to compare between these to add to the understanding of the postnatal development of somatosensory processing. Overall, whilst we found multiple results that supported previous single-unit and patch clamp studies we suggest that the dorsal horn has not matured in its processing of somatosensory inputs until young adulthood. Whilst this contradicts previous studies which predominantly suggest maturation has occurred by P28 it may reflect further development of dorsal horn circuits rather than activity of specifically WDR neurons. This suggests further studies are needed to determine the development of dorsal horn processing in later postnatal life and early adulthood.

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Appendices

MSP_Pipeline Script

```
params.resolution = 100; % Milliseconds
params.pre_stim = 5; % Seconds
params.stimulus_duration = 5; % Seconds. Use 0 for electrical stimuli
params.post_stim = 60; % Seconds

% Optional parameters. Will default to 1 if not declared here.
params.n_stims_group = 3 % Number of stimuli per group.
%params.n_base = 20; % Number of stimuli to calculate mean baseline_matrix

MSP_Core

% Enable the appropriate script for the experiment
mode = 'Physiological'; % Choose 'None', 'Electrical' or 'Physiological'
or add another

if strcmp(mode, 'None') ==  1
  display 'No post-processing script selected.'
  return

elseif strcmp(mode, 'Electrical') ==  1
  MSP_Elec_Latency
  params.experiment_type = 'Drug'; % Choose 'Amplitude', 'Duration',
'Windup', or 'Drug'
  MSP_Elec_Sorting

elseif strcmp(mode, 'Physiological') ==  1
  params.stim_style = 'Alternate'; % % Choose 'Alternate', or 'Continuous'
  Etho_MSP_SecSub

else
  display 'Error selecting post-processing script.'
end
```

MSP_Core Script

```
if isfield(params, 'n_stims_group') == 0
  params.n_stims_group = 1;
end

if isfield(params, 'n_base') == 0
  params.n_base = 1;
end

params.epoch_length = params.pre_stim + params.post_stim +
params.stimulus_duration;
data.bins = [-
params.pre_stim:(0.001*params.resolution):(params.stimulus_duration +
params.post_stim)];
time = data.bins + ((params.resolution/1000) / 2);
```

```
data.time = time(1:length(time)-1);
data.time_ms = data.time *1000;

display 'Analysis parameters:'
display(['     Number of stimuli per group: ',
num2str(params.n_stims_group)])
display(['     Epoch length: ', num2str(params.epoch_length), ' s'])
display(['     Bin resolution: ', num2str(params.resolution), ' ms'])
display(['     Bin range: ', num2str(data.bins(1)), ' ms to ',
num2str(data.bins(end)), ' ms'])
display(['     Baseline correction stimuli: ', num2str(params.n_base)])

% Checking number of events
params.n_groups = length(Stimulus_Event)/params.n_stims_group;
integ = rem(params.n_groups,1);
if integ ~= 0
    display 'Incorrect number of stimuli'
    return
end

display 'Sorting channels...'; tic,
MSP_Arrange
data.depth = [1:16];
data.raw = arranged_data;
data.stims = Stimulus_Event;
clearvars -except data params

display 'Finding stimuli and binning data...';
data.binned = zeros(length(data.time),16,length(data.stims));
for stimulus = 1:length(data.stims)
     stim_start = data.stims(stimulus) - params.pre_stim;
     stim_stop = data.stims(stimulus) + (params.stimulus_duration +
params.post_stim);
     for channel = 1:16
          start_vector = find_ind(stim_start, data.raw(:,channel));
          end_vector = find_ind(stim_stop, data.raw(:,channel));
          unbinned = data.raw(start_vector:end_vector,channel);
          unbinned = unbinned - data.stims(stimulus);
          for bin = 1:length(data.time)
               count(bin) = sum(unbinned>data.bins(bin)) -
sum(unbinned>data.bins(bin+1));
          end
          data.binned(:,channel,stimulus) = count;
     end
end
clearvars -except data n_stims_group params

display 'Completing baseline subtraction...';
baseline = find_ind((0.1 * -params.pre_stim), data.time);
data.binned_bs = zeros(size(data.binned));
baseline_matrix =
mean(mean(data.binned(1:baseline,:,1:params.n_base),3),1);

for stimulus = 1:length(data.stims)
     for channel = 1:16
          data.binned_bs(:,channel,stimulus) =
data.binned(:,channel,stimulus) - baseline_matrix(channel);
     end
end
clearvars -except data params
```

MSP_Arrange Script

```
vars = whos;
for i = 1:length(vars)
    var_max(i,:) = getfield(vars(i), 'size');
end
var_max = max(var_max(:,1));

arranged_data = NaN(var_max,16);
arranged_data(1:length(sig021i),1) = sig021i;
arranged_data(1:length(sig020i),2) = sig020i;
arranged_data(1:length(sig022i),3) = sig022i;
arranged_data(1:length(sig019i),4) = sig019i;
arranged_data(1:length(sig023i),5) = sig023i;
arranged_data(1:length(sig018i),6) = sig018i;
arranged_data(1:length(sig024i),7) = sig024i;
arranged_data(1:length(sig017i),8) = sig017i;
arranged_data(1:length(sig029i),9) = sig029i;
arranged_data(1:length(sig028i),10) = sig028i;
arranged_data(1:length(sig030i),11) = sig030i;
arranged_data(1:length(sig027i),12) = sig027i;
arranged_data(1:length(sig031i),13) = sig031i;
arranged_data(1:length(sig026i),14) = sig026i;
arranged_data(1:length(sig032i),15) = sig032i;
arranged_data(1:length(sig025i),16) = sig025i;
```

MSP_Elec_Latency

```
display 'Mode: Electrical';
latencies = {'Pre-Stimulus', 'A-Beta', 'A-Delta', 'C-Fibre', 'Post-
Discharge'};
params.latencies = [-100, -1; 3, 11; 11, 90; 90, 300; 300, 800]; %
Milliseconds
for i = 1:5
  params.latency_index(i,1) = find_ind(params.latencies(i,1),
data.time_ms);
  params.latency_index(i,2) = find_ind(params.latencies(i,2),
data.time_ms);
end
for i = 1:size(params.latency_index,1)
  l1 = round(data.time_ms(params.latency_index(i,1)));
  l2 = round(data.time_ms(params.latency_index(i,2)));
  d = sprintf('   %s: %d ms to %d ms', char(latencies(i)), l1, l2);
  disp(d)
end
clearvars d i l1 l2 latencies

[counts.sum, counts.mean, counts_bc.sum, counts_bc.mean] =
deal(zeros(5,16, length(data.stims))); % (latency, channel, stimulus)
for s = 1:length(data.stims)
  for c = 1:16
    for l = 1:length(params.latencies)
        counts.sum(l, c, s) =
sum(data.binned_bs(params.latency_index(l,1):params.latency_index(l,2), c,
s));
        counts.mean(l, c, s) =
```

```
mean(data.binned_bs(params.latency_index(l,1):params.latency_index(l,2),
c, s));
      if l == 1 % Don't correct baseline itself
        counts_bc.sum(l, c, s) = counts.sum(l, c, s);
        counts_bc.mean(l, c, s) = counts.mean(l, c, s);
      else
        counts_bc.sum(l, c, s) = counts.sum(l, c, s) - (counts.mean(1,c,s)
* (params.latency_index(l,2) - params.latency_index(l,1)));
        counts_bc.mean(l, c, s) = counts.mean(l, c, s) -
counts.mean(1,c,s);
      end
    end
  end
end
clearvars l c s
```

MSP_Elec_Sorting

```
if isfield(params, 'experiment_type') == 0
  display 'No experiment type declared, terminating early.'
  return
end

if strcmp(params.experiment_type, 'Threshold') ||
strcmp(params.experiment_type, 'Drug')  ==  1
  [sorted.sum, sorted.mean, sorted_bc.sum, sorted_bc.mean] =
deal(zeros(5,16,params.n_groups));
  start = 1; stop = params.n_stims_group;
  for group = 1:params.n_groups
    sorted.sum(:,:,group) = mean(counts.sum(:,:,start:stop),3);
    sorted.mean(:,:,group) = mean(counts.mean(:,:,start:stop),3);
    sorted_bc.sum(:,:,group) = mean(counts_bc.sum(:,:,start:stop),3);
    sorted_bc.mean(:,:,group) = mean(counts_bc.mean(:,:,start:stop),3);
    start = start + params.n_stims_group; stop = stop +
params.n_stims_group;
  end
  clearvars start stop group

elseif strcmp(params.experiment_type, 'WindUp') ==  1
  counts.sum(6,:,:) = sum(counts.sum(4:5,:,:),1);
  counts_bc.sum(6,:,:) = sum(counts_bc.sum(4:5,:,:),1);
  params.latency_index(6,:) = [params.latency_index(4,1),
params.latency_index(5,2)];

  start = 1; stop = params.n_stims_group;
  for group = 1:params.n_groups
    sorted.sum(:,:,:,group) = counts.sum(:,:,start:stop);
    sorted.mean(:,:,:,group) = counts.mean(:,:,start:stop);

    start = start + params.n_stims_group; stop = stop +
params.n_stims_group;
  end

  for group = 1:params.n_groups
    for channel = 1:16
      for latency = 1:6
        sorted.psc(latency, channel, :, group) = sorted.sum(latency,
channel, :, group) - (sorted.mean(1,channel,1,group) *
```

```
(params.latency_index(latency,2) - params.latency_index(latency,1))); %
psc = pre-stimulus corrected
      end
    end
  end

  [out.sum, out.psc] = deal(zeros(6,16,20,2));
  out.sum(:,:,:,1) = sorted.sum(:,:,:,1); % Take control sequence
  out.sum(:,:,:,2) = mean(sorted.sum(:,:,:,2:4),4); % Mean of 3 wind up
trains

  out.psc(:,:,:,1) = sorted.psc(:,:,:,1); % Take control sequence
  out.psc(:,:,:,2) = mean(sorted.psc(:,:,:,2:4),4); % Mean of 3 wind up
trains

  clearvars start stop temp temp_bc
else
  display 'No appropriate experiment type declared. Sorting aborted.'
  return
end
```

Etho_MSP_SecSub

```
%%% Baseline Correction
bc_ind = find_ind(0.1*(-params.pre_stim), data.time);
for stimulus = 1:size(data.binned_bs,3)
  for channel = 1:size(data.binned_bs,2)
    bc_coeff = mean(data.binned_bs(1:bc_ind,channel, stimulus),1);
    data.binned_bc(:,channel,stimulus) =
data.binned_bs(:,channel,stimulus) - bc_coeff;
  end
end
clearvars bc_ind bc_coeff stimulus channel

%%% Subsecond Parameters
sub_start = -1;
sub_finish = params.stimulus_duration +1; % 5s stimulus + 1 second
start_ind = find_ind(sub_start, data.time);
stop_ind = find_ind(sub_finish, data.time);

subsecond.time_ms = data.time_ms(start_ind:stop_ind);
subsecond.binned = data.binned(start_ind:stop_ind, :, :);
subsecond.binned_bc = data.binned_bc(start_ind:stop_ind, :, :);
clearvars sub_start sub_finish start_ind stop_ind

%%% Second
coeff = 10; % periods to sum
start = 1; stop = coeff;
for i = 1:(length(data.time_ms)/coeff)
  for channel = 1:16
    for group = 1:size(data.binned,3)
      second.binned(i,channel,group) =
sum(data.binned(start:stop,channel,group));
      second.binned_bc(i,channel,group) =
sum(data.binned_bc(start:stop,channel,group));
    end
  end
  second.time(i) = mean(data.time(start:stop));
```

```
  start = start + coeff; stop = stop + coeff;
end
clearvars channel coeff group i start stop data

%%% Subsecond

if isfield(params, 'stim_style') == 1 && params.n_stims_group ~= 1
  if strcmp(params.stim_style, 'Alternate') || strcmp(params.stim_style,
'Continuous') ==  1
    [second.grouped, second.grouped_bc]  =
deal(zeros(size(second.binned,1), size(second.binned,2),
(size(second.binned,3)/params.n_stims_group)));
    [subsecond.grouped, subsecond.grouped_bc]  =
deal(zeros(size(subsecond.binned,1), size(subsecond.binned,2),
(size(subsecond.binned,3)/params.n_stims_group)));

    if strcmp(params.stim_style, 'Alternate') == 1
      group_vec =
[1:params.n_groups:(params.n_stims_group*params.n_groups)];
    elseif strcmp(params.stim_style, 'Continuous') ==  1
      group_vec = [1:1:params.n_stims_group];
    end

    for i = 1:params.n_groups
      second.grouped(:,:,i) = mean(second.binned(:,:,group_vec),3);
      second.grouped_bc(:,:,i) = mean(second.binned_bc(:,:,group_vec),3);
      subsecond.grouped(:,:,i) = mean(subsecond.binned(:,:,group_vec),3);
      subsecond.grouped_bc(:,:,i) =
mean(subsecond.binned_bc(:,:,group_vec),3);
      if strcmp(params.stim_style, 'Alternate') == 1
        group_vec = group_vec + 1;
      elseif strcmp(params.stim_style, 'Continuous') ==  1
        group_vec = group_vec + params.n_stims_group;
      end
    end

clearvars -except subsecond second
end
```

Elec_Combine

```
function output = Elec_Combine(matrix)
% function output = Elec_Combine(matrix)
% Matrix is the variable name to take from each file: e.g. 'sorted.mean'
or 'sorted_bc.sum'
% Converts sorted_x(latency, channel, group) to sorted_x(animal, latency,
channel, group)

cd 'R:\BMS-Hathway-Group\Emma Battell\MEA data'
[filelist, path] = uigetfile('MultiSelect', 'on');
n_files = length(filelist);
cd(path)

% Load in desired matrices from all files
for file = 1:n_files
  load(filelist{file});
  if length(size(eval(matrix))) == 2
    temp_unique_name(file,:,:) = eval(matrix);
```

```
  elseif length(size(eval(matrix))) == 3
    temp_unique_name(file,:,:,:) = eval(matrix);
  elseif length(size(eval(matrix))) == 4
    temp_unique_name(file,:,:,:,:) = eval(matrix);
  end
end
clearvars -except filelist matrix n_files path temp_unique_name
output = temp_unique_name;
```