What the young brain tells the spinal cord: top down modulation of dorsal horn sensory systems during postnatal development

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Declaration

The work presented in this thesis was carried out in the Department of Neuroscience, Physiology and Pharmacology at University College London between September 2012 and January 2016. I, Frederick Joseph Schwaller, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis

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Abstract

The brain can endogenously and powerfully modulate the processing of somatosensory information in the spinal cord. In adults, the rostroventral medulla (RVM) can inhibit and facilitate somatosensory processing in the adult dorsal horn, providing powerful control of pain behaviours. In neonates, balanced descending control of processing of dorsal horn activity is immature. Here, I examine the anatomical and functional maturation of descending control of spinal sensory circuitry in rats and hypothesise that descending serotonergic neurons in the RVM provide ongoing descending facilitation of spinal sensory networks in young animals.

In chapter 2, I demonstrate that cutaneous noxious stimulation activates neurons in regions of the brainstem which receive sensory inputs from the dorsal horn at P4; eight days before noxious-evoked neuronal activation in descending modulatory nuclei. In chapter 3, silencing the RVM unmasked descending facilitation of nociceptive dorsal horn neuron electrophysiological activity in uninjured P8 and P21 rats, but unmasked descending facilitation at P40. Thus, there is a switch from ongoing descending facilitation to inhibition between P21 and P40.

Experiments in chapter 4 demonstrate anatomical maturation of descending serotonergic pathways from the RVM to the spinal cord during postnatal development. In chapter 5, the function of these pathways was investigated. Here, deletion of descending serotonergic fibres or blockade of spinal 5-HT_3Rs unmasked background serotonergic facilitation of tactile and noxious dorsal horn neuron electrophysiological activity at P8 and P21. In adults, 5-HT/5-HT_3Rs also facilitate tactile inputs in the dorsal horn, but net modulation of noxious inputs switches to be inhibitory.

In conclusion, a change in function of descending modulatory pathways arising from the brainstem occurs during postnatal development: in young rats, descending modulatory pathways enhance the saliency of low and high threshold mechanical inputs in the dorsal horn, whilst balanced inhibition and excitation of high and low threshold inputs occurs in adulthood.

List of abbreviations

5-HT	5-hydroxytryptamine, serotonin
5-HTR	5-hydroxytryptamine receptor
5-HTT/SERT	5-hydroxytryptamine transporter/serotonin reuptake transporter
ACC	anterior cingulate cortex
AD	after discharge
ANOVA	analysis of variance
AMPA	alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ATP	adenosine triphosphate
BDNF	brain-derived neurotrophic factor
Ca^{2+}	calcium
CCI	chronic constriction injury
CCK	cholecystokinin
CL	central lateral thalamic nucleus
CFA	complete Freund's adjuvant
CGRP	calcitonin gene-related peptide
CNS	central nervous system
CPM	conditioned pain modulation
DAMGO	[D-Ala^2, N-$MePhe^4$, Gly-ol]-enkephalin
DLF	dorsolateral funiculus
DNIC	diffuse noxious inhibitory control
DRG	dorsal root ganglia
DRN	dorsal raphe nucleus
DRt	medullary dorsal 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
GAD65/67	glutamate decarboxylase (65kDa and 67kDa isoforms)
GiA	gigantocellularis reticularis nucleus alpha
GlyR	glycine receptor

GlyT2	glycine transporter 2
GPCR	G protein-coupled receptor
IB4	isolectin B4
K^+	potassium
L	lumbar spinal cord segment
LII	lamina II
LC	locus coeruleus
LPGi	lateral paragigantocellular nucleus
MAPK	mitogen activated protein kinase
mEPSC	miniature excitatory postsynaptic current
mIPSC	miniature inhibitory postsynaptic current
MRGPRD	mas-related G-protein coupled receptor member D
mRNA	messenger ribonucleic acid
Na^+	sodium
NaV	voltage gated sodium channel
NeuN	neuronal nuclear antigen
NICU	neonatal intensive care unit
NGF	neuronal growth factor
NK1	neurokinin 1
NMDA	N-methyl-D-aspartate
nNOS	neuronal nitric oxide synthase
NPY	neuropeptide Y
NTS	solitary nucleus
P	postnatal day
PAG	periaqueductal grey
PB	parabrachial
PB	phosphate buffer
pERK	phosphorylated extracellular signal-regulated kinase
PFA	paraformaldehyde
PKA	protein kinase A
PKC	protein kinase C
Po	posterior group of thalamic nuclei
PV	parvalbumin
Py	pyramidal tract
RF	receptive field

RMg	raphe magnus
ROb	raphe obscurus
RORα	RAR-related orphan receptor alpha
RP	raphe pallidus
RT	room temperature
RVM	rostroventral medial medulla
SEM	standard error of the mean
SOM	somatostatin
SNI	spared nerve injury
SNL	spinal nerve ligation
SP	substance P
SPA	stimulus-produced analgesia
SRD	subnucleus reticularis dorsalis
STT	Spinothalamic tract
TLR	toll-like receptor
TNFα	tumour necrosis factor alpha
TrkA	neurotrophic tyrosine kinase receptor type 1
TRPV1	transient receptor potential cation channel subfamily V member 1
TPH	tryptophan hydroxylase
TSA	tyramide signal amplification
TTBS	triton Tris buffered saline
vFh	von Frey hair
VGLUT	vesicular glutamate transporter
vlPAG	ventrolateral periaqueductal grey
VMpo	posterior ventral medial nucleus (thalamus)
VPL	ventral posterior lateral nucleus (thalamus)
VPM	ventral posterior medial nucleus (thalamus)
VTA	ventral tegmental area
WDR	wide dynamic range

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Chapter 1
General Introduction

1.1 Introduction

From detection of tactile and noxious stimuli by nerve endings in the skin, muscles, joints to the touch or pain perceptions maintained in networks in the brain, neural representations of sensory stimuli are modulated, moderated and maintained in multiple regions of the central nervous system (CNS). The spinal dorsal horn is the first integrative point of primary afferent sensory information from the body in the CNS before transmission to higher brain centres or to spinal motor circuits. In adult mammals, somatosensory inputs to the spinal cord are modulated by local segmental inhibitory and excitatory interneurons, as well as from descending connections from higher centres of the brain. Modulation of sensory inputs at the level of the spinal cord thus provides powerful control of the gain of sensory information projecting to the cortex and to spinal motor circuits.

Individual pain experiences can vary hugely: attention, distraction, anxiety, mood, past experiences and many other factors can shape our subjective pain experience during different behavioural states. Descending connections are likely to be pivotal neural pathways that drive this endogenous pain modulation. Spatially, this allows for precise modulation of sensory inputs at specific synapses from different body regions; and temporally allows for both rapid moment to moment modulation which can be refined to individual stimuli or trains of stimuli (Fields et al., 1983) and for longer term modulation during different behavioural states such as feeding or sleeping (Foo and Mason, 2005; Mason, 2011). A key aspect of descending modulation is the ability to alter the saliency of different somatosensory inputs to the CNS; for example, descending facilitation of nociceptive inputs to the spinal cord enhances the saliency of noxious stimuli in relation to non-noxious stimuli during chronic pain states. Similarly, descending modulation of tactile inputs in the spinal cord changes the relative strength of sensory control of motor circuits and limb movements (Bourane et al., 2015; Brownstone et al., 2015).

At birth CNS processing and modulation of somatosensory information is immature in mammals. Mature balance of sensory inputs arises from activity-dependent strengthening and weakening of synapses in somatosensory circuits and pathways over the postnatal period. Human neonates are more sensitive to cutaneous tactile and noxious stimuli; reflexes are exaggerated in amplitude and duration in comparison to adults (Fitzgerald et al., 1988; Andrews and Fitzgerald, 1994; Cornelissen et al., 2013), suggesting immature excitatory and inhibitory balances in sensory-motor circuits.

Exaggerated cutaneous reflexes have also been observed in neonatal rodents (Fitzgerald et al., 1988; Walker et al., 2003). Experimentally, the rat is used as a model of human development to provide insight into the mechanisms which underlie the development of sensory systems. Direct age translation from rat to human is approximate, as rats develop rapidly after birth and are considered adults by around 6-8 weeks of age. At postnatal day (P) 3, rats correspond to 26-35 postconception weeks in human infants, and a P21 weanling rat is considered to be adolescent (McCutcheon and Marinelli, 2009). This thesis aims to investigate the anatomical and functional maturation of descending modulatory pathways from the brainstem to the spinal cord in the developing rat. This introductory chapter outlines the processing of cutaneous tactile and noxious stimuli in the adult and developing nervous system and will focus on the role of descending modulation of spinal sensory circuits.

1.2 Sensory processing in the adult spinal cord

The dorsal horn of the spinal cord is divided into six laminae which were originally defined from cytoarchitectural Nissl stains in the spinal cords of cats (Rexed, 1952), and rats (Molander et al., 1984). There are four major neuronal components of the dorsal horn: primary afferent inputs, local interneurons, projection neurons, and descending inputs from the brain. Cutaneous sensory inputs are somatotopically organised in the spinal cord, such that cutaneous receptive fields are organised rostro-caudally and medio-laterally (Molander and Grant, 1985). These 'body maps' are labile, especially during postnatal development (Beggs et al., 2002; Granmo et al., 2008), and under the control of excitatory and inhibitory signalling in the spinal cord. Sensory inputs carrying different modalities have distinct termination patterns in the spinal dorsal horn, with nociceptive inputs predominantly terminating in the superficial dorsal horn (laminae I and II; also known as the substantia gelatinosa), and non-noxious tactile and nociceptive inputs terminating in the deep dorsal horn (laminae III-V).

1.2.1 Primary afferent neurons

Aβ, Aδ and C type primary afferent sensory neurons are classified based on their myelination, diameter, conduction velocity (table 1.1), and expression of cell-specific markers. Myelinated A-fibres express neurofilament (Lawson et al., 1984) and toll-like receptor 5 (TLR5) (Xu et al., 2015), whilst C-fibres are generally split into two

subclasses: peptidergic C-fibres which express transient receptor cation channel 1 (TRPV1), calcitonin gene-related peptide (CGRP) and substance P (although some medium diameter DRG neurons also express these peptides (Gibson et al., 1984)); and non-peptidergic C-fibres which express isolectin B4 (IB4) and MRGPRD (Snider and McMahon, 1998; Zylka et al., 2005). Sensory transducing A-fibres are subdivided into Aβ and Aδ populations which detect innocuous tactile stimuli and noxious stimuli respectively. These primary afferent neurons have termination patterns which are distinct but overlap in the spinal dorsal horn: C fibres terminate in laminae I and II; Aδ fibres terminate in the superficial and deep dorsal horn; and Aβ fibres are more restricted to laminae III-V (Todd, 2010) (Fig. 1.1). Glutamate is the main excitatory neurotransmitter released by all primary afferent neurons, and is co-released with peptides such as substance P and/or CGRP in peptidergic C-fibres (De Biasi and Rustioni, 1988).

Fibre type	**Axon diameter (μm)**	**Myelination**	**Conduction velocity (ms^{-1})**
C	0.4-1.2 – Thin	Unmyelinated	0.5-2.0
Aδ	2-6 – Medium	Thin	12-30
Aβ	>10 - Thick	Thick	30-100

Table 1.1. Sensory transducing primary afferent neurons
Thin unmyelinated C fibres have smaller axon diameters and slower conduction velocities compared to thinly myelinated and thickly myelinated Aδ and Aβ fibres.

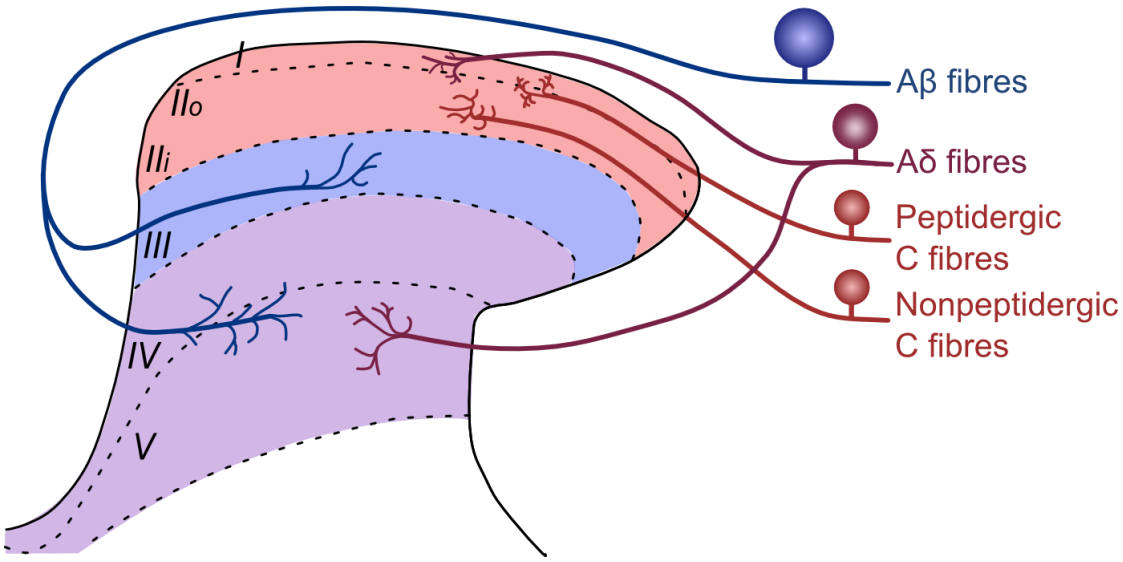

Fig 1.1. Termination patterns of primary afferent neurons in the spinal cord.
Nociceptive inputs from Aδ fibres and C fibres terminate in lamina I and II and tactile inputs from Aβ fibres terminate in the deeper laminae (III-V). Neurons in the superficial dorsal horn therefore preferentially receive nociceptive inputs (pink region), whilst neurons in lamina III almost exclusively receive non-noxious Aβ inputs (blue region). Noxious and non-noxious inputs from Aδ and Aβ fibres terminate in laminae IV-V, and many neurons in these deeper laminae display wide dynamic range properties (purple region).

1.2.2 Cutaneous mechanotransduction

The perceptual recognition and interpretation of a rich and complex tactile environment ultimately begins with activation of populations of mechanosensitive neurons with nerve endings in glabrous and hairy skin. Mechanical forces applied to the skin can range several orders of magnitude, and the detection of different intensities of stimuli begins with the activation of two populations of mechanoreceptive neurons: low-threshold mechanoreceptors (LTMRs) that respond to innocuous mechanical stimulation and high-threshold mechanoreceptors (HTMRs) that respond to harmful mechanical stimuli (Abraira and Ginty, 2013).

LTMRs are activated by innocuous mechanical forces, such as those applied by a brush stimulus in an experimental setting. Firing patterns of Aβ LTMRs (and smaller populations of Aδ and C LTMRs) in response to sustained innocuous mechanical stimuli can differ: slowly adapting LTMRs tend to fire for the duration of the stimulus where as rapidly adapting LTMRs fire on the stimulus on and offset. HTMRs include Aδ and C fibres that innervate the epidermis and respond to high intensity and harmful mechanical stimuli, such as those applied by a pinch stimulus. Like LTMRs, many

HTMRs are not selectively activated by mechanical stimuli and also respond to thermal stimuli.

An integrative view of mechanoreception and the perception of touch has been proposed by Abraira and Ginty (2013) based on evidence of mechanoreception in mammalian hairy and glabrous skin. These authors postulate that touch perception is ultimately a product of the activation of populations of LTMRs with end organs of distinct tuning properties, conduction velocities and unique spatial distributions. The patterns of LTMR firing are then integrated and decoded in a somatotopic manner in the spinal dorsal horn where sensory information is transmitted from deep dorsal horn projection neurons to the somatosensory cortices via the postsynaptic dorsal column (PSDC) and the spinocervical tract (SCT). A similarly integrative view of nociceptive sensory processing via HTMRs can also be postulated. Patterns of HTMR firing are integrated and decoded by dorsal horn neurons in the superficial and deep dorsal horn. Projection neurons then send ascending sensory information to somatosensory regions of the brain via the spinothalamic and spinoreticular tracts. Thus, neurons in the dorsal horn play a pivotal role in the processing and integration of specialised LTMR and HTMR sensory inputs. Whilst it is acknowledged that thermosensation and chemosensation are important aspects of sensory perception, this thesis will primarily focus on the central processing of cutaneous mechanical sensory inputs.

1.2.3 Projection neurons

Neurons in the dorsal horn carry somatosensory signals in ascending tracts to various regions of the brain including the brainstem, midbrain, thalamus and cortical structures (Fig. 1.2). The majority of projection neurons have cell bodies located in lamina I, and smaller populations reside in laminae III-V (Todd, 2010) (Fig. 1.3). Projection neurons constitute roughly 5% of neurons in lamina I of the rat lumbar spinal cord, of which 95% project to the parabrachial (PB) nucleus, 30% to the periaqueductal grey (PAG), 25% to the solitary nucleus (NTS) and 5% project to the thalamus (Spike et al., 2003). The majority (80%) of lamina I projection neurons express neurokinin-1 (NK1) receptor, the receptor for substance P, and receive sensory inputs from substance P-positive C-fibres and Aδ nociceptors (Todd et al., 2002; Todd, 2010; Baseer et al., 2014). Virtually all lamina I projection neurons (96-100%) respond to peripheral noxious stimuli, whilst a few cells also demonstrate wide-dynamic range (WDR) properties and respond to high and low threshold sensory inputs (Dostrovsky and Craig, 1996; Han et al., 1998; Bester et al., 2000b; Keller et al., 2007).

The deep dorsal horn also contains neurons which send ascending projections to multiple brain regions, including the PB nucleus, PAG, ventrolateral medulla, amygdala, hypothalamus and the globus pallidus (Hylden et al., 1986; Mouton and Holstege, 2000; Todd et al., 2002; Andrew et al., 2003; Braz et al., 2005). Some lamina V neurons also project to the ventral horn and are involved in sensory feedback control of motor circuits (Schouenborg et al., 1995). LTMR Aβ fibres and HTMR nociceptive Aδ fibres terminate extensively in the deeper laminae, meaning that heterogeneous populations of neurons respond to LTMR inputs or HTMR inputs, but the majority display WDR properties (Abraira and Ginty, 2013). LTMR inputs primarily convey tactile information to the brain via PSDC and SCT neurons in the deep dorsal horn. These populations of projection neurons are heterogeneous in their morphology and stimulus-response properties, however the majority of these neurons are activated by LTMR inputs from glabrous or hairy skin in the cat (Angaut-Petit, 1975; Brown et al., 1987).

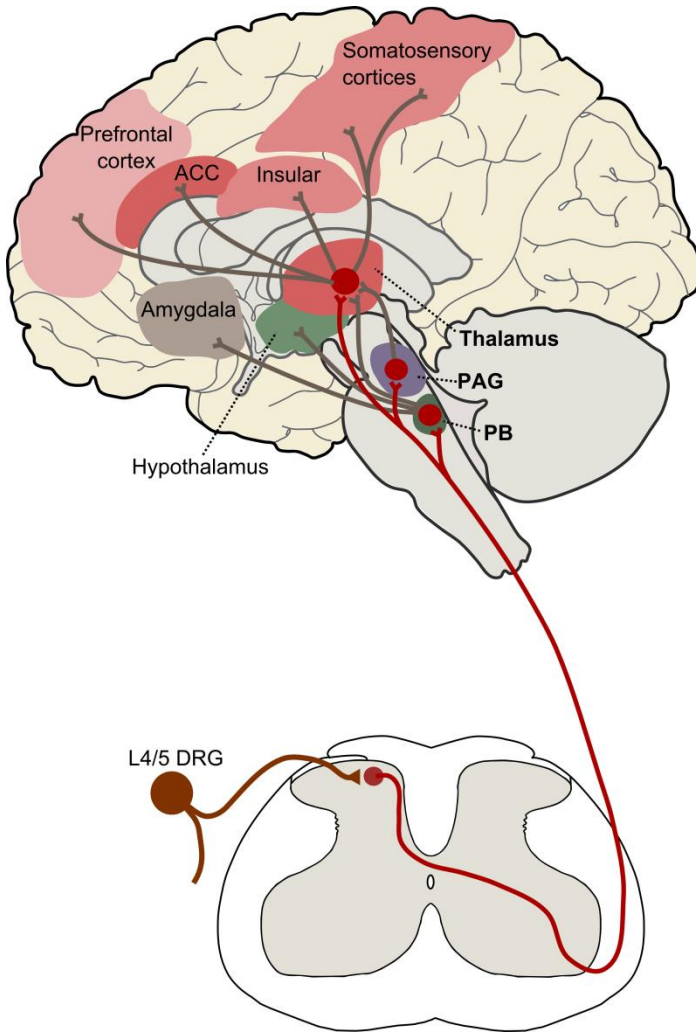

Fig 1.2. Ascending sensory pathways from the spinal cord.
Dorsal horn neurons predominantly project to the parabrachial nucleus (PB), the periaqueductal grey (PAG) and the thalamus (red pathways), although other brainstem nuclei such as the NTS also receive sensory inputs from the dorsal horn (not shown). Sensory information is transmitted to regions of the brain such as the anterior cingulate cortex (ACC), the somatosensory cortices and the ventral tegmental area (not shown) via secondary projections from the PB nucleus, PAG and thalamus (grey pathways).

Lamina V WDR neurons are known to have dendritic trees which extend dorsally into lamina II, with arborisations extending into laminae III and IV (Wei and Zhao, 1997). Monosynaptic inputs from substance P-containing C fibres have been reported on lamina II dendrites (De Koninck et al., 1992) and monosynaptic inputs from myelinated afferents have been reported on lamina III dendrites of lamina V neurons (Bráz and Basbaum, 2009). Additionally, a polysynaptic input pathway from unmyelinated afferents terminating in lamina II and ventrally spreading to lamina V neurons via local interneurons indicates direct and convergent processing of high threshold and low threshold sensory inputs (Bráz and Basbaum, 2009). A large number

of lamina V neurons which express NK1 receptors are projection neurons which terminate in the contralateral PB nucleus, however these neurons are part of a small subpopulation of lamina V neurons as few neurons in the deep dorsal horn express NK1 receptors (Todd et al., 2000). It is currently unclear what proportion of neurons in lamina V are projection neurons in the rat.

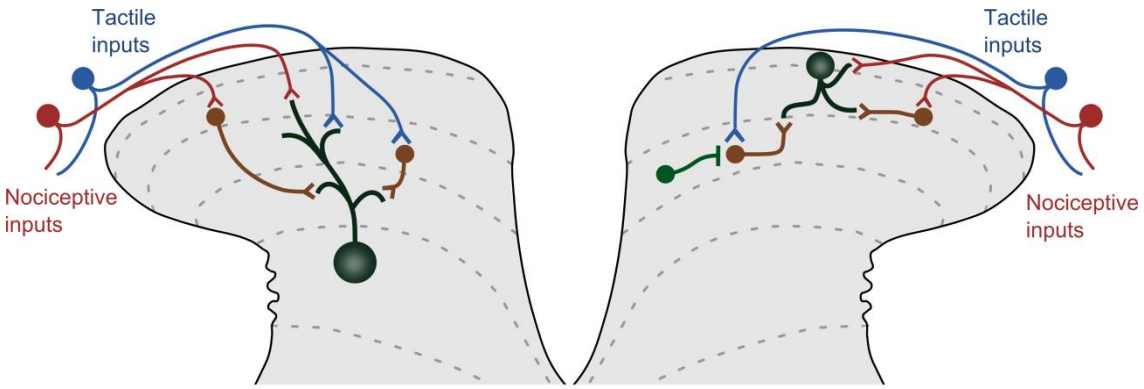

Fig 1.3. Projection neurons in the spinal dorsal horn.
There are two major populations of neurons situated in lamina V (left) and lamina I (right) which send ascending noxious information to the brain. These neurons receive a combination of monosynaptic inputs from primary afferent neurons and polysynaptic inputs via local interneurons (brown). Lamina V neurons are predominantly wide dynamic range neurons which are activated by both noxious and non-noxious inputs. Lamina I neurons are preferentially activated by noxious inputs. Tactile inputs can activate lamina I projection neurons, but these inputs are under inhibitory control from inhibitory interneurons (green; see section 1.2.4)

1.2.4 Interneurons and dorsal horn circuitry

The spinal dorsal horn generates a complex, gated circuit that is regulated by activity from nociceptive and non-nociceptive afferents. Gate control theory proposed that sensory processing in the spinal cord depends on the balance of activity of large (low threshold) and small diameter (high threshold) afferent fibres (Melzack and Wall, 1965). Both fibre types can excite transmission cells, but large diameter cells activate spinal inhibitory neurons which inhibit small diameter afferents (Fig. 1.4). Key to the gate control theory are the neurons in the spinal cord: transmission cells which either activate, or are, neurons which project to supraspinal sites; and interneuron populations which inhibit inputs onto transmission cells from large diameter fibres. Ultimately, the gated output from transmission cells activates action systems in the brain which drive attentional focus, behavioural reactions and pain perception.

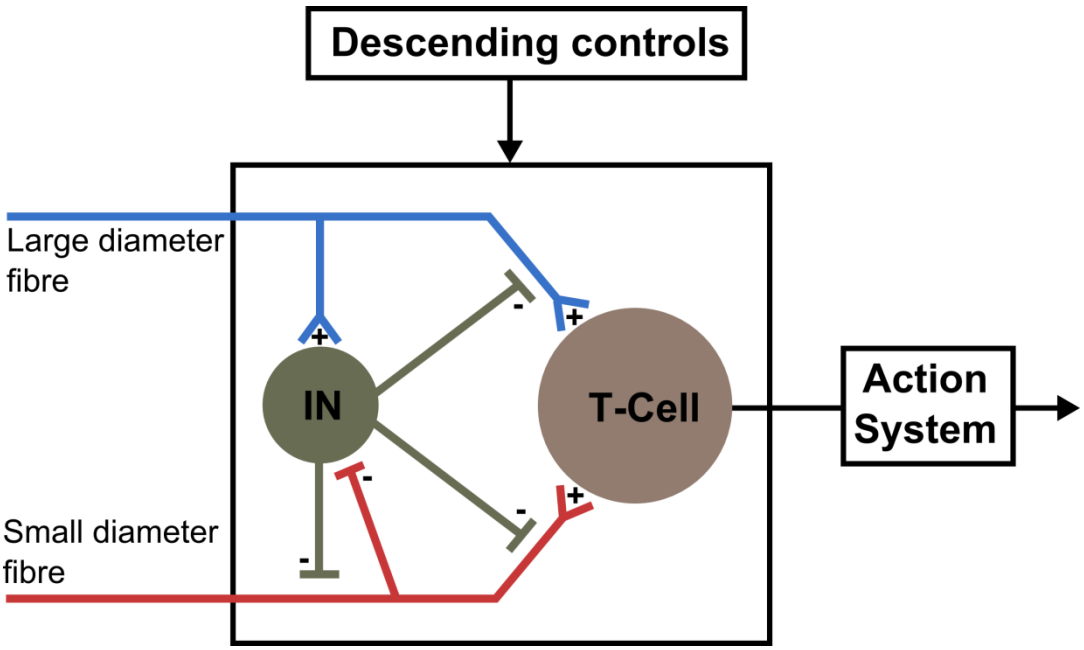

Fig 1.4 Gate control theory.
Melzack and Wall's gate control theory postulated that both large and small diameter primary afferent neurons activate transmission (T) cells in the spinal dorsal horn. Inputs from large diameter fibres (presumed non noxious) to T cells are tonically inhibited by feedforward activation of local inhibitory interneurons (IN), therefore small diameter inputs (presumed nociceptive) predominantly excite T-cells. T-cells activation engages an action system; be it driving motor reflexes, attention guidance and/or pain perception. This dorsal horn sensory circuit is also modulated by descending controls from the brain.

Inhibitory interneurons use GABA and/or glycine as neurotransmitters, and represent 25%, 30% and 40% of neurons in lamina I, II and III, respectively (Polgár et al., 2003). Excitatory interneurons in the dorsal horn can be identified by the expression of vesicular glutamate transporters (VGLUTs), most notably VGLUT2 (Todd et al., 2003). Grudt and Perl (2002) described four morphologically distinguishable types of interneuron: islet cells which are invariably GABA/glycinergic; radial and vertical cells which express glutamate; and central cells which include inhibitory and excitatory subsets (Maxwell et al., 2007; Yasaka et al., 2007; Todd, 2010). A range of different neurochemical markers have also been used to define populations of excitatory interneurons (protein kinase Cγ (PKCγ), somatostatin) and inhibitory interneurons (neuropeptide Y (NPY), galanin, parvalbumin, neuronal nitric oxide synthase (nNOS)).

Local spinal inhibitory neurons, in conjunction with descending inhibitory influences from the brainstem, modulate sensory inputs to the dorsal horn and moderate the output of projection neurons. Heterogeneous populations of inhibitory GABAergic and

glycinergic neurons are activated by LTMR and HTMR inputs from Aβ-fibres and from C and Aδ-fibres, driving sensory-evoked inhibition in the dorsal horn (Zhou et al., 2007, 2008; Duan et al., 2014). Inhibition of sensory inputs is achieved both presynaptically, through inhibitory axo-axonic synaptic inputs onto primary afferent terminals (Todd, 1996; Watson et al., 2002), and postsynaptically through inhibition of target dorsal horn neurons (Torsney and MacDermott, 2006).

Interneurons are involved in feedforward excitation and inhibition of projection neurons, and thus control the gain of sensory output from the dorsal horn. These feedforward mechanisms are important in providing polysynaptic sensory inputs onto projection neurons in lamina I and lamina V from high threshold C and Aδ-fibres and from low threshold Aβ-fibres. Early electrophysiological experiments involving dual recordings from lamina I and lamina II neurons suggested that there is a flow of sensory information from lamina II to projection neurons in lamina I (Price et al., 1979). Subsequent experiments have demonstrated C and Aδ polysynaptic excitatory inputs to lamina I projection neurons via lamina II central and vertical cells (Lu and Perl, 2005) and Aβ inputs to lamina I NK1R+ neurons which are tonically inhibited by GABA/glycinergic neurotransmission (Torsney and MacDermott, 2006).

Recently, a number of different research groups have identified several populations of interneurons which are crucial in integrating nociceptive and tactile inputs in the spinal dorsal horn. These interneuron populations involved in mechanoreception include:

1. **Parvalbumin (PV)+ inhibitory interneurons** are situated on the lamina II/III border, receive synaptic inputs from Aβ fibres and provide feedforward inhibition of local interneurons and Aβ terminals (Hughes et al., 2012). PV+ neurons inhibit Aβ-mediated firing of excitatory PKCγ interneurons, thereby inhibiting polysynaptic Aβ-inputs to projection neurons (Petitjean et al., 2015). Following peripheral nerve injury PV+ appositions onto PKCγ neurons are reduced, causing reduced inhibition of Aβ-inputs in the dorsal horn which correlates with tactile allodynia (Petitjean et al., 2015).
2. **Dynorphin (DYN)+ inhibitory interneurons** are situated in lamina I-V and receive C and A fibre inputs. These neurons are also important for tonic feedforward inhibition of Aβ-inputs to dorsal horn neurons, as ablating Dyn+ neurons in the dorsal horn increases Aβ input-mediated EPSCs and action potential firing of lamina I-III neurons in the dorsal horn (Duan et al., 2014). Importantly, ablating Dyn+ neurons in uninjured mice causes

spontaneous development of mechanical allodynia, suggesting that these neurons normally inhibit tactile inputs onto nociceptive circuits.

3. **Somatostatin (SOM)+ excitatory interneurons** are spread from lamina I-III, but are predominantly in lamina II. All SOM+ neurons receive mono or polysynaptic C fibre inputs and/or Aδ inputs, and a subset of SOM+ neurons receive Aβ inputs that is under tonic GABAergic/glycinergic inhibition (Duan et al., 2014). Ablation of SOM+ neurons causes behavioural mechanical hyposensitivity in uninjured mice, and prevents Aβ fibre-mediated action potential firing in lamina II-III dorsal horn neurons, suggesting that SOM+ neurons are important for feedforward excitation of low and high-threshold mechanical inputs in the dorsal horn (Duan et al., 2014).
4. **VGLUT3** is a marker for C-low threshold mechanoreceptor (CLTMR) afferent fibres in adult animals, however it was recently found that transient expression of VGLUT3 in dorsal horn neurons between P5-20 is important in establishing mechanical pain states in adulthood (Peirs et al., 2015). Ablation of dorsal horn VGLUT3+ neurons at P10 prevent polysynaptic Aβ-mediated EPSCs in NK1R+ lamina I neurons, demonstrating that VGLUT3+ neurons are involved in Aβ-driven feedforward excitation of lamina I neurons (Peirs et al., 2015). Activation of VGLUT3+ dorsal horn neurons produced mechanical allodynia in uninjured mice, whilst mechanical hyposensitivity was produced by either conditional knockout or transient chemogenetic silencing of dorsal horn VGLUT3+ neurons. This feedforward excitation was found to be mediated via PKCγ and calretinin+ excitatory interneurons (Peirs et al., 2015).
5. **RORα excitatory interneurons** on the lamina II/III border are preferentially innervated and activated by LTMRs (Bourane et al., 2015). Ablation of RORα neurons in the dorsal horn impairs behavioural responses to light dynamic and static tactile inputs, as well as motor impairments in uninjured mice. Tracing experiments demonstrated that RORα neurons send axonal projections to ventral motor neurons, and receive synaptic inputs from descending corticospinal projection neurons (Bourane et al., 2015). Thus, dorsal horn RORα neurons receive and integrate peripheral tactile inputs and descending corticospinal inputs to influence motor control.

Collectively, these research papers have identified key interneuron populations and microcircuits that are proposed to gate noxious and non-noxious mechanical sensory inputs in the spinal cord (Fig. 1.5). The identification of Aβ input-mediated inhibition of Aβ-driven polysynaptic inputs to [presumed] projection neurons via local inhibitory interneurons strongly supports proposals made in the original gate control theory (Melzack and Wall, 1965). Anatomical evidence of descending inputs from corticospinal neurons onto RORα neurons was shown (Bourane et al., 2015), however the role of descending modulation from the brain on other populations of interneurons was not elucidated in these studies. An important part of the gate control theory is the descending influences from the brain, and it is likely that descending sensory modulatory projections from the brain provide important top-down control dorsal horn sensory microcircuits via modulation of the firing properties of interneuron populations.

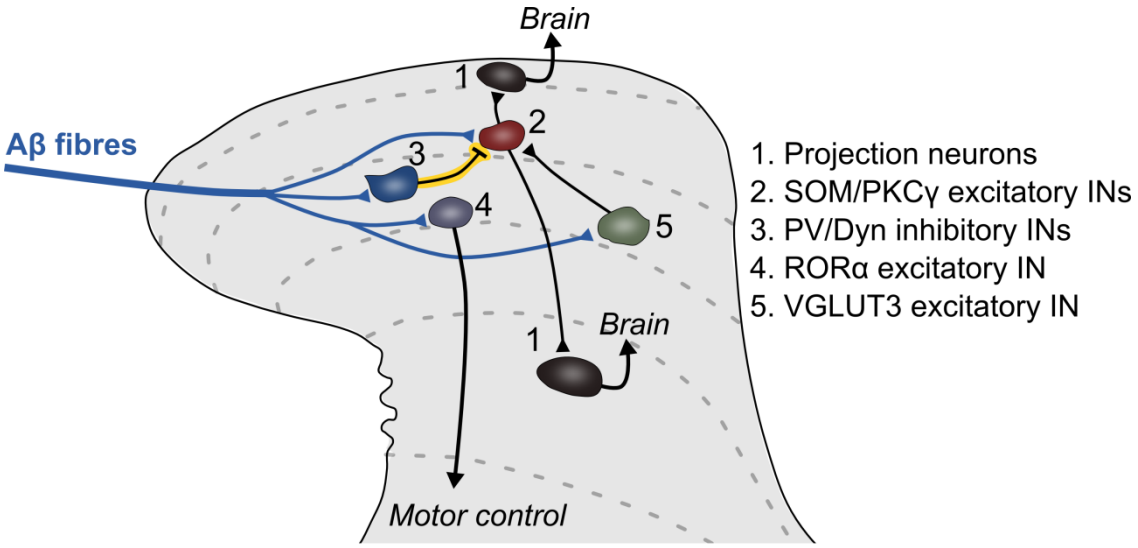

Fig 1.5. Tactile sensory circuits in the dorsal horn.
Aβ fibres are activated by non-noxious tactile inputs and make synaptic contacts in laminae III-V with local interneurons. Some of these interneurons are excitatory populations (somatostatin (SOM), protein kinase Cγ (PKCγ) (2) which provide polysynaptic Aβ inputs to projection neurons in lamina I and V (1) (Duan et al., 2014). These polysynaptic circuits are under Aβ input-driven tonic inhibitory control from parvalbumin (PV) and dynorphin (Dyn) expressing interneurons (3; highlighted in yellow) (Duan et al., 2014; Petitjean et al., 2015). Aβ inputs also activate RORα neurons which project to the ventral horn and modulate motor circuits (4) (Bourane et al., 2015). VGLUT3 is transiently expressed in the dorsal horn between P5-20 (5). These neurons are activated by Aβ inputs and are important in establishing the sensitivity of polysynaptic circuits involved in mechanical allodynia in adulthood (Peirs et al., 2015).

1.2.5 Receptive fields in the dorsal horn

Receptive fields are arranged somatotopically in the spinal dorsal horn and reflect afferent input from the periphery. For example, primary afferent neurons with dendritic terminals on the cutaneous plantar surface of the hindpaw have axonal projections which terminate in the medial lumbar 4-5 spinal dorsal horn in the rat (Molander and Grant, 1985). Regions of the dermatome are thus represented in the spinal cord by groups of dorsal horn neurons, many of which have overlapping receptive fields. The receptive field properties of dorsal horn neurons are determined by monosynaptic and polysynaptic sensory inputs and are influenced by excitatory and inhibitory controls from local interneurons and supraspinal descending modulatory neurons. Patch clamp experiments have demonstrated that dorsal horn neurons also display subthreshold excitatory postsynaptic currents (EPSCs) in response to stimulation of receptive fields which extend beyond the boundaries of suprathreshold receptive fields (Woolf and King, 1989; Kato et al., 2011). In addition to excitatory receptive fields, surrounding inhibitory fields can supress dorsal horn neuron firing activity when stimulated. These inhibitory receptive fields are situated on heterotopic body regions (Le Bars et al., 1979) and on the dermatome which locally surrounds the excitatory receptive fields (Kato et al., 2011).

Somatotopy in the dorsal deep horn is also a linked with the modular organisation of sensorimotor circuits in the spinal cord. Sensory inputs converging onto reflex encoding neurons in the deep dorsal horn are important for relaying sensory information to the ventral horn. Crucial in the link between sensory dermatomes and motor neuron pools in the ventral horn are reflex encoder neurons in lamina V. These neurons have sensory receptive fields which closely correlate with muscle receptive fields, and pass somatotopically organised sensory information from lamina III-IV to the ventral horn to drive motor reflexes and modulate motor commands (Levinsson et al., 2002; Schouenborg, 2003). Assessment of the type and strength of stimuli which cause the reflex and the spatial properties of the reflex are often used as a measure of the sensitivity of the polysynaptic connections in the spinal cord.

Receptive fields of dorsal horn neurons are not rigid, but can be transiently increased or decreased in size. Periods of strong nociceptive inputs to the dorsal horn, such as from application of the chemical irritant mustard oil (Woolf and King, 1989), punctate burns to the skin (McMahon and Wall, 1984), or tissue inflammation (Hylden et al., 1989), causes enlargement of dorsal horn neuron cutaneous receptive fields. Enlargement of the representation of damaged body regions during pain states therefore increases the saliency of the injured tissue. Early experiments demonstrated that cutaneous receptive

fields in the cat could be enlarged by spinal application of glutamate and shrunk by application of GABA or glycine (Zieglgänsberger and Herz, 1971), suggesting that the balance of excitatory and inhibitory neurotransmission in the dorsal horn tightly controls receptive field size of neurons. Enlargement of suprathreshold receptive fields is likely caused by potentiation of subthreshold EPSCs in subthreshold receptive fields (Kato et al., 2011). Thus, transient changes in receptive field size are dependent upon the strength of sensory inputs from the periphery and reflect changes in the excitability of dorsal horn neurons.

Activity of dorsal horn neurons in response to cutaneous stimulation largely depends on the primary afferent inputs received. The majority of neurons in the superficial dorsal horn are nociceptive specific and exhibit increased firing activity selectively to noxious stimulation. Indeed, 80% of projection neurons in lamina I are nociceptive specific, whilst 20% are classified as wide dynamic range (WDR) neurons and fire in response to both innocuous tactile and noxious mechanical stimulation (Keller et al., 2007). Apparent preference towards nociceptive specificity of superficial dorsal horn neurons may, however, be a feature of extracellular recordings where suprathreshold firing activity is the output, as subthreshold brush-evoked (EPSCs) can be evoked in the majority of cells (9/13) in the superficial dorsal horn in spinal slices (Kato et al., 2011).

1.3 Projection targets of dorsal horn neurons

Projection neurons in the spinal dorsal horn send ascending axons to multiple regions in the brain including the PB nucleus; PAG and various nuclei of the thalamus. Dorsal horn neurons also send sensory inputs to interneurons and motorneurons in the ventral horn of the spinal cord which drive and modulate sensory-evoked reflexes and motor commands. This thesis will focus on the PB nucleus and the PAG as major ascending targets of the spinal dorsal horn due to their hypothesised roles in the spinal-bulbo-spinal loop.

1.3.1 The Parabrachial Nucleus

Neurons in the superficial and deep spinal dorsal horn send ascending axons to the contralateral PB nucleus (Slugg and Light, 1994; Bernard et al., 1995; Craig, 1995; Feil and Herbert, 1995a; Spike et al., 2003). The PB nucleus is the major target nucleus of neurons in the superficial dorsal horn, as 95% of lamina I projection neurons have axons which terminate here (Spike et al., 2003). Electrophysiological studies in the cat

have demonstrated that the majority of neurons in the PB nucleus are preferentially activated by noxious stimuli, however a small proportion of neurons display wide-dynamic range properties, demonstrating that low and high threshold inputs ascend to the PB nucleus (Hylden et al., 1985). Fos is expressed in the PB nucleus following application of noxious stimuli in acute cutaneous and visceral pain models and during inflammation (Lantéri-Minet et al., 1993, 1994; Bellavance and Beitz, 1996; Hermanson and Blomqvist, 1996; Pinto et al., 2003); however touch-evoked Fos expression in the spinal dorsal horn and PB nucleus has, been observed following nerve crush in adult rats (Bester et al., 2000a), suggesting that sensory response properties of PB neurons changes during chronic pain states.

Neurons in the PB nucleus project to a number of brain regions associated with processing of noxious information including the RVM, PAG, hypothalamus, amygdala, and various thalamic nuclei involved in nociception (Bernard et al., 1993; Alden et al., 1994; Bester et al., 1999; Gauriau and Bernard, 2002a). Ascending nociceptive projections via the PB nucleus are postulated to drive affective and autonomic components of pain; actions which are mediated primarily through the amygdala, hypothalamus and the forebrain; whilst nociceptive projections to the PAG are thought to be important in the generation of stress induced analgesia and autonomic changes such as changes in heart rate and engagement of fight/flight responses associated with environmental stressors (Hunt and Mantyh, 2001).

1.3.2 The periaqueductal grey

The PAG receives sensory inputs from the dorsal horn as well as affective and autonomic inputs from various brain regions. Sensory inputs predominantly originate from the spinal dorsal horn and the PB nucleus, with ascending sensory inputs from the spinal dorsal horn terminating in the ventrolateral (vlPAG) and lateral (lPAG) regions of the PAG (Keay et al., 1997). Around 30% of lamina I projection neurons target the PAG (Spike et al., 2003). Sensory integrative regions of the cortex such as the anterior cingulate cortex and the insular also project to the PAG (Beitz, 1982a; An et al., 1998; Floyd et al., 2000); as does the central amygdala (Rizvi et al., 1991), hypothalamus (Bandler and Keay, 1996; Rizvi et al., 1996) and various brainstem nuclei including the locus coeruleus and the RVM (Beitz, 1982a; Herbert and Saper, 1992).

Major outputs of the PAG include the RVM and the locus coeruleus (Beitz, 1982b; Beitz et al., 1983; Cameron et al., 1995; Yin et al., 2014a), and these pathways are important in descending modulation of spinal sensory circuits. The lPAG and vlPAG

are particularly important regions of the PAG involved in sensory integration and modulation, as projections from the dorsal horn terminate here and projections to the RVM originate here (Beitz et al., 1983; Keay et al., 1997; Yin et al., 2014b). Other important projection sites of the PAG include the PB nucleus (Krout et al., 1998) and higher brain regions such as the ventral tegmental area (VTA) and the medial thalamus and the orbitofrontal cortex (Coffield et al., 1992; Cameron et al., 1995).

The PAG acts as an important integrative nucleus which mediates descending pain modulation; directly receiving ascending sensory inputs from the dorsal horn, and subsequently driving descending modulation of dorsal horn sensory inputs via recruitment of the RVM. Autonomic and affective inputs from regions such as the hypothalamus, amygdala and anterior cingulate cortex also contribute to endogenous pain modulation from the PAG (Bandler and Shipley, 1994). The PAG also has roles in fear behaviours and modulating motor reflex excitability (Koutsikou et al., 2015) which are thought to overlap with pain and stress modulatory roles of the PAG (Bandler and Shipley, 1994).

1.3.3 Thalamic nuclei

Another major output of somatosensory information from the spinal cord is to the thalamus. In primates, lamina I spinothalamic tract (STT) neurons project heavily to the posterior part of the ventral medial nucleus (VMpo), causing almost all neurons in the VMpo to be nociceptive or thermoreceptive (Craig et al., 1994). In contrast, STT neurons in the deep dorsal horn primarily project to the ventral posterior lateral nucleus (VPL), suggesting that different thalamic nuclei receive anatomically and functionally distinct somatosensory inputs (Craig, 2006). In awake humans, microstimulation within the VMpo causes discreet and localised sensations of pain or cooling, demonstrating the importance of this region in discriminative pain (Davis et al., 1999; Craig, 2003).

In the rat, there are few if any inputs from lamina I neurons to the VMpo. Instead, the densest output from lamina I in the rat is to the ventral posterolateral and posteromedial (VPL/VPM) and posterior group of thalamic nuclei (Po), whilst STT neurons in the deep dorsal horn conveying tactile and nociceptive information primarily project to the central lateral thalamic nucleus (CL) and the VPL (Gauriau and Bernard, 2004a). Populations of neurons in the Po are nociceptive specific, tactile specific or display WDR properties, suggesting that this region is important for the convergence of somatosensory inputs (Gauriau and Bernard, 2004b).

The functional anatomy of somatosensory inputs to thalamic nuclei indicates that pain and touch sensations are associated with multiple ascending pathways. Key to the sensory perceptual qualities following cutaneous stimulation are the complex thalamocortical connections. Interconnectivity with different cortical regions which are known to be involved in pain and touch perception in human imaging studies, such as the somatosensory cortices, insular, anterior cingulate cortex and prefrontal cortices, ultimately drive the sensory and affective qualities of pain and touch (Baliki and Apkarian, 2015).

1.4 Descending modulation of adult dorsal horn sensory systems

Descending control of sensory inputs is an important aspect of endogenous pain modulation (Fig. 1.6). Regions in the brainstem such as the RVM project to the spinal dorsal horn and excite or inhibit sensory circuits during acute and chronic pain states. Descending pain modulatory brainstem regions receive ascending sensory information, and thus contribute to a rapid feedback loop which is vital in moment to moment modulation of sensory inputs. This functionally defined and anatomically inferred feedback loop is termed 'the spinal-bulbo-spinal loop'. The RVM is an important brainstem modulatory site, but is not the only region which projects to the spinal dorsal horn. Other regions that project to the spinal dorsal horn and modulate sensory inputs include: the noradrenergic LC; the dopaminergic A11 nucleus of the hypothalamus (Koblinger et al., 2014); the medullary dorsal reticular nucleus (DRt) (Tavares and Lima, 1994); and the subnucleus reticularis dorsalis (SRD) (Villanueva et al., 1996). Whilst it is acknowledged that these nuclei have important roles in modulating spinal sensory circuits in adult mammals, this thesis will focus on the RVM as a major spinally-projecting descending pain modulatory nucleus.

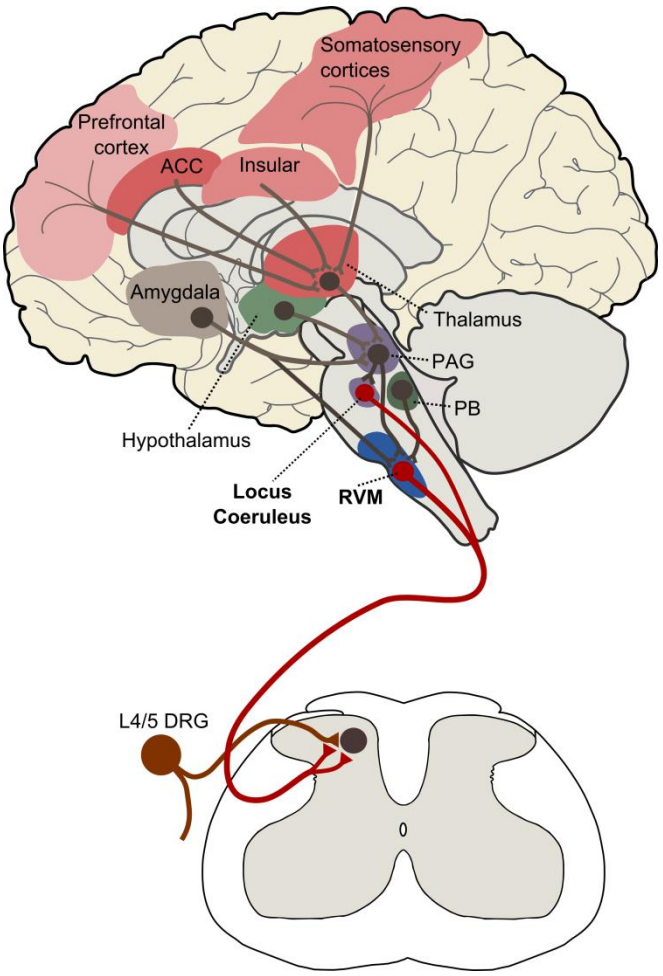

Fig 1.6. Descending sensory modulation from the brain.
The rostroventral medial medulla (RVM) and the locus coeruleus are two brainstem nuclei that include neurons which project to the spinal dorsal horn and modulate sensory information. Many areas of the brain including the anterior cingulate cortex (ACC), the insular and the amygdala are known to have important roles in endogenous pain modulation. In part, this is mediated by these regions (and others) projecting to the RVM and the locus coeruleus via the periaqueductal grey (PAG) (grey pathways). The PAG is a key integrative site which receives inputs from the spinal dorsal horn and higher centres of the brain and activates spinally projection neurons in the RVM to drive descending modulation of spinal sensory circuits (red pathways).

Top-down pain modulatory systems contributed to Melzack and Wall's 'Gate Control theory of pain' (Melzack and Wall, 1965), and subsequent experiments by Wall in 1967 demonstrated that lamina V dorsal horn neurons in cats are more responsive to noxious stimuli when the spinal cord is blocked, thus showing that descending pathways normally inhibit dorsal horn nociceptive inputs (Wall, 1967). Further evidence of a predominantly antinociceptive role of descending pathways in adults came from observations of stimulation-produced-analgesia (SPA) caused by electrically stimulating specific brainstem or midbrain nuclei. In these early experiments, electrical

stimulation of the PAG in awake rats led to potent analgesia during peripheral noxious mechanical and electrical stimulation (Reynolds, 1969; Mayer and Liebeskind, 1974). More recent work by Lumb and colleagues has built on these findings and demonstrated that glutamatergic excitation of PAG neurons strongly modulates A- and C-fibre evoked firing activity of deep dorsal horn neurons (McMullan and Lumb, 2006a; Waters and Lumb, 2008). There is also evidence for preferential inhibition of C-fibre inputs to the dorsal horn evoked during cutaneous thermal stimulation which arises from the PAG (McMullan and Lumb, 2006b; Leith et al., 2010)

Few PAG neurons directly project to the spinal dorsal horn (Basbaum and Fields, 1979; although see Mantyh and Peschanski, 1982 who found some direct projections from the PAG to the lumbar spinal cord) and it was not until later that the important role of the RVM was discovered to be involved in descending pain modulation. Chemical lesion of, or local anaesthetic injections into, the RVM abolished the analgesia produced by stimulation of the PAG (Behbehani and Fields, 1979; Aimone and Gebhart, 1986), demonstrating that descending modulation from the PAG requires the RVM in these experimental paradigms. Moreover, SPA can be evoked by electrically stimulating the RVM (Fields et al., 1977), and it is now known that stimulation of the RVM can facilitate as well as inhibit nocifensive withdrawal reflexes, depending on the stimulus strength or concentration of drug used (Zhuo and Gebhart, 1997).

PAG-RVM connectivity has also been shown to mediate endogenous pain modulation from higher brain regions. For example a pain modulatory pathway from the central amygdala to the RVM via the PAG has been proposed by Heinricher and colleagues. Injection of morphine into the central amygdala causes analgesia which can be blocked by lesions of the PAG. Moreover, lesioning the PAG disrupts changes to RVM On and Off cell firing properties caused by injection of morphine into the amygdala (McGaraughty and Heinricher, 2002; McGaraughty et al., 2004), suggesting that PAG-RVM connectivity is required for descending pain modulation from the amygdala.

DNIC: functional evidence of a spinal-bulbo-spinal loop

Diffuse noxious inhibitory control (DNIC), first described in anaesthetised rats (Le Bars et al., 1979), is the phenomenon whereby noxious stimulation applied to heterotopic body sites inhibits firing properties of dorsal horn neurons with receptive fields on somatotopically restricted sites such as the hindpaw. DNIC is mediated by several brainstem regions, including the SRD and the RVM (Villanueva and Le Bars,

1995; Okada-Ogawa et al., 2009). DNIC is driven by noxious heterotopic sensory inputs and relies upon ascending sensory feedforward activation of descending modulatory sites. The presence of DNICs is therefore mediated by a functional spinal-bulbo-spinal loop. Indirect evidence of locus coeruleus and RVM-mediated DNIC arises from pharmacological experiments in the rat; DNIC can be abolished following spinal application of α2-adrenoceptors, and enhanced following spinal application of 5-HT_3R antagonists (Bannister et al., 2015). Whilst it was presumed that noradrenergic neurotransmission arose from the LC, and serotonergic neurotransmission from the RVM, these authors did not look directly at the involvement of these brainstem nuclei in mediating and modulating DNIC. Anatomical tracing experiments in rats support the idea of generalised non-somatotopically organised RVM descending modulation, as 30-50% of RVM projection neurons have termination patterns in multiple segments of the spinal cord (Huisman et al., 1981). This 'pain inhibits pain' phenomenon has also been observed in humans, in the forms of conditioned pain modulation (CPM) and offset analgesia. In humans, functional magnetic resonance imaging (fMRI) data suggests that brainstem regions such as the RVM, PAG and SRD are activated during CPM and offset analgesia, suggesting involvement of these regions in endogenous pain modulation (Derbyshire and Osborn, 2009; Youssef et al., 2016).

1.4.1 The physiology of the RVM

1.4.1.1 Inputs and outputs

The RVM consists of several different brainstem nuclei, including the raphe magnus (RMg), lateral paragigantocellular nucleus (LPGi) and gigantocellular reticular nucleus alpha (GiA). The RVM acts, along with the PAG, as a crucial integrative nucleus; receiving inputs from both sensory and affective nociceptive-processing regions and driving descending modulation of spinal sensory circuits. The RVM is also known to have roles in autonomic control and thermoregulation, as blocking GABAergic transmission in the RVM increases the temperature of the paws and tail and decreases blood pressure and heart rate (Bitar et al., 2015). The RVM is therefore not a nucleus which selectively modulates somatosensation. With this in mind, it is important to separate somatosensory modulatory roles of RVM neurons that project to the spinal dorsal horn from roles of RVM neurons that project elsewhere in the CNS.

The majority of inputs to the RVM originate from the PAG, with additional inputs from the PB nucleus and the dorsal raphe nucleus (Beitz, 1982c; Hermann et al., 1997; Braz et al., 2009). Anatomical tracing experiments have described a BDNF+ pathway

from the lateral and ventrolateral PAG to the RVM (Yin et al., 2014b). This pathway could have functional importance in driving descending facilitation from the RVM, as intra-RVM BDNF injections activate TrkB receptors (primary expressed by serotonergic neurons in the RVM) and causes behavioural hypersensitivity (Wei et al., 2010). Populations of GABA, somatostatin and neurotensin-containing PAG neurons also project to the RVM (Beitz, 1982b; Beitz et al., 1983; Morgan et al., 2008a) and are likely to have important roles in activating RVM neurons and driving descending modulation of spinal dorsal horn sensory circuits.

Inputs from the limbic system have also been reported, primarily from neurons in the lateral, dorsal, paraventricular and preoptic nuclei of the hypothalamus and the central nucleus of the amygdala (Hermann et al., 1997; Murphy et al., 1999; Verner et al., 2008). There is some evidence of sparse ascending inputs from the trigeminal (Sugiyo et al., 2005) and the deep spinal dorsal horn (Braz et al., 2009) directly to the RVM, however there is no information regarding the role of these inputs in driving descending RVM modulation.

It is unlikely that the neural inputs to the RVM encode an exclusive correlate of sensory-driven information from the dorsal horn. Information from the PAG, the limbic system, and subsidiarily from the PB nucleus, likely represents integrated inputs which are driven by ascending sensory inputs but steered by autonomic regulation and neural correlates of conditioned 'memories' and aversion (Navratilova et al., 2013; Roy et al., 2014). Thus, the highly selected descending modulation from the RVM over spinal dorsal horn sensory circuitry could, hypothetically, reflect the net output of integrating sensory, attentional and affective top-down modulatory components of nociception and pain.

RVM neurons project to the spinal dorsal horn via the dorsolateral funiculus (DLF) (Basbaum et al., 1976; Basbaum and Fields, 1979) and have termination patterns in laminae I-II and in deeper laminae IV-VI (Light and Kavookjian, 1985; Antal et al., 1996; Aicher et al., 2012), forming synapses with dorsal horn neurons (Antal et al., 1996) and primary afferent terminals (Zhang et al., 2015). Other projection targets from the RVM include the hypothalamus and amygdala (Vertes, 1984; Hermann et al., 1996). The majority of spinally projecting RVM neurons are GABA/glycinergic or serotonergic, however other neurotransmitters are also expressed and presumably released in the spinal dorsal horn from RVM neuron axons. Variability between different papers using retrograde tracing combined with immunohistochemistry techniques makes it hard to quote exact figures for the proportions of spinally

projecting RVM neurons which express and release certain neurotransmitters. Hossaini et al., (2012) identified that 45% of RVM neurons which project to the lumbar dorsal horn contain GlyT2 and/or GAD67 mRNA, suggesting that nearly half of spinally projecting RVM neurons are inhibitory GABA/glycinergic neurons (Hossaini et al., 2012). However, others have found that only 9% of spinally projecting RVM neurons are GABA-immunoreactive (Reichling and Basbaum, 1990), and 10-15% of spinally projecting RVM neurons are GAD-immunoreactive (Jones et al., 1991). The proportion of descending RVM neurons which are serotonergic is more consistent in the literature; with between 31-46% of spinally projecting RVM neurons containing 5-HT or Tph (Bowker et al., 1981a; Kalyuzhny et al., 1996; Braz and Basbaum, 2008). Some evidence suggests that many neurons in the RVM contain both GABA and 5-HT (Millhorn et al., 1988), suggesting that these two populations may overlap; however others have not found evidence of GAD and 5-HT coexpression (Jones et al., 1991). Spinally projecting neurons also contain proenkephalin, the majority of which also express GABA (Zhang et al., 2015), neurotensin (Wang et al., 2014) and substance P (Bowker and Abbott, 1988).

Evidence for direct supraspinal modulation of primary sensory inputs has been described, and involves direct anatomical synaptic connectivity between RVM GABAergic/enkephalinergic neurons and primary afferent terminals (Zhang et al., 2015). 80% of RVM neurons which make synaptic connections with primary afferent neurons express or GABA, many of which also expressed proenkephalin, whilst 17% contain 5-HT. This direct descending modulation of afferent inputs at least partially drives inhibition of behavioural sensitivity to thermal or mechanical stimuli (Zhang et al., 2015). *In vitro* Ca^{2+} imaging in mice has also demonstrated direct RVM-derived 5-HT-mediated excitation of TRPV1+ primary afferent sensory terminals in the trigeminal dorsal horn (Kim et al., 2014a). Anatomical evidence of RVM neurons targeting axons is controversial, as previous evidence suggests that the majority of RVM neuron postsynaptic targets are dorsal horn neuron dendrites (78% of terminals), and few, if any, axo-axonic synaptic contacts on primary afferent neuron terminals were identified (Antal et al., 1996). Therefore, it is likely that RVM neurons primarily modulate dorsal horn neuron activity over primary afferent neuron activity to change processing of somatosensory information in the spinal cord.

1.4.1.2 On, Off and Neutral cells in the RVM

In the early 1980's, Fields and colleagues recorded firing properties of RVM neurons from lightly anaesthetised rats in response to heat stimulation of the tail (Fields et al., 1983). Two classes of cells were characterised: On cells which displayed increased firing activity during ballistic tail withdrawal from noxious thermal stimulation; and Off cells which displayed decreased firing activity during tail withdrawal. Changes in firing activity of On and Off cells closely correlated with tail flick withdrawal rather than the temperature of the stimulus. Based on these findings, the authors hypothesised that bidirectional pain modulation from the RVM is mediated by noxious sensory-evoked changes in On and Off cell firing properties. On and Off cells were later found to be sensitive to opioids, as microinjection of opioids into the PAG or RVM silences On cell activity and increases Off cell activity in anaesthetised rats (Fang et al., 1989; Heinricher et al., 1994, 2009). Thus, opioid analgesia is at least partly mediated by opioid-induced enhancement of tonic Off cell firing activity in the RVM. Additionally, the balance of RVM On and Off cell firing activity is hypothesised to influence nocifensive withdrawal thresholds in acute and chronic pain states (Heinricher et al., 2009; Mason, 2011, 2012).

Robust observations of changes in On and Off cell firing rates have often produced a shorthand of increased On cell and decreased Off cell firing being equated to pronociception, and increased Off cell firing being equated to antinociception. Importantly, the response properties of these cells are not restricted to pain behaviours and are dependent on the state of anaesthesia. RVM neuron recordings in awake rats have demonstrated that increased On cell and decreased Off cell firing activity is increased by noxious and non-noxious inputs alike, without correlating with nocifensive withdrawals (Oliveras et al., 1990; Leung and Mason, 1999). Additionally, Off cells fire continuously during slow wave sleep, and micro-arousals which were coupled with muscle activity were accompanied by decreased Off cell firing and increased On cell firing (Leung and Mason, 1999; Mason et al., 2001). Similarly, increased Off cell firing and decreased On cell firing has also been observed during bouts of micturition (Baez et al., 2005) and during eating and drinking (Foo and Mason, 2005; Mason and Foo, 2009). Thus, whilst strong evidence shows that RVM On and Off cells are important in descending pain modulation, these neurons are not selectively activated by noxious sensory inputs and changes in their firing properties are not a pure correlate of nocifensive withdrawals.

Neutral cells have also been described in the RVM. These neurons do not exhibit a change in firing activity during nocifensive withdrawal (Fields et al., 1983; Heinricher et al., 2009), and were hypothesised to have little role in descending modulation of nociception. Importantly, some Neutral cells contain 5-HT, but On and Off cells do not (Potrebic et al., 1994). Serotonergic (5-HT+) neurons in the RVM have been shown to exhibit changes in firing activity in response to noxious sensory stimulation (Gau et al., 2013), but these neurons are a distinct population of neurons from On and Off cells (Gao and Mason, 2000). Considering the inclusion of serotonergic neurons in the Neutral population of cells, and the well documented pain modulatory role of RVM serotonergic neurons (Suzuki et al., 2004b), it seems erroneous to exclude somatosensory modulatory roles of Neutral cells.

1.4.2 Descending RVM modulation during pain states: balance and timing

Pain in response to tissue damage and injury is protective; driving avoidance and guarding behaviours to safeguard the injured body region during healing. During most situations, descending controls have a distinct role during chronic pain states to increase the saliency of sensory inputs originating from the damaged region to drive these protective behaviours. Whilst the majority of this thesis focusses on descending controls during acute nociception, here I will briefly discuss changes in the function of the RVM during chronic pain states.

Injection of the local anaesthetic agent lidocaine into the RVM is one method that has been used to unmask endogenous descending modulation that represents the net output from the RVM. RVM lidocaine injection reduces behavioural hypersensitivity caused by hindpaw inflammation (Cleary and Heinricher, 2013), mustard oil application to the hindpaw (Kincaid et al., 2006), or by peripheral nerve injuries such as spinal nerve ligation (SNL) (Pertovaara et al., 1996; Burgess et al., 2002; Taylor et al., 2007; De Felice et al., 2011) and chronic constriction injury (CCI) (Okubo et al., 2013a). In anaesthetised animals, the effect of net RVM descending modulation on spinal dorsal horn neuron properties can also be unmasked following injection of lidocaine into the RVM by comparing firing properties to those from control animals. For example, RVM lidocaine injection reduces firing activity of dorsal horn neurons in mustard oil-treated rats compared to control rats (Pertovaara, 1998). Collectively, these behavioural and electrophysiological experiments suggest pronociceptive modulation arising from the RVM in injured animals.

Descending modulation from the RVM is not absolute in its directionality. By recording dorsal horn neuron properties before and after RVM lidocaine injection in the same anaesthetised rat, Bee and Dickenson (2007) demonstrated that the RVM heterogeneously inhibits, facilitates or does not change stimulus-evoked firing properties of individual dorsal horn neurons. Importantly, the proportion of cells which were inhibited or facilitated by RVM lidocaine injection changed following peripheral nerve injury (Bee and Dickenson, 2007). Net descending modulation from the RVM thus reflects changes in the balance of excitatory and inhibitory drive which is altered by events such as tissue or nerve injury.

This changing balance of facilitatory or inhibitory descending modulation from the RVM can be exemplified in the context of inflammatory pain. Injection of lidocaine into the RVM one hour after Complete Freund's Adjuvant (CFA) injection into the hindpaw attenuates CFA-induced behavioural hypersensitivity, but facilitates behavioural hypersensitivity when injected 3-10 days after CFA injection (Cleary and Heinricher, 2013). This time-dependent switch from endogenous descending facilitation to inhibition is mediated by changes in glutamate receptor activation: injection of a low dose of NMDA into the RVM in the first hours after CFA injection facilitates behavioural hypersensitivity; whilst injection of AMPA into the RVM attenuates behavioural hypersensitivity with increasing efficacy between 5 and 24 hours after inflammation (Guan et al., 2002, 2003, 2004). Increased phosphorylation of GluR1 subunits was observed in the RVM within 30 minutes after hindpaw CFA injection, and this effect could be blocked by local anaesthetic injections at the site of CFA injection (Guan et al., 2004). These papers demonstrate that enhanced sensory afferent input drive (caused by tissue inflammation) can change receptor signalling in the RVM which causes downstream changes in the balance of descending modulation of sensorimotor reflexes. Thus, a responsive and labile RVM is an important modulator of moment to moment sensory feedback via a spinal-bulbo-spinal loop which is tailored to a particular behavioural state.

1.4.3 Descending serotonergic modulation from the RVM

Serotonin-containing neurons in the RVM are located in the lateral paragigantocellular reticular nucleus (LPGi) and raphe magnus nucleus (RMg), which together constitute the B3 serotonergic group. Around 11% of all neurons in the RVM contain 5-HT (Marinelli et al., 2002), and retrograde tracing experiments in adult rats have found that between 31-46% of all RVM neurons which project to the lumbo-sacral spinal cord

contain 5-HT or the 5-HT synthesis rate limiting enzyme tryptophan hydroxylase (Tph) (Bowker et al., 1981a; Kalyuzhny et al., 1996; Braz and Basbaum, 2008). Anterograde tracing experiments labelling descending projections from neurons in the RVM have identified extensive labelling of 5-HT-containing axon terminals primarily in laminae I-II and also in laminae IV-X of the dorsal horn in the adult mouse (Liang et al., 2015), and rat (Rajaofetra et al., 1989; Jones and Light, 1990).

Electrical stimulation of the RVM causes the release of 5-HT in the spinal cord (Hammond et al., 1985) which binds to and activates different 5-HT receptor (5-HTR) subtypes expressed in the spinal dorsal horn (table 1.2). Like descending modulation from the RVM in general, serotonergic output from the RVM can be either pronociceptive or antinociceptive, depending on the injury state of the animal and on the subtype of 5-HTR activated in the spinal dorsal horn. Intrathecal administration of 5-HT can either reduce pain-like behaviours in response to noxious stimulation in mice and rats (Hylden and Wilcox, 1983; Schmauss et al., 1983), or cause biting and licking behaviours in mice (Fasmer and Post, 1983). RVM-derived 5-HT release in the dorsal horn is thought to partially mediate RVM stimulation produced analgesia, as intrathecal administration of the 5-$HT_{1/2}$R antagonist methysergide reduces this effect (Hammond and Yaksh, 1984). However, pronociception has also been demonstrated following optogenetic activation of Tph-expressing neurons in the RVM, as demonstrated by increased mechanical and thermal nocifensive withdrawal thresholds in Tph-2-channelrhodopsin transgenic mice (Cai et al., 2014).

Receptor subtype	**Intracellular mechanism**
5-HT_{1A}	Inhibitory GPCR, coupled to G_i/G_o
5-$HT_{2A, B \& C}$	Excitatory GPCR, coupled to $G_{q/11}$
5-HT_3	Excitatory ion channel (cation entry)
5-HT_7	Excitatory GPCR, positively coupled to G_s.

Table 1.2. 5-HT receptors expression in the adult spinal dorsal horn linked with modulation of spinal sensory inputs
Pharmacological and anatomical studies have identified four major 5-HT receptor subtypes which are expressed in the spinal dorsal horn. 5-$HT_{1,2,7}$ receptor subtypes are G-protein coupled receptors (GPCRs) and 5-HT_3 is an excitatory ionotropic channel. For reviews see Suzuki et al., (2004) and Wei et al., (2012).

RVM 5-HT neurons are also thought to partially mediate morphine analgesia, as this can be reduced following destruction of 5-HT terminals in the spinal cord by with 5,7-

dihydroxytryptamine (5,7-DHT) (Vogt, 1974; Mohrland and Gebhart, 1980). However, recently it was demonstrated that local depletion of 5-HT by focally injecting Tph2-shRNA into the RVM has no effect upon morphine-induced analgesia (Wei et al., 2010); suggesting that serotonergic RVM neurons, but not 5-HT itself, partially mediates morphine-induced analgesia. 5-HT-containing neurons in the RVM also coexpress other neurotransmitters such as enkephalin, substance P, neurotensin and GABA (Millhorn et al., 1987, 1988; Reddy et al., 1990; Wang et al., 2014), therefore it is likely that these neurotransmitters also mediate serotonergic neuron-mediated modulation of sensory circuits indirectly from activation of 5-HTR subtypes in the dorsal horn.

Descending serotonergic excitation and inhibition has also been described locally in the spinal dorsal horn. Focal application of 5-HT onto the surface of the dorsal horn inhibits C-fibre evoked firing properties of spinal dorsal horn neurons in anaesthetised rats; effects which could be mimicked by applying 5-HT_{1A}, 5-HT_{1B}, 5-HT_{2A} or 5-HT_{2C} agonists (Liu et al., 2007). However, destruction of 5-HT terminals in the spinal cord with 5,7-dihydroxytryptamine (5,7-DHT), or focal application of the 5-HT_3R antagonist ondansetron to the dorsal horn, decreases sensory-evoked dorsal horn neuron firing properties compared to control rats (Rahman et al., 2004a, 2006; Suzuki et al., 2004a), demonstrating that descending serotonergic modulation and spinal 5-HT_3Rs have a net endogenously facilitatory effect upon dorsal horn neurons in these experiments. *In vitro* patch clamp experiments in spinal cord slices show that 5-HT application can either potentiate or inhibit EPSCs in individual dorsal horn neurons, and inhibition of dorsal horn neuron EPSCs can be replicated by application of 5-HT_{1A}R agonists (Li and Zhuo, 1998a). 5-HT application can also increase the frequency of spontaneous IPSCs of dorsal horn neurons, and this effect can be blocked by application of 5-HT_{2A} and 5-HT_{2C} antagonists (Xie et al., 2012). Presumably, descending modulation from serotonergic RVM neurons upon dorsal horn neurons (Rahman et al., 2006) and pain behaviours (Cai et al., 2014) represents the net output of 5-HT-mediated modulation of spinal inhibitory and excitatory circuits.

Robust evidence supports the hypothesis that descending serotonergic modulation from the RVM is pronociceptive during chronic pain states. 5,7-DHT ablation of descending 5-HT terminals attenuates behavioural hyperalgesia following hindpaw inflammation (Carr et al., 2014), formalin injection (Svensson et al., 2006), spinal cord injury (Oatway et al., 2004) and peripheral nerve injury (Leong et al., 2011). Similarly, deletion of endogenous 5-HT in the RVM attenuates formalin and CFA-induced

hyperalgesia (Wei et al., 2010; Guo et al., 2014). Descending serotonergic pronociception in pain states is thought to be mediated (at least in part) by activation of 5-HT_3Rs in the spinal dorsal horn, as intrathecal injections of 5-HT_3R antagonists attenuate behavioural hypersensitivity caused by inflammatory pain states (Green et al., 2000; Svensson et al., 2006; Lagraize et al., 2010). Similarly, 5-HT_3R knockout mice show reduced formalin-evoked pain behaviours and serotonin-evoked itching behaviours compared to wild-type mice (Zeitz et al., 2002). Of note, injection of 5-HT_3R antagonists or 5-HT_3R knockout has no effect upon baseline thermal or mechanical withdrawal thresholds in uninjured adult animals (Zeitz et al., 2002; Lagraize et al., 2010; Guo et al., 2014), suggesting that 5-HT_3R-mediated pronociception is limited to chronic pain states in adult animals.

In neuropathic pain states, descending modulation from serotonergic RVM neurons is thought to be involved primarily in the maintenance, rather than the onset, of chronic pain following peripheral nerve injury. Transiently blocking 5-HT synthesis in RVM neurons or application of 5-HT_3R antagonists to the dorsal horn attenuates behavioural hypersensitivity 14 days after chronic constriction injury, but not before (Wei et al., 2010; Okubo et al., 2013a). Similarly, blocking 5-HT synthesis in the RVM or 5-HT_3Rs in the medullary dorsal horn reduces capsaicin-induced Ca^{2+} signalling in TRPV1 expressing trigeminal primary afferent neurons in brainstem slices (Kim et al., 2014a). Descending facilitation from serotonergic and non-serotonergic neurons in the RVM is also delayed after nerve injury, as injection of lidocaine into the RVM attenuates behavioural hypersensitivity 6-12 days, but not 3 days after SNL (Burgess et al., 2002). Selective depletion of 5-HT in the RVM replicates this RVM-mediated pro-nociception, whilst antagonism of spinal 5-HT_3Rs attenuates this effect (Okubo et al., 2013a).

1.5 The development of spinal and brainstem somatosensory connections in the postnatal period

Sensory systems undergo profound maturation in the postnatal period, causing processing of sensory inputs to differ in young and adult nervous systems. This introduction has illustrated that the balance of excitation and inhibition in parts of the adult central nervous system which receive and modulate somatosensory information is crucial for normal sensory feedback. Functional sensory systems in adulthood rely upon activity-dependent maturation of excitatory-inhibitory balance in sensory pathways during postnatal development. Perturbing the gain of activity-dependent

maturation during early postnatal life, either by sensory deprivation (e.g., visual deprivation (Wiesel and Hubel, 1963) or sensory overstimulation (e.g., neonatal tissue injury (Walker et al., 2009; Schwaller and Fitzgerald, 2014)), can permanently alter the developmental outcome of sensory systems. Thus, changes in excitatory-inhibitory balance during critical periods have a major impact on the developmental outcome of sensory systems. This section will outline what is currently known about processing of cutaneous somatosensory inputs in the nervous system in young mammals.

1.5.1 Excitability of spinal sensory networks in young animals

Neonatal sensory networks, such as those in the spinal cord, are more excitable than those in the healthy adult. As a result, young animals are more sensitive to cutaneous stimulation. Below is a summary of the behavioural evidence for this:

1. Behavioural withdrawal thresholds to mechanical stimulation of the skin are lower in neonatal rats and human infants, and extend to the non-noxious range (Fitzgerald et al., 1988; Andrews and Fitzgerald, 1994; Cornelissen et al., 2013; Zavitsanou et al., 2013). These mechanical withdrawal thresholds increase with postnatal age, with thresholds approaching adult-like ranges in the fourth postnatal week in rats (Fitzgerald et al., 1988).
2. Behavioural reflexes can be can sensitised to repeated innocuous mechanical stimuli in neonatal rats and humans (Jennings and Fitzgerald, 1998; Cornelissen et al., 2013), and repeated cutaneous stimulation is more likely to produce habituation than sensitisation in older rats and human infants (Fitzgerald et al., 1988).
3. At birth, reflexes in response to a noxious stimulus are not restricted to the stimulated limb and are often whole body movements. This has been observed both in neonatal rodents and humans (Fitzgerald et al., 1988; Cornelissen et al., 2013).
4. In comparison to adults, reflex responses to noxious stimuli are prolonged and continue long after punctate stimulation in rats during the first two weeks of life (Fitzgerald and Gibson, 1984). Similarly, noxious-evoked reflex responses are prolonged and are greater in magnitude (measured using EMG activity) in newborn infants (Cornelissen et al., 2013).
5. Excitability of neonatal reflexes correlates with activity of individual dorsal horn neurons. Cutaneous receptive fields of dorsal horn neurons are larger in neonatal animals; encompassing a relatively larger region of the hindpaw

compared to dorsal horn neurons in adult animals (Fitzgerald, 1985; Torsney and Fitzgerald, 2002).

Refinement of reflexes is hypothesised to result from Hebbian strengthening and weakening of synapses in the spinal cord during the postnatal period. This section of the introduction will outline developmental changes in peripheral and descending inputs into the spinal dorsal horn, and how these changes alter dorsal horn sensory networks.

1.5.2 Anatomical changes in afferent input to the dorsal horn during development

In rodents, sensory neurons in the dorsal root ganglia (DRG) grow dendritic processes into target tissues such as the skin and joints during embryonic stages of development. Growth and survival of DRG neurons and peripheral terminals is dependent upon neurotrophin signalling in the periphery, one source of which is pre-existing motor neuron axons (Usui et al., 2012). Sensory DRG neuron central terminals also enter the lumbar spinal dorsal horn during embryonic stages. RT97-expressing A-fibres are present in the lumbar dorsal horn from embryonic day (E) 15-17, whilst TrkA-expressing C-fibres are present dorsal horn from E18-20 (Jackman and Fitzgerald, 2000).

Peripheral sensory transduction is functional before birth; DRG neurons have functional peripheral terminals which respond to cutaneous touch, heat and chemical stimulation from E16 (Fitzgerald, 1987a). Moreover, DRG neurons are spontaneously active during late embryonic development, but this property is absent by birth (Fitzgerald, 1987a). Expression of tetrodotoxin-resistance voltage gated sodium channels ($Na_v1.9$ and $Na_v1.8$) in DRG neurons increases from E15 and reaches adult levels by P7 (Benn et al., 2001), suggesting that electrical properties of primary afferent neurons may change during late embryonic and early postnatal development. C-fibre responsiveness to different sensory modalities does change during postnatal development; a phenotypic switch from predominantly mechanoreceptive to mechanoreceptive and thermoreceptive polymodal populations of C-fibres occurs around P8-P10 (Jankowski et al., 2014).

Whilst peripheral afferent neuron terminals are present in the spinal dorsal horn at birth, the densities and distributions of axonal terminals and synaptic inputs change during the first weeks of life. C-fibre terminals are observed in laminae I and II from birth and A-fibre terminals are scattered throughout the superficial and deep dorsal horn over the first weeks of life. Gradually, pruning of A- fibre terminals and weak

synaptic inputs refines primary afferent neuron termination patterns to adult-like distributions by around P28 (Fig. 1.7) (Beggs et al., 2002; Fitzgerald, 2005; Granmo et al., 2008). Drawing from evidence in the visual system, it can be hypothesised that microglia have a key phagocytic role in pruning and refining termination patterns of sensory inputs in the dorsal horn, as is the case in the lateral geniculate nucleus in neonatal mice (Schafer et al., 2012).

Withdrawal of A fibres from the superficial dorsal horn is dependent on excitatory glutamatergic neurotransmission in the dorsal horn, as applying the NMDAR antagonist MK-801 to the dorsal horn prevents withdrawal of A-fibre terminals to the deeper laminae in adulthood (around P40; Beggs et al., 2002). Rearing pups in an environment with constant sensory stimulation in a vibrating cage also perturbs the postnatal withdrawal of A fibres from the superficial dorsal horn in adult rats (Granmo et al., 2008). These papers collectively demonstrate that maturation of adult body maps depends upon the strength of sensory inputs during early postnatal periods.

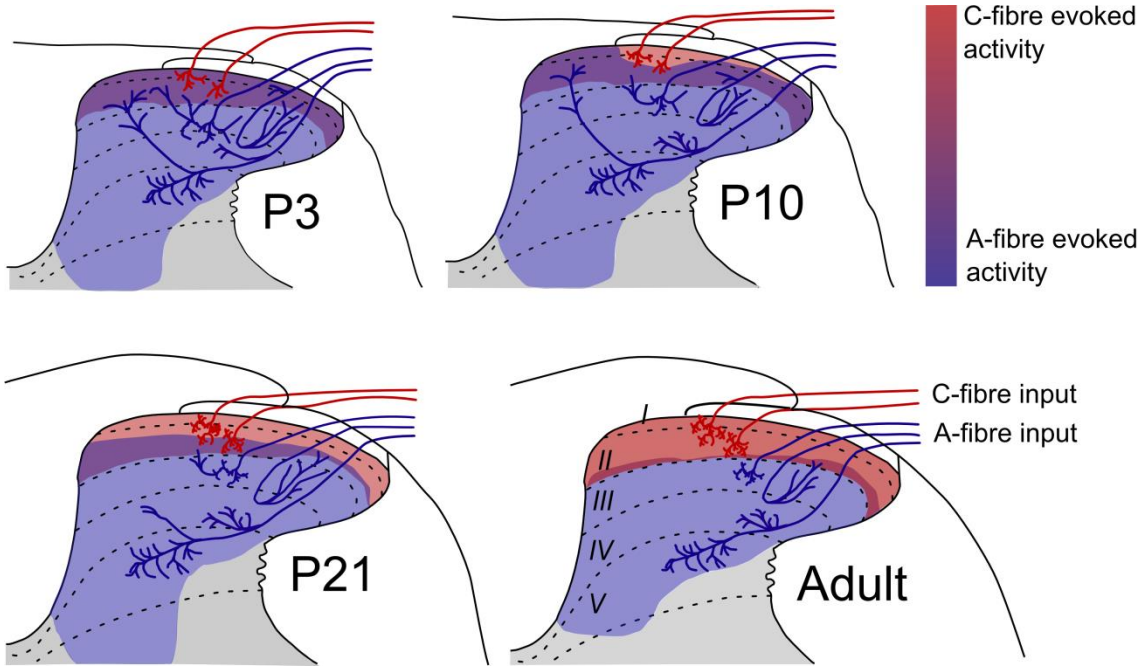

Fig 1.7. Primary afferent neuron termination patterns in the developing dorsal horn.
In young P3-P21 rats, A fibres (blue fibres) terminate in the superficial and deep dorsal horn. Monosynaptic Aβ inputs activate superficial and deep dorsal horn neurons in young rats (blue shading). By adulthood, A fibre terminals have withdrawn to the deeper laminae and monosynaptic Aβ inputs to superficial dorsal horn neurons are not observed. C-fibres (red fibres) terminate in laminae I-II from P3. These termination patterns do not change with age, but the strength of these C-fibre synaptic inputs increases with postnatal age. Note the depicted increase in size of the dorsal horn between P3-P21.

1.5.3 Cutaneous mechanotransduction in neonatal rodents

Mechanotransduction in afferent neurons is functional in neonatal rodents, and the majority of mechanoreceptive primary afferent types found in adult animals can be distinguished at early developmental ages. *In vivo* electrophysiological recordings from E16-20 rats *in utero* have demonstrated that LTMR and HTMR primary afferents have clear cutaneous receptive fields and respond to cutaneous touch and pressure (Fitzgerald, 1987). Slowly adapting and rapidly adapting LTMRs and HTMRs in glabrous skin are identified in P0 rats (Fitzgerald, 1987b), and cutaneous afferent fibres innervating hairy skin are specified into sensory neuron phenotypes observed in adults at P14 (Koltzenburg et al., 1997). Natural stimulation may result in lower primary afferent neuron firing frequencies carried at lower conduction velocities in neonatal rodents compared to in adults (Fitzgerald, 1987b); however, collectively, these findings suggest that natural mechanical stimuli applied to glabrous or hairy skin, such as innocuous brush or noxious pinch, likely evokes similar primary afferent neuron response patterns in neonatal and adult rodents. Whilst, for the most part, mechanoreception in the peripheral nervous system is comparable in neonates and adult rodents, evidence suggests that the processing of mechanical sensory inputs in the spinal dorsal horn differs in neonatal and mature animals. The following sections will discuss developmental changes in the central processing of somatosensory inputs.

1.5.4 Functional changes in afferent input to the dorsal horn during development

Whilst C-fibre terminals are present in the dorsal horn from birth, maturation of functional C-fibre synapses takes place over the first few postnatal weeks. In anaesthetised P7 rats, electrical C-fibre stimulation does not evoke firing activity of neurons in the deep dorsal horn, and only weakly increases neuronal firing activity in the superficial dorsal horn (Fitzgerald, 1985). Electrical C-fibre stimulation does increase Fos expression in the dorsal horn at P3, but age-dependent increases in C-fibre-evoked Fos expression are observed between P21-30, suggesting maturation of C-fibre inputs well into adolescence in the rat (Jennings and Fitzgerald, 1996). Similarly, cutaneous noxious mechanical and thermal stimuli increase Fos expression in the dorsal horn at P0, but noxious-evoked Fos-expression increases between P0-P14 (Yi and Barr, 1995).

Heat and capsaicin sensitive TRPV1 receptors are expressed in the DRG from birth, and TRPV1 expression does not change between P2-P10 (Guo et al., 2001). Chemical irritants which activate C-fibres such as capsaicin or menthol evoke membrane

depolarisations in dorsal horn neurons in spinal cord slices from P0 rats, and the frequency of stimulus-evoked EPSCs increases with postnatal age between P0-P11 (Baccei et al., 2003). This paper also showed that the proportion of neurons displaying capsaicin-evoked action potential firing, and the frequency of action potential firing, increased between P1-P5 (Baccei et al., 2003). These electrophysiology and Fos immunohistochemistry experiments demonstrate that there is considerable postnatal strengthening of multi-modal sensory C-fibre inputs to dorsal horn neurons in the first weeks of postnatal life.

Neonatal dorsal horn sensory networks are thought to be primarily activated by A-fibre inputs during the first postnatal weeks whilst C-fibre synapses are strengthened. Whilst noxious inputs increase Fos expression in the dorsal horn at all ages, non-noxious tactile inputs increase Fos expression at P3, but not at P21; demonstrating noxious input-specific Fos expression in the dorsal horn from P21 (Jennings and Fitzgerald, 1996). Additionally, the proportion of superficial dorsal horn neurons which display monosynaptic Aβ-evoked EPSCs decreases between P21-P60, whilst those that displayed polysynaptic EPSCs did not (Park et al., 1999; Nakatsuka et al., 2000). It is not currently known if polysynaptic Aβ inputs to superficial dorsal horn neurons are under tonic inhibitory control in young rats, as is the case in the adult dorsal horn (Torsney and MacDermott, 2006; Duan et al., 2014). As inhibitory neurotransmission in the neonatal dorsal horn is immature, it is likely that these polysynaptic Aβ circuits are not gated; providing further Aβ-mediated activation of superficial dorsal horn neurons. Thus, cutaneous LTMRs which are activated by innocuous mechanical stimuli such as brush activate both neurons in the superficial and deep dorsal horn in neonates. Brush-evoked activity then becomes restricted to deep dorsal horn neurons by the fourth postnatal week, suggesting distinct processing of low and high threshold sensory inputs by this age.

The high incidence of monosynaptic Aβ-fibre inputs to superficial dorsal horn neurons as late as P21 is consistent with unrefined termination patterns of A-fibres in the dorsal horn at this age (Beggs et al., 2002). As A-fibre termination patterns are refined to the deeper laminae in adult animals, monosynaptic Aβ-fibre inputs are restricted to neurons in lamina III-V. Age-dependent weakening of A-fibre inputs coincides with strengthening of C-fibre inputs to superficial dorsal horn neurons in the third and fourth postnatal weeks (Nakatsuka et al., 2000; Koch and Fitzgerald, 2013). It is hypothesised that dominant A-fibre mediated activation of superficial and deep dorsal horn neurons

in early life is crucial for activity-dependent maturation of both nociceptive and tactile sensory networks in the dorsal horn.

1.5.5 Excitatory and inhibitory neurotransmission in the immature dorsal horn

As previously outlined, spinal reflexes are hyperexcitable in neonatal animals compared to those in adulthood. This is hypothesised to be due to an excitatory-inhibitory imbalance which favours excitation. During the first postnatal weeks local and supraspinal excitatory control of spinal sensory networks diminishes whilst inhibitory processes are strengthened. Alterations in the expression of glutamatergic AMPA, kainate and NMDA receptors are apparent in the developing dorsal horn: protein expression of glutamate receptor subunits (e.g., GluR1 and NR1) in the dorsal horn decreases with postnatal age (Brown et al., 2002), suggesting that excitatory glutamatergic signalling may be increased in neonatal rats compared to adult rats. However, the amplitude of mEPSCs recorded from dorsal horn neurons in spinal cord slices dos not change between P0-P11, suggesting that intrinsic excitability of dorsal horn neurons does not change during this age range (Baccei et al., 2003).

Another source of excitation in the neonatal dorsal horn arises from the presence of excitatory interneurons which transiently express VGLUT3 between P5-P20 (Peirs et al., 2015). Monosynaptic Aβ inputs onto VGLUT3 neurons drives feedforward excitation of lamina I NK1R neurons in neonatal mouse spinal cord slices. Expression of VGLUT3 in these neurons is important in establishing mechanical sensitivity in adulthood, as ablation of these neurons prevents mechanical hypersensitivity following peripheral inflammation or nerve inflammation in adult mice (Peirs et al., 2015). It is not currently known which factors control the onset and offset of VGLUT3 expression in this population of excitatory interneurons.

An important feature of the developing CNS is spontaneous neuronal activity. Spontaneously active pacemaker neurons are present in the neonatal dorsal horn and have cell bodies in lamina I (Baccei, 2014). Oscillatory bust-firing activity of these neurons is independent from sensory inputs to the dorsal horn and relies upon intrinsic voltage-gated conductance mediated by Na^+ and Ca^{2+} channels (Li and Baccei, 2011). These spontaneously active neurons are glutamatergic, and are hypothesised to provide an endogenous source of excitation in the dorsal horn which drives the maturation of dorsal horn circuits. Additionally, a subpopulation of lamina I pacemaker neurons are projection neurons with terminals in the PB nucleus and the PAG, suggesting that

these neurons may have important roles in the maturation of supraspinal sensory networks (Li et al., 2014).

Major changes in the balance of local excitatory-inhibitory signalling in the developing dorsal horn are a result of maturation of GABAergic and glycinergic inhibitory transmission. GABAergic transmission is functional from P3, as application of $GABA_A$ receptor antagonists increases the cutaneous receptive field size of dorsal horn neurons *in vivo* (Bremner et al., 2006). Similarly, *in vitro* experiments demonstrated that $GABA_A$ receptor antagonism increases evoked action potential firing of dorsal horn neurons from P3 spinal cord slices (Bremner et al., 2006). $GABA_AR$-mediated inhibitory postsynaptic currents (IPSCs) have also been recorded from dorsal horn neurons in P0 spinal cord slices, demonstrating functional GABAergic inhibition at birth (Baccei and Fitzgerald, 2004). These IPSCs increased in amplitude between P10 and P21, suggesting age-dependent increases in the strength of GABAergic inhibitory synapses (Ingram et al., 2008).

Recent evidence suggests that immature glycinergic inhibitory signalling in the dorsal horn may be a major contributor to the excitability of sensory networks in young rats (Koch et al., 2012). Application of the glycine receptor (GlyR) strychnine to the dorsal horn increases tactile and noxious-evoked dorsal horn neuron firing properties at P21, demonstrating functional glycinergic inhibition at this age. In contrast, strychnine application unmasks glycinergic facilitation of tactile-evoked dorsal horn neuron firing properties at P3. The switch to glycinergic inhibition occurs around P14 and is dependent on functional C-fibres between P10-P13, as blocking capsaicin-sensitive C-fibre activity during this period prevented maturation of glycinergic inhibition at P14 (Koch et al., 2012). Thus, activity-dependent maturation of inhibitory circuits is driven by strengthening of C-fibre synapses in dorsal horn neurons in the first weeks of life. It is hypothesised that immature GABAergic and glycinergic inhibitory transmission in the dorsal horn plays a crucial role in permitting intrinsic excitability of spinal sensory circuits in the first weeks of life (Koch and Fitzgerald, 2013).

1.5.6 The postnatal development of ascending projections to the brain

Peripheral sensory inputs activate somatosensory regions of the brain in new born human infants. In term born infants, tactile and noxious sensory stimuli evoke distinct event-related potentials in the somatosensory cortex, as measured by electroencephalography (EEG) (Slater et al., 2010), but this is preceded by a developmental period of nonspecific event-related bursting before 35 gestational weeks

(Fabrizi et al., 2011). Functional magnetic resonance imaging (fMRI) experiments have also demonstrated that many regions of the brain associated with sensory and affective components of pain processing in adults display functional sensory-evoked activation in newborn infants (Goksan et al., 2015; Williams et al., 2015). In rodents, it is less well known when somatosensory inputs activate primary sensory and sensory integrative regions of the brain during development.

As discussed, sensory inputs activate neurons in the rodent spinal dorsal horn from birth. Retrograde tracing experiments have shown that projection neurons in lamina I send ascending axons to the PB nucleus and PAG from birth, however it is not known whether there is postnatal anatomical growth of these pathways (Li and Baccei, 2012). Hindpaw formalin injection increases Fos expression in the PB nucleus from P3, demonstrating functional connectivity between the dorsal horn and the PB nucleus from this age (Barr, 2011; Man et al., 2012). In contrast, noxious-evoked Fos expression is not observed in the PAG and thalamus until P14, suggesting later functional maturation of spino-PAG and spino-thalamic pathways (Barr, 2011).

There is a considerable lack of information about the connectivity of ascending projections from the dorsal horn to the brain. Anatomical and functional pathways from the dorsal horn to the brainstem have been described at P3, but it is not known if there is anatomical growth or functional strengthening of these connections. Similarly, it is not known when sensory inputs activate other sensory integrative and modulatory regions of the brain during development in the rodent. Tactile and noxious inputs do evoke local field potential bursting activities in the somatosensory cortex of anaesthetised neonatal rats (Chang and Fitzgerald, unpublished findings). It can therefore be assumed that synaptic connections of neuronal pathways between sensory afferent fibres and neurons in the somatosensory cortex are functional, but it is not known if sensory information is processed differently along these pathways in young and adult rats.

The presence of sensory-evoked neuron activity in the dorsal horn at E16-20 coincides with reflex movements in embryonic rats (Fitzgerald, 1987a), suggesting functional connectivity between sensory circuits in the dorsal horn and motor units in the ventral horn of the spinal cord before birth. Retrograde tracing experiments have since demonstrated anatomical connectivity between dorsal horn and ventral horn neurons in neonatal mice (Zampieri et al., 2014). Thus, whilst the presence of ventrally projecting sensory outputs from the dorsal horn neurons is well established, these

observations do not permit the conclusion that sensory information activates networks in the brain during this developmental period.

1.5.7 The postnatal development of descending modulation of dorsal horn circuits

The adult PAG-RVM descending modulatory pathway is driven by "bottom up" sensory inputs and "top down" inputs from higher centres of the brain, and provides controlled moment to moment inhibition or facilitation of spinal sensory inputs in a context-specific manner. Current evidence suggests that this balanced inhibitory and excitatory system is not mature in young animals, as descending modulation arising from the RVM predominantly facilitates low and high threshold inputs to the dorsal horn in the first weeks after birth before descending inhibitory transmission matures (Hathway et al., 2009a; Koch and Fitzgerald, 2013). As such, top down control of spinal sensory processing is hypothesised to serve a different role in uninjured young rats; amplifying low and high threshold inputs under all conditions, rather than providing highly regulated amplification or inhibition of noxious inputs as is the case in adulthood.

The first evidence of immature descending controls arose from experiments where the effects of electrical stimulation of the DLF on C-fibre evoked firing activity of dorsal horn neurons were investigated in rats of different postnatal ages (Fitzgerald and Koltzenburg, 1986). Electrical stimulation of descending axons in the DLF was found to weakly inhibit C-fibre-evoked firing properties of dorsal horn neurons at P12, but not before, and the strength of this descending inhibition increased between P12 and P24 (Fitzgerald and Koltzenburg, 1986). Early experiments demonstrated that descending modulation of pain behaviours from the PAG is also weak at P21, as SPA requires higher current intensity stimulation at P21 than in adult rats, and is absent at P7 and P14 (van Praag and Frenk, 1991).

Evidence of descending facilitation in young rats came later (Hathway et al., 2009a). In adults, low amplitude electrical stimulation of the RVM facilitates, whilst high amplitude stimulation inhibits sensory-evoked hindlimb reflexes and dorsal horn neuron firing properties. In contrast, stimulation of the RVM at any amplitude facilitates sensory-evoked reflexes and dorsal horn neuron firing properties at P3 and P21 (Hathway et al., 2009a). By P25-P30, high amplitude RVM stimulation fails to facilitate hindlimb reflexes, and by P35-40 high amplitude stimulation inhibits hindlimb reflexes and dorsal horn neuron properties, demonstrating that exogenously-evoked descending inhibition from the RVM matures between P25-P35 (Hathway et al.,

2009a) (Fig. 1.8). In adults, electrical stimulation of the RVM preferentially inhibits C-fibre-evoked firing properties of dorsal horn neurons, whilst RVM stimulation at P21 facilitates A-fibre evoked properties of dorsal horn neurons (Koch and Fitzgerald, 2014). Therefore, descending RVM modulation may target A-fibre inputs in young P21 rats to amplify processing of low threshold sensory inputs in the spinal dorsal horn. As primary afferent neurons were electrically stimulated in these experiments, it is unclear if descending modulation in young rats is non-selectively targeted to physiological non-noxious and noxious stimuli, or is targeted to noxious stimuli, as observed in adulthood.

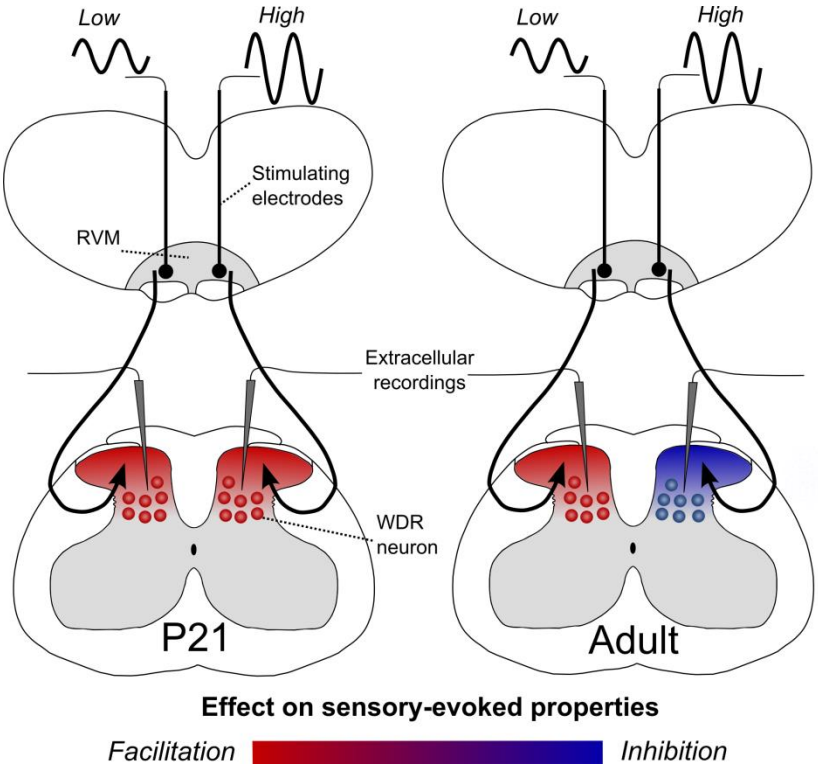

Fig 1.8. Descending RVM control of spinal sensory circuits changes during development. At P21, low of high amplitude electrical stimulation of the RVM facilitates (red) sensory evoked dorsal horn wide dynamic range (WDR) neuron properties and hindlimb reflexes. Between P21 and P40, descending inhibitory transmission matures, such that in adults high amplitude RVM stimulation inhibits (blue) sensory evoked dorsal horn neuron properties and hindlimb reflexes. This schematic diagram is based on data from Hathway et al., (2009).

Like in adults, endogenous opioid activity in the PAG-RVM modulates spinal reflexes in young rats; however, this opioidergic control has different functions in the first postnatal weeks and undergoes considerable postnatal maturation. Microinjection of μ-opioid receptor agonists (DAMGO) into the PAG inhibits hindlimb reflexes in adult

rats, but facilitates such reflexes at P21 and lacks efficacy at P10 (Kwok et al., 2013). Similarly, DAMGO injection into the RVM facilitates hindlimb reflexes at P21 and inhibits reflexes at P40 (Hathway et al., 2012). Importantly, chronic antagonism of μ-opioid receptors between P21-P28 prevented RVM stimulation produced inhibition of spinal reflexes in adult P40 rats, demonstrating that endogenous opioidergic transmission in the RVM is required for the maturation of descending inhibition form the RVM (Hathway et al., 2012).

In vitro patch clamp experiments in brainstem slices have demonstrated that properties of RVM neurons change during postnatal development (Li et al., 2015a). A higher proportion of RVM neurons are spontaneously active in P10-P21 slices compared to neurons in P30+ slices. Additionally, the probability of GABA release is lower in P30+ RVM neurons, and GABA evokes relatively smaller IPSCs than in P10-P21 neurons, thus demonstrating that inhibitory GABAergic transmission is enhanced in the RVM in young rats (Liang et al., 2015). It is currently unclear how enhanced GABAergic transmission in the RVM *in vitro* may mediate dominant facilitation of spinal sensory circuits *in vivo*. One hypothesis is that enhanced GABAergic transmission may cause disinhibition of spinally projecting RVM neurons, thereby exciting sensory networks in the dorsal horn.

Anatomical studies have suggesting that neurons in the brainstem innervate the dorsal horn from birth, as serotonergic terminals are found in the spinal dorsal horn at birth (Rajaofetra et al., 1989). More informative retrograde tracing experiments have demonstrated that there is a significant postnatal growth of serotonergic axons from the RVM into the lumbar spinal dorsal horn between P7-P14 (Tanaka et al., 2006). Whilst these experiments describe descending serotonergic inputs to the dorsal horn, they did not investigate the postnatal maturation of other neurotransmitter-containing spinally projecting RVM neurons. Thus, it is not currently known if neurons in the RVM project to the spinal dorsal horn from birth.

The majority of experiments investigating descending modulation in young rats have electrically or pharmacologically excited the PAG or RVM to exogenously excite descending pathways (van Praag and Frenk, 1991; Hathway et al., 2009a; Koch and Fitzgerald, 2013; Kwok et al., 2013). As such, there is a major gap in our understanding of the endogenous function of these pathways in young rats. Whilst the roles of opioids in descending modulation have begun to be investigated, other neuromodulators involved with descending modulation have not been investigated in young animals, especially the function neurotransmitter-receptor coupling at the level

of the spinal cord. In this thesis I aim to investigate the function of descending serotonergic modulation upon dorsal horn activity in young uninjured rats and test whether this serotonergic modulation changes during development. Downstream from descending serotonergic neurons, I will also investigate the functional role of a 5-HT receptor expressed in the spinal cord, 5-HT_3R, in modulation dorsal horn activity. Additionally, it is currently unknown if descending modulation can be evoked by cutaneous stimulation, thus acting as part of a spinal-bulbo-spinal loop in young rats. Some evidence suggests that pinch stimulation does not evoke descending inhibition until P21 (Boucher et al., 1998a), however it has not been investigated if descending facilitation can be evoked by cutaneous stimulation at younger ages. This thesis aims to further investigate the function of descending modulation of spinal processing of cutaneous tactile and noxious sensory inputs in young and adult rats, with primary focus on uninjured animals.

1.6 Thesis aims

The aims of this thesis were as follows:

1. To map the functional development of the spinal-bulbo-spinal loop by characterising when noxious stimuli active neurons in several brainstem and midbrain nuclei.
2. To investigate the endogenous function of descending RVM modulation over spinal dorsal horn sensory circuits in young and adult rats.
3. To map the anatomical development of descending serotonergic inputs to the dorsal horn.
4. To investigate the endogenous function of descending serotonergic modulation over spinal dorsal horn neurons in young and adult rats.

Chapter 2

Connectivity of ascending and descending sensory pathways during postnatal development

2.1 Introduction

The aim of this chapter is to map the postnatal development of anatomical and functional connectivity between ascending nociceptive pathways from the spinal dorsal horn and target regions in the brainstem and midbrain. In the adult, primary afferent neurons activate dorsal horn neurons including ascending projection neurons in the superficial and deep dorsal horn which have axonal terminals in the parabrachial (PB) nucleus and the periaqueductal grey (PAG) (Ossipov et al., 2010). These same nuclei project to and modulate neurons in nearby brainstem regions, such as the rostroventral medial medulla (RVM), which in turn project down to the spinal dorsal horn and modulate dorsal horn neuron excitability and sensory responses (Heinricher et al., 2009) (Fig 2.1A and B). This proposed 'spinal-bulbo-spinal loop' is not isolated from other parts of the central nervous system; but is modulated, moderated and balanced by inputs from other brain nuclei and cortical regions and thereby underlies the modulation of pain by neural correlates of attention, stress, fear, reward and expectation (Helmstetter and Tershner, 1994; Fairhurst et al., 2007; Bingel and Tracey, 2008; Rea et al., 2014). Despite the importance of this modulatory system, little is known about the postnatal development of the structural and functional connections within the spinal-bulbo-spinal loop.

2.2 Evidence for the spinal-bulbo-spinal loop in adult rodents

2.2.1 Ascending projections from the dorsal horn

Projection neurons in the spinal dorsal horn have cell bodies in lamina I and laminae III-IV, and send axonal projections to target various nuclei in the brain, including the PB nucleus, PAG, caudal ventrolateral medulla and the thalamus. The majority of ascending projections from the dorsal horn arise from lamina I; these neurons constitute roughly 5% of neurons in lamina I of the rat lumbar spinal cord, of which 95% project to the PB, 30% to the PAG, 25% to the NTS and 5% project to the thalamus (Spike et al., 2003). This chapter will focus on the PB nucleus and the PAG as important nuclei in the ascending arm of the spinal-bulbo-spinal loop.

PB Nucleus

The PB nucleus is a major target of spinal dorsal horn neuron axonal projections. Tracing experiments in adult rodents have demonstrated the densest output from the superficial spinal dorsal horn neurons is to the contralateral PBel nucleus (Slugg and Light, 1994; Craig, 1995; Feil and Herbert, 1995a), and from deep dorsal horn neurons to the PBil nucleus (Hylden et al., 1986; Bernard et al., 1995). Functionally, some PB

neurons recorded in the uninjured cat display wide-dynamic range properties (Hylden et al., 1985), however the majority of PB neurons in uninjured adult mammals selectively respond to noxious inputs (Lantéri-Minet et al., 1993, 1994; Bellavance and Beitz, 1996; Hermanson and Blomqvist, 1996; Pinto et al., 2003). Similarly, Fos expression in PB neurons can be evoked following peripheral noxious thermal (Bester et al., 1997) or formalin injection (Pertovaara et al., 1993). Outputs from the PB nucleus include the RVM, hypothalamic nuclei, the amygdala, and various thalamic nuclei involved in nociception (Bernard et al., 1993; Alden et al., 1994; Bester et al., 1999), and are thought to be a major contributor to emotional, autonomic and neuroendocrine features of pain experience (Gauriau and Bernard, 2002b).

PAG

The PAG is a structure which has important roles in modulating behavioural responses to painful, threatening or stressful stimuli. The PAG receives inputs from a large number of areas in the central nervous system, including the spinal dorsal horn. Tracing experiments in the adult rat, cat and monkey have shown that dorsal horn projection neurons terminate primarily in the contralateral ventrolateral and lateral segments of the PAG (Yezierski, 1988; Keay et al., 1997). Fos expression in the PAG can be evoked following cutaneous or deep tissue noxious stimulation in adult rodents (Keay and Bandler, 1993) or by social stressors such as exposure to predators (Canteras and Goto, 1999). Early experiments investigating the function of the PAG demonstrated that electrical stimulation of the PAG evokes inhibition of pain behaviours in rodents (Reynolds, 1969) and that this so called 'stimulation produced analgesia' (SPA) requires a functional RVM, as silencing RVM neurons (by locally injecting a local anaesthetic) prevented SPA evoked by stimulating the PAG (Aimone and Gebhart, 1986). Subsequent use of anterograde and retrograde anatomical tracing techniques identified extensive axonal projections from the PAG to the RVM which contain a range of neurotransmitters, including GABA, BDNF, somatostatin and neurotensin (Beitz, 1982c; Beitz et al., 1983; Van Bockstaele et al., 1991; Morgan et al., 2008b; Yin et al., 2014b). Other experiments have demonstrated that descending modulation arising from the PAG is not exclusive inhibitory, as glutamatergic stimulation of the PAG can inhibit or facilitate dorsal horn firing activity evoked by cutaneous noxious mechanical stimulation (Waters and Lumb, 2008). The PAG is therefore a key nucleus in the spinal-bulbo-spinal loop which integrates sensory and affective inputs to drive downstream descending modulation of dorsal horn sensory circuits via the RVM (Ossipov et al., 2010).

2.2.2 Descending projections from the brain

Many regions of the brain have been implicated in descending modulation of pain behaviours and spinal sensory circuits. Important brainstem regions such as the RVM and the locus coeruleus (LC) send axonal projections to the spinal dorsal horn to modulate sensory inputs. The dorsal raphe nucleus (DRN) also integrates sensory and affective inputs to modulate somatosensory ascending and descending nuclei, including the RVM (Wang and Nakai, 1994). This chapter will focus on the RVM and DRN as important nuclei in the descending arm of the spinal-bulbo-spinal loop.

RVM

Projections to spinal dorsal horn form synapses with primary afferent neuron terminals and dorsal horn neurons to modulate incoming sensory inputs (Heinricher et al., 2009). Inputs to the RVM originate from regions of the CNS known to (non-selectively) receive or modulate nociceptive information. The majority of inputs originate from the PAG, with additional inputs from the PB nucleus and the DRN (Beitz, 1982c; Hermann et al., 1997; Braz et al., 2009). Axonal projections from the RVM terminate throughout the spinal dorsal horn, with the highest density in laminae I-II and IV-V (Antal et al., 1996), and form functional synapses with dorsal horn neurons and primary afferent terminals to excite or inhibit nociceptive inputs to the dorsal horn (Kato et al., 2006; Kim et al., 2014a).

The responsivity of RVM neurons to peripheral noxious stimulation is well established. Electrophysiologically defined 'On' and 'Off' cells in the RVM change firing properties following peripheral noxious stimulation; respectively increasing and decreasing firing rates before subsequent nocifensive reflex withdrawal (Fields et al., 1983; Heinricher et al., 2009). Additionally, immunohistochemistry experiments have demonstrated that hindpaw inflammation or noxious stimuli increase Fos expression and pERK levels in the RVM (Imbe et al., 2005, 2008; Géranton et al., 2008; Gau et al., 2009). Thus, noxious-evoked activity in the RVM is hypothesised to be an important driver of descending modulation of somatosensation in the spinal cord and pain behaviours.

DRN

The DRN is a nucleus in the midbrain consisting largely of 5-HT containing neurons. It is known to be involved in physiological functions such as regulation of body temperature; cardiovascular function; sleep and motor behaviour; and, importantly, pain modulation (Wang and Nakai, 1994). In the adult, subcutaneous or visceral formalin injection increases Fos expression in both serotonergic and non-serotonergic neurons in the DRN (Chen et al., 2003). The DRN receives inputs from many regions

of the brain which are involved in nociceptive and pain circuitry including the PB nucleus, RVM and parafascicular nucleus of the thalamus (Sakai et al., 1977; Sim and Joseph, 1989; Braz et al., 2009). Major projection sites of the DRN include the PAG, RVM and the parafascicular nucleus of the thalamus (Andersen and Dafny, 1983a; Beitz et al., 1986; Reichling and Basbaum, 1991; Braz et al., 2009).

Early studies showed that electrical stimulation of the DRN causes behavioural analgesia and inhibition of spinal dorsal horn neuron activity, without motor or autonomic side effects (Oliveras et al., 1974, 1979). Additionally, lesion of the RVM partially reverses the antinociceptive effect of DRN stimulation (Yu et al., 1988); leading to the hypothesis that the descending antinociceptive role of the DRN is largely mediated via extensive connectivity to the RVM (Wang and Nakai, 1994). Electrical stimulation of the DRN is also known to inhibit noxious stimulus-evoked firing activity of neurons in the parafascicular nucleus of the thalamus (Andersen and Dafny, 1983a, 1983b). Thus, the DRN is situated in a key junction to target ascending nociceptive inputs directly via the thalamus and the spinal dorsal horn, both directly and via descending modulatory nuclei such as the RVM (Wang and Nakai, 1994).

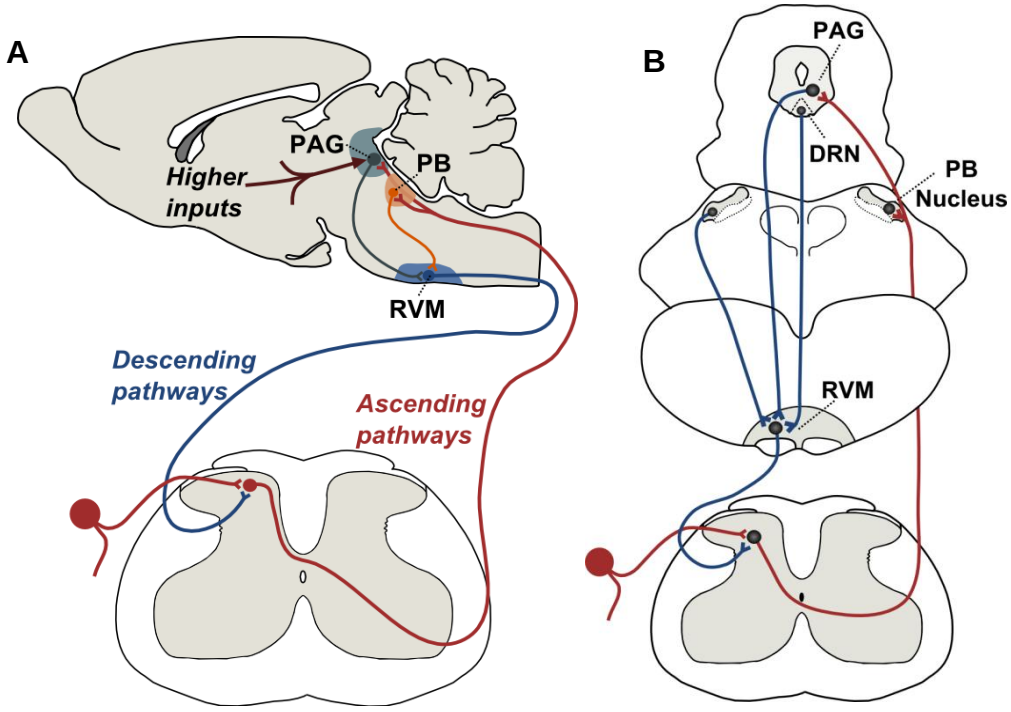

Fig 2.1 (previous page). Ascending and descending nociceptive connections in the 'spinal-bulbo-spinal loop'.
Ascending sensory projections from the spinal dorsal horn terminate primarily in the parabrachial (PB) nucleus and the periaqueductal grey (PAG). Descending projections from the brain to the spinal dorsal horn originate primarily in the rostroventral medial medulla (RVM). Sensory inputs to the RVM originate from the PAG and PB, and autonomic and affective inputs primarily, but not exclusively, originate from higher inputs to the PAG (A). The spinal-bulbo-spinal loop is a sensory driven and sensory modulating feedback circuit (B). Key nuclei in the brain include the PB nucleus, PAG and the RVM. The dorsal raphe nucleus (DRN) also plays an important role in descending modulation of spinal circuitry.

2.2.3 DNIC: functional evidence of a spinal-bulbo-spinal loop

Diffuse noxious inhibitory control (DNIC) is a phenomenon whereby application of strong noxious stimuli to one part of the body inhibits pain in multiple remote body regions. DNIC was first identified in anaesthetised rats, where noxious stimulation applied to heterotopic body sites inhibited noxious-evoked firing properties of dorsal horn neurons with receptive fields on the hindpaw (Le Bars et al., 1979). DNIC relies on functional ascending projections from the spinal dorsal horn, as substance P-saporin ablation of dorsal horn NK1-ir neurons (including projection neurons) prevented reduced Fos expression in the dorsal horn caused by dual forepaw and hindpaw noxious stimulation observed in control animals (Suzuki et al., 2002). Descending brainstem regions such as the subnucleus reticularis dorsalis (SRD) are also required for DNIC, however the role of the PAG-RVM in DNIC is disputed, as lesions of these regions had no effect on DNIC (Villanueva and Le Bars, 1995). More recent evidence has identified a key role of spinal 5-HT_3Rs in supressing DNIC, providing indirect evidence for the role of descending serotonergic RVM neurons in DNIC (Bannister et al., 2015). Similarly, inactivation of the RVM can restore DNIC in chronic morphine-treated rats (Okada-Ogawa et al., 2009). The presence of DNICs is therefore mediated by a functional spinal-bulbo-spinal loop, whereby heterotopic noxious inputs drive ascending recruitment of descending pain modulatory nuclei which causes inhibition of nociceptive inputs at the level of the dorsal horn.

2.3 Evidence for the spinal-bulbo-spinal loop in young rodents

2.3.1 Ascending projections from the dorsal horn

In the first postnatal week, neurons in the spinal dorsal horn and the somatosensory cortex are activated by peripheral sensory stimuli, demonstrating functional connectivity between the spinal cord and the somatosensory cortex from birth (Chang and Fitzgerald, unpublished findings). It is not currently known how sensory

information is encoded along this multi-synaptic pathway in young rats and whether processing of somatosensory information in brain regions differs in young and adult rats. Moreover, there is only limited information about the anatomical connectivity of these ascending pathways

Retrograde tracing experiments in the rat have demonstrated that projection neurons in the superficial lumbar dorsal horn project to the brainstem PB nucleus and midbrain PAG from birth (Li and Baccei, 2012), however these experiments did not investigate changes in dorsal horn neuron projections at different postnatal ages. Functional recruitment of neurons in the PB nucleus has been reported at P3 and P14, as demonstrated by an increase in Fos expression in the PB nucleus following hindpaw formalin injection (Barr, 2011; Man et al., 2012). Conversely, hindpaw formalin injection evoked Fos expression in the PAG and the midbrain or paraventricular thalamic nuclei was not observed until P14 (Barr, 2011;). These studies suggest that ascending sensory inputs from the dorsal horn activate neurons in the PB nucleus from P3, but ascending inputs do not activate neurons in the PAG until P14, despite the presence of anatomical connectivity between the dorsal horn and the PAG at birth. However, these experiments do not provide clear age-specific information about the ascending nociceptive recruitment of many parts of the brainstem and midbrain involved in processing sensory information such as the RVM and DRN.

2.3.2 Descending projections from the brain

Anatomical connections from brainstem regions to the spinal cord have been characterised in young animals. Using rabies virus-based transneuronal tracing techniques, a recent study has demonstrated that a large population of neurons in the P8 mouse RVM project to the spinal dorsal horn and form synapses with primary afferent terminals (Zhang et al., 2015). These descending RVM neurons were either GABAergic/glycinergic (80%) or serotonergic (17%), but a large population of GABAergic neurons also contained proenkephalin. In other retrograde tracing experiments Tanaka et al. (2006) injected the retrograde tracing agent cholera toxin B into the lumbar dorsal horn and quantified the proportion of serotonergic neurons in the RVM which project to the dorsal horn. The proportion of serotonergic RVM neurons that project to the lumbar dorsal horn increased from 6% at P7 to 29% at P14, demonstrating substantial growth of axonal projections to the dorsal horn between P7 and P14 (Tanaka et al., 2006). Whilst this postnatal growth of RVM neuron projections to the lumbar dorsal horn has been observed in the serotonergic modulatory system, it

is not known whether other neurotransmitter-containing RVM neurons follow the same developmental trajectory. Similarly, it is not known whether the descending modulatory sites in the brainstem, such as the RVM, project to the spinal dorsal horn from birth.

The functional development of descending sensory modulatory pathways has been investigated by previous researchers, but most studies have focussed on postnatal ages from the third postnatal week. Stimulation of the RVM evokes descending facilitation of dorsal horn circuitry at P21; exciting peripheral noxious stimulus-evoked dorsal horn neuron firing activity and hindlimb reflexes (Hathway et al., 2009a, 2012; Koch and Fitzgerald, 2014). Descending inhibition of nocifensive behaviours arising from the PAG (evoked by electrical stimulation of the PAG) is absent before and weak at P21 (van Praag and Frenk, 1991), and PAG μ-opioid receptor-mediated descending modulation of spinal reflex excitability has been found to be absent at P10, but present at P21 (Kwok et al., 2013). Of note, an important piece of evidence has suggested descending modulation arising from the RVM as early as P3; as ablating RVM neurons by focally injecting kainate into the RVM unmasks increases nocifensive behavioural thresholds (Hathway et al., 2009a), suggesting functional descending facilitation of sensory-motor thresholds at this age. Thus, evidence for functional and anatomical descending modulatory pathways from the brainstem to the spinal dorsal horn in the first postnatal week is limited but present.

A key aim of this chapter is to identify when noxious sensory inputs activate neurons in brainstem and midbrain regions involved in the ascending and descending arms of the spinal-bulbo-spinal loop. Current functional and anatomical evidence suggests that sensory inputs activate part of the ascending arm of the loop (the dorsal horn and PB nucleus, but not the PAG and thalamic nuclei) at birth (Barr, 2011; Li and Baccei, 2012; Man et al., 2012). Connections from the RVM to the spinal dorsal horn have been identified at the end of the first postnatal week (Tanaka et al., 2006) and descending modulation of sensory inputs has been observed at P3 (Hathway et al., 2009a). It is currently not known whether sensory inputs activate brainstem and midbrain regions involved in the descending arm of the loop, such as the RVM and DRN. Interestingly, DNIC, as observed by distal pinch stimulation-induced reduction in Fos expression evoked by heterotopic formalin injection, is absent at P12 but present at P21 (Boucher et al., 1998b), suggesting an absence of sensory-evoked descending inhibition before P21. Thus, whilst some current evidence suggests functional

ascending and descending arms of the spinal-bulbo-spinal loop from P3, it is less clear whether ascending projections drive descending pain modulation before P21.

2.4 Experimental aims

The primary aim of this chapter is to characterise when peripheral noxious stimulation activates Fos in several brainstem and midbrain regions involved in the spinal-bulbo-spinal loop during postnatal development. The key hypotheses are:

1. Ascending pathway nuclei: peripheral noxious stimulation increases Fos expression in the PB nucleus in the first postnatal week, but Fos expression in the PAG will not be observed until the end of the second postnatal week.
2. Descending pathway nuclei: peripheral noxious stimulation increases Fos expression in the RVM and DRN later than the ascending arm, at the end of the second postnatal week.

2.5 Methods

2.5.1 Animals

All experiments were performed in accordance with the United Kingdom Animal (Scientific Procedures) Act 1986. Reporting is based on the ARRIVE Guidelines for Reporting Animal Research developed by the National Centre for Replacement, Refinement and Reduction of Animals in Research, London, United Kingdom (Kilkenny et al., 2010). Male and female Sprague-Dawley rats at postnatal day (P) 4, 6 8, 12, 21, 26 and 40 were obtained from the Biological Services Unit, University College London. Rats aged <P40 are considered adult in this thesis (McCutcheon and Marinelli, 2009). Rats were bred and maintained in-house and exposed to the same caging, diet and handling throughout development. Litters were weaned at P21 into same sex cages of four littermates and were housed in 12h light/dark cycles at constant ambient temperature and humidity with free access to water and food.

2.5.2 Noxious mechanical stimulation and immunohistochemistry

Animals at P4, P8, P12, P21 and P40 (n=4 per age) were anaesthetised with isoflurane and maintained at a low level of anaesthesia sufficient to cause areflexia (1.8-2%). Hindpaw pinch stimulation was used as a mechanical noxious stimulus, and as such 'pinch stimulation' will be used as shorthand for 'mechanical noxious stimulation' in this chapter. Pinch stimulation was applied with a pair of F.S.T curved, serrated and blunt forceps (produce code 11152-10; tip 0.3mm) for 5 seconds on 6 points on the dorsal surface, followed by 6 points on the ventral surface of the left hindpaw over the course of 2 minutes. Control animals (n=4 per age) received anaesthesia for the same time period but no stimuli were applied. Rats were transcardially perfused 2 hours after pinch stimulation.

For perfusions, rats were re-anaesthetised with pentobarbitone sodium (500mg/kg) and perfused transcardially with heparinised saline (5000 IU/ml) followed by 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer. The brain was removed and postfixed overnight in 4% PFA and transferred to a 30% sucrose solution in 0.1 PBS containing 0.01% azide and stored at 4°C. Brain and spinal cord tissue was sectioned on a freezing microtome at 40µm and 30µm thicknesses.

Brainstem sections from pinched and naïve animals were double labelled for c-fos (rabbit, 1:20,000, Merck Millipore) and NeuN (to label neuronal nuclei; mouse, 1:500, Chemicon). Free floating sections were blocked with 3% goat serum in 0.3% Triton X-100 in 0.1M PBS for 1h at room temperature (RT). Sections were then incubated

overnight at room temperature with primary antibodies. The next day, sections were incubated in biotinylated anti-rabbit antibody (goat anti-rabbit; 1:400; Vector Stain) for 90mins. Sections were placed in ABC complex (1:125; Vector Stain, ABC elite kit, Vector Labs) for 30mins, followed by biotinylated tyramide (1:75; TSA Stain Kit; Perkin Elmer) for 7mins. Sections were then incubated in fluorescence Isothiocyanate (FITC; 1:600; Vector Stain) for 2 hours. For double labelling with NeuN, sections were incubated in mouse anti-NeuN (1:500; Chemicon) overnight before sections were incubated for 2 hours with goat anti-mouse Alexafluor 594 (1:250; Invitrogen).

All sections were mounted on gelatinised slides and were then cover slipped with Fluoromount (Sigma). Negative control stains omitting primary antibodies resulted in absence of positive immunofluorescence. Sections were viewed using a Leica DMR light microscope, photographed using a Hamamatsu C4742-95 digital camera and analysed with Volocity Software 6.3.

2.5.3 Immunoreactivity cell counting and fluorescence intensity measurements

NeuN and Fos-immunoreactive (Fos-ir) neurons were examined in the ventrolateral PAG (vlPAG), the parabrachial (PB) nucleus, the dorsal raphe nucleus (DRN) and the rostroventral medulla (RVM) (Fig 2.1B). Coordinates of these brain regions (in relation to Bregma) are based on the adult rat. Because the brain increases in volume during postnatal development, the same coordinates cannot be used in young rats; instead, key landmarks, described where possible, were used to identify brain regions of interest. In the PB area between Bregma -7.64mm and -8.30mm, the spinocerebellar tract, the inferior colliculus and the brachium conjunctivum were used as landmarks and rostro-caudal distribution of sections were determined with respect to the reference plane where the inferior colliculus merges with the pons. NeuN and Fos-ir neurons were counted in the contralateral mesencephalic PBel, PBsl and PBil and the pontine PBdl, PBel and PBil (Fig 2.2F); areas which are known to receive ascending input from the spinal dorsal horn in adult rat studies (Slugg and Light, 1994; Craig, 1995; Feil and Herbert, 1995b). In the PAG between Bregma -7.64mm and -8.72mm, NeuN and Fos-ir neurons were counted in the contralateral ventrolateral region (Fig 2.2E) which receives ascending input from the spinal dorsal horn (Keay et al., 1997) and includes neurons that directly project to the RVM (Yin et al., 2014a) in adult rats. NeuN and Fos-ir cells were counted In the DRN between Bregma -7.64mm and -8.30mm (Fig 2.2E). In the RVM between Bregma -11.60mm and -9.50mm, NeuN-ir cells were counted bilaterally in the nucleus raphe magnus (RMg), lateral paragigantocellular

nucleus (LPGi) and gigantocellular reticular nucleus alpha (GiA) (Fig 2.2D). NeuN and Fos-ir cells were not counted in the raphe pallidus due to its limited role in modulation of nociceptive circuitry. NeuN counts in the RVM were performed separately in the RMg, LPGi and GiA due to observations of different neuronal densities in the three regions, whereas Fos-ir counts were performed in the total RVM. Sections with the highest number of Fos-ir cells in each region were chosen and counted. NeuN or Fos-ir counts were performed in 5 sections per animal to create a mean for each animal. Sections were chosen based on anatomical landmarks and on sections with the highest Fos expression in the areas of interest.

2.5.4 Statistical analysis

Statistical analyses and graphing were performed using GraphPad Prism 6 (GraphPad software, La Jolla, CA, USA) and $P<0.05$ was considered statistically significant. Sample sizes (5 sections per animal, 4 animals per group) for testing were based on previously reported group differences in immunohistochemistry experiments (Bester et al., 2000b; Barr, 2011). All data sets were normally distributed therefore parametric statistical tests were used. Data are represented as means ± standard error of mean (SEM).

The mean pinch-induced Fos-ir and mean control Fos-ir in each brainstem region were compared at each age using unpaired student's t-test and two-way ANOVA followed by Bonferroni *post hoc* multiple comparisons test.

2.6 Results

2.6.1 Brainstem and midbrain nuclei neuronal density changes during development

Neuronal density was measured in the RVM, PB nucleus in the brainstem (Fig 2.2D and F), and the midbrain PAG and DRN (Fig 2.2E) at four different ages: P4, P12, P21 and P40. Neuronal cell bodies were visualised with NeuN and counted in a 200µm x 200µm x 40µm cuboid region of interest (ROI) in each brainstem and midbrain region. NeuN counts were performed in 4 sections per animal and a mean value was created for each animal, such that 1 n = 1 animal. NeuN counts were performed in 4 animals per age.

Quantification and analysis of variance (ANOVA) of NeuN counts in the PB nucleus demonstrated that age was a significant actor (One-way ANOVA, age comparison, $F(3,12)=7.74$, $P=0.004$), and Bonferroni post hoc analysis demonstrated that NeuN-ir counts were significantly higher at P4 compared to P12 (One-way ANOVA, age comparison, with Bonferroni post-hoc analysis, $P<0.01$; Fig 2.2A).

In the vlPAG, age was a significant factor (One-way ANOVA, age comparison, $F(3,12)=20.06$, $P=0.0001$), and Bonferroni post hoc analysis demonstrated that NeuN-ir counts were significantly higher at P4 compared to P12 (One-way ANOVA, age comparison, with Bonferroni post-hoc analysis, P4 vs. P12 $P<0.01$; Fig 2.2B). NeuN-ir counts also changed with age in the DRN and were significantly higher at P4 compared to P12 (One-way ANOVA, age comparison, $F(3,12)=12.48$, $P=0.0005$; Bonferroni post hoc analysis, P4 vs P12 $P<0.05$; Fig 2.2B).

NeuN-ir counts in ROIs in different regions of the RVM were not uniform, so counts were performed separately in the LPGi, GiA and RMg. Examples of ROIs in the different regions of the RVM are shown in Figure 2.2D. Analysis of variance demonstrated that age had a significant effect on NeuN-ir counts across the RVM (Two-way ANOVA, age comparison, $F(3,36)=129.0$, $P<0.0001$). In the LPGi, the RMg, and in the GiA, NeuN-ir counts were significantly higher at P4 compared to P12, and at P21 compared to P40 (Two-way ANOVA with Bonferroni post-hoc analysis, age comparison, $P<0.01$ to 0.001 at different age; Fig 2.2C).

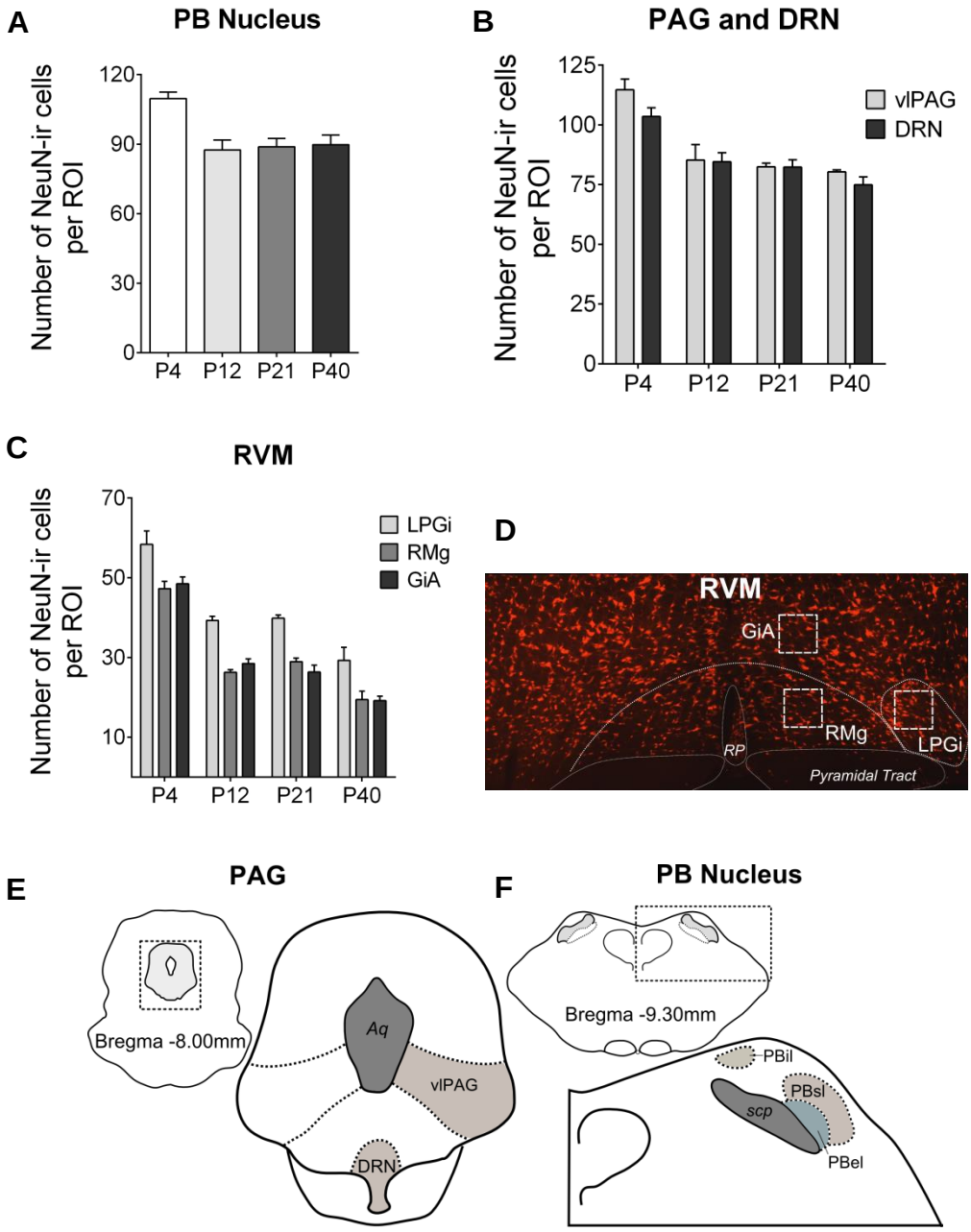

Fig 2.2. Neuronal density in brainstem and midbrain nuclei decreases with age.
NeuN-ir neurons were counted in a 200µm x 200µm x 40µm cuboid in the Parabrachial (PB) nucleus (A), periaqueductal grey (PAG; B), dorsal raphe nucleus (DRN; B) and the rostroventral medial medulla (RVM; C). The number of NeuN-ir cells in a region of interest (ROI) in the PB nucleus (A), PAG (B) and DRN (B) decreased between P4 and P12 (One-way ANOVA with Bonferroni post hoc analysis). NeuN counts were performed in three ROIs in the RVM: the lateral paragigantocellular nucleus (LPGi), raphe magnus (RMg) and the gigantocellular reticular nucleus alpha (GiA) (D) as neuronal density differs in these three regions. NeuN-ir counts were higher in each of the three regions at P4 compared to P12 and at P21 compared to P40 (C) (Two-way ANOVA with Bonferroni post hoc analysis). NeuN counts were performed in the ventrolateral (vl) PAG (E), the entirety of the DRN (E), and the entirety of the PB nucleus which includes the external lateral (PBel), superior lateral (PBsl) and internal lateral (PBil) regions of the PB nucleus, adjacent to the superior cerebellar peduncle (scp) (F).

2.6.2 Hindpaw pinch stimulation increases Fos expression in the PB nucleus from P4

Fos expression was mapped in the PB nucleus in naïve rats (n=4 per age) and in rats that received hindpaw pinch stimulation (n=4 per age) at postnatal ages P4, P8, P12, P21 and P40. Fos counts were performed in 4-5 sections per animal and a mean value was created for each animal, such that 1 n = 1 animal. Fos counts were performed in 4 animals per age. Because the density of neuronal cell bodies in the PB nucleus was found to decrease between P4 and P12, all statistical comparisons were performed within age; comparing Fos-ir counts in naïve vs pinch animals. A Two-way ANOVA with Bonferroni post-hoc test was used to compare naïve vs pinch Fos-ir counts at P12, P21 and P40 as the density of neurons in the PB did not change after P12. Unpaired student's t-tests were used to compare naïve vs pinch Fos-ir counts at P4 and P8.

Two-way ANOVA demonstrated that pinch stimulation significantly increased Fos counts in the PB nucleus compared to control at P12, P21 and in adult P40 rats, and that age was a significant factor (two-way ANOVA, pinch vs nave; $F(4,30)=35.32$, $P<0.0001$, with Bonferroni *post hoc* comparison $P<0.05$ to 0.001 at different ages; Fig 2.3E). At P4 and P8, unpaired Student's t-tests demonstrated significantly increased Fos-ir counts after pinch compared to naïve at these ages (unpaired Student's t-test, pinch vs naïve, $P<0.05$ and $P<0.01$ at P21 and P40; Fig 2.3E). Figure 2.3B shows the extent of Fos expression in the PB nucleus of adult P40 pinch rats and Figure 2.3B shows the distinct absence of Fos expression in P40 naïve rats. Similarly, Fos expression was observed in pinched P4 rats, and a smattering of Fos expression was seen in naïve P4 rats (Fig 2.3C and D). Hindpaw pinch stimulation increased the number Fos-ir cells in the PB nucleus to a similar and low amount at P4, P8 and P12 compared to P21 and P40 (Fig 2.3E).

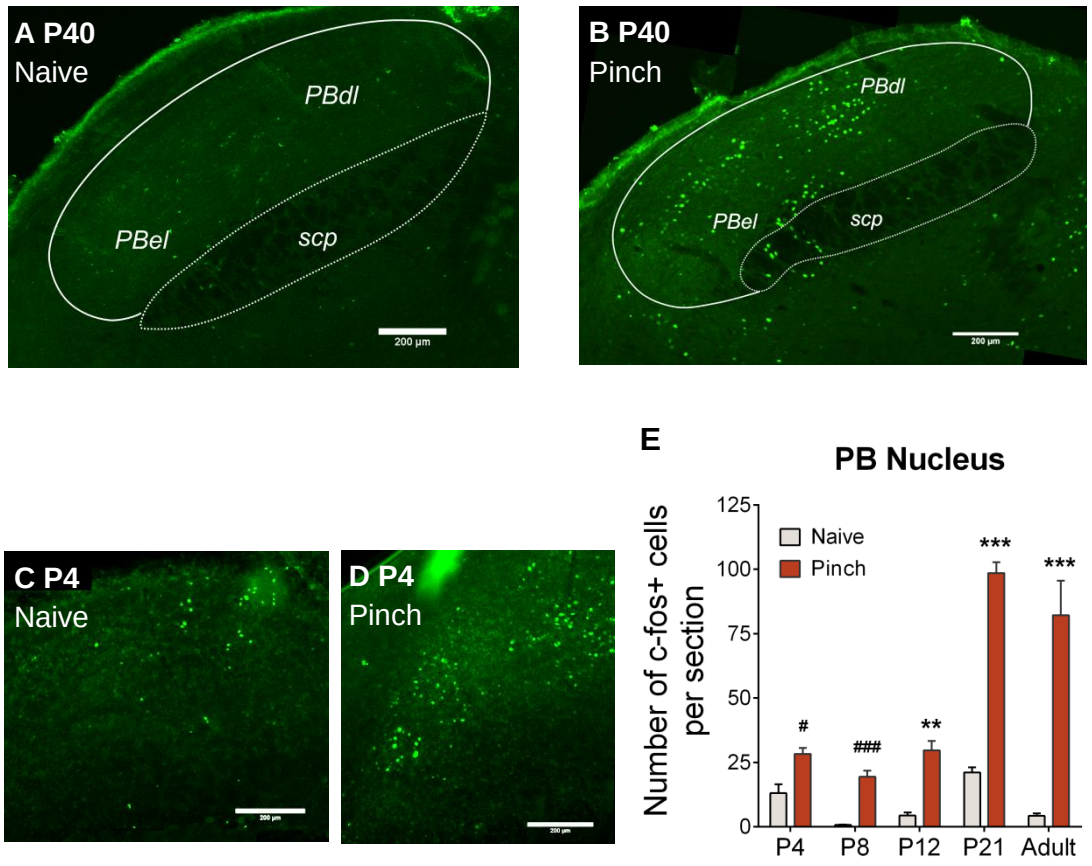

Fig 2.3. Pinch-evoked Fos expression in the Parabrachial nucleus is observed from P4.
Rats of several postnatal ages received hindpaw pinch stimulation under light anaesthesia (n=4 per age; control n=4 per age). Brainstem sections were then stained for c-fos. The number of c-fos-ir cells was low in the parabrachial (PB) nucleus in naïve adult P40 animals (A). In contrast, hindpaw pinch stimulation increased c-fos-ir cell counts in the entirety of the PB nucleus, including the external lateral PB (PBel) and dorsolateral PB (PBdl), adjacent to the superior cerebellar peduncle (scp) (B). A small number of c-fos-ir cells were observed in the PB nucleus of naïve P4 rats (C), and hindpaw pinch stimulation at this age further increased c-fos-ir cell counts (D). C-fos-ir cell counts in naïve and pinch animals was quantified in P4, P8, P12, P21 and adult P40 rats (E). Pinch stimulation increased c-fos-ir cell counts in all ages when compared to age-matched naïve animals. **,*** P<0.01 and 0.001 respectively; two-way ANOVA with Bonferroni post hoc analysis. #,### P<0.05 and 0.001 respectively; unpaired students t-test.

2.6.3 Hindpaw pinch stimulation increases Fos expression in the PAG from P12

Fos-ir counts were performed in the contralateral vlPAG in naive and pinched animals at the P4, P8, P12, P21 and P40 (n=4 pinch animals per age and n=4 naïve animals per age). Fos counts were performed in 4-5 sections per animal and a mean value was created for each animal, such that 1 *n* = 1 animal. Fos counts were performed in 4 animals per age. A Two-way ANOVA with Bonferroni post-hoc test was used to compare naïve vs pinch Fos-ir counts at P12, P21 and P40 as the density of neurons in the PAG did not change after P12. Unpaired student's t-tests were used to compare naïve vs pinch Fos-ir counts at P4 and P8.

At P4 and P8, Fos expression was low in the PAG, including the ventrolateral segment, in naïve and pinch animals (Figs. 2.4A, D and G). Fos expression was observed in the PAG from P12, but was mainly restricted to the contralateral vlPAG (Figs. 2.4B, E and H). Fos expression in pinched P21 rats was generally high throughout the extent of the PAG and the midbrain (Figs. 2.4C, F and I), whilst Fos expression at P12 and P40 was predominantly in the contralateral vlPAG in pinched rats, as observed at P12 and P40. Two-way ANOVA demonstrated a significant increase in Fos expression in pinch animals compared to naïve at P12, P21 and P40, and that age was a significant factor (two-way ANOVA, pinch vs nave; $F(4,30)=147.4$, $P<0.0001$, with Bonferroni *post hoc* comparison, $P<0.01$ to 0.001 at P12, P21 and P40; Fig 2.4L). At P4 and P8, unpaired Student's t-tests demonstrated no significant difference in Fos-ir counts after pinch compared to naïve at these ages (unpaired Student's t-test, pinch vs naïve, $P>0.05$ at P4 and P8; Fig 2.4L).

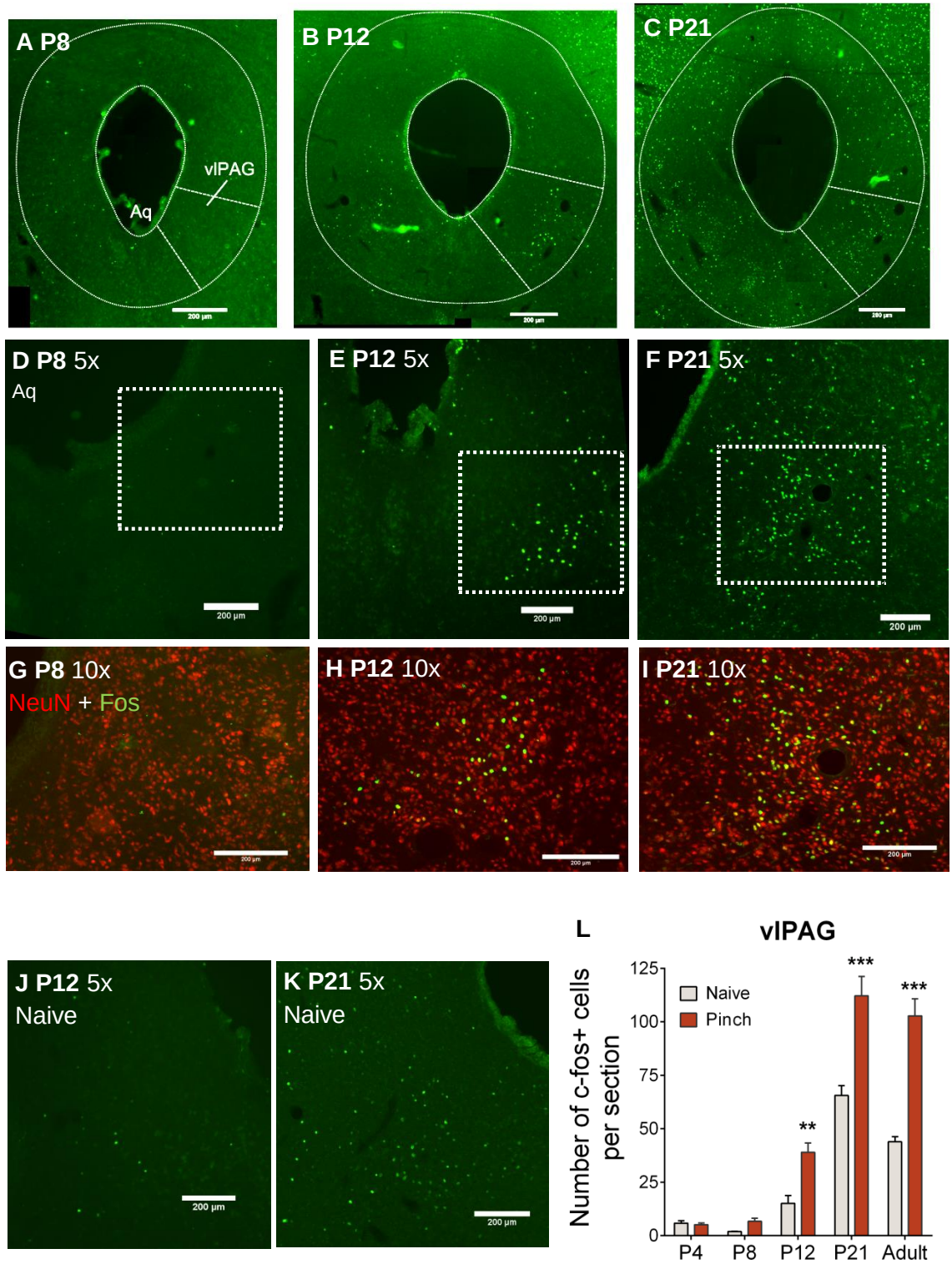

Fig 2.4. Pinch-evoked Fos activation in the PAG is observed from P12, but not earlier.
Fos counts were performed in the ventrolateral (vl) periaqueductal grey (PAG) in naïve and hindpaw pinched animals. Little, if any, Fos activation was observed in pinched P8 rats (A, D, G). A small amount of Fos activation was observed selectively in the vlPAG in pinched P12 rats (B, E, H). In contrast, substantial Fos activation was observed throughout the PAG of P21 pinched animals (C, F, I). Fos activation in naive control P12 and P21 animals are shown in J and K. Quantification of Fos activation in the PAG of naïve and pinch rats demonstrated that pinch stimulation significantly increased Fos activation compared to naïve at P12, P21 and in adult P40 rats (L).

2.6.4 Hindpaw pinch does not increase Fos expression in the DRN until P40

Fos-ir counts were performed in the DRN at postnatal ages P4, P8, P12, P21 and P40 in naïve (n=4 per age) and pinched (n=4 per age) rats. Fos counts were performed in 4-5 sections per animal and a mean value was created for each animal, such that 1 n = 1 animal. Fos counts were performed in 4 animals per age. A Two-way ANOVA with Bonferroni post-hoc test was used to compare naïve vs pinch Fos-ir counts at P12, P21 and P40 as the density of neurons in the DRN did not change after P12. Unpaired student's t-tests were used to compare naïve vs pinch Fos-ir counts at P4 and P8.

Very little Fos expression was observed in the DRN in both pinch and naive animals at P4, P8, P12 and P21 (Fig 2.5A). Fos expression in the DRN was high in pinched P40 rats (Fig 2.5B), and two-way ANOVA demonstrated that Fos expression in the DRN was significantly higher in pinched rats compared to naïve rats only at P40, and that age was a significant factor (two-way ANOVA, pinch vs nave; $F(4,30)=143.8$, $P<0.0001$, with Bonferroni *post hoc* comparison, $P<0.001$ at P40; Fig 2.5C). Similarly, unpaired Student's t-tests demonstrated no significant difference in Fos-ir counts after pinch compared to naïve at P4 and P8 (unpaired Student's t-test, pinch vs naïve, $P>0.05$ at P4 and P8; Fig 2.5C.

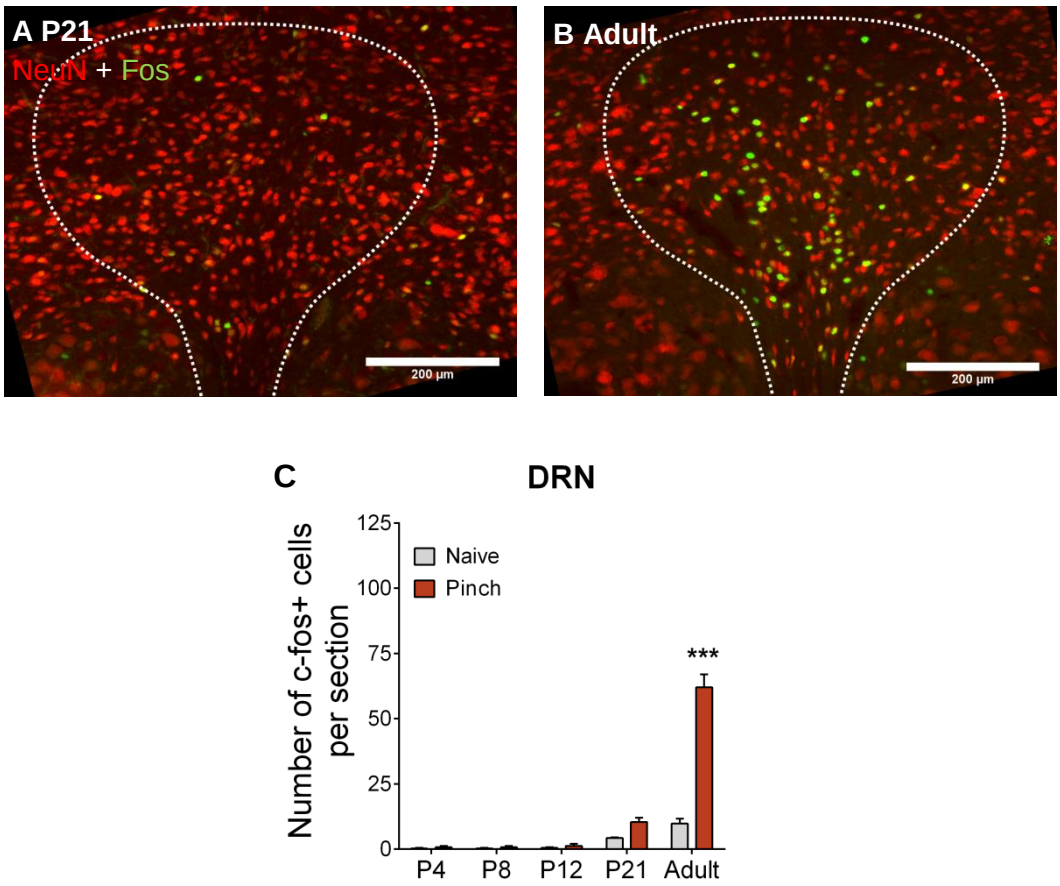

Fig 2.5. Pinch-evoked Fos expression in the DRN is absent until adulthood.
C-fos-ir counts were performed in the dorsal raphe nucleus (DRN) of naïve (n=4 per age) and hindpaw pinched rats (n=4 per age) of different postnatal ages; green = c-fos-ir cells and red = NeuN-ir cells. All images are from pinched rats. C-Fos expression was low, if present at all, at P21 (A) and in younger ages (C). Hindpaw pinch stimulation increased the number of c-fos-ir cells in adult P40 rats (B) compared to age matched naïve rats (C). Two-way ANOVA with Bonferroni post hoc analysis; *** $P<0.001$.

2.6.5 Hindpaw pinch stimulation increases Fos expression in the RVM from P12

Fos-ir counts were next performed in the RVM in naïve (n=4 per age) and pinched animals (n=4 per age) at postnatal ages P4, P8, P12, P21 and P40. Fos counts were performed in 4-5 sections per animal and a mean value was created for each animal, such that 1 n = 1 animal. Fos counts were performed in 4 animals per age. A Two-way ANOVA with Bonferroni post-hoc test was used to compare naïve vs pinch Fos-ir counts at P12, P21 and P40 as the density of neurons in the RVM did not change after P12. Unpaired student's t-tests were used to compare naïve vs pinch Fos-ir counts at P4 and P8.

Fos-ir cells were observed in high numbers in the RVM in both naïve and pinch rats at P4, P12, P21 and P40, but little Fos expression was observed in the RVM of naïve and pinched P8 rats (Figs 2.6A, B, C and D). Fos expression in pinched and naïve rats of all ages was randomly distributed throughout the RVM, with no obvious somatotopy or region-selective activation.

A two-way ANOVA demonstrated that pinch stimulation significantly increased the number of Fos-ir cells in the RVM compared to naïve at P12, P21 and in adult P40 rats, and that age was a significant factor (two-way ANOVA, pinch vs nave; $F(4,30)=19.04$, $P<0.0001$, with Bonferroni *post hoc* comparison, $P<0.05$ to 0.001 at different ages; Fig 2.6E). Unpaired Student's t-tests demonstrated no significant difference in Fos-ir counts after pinch compared to naïve at P4 and P8 (unpaired Student's t-test, pinch vs naïve, $P>0.05$ at P4 and P8; Fig 2.5C. Fos expression was equally high in naïve and pinched P4 rats (Fig 2.4J), and pinch-evoked Fos expression was apparent and high by P12 (Fig 2.6C) and Fos counts were as numerous as P21 (Fig 2.6D) and adult pinched rats (Fig 2.6E).

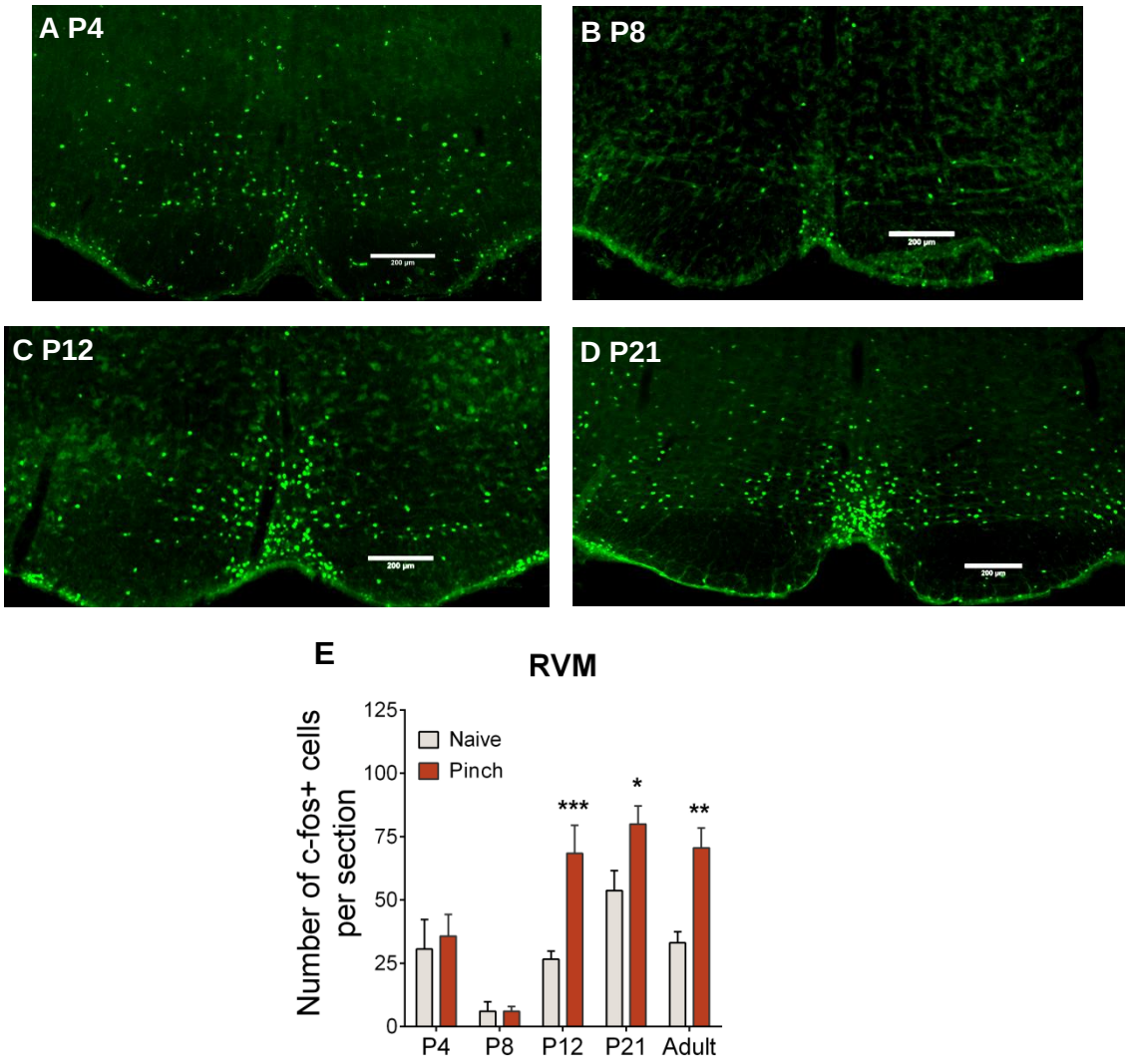

Fig 2.6. Pinch-evoked Fos expression in the RVM is observed from P12, but not earlier.
Fos counts were performed in the rostroventral medial medulla (RVM) of naïve and pinched rats of different ages (pinch n=4 per age; naïve n=4 per age). All images are from pinched rats. C-Fos expression was observed in naïve and pinched animals alike at P4 (A). In contrast, c-Fos expression was not observed at P8 (B). Pinch-evoked c-Fos expression was observed at P12 (C) and P21 (D) and in adult P40 rats. Quantification of the number of c-fos-ir cells revealed high c-Fos expression at P4 which was not evoked by hindpaw pinch stimulation (E). The number of c-fos-ir cells was significantly increased by pinch stimulation, compared to naïve, at P12, P21 and P40 (E). *,**,*** $P<0.05$, 0.01 and 0.001; Two-way ANOVA with Bonferroni post hoc analysis.

2.6.7 Summary of results

The findings from this chapter can be summarised as follows:

1. The neuronal density, as measured by NeuN-ir counts, in the brainstem PB nucleus, and the midbrain vlPAG and the DRN decreases between P4 and P12. In the RVM, neuronal density decreases in the RMg, LPGi and GiA between P4 and P12, and also decreases in these regions between P21 and P40.
2. Hindpaw pinch stimulation increased the number of Fos-ir cells in the PB nucleus at all postnatal ages from P4.
3. Hindpaw pinch stimulation did not increase the number of Fos-ir cells in the vlPAG until P12.
4. Similarly, hindpaw pinch stimulation did not increase the number of Fos-ir cells in the RVM until P12.
5. Hindpaw pinch stimulation did not increase the number of Fos-ir cells in the DRN until P40.

2.7 Discussion

The aim of this chapter was to test the hypothesis that functional connectivity between the dorsal horn and brainstem projection targets is observed in the first postnatal week, before sensory inputs activate brainstem regions which project to the dorsal horn. To test this, Fos expression evoked by hindpaw pinch stimulation was mapped in several brainstem and midbrain nuclei in the spinal-bulbo-spinal loop at several postnatal ages from P4 to P40.

2.7.1 Technical considerations

The oncogene *c-fos* and its protein form Fos have been used extensively as a marker of neuronal circuit function. In the pain field, the identification of Fos expression in the spinal dorsal horn following stimulation of primary sensory neurons (Hunt et al., 1987) has led to explosion of the use of Fos as a marker of activation of CNS nociceptive pathways following peripheral stimulation. Importantly, Fos expression following peripheral noxious stimulation occurs not just at the primary synapse in the spinal dorsal horn, but also throughout the extent of circuits involved in nociception and pain. Transcriptional activation of the immediate early oncogene *c-fos* occurs in neurons within minutes after stimulation, causing upregulation of Fos protein which peaks around 2 hours after stimulation. Upregulation of *c-fos* occurs downstream from growth factor-mediated activation of cell surface receptor tyrosine kinases; neuromodulators which activate GPCRs, leading to elevated intracellular cAMP; and the activation of cation ionotropic receptors which increase intracellular Ca^{2+} levels (West et al., 2002). *C-fos* encodes for the nuclear protein Fos which binds to nuclear proteins of the Jun family, creating a Fos-Jun complex which regulates downstream expression of target genes involved in long-term intracellular changes.

An important problem of using Fos as a marker of synaptic connectivity and function is that not all neurons express Fos when activated. Fos expression is not observed following peripheral noxious stimulation in DRG neurons (Hunt et al., 1987), or in ventral posterolateral thalamic neurons involved in nociception (Bullitt, 1990), suggesting that an absence of Fos expression may not necessarily indicate an absence of neuronal response to peripheral noxious stimulation. It is possible that absence of pinch-induced Fos expression observed in the PAG and RVM of neonatal rats in the present data is not due to an absence of functional connections between ascending pathways and the PAG-RVM, but rather due to immature intracellular pathways which induce *c-fos* and Fos. Extensive *c-fos* mRNA leading to reliable upregulation of

Fos protein has been reported in the developing midbrain and brainstem of postnatal rats from birth (MacDonald et al., 1990; Blumenfeld et al., 1992; Wagner et al., 1994), strongly suggesting that intracellular pathways involved in *c-fos* upregulation and induction of Fos protein are functional in neurons in the CNS from birth. Therefore, absence of pinch-induced Fos expression in the neonatal PAG and RVM, when Fos expression is observed in the same nuclei in mature rats, is likely to be due to immature synaptic connections between ascending axons and neurons within the PAG-RVM.

Fos is activated by inputs other than those from nociceptors and Fos expression cannot be considered a selective marker of nociception. Here, Fos expression was high around the brainstem (particularly at P21) and was not restricted to nuclei which are involved in nociception and pain modulation. It is important to consider that neurons in all the brain nuclei investigated in this chapter modulate circuits that are not involved in nociception; for example neurons in the PB nucleus are known to have roles in cardiorespiratory control (Mizusawa et al., 1995) and neurons in the vlPAG are involved in fear-potentiated startle motor responses (Monassi et al., 1999), fear conditioning-induced learning (Johansen et al., 2010) and REM sleep (Sastre et al., 1996). Indeed, extensive Fos expression was observed in the PB nucleus, PAG and RVM in the absence of noxious stimulation in naïve rats in data presented in this chapter. Statistical differences between naïve and pinch animals within postnatal ages identify Fos expression most likely caused by noxious stimulation. Isoflurane anaesthesia may have altered Fos activation in the CNS; however as both control and pinched animals were anaesthetised, possible effects of isoflurane on Fos levels were controlled for.

Noxious mechanical stimulation was the only modality of sensory input applied to the hindpaw in experiments in this chapter. As such, a short-hand has developed such that noxious mechanical stimulation is often simply referred to as 'noxious stimulation', or words to that effect. Neurons in the peripheral and central nervous system respond differently to types of stimuli, and it is likely that these differences have been missed in these experiments. The effect of stimulating the skin with innocuous tactile, chemical and thermal inputs would provide additional information about the maturation of the recruitment of the brain nuclei by other sensory stimuli.

2.7.2 Postnatal growth of brainstem nuclei in the spinal-bulbo-spinal loop

Neurogenesis in brainstem and midbrain regions stops before birth around embryonic day 15 (Bayer et al., 1993); therefore it is likely that as the central nervous system grows

in size during the first weeks of life, the density of neuronal cell bodies will decrease. Consistent with this, I found that neuronal density in the PB nucleus, vlPAG and DRN decreased between P4 and P12, but not at older ages. In the RVM, neuronal density in the LPGi, RMg and GiA decreased between P4 and P12, and also between P21 and P40. These data suggest that there is a growth of these brainstem and midbrain regions in the first 12 days of postnatal life which is reflected by a decrease in neuronal density during this period. Because the density of neuron cell bodies in all regions of interest decreased between P4-P12, Fos-ir counts were not statistically compared between ages.

Programmed neuronal cell death in the central nervous system during early postnatal development has been well characterised in mammals (Oppenheim, 1991), and has been observed in the neonatal brainstem (Machaalani and Waters, 2006). It is likely that small populations of neurons do undergo programmed cell death in the first weeks of life in the brainstem and midbrain regions analysed in results here. Therefore, decreased NeuN cell counts with age could also reflect normal neuronal cell death within regions of interest.

2.7.3 Ascending nociceptive pathways are partially functional from birth

The output from the dorsal horn to brainstem and midbrain nuclei is modulated by dorsal horn interneurons (Melzack and Wall, 1965). In the adult, this dorsal horn circuitry is currently a popular area of research and new studies have identified interneuron populations that have crucial roles in transmitting and modulating sensory information onto ascending projection neurons (Duan et al., 2014). Our understanding of these circuits in the neonate is minimal, however it is likely that these gating functions develop postnatally such that ascending sensory transmission to the neonatal brain differs from that in the adult.

Noxious peripheral stimulation activates dorsal horn neurons from birth; low threshold brush stimulation causes Fos expression in the dorsal horn at P3 which diminishes by P21, and noxious pinch stimulation reliably activates Fos from P3 onwards (Jennings and Fitzgerald, 1996). Additionally, projection neurons in lamina I have axonal terminals present in both the PB nucleus and the PAG from birth, and have biophysical membrane properties that allow stimulus-responsivity and faithful ascending nociceptive transmission (Li and Baccei, 2012). Thus, the foundations of a functional ascending pathway, starting with noxious recruitment of peripheral afferent fibres to dorsal horn-originated neurotransmission in the brainstem and midbrain, are present from birth. Experiments in this chapter demonstrated that noxious pinch stimulation

increased Fos expression in the PB nucleus from P4 and in the PAG from P12, demonstrating age differences in sensory-mediated activation of neurons in these two target regions of dorsal horn projection neurons.

The spino-parabrachial pathway

Experiments in this chapter demonstrated that hindpaw pinch stimulation activated Fos in the PB nucleus in P4 rats, over and above a low level of unevoked Fos expression in age-matched naïve animals. Pinch-evoked Fos expression was present between P4 and P12, but low compared to P21 and adult P40 animals, suggesting maturation of the strength of dorsal horn-originated inputs to the PB nucleus in the third postnatal week. In previous studies, Fos expression has been reported in the neonatal PB nucleus following hindpaw formalin injection in P3 rats (Barr, 2011; Man et al., 2012), suggesting functionality of ascending spino-PB pathways from birth.

Tracing experiments in adult rodents have demonstrated that neurons in the PB nucleus receive inputs from lamina I and deep laminae projection neurons. In the rat, the densest output from the superficial spinal dorsal horn is to the contralateral PBel nucleus (Slugg and Light, 1994; Craig, 1995; Feil and Herbert, 1995a) via the dorsolateral funiculus (DLF). Ascending inputs to the PBil nucleus predominantly, but not exclusively, originate from deep dorsal horn neurons (Hylden et al., 1986; Bernard et al., 1995). Whilst anatomical connections from lamina I neurons to the PB nucleus have been shown in newborn rats (Li and Baccei, 2012), the presence of direct connections from lamina IV-V to the PB nucleus have not been investigated in the neonate. The results presented in this chapter show that pinch-induced Fos expression was present throughout the PB nucleus, including the PBel and PBil. The simplest conclusion from these findings is that functional connections between both superficial and deep dorsal horn neurons and PB nuclei are present from birth.

A small number of PB neurons in the uninjured cat are activated by low and high threshold stimulation of the skin (Hylden et al., 1985), however the majority of PB neurons in uninjured adult mammals selectively respond to noxious inputs (Lantéri-Minet et al., 1993, 1994; Bellavance and Beitz, 1996; Hermanson and Blomqvist, 1996; Pinto et al., 2003). Experiments in this chapter only used a noxious mechanical stimulus to activate Fos in brainstem nuclei. Brush-evoked Fos expression has been demonstrated in the P3, but not P21 (Jennings and Fitzgerald, 1996), dorsal horn, thus it would be interesting to investigate whether tactile inputs also activate ascending sensory pathways in neonatal rats.

The Spino-PAG pathway

In experiments in this chapter, hindpaw pinch stimulation did not activate Fos in the PAG at P4 and P8 rats, but did at P12, suggesting that noxious sensory inputs do not activate neurons in the PAG until P12. These findings are surprising, as axonal projections from the dorsal horn to the PAG are present at P3 (Li and Baccei, 2012), and the presence of axonal projections from the dorsal horn to the PB nucleus at P3 (Li and Baccei, 2012) predicted activation of Fos in the PB nucleus by noxious sensory inputs. At P21 and P40, Fos expression was high throughout the PAG in both naïve and pinched rats, but a significant pinch-mediated Fos expression was observed in the vlPAG.

Late functional recruitment of PAG neurons has also been reported in the neonatal rat elsewhere. Fos immunohistochemistry studies have shown that formalin-induced Fos expression in the PAG is absent at P3, but is observed at P14 (Barr, 2011); and fear-induced Fos expression in the PAG (and freezing behaviour) is not observed until P14 (Wiedenmayer and Barr, 2001). Additionally, downstream effects of activating the PAG are absent in the first postnatal weeks: behavioural studies have demonstrated that PAG stimulation produced analgesia is not observed until P21 (van Praag and Frenk, 1991); and injection of kainate acid or k-opioid agonists into the PAG increases heart rate and defensive behaviours at P14 but not P7 (Goodwin and Barr, 1998, 2005). Hindlimb reflex experiments have also shown that injection of μ-opioid agonists into the PAG modulates hindlimb reflexes at P21 but not P10 (Kwok et al., 2013). Thus, functional signalling in the PAG may be immature or absent until the end of the second postnatal week.

In the adult, the PAG receives and integrates ascending sensory synaptic inputs from the dorsal horn and receives affective and autonomic inputs from other parts of the brain such as the hypothalamus and amygdala (Bandler and Shipley, 1994). Age-dependent increments in Fos expression in the PAG could indicate strengthening of synapses from other parts of the CNS. Indeed, as hindpaw formalin-induced Fos expression in the hypothalamus (which contains neurons that project to the PAG (Rizvi et al., 1996) increases dramatically after P14 (Barr, 2011), it is reasonable to suggest that maturation and strengthening of circuits involved in higher processing and modulation of nociception and pain may contribute to increase neuronal activation in the PAG following noxious events in later life. To summarise, a major change in PAG circuitry and neural connectivity occurs between P7 and P14, and whilst anatomical connections from the dorsal horn (or other regions of the CNS) to the PAG are present

from birth (Li and Baccei, 2012), functional synapses resulting in PAG neuron activation may not be mature until P12-P14.

2.7.4 RVM neurons are not responsive to ascending noxious inputs until P12

The present experiments show that pinch-induced Fos expression in the RVM was observed from P12, but not at P4 or P8. This time-line of pinch-induced Fos expression in the RVM closely matches the PAG, but not the PB nucleus. Thus, the maturation of sensory-driven PAG neuronal activation correlates with downstream activation of the RVM. These findings suggest the presence of a critical period between P8 and P12 when ascending nociceptive circuits mature and drive the recruitment of descending modulatory circuits originating in the PAG-RVM.

Previous experiments used focal electrical stimulation of the RVM to exogenously excite the RVM and recruit descending modulation of dorsal horn neurons or hindlimb flexion reflexes in P21 rats (Hathway et al., 2009a; Koch and Fitzgerald, 2014). These experiments do not elucidate whether descending RVM modulation is driven by ascending sensory inputs. One important piece of evidence points to endogenous descending modulation of spinal nocifensive reflexes in neonatal animals: ablation of RVM neurons by focal injection of kainate increases behavioural withdrawal thresholds at P3 (Hathway et al., 2009a). When taking into consideration that pinch stimulation did not increase Fos expression in the RVM at P4 in experiments in this chapter, these findings suggest that descending RVM modulation of spinal sensory circuits may occur in the absence of ascending sensory activation of RVM neurons. As pinch-evoked Fos expression in both the PAG and the RVM was also only present from P12, it is likely that sensory activation of PAG neurons is required for activation of RVM neurons at P12. The maturation of synaptic connections from the dorsal horn to the PAG and from the PAG to the RVM may therefore be crucial in connecting the ascending arm of the spinal-bulbo-spinal loop to the descending arm. Experiments in the next chapter will question further if descending RVM modulation of spinal sensory circuits can occur in the absence of ascending sensory recruitment of RVM neurons.

Interestingly, high Fos expression was observed in the RVM at P4. As the number of Fos-ir cells did not differ between pinch and naïve animals, and little to no Fos expression was observed at P8, these findings suggest that this age-dependent Fos expression was not driven by ascending sensory inputs. A lack of sensory-driven inputs to the RVM at P4 suggests the presence of intrinsically active circuits within the RVM. Spontaneously firing 'pacemaker' glutamatergic neurons have been well described in

the neonatal rat spinal-bulbo-spinal loop in the superficial spinal dorsal horn (Li and Baccei, 2012; Baccei, 2014; Li et al., 2014). These intrinsically bursting neurons may function to provide endogenous excitation of developing nociceptive circuits early in development. Whilst the presence of age-specific pacemaker neurons have not been identified in the neonatal RVM, patch-clamp experiments have demonstrated that the majority of RVM neurons in adult brainstem slices do not exhibit spontaneous firing activity as frequently observed in RVM neurons in P10-21 brainstem slices (Li et al., 2015b). Several other studies have also demonstrated multiple sub-populations of locally and spinally projecting RVM neurons from brainstem slices of P21 or younger rats that readily display spontaneous firing activity, (Zhang et al., 2006, 2007; Zhang and Hammond, 2010), however few studies have directly compared RVM neuronal properties in young and adult animals.

Intrinsically active pacemaker neuronal properties are an electrophysiological phenomenon, therefore the presence of 'unevoked' Fos expression in the neonatal RVM observed here can only provoke the suggestion of intrinsically active RVM circuits at this age. Robust correlations between Fos expression and spontaneous neuronal firing have been found in the neocortex during early postnatal development. Two-photon imaging of Fos-GFP expression in transgenic mice has demonstrated that higher Fos-expressing layer 2/3 pyramidal neurons exhibit higher spontaneous firing rates and fire more often during network activity in acute brain slices and *in vivo*, including in the absence of sensory inputs (Yassin et al., 2010). It is, however, unclear if enhanced Fos-activation is the cause or effect of enhanced spontaneous firing activity in these neurons. Future patch clamp experiments in neonatal RVM brainstem slices in combination with retrograde tracing techniques would be useful to identify the predicted presence of pacemaker neurons in the RVM which do or do not project to the spinal dorsal horn.

2.7.5 The DRN is not activated by peripheral noxious stimulation until adulthood

Experiments in this chapter demonstrated that hindpaw pinch stimulation did not increase Fos expression in the DRN in rats younger than P40, demonstrating that ascending noxious sensory inputs to the DRN are late to mature. Fos expression was generally very low or absent in the DRN of naïve and pinch P4, P8, P12 and P21 rats, suggesting absent or immature functional synaptic inputs from sensory regions.

Patch clamp experiments in neonatal mouse slices have demonstrated that serotonergic neurons in the DRN undergo considerable postnatal maturation in their intrinsic

membrane electrophysiological properties and morphology (Rood et al., 2014). DRN cells at P4 were found to be more hyperexcitable due to absence of K+ leak currents and absence of 5-HT_{1A} autoinhibition. Importantly, glutamatergic EPSC and GABAergic IPSC frequencies increase dramatically with postnatal age, and the proportion of DRN cells which respond to glutamate and GABA increases with age; most notably between P21 and P60 (Rood et al., 2014). When this is taken into account with data from the current chapter, it is likely that late postnatal maturation of afferent synaptic inputs to the DRN causes delayed noxious stimulus-evoked Fos expression in the adult rat. Tracing experiments would help to identify the ascending neurons that grow into the DRN after P21 and drive activation of DRN neurons.

Early studies showed that electrical stimulation of the DRN causes powerful behavioural analgesia and inhibition of spinal dorsal horn neuron activity, without motor or autonomic side effects (Oliveras et al., 1974, 1979). Additionally, lesion of the RVM partially reverses the antinociceptive effect of DRN stimulation (Yu et al., 1988); leading to the hypothesis that the descending antinociceptive role of the DRN is largely mediated via extensive connectivity to the RVM (Wang and Nakai, 1994). Electrical stimulation of the DRN is also known to inhibit noxious stimulus-evoked firing activity of neurons in the parafascicular nucleus of the thalamus (Andersen and Dafny, 1983a, 1983b). The antinociceptive role of DRN is likely mediated by targeting ascending nociceptive inputs directly via the thalamus and the spinal dorsal horn, both directly and via descending modulatory nuclei such as the RVM (Wang and Nakai, 1994). It is currently unknown whether the DRN modulates sensory-evoked behaviours and circuits in the spinal cord in young rats. It would be interesting to investigate whether the DRN can modulate spinal sensory circuits in the absence of sensory-evoked activation of DRN neurons before P40, as is proposed to be the case of descending RVM modulation in the absence of ascending sensory-evoked activation of RVM neurons in the first postnatal week.

Current evidence suggests that the adult DRN is exclusively antinociceptive in its output (Wang and Nakai, 1994). If this is indeed the case, then maturation of ascending sensory recruitment of the DRN during postnatal development could be a major factor which contributes to the onset of dominant descending inhibition of spinal nociceptive circuitry after P28 (Hathway et al., 2009a). Future experiments characterising the maturation of DRN recruitment of RVM descending modulatory pathways during postnatal development could help elucidate this idea.

2.8 Conclusions

In this chapter, Fos was used as a measurement of nociceptive inputs to selected brain regions. Peripheral noxious stimulation increased Fos expression in the PB nucleus from P4, demonstrating functional connectivity of spino-parabrachial connections in the first days of life. Conversely, the PAG and RVM, areas which are known to be involved in descending modulation of spinal sensory circuitry, do not receive ascending noxious inputs until P12 onwards. Noxious-evoked Fos expression in the DRN was not observed until adulthood (Fig 2.7). These findings demonstrate that ascending noxious inputs from the dorsal horn to the brain activate nuclei in the ascending arm of the spinal-bulbo-spinal loop in the first postnatal week, but do not activate nuclei in the descending arm of the loop until P12. The spinal-bulbo-spinal loop is therefore not 'closed' until this age. Subsequent maturation of connections from other brain regions, such as the DRN, may not contribute to the loop until adulthood. Based on current evidence, it is possible that descending RVM modulation of spinal sensory circuits at P3 (Hathway et al., 2009a) may not be driven in the absence of ascending sensory inputs to the RVM at this young age. Experiments in chapter 3 will investigate this hypothesis further.

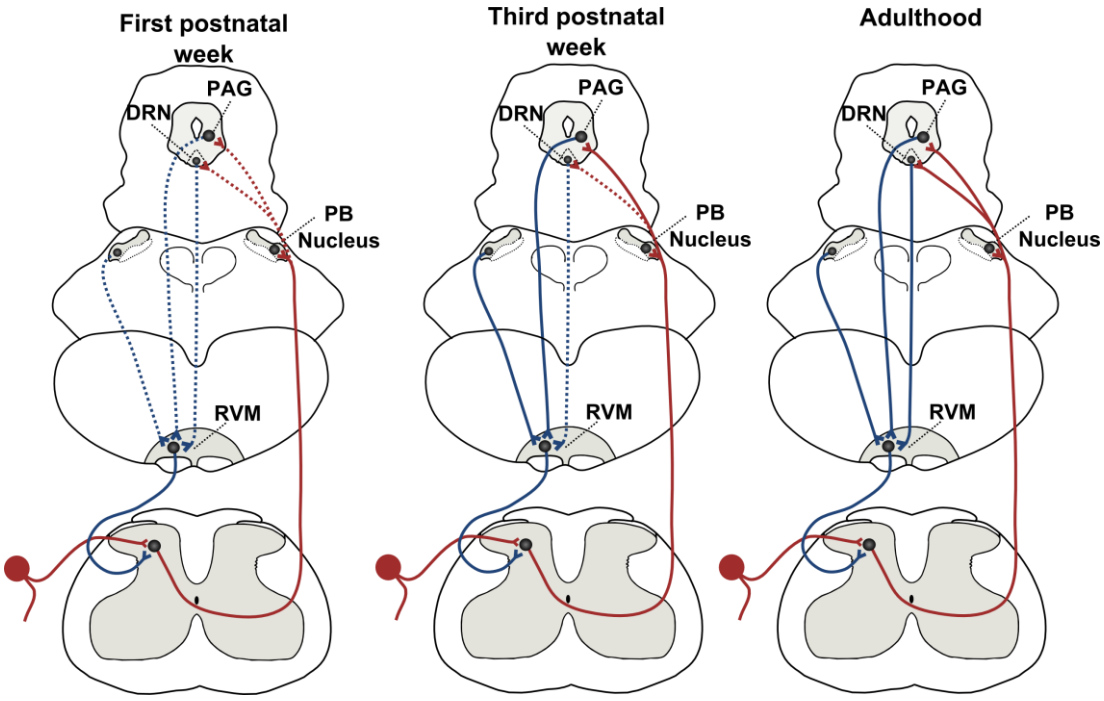

Fig 2.7. Changing connectivity of the spinal-bulbo-spinal loop during postnatal development
Noxious inputs activate dorsal horn neurons parabrachial (PB) neurons in the first postnatal week (solid red line). Other connections in the loop are not mature (dotted lines). By P12, noxious inputs from the dorsal horn activate neurons in the PB nucleus, periaqueductal grey (PAG) and the rostroventral medulla (RVM). By P40, neurons in the dorsal raphe nucleus (DRN) are also activated by noxious inputs and contribute to the spinal-bulbo-spinal loop.

Chapter 3

The functional development of descending RVM modulation of spinal sensory circuits

3.1 Introduction

In the previous chapter the anatomical and functional connectivity of the spinal-bulbo-spinal loop was characterised during development. It was established that functional connectivity of ascending pathways from the spinal cord to the parabrachial nucleus mature before the descending pathways, as demonstrated by a lack of pinch-induced Fos activation in the periaqueductal grey (PAG) and rostroventral medial medulla (RVM) before P12. This chapter will focus on investigating the function of RVM descending modulation of dorsal horn sensory circuitry during postnatal development

3.1.1 Descending modulation of acute nociceptive pain in adults

Endogenous descending control of sensory inputs is an important aspect of CNS pain processing. Descending pain modulation can arise from many parts of the brain including the PAG, insular cortex and amygdala, and is predominantly (but not exclusively) mediated via RVM-dorsal horn descending pathways (Braz and Basbaum, 2008; Sato et al., 2013; Ossipov et al., 2014). SPA can be evoked by stimulation of the RVM (Fields et al., 1977), and it is now known that stimulation of the RVM can facilitate as well as inhibit nocifensive withdrawal reflexes and dorsal horn neuron firing properties, depending on the stimulus strength or concentration of drug used (Zhuo and Gebhart, 1997). The balance of excitatory and inhibitory drive from the RVM is crucial in moment to moment modulation of nociceptive inputs to the spinal dorsal horn. Bi-modulatory outputs from the RVM are not a simple 'on-off' mechanism, but are rather tailored such that inhibition and facilitation can operate in parallel (Vanegas, 2004; Vanegas and Schaible, 2004; Cleary and Heinricher, 2013).

A recent paper has demonstrated the bi-modulatory, although predominantly facilitatory, nature of descending RVM modulation by recording from dorsal horn wide dynamic range (WDR) neurons in adult rats before and after microinjection of the local anaesthetic lidocaine into the RVM (Bee and Dickenson, 2007). In these experiments, RVM lidocaine injection inhibited noxious stimulus-induced firing in 64% of dorsal horn neurons, whereas 24% of neurons exhibited increased firing activity and 12% of neurons did not display altered firing activity. Following nerve injury, the proportion of dorsal horn neurons inhibited by RVM lidocaine increased to 81%. Thus, descending influences from the RVM are not uniform and are responsive to changes in afferent inputs and injury states. In this chapter I aim to investigate endogenous and net descending RVM modulation of dorsal horn neuron activity during cutaneous mechanical stimulation in uninjured rats.

3.1.2 Descending modulation of inflammatory pain states

Hindpaw injection complete freund's adjuvant (CFA), mustard oil or capsaicin causes robust and reversible behavioural hypersensitivity and sensitisation of hindlimb reflexes in neonatal rats (Marsh et al., 1999a, 1999b; Lidow et al., 2001; Walker et al., 2003, 2007). Sensitisation and ectopic growth of peripheral afferent fibres and activation of pERK in dorsal horn neurons has been shown to mediate inflammatory pain in young rats (Walker et al., 2003, 2007; Jankowski et al., 2014), however, to date, the role of brainstem descending modulation of inflammatory pain states has not been investigated in young animals. A secondary aim of this chapter is to establish the role of descending RVM modulation of inflammatory pain states in young animals by injecting lidocaine into the RVM of CFA-inflamed rats of several postnatal ages and measuring behavioural nocifensive thresholds.

In the adult rat, hindpaw inflammation causes sensitisation of neurons in the spinal dorsal horn (Woolf and King, 1990) and the RVM (Kincaid et al., 2006). In the RVM, presumed pronociceptive 'On'-cells display sustained and increased firing activity and presumed antinociceptive 'Off'-cells display reduced firing activity in the first hours and for several days after hindpaw CFA inflammation (Kincaid et al., 2006; Cleary and Heinricher, 2013). Prolonged and increased RVM On-cell firing may suggest a consistently pronociceptive output of the RVM during the transition from an acute to chronic inflammation pain state, however changes in On and Off-cell firing activity are an unreliable predictor of the output of descending RVM modulation.

RVM silencing and stimulation experiments have suggested a time-dependent role of RVM modulation of inflammatory pain states. Lidocaine injected into the RVM one hour after CFA inflammation attenuates tail-flick hypersensitivity, but facilitates tail-flick hypersensitivity when injected 3-10 days after inflammation (Cleary and Heinricher, 2013). Injection of lidocaine into the RVM immediately following hindpaw mustard oil application also attenuates the hyperexcitability of spinal dorsal horn neurons with receptive fields outside the area of mustard oil application (Pertovaara, 1998). Acute inflammatory phase descending RVM facilitation of inflammatory pain may be mediated by NMDAR activation in the RVM, as injection of a low dose of NMDA into the RVM within the first hours of CFA inflammation facilitates behavioural hypersensitivity (Guan et al., 2002). Similarly, intra-RVM NMDA antagonists attenuate behavioural hypersensitivity caused by mustard oil application (Urban et al., 1999b). Conversely, injection of AMPA into the RVM attenuates thermal

hypersensitivity with time-dependent increasing potency from 5 hours after CFA inflammation (Guan et al., 2002, 2003). Therefore, activation of NMDARs in the RVM drives descending facilitation of spinal sensory circuitry immediately following hindpaw inflammation, but activation of AMPARs in the RVM drives inhibition of spinal sensory circuitry at later time points after inflammation.

3.1.3 Descending pain modulation in young animals

In young animals, descending RVM modulation of spinal nociception is predominantly facilitatory. Electrical stimulation of the DLF weakly inhibits few dorsal neuron activity in the third postnatal week, but not before P12 (Fitzgerald and Koltzenburg, 1986), and electrical stimulation of the RVM only facilitates dorsal horn sensory neuron and hindlimb reflex activity at P21, whereas stimulus intensity-dependent reflex inhibition is observed from P28 (Hathway et al., 2009a, 2012; Koch and Fitzgerald, 2014). Thus, exogenous electrical recruitment of descending pathways from the RVM has consistently evoked strong and dominant facilitation, and weaker inhibition, of spinal sensory circuits. Whilst these studies have given invaluable data describing age-specific effects of exogenously activating descending pathways, they did not elucidate the endogenous or tonic role of the developing RVM during normal sensory feedback. An important piece of evidence suggests that the RVM exerts an endogenous facilitation of nocifensive behaviours at P3, as excitotoxic lesion of RVM neurons increases mechanical withdrawal thresholds at this age (Hathway et al., 2009a). Experiments in this chapter will build on these findings to investigate the role of endogenous RVM neuron activity upon sensory evoked electrophysiological properties of spinal dorsal horn neurons in a range of postnatal ages.

3.2 Experimental aims

The aims of this chapter are to investigate the role of RVM descending pathways in modulating spinal sensory circuitry during postnatal development in both uninjured and injured states. The key hypotheses are:

1) The RVM endogenously facilitates spinal dorsal horn neuron sensory-evoked firing and receptive field properties in young P8 and P21 rats.
2) In the adults, the RVM endogenously inhibits spinal dorsal horn neuron sensory-evoked firing and receptive field properties.
3) Descending excitatory drive from the RVM facilitates acute CFA-induced inflammatory behavioural hypersensitivity in young and adult rats.

3.3 Methods

3.3.1 Animals

All experiments were performed in accordance with the United Kingdom Animal (Scientific Procedures) Act 1986. Reporting is based on the ARRIVE Guidelines for Reporting Animal Research developed by the National Centre for Replacement, Refinement and Reduction of Animals in Research, London, United Kingdom (Kilkenny et al., 2010). Male and female Sprague-Dawley rats at postnatal day (P) 8, 12, 21 and 40 were obtained from the Biological Services Unit, University College London. Rats were bred and maintained in-house and exposed to the same caging, diet and handling throughout development. Litters were weaned at P21 into same sex cages of four littermates and were housed in 12h light/dark cycles at constant ambient temperature and humidity with free access to water and food.

3.3.2 Drugs

In RVM silencing electrophysiology and behaviour experiments, lidocaine hydrochloride monohydrate (Sigma-Aldrich, UK) was dissolved in saline to a concentration of 20mg/ml (2%) and microinjected into the RVM. Total volume of drug administered into the RVM was 0.7-1μL, depending on age and control animals received equivalent volumes of saline. The dose was based on previous studies injecting lidocaine into the adult RVM (Bee and Dickenson, 2007) and volumes were adjusted for the age of animals. In behavioural experiments, the skin overlying the injection site was then sutured with 5-0 suture (Ethicon), EMLA cream (AstraZeneca) was placed on the wound, before animals woke up for behavioural testing.

In inflammation experiments, Complete Freund's Adjuvant (CFA, heat-killed Mycobacterium tuberculosis in mineral oil, 1mg/ml, Sigma-Aldrich, Gillingham, UK) was injected subcutaneously into the left hindpaw plantar surface in P12, P21 and P40 rats. P12 and P21 rats received 25μg in 25μl and P40 rats received 100μg in 100μl; doses which were based on previous experiments in young (Walker et al., 2003; Lima et al., 2014) and adult rats (Guan et al., 2003, 2004; Cleary and Heinricher, 2013).

3.3.3 Electrophysiology Surgery

Rats were anaesthetised with isoflurane (induction 4% in medical O_2), tracheotomised and artificially ventilated under constant isoflurane anaesthesia (maintenance of 1.8% in medical O_2, Univentor Anaesthesia Unit 400; Royem Scientific, UK). The air flow and breathing rate were adjusted to the animal's sizes using a small animal ventilator

(model 687, Harvard Apparatus, MA, USA). Heart rate was constantly monitored via electrocardiogram. A homoeothermic blanket with feedback control (model 507220F, Harvard Apparatus, MA, USA) was used to maintain body temperature at physiological levels. The rat was mounted onto a stereotaxic frame (Kopf Instruments, Tujunga, CA, USA). A laminectomy was performed to expose the lumbar spinal cord, the vertebral column was secured with a clamp to the thoracic site and the dura and pia mater were removed. A film of mineral oil was used to cover the exposed spinal cord to prevent heat loss. The skull was exposed and bregma located to perform a small craniotomy for unilateral RVM microinjection.

Stereotaxic coordinates for the RVM were calculated as outlined previously (Hathway et al., 2009a): adult = lateral 0mm, antero-posterior 9.7mm, dorso-ventral -10.0mm; P21 = lateral 0mm, antero-posterior 9.2m, dorso-ventral -10.0mm; P8 = lateral 0mm, antero-posterior 8.0mm, dorso-ventral 7.5mm. At the end of experiments, animals were terminally anaesthetised with an intraperitoneal overdose of injection of pentobarbitone (Euthetal®, UK). The brain was dissected out, the cerebellum removed, and the brainstem was cut coronally with a scalpel blade to allow visual inspection of the tract mark made by the injection site into the RVM (Fig 3.2C). Data from animals with injection sites outside the RVM were excluded.

3.3.4 In vivo extracellular recordings in the dorsal horn

To isolate individual neurons in the dorsal horn, a 6µm tipped glass-coated carbon fibre microelectrode (Kation Scientific, Minneapolis, USA) was lowered through the spinal cord with an in vivo manipulator (Scientifica, UK) while stroking the plantar surface of the hindpaw as a search stimulus for dorsal horn wide dynamic range (WDR) neurons in lamina IV-VI (Fig 3.1A). All recorded WDR neurons had receptive fields in the glabrous skin. Mean recording depth at P8 was 440.6µm, at P21 was 586.8µm and at P40 was 569.8µm (see Fig 3.1C for range and distribution).

Cutaneous glabrous receptive fields to brush and pinch stimulation were mapped and the number of spikes per stimulus to brush, pinch and von Frey hair (vFh) stimulation of the receptive field were recorded. The brush stimulus used was a fine acrylic paintbrush with a 1mm tip, briefly applied to the centre of the hindpaw receptive field. Pinch stimulation in all ages was performed with a pair of F.S.T curved serrated forceps (product code 11152-10) with a 0.3mm tip. The centre of the receptive field was pinched until the arms of the forceps just began to bend and was applied for 2.5 seconds, providing consistent pinch stimuli. Because the relative size of the hindpaw is

smaller in P8 rats, brush and pinch stimuli unavoidably covered a relatively larger region of the hindpaw compared to older animals. Example brush and pinch-evoked spike activity is shown in figure 3.1D. Spontaneous neuronal activity was recorded for one minute. A vFh force calibration curve for vFhs (ranging from 0.18g to 6.70g or 9.80g) used in electrophysiology and behavioural experiments is shown in figure 3.2A. VFhs were applied to the centre of the hindpaw receptive field for 1 second. To avoid overstimulation and sensitisation of nociceptors during electrophysiology experiments, the maximum vFh forced applied to P8 rats was 6.70g, and to P21 and adult rats was 9.80g. Stimulus evoked potentials were digitalised using PowerLab 4/30 interface and isolated using the Chart 5 software spike histogram plug-in (AD Instruments Ltd, Oxford, UK).

In RVM lidocaine experiments, a 26 gauge 10µl syringe (Hamilton, Reno, NV, USA) was lowered into the RVM and 2% lidocaine solution or saline was injected slowly over a five minute period (Fig 3.1B) and the needle was left in place throughout the experiment. The experimenter was blinded to the drug administered. Lidocaine at a volume of 1.0µl should effectively suppress neuronal activity within a radius of 1.4-1.7mm within 4 minutes in adult rats (Sandkühler et al., 1987), and therefore 0.7µl would predictably supress neuronal activity within a radius of 1.0-1.2mm in younger rats. No changes in heart rate were observed following injection of saline or lidocaine into the RVM.

WDR neuron searching and recordings were performed 10 minutes after RVM microinjection and for up to 90 minutes; a timeframe which is within the period of maximal effect of lidocaine (Bee and Dickenson, 2007). Cell properties were compared as populations from lidocaine-treated animals (P8 = 22 cells from 4 animals; P21 = 24 cells from 4 animals; adult = 17 cells from 4 animals) and control animals (P8 = 21 cells from 6 animals; P21 = 28 cells from 4 animals; adult = 23 cells from 7 animals). The control cell population is a pooled group of cells from animals receiving RVM saline (P8 = 7 cells from 2 animals; P21 = 15 cells from 2 animals; adult (13 cells from 2 animal) and naïve animals which displayed indistinguishable cell properties.

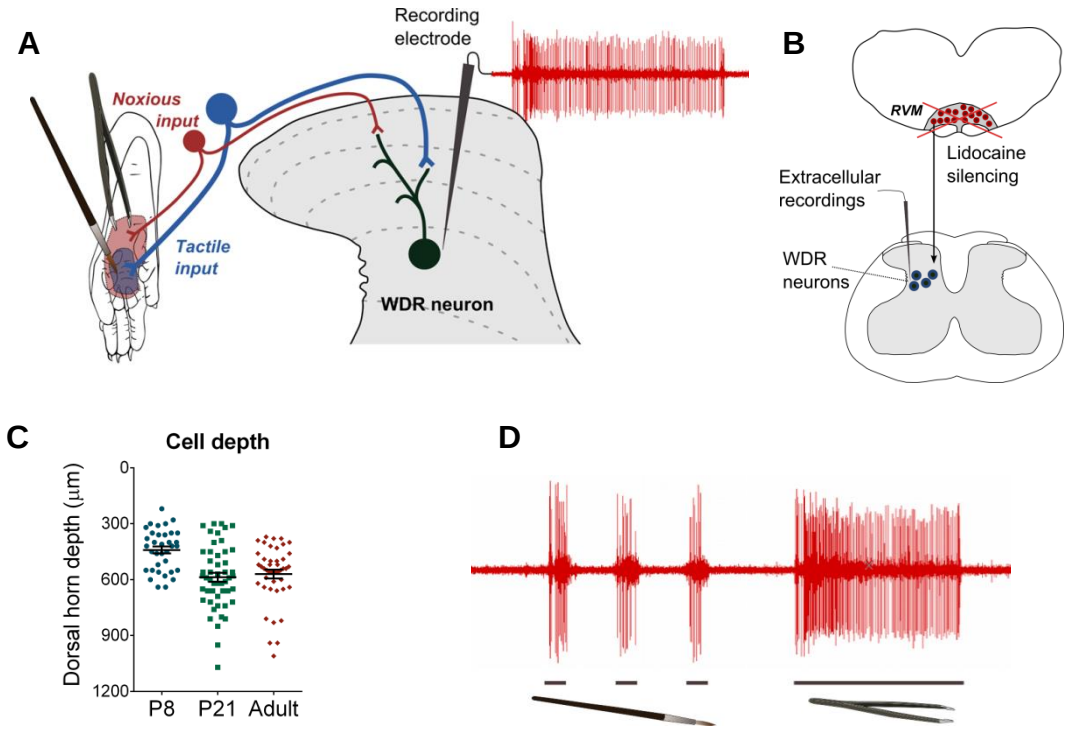

Fig 3.1. Schematic diagrams of electrophysiology experiment methodology.
In electrophysiology experiments, hindpaw brush or pinch stimulation evoked firing activity was recorded from dorsal horn wide dynamic range (WDR) neurons (A). Lidocaine was injected into the RVM to block RVM neuron activity (B). WDR neurons were recorded in the deep dorsal horn in P8, P21 and adult P40 rats (C). Brush and pinch evoked firing activity upon stimulation (black bars) in WDR neurons (D).

3.3.5 Behavioural Testing

Animals were habituated to the testing environment for 2 hours before baseline testing. For measurement of mechanical withdrawal threshold in the testing environment, animals were placed in individual Plexiglas cubicles on an elevated mesh platform. Left and right hindpaw plantar surfaces were stimulated with von Frey hair filaments and the reflex threshold was determined by using the up-down method (Chaplan et al., 1994). Briefly, a starting vFh filament was applied to the plantar surface which was roughly the average baseline withdrawal threshold for each age (P40 = 9.47g; P21 = 3.97g; P12 = 0.82g). Depending on the response of the animal, higher or lower force vFhs were then applied to the hindpaw to establish a series of 6-9 positive or negative reflex withdrawals. This pattern of responses was then converted such that data is expressed as log of the mean of the 50% reflex withdrawal threshold (Chaplan et al., 1994). Reflex withdrawal was defined as a full paw flick withdrawal away from the vFh filament. This method of establishing mechanical withdrawal thresholds with vFhs was

chosen to prevent overstimulation and sensitisation of the hindpaw (particularly in younger animals).

In CFA only experiments, vFh thresholds were measured at baseline, 1 hour, 1 day, 3 days, 7 days and 10 days after CFA injection. In CFA and RVM silencing experiments, 2% lidocaine was injected into the RVM 30 minutes after intraplantar injection of CFA. VFh stimuli were applied to plantar surface of the hindpaw which corresponded to the region of primary hyperalgesia. vFh thresholds were then measured 15, 30, 45, 60 and 90 minutes after RVM lidocaine injection to confirm return to normal CFA-induced behavioural hypersensitivity. Mechanical withdrawal thresholds were compared within age groups between CFA only control animals (P12 n=8, P21 n=7, P40 n=6) and CFA and RVM lidocaine animals (P12 n=4, P21 n=4, P40 n=4). The control group consists of pooled data from CFA only animals (P12 n=6, P12 n=5, P40 n=4) and CFA and RVM saline animals (n=2 per age). At the end of experiments, animals were terminally anaesthetised with an intraperitoneal injection of pentobarbitone (Euthetal®, UK). The brain was dissected out, the cerebellum removed, and the brainstem was cut coronally with a scalpel blade to allow visual inspection of the tract mark made by the injection site into the RVM (Fig 3.2C). Data from animals with injection sites outside the RVM were rejected.

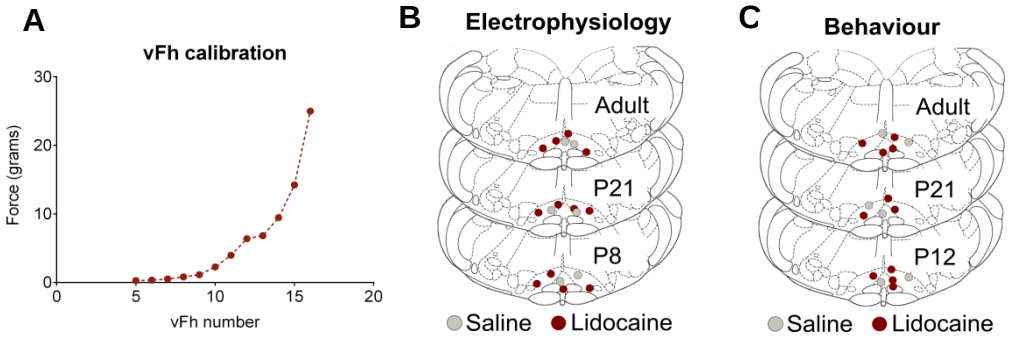

Fig 3.2. Recorded cell depths and brainstem injection sites.
Calibrated von Frey hairs (vFh) were used to stimulate the hindpaw in both electrophysiology and behaviour experiments (A). Lidocaine or saline injection sites were checked in electrophysiology (B) and behaviour (C) experiments.

3.3.6 Statistical analysis

Statistical analyses and graphing were performed using GraphPad Prism 6 (GraphPad software, La Jolla, CA, USA) and $P<0.05$ was considered statistically significant. Sample sizes for testing were based on previously reported group differences between

RVM/PAG lidocaine/stimulated animals in electrophysiology experiments (Waters and Lumb, 1997; Bee and Dickenson, 2007; Koch and Fitzgerald, 2014) and behaviour experiments (Cleary and Heinricher, 2013). Data are represented as means ± standard error of mean (SEM).

In electrophysiology experiments, evoked cell response values are expressed as the mean of three stimuli. For pinch after discharge (AD) values, spikes were counted in a 1s bin during the peak time of pinch after-stimulus spike activity. WDR neuron recordings from lidocaine-treated animals were pooled and treated as one population of neurons for each age. Data from naïve animals were combined with data from RVM saline treated animals as a control group, as there were no statistical differences between them at any age (data not shown). In normally distributed data sets, group differences between ages and between control and lidocaine-treated animals within age groups were tested with unpaired Student's t-tests and one-way analysis of variance (ANOVA) followed by Bonferroni *post hoc* multiple comparisons tests. Data sets which were not normally distributed were compared with Mann-Whitney and Kruskal-Wallis tests. In vFh experiments, Two-way repeated measures ANOVA were used within the age groups followed by Bonferroni *post hoc* multiple comparison test to compare differences in responses to increasing vFh force in control animals and lidocaine-treated animals. Dorsal horn neuron receptive fields were drawn on a template during recording and then imported and expressed as a percentage of the total area of the hindpaw plantar surface using Inkscape (version 0.48 www.inkscape.org).

In CFA behaviour experiments, ipsilateral and contralateral mechanical withdrawal thresholds were compared using Two-way repeated measures ANOVA followed by Bonferroni *post hoc* multiple comparison test. In CFA and RVM lidocaine behaviour experiments, data from CFA only animals were combined with data from CFA and RVM saline treated animals as a control group, as there were no statistical differences between them at any age (data not shown). Two-way repeated measures ANOVA was used within age groups followed by Bonferroni *post hoc* multiple comparison tests to compare differences in mechanical withdrawal threshold in CFA only control animals and CFA and lidocaine-treated animals.

3.4 Results

3.4.1 Control animal dorsal horn WDR neuronal activity at different postnatal ages

A total of 72 deep dorsal horn WDR neurons were recorded in uninjured rats of different postnatal ages for this study: 23 cells from 6 P8 rats; 28 cells from 4 P21 rats; and 21 cells from 7 adult P40 rats. Comparisons of dorsal horn neuron firing and receptive field properties at three postnatal ages demonstrated significant changes in neuronal processing of cutaneous mechanical stimulion during development. Changes in WDR neuron firing activity in response to brush, pinch and vFh stimulation of the cutaneous receptive field, and changes in the relative size of the receptive fields, were observed when comparing cell populations between the three postnatal ages.

Spontaneous firing was very low in all ages and did not differ between ages (Kruskal-Wallis test with Dunn's *post hoc* analysis, P8 vs P21 vs P40; Fig 3.3A). A one-way ANOVA of brush-evoked firing activity between ages demonstrated that age was a significant factor (One-way ANOVA, P8 vs P21 vs P40; $F(2,69)=3.66$, $P=0.031$) and Bonferroni post hoc analysis demonstrated that brush-evoked firing activity was significantly lower at P8 than at P21, but not when compared to adult P40 rats (One-way ANOVA with Bonferroni *post hoc* analysis, P8 vs P21 $P<0.05$; P8 and P21 vs P40 $P>0.05$; Fig 3.3B). Hindpaw cutaneous brush receptive field sizes were not significantly different between the ages (Kruskal-Wallis test with Dunn's *post hoc* analysis, P8 vs P21 vs P40; Fig 3.3C).

Pinch-evoked firing activity changed significantly with postnatal age (One-way ANOVA, P8 vs P21 vs P40; $F(2,69)=17.93$, $P<0.0001$) and Bonferonni post hoc analysis demonstrated significantly lower pinch-evoked firing activity at P8 compared to P21 and P40 (One-way ANOVA with Bonferroni *post hoc* analysis, P8 vs P21 and P40 $P<0.001$ and $P<0.01$; Fig 3.3D). Additionally, pinch-evoked firing activity at P21 was significantly higher than at P40 (One-way ANOVA with Bonferroni *post hoc* analysis, P21 vs P40 $P<0.05$; Fig 3.3D). Hindpaw cutaneous pinch receptive fields were significantly and relatively larger at P8 and P21 compared to P40 (Kruskal-Wallis test with Dunn's *post hoc* analysis, P8 vs P21 vs P40; Fig 3.3E). Post pinch stimulus after discharge was rarely observed at P8, and was significantly higher at P21 and P40 (Kruskal-Wallis test with Dunn's *post hoc* analysis, P8 vs P21 and P40 $P<0.001$ and $P<0.05$; Fig 3.3F).

WDR neurons at all ages reliably coded for stimulus intensity (Fig 3.3G), firing more frequently upon stimulation with increasing forces of mechanical vFh stimuli. A three-way repeated measures ANOVA was performed on the P8, P21 and P40 vFh stimulus

response curves with data from vFhs 0.18g to 6.69g and revealed that age was a significant factor (Three-way repeated measures ANOVA, P8 vs P21 vs P40; $F(2,61)=4.2l$, $P=0.019$). Bonferroni post hoc analysis revealed that vFh evoked firing activity was significantly higher at P21 compared to P8 at 1.11g, 4.23g and 6.69g forces, and higher in adults compared to P8 at 6.67g (Three-way repeated measures ANOVA with Bonferroni *post hoc* analysis, P8 vs P21 vs P40, $P<0.05$ to 0.01; Fig 3.3G). A two-way repeated measures ANOVA was also performed comparing the full range of vFh stimuli (0.18g to 9.77g) between P21 and adult data sets and revealed that age was a not significant factor (Two-way repeated measures ANOVA, P21 vs P40; $F(1,47)=2.01$; $P=0.16$).

The variability of the populations of WDR neuron data sets also changed with age. For example, pinch-evoked firing activity ranged from 23.00 to 98.00 spikes per stimulus at P8; 28.67 to 292.33 at P21; and 35.33 to 258.33 in adult data sets. The SEM was lower at P8 compared to P21 and adult brush and pinch-evoked and pinch AD firing data sets. For example, the SEM for pinch-evoked firing activity was: P8 = 4.03; P21 = 12.41; adult = 11.35.

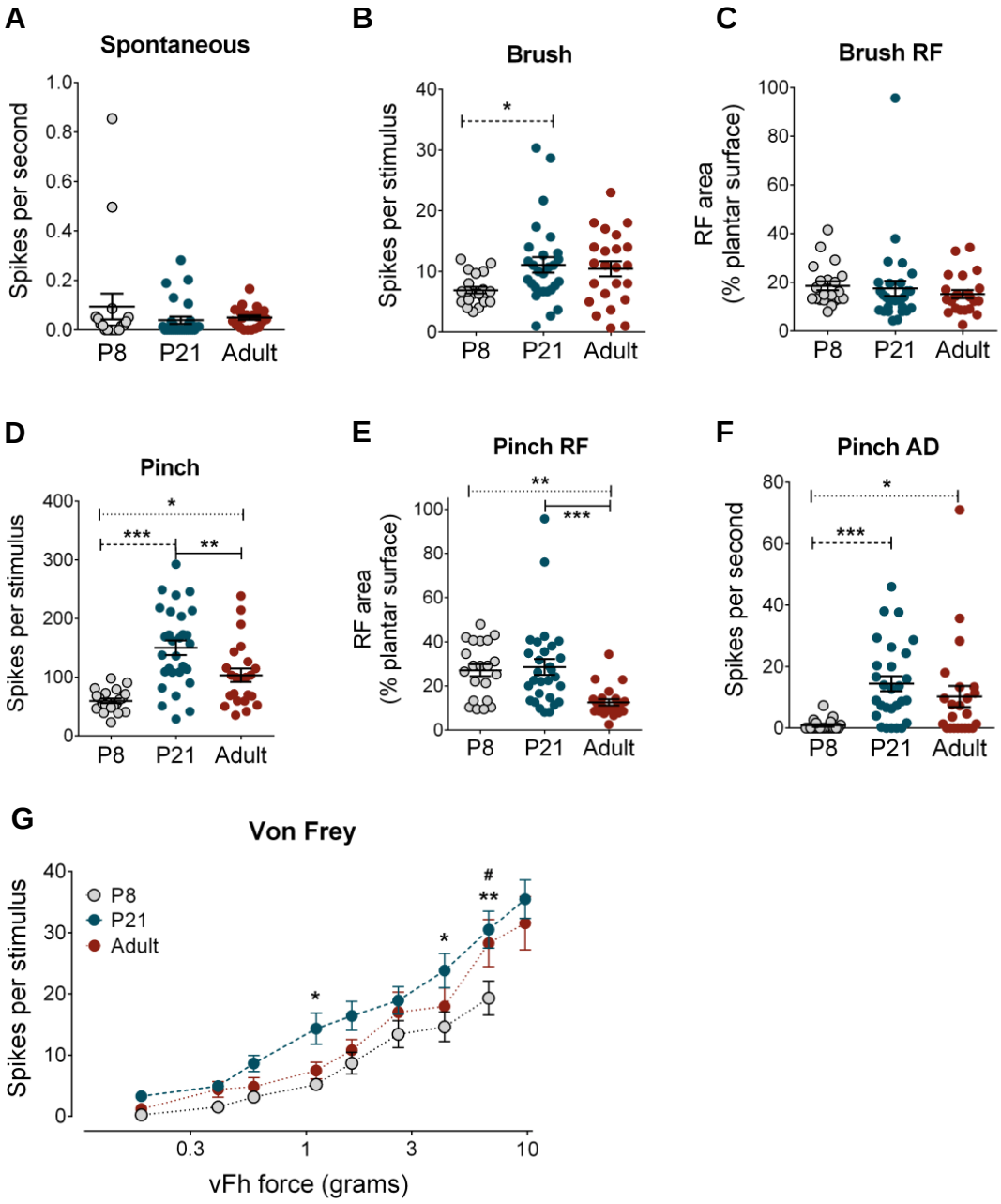

Fig 3.3. In vivo dorsal horn WDR neuron recordings in naïve P8, P21 and adult rats.
Peripheral stimulus-evoked dorsal horn WDR neuron properties were compared as populations between P8 (n=23), P21 (n=28) and adult P40 (n=21) rats. Spontaneous activity remained low and unchanged with age (A). Brush-evoked firing was significantly lower in P8 than P21 and adult rats (B) but brush receptive fields (RF) did not change with age (C). Pinch-evoked firing changed significantly with age (D). Pinch RFs were significantly and relatively smaller in adult rats than at P21 and P8 (E). Pinch after discharge (AD) was significantly lower at P8 compared to P21 and adult rats (F). von Frey hair (vFh) evoked firing activity was significantly higher at 1.1g, 4.2g and 6.7g vFhs at P21 compared to P8 (*), and at 6.7g in adults compared to P8 (#) (G). *, #, **,*** P<0.05, 0.05, 0.01 and 0.001 respectively, see text for details of statistical analyses.

3.4.2 Comparison of dorsal horn WDR neuron activity in P8 control and RVM lidocaine rats

To test whether tonic activity of descending modulation from the RVM influences dorsal horn neuron activity in uninjured young rats, lidocaine was microinjected into the RVM of a separate group of P8 animals. Following injection of 2% lidocaine (0.7µl) into the RVM of P8 rats (n=22 from four rats) the same dorsal horn properties were recorded as above. A total of 43 neurons were recorded from in these experiments; 22 from four RVM lidocaine treated rats, and 21 from six control rats (which includes 7 cells from two RVM saline control rats and 14 from four naïve rats). WDR neuron firing and receptive field properties were compared as whole populations between control and RVM lidocaine treated animals.

There was no significant difference in spontaneous spike activity of WDR neurons in control and RVM lidocaine treated animals (Mann-Whitney test, Fig 3.4A). Similarly, brush-evoked firing activity and brush receptive field size was not significantly different in RVM lidocaine animals compared to control (Mann-Whitney test, Figs 3.4B and C). At P8, the mean number of spikes per pinch stimulus in RVM lidocaine animals was significantly lower in than control animals (unpaired Student's t-test, $P<0.001$; Fig 3.4D). The mean pinch receptive field size was also significantly and relatively smaller in RVM lidocaine animals compared to control animals at P8 (unpaired Student's t-test, $P<0.05$; Fig 3.4E). Pinch after discharge was not significantly different between control and RVM lidocaine animals (Mann-Whitney test, Fig 3.4F).

Comparison of stimulus response curves between RVM lidocaine and control animals at P8 revealed no significant effect of RVM lidocaine microinjection on vFh evoked firing activity (Two-way ANOVA with Bonferroni *post hoc* analysis, control vs. lidocaine, $F(1,29)=0.378$, $P=0.543$; Fig 3.4G).

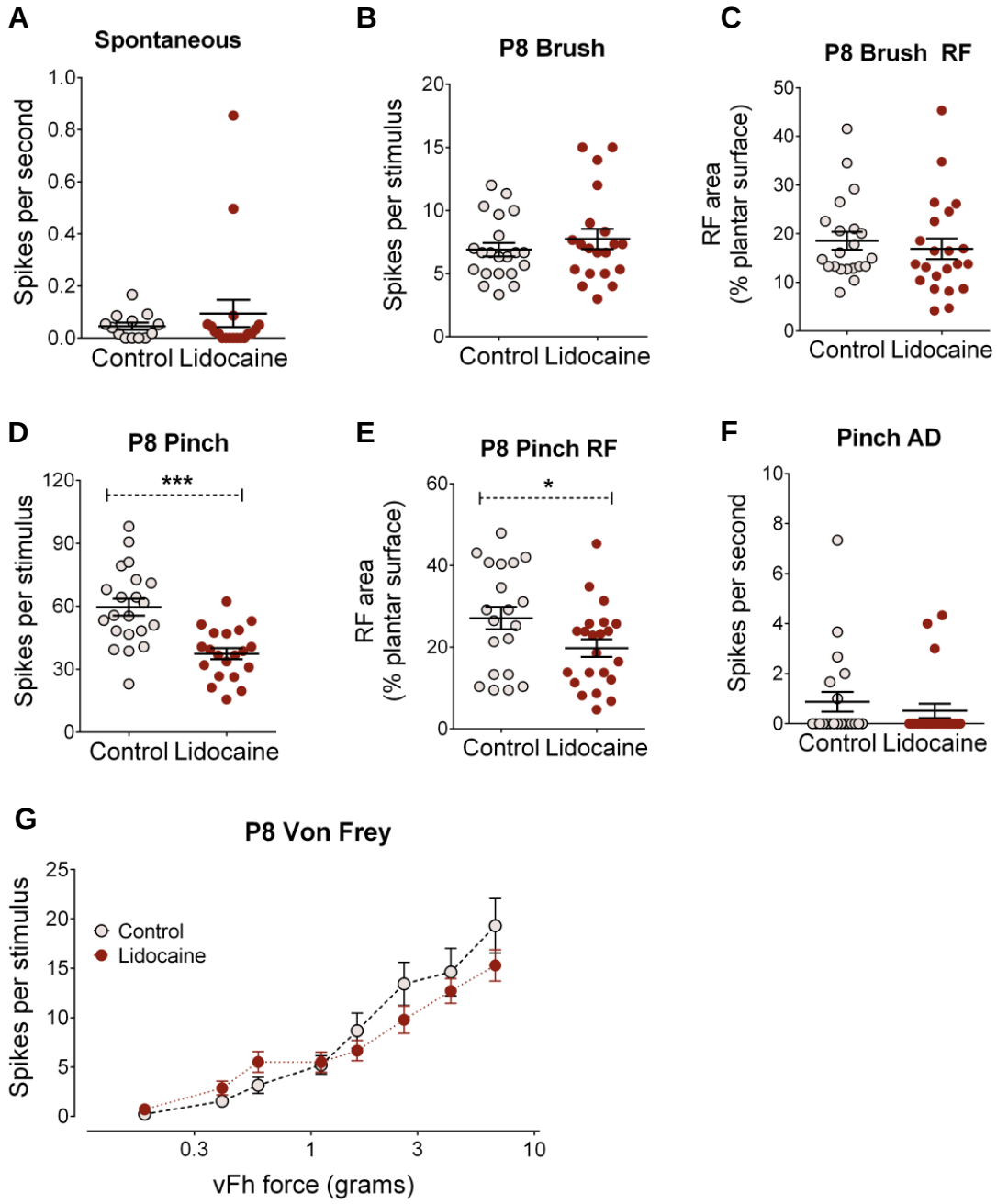

Fig 3.4. Injection of lidocaine into the RVM at P8 inhibits dorsal horn WDR neuron firing. Peripheral stimulus-evoked dorsal hon WDR neuron properties were compared between populations of neurons from RVM lidocaine treated rats (n=22 from 4 rats) and control rats (n=21 from 6 rats) at P8. Spontaneous activity remained low and unchanged in RVM lidocaine treated animals (A). Brush-evoked firing (B) and brush receptive fields (RF) (C) did not significantly differ between control and RVM lidocaine animals. Pinch-evoked firing (D) was significantly lower and pinch RFs (E) were significantly and relatively smaller in RVM lidocaine animals compared to control but pinch after discharge (AD) did not significantly differ between groups (F). von Frey hair (vFh) evoked firing activity did not significantly differ between control and RVM lidocaine groups (G). *,*** P<0.05 and 0.001 respectively, unpaired Student's t-test.

3.4.3 Comparison of dorsal horn WDR neuron activity in P21 control and RVM lidocaine rats

Next, the effect of injecting lidocaine into the RVM upon WDR neuron activity was investigated in P21 rats. Cutaneous receptive fields and evoked spike activity of WDR neurons were recorded in RVM lidocaine animals (n=21 from 4 rats) and control animals (n=26 from 4 rats; including n=15 from 2 RVM saline rats and n=11 from 2 naïve rats). The same stimulation parameters were used as above.

There was no significant difference in spontaneous spike activity of WDR neurons in control and RVM lidocaine treated animals (Mann-Whitney test, Fig 3.5A). Similarly, brush-evoked firing activity and brush receptive field size was not significantly different in RVM lidocaine animals compared to control (unpaired Student's t-test, Figs 3.5B and C). Mean pinch-evoked firing activity was significantly lower and mean pinch receptive field size was significantly and relatively smaller in RVM lidocaine animals compared to control animals (unpaired Student's t-test, $P<0.01$ and Mann-Whitney test, $P<0.05$, respectively; Figs 3.5D and 3.5E). There was no significant difference in pinch after discharge between control and RVM lidocaine animals (unpaired Student's t-test, Fig 3.5F).

A two-way repeated measures ANOVA revealed a significant effect of lidocaine treatment on vFh evoked firing activity (Two-way repeated measures ANOVA, control vs. lidocaine, $F(1,47)=8.488$, $P<0.01$; Fig. 4B). Bonferroni post-hoc analysis revealed significant differences between RVM lidocaine and control cells at 1.1g, 1.6g, 6.7g and 9.8g vFhs (Two-way repeated measures ANOVA with Bonferroni post-hoc analysis, control vs. lidocaine, $P<0.01$ to 0.05 at different vFh forces; Fig 3.5G).

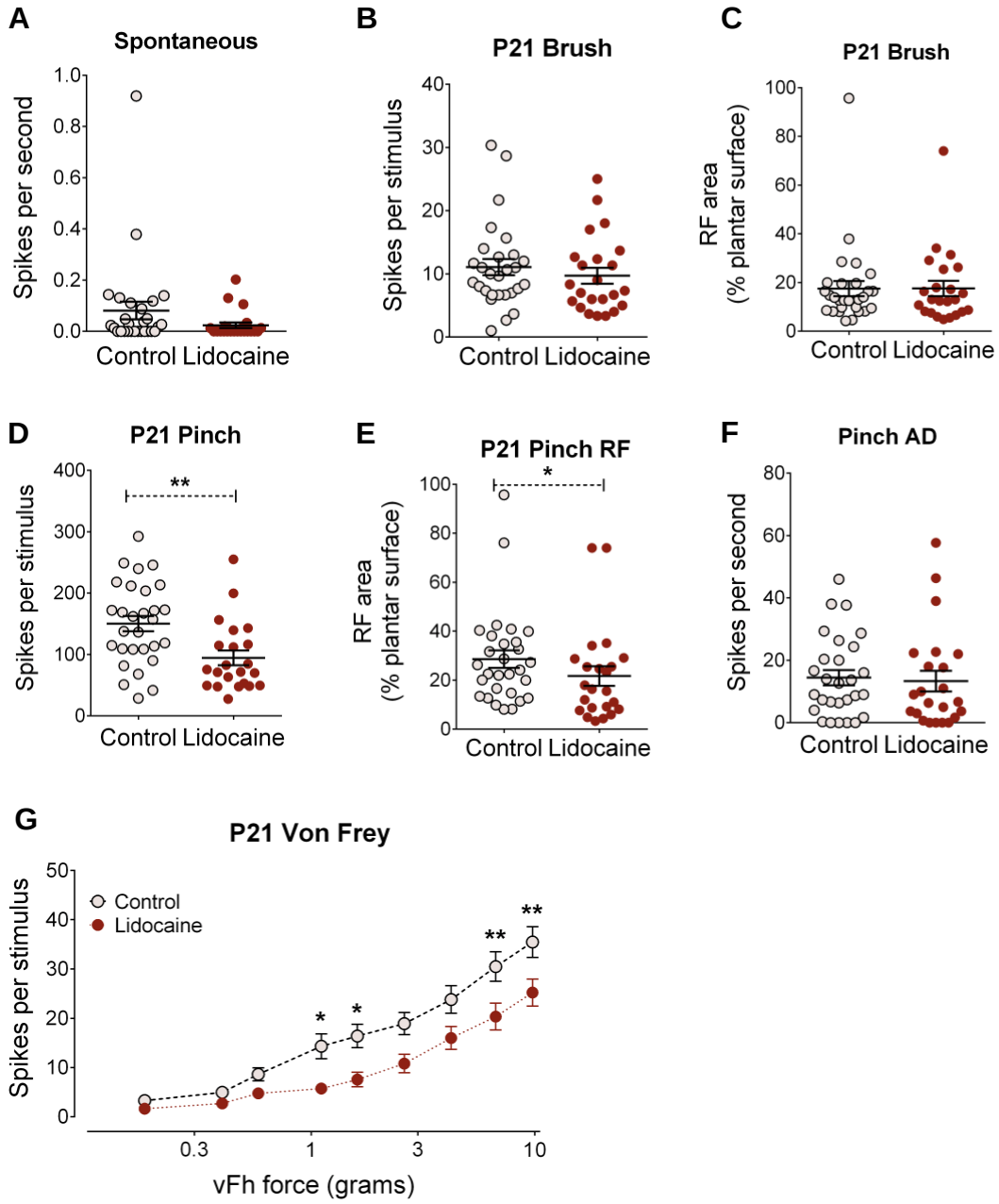

Fig 3.5. Injection of lidocaine into the RVM at P21 inhibits dorsal horn WDR neuron firing. Peripheral stimulus-evoked dorsal horn WDR neuron properties were compared between populations of neurons from RVM lidocaine treated rats (n=21 from 4 rats) and control rats (n=26 from 4 rats) at P21. Spontaneous activity remained low and not significantly different between RVM lidocaine treated animals (A). Brush-evoked firing (B) and brush receptive fields (RF) (C) did not significantly differ between control and RVM lidocaine animals. Pinch-evoked firing (D) was significantly lower and pinch RFs (E) were significantly and relatively smaller in RVM lidocaine animals compared to control. Pinch after discharge (AD) did not significantly differ between groups (F). von Frey hair (vFh) evoked firing activity was significantly lower in RVM lidocaine animals at 1.1g, 1.6g, 6.9g and 9.8g vFhs (G). *,**, *** $P<0.05$, $P<0.01$ and 0.001 respectively, see text for details of statistical analyses.

3.4.4 Comparison of dorsal horn WDR neuron activity in adult control and RVM lidocaine rats

Next, the effect of injecting lidocaine into the RVM upon WDR neuron activity was investigated in adult P40 rats. Cutaneous receptive fields and evoked spike activity of WDR neurons were recorded in RVM lidocaine animals (n=17 from 4 rats) and control animals (n=23 from 7 rats; including n=13 from 2 RVM saline rats and n=10 from 5 naïve rats). The same stimulation parameters were used as above.

There was no significant difference in spontaneous spike activity of WDR neurons in control and RVM lidocaine treated animals (Mann-Whitney test, Fig 3.6A). Similarly, brush-evoked firing activity and brush receptive field size was not significantly different in RVM lidocaine animals compared to control (unpaired Student's t-test, Figs 3.6B and C). In adults, mean pinch-evoked firing activity was significantly higher, pinch after discharge was significantly higher and the mean pinch receptive field size was significantly and relatively larger in RVM lidocaine animals compared to control animals (Mann-Whitney test, $P<0.01$, $P<0.05$ and $P<0.05$ respectively; Figs 3.6D, 3E and 3F).

Injection of lidocaine into the RVM did not significantly alter vFh-evoked firing activity compared to control (Two-way ANOVA with Bonferroni post-hoc analysis, control vs. lidocaine, $F(1,35)=1.361$, $P=0.251$; Fig 3.6G).

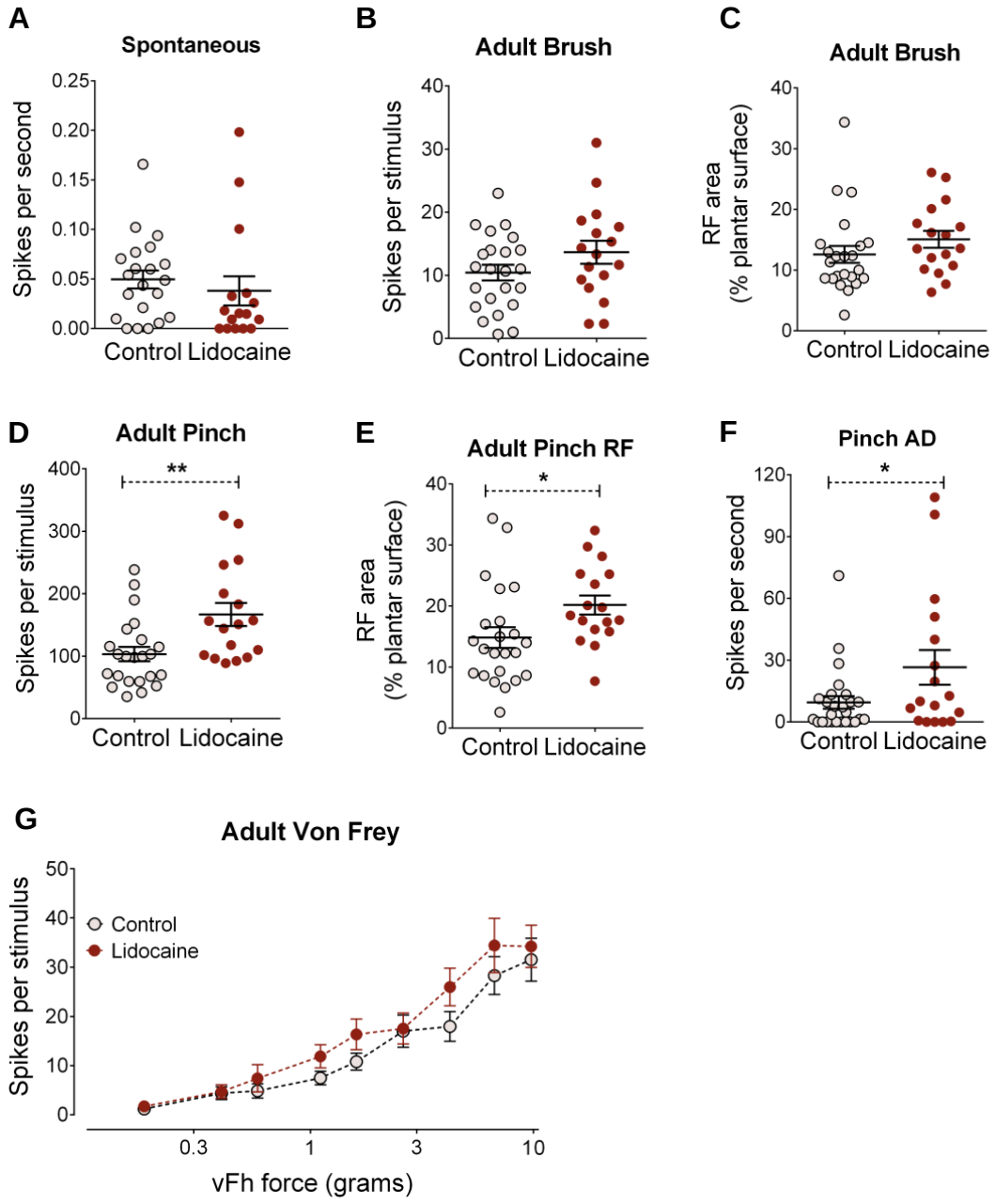

Fig 3.6. Injection of lidocaine into the RVM in adults facilitates dorsal horn WDR neuron firing.

Peripheral stimulus-evoked dorsal horn WDR neuron properties were compared between populations of neurons from RVM lidocaine treated rats (n=17 from 4 rats) and control rats (n=23 from 7 rats) in adults. Spontaneous activity remained low and did not significantly differ between RVM lidocaine treated animals (A). Brush-evoked firing (B) and brush receptive fields (RF) (C) did not significantly differ between control and RVM lidocaine animals. Pinch-evoked firing (D) was significantly higher and pinch RFs (E) were significantly and relatively larger in RVM lidocaine animals compared to control. Additionally, pinch after discharge (AD) was significantly higher in RVM lidocaine animals (F). von Frey hair (vFh) evoked firing activity was not significantly different between RVM lidocaine and control groups (G). *,** $P<0.05$ and 0.01 respectively, see text for details of statistical analyses.

3.4.5 Hindpaw injection of CFA causes lasting mechanical hypersensitivity in P12, P21 and adult rats

The activity of the RVM is known to be altered in adult inflammatory pain states and contributes to pain behaviours (Cleary and Heinricher, 2013). The aims of the following experiments were to investigate the effect of injecting lidocaine into the RVM upon behavioural mechanical withdrawal thresholds following hindpaw inflammation. In preliminary experiments, inflammatory pain states were induced in control P12, P21 and adult P40 rats by subcutaneous injection of Complete Freund's Adjuvant (CFA) into the plantar surface of the left hindpaw. P12 rather than P8 rats were used because experiments in chapter two demonstrated that hindpaw pinch stimulation increases Fos activation in the RVM of P12 but not P8 animals, suggesting that ascending nociceptive inputs activate neurons in the RVM from P12. Here, I aimed to establish the role of descending RVM projections in inflammatory pain states at postnatal ages when descending circuitry is hypothesised to be influenced by ascending sensory inputs in young and older rats.

25µl (containing 25µg) of CFA was injected into the left hindpaw of 6 P12 and 5 P21 rats and 100µl (containing 100µg) of CFA was injected into the left hindpaw of 4 adult P40 rats. VFh evoked mechanical withdrawal thresholds of left and right hindpaws were measured at baseline, 1 hour after CFA injection and for up to 10 days after.

Intraplantar injection of CFA into the left hindpaw of P12 and P21 rats decreased ipsilateral mechanical vFh thresholds compared to contralateral thresholds 1hr after inflammation and showed no sign of recovery 10 days after inflammation (Two-way repeated measures ANOVA, P21 ipsi vs. contra, $F(1,10)=309.7$, $P<0.001$, with Bonferroni *post hoc* test, $P<0.001$ at all timepoints; Fig 3.7A), (Two-way repeated measures ANOVA, P21 ipsi vs. contra, $F(1,8)=1756$, $P<0.001$, with Bonferroni *post hoc* test, $P<0.001$ at all timepoints; Fig 3.7B). Intraplantar injection of CFA into the left hindpaw of adult P40 rats caused a similarly prolonged reduction in mechanical withdrawal threshold (Two-way repeated measures ANOVA, P40 ipsi vs. contra, $F(1,6)=115.7$, $P<0.001$, with Bonferroni *post hoc* test, $P<0.001$ at all timepoints; Fig 3.7C).

A three-way repeated measures ANOVA of the ipsilateral vFh thresholds across time and between the three ages demonstrated a significant effect of age on CFA-induced vFh withdrawal threshold (Three-way repeated measures ANOVA, P12 vs P21 vs adult; $F(2,12)=62.90$; $P<0.001$).

A one-way ANOVA comparison of baseline mechanical withdrawal thresholds in P12, P21 and adult P40 rats demonstrated that age was a significant factor and Bonferroni post hoc analysis demonstrated that thresholds were lower in younger animals and significantly increased with age (One-way ANOVA, P12 vs P21 vs P40, $F(2,12)=19.14$; $P<0.001$; with Bonferroni *post hoc* test $P<0.001$ across all ages; Fig 3.7D). Before baseline data was converted and normalised as described by Chaplan et al. (1994), vFh evoked nocifensive hindpaw withdrawal thresholds were most commonly observed at the following forces: P12 = 0.82g to 3.97g; P21 = 2.27g to 6.39g; adult = 6.89g to 25.00g.

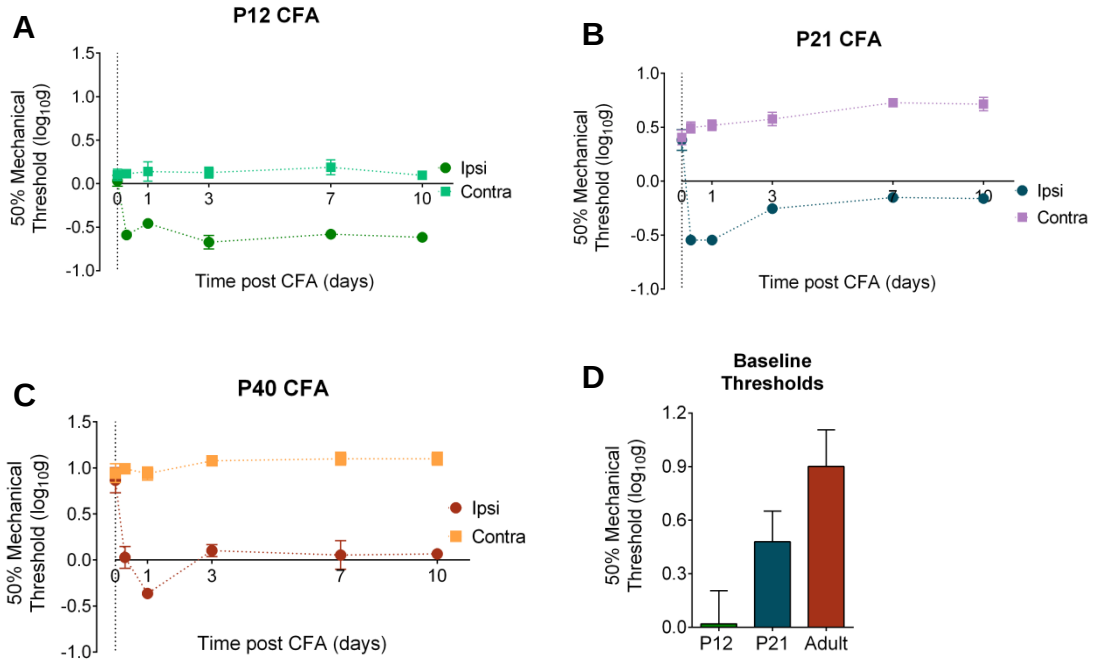

Fig 3.7. Hindpaw CFA inflammation reduces behavioural vFh-evoked withdrawal thresholds
Complete Freund's adjuvant (CFA) was injected into the left hindpaw of P12 (n=6), P21 (n=5) and adult P40 (n=4) rats. Ipsilateral and contralateral vFh withdrawal thresholds were established at baseline and for up to 10 days after CFA injection. Injection of CFA into the hindpaw reduced ipsilateral vFh withdrawal thresholds in P12 rats (A), P21 rats (B) and adult rats (C) compared to contralateral. Baseline mechanical withdrawal thresholds recorded before CFA inflammation increased with postnatal age (D).

3.4.6 Injection of lidocaine into the RVM increases vFh thresholds in CFA-treated P21 and adult animals

Next, the role of RVM activity upon CFA-induced behavioural hypersensitivity was investigated at different ages by microinjecting 2% lidocaine into the RVM. CFA was injected into the left hindpaw of P12 (n=4), P21 (n=4) and adult P40 (n=4) rats 30 minutes prior to RVM lidocaine injection and mechanical withdrawal thresholds were established up to 90 minutes after RVM lidocaine injection (Fig 3.8D). Time=0 therefore represents the time of RVM lidocaine injection. Additionally, saline was injected into the RVM of control animals (n=2 per age), and vFh threshold data after CFA injection was compared to age matched data from animals which only received hindpaw CFA injection. As data from RVM saline+CFA animals matched data from CFA only control animals, these data were pooled to create CFA-only control groups (P12 n=8, P21 n=7, adult n=6) which were compared to CFA-lidocaine animals.

In P12 animals, RVM lidocaine did not significantly change mechanical withdrawal thresholds compared to CFA-only control animals (Two-way repeated ANOVA, P12 CFA-only vs CFA-lidocaine, $F(1,10)=2.270$, $P=0.131$, with Bonferroni *post hoc* test, $P>0.05$; Fig 3.8A). At P21, two-way ANOVA with Bonferroni post hoc analysis revealed that RVM lidocaine was a significant factor, and CFA-lidocaine animals had significantly higher vFh withdrawal thresholds compared to age-matched CFA-only animals at 15, 30, 45 and 60 minutes after RVM lidocaine injection (Two-way repeated measures ANOVA, P21 CFA-only vs. CFA-lidocaine, $F(1,9)=17.73$, $P<0.01$, with Bonferroni *post hoc* test, $P<0.01$ to $P<0.001$ at different time points; Fig 3.8B). Similarly in adult animals, two-way ANOVA with Bonferroni post hoc analysis revealed that RVM lidocaine was a significant factor, and CFA-lidocaine animals had significantly higher vFh withdrawal thresholds compared to age-matched CFA-only animals at 15, 45 and 60 minutes after RVM lidocaine injection (Two-way repeated measures ANOVA, P40 CFA-only vs. CFA-lidocaine, $F(1,8)=8.578$, $P<0.05$, with Bonferroni *post hoc* test, $P<0.05$ at different time points; Fig 3.8C). By 90 minutes after RVM lidocaine injection, mechanical withdrawal thresholds decreased to CFA-only animal levels in both P21 and adult animals (Figs 3.8B and C).

Importantly, microinjection of lidocaine or saline into the RVM had no significant effect on contralateral vFh withdrawal thresholds at any age when compared to baseline, demonstrating that anaesthesia and surgery had no effect on vFh thresholds (Figs 3.8A, B and C).

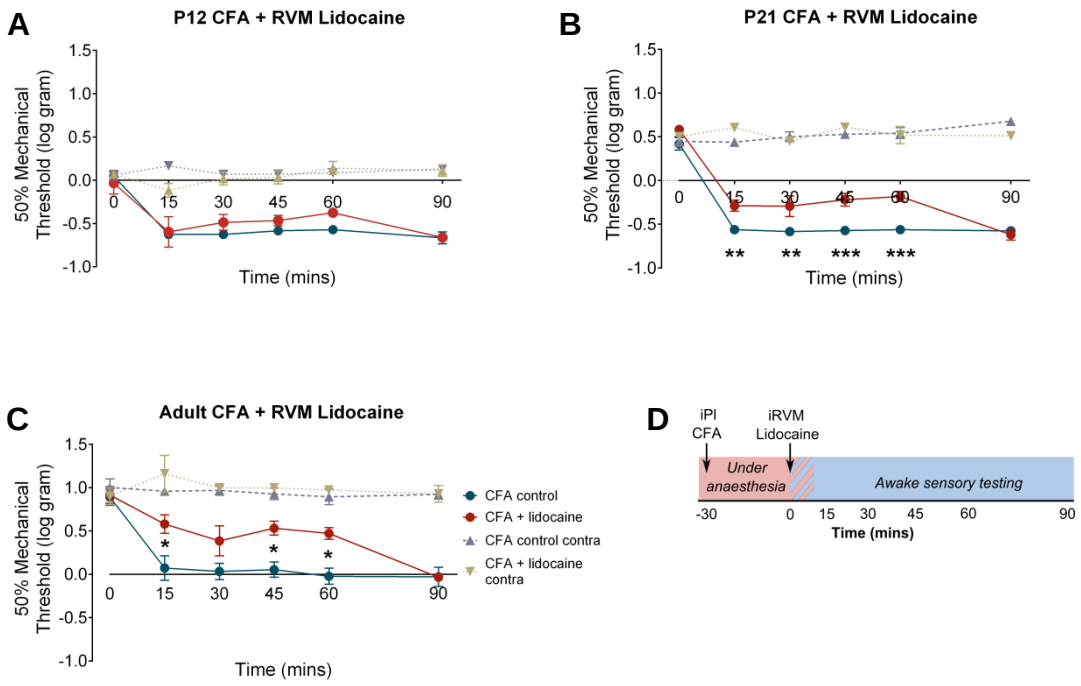

Fig 3.8. RVM lidocaine reduces CFA-induced mechanical hypersensitivity at P21 and P40. Complete Freund's adjuvant (CFA) was injected into the left hindpaw of anaesthetised control animals (P12 n=8, P21 n=7, adult n=6). In lidocaine treated animals, this CFA injection was followed by microinjection of lidocaine into the RVM of P12 (n=4), P21 (n=4) and adult (n=4) rats. Rats were then woken up and vFh stimuli were applied to the ipsilateral and contralateral hindpaws, as illustrated (D). VFh withdrawal thresholds of P12 animals injected with intra plantar (iPl) CFA+RVM lidocaine did not significantly differ compared to control animals which only received iPl CFA ± RVM saline (A). At P21 (C) and in adult P40 rats (D), CFA+RVM lidocaine animals had significantly higher vFh withdrawal thresholds for 60 minutes after lidocaine injection compare to control animals before returning to control levels after 90 minutes. *,**, *** $P<0.05$, $P<0.01$ and 0.001 respectively, Two-way repeated measures ANOVA with Bonferroni *post-hoc* analysis.

3.4.7 Summary of results

The data presented above can be summarised as follows:

1. Deep dorsal horn WDR neuron firing activity and cutaneous hindpaw receptive field properties change with postnatal age. At P8, brush and pinch and vFh-evoked firing activities were low compared to P21 and adult P40 rats. The relative size of cutaneous hindpaw pinch receptive fields also reduced between P21 and P40.
2. At P8 and P21, injecting lidocaine into the RVM decreased deep dorsal horn WDR neuron pinch-evoked firing and receptive field properties. At P21, WDR neuron vFh-evoked firing activity was also reduced in RVM lidocaine rats.
3. Injecting lidocaine into the RVM of adult rats increased deep dorsal horn WDR neuron pinch-evoked firing activity and receptive field size.
4. Intraplantar injection of CFA produced lasting behavioural mechanical hypersensitivity at P12, P21 and P40.
5. Injecting lidocaine into the RVM 30 minutes after hindpaw CFA inflammation increased vFh-evoked behavioural withdrawal thresholds compared to CFA-only animals, both at P21 and in adult rats. RVM lidocaine had no effect on CFA-induced mechanical hypersensitivity at in P12 rats.

3.5 Discussion

In this chapter I aimed to investigate the role of the RVM in modulating spinal sensory circuitry during development in uninjured and injured animals. Previous studies investigating the role of RVM descending modulation during postnatal development have exogenously activated RVM neurons using electrical stimulation protocols (Hathway et al., 2009a, 2012; Koch and Fitzgerald, 2014). Here, I focally injected lidocaine into the RVM to investigate the endogenous and ongoing role of RVM descending modulation over spinal sensory circuitry during development.

3.5.1 Technical considerations

By searching for deep dorsal horn WDR neurons with hindpaw cutaneous receptive fields I sought to record from a homogenous population of neurons which can be reliably and robustly compared across numerous ages and conditions. Neuronal populations sampled in data presented in this chapter may not represent the dorsal horn as a whole but do represent a population of deep dorsal horn neurons with common electrophysiological properties. In doing so, developmental changes in electrophysiological firing and receptive field properties of more superficial spinal dorsal horn neurons may have been overlooked.

When searching for and recording from deep dorsal horn WDR neurons in electrophysiology experiments, only cells with receptive fields on the glabrous plantar surface of the hindpaw were chosen. The afferent inputs from hairy and glabrous skin are notably different. For example: a larger proportion of C-polymodal nociceptors innervate the hairy skin compared to the glabrous skin of the rat hind foot (Leem et al., 1993), and hairy skin C-nociceptors are more readily sensitised by conditioning stimuli compared to those that innervate glabrous skin (Andrew and Greenspan, 1999). Moreover, thermal hyperalgesia caused by CFA-induced inflammation of glabrous foot skin outlasts that caused by inflammation of hairy skin (Drake et al., 2014). This shorter lasting thermal hyperalgesia following hairy-skin inflammation could be reversed by intrathecal administration of the α-2 adrenoceptor antagonist yohimbine, suggesting that descending noradrenergic influences from the brainstem differently modulate hairy and glabrous skin afferent inputs (Drake et al., 2014). The selection of WDR neurons with glabrous receptive fields in experiments in this chapter therefore restricts conclusions being extended to neurons receiving afferent inputs from hairy skin.

When performing between-age comparisons of spike activity using statistical methods in these data sets, some considerations must be acknowledged. Changing skin thicknesses and paw sizes during development means that the magnitude of stimulus-evoked peripheral nociceptor activation may differ across the ages tested. At the primary afferent neuron synapse, dorsal horn WDR neuron spike activity may not reflect the same relative degree of primary afferent inputs across the ages. Younger animals may therefore receive a greater degree of afferent input from the same pinch stimulus used for older animals, which may limit statistical comparisons of WDR neuron spike activity between ages.

Sex-related differences in nociceptive circuitry and pain states have been a major focus of research over the last two decades. Prevalence of chronic pain syndromes is generally higher in females (for review see Mogil, 2012), and laboratory studies have identified key sex-related differences in nervous, hormonal and immune system processing of chronic pain states (LaPrairie and Murphy, 2010; Sorge et al., 2015). In the present data, sex-related differences were not investigated despite the combined use of male and female rats. In all electrophysiology data sets, a population of WDR neurons was recorded from both male and female rats; however too few animals were used to investigate sex-related differences using statistical analyses. Some sex-related differences in WDR neuron processing of formalin-induced persistent nociception have been shown in adult rats (You et al., 2006), but a paucity of data precludes firm conclusions being drawn. Indeed, previous studies exclusively used male rats (Bee and Dickenson, 2007), used males and female rats but did not investigate sex-related differences (Koch et al., 2012; Koch and Fitzgerald, 2014), or did not state the sex of rats used (Hathway et al., 2009a). Sex-related differences may have been overlooked in these studies and in the present data sets and in those of other chapters, however investigating these differences was not the main aim of this thesis.

A possible confound for data in this chapter arises from the acidity of lidocaine. The lidocaine injectate used in this chapter had a pH of 5.4, and was not buffered to a physiological pH of 7.4. The preparation of the lidocaine solution was taken from Bee and Dickenson (2007) who did not adjust for the acidity. Many others have also not adjusted for, or did not state, the acidity of lidocaine when injecting into the RVM and PAG (Pertovaara et al., 1996, 1997; Pertovaara, 1998; Cleary and Heinricher, 2013; Wang et al., 2013). The brainstem, including the RVM, contains an abundance of acid sensing ion channels (ASICs) which contribute to chemosensitivity of respiratory control neurons (Cao et al., 2009; Huda et al., 2012). It is possible that the acidity of

the lidocaine injectate activated ASICs in the brainstem, causing cation influx and depolarisation of RVM neurons. However, lidocaine has been shown to *inhibit* ASIC1 in cultured cortical neurons, regardless of the pH (Lin et al., 2011), thus it is unlikely that the acidic lidocaine injectate used here would activate ASICs in the RVM. Unbuffered lidocaine injection into the spinal cord causes reliable conduction block (silencing) within 4 minutes (Sandkühler et al., 1987), and as WDR neuron recordings were performed 15 minutes after RVM lidocaine injection in these experiments, it is likely that RVM neuronal activity was silenced at this time point. Additionally, lidocaine was injected slowly over a five minute period, meaning that the mildly acidic solution will be rapidly buffered, especially as isoflurane increases cerebral blood flow (Cucchiara et al., 1974; Olsen et al., 1994). Importantly, no changes in heart rate were observed following RVM lidocaine injection.

Without the combined injection of a dye and lidocaine, it was not known how far the lidocaine solution spread in the RVM. Based on previous injections in the adult, I predicted that 1.0µl and 0.7µl of lidocaine solution would supress neuronal activity within a radius of ~1.5mm and 1mm respectively (Sandkühler et al., 1987). 2% lidocaine solution has shown to be an effect method of silencing neuronal activity (Sandkühler et al., 1987), however it was not confirmed in this chapter whether the effects of lidocaine injection on dorsal horn neuron electrophysiological properties were due to silencing of RVM neuron activity. It is inevitable that lidocaine will also silence fibres of passage which run through the brainstem; therefore these effects cannot be discounted.

3.5.2 Deep dorsal horn neuron electrophysiological properties change during postnatal development

Here, I recorded sensory-evoked firing activity and receptive field sizes of deep dorsal horn WDR neurons at P8, P21 and P40. Generally, brush, vFh and pinch-evoked firing responses and the propensity for pinch stimulus after-discharge were lower at P8 compared to P21 and P40. Additionally, in agreement with previous data, hindpaw cutaneous pinch receptive fields were relatively larger in P8 and P21 rats compared to adult P40 rats.

The neonatal dorsal horn is dominated by A-fibre afferent inputs which have expansive terminal patterns diffusely distributed throughout the superficial and deep dorsal horn before near adult-like distribution of A-fibre terminals is established by P21-P28 (Beggs et al., 2002; Granmo et al., 2008). Electrophysiology experiments have also

demonstrated that neurons in lamina II receive a greater degree of monosynaptic Aβ inputs in neonatal animals (Park et al., 1999; Nakatsuka et al., 2000). High threshold C-fibre synaptic input to the dorsal horn matures slowly over the first postnatal weeks. Stimulation of peripheral C-fibres by heating or mustard oil application evokes little Fos expression in the neonatal dorsal horn, but increasingly evokes Fos and pERK activity during the second postnatal week (Yi and Barr, 1995; Walker et al., 2007). It is reasonable to argue that low pinch-evoked WDR neuron firing in P8 animals may reflect immature C-fibre synapses at this age. Indeed, C-fibre stimulation fails to activate deep dorsal horn neurons until P7-P8, but activates superficial dorsal horn neurons in the first days of life (Fitzgerald, 1985). As monosynaptic C-fibre synapses and polysynaptic C-fibre driven circuits are strengthened in the second and third postnatal weeks, pinch-evoked firing activity of deep dorsal horn neurons increases with age. A large component of pinch-evoked firing activity is, however, mediated by Aδ-inputs which likely drive nociceptive responses in neonatal animals.

Relatively large cutaneous receptive fields are a hallmark property of neonatal dorsal horn neurons (Fitzgerald, 1985; Fitzgerald and Jennings, 1999; Torsney and Fitzgerald, 2002). Indeed, pinch receptive field sizes here were relatively large and diffuse at P8 and P21 and were refined to a smaller area of the hindpaw in adults. Dorsal horn neuron cutaneous receptive fields are labile in the adult, and can be acutely enlarged by a brief nociceptive afferent barrage (Woolf and King, 1990) or by hindpaw inflammation and skin incision (Ren and Dubner, 1996; Zahn and Brennan, 1999). Moreover, inflammation in the first postnatal week can permanently expand dorsal horn neuron receptive fields in adulthood (Torsney and Fitzgerald, 2003). The mechanisms underlying the refinement of neonatal dorsal horn neuron receptive fields are likely to be akin to those regulating general excitability in the developing dorsal horn. Firstly, extensive sprouting of A-fibres in the neonatal superficial and deep dorsal horn are pruned and reorganised to terminate in laminae III-IV by P28 (Beggs et al., 2002; Granmo et al., 2008). These large and diffuse termination patterns of A-fibres may contribute to extensive dorsal horn neuron cutaneous receptive fields during the first weeks of life, especially when strong Aδ-inputs are thought to dominate during a period of C-fibre synapse strengthening (Koch and Fitzgerald, 2013). Other major contributing factors are presumably through the onset of local inhibitory controls from the second postnatal week (Koch et al., 2012) and descending inhibitory controls over the subsequent weeks (Fitzgerald and Koltzenburg, 1986).

3.5.3 The RVM provides background descending facilitation in uninjured young rats

Here, I focally injected lidocaine into the RVM at different ages to investigate the endogenous and ongoing role of the RVM in modulating dorsal horn sensory inputs during postnatal development. At P8 and P21, injecting lidocaine the RVM reduced pinch-evoked dorsal horn neuron firing and receptive field size, demonstrating that the RVM exerts an endogenous and net facilitation of dorsal horn WDR neuron processing of nociceptive inputs in young animals. Because RVM stimulation selectively facilitates deep dorsal horn neuron processing of A-fibre (presumed Aβ and Aδ) inputs in P21 rats (Koch and Fitzgerald, 2014), I hypothesised that brush-evoked WDR neuron firing would be altered following RVM lidocaine. Aβ-fibre (and presumably low threshold C-tactile fibre) mediated brush-evoked firing activity and receptive fields were not changed by RVM lidocaine at any age, however, some facilitation of WDR neuron processing of low-force punctate mechanical stimuli in the non-noxious range was observed in P21 rats, suggesting some descending modulation of punctate non-noxious mechanical inputs.

In young animals, exogenous electrical stimulation of the RVM facilities dorsal horn neuron firing activity (Koch and Fitzgerald, 2014) and spinal reflexes (Hathway et al., 2009a, 2012) but drives pronociception or antinociception in adults (Zhuo and Gebhart, 1997). Stimulation-evoked RVM descending inhibition of C-fibre inputs observed in adults is absent in young P21 animals, and instead targets and facilitates A-fibre inputs (Koch and Fitzgerald, 2014). Microinjection of the μ-opioid receptor agonist DAMGO into the PAG of P21 animals also evokes descending facilitation of spinal reflexes, and appears to be specific to that age, as DAMGO did not change spinal reflex excitability at P10, but inhibited spinal reflexes in adult rats (Kwok et al., 2013). Data in this chapter strongly agrees with these previous findings that descending brainstem areas predominantly excite spinal nociceptive circuits in young animals; however, this is drawn from experiments that primarily *evoked* descending modulation by directly stimulating the RVM or PAG. A modicum of data about the endogenous role of the RVM has shown that pharmacologically ablating RVM neurons in P3 and P21 rats reduces behavioural withdrawal thresholds (Hathway et al., 2009a), again demonstrating descending facilitation, but separation of the effects on sensory and motor circuitry is challenging in these results. By injecting lidocaine into the RVM to silence RVM neuron activity, results in this chapter give direct evidence for endogenous descending RVM modulation of spinal dorsal horn neuron populations in neonatal rats.

In the previous chapter I demonstrated that noxious recruitment of the ascending arm of the spinal-bulbo-spinal loop does not increase Fos immunoreactivity in the PAG and RVM until P12. One important finding in this chapter is that injecting lidocaine into the RVM to block RVM neuron activity at P8 unmasks ongoing net descending facilitation that: does not require bottom up or top down recruitment; is seemingly selective to noxious inputs; and does not modulate the spontaneous activity of dorsal horn neurons. Without the capacity to be recruited by feedforward activation, the immature RVM would presumably be intrinsically active. Spontaneously firing pacemaker neurons have been well described in the neonatal spinal cord (Baccei, 2014), and a greater propensity for spontaneous firing activity has been observed in the neonatal RVM compared to adulthood (Li et al., 2015b). It is therefore reasonable to suggest that in the immature state of a 'sensory unreactive' RVM, intrinsically active circuits within the RVM drive descending facilitation in the first days of postnatal life. Further experiments using patch clamp techniques in the neonatal RVM could provide additional information about this hypothesis.

3.5.4 The impact of early descending facilitation of spinal sensory circuits

A state of endogenous RVM descending facilitation would provide ongoing excitation of deep dorsal horn nociceptive inputs. It could be postulated that this ongoing descending RVM facilitation may amplify Aδ-input driven excitation of deep dorsal horn neurons during the first weeks of life and increase general excitability of the developing sensory circuitry. Lamina V is a major output region of the dorsal horn, including distinct neuronal populations which project to the ventral horn (Schouenborg et al., 1995) and to the PB nucleus (Todd et al., 2000) which respectively drive sensory-motor reflex circuitry and ascending nociceptive circuits. Selective facilitation of deep dorsal horn processing of noxious Aδ-driven inputs provides a mechanism for strengthening modality-specific pathways involved in nociception, pain and nocifensive reflex behaviours but not non-noxious tactile inputs. The RVM therefore may act as a saliency driver of nociceptive stimuli in neonatal animals and importantly amplifies processing of nociceptive stimuli above the level of heightened excitability of low-threshold inputs during the first weeks of life.

The onset of peripheral noxious stimulation-induced PAG and RVM neuron activation at P12 (see chapter 2) coincides with C-fibre synaptic strengthening (Koch et al., 2012), therefore establishment of C-fibre-mediated recruitment of descending RVM pathways may further drive dorsal horn sensory circuit maturation as part of a positive feedback

loop. As C-fibre mediated pathways mature, the PAG-RVM becomes more responsive to ascending nociceptive inputs and becomes a saliency detector (a hypothesis coined by Hellman and Mason, 2012) as well as a saliency driver; providing modulation of inputs to the dorsal horn as part of a complete and responsive spinal-bulbo-spinal loop. Preferential descending PAG facilitation of dorsal horn neurons which only receive A-fibre inputs and PAG inhibition of dorsal horn neurons which receive A- and C-fibre inputs has been reported in adult rats, (Waters and Lumb 2008). Current evidence suggests that descending modulation arising from the RVM has a degree of selectivity in young rats too: descending facilitation is selective to A-fibre mediated dorsal horn neuron activity during the first weeks of life (Koch and Fitzgerald, 2014), and C-fibre evoked deep dorsal horn activity is predominantly inhibited (albeit weakly) from P12 (Fitzgerald and Koltzenburg, 1986). Moreover, facilitation of deep dorsal horn processing of punctate tactile, but not dynamic brush, inputs observed at P21 suggests some facilitation of non-noxious inputs (presumed to be mediated by LTMR Aβ-fibre and/or C-tactile fibres) at this age. Descending inhibitory drive matures in parallel with strengthening of C-fibre synapses in the young rat, suggesting that these the maturation of these two systems are linked.

Figure 3.9 illustrates a proposed model of descending modulation of dorsal horn circuitry during development. This theory of a saliency driving and nociceptive selective descending facilitatory system in young animals stems predominantly from functional data, but unfortunately the anatomical connectivity of nociceptive circuitry in young animals is not well understood.

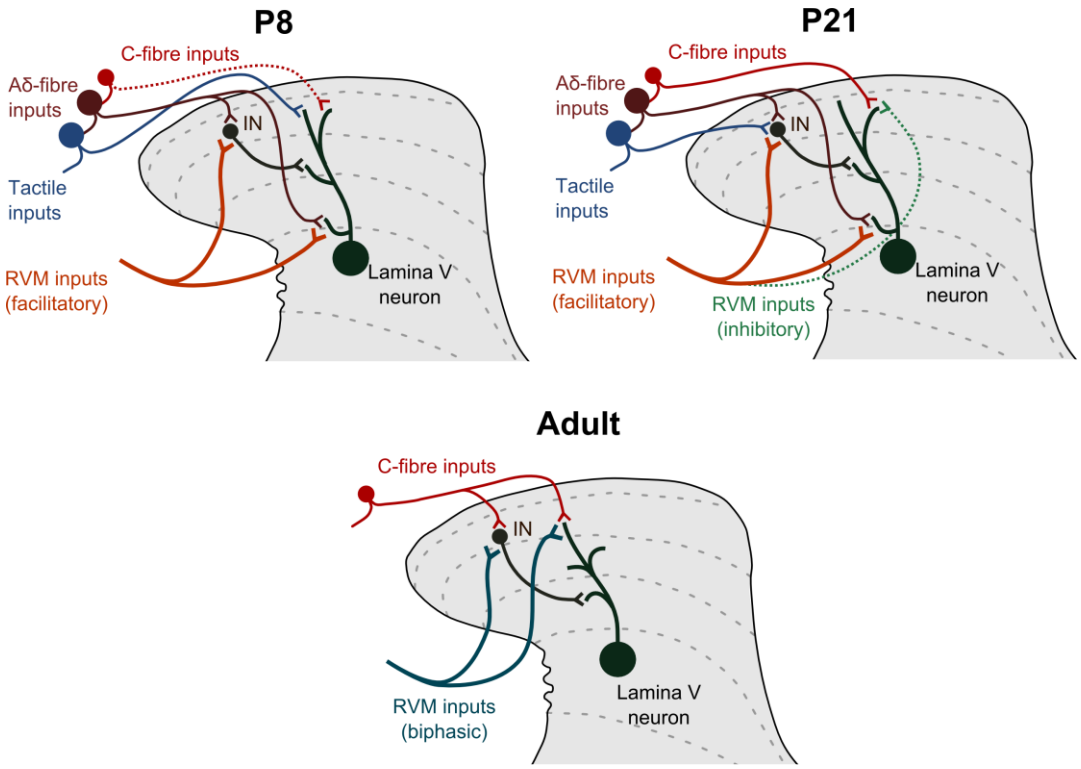

Fig 3.9. A proposed model of descending RVM neuron modulation of deep dorsal horn neuron activity during development.
At P8, RVM neurons target Aδ-fibre driven nociceptive processing in deep dorsal horn WDR neurons. C-fibre inputs are weak (dotted light red line) and descending inhibition is absent at this age. In adolescent P21 rats, RVM neurons target non-C-fibre driven (presumed Aδ-fibre) nociceptive processing in WDR neurons. Some descending facilitation of WDR neuron processing of punctate tactile, but not dynamic brush, inputs is observed at this age. Weak descending inhibition (dotted green line) of C-fibre inputs is observed at P21. In comparison, WDR neuron processing of C-fibre inputs are selectively inhibited or facilitation in adult animals.

3.5.5 Mechanisms underlying postnatal maturation of descending inhibition of spinal nociception

Here, I demonstrated that injecting lidocaine into the RVM unmasks net descending inhibition of pinch-evoked WDR neuron firing activity in adult animals. It is important that the results in this chapter are not interpreted to suggest that the biphasic role of the RVM during development is absolute, switching from facilitation to inhibition. Descending RVM modulation is not a simple 'on-off' mechanism, but rather a system where inhibition and facilitation can operate in parallel to produce a balanced net outcome. The aim of a population based analysis in these experiments was to unmask the net effect of descending modulation, but without intra-cell baseline comparisons, the range of effects of silencing the RVM has upon individual dorsal horn neurons cannot be educed (see Bee and Dickenson, 2007). It is therefore unlikely that *all* adult or P21 WDR neurons sampled in the current data were inhibited or facilitated,

respectively, following RVM silencing. Even though the net effect of descending modulation from the RVM at P21 is facilitation, weak descending inhibition can be evoked at this age by electrical stimulation of the PAG (van Praag and Frenk, 1991) or the DLF (Fitzgerald and Koltzenburg, 1986).

Pharmacological or electrical stimulation of the RVM or PAG in the adult rat reliably inhibits deep dorsal horn neurons with strong C-fibre inputs (McMullan and Lumb, 2006a, 2006b; Waters and Lumb, 2008; Koch and Fitzgerald, 2014). Whilst this study cannot differentiate between A and C-fibre recruitment during pinch-stimulation, it is likely that C-fibre inputs are predominantly targeted by descending RVM neurons in the adult.

Interestingly, the onset of descending inhibition during development is dependent upon functional C-fibre inputs. Neonatal ablation of C-fibres by capsaicin treatment has been shown to result in a deficit in descending inhibition of nocifensive reflexes when the animal reaches maturity (Zhuo and Gebhart, 1994). Collectively, these findings can be interpreted in two ways: firstly that descending inhibition is driven by strong C-fibre inputs, and as these circuits mature over the second and third postnatal weeks, so does the strength of descending inhibition of spinal sensory circuits; secondly, descending inhibitory synapses target C-fibre synapses and deep dorsal horn neurons with strong C-fibre inputs, and as C-fibre synapses mature, descending RVM neurons target and inhibit these synapses. Thus, the increasing strength of descending inhibition during development correlates with the timing of strengthening dorsal horn C-fibre inputs, and vice versa.

The development of descending inhibitory modulation of dorsal horn sensory circuitry has also been shown to be dependent on constitutive opioidergic activity in the RVM. Blocking opioidergic activity in the RVM between P21 and P28, but not earlier, prevents the normal maturation of descending inhibition (Hathway et al., 2012). Indeed, substantial changes in endogenous opioid signalling takes place in the PAG and dorsal horn through development and may underlie this opioid-mediated maturation (Kwok et al., 2013). GABAergic transmission in the RVM also changes during development. Patch clamp experiments in the RVM from young (P10-21) and adult rat brainstem slices have demonstrated that the probability of GABA release is higher in the young RVM (Li et al., 2015b). Additionally, endocannabinoids tonically reduce GABA release in the adult RVM neurons, but fail to do so in young RVM neurons (Li et al., 2015b), suggesting that endocannabinoid-mediated GABA release may be crucial for maintaining the normal balance of excitation and inhibition in the

adult RVM. In young animals, increased GABA release may disinhibit descending RVM neurons and drive ongoing descending facilitation of dorsal horn neuron nociceptive inputs. Whilst no major differences were observed between RVM On and Off cell firing properties in P21 and adult animals (Devonshire et al., 2015), it is still possible that changes in the proportion and connectivity of the two cell types may change with age and influence descending modulatory output from the RVM.

3.5.6 The RVM facilitates behavioural hypersensitivity during an acute peripheral inflammatory state in P21 and P40, but not P12 rats

Experiments in this chapter demonstrated that injection of lidocaine into the RVM reduced mechanical hypersensitivity caused by prior intraplantar CFA injection in adult rats. This attenuation of mechanical hypersensitivity was selective for the inflamed ipsilateral limb and lasted for 60 minutes before mechanical thresholds returned to the same readily excitable level observed in CFA-only control animals. The same effect was observed at P21, demonstrating that the RVM both responds and contributes to acute inflammatory pain states in adolescent rats. Silencing the RVM at P12 had no effect on CFA-induced mechanical hypersensitivity, suggesting the onset of inflammation does not drive descending facilitation from the RVM at this age.

In early stages of inflammatory pain states, the adult RVM exerts a pronociceptive effect (Urban et al., 1996, 1999b; Kincaid et al., 2006; Cleary and Heinricher, 2013) by exciting dorsal horn neuron responses to sensitised peripheral noxious inputs (Pertovaara, 1998). Experiments in adult rats in this chapter support these previous findings. A body of evidence has demonstrated that this is mediated by NMDA receptor-mediated activity in the RVM (Coutinho et al., 1998; Urban et al., 1999a; Guan et al., 2002). Conversely, during the later chronic stage of inflammatory pain states, the RVM exerts an antinociceptive effect (Cleary and Heinricher, 2013) which is mediated by delayed recruitment of AMPA/kainate and NMDA receptors in the RVM (Coutinho et al., 1998; Guan et al., 2002, 2003, 2004).

Of note, injection of lidocaine into the RVM had no effect on uninjured contralateral withdrawal thresholds of animals at any age, suggesting that descending facilitatory pathways may not excite ballistic nocifensive withdrawal thresholds in uninjured body regions. Previously, kainate ablation of RVM neurons has been shown to have an antinociceptive effect in uninjured P3 and P21 animals, but not at P40 (Hathway et al., 2009a), demonstrating age-specific descending RVM facilitation of acute nocifensive behaviours. Figure 3.10 illustrates the proposal that descending facilitatory control of

spinal noxious activity in the early postnatal period arises from spontaneous activity within the RVM and only later in development can this descending activity be modulated by ascending sensory inputs in a spinal-bulbo-spinal loop.

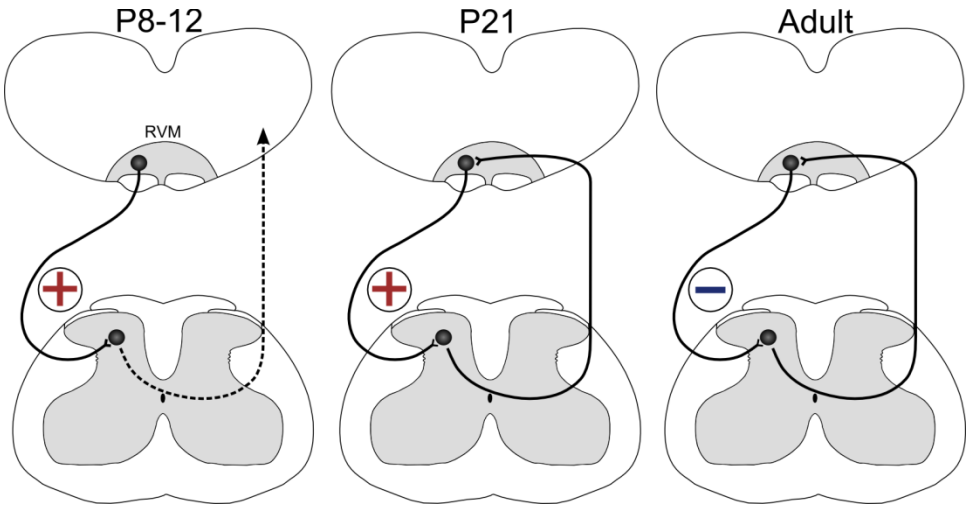

Fig 3.10. A proposed model of endogenous descending modulation during postnatal development.
At P8-12, net descending facilitation of dorsal horn circuitry occurs in the absence of ascending inputs to the RVM. At P21 the RVM is recruited by ascending inputs and is still facilitatory, but in adults this responsive descending modulation is predominantly inhibitory.

3.6 Conclusions

Here, in agreement with previous findings, I report that the RVM predominantly facilitates deep dorsal horn neuron nociceptive inputs in young animals and inhibits activity in adult animals, strongly agreeing with previous findings. Importantly, RVM descending facilitation of dorsal horn neuron excitability is apparent at P8, before RVM neurons are activated by peripheral noxious stimulation, suggesting the presence of ongoing descending facilitation in young animals which does not require nociceptive afferent input to the RVM. Moreover, RVM-mediated pronociception was observed in inflamed P21 and adult, but not P12 rats; providing further evidence that the RVM does not respond to nociceptive inputs in the first postnatal weeks. The aim of the next chapters will be to investigate the role of descending serotonergic modulation in descending RVM modulation of spinal sensory circuitry in young and adult rats.

Chapter 4

Connectivity of descending serotonergic pathways during postnatal development

4.1 Introduction

Descending RVM serotonergic modulation of spinal dorsal horn sensory inputs has been well described in adult pain literature, and particularly robust evidence has identified a pro-nociceptive role of spinal 5-HT_3 receptors (5-HT_3Rs) in animal models of chronic pain (Suzuki et al., 2004b; Okubo et al., 2013b; Kim et al., 2014b). In the following chapters I aim to test the hypothesis that dominant descending facilitation observed at P8 and P21 is mediated by descending serotonergic neurons and downstream activation of spinal 5-HT_3Rs. This chapter will focus on characterising the anatomical connectivity of spinally projecting RVM serotonergic neurons and 5-HT_3R targets in the spinal dorsal horn at different postnatal ages. The function of these pathways will be the focus of chapter 5.

4.1.1 Descending RVM serotonergic pathways modulate spinal sensory circuits in adult animals

Serotonin-containing neurons in the RVM are located in the lateral paragigantocellular reticular nucleus (LPGi) and raphe magnus nucleus (RMg), which together constitute the B3 serotonergic group. Around 11% of all neurons in the RVM contain 5-HT (Marinelli et al., 2002), and retrograde tracing experiments in adult rats have found that between 31-46% of all RVM neurons which project to the lumbo-sacral spinal cord contain 5-HT or the 5-HT synthesis rate limiting enzyme tryptophan hydroxylase (TPH) (Bowker et al., 1981a; Kalyuzhny et al., 1996; Braz and Basbaum, 2008). Anterograde tracing of descending projections from neurons in the RVM have identified extensive labelling of 5-HT-containing axon terminals primarily in laminae I-II and also in laminae IV-X of the dorsal horn in the adult mouse (Liang et al., 2015), and rat (Rajaofetra et al., 1989; Jones and Light, 1990).

Immunohistochemical staining of 5-HT transporter (5-HTT) has also been used to label serotonergic neuron axonal terminals in the spinal dorsal horn, with distribution patterns which strongly correlate with 5-HT staining (Sur et al., 1996). Importantly, 5-HT immunoreactivity in the lumbar spinal dorsal horn can be permanently abolished following cordotomy; demonstrating that most, if not all, serotonergic inputs originate from the brain (Bullitt and Light, 1989). Retrograde tracing experiments have since shown that the RVM supplies the majority of direct serotonergic inputs to the spinal dorsal horn from the brain (Braz and Basbaum, 2008).

Serotonergic neurons in the RVM are a major contributor to descending modulation of spinal sensory processing in adult animals. Early behavioural studies demonstrated a

predominantly antinociceptive role of descending 5-HT neurotransmission in uninjured adult animals. Morphine analgesia can be reduced by destruction of 5-HT terminals in the spinal dorsal horn with 5,7-dihydroxytryptamine (5,7-DHT) (Vogt, 1974; Mohrland and Gebhart, 1980). RVM electrical stimulation increases endogenous 5-HT release in the spinal cord (Hammond et al., 1985), and RVM stimulation-produced analgesia (SPA) can be reduced by intrathecal administration of the 5-$HT_{1/2}$R antagonist methysergide (Hammond and Yaksh, 1984). Similarly, *in vivo* electrophysiological recordings in rats have demonstrated that application of 5-HT onto the dorsal horn inhibits C-fibre evoked firing activity of spinal dorsal horn WDR neurons (Liu et al., 2007). On the other hand, intrathecal administration of 5-HT in mice has been reported to cause biting and licking behaviours which are indicative of a pain state (Fasmer and Post, 1983).

Pro-nociceptive 5-HT neurotransmission in adult animals has also been reported elsewhere. Optogenetic activation of TPH-expressing neurons (presumed serotonergic) in the RVM decreased mechanical and thermal nocifensive withdrawal thresholds in Tph-2-channelrhodpsin transgenic mice (Cai et al., 2014). 5,7-DHT ablation of descending 5-HT terminals reduced spinal dorsal horn neuron firing properties, demonstrating normal excitation of these neurons, in naïve rats and following spinal nerve ligation (Rahman et al., 2006). On the other hand, attenuation of behavioural hyperalgesia following 5,7-DHT ablation of descending 5-HT terminals has been found in inflammatory pain models (Carr et al., 2014), formalin test (Svensson et al., 2006), spinal cord injury (Oatway et al., 2004) and peripheral nerve injury (Leong et al., 2011), but not in uninjured animals (Leong et al., 2011). Collectively, robust evidence points towards a pro-nociceptive role of descending 5-HT neurotransmission following peripheral inflammatory or nerve injury in adult rats (Oatway et al., 2004; Svensson et al., 2006; Okubo et al., 2013b). In comparison, conflicting evidence in control animals precludes firm conclusion being drawn about the function of descending serotonergic pain modulation. Experiments in this chapter focus on descending modulation in naïve animals.

4.1.2 5-HT receptor expression in the adult spinal dorsal horn

Multiple different 5-HT receptor subtypes are expressed in the spinal dorsal horn which, when activated, modulate dorsal horn neuron electrophysiological properties or pain related behaviours (see table 4.1).

Receptor subtype	Intracellular mechanism
5-HT_{1A}	Inhibitory GPCR, coupled to G_i/G_o
5-$HT_{2A, B \& C}$	Excitatory GPCR, coupled to $G_{q/11}$
5-HT_3	Excitatory ion channel (cation entry)
5-HT_7	Excitatory GPCR, positively coupled to G_s.

Table 4.1. 5-HT receptors expression in the adult spinal dorsal horn linked with modulation of spinal sensory inputs
Pharmacological and anatomical studies have identified four major 5-HT receptor subtypes which are expressed in the spinal dorsal horn. For reviews see Suzuki et al., (2004) and Wei et al., (2012).

One receptor subtype, 5-HT_3R, has emerged from the literature in recent years with demonstrably robust spinal excitatory and pain facilitatory roles, most notably in adult inflammatory and neuropathic pain states (Zeitz et al., 2002; Suzuki et al., 2004b; Svensson et al., 2006; Okubo et al., 2013b). 5-HT_3Rs are expressed on nociceptive DRG neurons (Zeitz et al., 2002) and their central terminals in the spinal dorsal horn (Maxwell et al., 2003; Kim et al., 2014b), and on excitatory and inhibitory dorsal horn interneurons (Conte et al., 2005; Huang et al., 2008; Fukushima et al., 2009; Guo et al., 2014). There is good evidence for a descending modulatory pathway starting with the activation of RVM serotonergic neurons, release of 5-HT in the dorsal horn, activation of 5-HT_3Rs and subsequent excitation of primary afferent neurons and dorsal horn neurons having a key role in maintaining chronic pain states caused by peripheral nerve injury (Okubo et al., 2013b; Guo et al., 2014; Kim et al., 2014b). Importantly, endogenous activation of 5-HT_3Rs has a minimal effect on pain behaviours in uninjured control animals (Dogrul et al., 2009; Peters et al., 2010). To date, the expression and function of spinal 5-HT_3Rs has not been investigated in young rats. Due to the well-established pronociceptive role of 5-HT_3Rs in adult pain states, I hypothesise that endogenous 5-HT_3R activation mediates descending facilitation of dorsal horn neurons in uninjured young, but not adult, rats.

5-HT+ fibres invade the caudal spinal cord from E19, and the density of 5-HT+ fibres increases after birth (Bregman, 1987; Rajaofetra et al., 1989), suggesting that brainstem serotonergic regions project to the spinal cord before birth. Retrograde tracing experiments have also identified considerable postnatal growth of serotonergic axonal projections from the brainstem to the spinal cord between P7 and P14 (Tanaka et al., 2006). Similarly, 5-HT immunoreactivity increases in the lumbar spinal cord with postnatal age (Bregman, 1987), however these studies did not discuss in detail specific

projections from the RVM or changes in the innervation of dorsal horn laminae with age. Experiments in this chapter therefore aim to characterise the anatomical maturation of serotonergic spinally-projecting RVM neurons and their spinal 5-HT_3R targets during postnatal development. Experiments in chapter 2 demonstrated that peripheral noxious stimulation increases Fos expression in the RVM from P12. Here I build on these findings to investigate whether serotonergic neurons in the RVM are activated by noxious stimuli at different postnatal ages. Investigating the anatomical connectivity of descending serotonergic pathways will form the basis of experiments in chapter 5 which will test the function of these pathways in young and adult rats.

4.2 Experimental aims

The aims of this chapter are to characterise the functional and anatomical connectivity of the serotonergic pathway from the RVM to the spinal dorsal horn. The key hypotheses are:

1. Serotonergic RVM neurons project to the neonatal spinal dorsal horn and this projection increases with postnatal age.
2. Serotonergic RVM neurons are activated following peripheral noxious stimulation; however, like in chapter 2, this may not develop until in the second postnatal week.
3. The density and distribution of 5-HT terminals in the spinal dorsal horn changes with postnatal age, reflecting the arrival of descending serotonergic RVM neurons.
4. 5-HT_3Rs are expressed in the spinal dorsal horn of rats of all ages.

4.3 Methods

4.3.1 Animals

All experiments were performed in accordance with the United Kingdom Animal (Scientific Procedures) Act 1986. Reporting is based on the ARRIVE Guidelines for Reporting Animal Research developed by the National Centre for Replacement, Refinement and Reduction of Animals in Research, London, United Kingdom (Kilkenny et al., 2010). Male and female Sprague-Dawley rats at postnatal day (P) 0, 4, 6, 7, 8, 12, 14, 16 21, 26 and 40 were obtained from the Biological Services Unit, University College London. Rats were bred and maintained in-house and exposed to the same caging, diet and handling throughout development. Litters were weaned at P21 into same sex cages of four littermates and were housed in 12h light/dark cycles at constant ambient temperature and humidity with free access to water and food.

4.3.2 Retrograde tracing

In retrograde labelling experiments, rats aged P0, P6 and P12 (n=4 per age) were anaesthetised with isoflurane (induction 4% in medical O_2, maintenance 2.5%). An incision was made over the lumbar 4-5 spinal dorsal horn and a small bilateral laminectomy was then made to allow sufficient room for injection of retrograde tracers. A 500nl injection of Microspheres (Retrobeads, Lumafluor) was made directly into the left lumbar 4-5 spinal dorsal horn using a 26 gauge Hamilton syringe. The needle was left in place for 30 seconds before withdrawal. P26 animals received intraspinal injection of 1µl of FluoroGold (Fluorochrome, n=4) instead of Microspheres, due to poor tracing efficacy of Microspheres in adult animals. The muscle and skin overlying the injection site was then sutured with 5-0 suture (Ethicon), EMLA cream (AstraZeneca) was placed on the wound, and animals were returned to the mothers or back to home cages. Animals were sacrificed and transcardially perfused 4 days after intraspinal injections of retrograde tracers.

Lumbar spinal cords were sectioned and observed under a fluorescence light microscope to ensure that >50% of the dorsal horn in the left hemisphere of the lumbar 4-5 spinal cord contained Microsphere or FluoroGold retrograde dye.

4.3.3 Pinch stimulation and immunohistochemistry

Animals at P4, P8, P12, P21 and P40 (n=4 per age) were anaesthetised with isoflurane and maintained at a low level of anaesthesia sufficient to cause areflexia (1.8-2%). The pinch stimulus was applied with a pair of F.S.T curved, serrated forceps (product code

11152-10; tip 0.3mm) for 5 seconds on 6 points on both the dorsal and ventral surface of the left hindpaw over the course of 1 minute. Control animals (n=4 per age) received the same length of anaesthesia. Rats were transcardially perfused 2 hours after pinch stimulation or anaesthesia. Separate groups of rats (aged P0, P6, P12 and P26) received intraspinal injection of retrograde tracers and were perfused 4 days later. For spinal cord 5-HTT and 5-HT_3R immunohistochemistry quantification experiments rats, naïve P7, P14, P21 and P40 rats (n=4 per age) were perfused.

For perfusions, rats were anaesthetised with pentobarbitone sodium (500mg/kg) and perfused transcardially with heparinised saline (5000 IU/ml) followed by 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer. The brain and lumbar spinal cord were removed and postfixed overnight in 4% PFA and transferred to a 30% sucrose solution in 0.1 PB containing 0.01% azide and stored at 4°C. Brain and spinal cord tissue was sectioned on a freezing microtome at 40µm and 30µm thicknesses.

For TSA amplification immunostaining (brainstem c-fos (rabbit, 1:20,000, Chemicon), spinal cord 5-HTT (rabbit, 1:10,000, Immunostar) or spinal cord 5-HT_3R (rabbit, 1:1000, Millipore), free floating sections were blocked with 3% goat serum in 0.3% Triton X-100 in 0.1M PBS for 1h at room temperature (RT). Sections were then incubated overnight at room temperature with primary antibodies. The next day, sections were incubated in biotinylated anti-rabbit antibody (goat anti-rabbit; 1:400; Vector Stain) for 90mins. Sections were placed in ABC complex (1:125; Vector Stain, ABC elite kit, Vector Labs) for 30mins, followed by biotinylated tyramide (1:75; TSA Stain Kit; Perkin Elmer) for 7mins. Sections were then incubated in fluorescence Isothiocyanate (FITC; 1:600; Vector Stain) for 2 hours.

For brainstem double labelling c-fos with NeuN or TPH, sections then were incubated in mouse anti-NeuN (1:500; Chemicon) or mouse anti-TPH (1:500; Sigma-Aldrich) overnight. For spinal cord double labelling 5-HT_3R with NeuN or Iba1, sections were incubated in mouse anti-NeuN (1:500) or mouse anti-Iba1 (1:100) overnight. Brainstem and spinal cord sections were then incubated for 2 hours with goat anti-mouse Alexafluor 594 (1:250; Invitrogen).

In retrograde tracing experiments, brainstem sections were blocked with 3% goat serum in 0.3% Triton X-100 in 0.1M PBS for 1h at RT, incubated overnight with TPH (1:500), washed thrice, then incubated with goat anti-mouse Alexafluor 594 (1:250).

All sections were mounted on gelatinised slides and were then cover slipped with Fluoromount (Sigma). Negative control stains omitting primary antibodies resulted in no immunofluorescence, demonstrating no non-specificity of any protocol. Sections

were viewed using a Leica DMR light microscope, photographed using a Hamamatsu C4742-95 digital camera and analysed with Volocity Software 6.3.

4.3.4 Immunoreactivity cell counting and fluorescence intensity measurements

In brainstem Fos and TPH immunohistochemistry staining experiments, Fos-ir, TPH-ir and Fos-ir & THP-ir cells were counted in the RVM of naïve and pinched rats. Two populations of neurons were then analysed: one population which was the percentage of serotonergic neurons which are activated, calculated as the number of TPH-ir & Fos-ir cells divided by the number of TPH-ir cells; the second population was the percentage of activated neurons which are serotonergic, calculated as the number of TPHir & Fos-ir cells divided by the number of Fos-ir cells. The raw Fos-ir cell counts were those used in experiments in chapter 2. Counts were performed in 4 sections per animals and were averaged to create one *n* per animal

In retrograde labelling experiments, TPH-ir and Microsphere/Fluorogold & TPH-ir cells were counted in the RVM. Data is expressed as the percentage of TPH-ir cells which were retrogradely labelled, and was calculated as the number of Microsphere/FluoroGold & THP-ir cells divided by the number of TPH-ir cells. Counts were performed in 4 sections per animals and were averaged to create one *n* per animal. Sections were chosen based on the presence and distribution of TPH-ir cells in the RVM.

For 5-HTT quantification, fluorescence intensity was measured in ROIs in the spinal dorsal horn. A global region of interest of 100μm x 50μm was determined and kept constant throughout the analysis (See Fig 3E). Within this area, a region of 10μm x 30μm was chosen as the sampled sub-region of interest for determining the mean intensity of 5-HTT of laminae I, II and III. A larger area of 30μm x 30μm was chosen for laminae IV-V. Intensities were performed in 4-5 sections per animals and were averaged to create one *n* value per animal. These intensity measurements were performed by Alexandros Kanellopoulos as part of his MSc dissertation.

For 5-HT_3R quantification, mean fluorescence intensity was measured in two 100μm x 100μm ROIs; one ROI in the superficial dorsal horn (laminae I and II), and one ROI in the deep dorsal horn (laminae III-V). The mean intensity of 5-HTT or 5-HT_3R for each sub-region of interest was measured using ImageJ/Fiji image analysis software. The intensities of 4-5 sections per animal were averaged to create one *n* value per animal.

ROIs were not adjusted for age, and as the spinal cord grows with postnatal age, the same size ROI in a P7 spinal cord will incorporate a relatively larger area of a lamina

being measured compared to the spinal cord of P21 or P40. However, as the *mean* intensity of 5-HTT or 5-HT_3R per ROI was measured, the changing scale of the spinal dorsal horn with age should not preclude data comparisons between ages.

4.3.5 Statistical analysis

Statistical analyses and graphing were performed using GraphPad Prism 6 (GraphPad software, La Jolla, CA, USA) and $P<0.05$ was considered statistically significant. Sample sizes for testing were based on previously reported group differences in immunohistochemistry experiments (Bester et al., 2000b; Barr, 2011). All data sets were normally distributed therefore parametric statistical tests were used. Data are represented as means ± standard error of mean (SEM).

The mean pinch-induced Fos-ir and mean control Fos-ir in each brainstem region were compared at each age using unpaired student's T-test and two-way ANOVA followed by Bonferroni *post hoc* multiple comparisons test. Between-age comparisons of TPH-ir neurons and Microsphere/FluoroGold + TPH-ir cells in the RVM were performed using one-way ANOVA with Bonferroni *post hoc* multiple comparisons test.

4.4 Results

4.4.1 Hindpaw noxious pinch increases Fos expression in RVM serotonergic neurons from P12

To investigate whether serotonergic neurons in the RVM are activated by hindpaw pinch stimulation during postnatal development, double immunohistochemical staining of brainstem sections with Fos and TPH was performed in the RVM of P4, P8, P12, P21 and P40 naïve and pinched rats. Fos and TPH counts were performed in 4-5 sections per animal and a mean value was created for each animal, such that 1 n = 1 animal. Fos counts were performed in 4 animals per age. A Two-way ANOVA with Bonferroni post-hoc test was used to compare naïve vs pinch Fos-ir counts at P12, P21 and P40 as the density of neurons in the RVM did not change after P12 (chapter 2). Unpaired student's t-tests were used to compare naïve vs pinch Fos-ir counts at P4 and P8.

Fos-ir counts in the RVM are the same as used in chapter 2. Example Fos staining images from pinched P4, P8, P12, P21 and P40 rats are shown in figures 4.1A, B, C, D and E respectively. TPH staining images from the same RVM sections are shown in figures 4.1A', B', C', D' and E', and the Fos & TPH double stain overlay images are shown in figures 4.1A'', B'', C'', D'' and E''. High power magnification of Fos-ir and TPH-ir cells from the P4 RVM is shown in figure 4.1H. Fos-ir and TPH-ir colabelled cells were observed in the RVM of control and pinched rats aged P4, P12, P21 and P40, demonstrating activated serotonergic neurons in the RVM from P4, but rarely at P8 due to low levels of Fos-ir cells at this age.

Firstly, I questioned whether the proportion of activated RVM serotonergic neurons changes with age. The proportion of RVM serotonergic neurons which are activated was calculated as the percentage of TPH-ir cells which colocalised with Fos. Two-way ANOVA comparing the percentage of TPH-ir cells which colocalised with Fos in naïve and pinch animals at different postnatal ages demonstrated that age was a significant factor. Bonferroni post hoc analysis demonstrated that the percentage of TPH-ir cells which colocalised with Fos was significantly higher in pinch animals compared to age matched naïve animals at P21 and P40 (Two-way ANOVA, pinch vs naïve, $F(4,30)=19.04$, $P<0.0001$, with Bonferroni *post hoc* comparison, $P<0.05$ and 0.01 at P21 and P40; Fig 4.1F). Whilst a two-way ANOVA did not find significance between naïve and pinch at P12, an unpaired Student's t-test did find significantly higher percentage of TPH-ir cells which express Fos in pinched rats compared to naïve rats at P12 (unpaired Student's t-test, P12 pinch vs naïve, $P<0.01$; Fig 4.1F). At P4, an

average of 18.65% (naïve) and 16.45% (pinch) of TPH-ir neurons colocalised with Fos, although these groups did not significantly differ (unpaired Student's t-test, $P>0.05$). At P8, little to no TPH-ir cells colocalised with Fos due to the low numbers of Fos-ir cells at this age, and there was no significant effect of pinch on TPH/Fos counts (unpaired Student's t-test, $P>0.05$). These results indicate that RVM serotonergic neurons are activated by hindpaw pinch stimulation from P12.

Next, I tested whether the proportion of activated serotonergic neurons changes in relation to activated non-serotonergic neurons in the RVM under different conditions. The proportion of activated neurons that are serotonergic was calculated as the percentage of total Fos-ir cells which colocalised with TPH. A two-way ANOVA with Bonferroni post hoc analysis showed that pinch did not significantly alter the percentage of Fos-ir cells that were TPH+ at any age (Two-way ANOVA, pinch vs naïve, with Bonferroni *post hoc* analysis, $P>0.05$ Fig 4.1G), although age was a significant factor (Two-way ANOVA, pinch vs naïve, $F(4,30)=176.1$, $P<0.0001$). Similarly, no significant differences in the proportion of Fos-ir cells which colocalised with TPH was found at P4 or P8 (unpaired Student's t-test, $P>0.05$). Thus, whilst more serotonergic neurons are activated following pinch stimulation compared to control from P12, the proportion of activated RVM neurons which are serotonergic compared to those which are non-serotonergic does not change following pinch stimulation.

Interestingly, the proportion of Fos-ir cells which colocalised with TPH was much higher at P4 compared to P12, P21 and P40: a high proportion of Fos-ir cells which colocalised with TPH in both naïve and pinched (66.79% and 59.45% respectively) P4 animals (Fig 4.1G). These secondary observations indicate that the majority of RVM neurons which are active at baseline conditions at P4 are serotonergic.

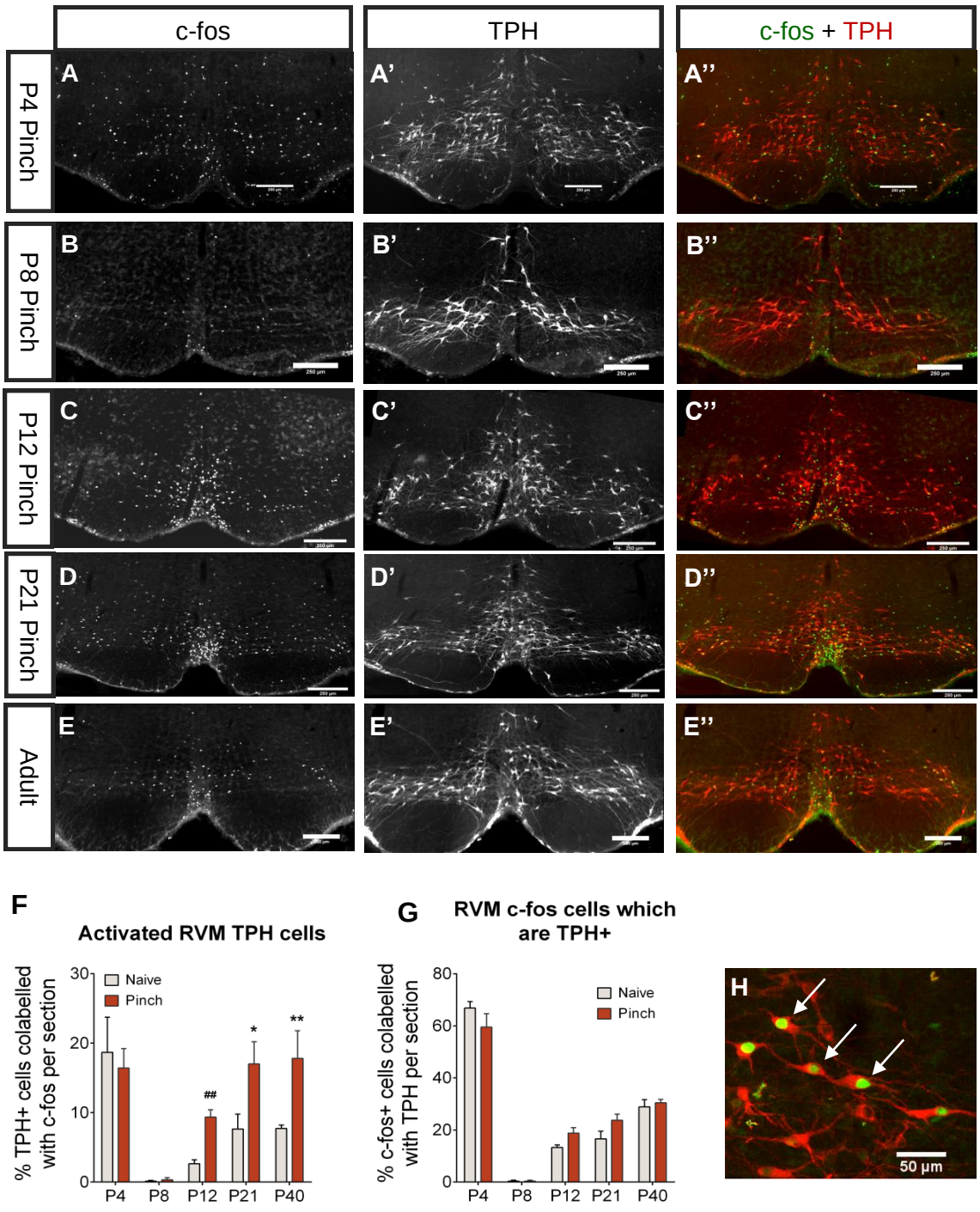

Fig 4.1. The proportion of pinch-evoked Fos and TPH-ir cells in the RVM changes with postnatal age.

C-fos and TPH counts were performed in the (RVM) of naïve and pinched rats of different postnatal ages (n=4 per age). Numerous c-fos-ir cells were observed at P4 (A), P12 (C), P21 (D) and P40 (E) but few were seen at P8 (B). TPH-ir cells were observed in the RVM at all ages, and the distribution of TPH-ir cells did not change with age (A', B', C', D' and E'). Overlay of green (c-fos) and red (TPH) channels for each age are shown in A'', B'', C'', D'' and E''. High powered magnification (20x) of TPH and c-fos-ir cells are shown in H from the P4 RVM, and arrows indicate colocalised cells. Hindpaw pinch significantly increased the percentage of TPH-ir + c-fos-ir colocalised cells at P12, P21 and P40 compared to naïve (F). Hindpaw pinch had no effect on the percentage of c-fos-ir cells which colocalised with TPH at any age (G). Two-way ANOVA with Bonferroni post hoc analysis *,** $P<0.05$, 0.01; students T-test ## $P<0.01$.

4.4.2 The proportion of retrogradely labelled serotonergic RVM neurons increases in the second postnatal week

Next, I questioned whether the proportion of serotonergic neurons in the RVM which project to the lumbar dorsal horn changes with age. Four days after injection of Microspheres (P0, P6 and P12) or FluoroGold (P26) lumbar 4-5 spinal dorsal horn (n=4 per age), rats were perfused and brainstem tissue was taken, section and immuno-stained with TPH antibody to label serotonergic neurons.

Firstly, the average number of TPH-ir cells in the RVM did not significantly change with age (One-way ANOVA age comparison, $F(3,12)=1.39$, $P=0.29$; Fig 4.2C). Retrograde labelling data is expressed as the percentage of TPH-ir neurons which contain either Microspheres (at P4, P10 and P16) or FluoroGold (at P30). At all ages, Microsphere/FluoroGold + TPH-ir cells were distributed around the raphe magnus (RMg) and the two lateral paragigantocellular nuclei (LPGi) in the RVM (See fig 4.2A and B for examples at P4 and P10). Retrogradely labelled neurons were also observed in the raphe pallidus (RP). Microsphere/FluoroGold labelled neurons which did not colocalise with TPH were found primarily in the gigantocellular reticular nucleus alpha (GiA), which contains no serotonergic neurons. Some Microsphere/FluoroGold labelled neurons were also found in the RMg, LPGi and RP. No obvious differences in the distribution of Microsphere/FluoroGold labelled neurons, TPH-ir neurons, or Microsphere/FluoroGold + TPH-ir neurons were found in the RVM between P4, P10, P16 or P30 rats. Examples of TPH-ir cells, Microsphere-ir cells and TPH-ir and Microsphere colocalised cells are shown in figure 4.2A'.

Quantification of the percentage of Microsphere/FluoroGold + TPH-ir cells at these 4 ages demonstrated a postnatal increase in the proportion of TPH-ir cells which were retrogradely labelled. Age was a significant factor (One-way ANOVA, comparison of ages, $F(3,12)=18.73$, $P<0.001$), and Bonferroni post hoc analysis demonstrated that the percentage of Microsphere/FluoroGold + TPH-ir cells significantly increased between P10 and P16, but not between P4 and P10, or between P16 and P30 (One-way ANOVA, comparison of ages, with Bonferroni post hoc analysis, P10 vs P16 $P<0.01$; Fig 4.2D).

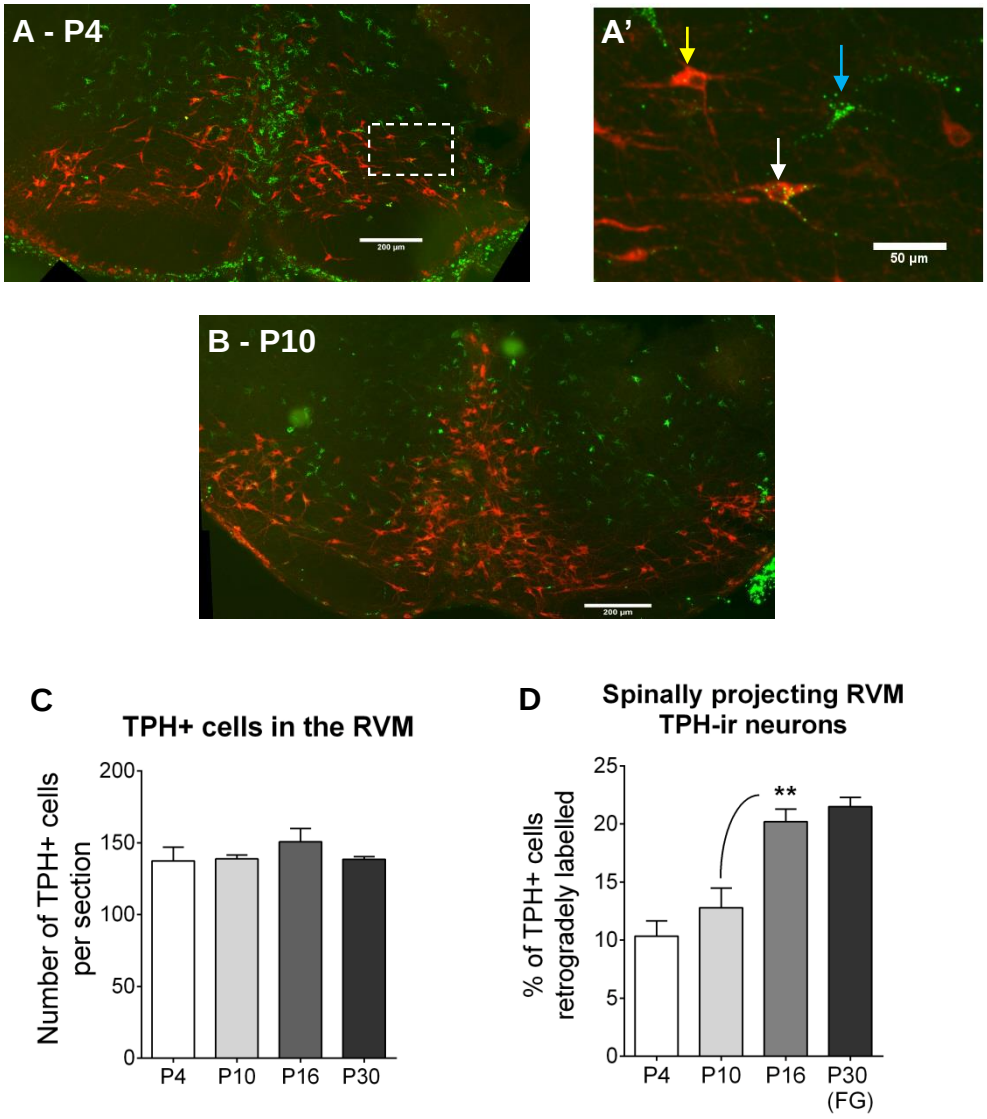

Fig 4.2. TPH-ir RVM neurons project to the lumbar dorsal horn from birth.
Retrograde tracing Microspheres (P0 (n=4), P6 (n=4) and P12 (n=4)) or FluoroGold (FG) (P26 (n=4)) was injected into the left lumbar 4-5 spinal dorsal horn. Brainstem tissue was taken 4 days later and stained with tryptophan hydroxylase (TPH). Microspheres (green) were present throughout the RVM at P4 (A) and P10 (B). Example of Microsphere labelled cells (blue arrow), TPH-ir cells (yellow arrow) and Microsphere and TPH-ir colocalised cells (white arrow) are shown in A', which is an excerpt from A. Quantification of TPH-ir cells in the RVM demonstrated no change of TPH-ir cells with age (C). There was a significant increase in the proportion of TPH-ir neurons which were colocalised with Microspheres between P10 and P16 (D). One way ANOVA with Bonferroni Post hoc analysis, ** $P<0.01$.

4.4.3 The distribution and density of 5-HTT in the spinal dorsal horn changes with postnatal age

Because the proportion of TPH-ir neurons in the RVM which project to the spinal dorsal horn increased between P10 and P16, I hypothesised that the distribution and density of serotonergic neuron terminals in the dorsal horn would also change with postnatal age. Here, 5-HTT was used to label serotonergic neuron transporter protein (presumed serotonergic axonal terminals) in lumbar spinal dorsal horn sections at P7, P14, P21 and P40 (n=4 per age).

Immunostaining 5-HTT revealed marked changes in the distribution and density of presumed serotonergic fibres in the spinal dorsal horn with postnatal age. At P7, 5-HTT staining was sparse in laminae I and II but was observed in deeper laminae III-V (Fig 4.3A). By P14, a thin band of 5-HTT staining in lamina I and increasing density of staining in laminae III-V can be seen, and a clear absence 5-HTT staining can be seen in lamina II (Fig 4.3B). At P21 and P40, this lamina II band of low 5-HTT density is still observed, and the density of 5-HTT staining in lamina I increases (Figs 4.3C and D). These results indicate that serotonergic terminals innervate the deep, but not the superficial lumbar dorsal horn from P7. The density of serotonergic terminals in the superficial dorsal horn increases with age, suggesting that the serotonergic innervation of the dorsal horn occurs in a ventral to dorsal trajectory during postnatal development.

Measurement and quantification of the mean intensity of 5-HTT staining were performed in 4 ROIs in laminae I, II, III and IV-V at P7, P14, P21 and P40 (Fig 4.3D). Two-way ANOVA comparing the effect of age and laminae of 5-HTT intensity demonstrated a significant effect of age (Two-way repeated measures ANOVA, age comparison, $F(3,12)=208.0$, $P<0.0001$). Bonferroni post-hoc analysis comparing the mean 5-HTT intensity within individual lamina between ages demonstrated significant increases in 5-HTT in all laminae at every age, except for laminae II and IV-V between P7 and P14 (Two-way repeated measures ANOVA, age and lamina comparison with Bonferroni *post hoc* analysis, $P<0.05$ to 0.001 at different laminae and ages; Fig 4.3E) (see table 4.2).

Data was also expressed as the percentage of 5-HTT staining within each lamina as a percentage of the total 5-HTT staining in laminae I-V (Fig 4.3F). The percentage of 5-HTT staining intensity in lamina I and II increased with postnatal age in parallel with a decrease in the percentage of 5-HTT staining intensity in deeper laminae. These results indicate that serotonergic fibres predominantly innervate the deep dorsal horn at P7

and P14, and that the density of serotonergic terminals in each lamina is more equivalent by P40.

Dorsal horn lamina	Bonferroni's multiple comparisons test	Mean 5-HTT Intensity Diff.	Significance
Lamina I	P7 vs. P14	-9.71	***
	P14 vs. P21	-10.59	***
	P21 vs. Adult	-12.15	***
Lamina II	P7 vs. P14	-1.691	**ns**
	P14 vs. P21	-10.58	***
	P21 vs. Adult	-11.47	***
Lamina III	P7 vs. P14	-9.009	***
	P14 vs. P21	-7.008	***
	P21 vs. Adult	-10.1	***
Laminae IV-V	P7 vs. P14	2.852	**ns**
	P14 vs. P21	-12.46	***
	P21 vs. Adult	-4.333	*

Table 4.2. Summary of statistical analysis of 5-HTT density in dorsal horn laminae.
5-HTT was quantified in regions of interest in laminae I, II, III and IV-V in rats of different postnatal ages (n=4 per age). A two-way ANOVA with Bonferroni *post hoc* analysis was performed to compare the effect of age on the density of 5-HTT staining within each laminae. Statistical analysis was performed using Prism GraphPad 6.

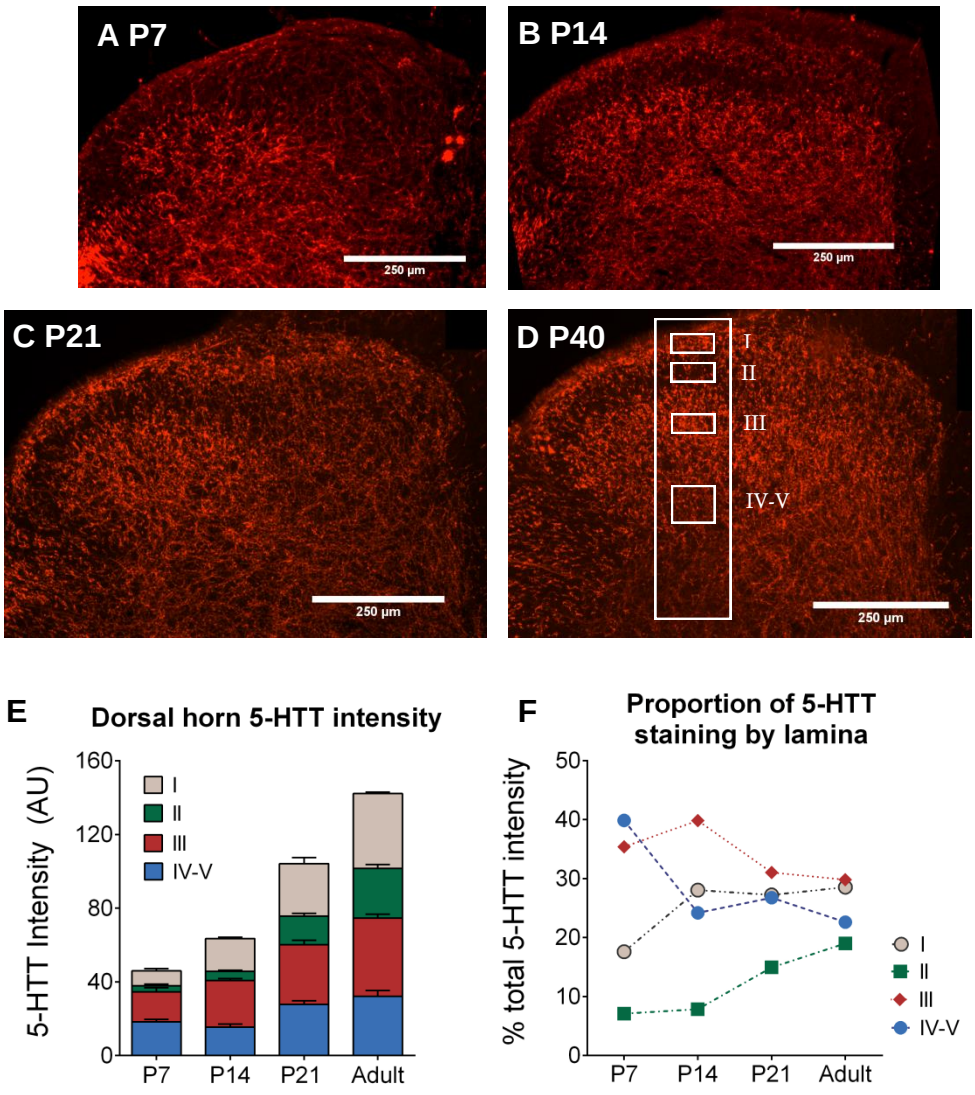

Fig 4.3. Dorsal horn 5-HTT staining density and distribution changes with postnatal age. Spinal cord sections from P7, P14, P21 and P40 rats (n=4 per age) were stained with 5-HTT antibody. 5-HTT staining in the superficial dorsal horn is low at P7 (A) and P14 (B), and increases at P21 (C) and P40 (D). 5-HTT staining in the deep dorsal horn is present from P7 (A) and increases with age. a distinct 5-HTT absent band can be seen at all ages. Mean intensity of 5-HTT was quantified in regions of interest in laminae I, II, III and IV-V in rats of different postnatal ages, as exemplified in D. Mean intensity of 5-HTT staining in all laminae increases with postnatal age (E) (Two-way ANOVA with Bonferroni post-hoc analysis; see text and table 1 for details). The proportion of 5-HTT staining in each lamina, when expressed as a percentage of total dorsal horn 5-HTT staining, changed with postnatal age (F).

4.4.4 The density and distribution of 5-HT_3Rs in the spinal dorsal horn does not change with postnatal age

Spinal cord tissue was taken from naïve P7, P14, P21 and P40 rats (n=4 per age) and immunostained for 5-HT_3R. Double immuno-staining was performed with NeuN to label neuronal cell bodies and Iba1 to label microglia. 5-HT_3R mean intensity measurements were performed in ROIs in the superficial dorsal horn (laminae I and II), and the deep dorsal horn (laminae III-V).

A thick band of 5-HT_3R staining was observed in laminae I and II, and a smattering of low density staining was observed in the deeper laminae III-V in rats of all ages at P7, P14, P21 and P40 (Figs 4.4A, B, C and D). The density and distribution did not appear to change with postnatal age. At P7, 5-HT_3R staining appeared to be localised in small dense matters which appear like cell bodies (Fig 4.4A), however double labelling of 5-HT_3R with NeuN or Iba1 found little or no colocalisation with neuronal and microglial cell bodies, respectively, at P7 (Figs 4.4E and F) or any other age (data not shown).

Two-way ANOVA with Bonferroni post-hoc analysis comparing the effect of age and laminae of 5-HT_3R intensity demonstrated no significant effect of age on 5-HT_3R intensity (Two-way repeated measures ANOVA, age comparison, $F(3,12)=1.09$ $P=0.39$; Fig 4.4G).

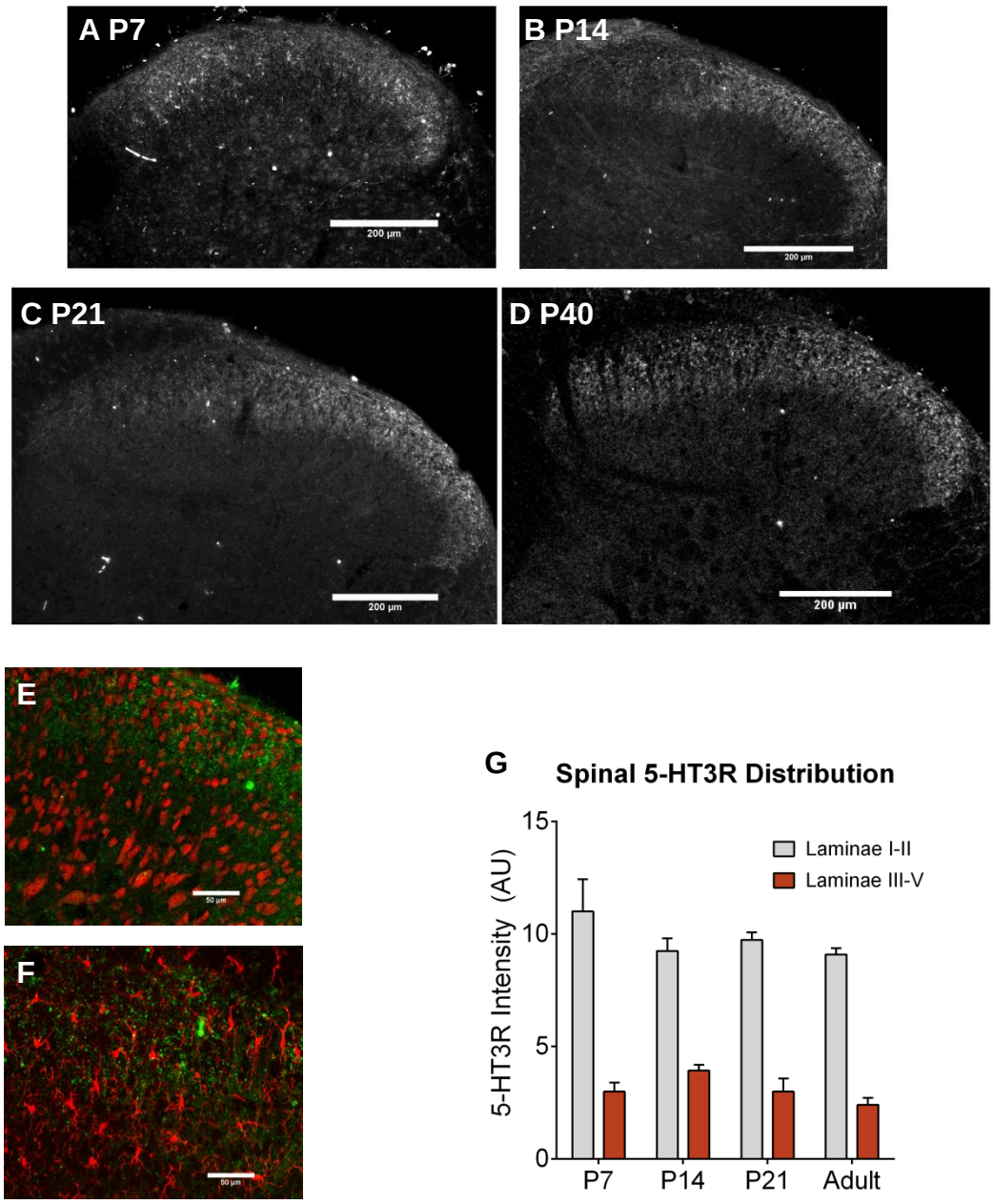

Fig 4.4. Dorsal horn 5-HT$_3$R staining density and distribution does not change with postnatal age.

Spinal cord sections from P7, P14, P21 and P40 rats (n=4 per age) were stained with 5-HT$_3$R antibody. A dense band of 5-HT$_3$R staining can be observed in the superficial dorsal horn at P7 (A), P14 (B), P21 (C) and P40 (D). Double staining with NeuN (E) (red; to stain neuronal nuclei) or Iba1 (F) (red; to stain microglia) revealed no colocalisation with 5-HT$_3$R (green) at P7. Mean intensity of 5-HT$_3$R was quantified in two regions of interest in laminae I-II and III-V in rats of different postnatal ages (G). Mean intensity of 5-HT$_3$R staining in the superficial and deep laminae does not change with postnatal age (Two-way ANOVA with Bonferroni post-hoc analysis).

4.4.5 Summary of results

The findings from this chapter can be summarised as follows:

1. Hindpaw pinch stimulation significantly increased the proportion of serotonergic RVM cells which express Fos at P12, P21 and P40, compared to naive. No increase was seen at P4 and P8.
2. A secondary observation from these results indicated that in naïve and pinched P4 rats, ~60% of Fos-ir neurons colocalised with TPH in the RVM, suggesting that the majority of activated RVM neurons in baseline conditions at P4 are serotonergic.
3. A large population (90%) of non-serotonergic and a small proportion (10%) of serotonergic cells were retrogradely labelled from the lumbar dorsal horn from birth. The proportion of retrogradely labelled serotonergic RVM neurons increased between P10 and P16, demonstrating postnatal growth of serotonergic axons from the RVM to the lumbar spinal cord.
4. The density and distribution of 5-HTT-ir serotonergic terminals in the lumbar spinal dorsal horn changed with postnatal age. 5-HTT staining was observed in the deep dorsal horn, but not the superficial dorsal horn at P7. The density of 5-HTT increased in both the superficial and deep dorsal horn with a developmental trajectory which suggests that serotonergic innervation of the dorsal horn occurs ventral to dorsal with postnatal age.
5. 5-HT_3R staining was found in a dense band in laminae I and II and sparsely in deeper laminae at P7, and did not change with postnatal age.

4.5 Discussion

In this chapter I aimed to characterise the anatomical connectivity of serotonergic RVM inputs to the spinal dorsal horn during postnatal development using a combination of retrograde tracing techniques and immunohistochemistry. Results in this chapter indicate that serotonergic RVM neuron growth into the lumbar spinal dorsal horn undergoes considerably postnatal maturation which is manifested as an increased proportion of spinally projecting serotonergic neurons in the RVM and increased density of serotonergic terminals in the spinal dorsal horn with postnatal age. The distribution of 5-HT_3Rs in the spinal dorsal horn was also mapped, and did not change with postnatal age. This chapter forms the basis for chapter 5 where the function of these pathways will be investigated at several postnatal ages.

4.5.1 Technical considerations

In retrograde tracing experiments two different tracers, Microspheres and FluoroGold, were used to label RVM neurons which project to the lumbar 4/5 spinal dorsal horn. Multiple pilot attempts to successfully retrogradely label RVM neurons with FluoroGold in neonatal animals failed, so an alternative retrograde tracer, Microspheres, was used instead. However, Microspheres have been documented to have poor labelling efficacy in mature animals (Katz and Iarovici, 1990), as indeed found in pilot studies, leading to the joint use of FluoroGold to label RVM neurons in mature animals and Microspheres to label RVM neurons in young animals. A similar proportion of spinally projecting serotonergic RVM was found in P16 animals which had received injection of Microspheres, and in P30 animals which had received injection of FluoroGold. These experiments suggest that the labelling efficacy of the two tracers is similar, although properly controlled comparisons were not performed here. Injection sites in the lumbar spinal cord were checked, and if Microspheres/FluoroGold was in <50% of the left dorsal horn, data from these animals were rejected.

Technical considerations regarding the use of Fos as a marker of neuronal activation, and regarding the use of pinch as a noxious stimulus are outlined in chapter 2 (2.5.1) and also apply to experiments in this chapter. Combination of retrograde tracing techniques and pinch-evoked Fos immunoreactivity in the RVM were avoided, as intraspinal injection of retrograde tracing agents damages the superficial dorsal horn and would presumably perturb ascending neurotransmission from the dorsal horn to the brainstem.

4.5.2 RVM serotonergic neurons are activated by cutaneous noxious stimulation

Experiments in this chapter demonstrated that hindpaw pinch stimulation increased the proportion of activated serotonergic neurons from P12 compared to naive. These results build on those from chapter 2 by identifying populations of pinch-responsive serotonergic neurons at P12, P21 and P40. In adults, serotonergic neurons in the RVM respond rapidly to peripheral noxious sensory inputs. Application of thermal stimuli above 50°C to the hindpaw increases serotonergic RVM neuron firing activity (Gau et al., 2013) and Fos expression (Gau et al., 2009). Similarly, hindpaw Complete Freund's Adjuvant (CFA) inflammation induces a rapid pERK activation profile in (Imbe et al., 2005, 2008; Geranton et al., 2010), and enhanced firing activity of RVM serotonergic neurons (Zhang and Hammond, 2010), demonstrating that brief or sustained peripheral nociceptor activation activates RVM serotonergic neurons. The sensitivity of RVM serotonergic neurons to peripheral stimulation has not previously been investigated in young animals. Experiments here agree that serotonergic neurons in the RVM are activated by natural pinch stimulation in the adult, and extend these findings to include young animals older than P12. Whether descending modulation arises from serotonergic RVM neurons in young rats cannot be elucidated from these findings.

Patch clamp experiments in the RVM have been performed on brainstem slices from young animals and have demonstrated pharmacological and electrical responsivity of RVM serotonergic and non-serotonergic neurons alike. Zhang et al., (2006) recorded RVM neurons membrane properties from P9-18 brainstem slices, and identified three types of serotonergic and non-serotonergic neurons in the RVM that project to the spinal cord. Additionally, serotonergic neurons could be distinguished from non-serotonergic RVM neurons, based on their higher membrane resistance, greater action potential half-width and lower propensity for fast action potential spiking (Zhang et al., 2006). Populations of spinally projecting and non-spinally projecting RVM TPH-ir neurons recorded from P10-18 mice brainstem slices have also been shown to respond to μ and k-opioid agonists (Marinelli et al., 2002). Whilst intrinsic firing properties of RVM serotonergic neurons from young animals were observed in these experiments, their occurrence in brainstem slice preparations precludes our understanding of how these neurons respond to peripheral stimulation. It remains to be determined if RVM neurons (serotonergic or otherwise) display changes in membrane and firing properties following peripheral noxious stimulation in rats younger than P9, although based on findings in this chapter, it is hypothesised that sensory inputs do not activate RVM neurons at this age.

A high proportion of Fos-ir neurons (~60%) in the naïve P4 RVM were found be serotonergic. This proportion of Fos-ir serotonergic neurons is much higher than that found in older animals (20-25%). As discussed in chapter 2, high Fos-expressing cortical pyramidal neurons exhibit higher spontaneous firing rates *in vivo* and *in vitro* (Yassin et al., 2010), and spontaneously active 'pacemaker' neurons have been well described in the neonatal spinal dorsal horn (Baccei, 2014). If indeed high Fos-immunoreactivity in the P4 RVM in the absence of peripheral noxious stimulation is indicative of hypothesised spontaneous firing activity, results here suggest a particularly important role of serotonergic neurons. Electrophysiological recordings used in combination of juxtacellular labelling techniques to identify serotonergic neurons would help to clarify the possible presence of pacemaker neurons in the RVM at P4.

4.5.3 The proportion of spinally projecting serotonergic RVM neurons increases in the second postnatal week

By injecting the retrograde tracer Microspheres into the newborn lumbar spinal dorsal horn, I demonstrated that a substantial number of neurons in the RVM have terminals in the spinal dorsal horn from birth. Colabelling of Microspheres with TPH demonstrated that 10-12% of serotonergic neurons have terminals in the lumbar dorsal horn in the first postnatal week. Between P10-16, the proportion of serotonergic neurons which project to the lumbar dorsal horn increased to 20%, demonstrating substantial axonal growth into the lumbar dorsal horn between these ages. Injection of FluoroGold into the lumbar spinal dorsal horn at P26 identified a similar proportion of RVM serotonergic neurons (21.5%) with terminals in the spinal dorsal horn at P30. These findings suggest that there is a substantial prenatal growth of RVM serotonergic axonal terminals into the lumbar dorsal horn which is observed at birth; however a second wave of growth occurs in the second postnatal week which is manifested as an increased proportion of RVM serotonergic neurons with axonal projections in the lumbar dorsal horn.

These findings are in agreement with a previous paper which demonstrated an increase in the proportion of cholera toxin B retrogradely labelled spinally projecting 5-HT+ neurons in the B3 serotonergic group from 6% to 29% at P7 and P14 (Tanaka et al., 2006). In contrast, serotonergic fibres from the B1 (raphe pallidus; RPa) and the B2 group (raphe obscurus; ROb) were found to be present in the lumbar ventral horn from P3 and did not increase in density with age (Tanaka et al., 2006). Retrograde tracing experiments used in combination with patch clamp recordings in P10-18 brainstem

slices identified as many as 24/60 spinally projecting TPH+ neurons (40% of all TPH neurons) in the RVM. Thus, the use of Microspheres and FluoroGold as retrograde tracing agents in this chapter may underestimate the proportion of spinally projecting RVM neurons.

4.5.4 The distribution and density of 5-HTT changes with postnatal age

Immunohistochemical experiments have used 5-HTT as a marker of serotonergic terminals, which is known to have comparable distribution to 5-HT+ fibres in the adult spinal dorsal horn (Sur et al., 1996). Here, I demonstrated that the distribution and density of serotonergic terminals in the spinal dorsal horn changes extensively with postnatal age. At P7, 5-HTT was sparse in the superficial dorsal horn but was densely observed in the deep dorsal horn. By P14, a thin band of 5-HTT immunoreactivity was observed in lamina I, but a band of 5-HTT absence was observed in lamina II. This band was also observed at P21 and P40, when the density of 5-HTT continued to increase in lamina I and the deep laminae III-V.

5-HT+ fibres grow into the spinal cord from the brainstem in a rostral to caudal, and ventral to dorsal manner (Bregman, 1987; Rajaofetra et al., 1989). They first begin to invade the rat caudal ventral spinal cord from E15, and enter the lumbar dorsal horn from E20 (Rajaofetra et al., 1989). Extensive invasion and branching of 5-HT+ fibres occurs in the cervical, thoracic and lumbar dorsal horns after birth during the first three postnatal weeks, before adult-like patterns are observed. This timescale of invasion and branching of 5-HT+ fibres in the lumbar spinal cord closely matches the age-dependent increase in distribution and density of 5-HTT-immunoreactivity observed here. Additionally, the postnatal increase in serotonergic terminal distribution in the lumbar dorsal horn correlates with the increase in number of spinally projecting retrogradely labelled neurons in the RVM, as observed here and previously (Tanaka et al., 2006).

Several lines of evidence have shown that serotonergic fibres originate exclusively from the CNS and not from the periphery. 5-HT immunoreactivity in the lumbar spinal dorsal horn can be permanently abolished following cordotomy; demonstrating that most, if not all, serotonergic inputs originate within the brain (Bullitt and Light, 1989). Interestingly, dorsal rhizotomy at the level of C5-T2 causes a reduction in 5-HT levels in the caudal T3-L5 segments of the spinal cord, but not at the level of the dorsal rhizotomy (Colado et al., 1988), demonstrating that few, if any, primary afferent inputs contain 5-HT. Additionally, neonatal capsaicin treatment to destroy TRPV1+ inputs extends the 5-HT distribution in the adult deep dorsal horn (Marlier et al., 1990),

suggesting that destruction of primary afferent inputs to the dorsal horn (either by dorsal rhizotomy or neonatal capsaicin treatment) perturbs descending serotonergic innervation of the spinal cord.

Earlier tracing studies using horseradish peroxidase (HRP) injected into the spinal dorsal horn suggested that retrogradely labelled serotonergic neurons in multiple Raphe nuclei project (including the B1-B3, B7 and B8 groups) to the spinal dorsal horn, however these experiments are confounded by the anterograde labelling actions of HRP (Bowker et al., 1981b). More recent experiments using a combination of FluoroGold retrograde tracing in transgenic mice have demonstrated that the majority of serotonergic terminals in the spinal dorsal horn originate from the RVM, with few inputs from other brainstem and midbrain raphe nuclei (Braz and Basbaum, 2008). In the adult mouse, sparse serotonergic inputs rom the RP and ROb do terminate in the lumbar dorsal horn, but primarily in the ventral horn; however the majority of serotonergic terminals in the dorsal horn were also found to originate from the RMg in the RVM (Liang et al., 2015). It is therefore likely that the majority of 5-HTT-ir terminals observed in experiments in this chapter originated from serotonergic RVM neurons in the B3 group. A summary figure outlining the hypothesised postnatal maturation of descending serotonergic projections from the RVM to the spinal dorsal horn is outlined in figure 4.5.

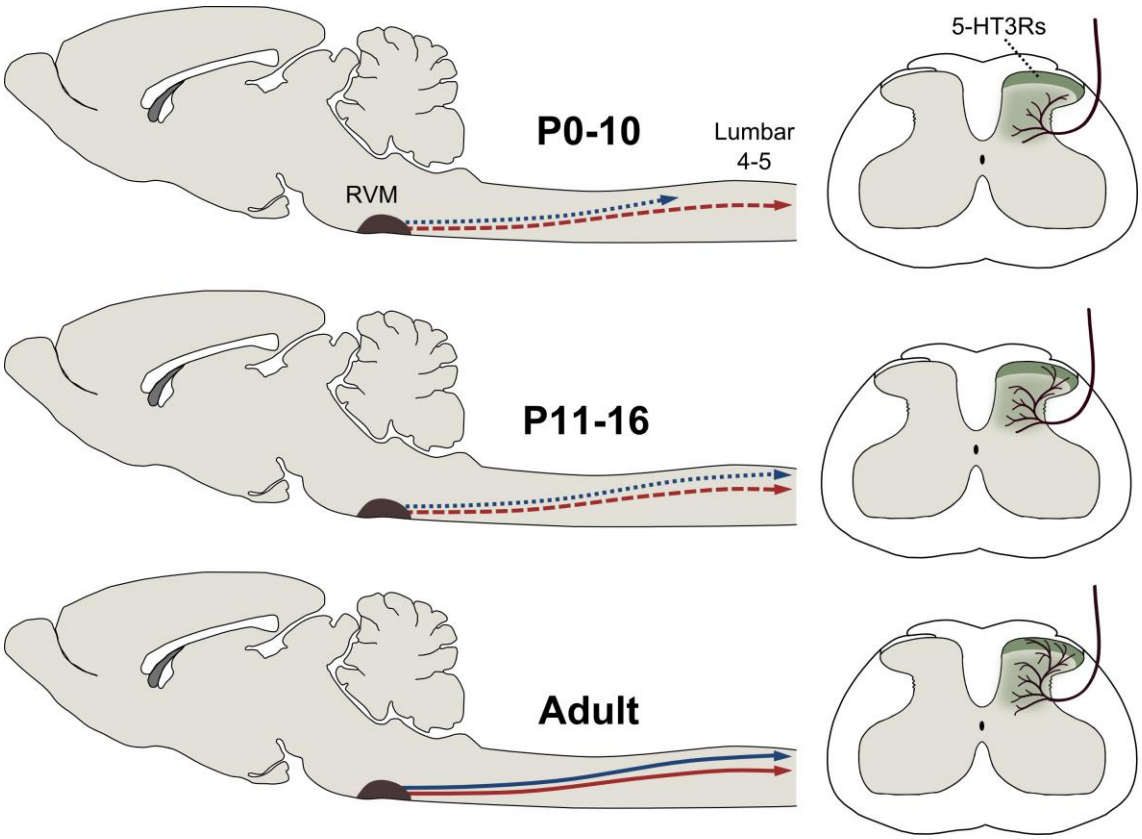

Fig 4.5. A proposed model of the postnatal development of RVM serotonergic innervation of the spinal dorsal horn.
In the first 10 days of postnatal life, a small population of serotonergic neurons in the RVM project to the lumbar spinal dorsal horn (dashed red line). Axonal terminals are sparsely distributed mainly in the deep dorsal horn and little serotonergic innervation of the superficial dorsal horn is observed. 5-HT_3Rs are expressed in the superficial dorsal horn from this age. A rapid growth of axonal projections of RVM serotonergic neurons to the lumbar dorsal horn occurs between P11-P16 (dotted blue lines). Serotonergic terminal density also increases in the superficial and deep dorsal horn during this period. Between P16 and P40 (adulthood), the density of serotonergic terminals in the superficial and deep spinal dorsal horn increases to reach mature levels, but the number of retrogradely labelled serotonergic RVM neurons does not increase (solid lines).

4.5.5 The distribution and density of 5-HT_3R does not change with postnatal age

Here, I immunostained 5-HT_3Rs in the lumbar dorsal horn of rats of several postnatal ages. A think band of 5-HT_3R staining was observed in laminae I and II, and background staining was observed in deeper laminae of the spinal dorsal horn at all ages and did not change with age. 5-HT_3R immunoreactivity at all ages was observed as punctae or fibrous-like projections and did not colocalise with NeuN or Iba1, demonstrating little to no expression of 5-HT_3Rs in the neuronal cytoplasm or on microglia.

In adults rodents, in situ hybridisation experiments identified 5-HT_3R mRNA in the brain, spinal cord and a subset of DRG neurons (Tecott et al., 1993), and later

immunohistochemistry experiments demonstrated dense labelling of 5-HT_3Rs in the superficial dorsal and sparser labelling in the deep dorsal, intermediate and the ventral spinal cord (Morales et al., 1998). Later experiments demonstrated that 10% of 5-HT_3R immunoreactivity is observed on peptidergic primary afferent C-fibres (CGRP+) in the spinal dorsal horn, with a smaller overlap (3%) of non-peptidergic C-fibres (IB4+) (Maxwell et al., 2003). A low overlap (2%) was observed on GAD+ dorsal horn neurons, suggesting that the majority of terminals which express 5-HT_3Rs are from excitatory interneurons (Maxwell et al., 2003; Conte et al., 2005). A recent paper demonstrated that 5-HT_3Rs are expressed on fractalkine-expressing neurons in the adult dorsal horn, and activation of 5-HT_3Rs causes release of fractalkine and downstream activation of CX3CR1 receptors on microglia (Guo et al., 2014), providing an important descending modulatory link between serotonergic neurons in the RVM and spinal microglia. Collectively, these studies performed in adult rodents demonstrate a wide range of cell types which express 5-HT_3Rs in the spinal dorsal horn. Whilst the distribution of 5-HT_3R expression was not found to change between P7 and P40 here, changes in cell-type 5-HT_3R expression during postnatal development may have been overlooked.

In vitro calcium-imaging experiments in anaesthetised adult mice have identified functional 5-HT_3Rs on trigeminal primary afferent fibres in the spinal dorsal horn analog medullary subnucleus caudalis (Vc) (Kim et al., 2014b). Application of a 5-HT_3R agonist increased Ca^{2+} fluorescence signals in primary afferent terminals, and application of a 5-HT_3R antagonist reduced primary afferent terminals Ca^{2+} fluorescence signals evoked by capsaicin, and reduced the frequency of mEPSCs in Vc neurons in brainstem slices (Kim et al., 2014b). Activation of 5-HT_3Rs on both primary afferent neuron terminals and on dorsal horn neurons therefore enhances processing of nociceptive inputs in the dorsal horn. There is little evidence of 5-HT_3R-mediated facilitation of low-threshold inputs in the adult dorsal horn and it remains unclear if 5-HT_3R activation selectively excites nociceptive circuits over non-noxious circuits.

4.6 Conclusions

In this chapter, the anatomical connectivity of descending serotonergic RVM pathways and the spinal dorsal horn was characterised during postnatal development. Results here indicate that spinally projecting RVM neurons undergo considerable postnatal growth during postnatal development: retrograde labelling experiments demonstrated that the proportion of serotonergic RVM neurons which project to the lumbar dorsal horn increased in the second postnatal week. Additionally, the density of serotonergic terminals in the lumbar spinal dorsal horn increased in a ventral to dorsal trajectory between P7-P40. 5-HT_3Rs are expressed in the superficial spinal dorsal horn from P7, and the distribution and density of this expression did not change with age. In accordance with results from chapter 2, results here also indicated that serotonergic neurons in the RVM are activated by cutaneous noxious mechanical stimulation from P12, but not before. The function of the descending serotonergic pathways and spinal 5-HT_3Rs during postnatal development will be investigated in the next chapter.

Chapter 5

Serotonergic modulation of dorsal horn neurons during postnatal development

5.1 Introduction

In the previous chapter I characterised the anatomical connectivity of RVM serotonergic neurons in the spinal dorsal horn, and the distribution of spinal 5-HT_3R at several postnatal ages. In chapter 3, I demonstrated that neurons in the RVM predominantly facilitate spinal dorsal horn neuron activity in young P8 and P21 rats. Here, I test the hypothesis that this early dominant descending RVM facilitation observed in young rats is mediated by serotonergic signalling via activation of 5-HT_3Rs in the spinal dorsal horn. The aim of this chapter is to investigate the functional effect of descending serotonergic control upon dorsal horn neuron electrophysiological activity. This was done by ablating descending serotonergic fibres or spinal blockade of 5-HT_3Rs

5.1.1 Descending RVM serotonergic modulation of spinal sensory circuits in uninjured adult animals

Serotonergic neurons are a key population of neurons in the RVM which modulate spinal sensory circuits. Early experiments suggested that serotonergic neurons in the RVM do not belong to electrophysiologically defined On or Off cell classes; as only Neutral cells demonstrated 5-HT immunoreactivity (Potrebic et al., 1994), and a small proportion of serotonergic RMg neurons were weakly activated by thermal stimulation of the tail (Gao and Mason, 2000). However, large proportions of serotonergic neurons in the LPGi and subsidiarily in the RMg were found to be strongly activated or inhibited by high threshold cutaneous mechanical and thermal stimulation, but not low threshold inputs (Gau et al., 2013). Whilst it is less clear if RVM serotonergic neurons can be classified as On, Off or Neutral cells, their responsivity to sensory inputs (presumed indirect) and roles in functional modulation of dorsal horn sensory circuits is well documented. The responsivity of serotonergic RVM neurons to noxious stimuli and their known role in descending modulation suggests that these are a neurochemically defined population of neurons in the descending arm of the spinal-bulbo-spinal loop

Convincing and repeatable evidence has demonstrated that depletion of spinal 5-HT in the spinal cord, either by destruction of serotonergic neurons by injection of 5,7-DHT or by shRNA interference of Tph-2 in RVM neurons (Wei et al., 2010) has no effect on acute nociceptive behavioural thresholds (Mohrland and Gebhart, 1980; Svensson et al., 2006; Wei et al., 2010; Leong et al., 2011; Carr et al., 2014), suggesting a limited role of endogenous descending serotonergic modulation of nociceptive reflexes.

However, descending modulation from serotonergic RVM neurons can be evoked in uninjured adult rats, as RVM stimulation produced analgesia (SPA) observed in the hot plate test and radiant heat test is abolished following depletion of spinal 5-HT (Wei et al., 2010) or reduced following 5,7-DHT treatment (Liu et al., 1988). RVM SPA can also be abolished by intrathecal injection of the non-selective 5-$HT_{1/2}$ receptor antagonist methysergide (Hammond and Yaksh, 1984). Direct activation of spinal 5-HTRs by local application of 5-HT has to been found to decrease C-fibre evoked spiking activity of dorsal horn neurons (Liu et al., 2007). Similarly, intrathecal injection of 5-HT has been reported to be antinociceptive in hot plate and tail flick tests in rats (Schmauss et al., 1983), and blocks biting and scratching behaviours caused by intrathecal injections of substance P when injected in low concentrations in mice (Hylden and Wilcox, 1983).

Collectively, these findings suggest that evoked, but not endogenous, descending 5-HT transmission in the spinal dorsal horn drives inhibition of spinal nociceptive circuits in uninjured adult animals. However, descending facilitatory drive from RVM serotonergic neurons has also been reported in uninjured adult animals: optogenetic activation of serotonergic neurons in the RVM has recently been shown to decrease mechanical and thermal withdrawal thresholds in adult mice (Cai et al., 2014). Additionally, 5,7-DHT injection in adult rats reduced noxious vFh and heat-evoked dorsal horn WDR neuron firing activity compared to control rats, unmasking an excitatory role of serotonergic neurons in the dorsal horn (Rahman et al., 2006). Similarly, blocking endogenous spinal 5-HT_3R activation by applying high doses of ondansetron reduces dorsal horn WDR neuron firing activity in response to high force vFh stimulation of the hindpaw (Rahman et al., 2004a). Thus, like overall descending modulation from the RVM, evidence suggests changeable descending serotonergic modulation of spinal sensory processing and pain behaviours.

5.1.2 Evidence for RVM serotonergic neuron and spinal 5-HT_3R-mediated pronociception in adult chronic pain models

Extensive evidence points towards a key pro-nociceptive role of RVM serotonergic neurons in the spinal and medullary dorsal horn in chronic pain states in adults. Depletion of 5-HT in the RVM by local injection of Tph-2 shRNA, or application of 5-HT_3R antagonists to the medullary dorsal horn had no effect on behavioural mechanical hypersensitivity 5 days after orofacial nerve injury, but attenuated Ca^{2+}-signals in TRPV1+ primary afferent terminals in medullary dorsal horn slices; and *in*

vivo medullary dorsal horn firing properties and behavioural mechanical hypersensitivity 14 days after injury in adult rats and mice (Okubo et al., 2013; Kim et al., 2014a). These authors propose a key role for 5-HT-5HT_3R signalling which drives the maintenance, but not the onset, of neuropathic pain states following orofacial nerve injury.

Ablation of descending serotonergic inputs to the dorsal horn by intrathecal injection of 5,7-DHT reverses increased vFh-evoked firing activity of dorsal horn neurons following spinal nerve injury (Rahman et al., 2006); an effect which can be mimicked following spinal application of the 5-HT_3R antagonist ondansetron (Suzuki et al., 2004a). Blocking 5-HT_3Rs in the spinal cord also reduces sustained firing activity of dorsal horn neurons (Green et al., 2000) and behavioural hypersensitivity (Svensson et al., 2006) in response to hindpaw formalin injection in adult rats. Importantly, in uninjured adult rats, blocking spinal 5-HT_3Rs only reduces dorsal horn firing activity during high intensity noxious mechanical stimulation (Rahman et al., 2004b), and does not alter behavioural nocifensive withdrawal thresholds (Lagraize et al., 2010; Guo et al., 2014). Based on these findings, a current hypothesis posits that 5-HT_3R activation drives excitation of spinal sensory circuits and pain behaviours in chronic pain states, but not during acute nociception in adulthood. To date, the functional effect of 5-HT_3R mediation upon sensory processing in the spinal dorsal horn has not been investigated in young animals.

5.1.3 Descending modulation of spinal sensory circuits in young animals

Descending RVM modulation of spinal sensory circuits changes during postnatal development. RVM-mediated facilitation of dorsal horn neurons and hindlimb nociceptive reflexes dominates during the first 4-5 weeks of life whilst descending inhibitory transmission gradually matures (Hathway et al., 2009a, 2012; Koch and Fitzgerald, 2014). In chapter 3, I demonstrated that this early descending facilitation of dorsal horn neuron firing and receptive field properties is continuous and can be unmasked by silencing RVM neurons. Experiments in chapter 4 demonstrated anatomical maturation of axonal projections into the lumbar dorsal horn from RVM serotonergic neurons over the first weeks of postnatal life. In contrast, the distribution of 5-HT_3R immunoreactivity in the dorsal horn did not change with age. In this chapter, I test the hypothesis that in the first weeks of life, a continuous background descending facilitation of dorsal horn neurons is provided by brainstem serotonergic neurons and that this facilitation is mediated by 5-HT_3Rs in the spinal dorsal horn.

Furthermore, I test the hypothesis that this background serotonergic 5-HT_3R facilitation declines with age and is not observed in healthy adult rats.

5.2 Experimental aims

The aims of this chapter are to investigate the effect of ablating serotonergic neurons with axons descending to the lumbar dorsal horn and of blocking spinal 5-HT_3Rs on dorsal horn neuron electrophysiological properties in young and adult animals. Additionally, I will question whether descending RVM facilitation can be abolished by ablating descending serotonergic neurons. I will also test whether ablating descending serotonergic fibres in in the first postnatal week affects the onset of descending inhibitory drive in adulthood. The key hypotheses are:

1. Descending RVM serotonergic neurons provide a background facilitation of dorsal horn neuron activity in young rats aged P8 and P21.
2. Serotonergic facilitation of dorsal horn neuron activity in young rats is mediated by 5-HT_3Rs in the dorsal horn.
3. This background descending serotonergic 5-HT_3R signalling declines over development and is not present in healthy adults
4. In the absence of descending serotonergic fibres in P21 rats, it is not possible to evoke descending modulation of dorsal horn neuron activity from the RVM.
5. The development of the descending modulatory system is impaired in the absence of descending serotonergic fibres during postnatal development.

5.3 Methods

5.3.1 Animals

All experiments were performed in accordance with the United Kingdom Animal (Scientific Procedures) Act 1986. Male and female Sprague-Dawley rats at postnatal day (P) 8, 16, 21 and 40 were obtained from the Biological Services Unit, University College London. Rats were bred and maintained in-house and exposed to the same caging, diet and handling throughout development. Litters were weaned at P21 into same sex cages of four littermates and were housed in 12h light/dark cycles at constant ambient temperature and humidity with free access to water and food.

5.3.2 Drugs

In descending RVM serotonergic neuron ablation experiments, 5,7-di-hydroxytryptamine creatine sulphate (5,7-DHT) (Sigma-Aldrich, UK) was dissolved in saline to a concentration of 3mg/ml. To prevent noradrenergic neuron toxicity, rats were pre-treated with Desipramine (Sigma-Aldrich) dissolved in saline at a concentration of 5mg/ml. Desipramine (25mg/kg) was intraperitoneally (I.P) injected 1 hour before 5,7-DHT or saline injection. For intrathecal injections, rats were deeply anaesthetised with isoflurane and low lumbar spinous processes were visualised through a small midline skin incision. A 30-guage needle was passed in the midline through the lumbar (L) 4/5 or L5/6 intervertebral space to perform intrathecal injection. The same injectate dose and volume of 5,7-DHT (60µg in 20µl) was used in all ages to insure sufficient spread of injectate to the lumbar 4/5 spinal cord (Westin et al., 2010). Control animals received equivalent volumes of saline. The skin overlying the injection site was then sutured with 5-0 suture (Ethicon), EMLA cream (AstraZeneca) was placed on the wound, and animals were returned to the mothers or back to home cages for five days before electrophysiology experiments.

In 5-HT_3R antagonism experiments, the selective 5-HT_3R antagonist Ondansetron Hydrochloride (Tocris Biosciences, UK) was dissolved in saline to a concentration of 0.4µg/µl, 2µg/µl or 10µg/µl. A 50µl solution containing 2µg, 10µg or 50µg of Ondansetron was then applied to the surface of the L4/5 spinal dorsal horn during electrophysiology experiments and dorsal horn neuron activity was recorded for up to 1 hour to ensure recordings were performed during the period of maximum effect of ondansetron (Rahman et al., 2004).

5.3.3 Electrophysiology Surgery

Rats were anaesthetised with isoflurane (induction 4% in medical O_2), tracheotomised and artificially ventilated under constant isoflurane anaesthesia (maintenance of 1.8% in medical O_2, Univentor Anaesthesia Unit 400; Royem Scientific, UK). The air flow and breathing rate were adjusted to the animal's sizes using a small animal ventilator (model 687, Harvard Apparatus, MA, USA). Heart rate was constantly monitored via electrocardiogram. A homoeothermic blanket with feedback control (model 507220F, Harvard Apparatus, MA, USA) was used to maintain body temperature at physiological levels. The rat was mounted onto a stereotaxic frame (Kopf Instruments, Tujunga, CA, USA). A laminectomy was performed to expose the lumbar spinal cord, the vertebral column was secured with a clamp to the thoracic site and the dura and pia mater were removed. A film of mineral oil was used to cover the exposed spinal cord to prevent heat loss. In ondansetron experiments, mineral oil was soaked away prior to application of drug solution. In RVM stimulation experiments, the skull was exposed and bregma located to perform a small craniotomy for unilateral RVM microinjection. Stereotaxic coordinates for the P21 RVM were calculated as outlined previously (Hathway et al., 2009a): lateral 0mm, antero-posterior 9.2m, dorso-ventral -10.0mm.

In ondansetron experiments, animals were terminally anaesthetised with pentobarbitone sodium (500mg/kg). In 5,7-DHT experiments, animals were terminally anaesthetised with pentobarbitone sodium (500mg/kg) and perfused transcardially with heparinised saline (5000 IU/ml) followed by 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer. The brain was removed and postfixed overnight in 4% PFA and transferred to a 30% sucrose solution in 0.1 PB containing 0.01% azide and stored at 4°C. To inspect stimulation sites, the cerebellum was removed, and the brainstem was cut coronally with a scalpel blade to allow visual inspection of the tract mark made by the injection site into the RVM (Fig 5.1C). Data from animals with stimulation sites outside the RVM were rejected. Spinal cords were sectioned and processed for immunohistochemical staining of 5-HT transporter protein (5-HTT) to confirm serotonin fibre ablation in the lumbar spinal cord (Figs 5.1D, E and F).

5.3.4 In vivo extracellular recordings in the dorsal horn

To isolate individual neurones in the dorsal horn, a 6µm tipped glass-coated carbon fibre microelectrode (Kation Scientific, Minneapolis, USA) was lowered through the spinal cord with an in vivo manipulator (Scientifica, UK) while stroking the plantar surface of the hindpaw as a search stimulus for dorsal horn wide dynamic range

(WDR) neurons in lamina IV-VI. All recorded WDR neurons had receptive fields in the glabrous skin. Mean recording depth at P8 was 405.4μm, at P21 was 529.7μm and at P40 was 577.4μm (see Fig 5.1B for range and distribution).

Cutaneous glabrous receptive fields to brush and pinch stimulation were mapped and the number of spikes per stimulus to brush, pinch and von Frey hair (vFh) stimulation of the receptive field were recorded. The brush stimulus used was a fine acrylic paintbrush with a 1mm tip, briefly applied to the centre of the hindpaw receptive field. Pinch stimulation in all ages was performed with a pair of F.S.T curved serrated forceps (product code 11152-10) with a 0.3mm tip. The centre of the receptive field was pinched until the arms of the forceps just began to bend and was applied for 2.5 seconds, providing consistent pinch stimuli. Because the relative size of the hindpaw is smaller in P8 rats, brush and pinch stimuli unavoidably covered a relatively larger region of the hindpaw compared to older animals. Spontaneous neuronal activity was recorded for one minute. A force calibration curve for vFhs (ranging from 0.18g to 6.70g or 9.80g) used in electrophysiology and behavioural experiments is shown in chapter 3 figure 3.2A. VFhs were applied to the centre of the hindpaw receptive field for 1 second. To avoid overstimulation and sensitisation of nociceptors, the maximum vFh forced applied to P8 rats was 6.70g, and to P21 and adult rats was 9.80g. Stimulus evoked action potentials were digitalised using PowerLab 4/30 interface and isolated using the Chart 5 software spike histogram plug-in (AD Instruments Ltd, Oxford, UK).

In RVM microstimulation experiments in P21 rats, WDR neuron baseline brush, pinch and vFh receptive fields and firing activity were initially characterised in the absence of electrical stimulation for baseline recordings. Then, trains of electrical stimuli of 500μs pulse width were applied at 10Hz, at 10 and 100μA using a stimulus isolator (NeuroLog). A second set of WDR neuron baseline firing properties was performed after electrical stimulation (see figure 5.1A for summary). These stimulation parameters reliable evoke descending excitation (10μA) and inhibition (100μA) of dorsal horn neuron and hindlimb or tail reflex activities in adult rats (Zhuo and Gebhart, 1997; Hathway et al., 2009a; Koch and Fitzgerald, 2014).

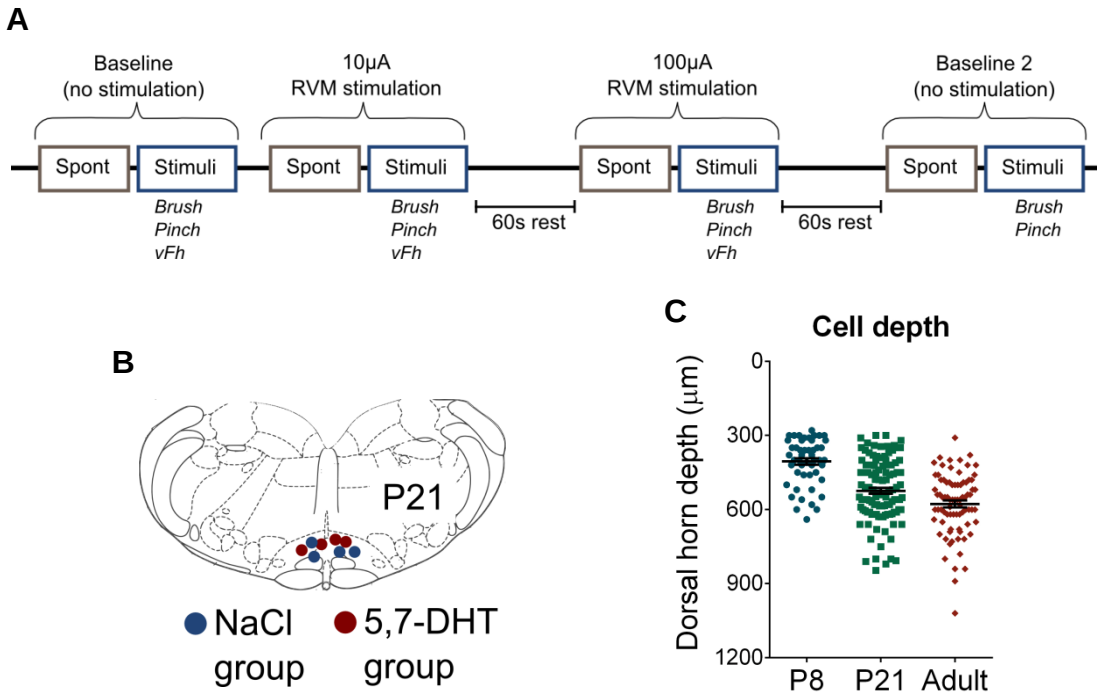

Fig 5.1. Electrophysiology experimental protocol and cell depths.
In RVM electrical stimulation experiments in P21 rats, dorsal horn wide neuron baseline spontaneous (spont) and sensory-evoked firing and receptive field properties were recorded (A). The same properties were recorded again during 10µA and 100µA stimulation of the RVM. Baseline WDR neuron properties were recorded again, after the stimulation bouts. Electrode placements in these experiments were checked (B). A total of 292 dorsal horn neurons were recorded for this chapter at P8, P21 and in adult P40-45 rats. The spread and mean cell depths at each age are shown in C.

5.3.5 Behavioural Testing

To test potential effects of neonatal (P8) ablation of descending RVM fibres on adult nocifensive behaviours, baseline mechanical withdrawal thresholds were measured 35 days after intrathecal 5,7-DHT (and desipramine) injection. Animals were habituated to the testing environment for 2 hours before baseline testing. For measurement of mechanical withdrawal threshold in the testing environment, animals were placed in individual Plexiglas cubicles on an elevated mesh platform. Left and right hindpaw plantar surfaces were stimulated with von Frey hair filaments and the reflex threshold was determined by using the up-down method (Chaplan et al., 1994). A starter filament of 9.47g was applied to the plantar surface, and depending on the response of the animal, higher or lower force vFhs were then applied to the hindpaw to establish a series of 6-9 positive or negative reflex withdrawals. Data is expressed as log of the mean of the 50% reflex withdrawal threshold. Mechanical withdrawal thresholds of

P8+35d 5,7-DHT treated animals (n=4) were compared with baseline mechanical thresholds of P40 animals (n=6) from chapter 2.

5.3.6 Immunohistochemistry

To confirm that intrathecal injections of 5,7-DHT successfully ablated descending serotonergic fibres, animals were transcardially perfused after electrophysiology experiments. Spinal cord sections were cut on a freezing microtome in 30µm sections and immunohistochemically labelled with a serotonin transporter (5-HTT) antibody.

Free floating sections were blocked with 3% goat serum in 0.3% Triton X-100 in 0.1M PBS for 1h at RT. Sections were then incubated overnight at room temperature with rabbit anti-5-HTT antibody (1:10,000, Immunostar). The next day, sections were incubated in biotinylated anti-rabbit antibody (goat anti-rabbit; 1:400; Vector Stain) for 90mins. Sections were placed in ABC complex (1:125; Vector Stain, ABC elite kit, Vector Labs) for 30mins, followed by biotinylated tyramide (1:75; TSA Stain Kit; Perkin Elmer) for 7mins. Sections were then incubated in fluorescence visualiser Cy3 (1:600; Vector Stain) for 45 minutes. Sections were mounted on gelatinised slides and were then cover slipped with Fluoromount (Sigma). Negative control stains omitting primary antibodies resulted in no immunofluorescence, demonstrating no non-specificity of any protocol. Sections were viewed using a Leica DMR light microscope, photographed using a Hamamatsu C4742-95 digital camera.

5.3.7 Statistical analysis

Statistical analyses and graphing were performed using GraphPad Prism 6 (GraphPad software, La Jolla, CA, USA) and $P<0.05$ was considered statistically significant. Sample sizes for testing were based on previously reported group differences between RVM silenced/stimulated animals in electrophysiology experiments (Waters and Lumb, 1997; Bee and Dickenson, 2007; Koch and Fitzgerald, 2014). Data are represented as means ± standard error of mean (SEM). Evoked cell response values are expressed as the mean of three stimuli. For pinch after discharge (AD) values, spikes were counted in a 1s bin during the peak time of pinch after-stimulus spike activity. Dorsal horn neuron receptive fields were drawn on a template during recording and then imported and expressed as a percentage of the total area of the hindpaw plantar surface using Inkscape (version 0.48 www.inkscape.org).

In experiments without RVM electrical stimulation, population-based statistical comparisons were performed. WDR neuron recordings from 5,7-DHT-treated or

ondansetron-treated animals were pooled and treated as one population of neurones for each age. Data from naïve animals were combined with data from RVM saline treated animals (from chapter 3) as a control group. The control cell population is a pooled group of cells from animals receiving RVM saline (P8 = 7 cells form 2 animals; P21 = 15 cells from 2 animals; adult (13 cells from 2 animal) and naïve animals which displayed the same cell properties. In normally distributed data sets, group differences between drug treated (5,7-DHT or ondansetron) and between control animals within age groups were tested with unpaired Student's t-tests or one-way analysis of variance (ANOVA) followed by Bonferroni *post hoc* multiple comparisons tests. Data sets which were not normally distributed were compared with Mann-Whitney or Kruskal-Wallis tests. In vFh experiments, Two-way repeated measures ANOVA were used within the age groups followed by Bonferroni *post hoc* multiple comparison test to compare differences in responses to increasing vFh force in control animals and drug-treated animals.

In experiments with RVM electrical stimulation in P21 rats, within subject, repeated measures statistical comparisons were used. The effect of RVM stimulation at different amplitudes (10μA and 100μA) on WDR neuron firing and receptive field properties were compared to baseline recordings from the same cell. Brush and pinch-evoked firing activities were compared to two baselines; one before and one after electrical stimulation bouts to ensure changes in WDR neuron firing properties were not long term after RVM stimulation and repeated hindpaw stimulation. All other comparisons were compared to baseline conditions before electrical stimulation bouts. Repeated measures one-way or two-way ANOVAs with Bonferroni *post hoc* analysis were performed to analyse within-cell and between population (baseline vs 10μA or 100μA) changes caused by RVM stimulation. Data sets which were not normally distributed were compared with repeated measures Friedman Tests. Brush and pinch-evoked firing activity data from saline and 5,7-DHT treated animals was also expressed as the percentage change from baseline 1 firing activity. Cells were classified as facilitated or inhibited by RVM stimulation if firing rates increased or decreased by >20% of baseline values, respectively. This threshold was chosen as it is above the normal level of variability observed between the two baseline recordings for each stimulus modality.

In behaviour experiments, mechanical withdrawal thresholds were compared using one-way ANOVA with Bonferroni *post hoc* analysis.

5.4 Results

5.4.1 Intrathecal injection of 5,7-DHT depletes descending serotonergic fibres in the lumbar spinal cord

To test whether intrathecal injections of 5,7-DHT ablate descending serotonergic terminals in the lumbar spinal dorsal horn, spinal cord sections were taken after electrophysiology experiments and labelled with 5-HTT antibody immunostaining at three different ages. 5,7-DHT injection in P4 rats caused a major reduction in 5-HTT immunoreactivity at P8 compared to control animals, however some 5-HTT labelled axons were observed in the deep dorsal horn (Fig 5.2A and B). The presence of 5-HTT immunoreactive fibres in these animals likely demonstrates later growth of serotonergic axonal terminals into the lumbar dorsal horn during the first two weeks of postnatal life (as observed in chapter 4). 5,7-DHT injection at P16 abolished 5-HTT immunoreactivity in the spinal cord at P21, and similarly, 5,7-DHT injection at P40 abolished 5-HTT immunoreactivity at P45-47 compared to control animals (Fig 5.2C and D).

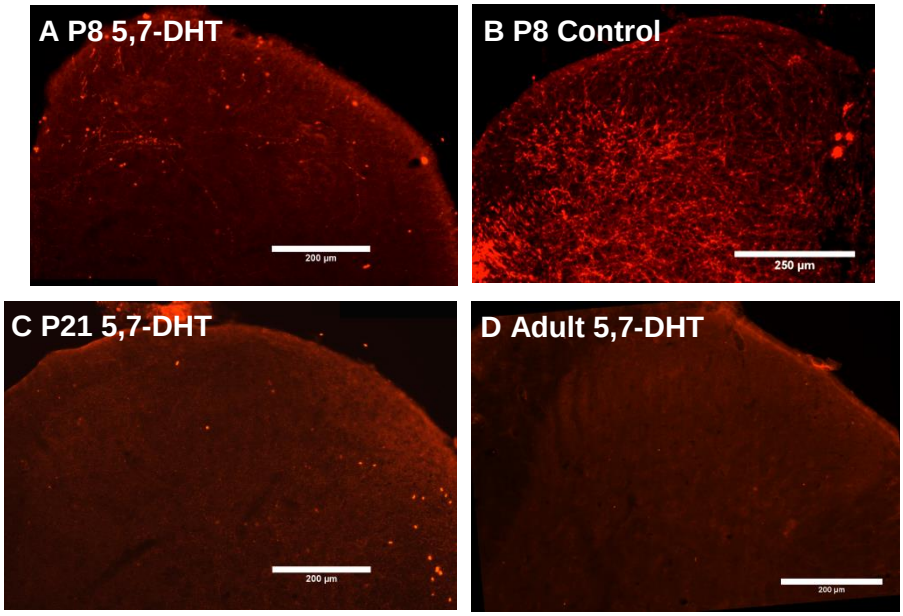

Fig 5.2. 5,7-DHT depletes 5-HTT immunoreactivity in the dorsal horn
5,7-DHT was intrathecally injected at P4, P16 or P40 and spinal cord tissue was taken 4-7 days later. 5,7-DHT injection ablated at P4 dramatically reduced 5-HTT staining in the dorsal horn at P8 (A) compared to control (B), however some 5-HTT+ fibres can still be seen. 5,7-DHT injection at P16 caused an absence of 5-HTT staining in the dorsal horn at P21 (C), and 5,7-DHT injection at P40 caused an absence of 5-HTT in the dorsal horn at P45-47 (D).

5.4.2 Short term depletion of descending serotonergic fibres at three different ages

Next, 5,7-DHT was intrathecally injected in three groups of animals at P4, P16 or at P40 and single unit dorsal horn WDR recordings were performed in control and 5,7-DHT treated rats at P8, P21 and P45-47. The aims of these experiments were to investigate whether descending serotonergic neurons modulate dorsal horn neuron processing tactile and noxious mechanical inputs in young rats, and to test whether this descending modulation changes with postnatal age.

5.4.2.1 Intrathecal 5,7-DHT injection at P4 unmasks serotonergic facilitation of brush and pinch evoked dorsal horn WDR neuron activity at P8

To test whether descending serotonergic projections to the spinal dorsal horn modulate dorsal horn neuron firing properties in neonatal animals, 5,7-DHT was intrathecally injected at P4. Single unit dorsal horn WDR neuron recordings were then performed 4 days later. A total of 41 deep dorsal horn WDR neurons were recorded in P8 rats for this study: 24 cells from six control rats, and 17 cells from four 5,7-DHT injected rats. Spontaneous firing activity was low in both control and 5,7-DHT-treated rats, and did not differ between the two groups (Mann-Whitney test, Fig 5.3A). Brush-evoked firing activity was significantly lower in 5,7-DHT-treated rats compared to control (unpaired Student's t-test, control vs 5,7-DHT, $P<0.05$; Fig 5.3B), however brush-receptive field size was not significantly different in the two groups (unpaired Student's t-test, Fig 5.3C). Pinch-evoked firing activity was also significantly lower in 5,7-DHT-treated rats compared to control (unpaired Student's t-test, control vs 5,7-DHT, $P<0.01$; Fig 5.3D). Pinch-evoked after discharge firing activity was absent in 5,7-DHT-treated rats, and was therefore significantly lower compared to control (unpaired Student's t-test, control vs 5,7-DHT, $P<0.05$; Fig 5.3F). Pinch receptive field size was not significantly different in 5,7-DHT-treated and control rats (unpaired Student's t-test, Fig 5.3E).

Comparison of stimulus response curves between 5,7-DHT-treated and control animals at P8 revealed no significant effect of 5,7-DHT on vFh evoked firing activity (Two-way repeated measures ANOVA with Bonferroni *post hoc* analysis, control vs. 5,7-DHT, $F(1,26)=2.070$, $P=0.162$; Fig 5.3G).

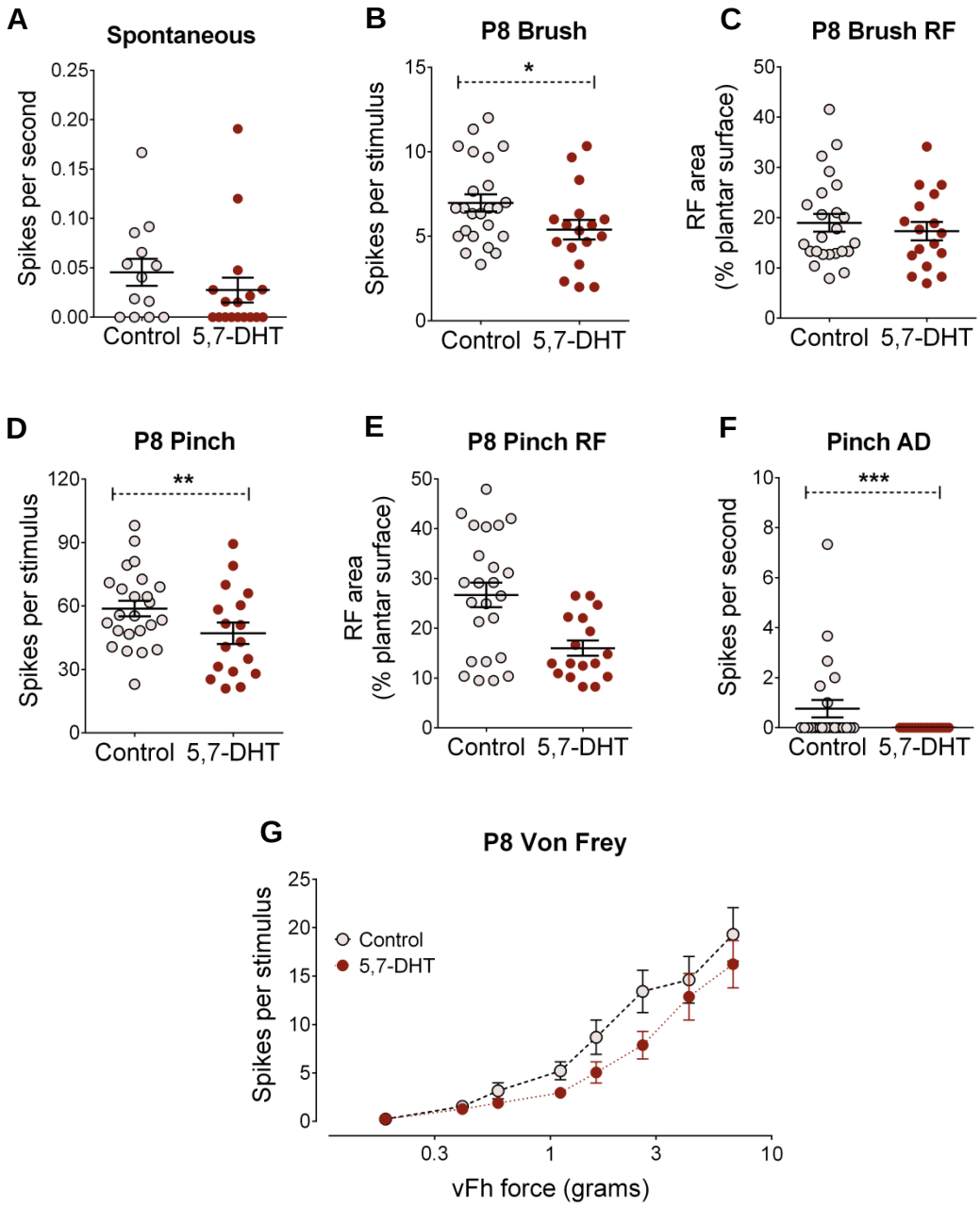

Fig 5.3. Intrathecal injection of 5,7-DHT at P4 decreased WDR neuron activity at P8
5,7-DHT was injected intrathecally at P4 and dorsal horn recordings were performed at P8 (n=17), and were compared to control animals (n=24). Spontaneous firing activity did not significantly differ between groups (A). Brush-evoked firing activity was significantly lower in 5,7-DHT treated rats compared to control (B), but brush receptive field (RF) size did not (C). Pinch-evoked firing was significantly lower in 5,7-DHT treated rats (D), as was pinch after discharge (AD) (F), but pinch receptive field size did not differ (E). von Frey hair (vFh) evoked firing activity did not differ between groups (G). Unpaired student's t-test, *,** P<0.05 and 0.01.

5.4.2.2 Intrathecal 5,7-DHT injection at P16 unmasks serotonergic facilitation of brush and pinch evoked dorsal horn WDR neuron activity at P21

Next, the effect of intrathecally injecting 5,7-DHT at P16 upon WDR neuron activity at P21 was investigated. A total of 65 cells were recorded in these experiments: 26 cells from four control rats, and 39 cells from seven 5,7-DHT treated rats.

Spontaneous firing activity was low in both control and 5,7-DHT treated rats, and did not differ between the two groups (unpaired Student's t-test; Fig 5.4A). Brush-evoked firing activity was significantly lower in 5,7-DHT-treated rats compared to control (unpaired Student's t-test, control vs 5,7-DHT, $P<0.01$; Fig 5.4B), however brush-receptive field size was not significantly different in the two groups (unpaired Student's t-test, Fig 5.4C).

Pinch-evoked firing activity was significantly lower in 5,7-DHT-treated rats compared to control (unpaired Student's t-test, control vs 5,7-DHT, $P<0.001$; Fig 5.4D). Pinch-evoked after discharge firing activity was also significantly lower compared to control (unpaired Student's t-test, control vs 5,7-DHT, $P<0.05$; Fig 5.4F). Moreover, average pinch receptive field size were significantly and relatively smaller in 5,7-DHT-treated rats compared to control rats (unpaired Student's t-test, control vs 5,7-DHT, $P<0.05$; Fig 5.4E).

A two-way repeated measures ANOVA revealed a significant effect of 5,7-DHT-treatment on vFh evoked firing activity (Two-way repeated measures ANOVA, control vs. 5,7-DHT, $F(1,60)=10.29$, $P<0.01$; Fig 5.4G). Bonferroni post-hoc analysis revealed significant differences between RVM 5,7-DHT and control rats at 4.23g and 9.77g vFhs (Two-way repeated measures ANOVA with Bonferroni *post-hoc* analysis, control vs. 5,7-DHT, $P<0.01$ to 0.01 at 4.23g and 9.77g vFh forces; Fig 5.4G).

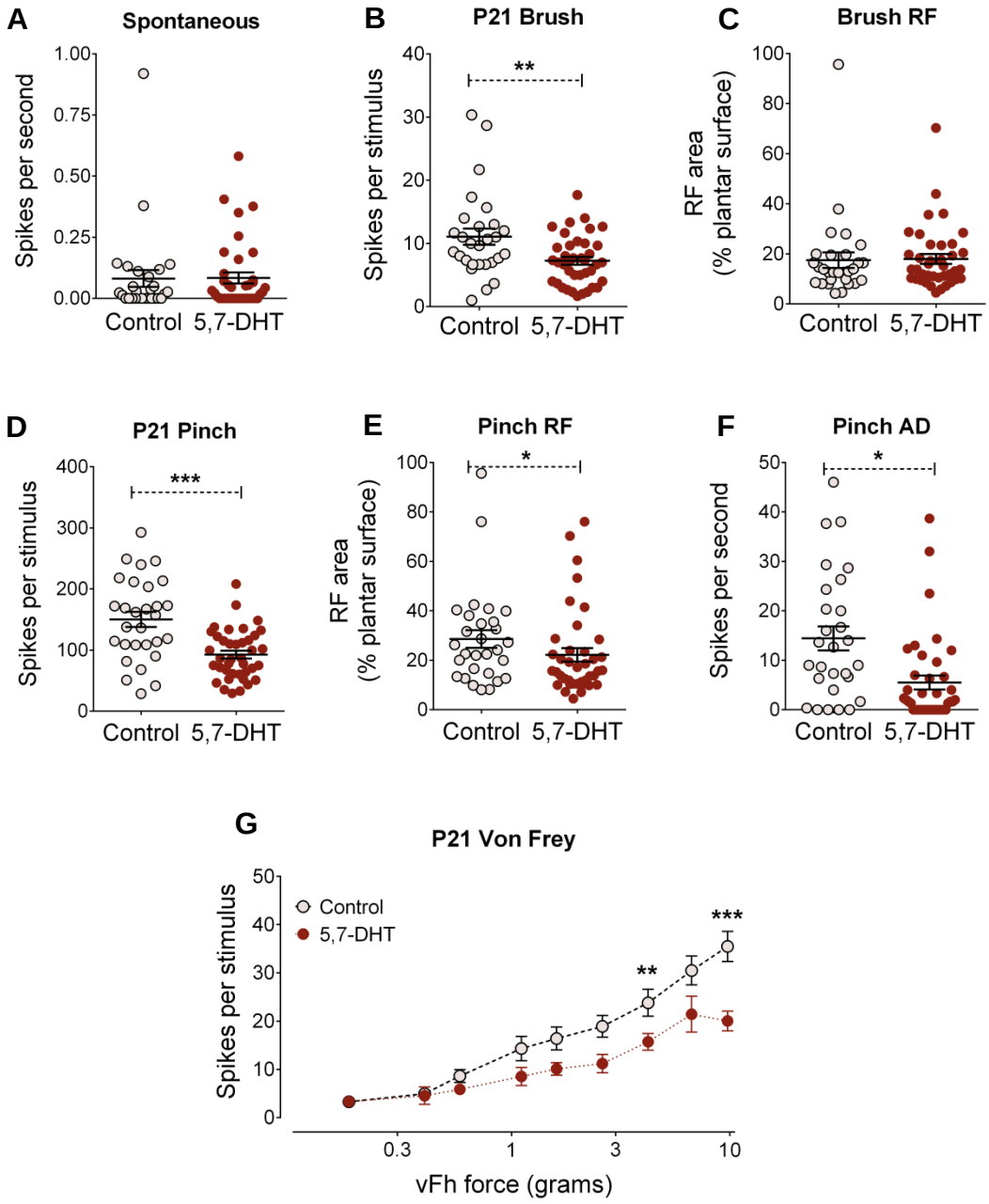

Fig 5.4. Intrathecal injection of 5,7-DHT at P16 decreased WDR neuron activity at P21
5,7-DHT was injected intrathecally at P16 and dorsal horn recordings were performed at P21 (n=39), and were compared to control animals (n=26). Spontaneous firing activity did not significantly differ between groups (A). Brush-evoked firing activity was significantly lower in 5,7-DHT treated rats compared to control (B), but brush receptive field (RF) size did not (C). Pinch-evoked firing was significantly lower in 5,7-DHT treated rats (D), as was pinch receptive field size (E) and pinch after discharge (AD) (F). Von Frey hair (vFh) evoked firing activity was significantly lower in 5,7-DHT treated rats at vFhs 4.23g and 9.77g (G). Unpaired student's t-test, and Two-way repeated measures ANOVA with Bonferroni post hoc analysis, *,**,*** P<0.05, 0.01 and 0.001.

5.4.2.3 Intrathecal 5,7-DHT injection at P40 unmasks serotonergic facilitation and inhibition in adult rats

Next, the effect of intrathecally injecting 5,7-DHT at P40 upon WDR neuron activity at P45-47 was investigated. A total of 46 cells were recorded in these experiments: 23 cells from seven control rats, and 23 cells from four 5,7-DHT treated rats.

Spontaneous firing activity was significantly lower in 5,7-DHT-treated rats compared to control (unpaired Student's t-test, control vs 5,7-DHT, $P<0.01$; Fig 5.5A). Brush-evoked firing activity was not significantly different between control and 5,7-DHT groups (unpaired Student's t-test; Fig 5.5B), but average brush receptive field was size significantly and relatively smaller in 5,7-DHT treated rats compared to control (unpaired Student's t-test, control vs 5,7-DHT, $P<0.05$; Fig 5.5C).

Pinch-evoked firing activity was significantly higher in 5,7-DHT-treated rats compared to control (unpaired Student's t-test, control vs 5,7-DHT, $P<0.01$; Fig 5.5D), however pinch receptive field size and pinch after discharge were not significantly different between the two groups (unpaired Student's t-test; Figs 5.5E and F).

Comparison of stimulus response curves between 5,7-DHT-treated and control animals at P40 revealed no significant effect of 5,7-DHT on vFh evoked firing activity (Two-way repeated measures ANOVA with Bonferroni *post hoc* analysis, control vs. 5,7-DHT, $F(1,41)=0.050$, $P=0.822$; Fig 5.5G).

These experiments demonstrate that ablation of descending serotonergic terminals in the dorsal horn unmasks descending serotonergic facilitation of tactile inputs in rats of all ages. In comparison, serotonergic modulation of pinch inputs changes from facilitation to inhibition with postnatal age.

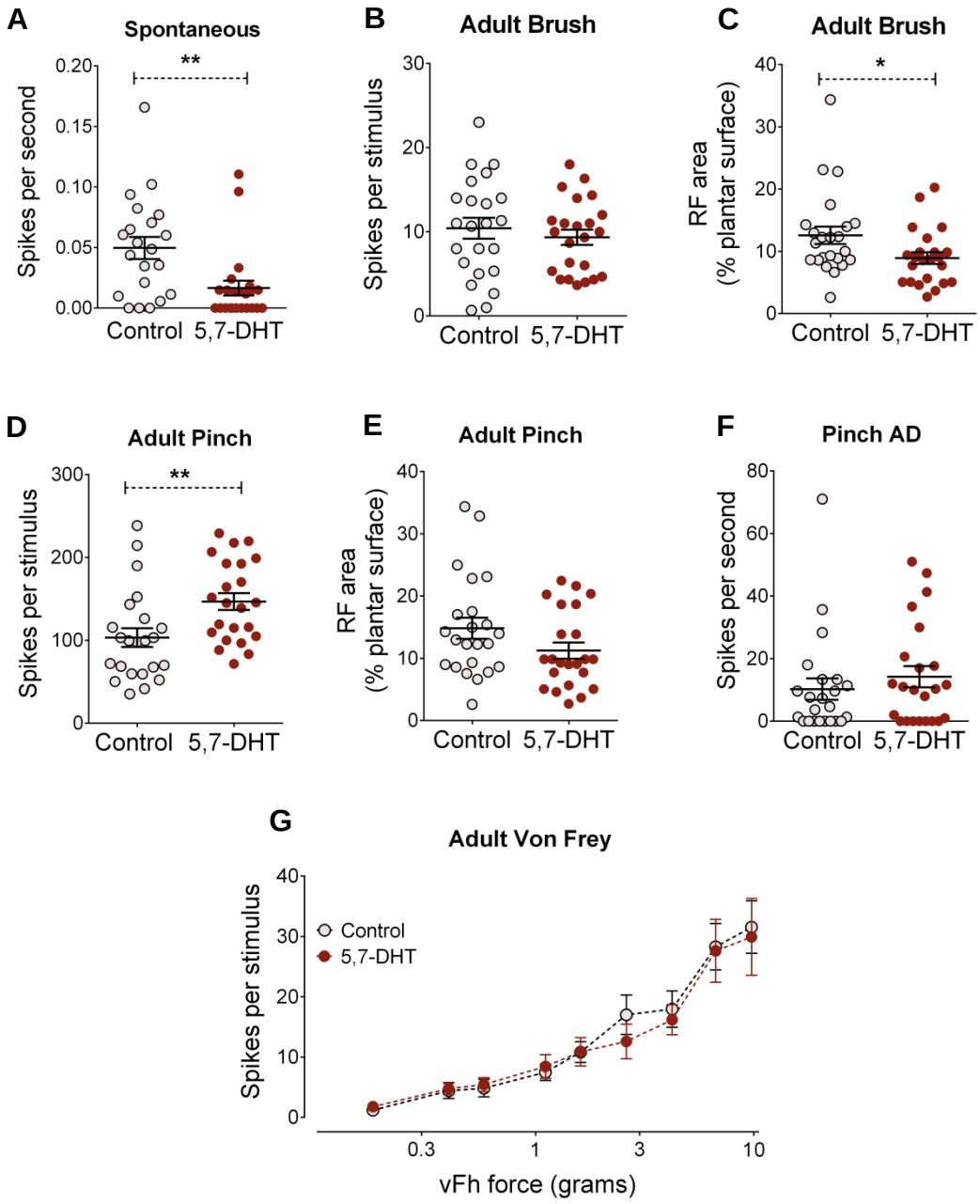

Fig 5.5. Intrathecal injection of 5,7-DHT at P40 facilitated and inhibited WDR neuron activity at P45-47

5,7-DHT was injected intrathecally at P40 and dorsal horn recordings were performed at P45-47 (n=23), and were compared to control animals (n=23). Spontaneous firing activity was significantly lower in 5,7-DHT treated rats (A). Brush-evoked firing activity did not significantly differ (B), but brush receptive field (RF) size was significantly lower in 5,7-DHT treated rats (C). Pinch-evoked firing was significantly higher in 5,7-DHT treated rats (D), but pinch receptive field size (E) and pinch after discharge (AD) (F) did not differ between groups. von Frey hair (vFh) evoked firing activity did not differ between groups (G). Unpaired student's t-test, *,** $P<0.05$ and 0.01.

5.4.3 RVM electrical stimulation increases mean pinch-evoked dorsal horn WDR neuron activity in saline-treated P21 rats

In the next sets of experiments, I tested the hypothesis that depletion of serotonergic neurons prevents descending facilitation of dorsal horn neurons caused by electrical stimulation of the RVM at P21. Electrical stimulation of the RVM at 10μA or 100μA previously been shown to cause excitation of dorsal horn neurons at P21 (Koch and Fitzgerald, 2014). In these first experiments, the effect of electrical stimulation of the RVM upon dorsal horn neuron properties was investigated in saline treated control P21 rats. WDR neuron firing activity was recorded at baseline, and during 10μA and 100μA electrical stimulation of the RVM. A total of 14 cells were recorded from four animals, however cells were lost in 3 instances during the 100μA stimulation bout and before the second baseline recording period, creating an *n* of 11 from four rats for these data sets. Because data sets were uneven, paired t-tests were used to compare baseline vs. 10μA or 100μA stimulation bouts. Electrode placement sites in the RVM were checked after electrophysiological recordings (Fig 5.1B).

Mean spontaneous firing activity was not significantly different during 10μA or 100μA stimulation compared to control (paired t-test, baseline vs. 10μA or 100μA, Fig 5.6A). Mean brush receptive field size during 10μA or 100μA stimulation and was not significantly different to baseline (paired t-test, baseline vs. 10μA or 100μA, Fig 5.6B). Similarly, mean brush-evoked firing activity was not significantly different during 10μA or 100μA stimulation when compared to baseline 1 or baseline 2 (paired t-test, baseline 1 or 2 vs. 10μA or 100μA, Fig 5.6D).

The mean pinch receptive field size was significantly larger during both 10μA or 100μA stimulation bouts compared to baseline (paired t-test, baseline vs. 10μA or 100μA, $P<0.01$ and $P<0.05$; Fig 5.6C). During 10μA RVM stimulation, mean pinch-evoked firing activity was significantly higher when compared to baseline 1 and baseline 2 (paired t-test, baseline 1 or 2 vs. 10μA, $P<0.01$; Fig 5.6E). Mean pinch-evoked firing activity was significantly higher during 100μA stimulation only when compared to baseline 2 (paired t-test, baseline 1 or 2 vs. 100μA, $P<0.001$; Fig 5.6E). Pinch after discharge firing did not significantly during 10μA or 100μA stimulation when compared to baseline (paired t-test, baseline vs. 10μA or 100μA, Fig 5.6F).

Three-way repeated measures ANOVA comparing the effect of 10μA or 100μA RVM stimulation on vFh-evoked firing activity demonstrated no significant effect of RVM stimulation compared to baseline (three-way repeated measures ANOVA with

Bonferroni *post hoc* analysis, baseline vs. 10µA or 100µA; F(2,26)=0.4021, P=0.672; Fig 5.6G).

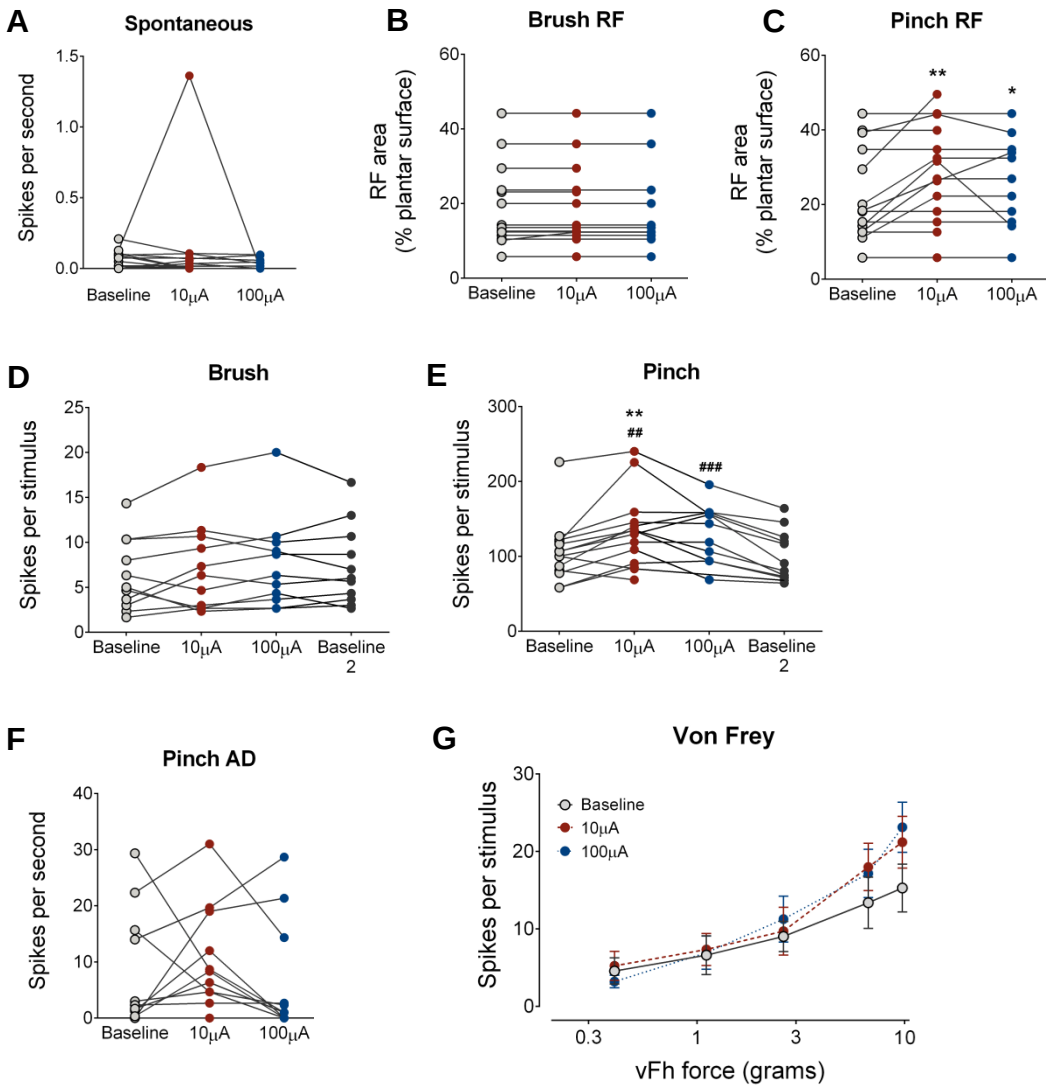

Fig 5.6. Electrical stimulation of the RVM in saline-treated P21 rats increases mean pinch-evoked dorsal horn WDR neuron activity

Saline was injected intrathecally at P16 and dorsal horn wide dynamic range (WDR) neuron recordings were performed at P21 (n=14). WDR neuron properties were recorded at baseline and during 10µA and 100µA stimulation of the RVM. No significant effect of stimulating the RVM at 10µA and 100µA on mean properties of WDR neurons was found compared to baseline for spontaneous activity (A) or brush receptive field (RF) size (B). Pinch RF size significantly increased during 10µA and 100µA RVM stimulation bouts compared to baseline (C). Brush evoked firing activity did not change upon RVM stimulation (D). Pinch evoked firing activity was significantly higher during 10µA stimulation compared to baseline 1 and 2, and was significantly higher during 100µA compared to baseline 2 only (E). Pinch after discharge (AD) (F) or von Frey hair (vFh) evoked firing activity did not change during RVM stimulation. See text for details of statistical analyses. *,** P<0.05 and 0.01 compared to baseline 1; ##, ### P<0.01 and 0.001 compared to baseline 2.

5.4.4 RVM electrical stimulation does not change mean dorsal horn WDR neuron properties in 5,7-DHT-treated P21 rats

Next, the effect of electrical RVM stimulation on dorsal horn neuron properties was investigated in young rats lacking descending serotonergic fibres. In these experiments, 5,7-DHT was intrathecally injected at P16 and dorsal horn WDR neuron recordings were performed at P21. Sensory-evoked WDR neuron firing and receptive field properties were recorded at baseline and during 10µA and 100µA electrical stimulation of the RVM, and again after electrical stimulation bouts for a second baseline (see methods Fig 5.1). A total of 20 cells were recorded from four rats. Absence of 5-HTT immunostaining in the lumbar spinal cord confirmed successful 5,7-DHT injections, and electrode tract marks in the RVM confirmed correct placement of stimulating electrodes (Fig 5.1B).

One-way repeated measures ANOVA comparing spontaneous firing activity at baseline, during 10µA and during 100µA RVM stimulation demonstrated no significant mean differences between the groups (One-way repeated measures ANOVA with Dunnett's *post hoc* comparison, baseline vs. 10µA or 100µA; (F1.212, 21.81)=1.821, P=0.192; Fig 5.7A). No significant mean differences were found between baseline recordings and at 10µA and 100µA stimulation for brush receptive field (One-way repeated measures ANOVA with Dunnett's *post hoc* comparison, baseline vs. 10µA or 100µA; (F1.645, 31.25)=1.756, P=0.193; Fig 5.7B) or pinch receptive field sizes (Friedman test, P=0.778; Fig 5.7C). Mean brush-evoked firing activity was unchanged during both 10µA and 100µA stimulation parameters compared to baseline 1 and baseline 2 (One-way repeated measures ANOVA with Bonferroni *post hoc* comparison, baseline vs. 10µA vs. 100µA vs. baseline 2; F=(2.555, 46.00)=2.085, P=0.120; Fig 5.7D). The same was observed for pinch-evoked firing activity (One-way repeated measures ANOVA with Bonferroni *post hoc* comparison, baseline vs. 10µA vs. 100µA vs. baseline 2; F=(2.119, 28.15)=0.763, P=0.480; Fig 5.7E) and pinch after discharge (One-way repeated measures ANOVA with Dunnett's *post hoc* comparison, baseline vs. 10µA or 100µA; F=(1.66, 29.99)=1.86, P=0.178; Fig 5.7F).

To test the effect of RVM stimulation at different amplitudes on stimulus response vFh-evoked firing activity, a repeated measures three-way ANOVA was performed and demonstrated no significant effect of stimulation on vFh-evoked firing at baseline compared to 10µA or 100µA stimulation (Three-way repeated measures ANOVA, baseline vs. 10µA and 100µA, F(2,36)=1.260, P=0.296; Fig 5.7G).

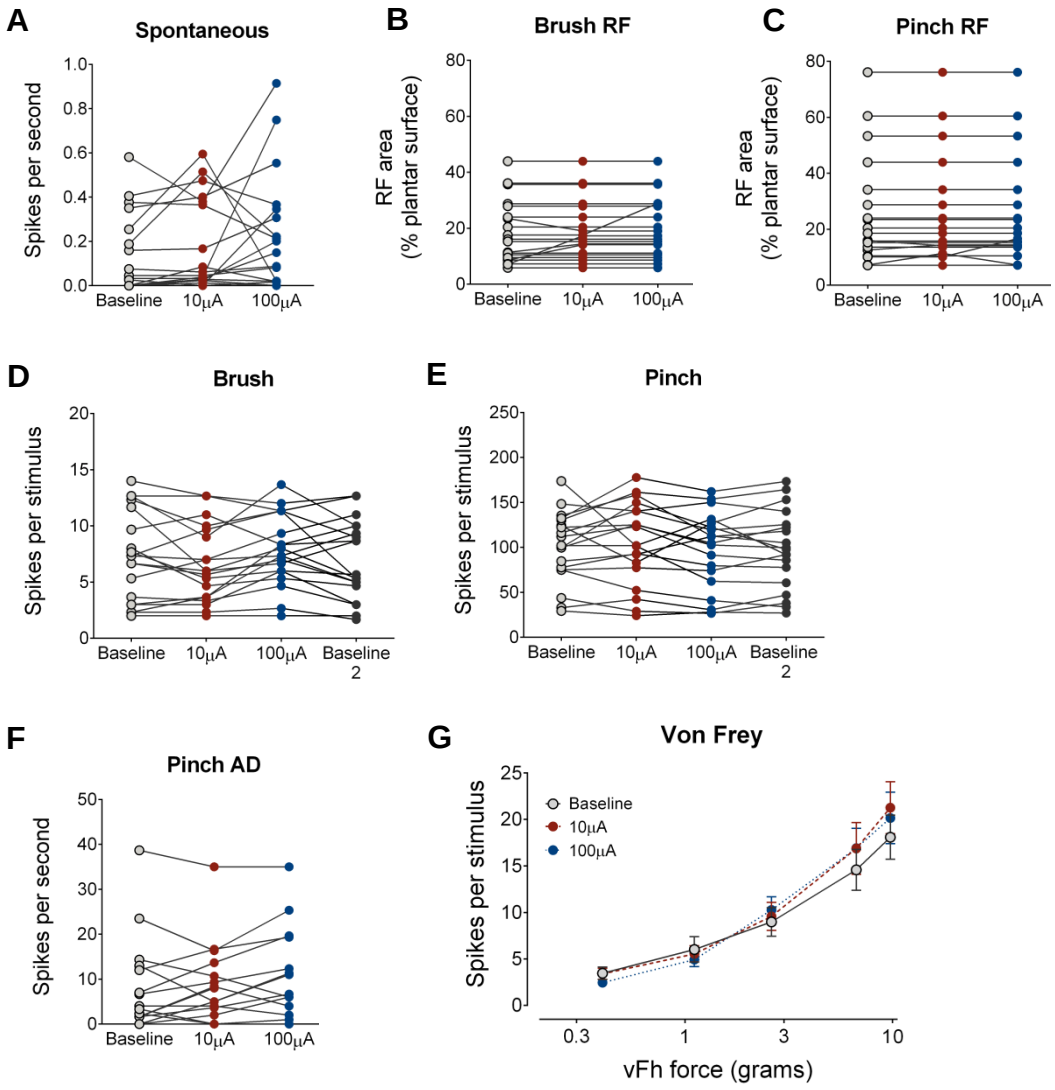

Fig 5.7. Electrical stimulation of the RVM in 5,7-DHT treated P21 rats does not change mean dorsal horn WDR neuron properties.
5,7-DHT was injected intrathecally at P16 and dorsal horn neuron recordings were performed at P21 (n=20). WDR neuron properties were recorded at baseline and during 10μA and 100μA stimulation of the RVM. No significant effect of stimulating the RVM at 10μA and 100μA on mean firing properties of WDR neurons was found compared to baseline for any parameter measured, including: spontaneous activity (A); brush receptive field (RF) size (B); pinch RF size (C); brush evoked firing activity (D); pinch evoked firing activity (E); pinch after discharge (AD) (F); or von Frey hair (vFh) evoked firing activity. See text for details of statistical analyses.

5.4.5 Comparing relative effects of RVM electrical stimulation on dorsal horn WDR neuron properties in 5,7-DHT and saline treated P21 rats

In the previous sections, the effect of RVM stimulation was analysed as mean changes in populations of WDR neuron properties in 5,7-DHT and saline-treated P21 rats compared to baseline. Analysis of mean cell population changes may overlook more subtle changes to individual neurons in the dorsal horn during RVM stimulation. Here, data is expressed as the percentage change of firing activity during RVM stimulation compared to baseline to compare the effects on individual dorsal horn neuron properties in saline and 5,7-DHT treated P21 rats.

Brush-evoked firing activity in saline and 5,7-DHT treated animals was normalised to baseline, such that data is expressed as the percentage change in firing activity from baseline 1. Of note, the percentage change of brush-evoked activity is distorted as few spikes are evoked by brush stimulation, thus percentage changes are larger compared to pinch-evoked activity. In saline-treated animals, 10μA and 100μA stimulation changed firing activity of the majority of individual WDR neurons, with a large spread of data indicating separate populations of WDR neurons which were facilitated or inhibited by 10μA or 100μA RVM stimulation (Fig 5.8A). Populations of facilitated, inhibited or unchanged cells were equally spread such that there was no mean change in the percentage change in firing activity compared to baseline. In 5,7-DHT-treated animals, 10μA or 100μA stimulation did change firing activity in some cells, but the majority of cells exhibited changes in firing activity which were within the normal range of variability (Fig 5.8A). This normal range of variability is defined as the percentage change of firing activity between baseline 1 and baseline 2, which was <20%. No mean change in the percentage change in firing activity compared to baseline was seen during 10μA or 100μA stimulation in 5,7-DHT-treated rats. The variability of the data sets was also lower than in saline-treated rats, indicating fewer cells which were inhibited or facilitated during RVM stimulation bouts.

Normalisation of pinch-evoked firing activity compared to baseline in saline-treated animals demonstrated that the majority of cells displayed relatively increased firing activity during either 10μA or 100μA stimulation bouts (Fig 5.8B). Indeed, the mean change in the percentage change in firing activity increased to 128% and 122%, respectively. Quantification of the number of cells which displayed a change in firing activity >20% from baseline demonstrated that pinch firing activity was facilitated in 10/14 and 6/11 cells following 10μA and 100μA stimulation, and no cells were inhibited (Fig 5.7C). In 5,7-DHT treated animals, the majority of cells exhibited

changes in firing activity which were within the normal range of variability during 10μA or 100μA stimulation bouts (Fig 5.8B). This is manifested as relatively large populations of neurons with firing activity changes less than 20% compared to baseline; however, a few outlier cells were facilitated or inhibited during 10μA or 100μA RVM stimulation bouts (Fig 5.8C). When the mean change in pinch-evoked firing activity was compared between saline 10μA vs 5,7-DHT 10μA groups, unpaired student's t-test demonstrated significantly increased firing activity in saline-treated rats (unpaired student's t-test, saline 10μA vs. 5,7-DHT 10μA, $P<0.01$, Fig 5.8B). The same was true when comparing saline 100μA vs 5,7-DHT 100μA groups (unpaired student's t-test, saline 100μA vs. 5,7-DHT 100μA, $P<0.01$, Fig 5.8B).

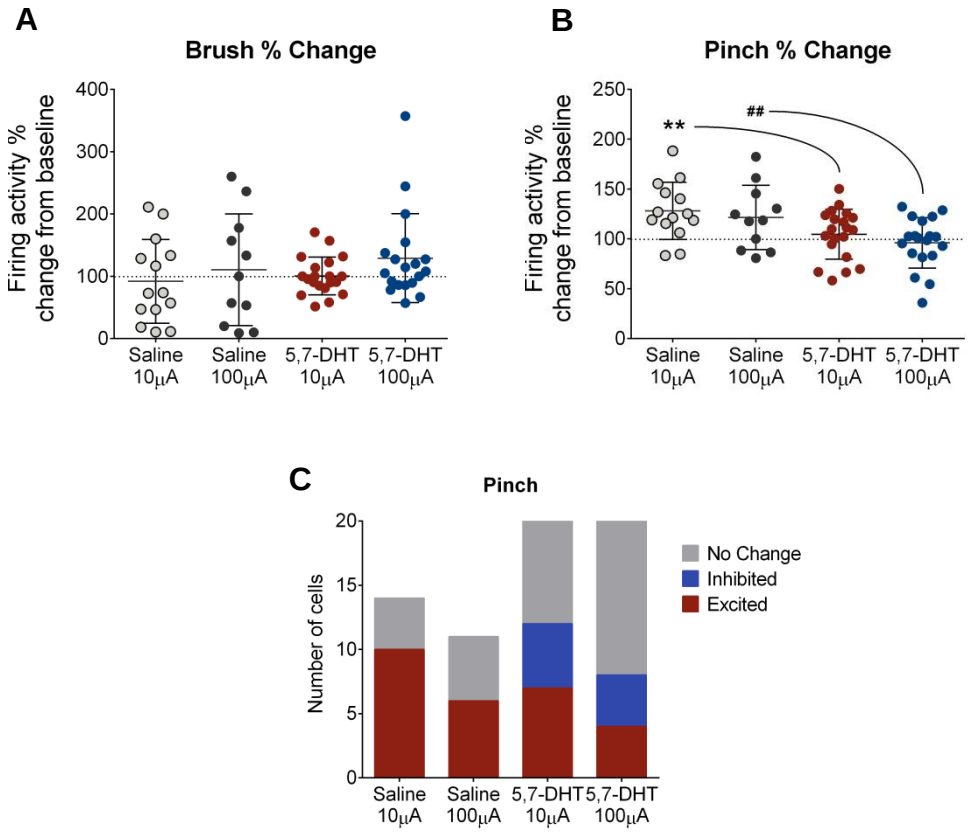

Fig 5.8. Electrical stimulation of the RVM changes individual dorsal horn WDR neuron firing properties at P21.
Brush and pinch-evoked dorsal horn WDR neuron firing activity during 10μA and 100μA RVM stimulation was recorded from 5,7-DHT (n=20) or saline (n=14) treated animals. Brush (A) and pinch-evoked (B) firing activity of individual cells during 10μA and 100μA stimulation is expressed as the percentage change from baseline. The mean percentage change of pinch-evoked firing activity at 10μA and 100μA in saline animals was significantly different to 10μA and 100μA in 5,7-DHT treated animals; unpaired student's t-test, ** $P<0.01$ 10μA vs 10μA; ## $P<0.01$ 100μA vs 100μA. Cells were classified as being inhibited or excited during 10μA and 100μA stimulation if firing activity was >20% less or more than baseline firing activity (C).

5.4.6 The long-term effects of intrathecal 5,7-DHT injection at P7 upon dorsal horn WDR neuron properties at P40-45

The aim of the following experiments was to investigate the long term effects of ablating descending serotonergic fibres in neonatal animals upon dorsal horn electrophysiological properties in adulthood. 5,7-DHT or saline was intrathecally injected at P7 rather than P0 to ablate the early and later growths of serotonergic fibres into the lumbar spinal cord (as described in chapter 4). 5-HTT immunostaining after electrophysiological recordings at P40-P45 demonstrated an absence of 5-HTT immunoreactivity in the lumbar spinal dorsal horn. A total of 61 cells were recorded in these experiments: 23 cells from seven naive control animals, 24 cells from four neonatal 5,7-DHT treated rats, and 14 cells from two neonatal saline treated rats. Cells from 5,7-DHT treated rats were compared to both naïve control and neonatal saline control groups.

Mechanical behavioural withdrawal thresholds were measured before electrophysiology experiments in neonatal 5,7-DHT and neonatal saline-treated adult rats and were compared to naïve adult rats. One way ANOVA demonstrated that mechanical withdrawal thresholds were not significantly different between the three groups (one-way ANOVA with Bonferroni *post hoc* analysis, Fig 5.9H).

Spontaneous activity of dorsal horn WDR neurons was low in control, neonatal 5,7-DHT and neonatal saline treated adult rats, and did not differ between the groups (one-way ANOVA with Bonferroni *post hoc* analysis, Fig 5.9A). Brush-evoked firing activity was significantly lower in neonatal 5,7-DHT-treated adult rats compared to control, but not neonatal-saline treated adult rats groups (one-way ANOVA with Bonferroni *post hoc* analysis, neonatal 5,7-DHT vs. control $P<0.05$; Fig 5.9B). Brush receptive field size did not differ between the three groups (Fig 5.9C).

Pinch-evoked firing activity was significantly higher in neonatal 5,7-DHT treated adult rats compared to both naïve control and neonatal saline-treated adult rats groups (one-way ANOVA with Bonferroni *post hoc* analysis, neonatal 5,7-DHT vs. control $P<0.001$; neonatal 5,7-DHT vs. neonatal saline $P<0.01$ Fig 5.9D). Pinch receptive fields were significantly larger in neonatal 5,7-DHT treated adult rats compared to control, but not neonatal saline-treated adult rats groups (one-way ANOVA with Bonferroni *post hoc* analysis, neonatal 5,7-DHT vs. control $P<0.05$ Fig 5.9E) . Pinch after discharge did not significantly differ between the three groups (Fig 5.9F). Repeated measures three-way ANOVA with Bonferroni *post hoc* analysis comparing vFh-evoked firing activity between control, neonatal 5,7-DHT treated and neonatal

saline treated adult rats demonstrated no significant effect of drug treatment between the groups (Three way repeated measures ANOVA, neonatal 5,7-DHT vs. control vs. neonatal saline, F(2,51)=0.042, P=0.979; Fig 5.9G).

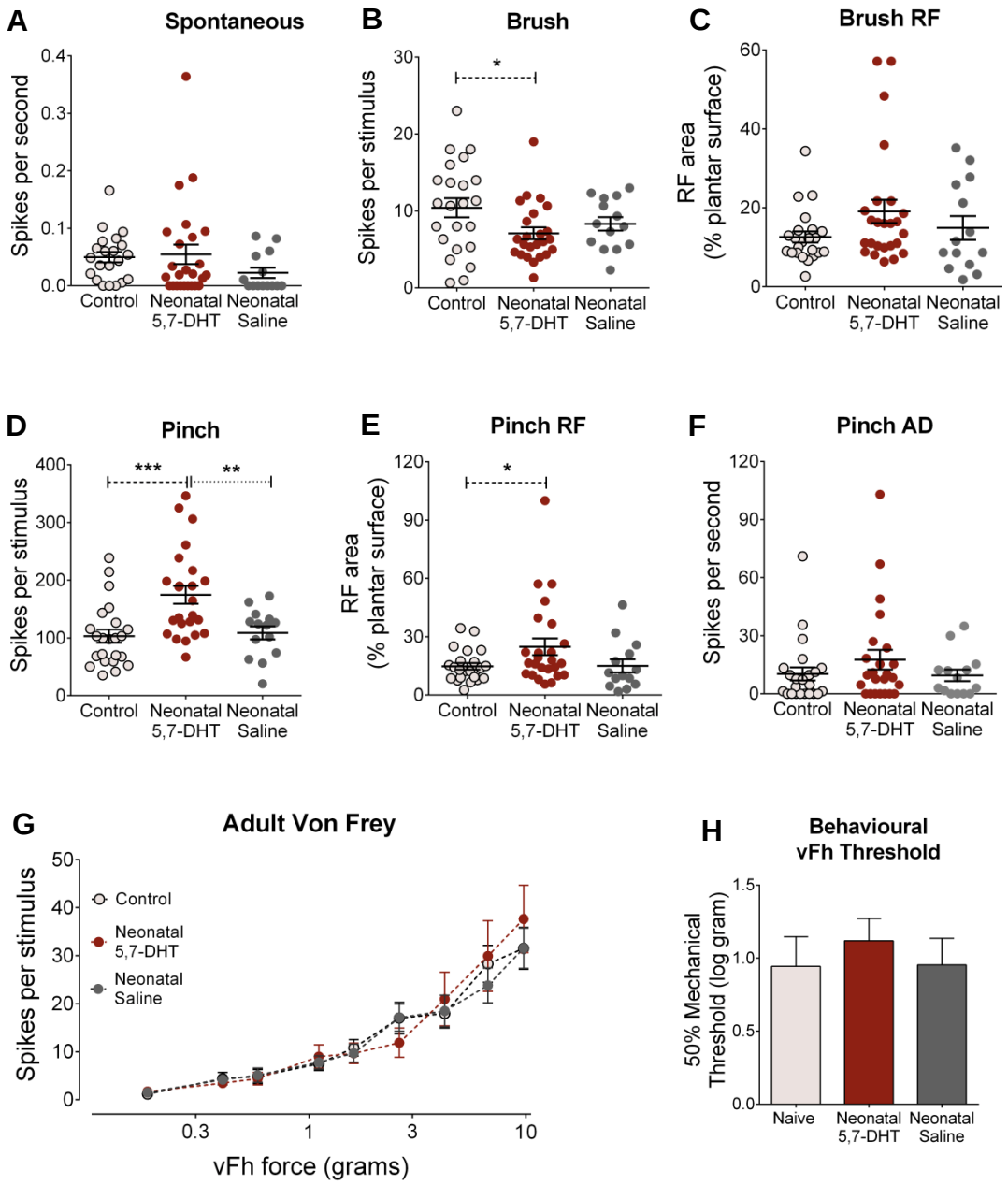

Fig 5.9 (previous page). Intrathecal injection of 5,7-DHT at P7 changed dorsal horn WDR neuron properties at P45-47
5,7-DHT was injected intrathecally at P7 and dorsal horn recordings were performed at P45-47 (n=24), and were compared to naive animals (n=23) and control animals which received intrathecal injection of saline at P7 (n=14). Spontaneous firing activity did not differ between groups (A). Brush-evoked firing was significantly lower in animals neonatally trated with 5,7-DHT or saline (B), but brush receptive field (RF) size did not differ (C). Pinch-evoked firing was significantly higher in 5,7-DHT treated rats compared to naïve and neonatal control rats (D), but pinch receptive field size (E) and pinch after discharge (AD) (F) did not differ between groups. von Frey hair (vFh) evoked firing activity did not differ between groups (G). Behavioural nocifensive withdrawal thresholds measured at P45 were not different between groups. One – way ANOVA with Bonferroni post-hoc test, *,** P<0.05 and 0.01.

5.4.7 Spinal application of the 5-HT_3R antagonist ondansetron decreases dorsal horn WDR neuron firing activity at P8

In the next sets of experiments, I investigated the effect of blocking spinal endogenous 5-HT_3R activation on dorsal horn WDR neuron firing properties in rats at several postnatal ages. In these experiments, 50µg of Ondansetron in 50µl of saline (Green et al., 2000) was applied to the surface of the dorsal horn in P8 rats before recording WDR neuron responses to stimulation of hindpaw cutaneous receptive fields for up to one hour. A total of 44 cells were recorded in these experiments: 24 cells from six control rats and 20 cells from five ondansetron-treated rats.

Spontaneous firing activity was low in control and ondansetron-treated animals, and did not significantly differ between the two groups (Mann-Whitney test, control vs ondansetron, Fig 5.10A). Brush-evoked firing activity did not significantly differ between control ondansetron-treated rats (unpaired student's t-test, Fig 5.10B), and nor did brush receptive field size (unpaired student's t-test, Fig 5.10C).

Mean pinch-evoked firing activity was significantly lower in ondansetron-treated rats compared to control (unpaired student's t-test, P<0.01, Fig 5.10D), but pinch receptive field size did not differ between the two groups (unpaired student's t-test, Fig 5.10E). Pinch after discharge absent in all but two cell in ondansetron-treated animals, and was significantly lower compared to control (unpaired student's t-test, Fig 5.10F).

Comparison of vFh stimulus response curves in ondansetron and control rats demonstrated no significant effect of lidocaine on vFh-evoked firing activity (Two-way repeated measures ANOVA, control vs. ondansetron, $F(1,28)=1.178$, $P=0.193$; Fig 5.10G).

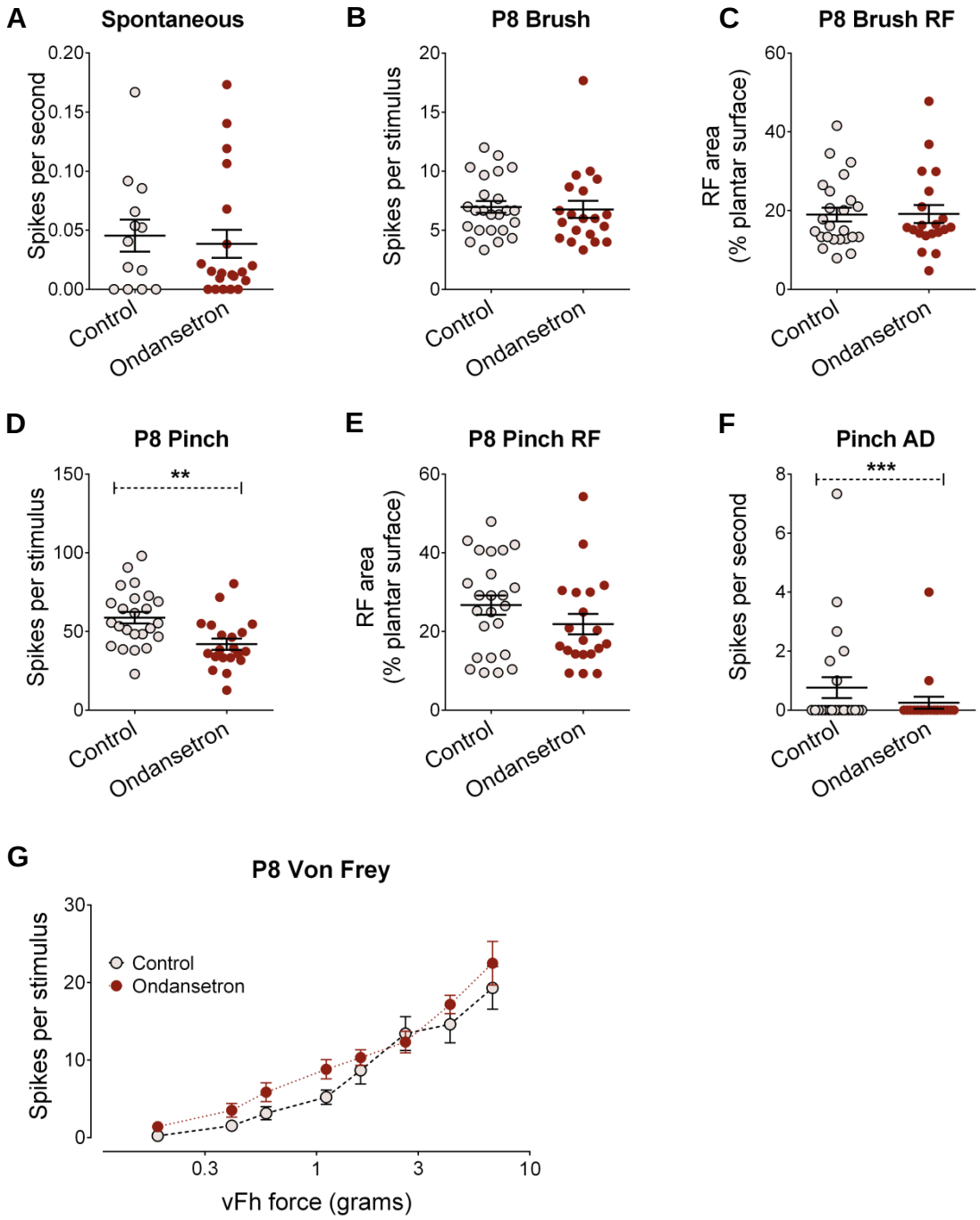

Fig 5.10. Spinal application of ondansetron at decreases dorsal horn WDR neuron activity at P8

The 5-HT_3R antagonist ondansetron (50µg) was applied to the surface of the spinal cord and dorsal horn neuron recordings were performed at P8 (n=20), and were compared to control animals (n=24). Spontaneous firing activity did not significantly differ between groups (A). Brush-evoked firing activity (B) and brush receptive field (RF) size (C) did not significantly differ between ondansetron treated rats compared to control. Pinch-evoked firing was significantly lower in ondansetron treated rats (D), as was pinch after discharge (AD) (F), but pinch receptive field size did not differ (E). von Frey hair (vFh) evoked firing activity did not differ between groups (G). Unpaired student's t-test, *,** $P<0.05$ and 0.01.

5.4.8 Spinal application of ondansetron decreases brush and pinch-evoked dorsal horn WDR neuron activity in a dose dependent manner at P21

Next, the effects of applying the 5-HT_3R antagonist ondansetron on dorsal horn WDR neuron properties were investigated at P21, and in this case three different doses of ondansetron were applied to the spinal cord. A total of 83 cells were recorded in these experiments. 50µl of ondansetron of three different doses was applied to the surface of the dorsal horn: 2µg (n=18 from three rats), 10µg (n=19 from three rats) or 50µg (n=20 from four rats). 26 cells were recorded from four control rats.

Spontaneous firing activity was not significantly different in any group (2, 10 or 50µg ondansetron) compared to control (one-way ANOVA with Dunnett's *post hoc* test; $F(3,82)=2.30$, $P=0.084$; Fig 5.11A). There were no significant differences in brush receptive field sizes (one-way ANOVA with Dunnett's *post hoc* test; $F(3,82)=1.827$, $P=0.149$; Fig 5.11B) or in pinch receptive field sizes (one-way ANOVA with Dunnett's *post hoc* test; $F(3,82)=1.397$, $P=0.250$; Fig 5.11C) in any group compared to control.

Brush-evoked firing activity was significantly lower in 2µg and 50µg ondansetron groups, but not 10µg, compared to control (one-way ANOVA, control vs 2µg, 10µg or 50µg; $F(3,82)=5.455$, $P=0.002$; with Dunnett's *post hoc* test, 2µg $P<0.05$ and 50µg $P<0.01$ Fig 5.11D). Pinch-evoked firing activity was significantly lower in 10µg and 50µg ondansetron groups, but not 2µg, compared to control (one-way ANOVA, control vs 2µg, 10µg or 50µg; $F(3,82)=9.855$, $P<0.0001$; with Dunnett's *post hoc* test, 10µg and 50µg $P<0.001$ Fig 5.11E). No significant differences in pinch after discharge were found between 2, 10 or 50µg ondansetron groups compared to control (one-way ANOVA with Dunnett's *post hoc* test; $F(3,82)=2.525$, $P=0.063$; Fig 5.11F).

VFh-evoked firing stimulus response curves following application of different doses of ondansetron were then compared to control. Two-way ANOVA comparing control and 2µg ondansetron demonstrated no effect of drug treatment (Two-way repeated measures ANOVA, control vs. 2µg, $F(1,39)=3.422$, $P=0.072$, Fig 5.11G). Similarly, there was no effect of drug treatment when comparing control and 10µg ondansetron groups (Two-way repeated measures ANOVA, control vs. 10µg, $F(1,41)=2.963$, $P=0.093$, Fig 5.11G). There was a significant effect of treatment when comparing control and 50µg ondansetron groups, and Bonferroni *post hoc* analysis demonstrated significantly lower vFh-evoked firing activity in the 50µg group at vFh forces 1.11g, 1.61g, 2.61g, 4.23g, 6.69g and 9.77g (Two-way repeated measures ANOVA, control vs. 50µg, $F(1,42)=21.31$, $P<0.0001$, with Bonferroni *post hoc* analysis, $P<0.01$ to $P<0.001$ at different vFhs; Fig 5.11G).

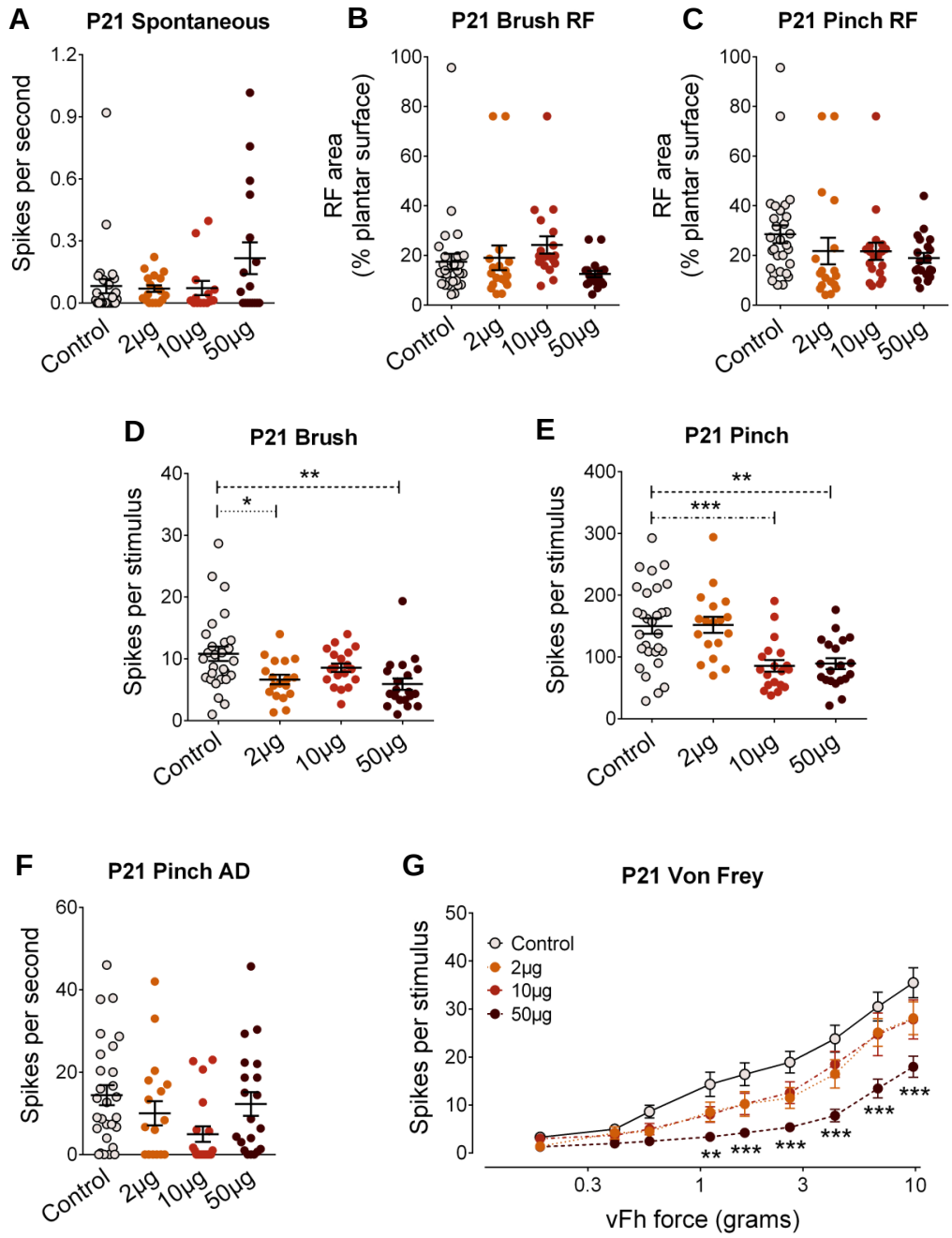

Fig 5.11. Spinal application of ondansetron dose-dependently decreased dorsal horn WDR neuron activity at P21.

The 5-HT_3R antagonist ondansetron was applied to the surface of the spinal cord and dorsal horn neuron recordings were performed at P21 and were compared to control animals (n=24). Ondansetron was applied at three different concentrations: 2µg (n=18), 10µg (n=19), or 50µg (n=20). Spontaneous firing activity did not significantly differ between groups (A). Brush (B) and pinch (C) receptive field (RF) sizes did not significantly differ between ondansetron treated rats compared to control. Brush-evoked firing was significantly lower following in 2µg or 50µg treated rats compared to control (C). Pinch-evoked firing activity was significantly lower in 10 and 50µg treated rats compared to control. Pinch after discharge (AD) did not differ between groups (E). One-way ANOVA with Dunnett's post-hoc test. Von Frey hair (vFh) evoked firing activity was significantly lower in 50µg treated rats compared to control at vFh forces from 1.11 to 9.77g (G). Two-way ANOVA with Bonferroni post-hoc test *,**,*** $P<0.05$, 0.01 and 0.001.

5.4.9 Spinal application of ondansetron dorsal horn dorsal horn WDR neuron decreases brush receptive field size at P40

The effect of blocking spinal 5-HT_3Rs on dorsal horn WDR neuron electrophysiological properties was also investigated in adult P40 rats. A single dose of 50μg of ondansetron was applied to the spinal dorsal horn in these experiments. A total of 44 cells were recorded in these experiments: 23 cells from 7 control rats and 21 cells from 4 ondansetron treated rats.

Spontaneous firing activity was low in both control and ondansetron treated animals, and there was a significant reduction in spontaneous firing in ondansetron treated rats when compared to control (Mann-Whitney test, $P<0.05$, Fig 5.12A). Brush-evoked firing activity was not significantly different between control and ondansetron groups (unpaired student's t-test, Fig 5.12B), but brush receptive fields were significantly and relatively smaller in ondansetron treated rats (unpaired student's t-test, $P<0.05$, Fig 5.12C).

No significant differences between control and ondansetron treated animals were found in pinch-evoked firing activity (unpaired student's t-test, Fig 5.12D), pinch receptive field size (unpaired student's t-test, Fig 5.12E), or pinch after discharge firing activity (unpaired student's t-test, Fig 5.12F).

Two-way ANOVA demonstrated that there was no significant effect of drug treatment on vFh-evoked firing activity stimulus response curves when comparing control and ondansetron groups (Two-way repeated measures ANOVA with Bonferroni *post hoc* analysis, control vs. ondansetron, $F(1,36)=3.488$, $P=0.070$, Fig 5.12G).

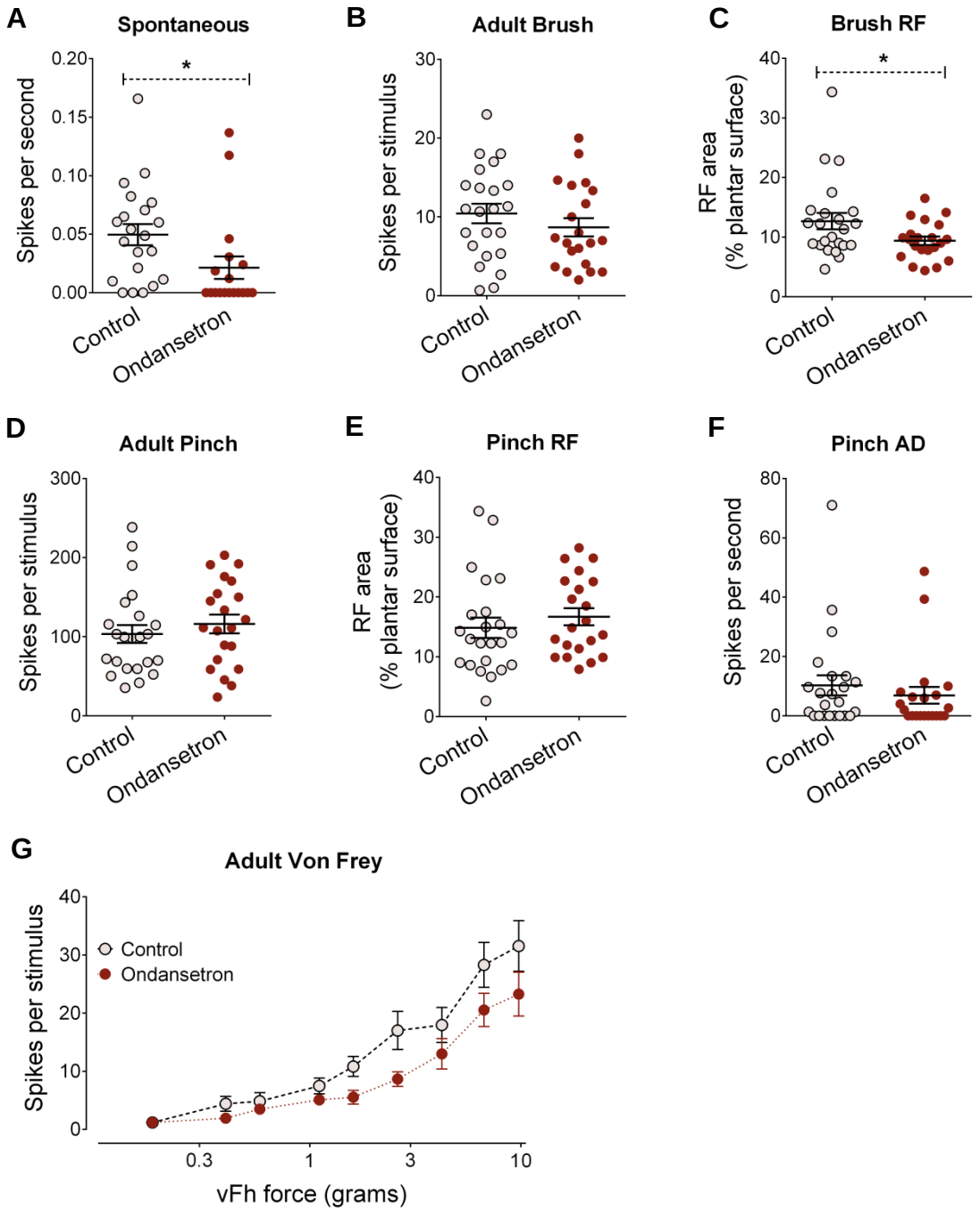

Fig 5.12. Spinal application of ondansetron decreased dorsal horn WDR neuron brush receptive field size at P40

The 5-HT_3R antagonist ondansetron (50µg) was applied to the surface of the spinal cord and dorsal horn neuron recordings were performed at P40 (n=21), and were compared to control animals (n=23). Spontaneous firing activity was significantly lower in ondansetron treated animals compared to control (A). Brush receptive field (RF) size was also significantly smaller in ondansetron treated animals (C). Brush-evoked firing activity (B), pinch-evoked firing activity, pinch receptive field size and pinch after discharge (AD) did not significantly differ between ondansetron treated rats compared to control. Von Frey hair (vFh) evoked firing activity also did not differ between groups (G). Unpaired student's t-test, * $P<0.05$.

5.4.10 Summary of results

The data presented here can be summarised as follows:

1. Intrathecal injection of 5,7-DHT at P4 ablated the majority of 5-HTT immunoreactive fibres in the spinal cord, and caused a reduction in brush and pinch-evoked firing activity of dorsal horn dorsal horn neurons at P8.
2. Intrathecal injection of 5,7-DHT at P16, which ablated all 5-HTT+ fibres in the dorsal horn, reduced brush-evoked firing, brush receptive field size, pinch-evoked and vFh-evoked firing activity of dorsal horn neurons at P21.
3. Intrathecal injection of 5,7-DHT at P40, which ablated all 5-HTT+ fibres in the dorsal horn, decreased brush receptive field size, but increased pinch-evoked firing activity of dorsal horn neurons in P45-47 adult animals. Therefore, there is a change in control of descending serotonergic control of high-threshold dorsal horn activity from excitation to inhibition between P21-P45, but low-threshold activity was always excited.
4. Electrical stimulation of the RVM at P21 predominantly facilitated dorsal horn dorsal horn neuron firing properties in saline-treated animals. In 5,7-DHT treated animals, electrical stimulation of the RVM did not change the excitability of the majority of WDR neurons.
5. Injection of 5,7-DHT at P7 decreased brush-evoked firing activity, but increased pinch-evoked firing activity and receptive field size of dorsal horn neurons at P40-45, suggesting that depletion of 5-HT in early life does not stop descending inhibition of noxious activity from developing.
6. Spinal application of the 5-HT_3R antagonist ondansetron (50μg) decreased pinch-evoked firing activity of dorsal horn neurons at P8.
7. Higher doses of ondansetron applied to the spinal cord decreased pinch-evoked and vFh-evoked firing activity of dorsal horn neurons in P21 animals. Decreased brush-evoked firing activity was observed following application of low (2μg) and high (50μg) concentrations of ondansetron.
8. Spinal application of 50μg ondansetron decreased brush receptive field sizes in adult P40 animals but did not affect pinch or vFh-evoked dorsal horn neuron responses. Thus, 5-HT_3R-mediated facilitation of low-threshold activity in the dorsal horn continues into adult life.

5.5 Discussion

In this chapter I aimed to investigate the role of descending serotonergic modulation of spinal sensory processing during postnatal development. In chapter 3, I demonstrated that ongoing descending facilitation of dorsal horn neurons in young uninjured rats switches to be inhibitory in adulthood. I hypothesised that this descending facilitation in young rats and inhibition in adult rats is mediated by descending serotonergic neurons. Additionally, I hypothesised that this proposed descending serotonergic facilitation is mediated by spinal 5-HT_3Rs selectively in young rats. To test this, the effects of ablating descending serotonergic terminals or blocking spinal 5-HT_3Rs were investigated upon spinal dorsal horn WDR neuron sensory-evoked properties at several postnatal ages. I identified a key role for endogenous 5-HT-5-HT_3R signalling in mediating dominant descending RVM facilitation of spinal dorsal horn neurons in young animals.

5.5.1 Technical considerations

Intrathecally injecting 5,7-DHT has been previously used as a technique to permanently ablate descending serotonergic terminals in the adult spinal cord (Rahman et al., 2006; Géranton et al., 2008). In experiments in this chapter, 5,7-DHT was intrathecally injected into rats of several postnatal ages to ablate serotonergic terminals in the spinal cord in young and adult rats. Ablation of serotonergic terminals was confirmed by an absence of 5-HTT immunoreactivity in the lumbar spinal cord, however it was not known if 5,7-DHT injection ablated serotonergic neuron cell bodies. Additionally, non-selective toxic actions of 5,7-DHT on non-serotonergic monoaminergic descending fibres was prevented by systemic pre-treatment with desipramine, a noradrenergic reuptake inhibitor.

Here, the same concentration of 5,7-DHT (60µg in 20µl) was used in all ages, and was therefore not corrected for the weight of the animal. This dose is based on previous experiments in which 60µg of 5,7-DHT in 20µl of saline was intrathecally injected to ablate descending serotonergic terminals in the adult spinal cord prior to dorsal horn electrophysiological recordings (Rahman et al., 2006), and on previous experiments in which P3 rats received intraventricular injections of 60-80µg 5,7-DHT to ablate serotonergic neurons in the midbrain (Piechal et al., 2012; Rok-Bujko et al., 2012). Intrathecal route of administration was chosen to ensure that only serotonergic neurons which project to the spinal cord were ablated. An injection volume of 20µl was used in all ages to ensure that injectate spread from the cauda equina to the lumbar spinal cord.

This volume is based on previous experiments which demonstrated that successful spread of injectate in the spinal cord in neonatal rats requires higher volumes relative to body mass compared to adults (Westin et al., 2010).

Many 5-HT-containing neurons, including those that project from the RVM to the spinal cord, contain various other neurotransmitters such as: substance P and enkephalin (Bowker et al., 1981a; Reddy et al., 1990); neurotensin (Wang et al., 2014); somatostatin (Bowker and Abbott, 1988); and GABA (Millhorn et al., 1987, 1988; but see Jones et al., ((1991)) who did not find colocalisation of GAD with 5-HT). It is important to consider that injection of 5,7-DHT will not selectively deplete endogenous 5-HT levels, but will also deplete co-expressed neurotransmitters. It is not clear in these experiments whether the effects of ablating serotonergic neurons are caused by depletion of 5-HT or loss of other neurotransmitters, therefore the aims of experiments in this chapter were to investigate the aims of ablating 5-HT-containing *neurons* rather than selectively depleting endogenous 5-HT. As discussed in chapter 4, the majority of descending 5-HT and 5-HTT containing terminals in the spinal dorsal horn originate from the RVM (Braz and Basbaum, 2008), however neurons in the raphe pallidus and raphe obscurus do send sparse projections to the lumbar dorsal horn (Liang et al., 2015). Therefore, whilst the majority of serotonergic terminals in the dorsal horn which were ablated by 5,7-DHT had cell bodies in the RVM, it is likely that terminals from dorsal horn projecting neurons in other caudal raphe nuclei were also ablated.

Experiments in this chapter involved electrically stimulating the RVM at different intensities. Electrical stimulation using bipolar electrodes depolarises local RVM neuronal cell bodies but also has non-desired effects on fibres of passage (Zhuo and Gebhart, 1997). Other experiments have demonstrated comparable descending inhibition and excitation upon microinjections of excitatory amino acids into the RVM which avoids these non-specific effects (Zhuo and Gebhart, 1997; Schwaller et al., 2015). Electrical stimulation protocols were chosen in these experiments to reliably investigate the effects of both low and high intensity RVM stimulation upon the electrophysiological properties of the same dorsal horn neuron compared to internally controlled baseline measurements before and after RVM stimulation, however the effects of stimulating fibres of passage cannot be discounted.

To examine the effects of RVM electrical stimulation upon sensory-evoked properties of dorsal horn WDR neurons, intra-cell changes were characterised by comparing mean properties at baseline and during 10 or 100μA stimulation bouts. Pinch or brush-evoked properties of cells were classed as inhibited or facilitated by RVM stimulation if

firing rates were reduced or increased relative to baseline by 20%. Previous experiments have used a 10% (Koch and Fitzgerald, 2014) or a 15% (Bee and Dickenson, 2007) change in firing activity as a threshold for excitation classification. A threshold of 20% was chosen as it is above on the normal variability of neuronal responses between two baseline measurements before and after RVM stimulation (data not shown). These classification criteria take into account intra-cell variability and controls for experimental problems such as electrode drift.

5.5.2 Descending serotonergic neurons inhibit nociception in the dorsal horn in uninjured adult rats

Findings in this chapter support the classical view that descending serotonergic neurons are predominantly antinociceptive in uninjured adult animals. Pinch-evoked dorsal horn neuron firing activity in 5,7-DHT treated adult rats was higher than control rats, unmasking net endogenous serotoninergic neuron mediated descending inhibition of noxious inputs to dorsal horn neurons in adult rats. These data build on those from RVM lidocaine silencing experiments in chapter 3 and suggest that descending serotonergic neurons in the RVM are a major source of descending inhibition of dorsal horn neuron properties from the RVM in adult rats.

Earlier behavioural experiments have demonstrated antinociceptive roles of descending 5-HT neurotransmission when serotonergic transmission is evoked by external manipulations: RVM stimulation-produced analgesia can be reduced by intrathecal administration of 5-$HT_{1/2}$R antagonist methysergide (Hammond and Yaksh, 1984); and intrathecal administration of 5-HT reduces pain-like behaviours following noxious thermal or mechanical stimulation (Schmauss et al., 1983). Similarly, exogenous 5-HT application to the dorsal horn decreased C-fibre evoked responses of dorsal horn WDR neurons (Liu et al., 2007); effects which could be mimicked by applying 5-HT_{1A}, 5-HT_{1B}, 5-HT_{2A} or 5-HT_{2C} agonists (Liu et al., 2007). Thus, robust evidence points towards an antinociceptive role of 5-HT signalling in the dorsal horn. Results here concur, and suggest that this descending inhibition is at least partially mediated by spinally projecting raphe-spinal serotonergic neurons, the majority of which have cell bodies in the RVM.

On the other hand, there is evidence for descending serotonergic facilitation in uninjured adult rats, as 5,7-DHT ablation of descending serotonergic neurons decreases thermal and vFh-evoked firing properties of dorsal horn neurons compared to control adult rats (Rahman et al., 2006) and optogenetic activation of serotonergic neurons in

the reduces thermal and mechanical behavioural withdrawal thresholds in uninjured adult mice (Cai et al., 2014). Others have shown that depletion of endogenous 5-HT levels in the spinal cord by intrathecal injection of 5,7-DHT or RVM injection of Tph-2 shRNA has no effect on behavioural withdrawal thresholds in adult rodents (Mohrland and Gebhart, 1980; Svensson et al., 2006; Wei et al., 2010; Leong et al., 2011; Carr et al., 2014). This is consistent with my own data in 5,7-DHT treated and control adult rats and suggests that serotonergic RVM neurons do not modulate nocifensive reflexes in the absence of exogenous RVM stimulation.

Many RVM 5-HT+ neurons co-express (and presumably co-release) other neurotransmitters such as GABA and enkephalin (Bowker et al., 1981a; Millhorn et al., 1988). Morphine-induced analgesia is blocked following ablation of serotonergic neurons in adult rats (Vogt, 1974; Mohrland and Gebhart, 1980), however morphine-induced analgesia is not blocked following depletion of endogenous 5-HT in the RVM without ablation of the neurons (Wei et al., 2010); suggesting that serotonergic neurons, but not 5-HT-5-HTR signalling per se, mediate morphine-induced analgesia at the spinal cord level. It is therefore likely that neurotransmitters co-released with 5-HT from descending serotonergic RVM neurons are also involved with modulation of acute noxious inputs to spinal sensory circuits. As experiments in this chapter used 5,7-DHT to ablate serotonergic neurons, the effects of depleting 5-HT cannot be dissociated from other neurotransmitter systems in these results without the use of selective 5-HTR subtype and other neurotransmitter receptor antagonists (e.g., $GABA_A$).

5.5.3 Descending serotonergic inputs facilitate low and high threshold inputs in the dorsal horn in young rats

Results in chapter 3 demonstrated that descending modulation arising from the RVM in young rats is predominantly facilitatory. I hypothesised that this descending facilitation is mediated by descending serotonergic neurons. In 5,7-DHT treated P8 and P21 rats, brush and pinch-evoked firing activity of dorsal horn WDR neurons was reduced compared to age-matched control rats. These data demonstrate that serotonergic neurotransmission provides background facilitation of low and high-threshold cutaneous sensory inputs to deep dorsal horn neurons in young animals. These results build upon those in chapter 3 which demonstrated ongoing descending RVM facilitation of noxious activity in the dorsal horn at P8 and P21, and suggest that descending serotonergic neurons are an important population of neurons which

mediate this descending facilitation in young rats. However, results here demonstrated that descending serotonergic modulation is not restricted to facilitating high-threshold noxious inputs, but also facilitates low-threshold tactile inputs in the young uninjured rat.

Another aim of this chapter was to investigate the relative contribution of RVM serotonergic neurons to descending modulation from the RVM in young P21 rats; the hypothesis being that ablating descending serotonergic neurons would prevent descending facilitation of dorsal horn neurons evoked by electrical stimulation of the RVM. Electrical stimulation of the RVM of 10 or 100μA intensity has previously been shown to facilitate A- and C-fibre evoked dorsal horn neuron firing in P21 rats (Hathway et al., 2009a; Koch and Fitzgerald, 2014); stimulation parameters which can facilitate and inhibit dorsal horn neuron firing activity in adult animals (Zhuo and Gebhart, 1997). Data from saline-treated P21 animals in this chapter concur with the findings of Koch and Fitzgerald (2014), as electrical stimulation of the RVM increased mean pinch-evoked properties of dorsal horn WDR neurons, and large populations of individual dorsal horn WDR neurons were facilitated by 10 or 100μA stimulation of the RVM compared to baseline.

In contrast, electrical stimulation of the RVM did not change mean sensory-evoked dorsal horn neuron activity in 5,7-DHT-treated rats, demonstrating that ablating descending serotonergic neurons prevented descending facilitation evoked from the RVM in young rats. However, it is unlikely that descending serotonergic transmission is the only source of descending RVM modulation of spinal sensory circuits. Indeed, electrical stimulation in 5,7-DHT treated rats did still change the firing activity of a small population of dorsal horn neurons, demonstrating descending modulation from non-serotonergic RVM neurons. Of note, electrical stimulation of the RVM inhibited individual dorsal horn neurons in 5,7-DHT treated rats but did not in saline-treated rats, suggesting that ablating descending serotonergic facilitation arising from the RVM unmasks weak inhibitory drive in young P21 rats. Thus, under normal conditions, descending serotonergic facilitation masks inhibition from non-serotonergic neurons at P21. I hypothesise from these findings that RVM serotonergic neurons are the major source of descending facilitation in young rats.

Evidence of serotonergic facilitation of dorsal horn neurons in young rats has been reported elsewhere. *In vitro* patch clamp experiments performed in spinal cord slices from P2-17 rats have demonstrated that 5-HT application at low doses can potentiate superficial dorsal horn cell EPSCs, and unmasked silent glutamate synapses by

inducing EPSCs to appear (Li and Zhuo, 1998b). Li and Zhuo (1998) postulate that 5-HT-mediated recruitment of silent synapses on WDR neurons may enhance responses to low and high-threshold sensory stimuli. As ablating serotonergic neurons did unmask facilitation of low and high-threshold sensory inputs in the dorsal horn in this chapter, results here provide evidence to support this hypothesis.

Inhibitory effects of 5-HT application to dorsal horn neurons have been observed in *in vitro* patch clamp experiments from neonatal rat spinal cord preparations. High doses of 5-HT can inhibit dorsal horn neuron EPSCs, and 5-HT_{1A}R agonist application inhibits EPSCs induced by dorsal root stimulation in spinal cord slices from P2-17 rats (Li and Zhuo, 1998b). 5-HT has also been shown to potentiate glycine-induced inward Cl- currents in dorsal horn cells in P14 spinal cord slices; an effect that can be blocked by applying the 5-HT_2R antagonist ketanserine (Li et al., 2002). However, glycine-mediated inhibition of dorsal horn neurons is absent in the first two weeks of postnatal life *in vivo* (Koch et al., 2012), suggesting that 5-HT mediated potentiation of glycinergic currents at P14 observed in spinal cord slice preparations may have limited function *in vivo*. It is likely that inhibitory effects of 5-HT observed in these experiments in young rats are masked by serotonergic facilitation of sensory-evoked dorsal horn properties via activation of 5-HT_3Rs. It would be interesting to investigate the role of 5-HT in potentiating glycine currents in early postnatal development; and whether 5-HT neurotransmission is required for the developmental onset of glycinergic inhibition in the dorsal horn.

Behavioural experiments have also suggested that serotonergic neurotransmission in the spinal cord can be antinociceptive in early development, as intrathecal injection of 5,7-DHT at P1 reduces baseline thermal withdrawal thresholds and morphine-induced increases in thermal thresholds at P7-P14 (Giordano and Barr, 1988). However, 5-HT depletion in, or administration of 5-HT to, neonatal spinal cord preparations is known to disrupt locomotor patterns recorded from ventral roots (Pearlstein et al., 2005); therefore the effects of 5-HT depletion on sensory circuits are hard to untangle from motor circuits when measuring sensory-motor reflex behaviours. Nevertheless, it is important to acknowledge that descending serotonergic facilitatory modulation from the RVM is not absolute in young rats, and that inhibitory modulation is functional, albeit weak.

5.5.4 Descending serotonergic facilitation of dorsal horn neurons in young rats is mediated by spinal 5-HT$_3$Rs

In adults, several classes of 5-HTRs have been shown to be involved in modulating pain behaviours and sensory processing in the dorsal horn (Liu et al., 2007). Robust evidence supports the notion that 5-HT$_3$Rs have a strong facilitatory role in adult pain states (Green et al., 2000; Zeitz et al., 2002; Dogrul et al., 2009; Kim et al., 2014a) but not in uninjured adult rats (Zeitz et al., 2002; Guo et al., 2014; Yang et al., 2014). Because of these observations, I hypothesised that 5-HT$_3$Rs would mediate dominant descending serotonergic facilitation in young animals at the level of the spinal cord as 5-HT$_3$R expression was found at P7 (chapter 4). This was indeed found to be the case at P8 and P21. At P8, pinch-evoked firing activity of dorsal horn neurons was lower in 50μg ondansetron treated compared to control, and at P21, clear dose-response curves were elicited following application of 2, 10 and 50μg ondansetron to the spinal cord. Ondansetron-mediated reduction of brush-evoked firing activity was observed in 2 and 50μg-treated rats, whilst reduction of pinch-evoked firing activity was observed only at higher doses of 10 and 50μg. VFh-evoked firing activity was also lower in 50μg ondansetron-treated rats at low and high force vFhs compared to control. These findings suggest that 5-HT$_3$R signalling is an important mechanism which mediates dominant descending RVM facilitation of both low and high-threshold cutaneous sensory inputs during early life in uninjured rats.

Importantly, ondansetron had no effect on vFh or pinch-evoked firing activity in adult animals, suggesting that 5-HT$_3$R does not modulate noxious-evoked activity in uninjured adult rats. These findings are in agreement with behavioural finds that 5-HT$_3$R antagonists do not change baseline mechanical or thermal withdrawal thresholds (Lagraize et al., 2010; Guo et al., 2014); and with electrophysiological findings that antagonism of 5-HT$_3$Rs in the spinal cord does not change C-fibre evoked responses of dorsal horn WDR neurons in adult rats (Liu et al., 2007). However, there is some evidence of 5-HT$_3$R-mediated facilitation of dorsal horn neuron properties in uninjured adult rats (Rahman et al., 2004b; Suzuki et al., 2004a; Bannister et al., 2015). In uninjured or sham operated rats, application of 50 or 100μg ondansetron to the spinal cord has been shown to inhibit firing activity of dorsal horn neurons in response to vFh stimulation of the hindpaw (Rahman et al., 2004b; Suzuki et al., 2004a). Importantly, vFh-evoked firing activity following ondansetron application was only inhibited in response to high vFh forces (>25g) in these papers. These electrophysiological experiments suggest that endogenous activation of spinal 5-HT$_3$Rs only excites dorsal

horn neurons during high intensity (presumed noxious) stimulation of primary afferent nociceptors in uninjured conditions. It is likely that mechanical stimuli used in experiments in this chapter were too moderate in force to recruit endogenous 5-HT_3R-mediated facilitation of pinch or vFh-evoked dorsal horn neuron properties.

A logical next question is to ask what mechanisms reduce 5-HT-mediated facilitation of dorsal horn circuits with postnatal age. Strong evidence suggests that 5-HT_{2A}, 5-HT_{2C} and 5-HT_7 receptor signalling in the dorsal horn mediates descending serotonergic inhibition of pain behaviours (Hammond and Yaksh, 1984; Dogrul et al., 2009; Viguier et al., 2013). Future immunohistochemistry experiments and electrophysiology experiments could investigate possible changes expression and function of these receptors in the dorsal horn with postnatal age. It could be hypothesised that increased 5-HT_2 and 5-HT_7 receptor expression and function with postnatal age may enhance pre and postsynaptic inhibition of sensory inputs to dorsal horn neurons and diminish 5-HT/5-HT_3R-mediated excitation. Developmental changes in RVM microcircuits may also determine the onset of descending inhibition form the RVM. Indeed, adult descending inhibition has also been shown to be dependent on constitutive opioidergic activity in the RVM, as blocking opioidergic activity in the RVM between P21 and P28, but not earlier, prevents the normal maturation of descending inhibition (Hathway et al., 2012). K and μ-opioid receptors are expressed on spinally projecting serotonergic neurons in the RVM (Marinelli et al., 2002), therefore maturation of opioidergic signalling in the RVM may moderate and modulate the excitability of these neurons and reduce 5-HT/5-HT_3R-mediated dorsal horn excitation of noxious inputs in adulthood.

5.5.5 Descending serotonergic neurons and spinal 5-HT_3Rs facilitate brush-evoked properties of dorsal horn neuron in all ages

A surprising and novel finding in this chapter was the evidence of descending serotonergic facilitation of tactile inputs in the dorsal horn in rats of all ages. In 5,7-DHT ablation experiments, dorsal horn WDR neuron brush-evoked firing activity was lower or brush receptive field sizes were relatively smaller compared to control at all ages. Similarly, application of ondansetron to the spinal cord of P21 (2 or 50μg) and adult rats (50μg) decreased dorsal horn WDR neuron brush-evoked firing activity or brush receptive field sizes; collectively demonstrating 5-HT/5-HT_3R-mediated facilitation of low-threshold inputs in young and adult rats. Reduced brush-evoked properties were coincidental with increased pinch-evoked responses in 5,7-DHT-treated

adult rats and unchanged pinch-evoked responses in ondansetron-treated adult rats; suggesting a component of 5-HT/5-HT_3R neurotransmission which selectively targets low-threshold inputs.

Serotonergic facilitation of low-threshold inputs to the dorsal horn has been observed in previous electrophysiological experiments. Dorsal horn neuron firing activity to non-noxious hindpaw stimulation with low force vFhs and 35-40°C heated water was slightly reduced in 5,7-DHT treated uninjured adult rats, and was strongly reduced in 5,7-DHT treated and nerve injured adult rats (Rahman et al., 2006). In contrast, evidence of 5-HT_3R-mediated facilitation of low-threshold inputs is limited, even in nerve-injured adult rats (Rahman et al., 2004a, 2004b; Okubo et al., 2013a). A current hypothesis is that endogenous 5-HT_3R-mediated facilitation of sensory inputs is weak in uninjured adults, and targets high threshold inputs (Rahman et al., 2004b), but is strong in adults with inflammatory or nerve injury, and targets low to high threshold inputs (Zeitz et al., 2002; Rahman et al., 2004a; Okubo et al., 2013a). The findings in this chapter identify a novel 5-HT_3R-mediated facilitation of low-threshold inputs to deep dorsal horn neurons which is observed in uninjured rats of all ages and is distinct from previously described 5-HT_3R-mediated facilitation of high-threshold inputs to dorsal horn neurons in injured adults.

Spinal cord slice *in vitro* patch clamp experiments have suggested that 5-HT_3R activation could have roles in antinociception, as 5-HT_3R agonist application can increase the frequency and amplitude of dorsal horn neuron IPSCs (Xie et al., 2012). *In vivo* patch clamp experiments dispute this, as blocking 5-HT_3Rs had no effect on RVM electrical stimulation-induced increases in dorsal horn neuron IPSCs (Kato et al., 2006). Whilst 5-HT_3R-mediated facilitation of inhibitory signalling in the dorsal horn may be present, an extensive body of evidence has repeatedly shown that this effect is masked by dorsal horn neuron excitatory and pronociceptive output of 5-HT_3R signalling in the spinal cord. Observations of endogenous 5-HT_3R-mediated facilitation of brush-evoked properties of dorsal horn neurons agree with these findings.

In vivo electrophysiology recordings performed in this chapter provide information about the effect of blocking spinal 5-HT_3Rs on populations of dorsal horn neurons. One cannot conclude from these experiments whether changes in deep dorsal horn neuron firing activity observed following application of ondansetron were due to blocking 5-HT_3Rs on sampled dorsal horn neurons or inputting primary afferent terminals (as described by Kim et al., 2014); or due to downstream effects caused by blocking 5-

HT_3Rs on upstream polysynaptic circuits involving primary afferent terminals and superficial dorsal horn neurons.

5.6 Conclusions

Here, I identified a key 5-HT/5-HT$_3$R-mediated mechanism that drives descending facilitation of sensory inputs to deep dorsal horn neurons in young rats. Spinally projecting serotonergic neurons were found to be a major source of descending facilitation from the RVM in young rats, and ablating these neurons unmasked previously silent descending inhibition. Descending serotonergic neurons were found to have a predominantly inhibitory effect on noxious stimulus-evoked deep dorsal horn neuron properties in adult rats. Additionally, 5-HT/5-HT$_3$R-mediated facilitation of low threshold brush-evoked responses of deep dorsal horn neurons was observed in rats of all ages. I propose that 5-HT/5-HT$_3$R-mediated facilitation of dorsal horn nociceptive networks is dependent on the excitability of the spinal dorsal horn nociceptive networks; as this facilitation is only observed in young rats and in adult rats with chronic pain conditions. A summary figure outlining the hypothesised role of descending RVM serotonergic modulation of sensory inputs to the dorsal horn in young and adult animals is outlined in figure 5.13.

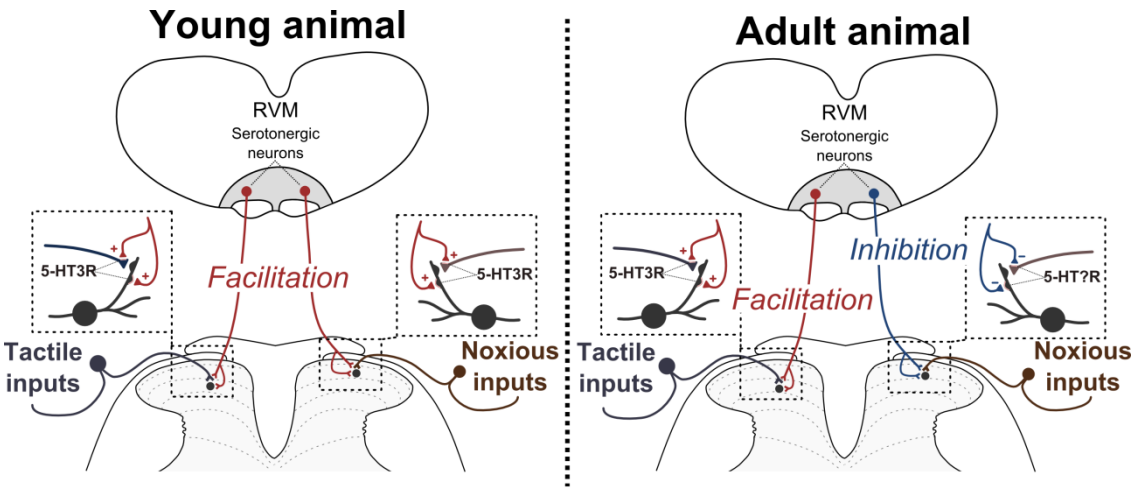

Fig 5.13. A proposed model of descending serotonergic modulation of sensory inputs to the dorsal horn in uninjured young and adult animals.
In young animals, descending serotonergic modulation facilitates low threshold tactile and high threshold [presumed noxious] inputs to the dorsal horn via recruitment of 5-HT$_3$Rs expressed on primary afferent terminals and dorsal horn neurons. In adult animals, descending serotonergic and 5-HT$_3$R-mediated facilitation of tactile inputs is also observed; however, noxious inputs are inhibited by descending serotonergic neurons via a different 5-HT receptor subtype.

Chapter 6
General Discussion

6.1 Background to this thesis

The research described in this thesis addresses how the developing brain modulates cutaneous sensory circuits in the developing spinal cord. In the adult, descending projections from the brainstem provide powerful modulation of spinal sensory networks. This descending modulation can be evoked by cutaneous noxious stimulation, as observed by the phenomenon of descending noxious inhibitory control (DNIC) in rodents (Bannister et al., 2015) and most likely during conditioned pain modulation (CPM) in humans (Yarnitsky, 2010). In healthy adults it is proposed that rapid sensory feedback modulation of somatosensory processing, either excitatory or inhibitory, can occur in response to different environmental cues. Furthermore the same system is thought to mediate the effects of attention, anxiety, mood, expectation and stress upon pain experience (Bingel and Tracey, 2008). This endogenous pain modulation acts via brainstem nuclei which send direct axonal projections to the spinal cord dorsal horn and act as important mediators between higher brain function and cutaneous sensory circuits in the spinal cord. Thus in healthy adults, nociception and pain are regulated by the balance of excitatory and inhibitory drive upon dorsal horn, descending from brainstem regions such as the rostroventral medulla (RVM).

When I began this research, it was known that in young rats, this descending modulation is not mature and that descending inhibition over dorsal horn activity is not apparent until ~ P30 (Hathway et al., 2009b). Before this age, descending excitation dominates over weaker inhibition in the dorsal horn. Imbalanced excitatory/inhibitory transmission is a striking feature of the neonatal dorsal horn: local interneurons and descending inputs from the brain contribute to enhance sensory inputs from low and high-threshold sensory inputs in the first postnatal weeks. Enhanced excitation in the neonatal dorsal horn is hypothesised to potentiate sensory inputs to the neonatal dorsal horn and promote activity-dependent maturation of spinal and supraspinal sensory pathways and networks (Koch and Fitzgerald, 2013). It has been proposed that lack of inhibitory control of sensory information in the neonatal dorsal horn is the reason why behavioural responses to tactile and noxious stimuli are hyperexcitable in human and rodent infants (Fitzgerald et al., 1988; Fitzgerald, 2005).

Little direct evidence existed, however, about the functional role of descending excitation upon dorsal horn circuits in young animals or about when and how the feedback loop, from spinal cord to brainstem and back to spinal cord, develop over the postnatal period. Furthermore nothing was known of the brainstem neuronal

populations involved in the changing postnatal profile of descending control or of the part played by descending serotonergic pathways in the maturation of infant spinal sensory circuits. This thesis has filled many of the gaps in our knowledge of the maturation of top down somatosensory control. In this chapter, I will discuss and speculate on the implications of the findings in this thesis in the wider context of the developing nervous system.

6.2 Summary of the results in this thesis

Experiments in Chapter 2 mapped the age at which noxious mechanical stimulation activates neurons in several regions of the brainstem and midbrain which are involved in the spinal-bulbo-spinal loop in rats. Pinch stimulation of the hindpaw was used as a noxious stimulus and Fos expression was used as a measure of neuronal activation. Results showed that:

- Hindpaw pinch stimulation activated neurons in the PB nucleus from P4, demonstrating functional ascending nociceptive pathways from the dorsal horn to the PB nucleus from this age.
- In contrast, pinch-evoked neuronal activation was not observed in the PAG or RVM until P12 and in the DRN until P40.

These findings build on previous work which investigated noxious stimulus-evoked activation in the neonatal brain (Barr, 2011; Man et al., 2012), and suggest that brainstem regions involved with descending modulation of sensory circuits do not receive nociceptive mechanical sensory inputs until the end of the second postnatal week.

Chapter 3 investigated the changing postnatal role of descending RVM activity upon spinal dorsal horn neuron electrophysiological properties. While electrical stimulation and ablation experiments had shown a dominant facilitation of spinal sensory reflexes from the RVM at P3–P30 (Hathway et al., 2009b), it was not clear whether the RVM directly modulates sensory neurons in the dorsal horn in an age dependent manner. Lidocaine was injected into the RVM to silence RVM neuronal activity and test the effect upon dorsal horn cell properties at P8 or P21

Results showed that:

- RVM activity normally endogenously facilitates dorsal horn neuron pinch-evoked firing and receptive field size in young rats aged P8 and P21.

- This descending facilitation was observed at an age when pinch-evoked neuronal activation in the RVM is absent (P8, shown in chapter 2), suggesting that early descending facilitation from the RVM is driven independently of sensory inputs.
- In contrast, RVM activity normally endogenously inhibits dorsal horn neuron pinch-evoked firing and receptive field size in adult rats, consistent with previous studies (Fields et al., 1977).
- In a persistent inflammatory pain model, RVM activity inhibits behavioural hypersensitivity at P21 and in adults, but has no effect upon mechanical hypersensitivity in P12 rats.

Chapters 4 and 5 focussed on investigating the structural and functional maturation of descending serotonergic modulation of spinal sensory networks. Chapter 4 used retrograde tracing experiments and immunostaining to map the development of serotonergic RVM neuron axonal projections to the lumbar dorsal horn. The results showed that:

- RVM neurons, including serotonergic neurons, project to the dorsal horn from birth.
- The proportion of serotonergic RVM neurons that project to the lumbar dorsal horn increases between P10-P16,
- The density and distribution of serotonergic terminals in the lumbar dorsal horn increases from P7-P40.
- The density and the distribution of 5-HT_3R expression in the superficial spinal dorsal horn does not change with postnatal age.

Chapter 5 investigated the role of serotonergic raphe-spinal neurons and spinal 5-HT_3Rs in the endogenous modulation of sensory-evoked dorsal horn neuron properties at several postnatal ages. First, descending serotonergic neurons were ablated by intrathecal injection of the toxin 5,7-DHT. Results showed that

- Ablation of descending serotonergic neurons decreased brush and pinch-evoked firing of dorsal horn neurons at P8 and P21, unmasking endogenous serotonergic *facilitation* of both low and high-threshold inputs in the dorsal horn in young rats
- On the other hand, ablation of descending serotonergic neurons at P40 had differing effects upon low and high-threshold inputs in the dorsal horn:

  - increasing pinch-evoked firing of dorsal horn neurons, unmasking endogenous descending serotonergic *inhibition* of high threshold inputs in adult rats.
  - decreasing the size of brush evoked receptive fields of dorsal horn neurons, unmasking endogenous descending serotonergic *facilitation* of low threshold inputs in adult rats.
- Electrical stimulation of the RVM in saline-treated P21 rats facilitated high-threshold inputs in the dorsal horn. However, ablation of descending serotonergic neurons suppressed descending facilitation evoked by electrical stimulation of the RVM, demonstrating that RVM serotonergic neurons are a major component of descending RVM facilitation in P21 rats.

Next, the role of spinal 5-HT_3Rs upon spinal nociception was investigated in young and adult animals using intrathecal administration of ondansetron. Results showed that:

- 5-HT_3R activity has no effect upon pinch-evoked properties of dorsal horn neurons at P40
- 5-HT_3R-mediates endogenous *facilitation* of brush receptive fields at P40,
- 5-HT_3Rs mediate endogenous *facilitation* of both brush and pinch evoked dorsal horn activity at P8 and P21, demonstrating that activation of spinal 5-HT_3Rs endogenously facilitates sensory transmission in the first weeks of life.

6.3 The spinal-bulbo-spinal loop during postnatal development

The concept of a spinal-bulbo-spinal loop originates from observations that cutaneous noxious stimulation can drive diffuse endogenous pain modulation (Le Bars et al., 1979). The loop can be loosely divided into two parts or arms: the ascending projections from the spinal dorsal horn to various nuclei in the brain; and the descending projections from brainstem nuclei to the dorsal horn, with anatomical connectivity between the two arms being inferred from anatomical and physiological findings. The hypothesis of a spinal-bulbo-spinal loop stems from adult research, and little is known about the ontogeny of this loop. When ascending pathways transmit sensory information to different brain regions and when sensory inputs can recruit descending modulation are questions that are yet to be fully answered. Some evidence suggested that sensory inputs may not recruit descending inhibitory drive in neonatal rats: sensory-evoked descending modulation is not observed until P21, as distal pinch

stimulation reduces c-fos expression induced by heterotopic formalin injection at P21, but not at P12 (Boucher et al., 1998a). These findings indicated that the spinal-bulbo-spinal loop is not mature in the first weeks of postnatal life.

Data presented in this thesis suggests that ascending sensory inputs activate neurons in different brainstem and midbrain regions from different postnatal ages. Inferred from these findings and from those elsewhere (Barr, 2011), it can be postulated that ascending nociceptive transmission to higher brain regions may be relayed primarily via the PB nucleus, but not the PAG or the paraventricular thalamus in the first postnatal week. In the adult, neurons in the PB project to the PAG, RVM, amygdala, hypothalamus and various thalamic nuclei (Bernard et al., 1993; Alden et al., 1994; Bester et al., 1999; Gauriau and Bernard, 2002a). Peripheral noxious stimulation in the first postnatal week failed to activate neurons in the PAG and RVM, suggesting that projections from the PB nucleus to these regions are not functional in neonates

Neurons in the PAG and RVM are not activated by peripheral mechanical noxious stimuli until P12. Importantly, descending modulation of dorsal horn neuron properties is observed in the first postnatal week, suggesting that descending RVM modulation may occur independently from sensory recruitment of descending pathways. Results in Chapter 3 also demonstrated that the RVM facilitates persistent behavioural hypersensitivity in inflamed rats at P21 and P40, but not at P10-12, strengthening the hypothesis that descending RVM modulation is not driven by sensory inputs before P12.

Upstream of the RVM, electrical stimulation of, or opioid injection into, the PAG has little effect on pain behaviours and hindlimb nocifensive reflexes before P21 (van Praag and Frenk, 1991; Goodwin and Barr, 2005; Kwok et al., 2013). These findings strengthen the hypothesis that descending projections from the PAG do not influence descending modulation arising from the RVM in the first two to three postnatal weeks. Before this, the RVM can modulate spinal sensory processing, suggesting that the RVM is "cut off" from sensory and autonomic afferent inputs from higher regions of the brain and intrinsically drives descending modulation at this age. Once top-down afferent inputs to the RVM mature, endogenous pain modulatory networks may, hypothetically, have the ability to drive descending modulation from the brainstem and provide balanced but changeable inhibition and excitation of spinal sensory networks. Interestingly, the maturation of pain modulation from the PAG coincides with the onset of descending inhibition from the brainstem (Fitzgerald and Koltzenburg, 1986;

van Praag and Frenk, 1991), suggesting that these higher inputs are required for the maturation of descending inhibition of dorsal horn nociceptive activity.

These finding have led to a secondary hypothesis that maturation of connections to the RVM from regions such as the PB nucleus and PAG permits noxious stimulus-induced activation of the RVM from P12, thus 'closing' the spinal-bulbo-spinal loop and allowing sensory inputs to drive descending pain modulation. Testing the validity of this requires a better knowledge of anatomical and functional connectivity of sensory modulatory pathways in the brain (e.g., between the PAG and RVM) during postnatal development. A summary diagram in figure 6.1 illustrates this hypothesis.

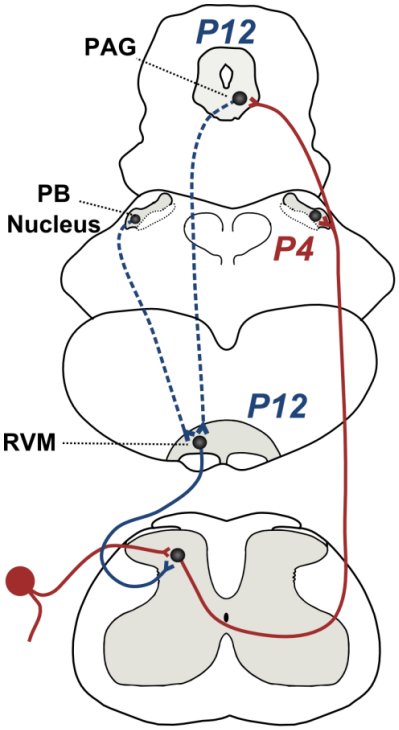

Fig 6.1. The spinal-bulbo-spinal loop in young rodents
Noxious sensory inputs activate neurons in the dorsal horn and the parabrachial (PB) nucleus at P4, but not until P12 in the periaqueductal grey (PAG) and rostroventral medulla (RVM). Solid lines indicate known anatomical connections between regions (Li and Baccei 2012; this thesis) and dashed lines indicate inferred connectivity from Fos immunohistochemistry experiments (chapter 2). Importantly, descending RVM modulation of spinal dorsal horn activity is functional in the first postnatal week, before sensory activation of RVM neurons is observed.

DNIC is an important example of endogenous pain modulation which is hypothesised to be mediated via a spinal-bulbo-spinal loop. CPM and offset analgesia paradigms are human analogues of DNIC which are used as an equivocal measure of endogenous pain modulation in human research (Yarnitsky, 2010). The majority of research using these paradigms has focussed largely on adult populations, but several groups have

recently investigated CPM in children. Children aged between 7-18 years of age display endogenous pain inhibition in CPM paradigms where cold water immersion is used as a conditioning stimulus to reduce pressure and thermal pain ratings (Goffaux et al., 2008; Evans et al., 2013; Tsao et al., 2013; Williams et al., 2013). Interestingly, children aged 12-17 years exhibited greater CPM than young children aged 8-11 years, (Tsao et al., 2013), suggesting that the strength of endogenous pain inhibition arising from the brain increases with postnatal age. These findings are consistent with animal data (Fitzgerald and Koltzenburg, 1986; van Praag and Frenk, 1991), and give support to the hypothesis that endogenous pain inhibitory transmission increases in potency with postnatal age.

Here we have demonstrated that, in rodents, immature descending inhibition is preceded by dominant descending facilitation at early postnatal ages, but this has not yet investigated in humans. Testing whether endogenous pain modulation is present in younger infants would be challenging. CPM paradigms rely on subjects understanding the testing protocols and being able to report changes in perceived pain verbally, therefore it is challenging to measure CPM in young children without basic linguistic skills. Two important translational questions from this thesis regarding endogenous pain modulation in children therefore remain: (1) is endogenous pain modulation observed in human neonates and infants? (2) does endogenous pain modulation change from excitation to inhibition in childhood?

6.4 Descending modulation of pain during development

Pain can be viewed in the context of a cost/benefit reward system (Mason, 2011; Navratilova and Porreca, 2014). Key to this hypothesis is the endogenous pain modulatory system, including descending modulatory pathways from the RVM to the spinal cord, which can increase or decrease the saliency of noxious stimuli in different situations. For example, descending inhibition from the RVM during feeding is hypothesised to override the aversive nature of noxious stimuli and maintain the reward of ingesting food and water to preserve feeding behaviours (Foo and Mason, 2005; Foo et al., 2009). However, the role of descending modulation changes upon tissue inflammation, whereupon descending facilitation arising from the RVM increases the saliency of primary afferent sensory inputs from the injured site and contributes to behavioural hypersensitivity (Vanegas and Schaible, 2004). Descending modulation from the RVM can be seen to represent the net output of upstream

recruitment of RVM neurons. These afferent inputs to the RVM originate from regions such as the spinal cord, amygdala, hypothalamus and the DRN, primarily via the PAG, and are proposed to convey sensory, affective and autonomic information to the RVM (Hermann et al., 1997; McGaraughty et al., 2004; Morgan et al., 2008b).

Experiments in this thesis are in agreement with the hypothesis of responsive and adaptive descending modulation which is tailored to the behavioural state of the animal. Electrophysiological recordings from uninjured anaesthetised adult rats in this thesis demonstrated endogenous descending inhibition of pinch-evoked dorsal horn neuron properties which arises from the RVM (chapter 3) and from raphe-spinal serotonergic neurons (chapter 5). In contrast, descending facilitation of nocifensive withdrawal thresholds was observed in adults with hindpaw inflammation (chapter 3). Thus in adults, descending modulatory drive decreases the saliency of nociceptive inputs in uninjured animals; however following tissue inflammation, descending modulation from the RVM increases the saliency of nociceptive inputs.

Several papers published from Bridget Lumb and colleagues have demonstrated that there is a degree of selectivity of descending modulation of the dorsal horn which arises from the PAG. Of note, pinch-evoked activity of the majority of deep dorsal horn neurons that receive both A and C-fibre inputs are inhibited following injection of the glutamate agonist DLH into the PAG. In contrast, injection of DLH into the PAG facilitated pinch-evoked activity of the majority of dorsal horn neurons that receive only A-fibre inputs (Waters and Lumb, 2008). Experiments in this thesis also provide evidence of descending RVM facilitation of A-fibre inputs in the dorsal horn in uninjured neonatal and adult rats: non-noxious tactile inputs to the dorsal horn, presumably carried by LTMR Aβ fibres, were facilitated by descending serotonergic neurons (chapter 5). Additionally, evidence in this thesis supports the hypothesis proposed by Lumb and colleagues that nociceptive Aδ and C-fibre inputs to the dorsal horn are preferentially inhibited by descending brainstem pathways in uninjured animals (Leith et al., 2007; Waters and Lumb, 2008), as pinch-evoked activity in the dorsal horn was inhibited by RVM neurons and raphe-spinal serotonergic neurons in uninjured adult rats (chapters 3 and 5).

In the first postnatal weeks, descending inhibition of C-fibre inputs is weak and descending RVM pathways preferentially facilitates dorsal horn neurons during A-fibre stimulation and (Fitzgerald and Koltzenburg, 1986; Koch and Fitzgerald, 2014). Thus, descending modulation of spinal sensory circuits in young animals lacks selectivity and is not bimodulatory. This period of weak descending inhibitory drive from the RVM

coincides with the strengthening of C-fibre inputs in the dorsal horn (Fitzgerald and Koltzenburg, 1986; Baccei and Fitzgerald, 2004), suggesting that the maturation of descending inhibition may be linked with the strengthening of C-fibre inputs in the dorsal horn.

In the adult, this cost/benefit reward system is reliant upon endogenous pain modulatory pathways which integrate extrospective and introspective information. By tailoring the processing of sensory information to the state of the animal, by selectively increasing or decreasing the saliency of somatosensory stimuli, descending pain modulatory pathways change behavioural outputs. Because these pain modulatory pathways in neonatal rats may not response to external cues and non-selectively increase the saliency of somatosensory inputs, it follows that pain in the neonate is not part of a functional cost/benefit reward system. The absence of such a system may seem maladaptive for survival, but this is not so surprising during the first weeks of postnatal life when rat pups are highly dependent on maternal support for food, shelter and protection. It is only when sensorimotor pathways develop and complex goal orientated behaviours are observed towards the end of the third postnatal week that selective and responsive pain modulation is necessary and, indeed, observed.

6.5 The discovery that descending serotonergic pathways facilitate tactile inputs in the dorsal horn at all ages

6.5.1 Raphe-spinal serotonergic descending modulation

Based on earlier findings that RVM electrical stimulation facilitates A-fibre-evoked firing responses in P21 rats (Koch and Fitzgerald, 2014), I hypothesised that RVM neurons would endogenously facilitate processing of tactile inputs in the dorsal horn. In chapter 3, injection of lidocaine into the RVM failed to change brush evoked firing activity or receptive field size of dorsal horn neurons at any age compared to control. Similarly, in chapter 5, electrically stimulating the RVM did not change mean brush firing activity of dorsal horn neurons in P21 saline-treated rats. However, results in Chapter 5 unmasked endogenous serotonergic facilitation of brush-evoked dorsal horn neuron activity at all ages. This serotonergic facilitation of tactile inputs in the dorsal horn was mediated by endogenous 5-HT_3R activation.

The origin of descending serotonergic control is unclear in these experiments, as intrathecal injection of 5,7-DHT likely ablated descending serotonergic fibres with cell bodies in several caudal raphe nuclei such as the RP, ROb as well as those in the RMg

and LPGi in the RVM. Whilst the majority of serotonergic terminals in the dorsal horn originate from neurons with cell bodies in the RVM (Braz and Basbaum, 2008), the effects of ablating spinally projecting RP and ROb serotonergic neurons cannot be discounted. Serotonergic modulation of low-threshold sensory inputs in the dorsal horn of uninjured adult rats has been reported elsewhere (Rahman et al., 2006), however these results were not discussed in detail and have been overlooked in the literature. There are two possible reasons why overall silencing or activation of RVM failed to alter brush evoked properties of dorsal horn neurons, whilst selective ablation of descending serotonergic terminals in the dorsal horn did:

(i) Descending serotonergic facilitation of tactile inputs arises from a relatively small subpopulation of RVM neurons whose activity is easily masked

Injection of lidocaine into or electrical stimulation of the RVM changes the properties of heterogeneous populations of neurons, the majority of which do not project to the spinal cord. *If* spinally projecting serotonergic RVM neurons modulate low-threshold inputs in the dorsal horn, it is likely that lidocaine injection or electrical stimulation would change the properties of other populations of RVM neurons and mask or inhibit the effects of this relatively small population of neurons in the RVM. The idea that spinally-projecting RVM serotonergic neurons can endogenously modulate low-threshold inputs in the dorsal horn infers that this population of neurons can be selectively activated by higher centres. Our current understanding of the connectivity of descending modulatory pathways in the brainstem is limited; therefore it is not known if populations of spinally-projecting RVM neurons can be preferentially or selectively recruited by higher centres to provide selective descending modulation of particular dorsal horn neuronal inputs.

There is evidence that TrkB receptors are preferentially expressed on spinally projecting serotonergic neurons in the RVM, and that deletion of endogenous 5-HT in the RVM prevents behavioural pain hypersensitivity caused by intra-RVM injections of BDNF (Wei et al., 2010). Perhaps then, BDNF release in the RVM may activate TrkB receptors preferentially expressed on spinally-projecting serotonergic neurons and evoke downstream 5-HT release in the dorsal horn. BDNF+ neurons in the vlPAG are known to project to the RVM (Yin et al., 2014a), suggesting that descending inputs from the PAG may be important in driving serotonergic descending modulation from the RVM. Experiments involving selective optogenetic and chemogenetic activation or silencing of descending serotonergic RVM pathways could be performed to further

investigate this BDNF-TrkB/serotonergic neuron descending sensory modulatory pathway.

(ii) Serotonergic facilitation of tactile inputs arises from nuclei outside of the RVM

Serotonergic neurons in the RP and ROb are known to terminate in the intermediate and ventral horn, whilst those in the RVM terminate in the dorsal horn (Liang et al., 2015). Inferred from these anatomical descriptions is divergent functionality of the raphe nuclei; with modulation of nociceptive inputs originating from the RVM and modulation of motor circuits from the RP and ROb (Jacobs et al., 2002). It is unlikely that there is a strict divergence of function of different raphe nuclei, as there is overlap in the termination patterns of raphe-spinal neurons from different nuclei: neurons in the RP and ROb have sparse axonal terminals in the deep dorsal horn, and neurons in the RVM have sparse terminals in the ventral horn (Liang et al., 2015).

Firing activity of serotonergic neurons in the RP and ROb is tightly coupled with locomotor activity, as the firing rate of RP and ROb neurons strongly correlated with the speed of treadmill running and returned to baseline firing patterns when locomotor activity ceased (Veasey et al., 1995). Additionally, application of 5-HT or 5-HTR agonists can facilitate or inhibit proprioceptive inputs to interneurons in the intermediate spinal cord involved in motor circuits (Jankowska et al., 1994, 2000; Hammar et al., 2004), suggesting that serotonergic projections from the RP and ROb can modulate low-threshold sensory recruitment of spinal motor circuits.

Based on this evidence and on findings that stimulation/silencing of RVM neurons did not change brush-evoked properties of dorsal horn neurons, I hypothesise that endogenous serotonergic facilitation of cutaneous tactile inputs in the deep dorsal horn arises from neurons in the RP and ROb (Fig 6.2A). Facilitation of tactile inputs arising from RVM neurons cannot be ruled out however, especially in young rats when electrical stimulation of the RVM can change A-fibre evoked properties of dorsal horn neurons (Koch and Fitzgerald, 2014).

Jacobs et al (2002) hypothesise that the primary role of the medullar serotonergic system is to provide coordination of motor and sensory processes which are activated with central motor commands and modulate a behavioural output. It remains to be discovered whether serotonergic facilitation of tactile inputs in the dorsal horn is activated by upstream central commands and contributes to downstream changes in motor and behavioural outputs.

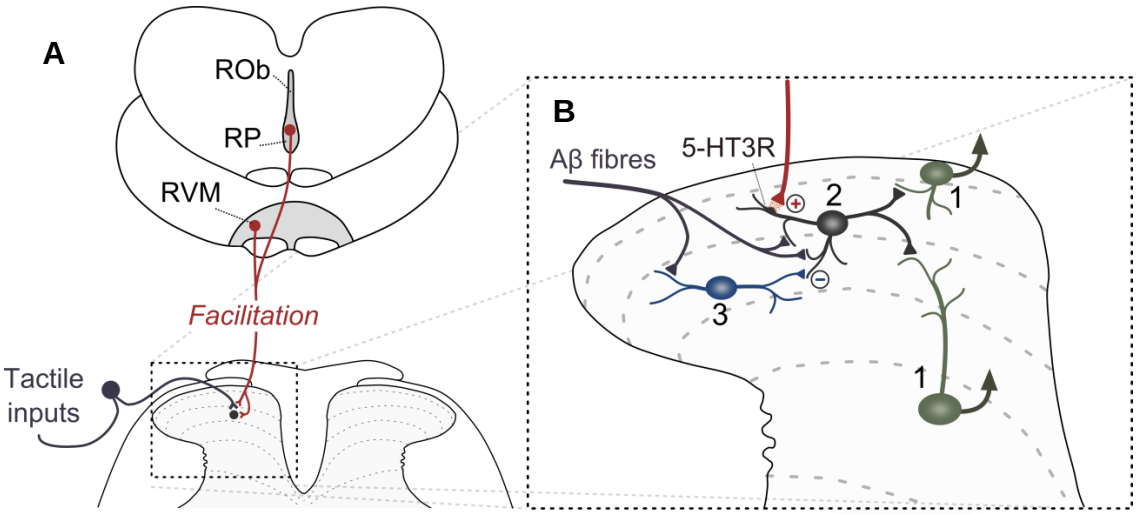

Fig 6.2. Serotonergic facilitation of tactile inputs in the neonatal and adult dorsal horn
Descending serotonergic facilitation of tactile inputs in the dorsal horn arises from serotonergic neurons in the rostroventral medulla (RVM), raphe pallidus (RP) or the raphe obscurus (ROb) (A). Serotonergic facilitation of tactile inputs is mediated via activation of 5-HT_3Rs in the dorsal horn (B). Aβ inputs activate excitatory (2) and inhibitory (3) interneurons in laminae II and III. Excitatory interneurons provide feedforward activation of projection neurons in the superficial and deep dorsal horn (1). The majority of 5-HT_3Rs are expressed on excitatory interneurons in the adult dorsal horn (Maxwell et al, 2005); therefore it is likely that these neurons are targeted by descending serotonergic inputs which facilitate processing of low threshold inputs.

6.5.2 The role of spinal 5-HT_3Rs in modulating processing of tactile inputs

An advantage of studying the descending serotonergic system in the spinal cord is that the only known source of 5-HT release in the dorsal horn is from serotonergic neurons in the raphe nuclei. Thus, investigations into the expression of 5-HTRs in the dorsal horn provide a *de facto* marker of neurons which are under direct descending serotonergic control. This cannot be said for the descending GABA/glycinergic system as both local interneuron populations and supraspinal RVM neurons release GABA/glycine in the dorsal horn. Experiments in this thesis demonstrated that serotonergic facilitation of low-threshold inputs in the dorsal horn was mediated by endogenous activation of 5-HT_3Rs. In the adult, the majority of 5-HT_3R expression (85%) is found on excitatory VGLUT2+ interneurons in the dorsal horn (Maxwell et al., 2003), and it is possible that 5-HT_3R-mediated facilitation of low-threshold and inputs is mediated by 5-HT_3R expression on excitatory interneuron populations which receive low-threshold sensory inputs. Likely candidates are somatostatin+, VGLUT+ or PKCγ+ excitatory interneurons which receive monosynaptic Aβ inputs and provide feedforward excitation of projection neurons (which is usually inhibited) (Duan et al., 2014; Peirs et al., 2015). Experiments in these papers were primarily conducted in adult

rodents, and expression of markers of interneuron subtypes and the physiological roles of these populations are largely unknown in neonatal rodents.

Unpublished findings from the Fitzgerald lab have shown that parvalbumin expression in dorsal horn neurons is absent until P14, and expression does not reach mature patterns until P21. Similarly, nNOS expression increases from P3 and reaches adult levels by P21 (Hirschberg and Fitzgerald, unpublished data). These findings demonstrate that these classes of inhibitory interneurons undergo changes in their neurochemical phenotype during the first weeks of life. Others have shown that the number of PKCγ excitatory interneurons in the lamina II of the dorsal horn increases between P7 and P21 (Sweitzer et al., 2004). It is difficult to draw firm conclusions about the function of these interneuron populations, but it can be hypothesised that the excitatory and inhibitory functions of these interneurons in dorsal horn sensory circuits is not mature until at least the fourth postnatal week.

The first three weeks of postnatal life are also marked by the transient expression of VGLUT3 in a population of excitatory interneurons between P5-P20 (Peirs et al., 2015). These neurons receive monosynaptic Aβ fibre inputs and provide feedforward excitation of lamina I neurons via PKCγ or calretinin excitatory interneurons. The presence of polysynaptic Aβ fibre-mediated excitation of projection neurons in young rats also coincides with high prevalence of monosynaptic Aβ inputs to neurons in the neonatal superficial dorsal horn (Park et al., 1999). Glycinergic/GABAergic transmission from inhibitory neurons during these first postnatal weeks only weakly inhibits monosynaptic and polysynaptic excitation of dorsal horn neurons (Baccei and Fitzgerald, 2004; Ingram et al., 2008). VGLUT3 (and other) excitatory interneurons likely contribute to provide additional excitatory drive in neonatal dorsal horn sensory networks during a period of immature glycinergic/GABAergic inhibitory transmission.

The role of descending modulatory control over specific dorsal horn neuron subtypes is not well understood, even in the adult literature. In theory, descending control over functionally distinct populations of dorsal horn neurons would provide powerful control over the processing of different sensory modalities in the dorsal horn. I hypothesise that in young and adult animals, descending serotonergic neurons, mediated by 5-HT_3Rs, excite low-threshold input feedforward pathways and permits polysynaptic Aβ fibre activation of dorsal horn neurons. In contrast, descending serotonergic modulation of high-threshold inputs in the spinal dorsal horn becomes tuned in adulthood, either inhibiting or facilitating processing of noxious inputs, depending upon the behavioural state of the animal.

Future experiments investigating 5-HT_3R expression in dorsal horn neuron subtypes (as defined by neurochemical markers such as PKCγ or calretinin) and the effects of ablating or exogenously driving activation of 5-HT_3Rs on these interneuron populations at different postnatal ages would help to elucidate the role of descending serotonergic modulation of dorsal horn sensory circuits. Figure 6.3 illustrates a hypothesis of descending serotonergic excitation of dorsal horn circuits in young rodents.

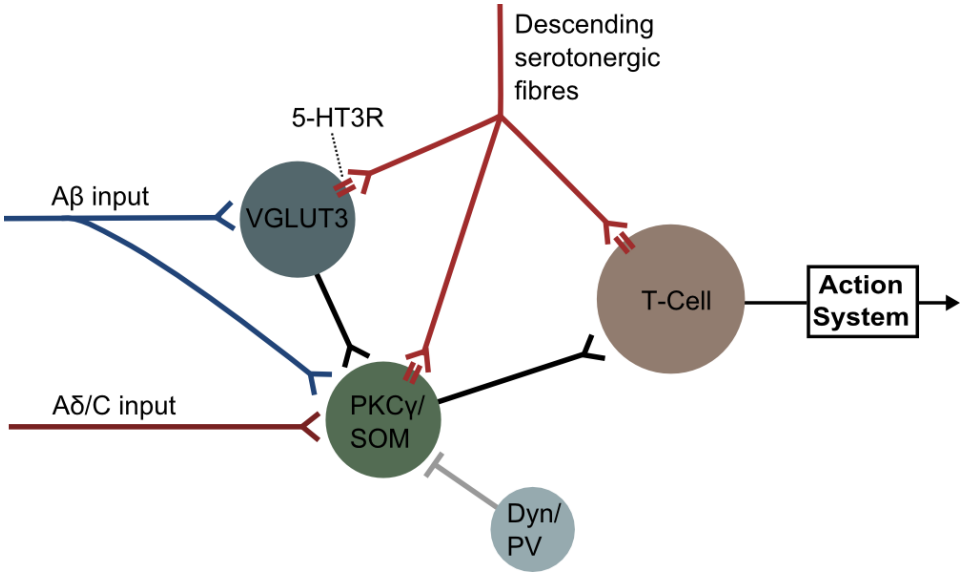

Fig 6.3. Hypothesised serotonergic activation of sensory networks in the dorsal horn of rats aged P8-P21
Excitatory VGLUT3, PKCγ, and somatostatin (SOM) interneuron populations are proposed to provide feedforward excitatory drive to transmission (T) cells (Piers et al, 2015; Duan et al, 2014). In the neonatal dorsal horn, inhibitory transmission (proposedly from dynorphin+ (Dyn) or parvalbumin (PV) populations) is immature, leading to greater excitation. Descending serotonergic facilitation in the young rat is mediated via activation of 5-HT_3Rs in the dorsal horn. Expression and activation of 5-HT_3Rs on excitatory interneuron or T-cells populations, but not inhibitory interneurons, may provide facilitation of low and high-threshold sensory processing in the dorsal horn.

6.6 Conclusions

Endogenous pain controls, acting via descending pathways, have a powerful impact on the processing of sensory stimuli in the spinal cord. In humans, expectation of pain relief can significantly reduce the size of cutaneous secondary hyperalgesia, thought to be maintained in the spinal cord, thus suggesting that placebo analgesia can influence the processing of noxious inputs in the spinal cord (Matre et al., 2006). By increasing or

decreasing the gain of sensory processing, descending controls can modulate the output from the dorsal horn; either to action centres in the brain or to motor circuitry in the ventral horn. In the adult, descending controls can be recruited by ascending sensory pathways, thus creating a spinal-bulbo-spinal loop which provides rapid feedback modulation of sensory processing in the dorsal horn. In the first postnatal week, descending modulation of dorsal horn neurons arising from the RVM may act independently from ascending noxious sensory recruitment of PAG-RVM networks, suggesting that ongoing descending RVM modulation is driven by intrinsic activity within the RVM (Schwaller et al., 2015).

In the first weeks of life, descending RVM modulation of spinal sensory processing is predominantly facilitatory (Hathway et al., 2009b, 2012; Koch and Fitzgerald, 2014). These first postnatal weeks are marked by weak inhibitory transmission, both from local GABAergic/glycinergic interneurons and from supraspinal neurons in the RVM, causing an imbalance of excitatory/inhibitory transmission in the dorsal horn which could drive the characteristic sensitivity of neonatal sensory-motor circuits. As supraspinal and spinal inhibitory signalling matures, so does the fidelity and control of spinal sensory processing and sensory-evoked motor reflexes. The onset of descending excitatory-inhibitory balance arising from the brainstem is one aspect of the "maturity" of the system. Another aspect arises from viewing the RVM as part of a larger endogenous pain modulatory system, and is concerned with the connectivity and recruitment of descending brainstem modulation from higher brain regions. One hypothesis that has emerged from this thesis is that descending facilitation arising from the RVM is not a result of ascending sensory inputs or from higher brain centres in neonatal rats. Once higher connections from brain regions involved with pain modulation (e.g., the PAG and the amygdala) are able to drive descending modulation from the RVM, then the system can be viewed as mature. This top-down aspect of descending modulation is currently poorly understood both in adults and during development.

The maturation of sensory processing in the CNS is dependent on sensory inputs from sensory organs. Consistently, activity-dependent strengthening and weakening of synaptic connections in developing the spinal cord is dependent on peripheral sensory inputs (Waldenström et al., 2003; Granmo et al., 2008). The postnatal maturation of descending inhibitory modulation is influenced by the strength of afferent inputs in young rats. Two studies have shown that ablating C-fibres in neonatal rats prevents the normal development of descending inhibition of noxious stimuli in adulthood (Cervero

and Plenderleith, 1985; Zhuo and Gebhart, 1994). Interestingly, neonatal hind or forepaw plantar incision at P3 prevents descending facilitation of hindlimb reflexes caused by electrical stimulation of the RVM in adult rats, suggesting that neonatal injury impairs descending excitatory transmission and/or strengthens descending inhibitory transmission later in life (Walker et al., 2015). Thus, the strength of noxious inputs during the first weeks of life impacts the strength of descending inhibition in adulthood.

Descending facilitatory drive in the first postnatal weeks may be an important source of excitation which acts to reinforce and strengthen functionally relevant synapses in the dorsal horn. Serotonergic transmission in the neonatal spinal cord has already been shown to promote maturation of sensory-motor circuits: in rat P2-17 spinal cord slices, 5-HT enhances dorsal horn neuron EPSCs by accelerating the maturation of silent glutamatergic synapses (Li and Zhuo, 1998b). In the neonatal spinal ventral horn, serotonergic transmission increases the excitability of intersegmental commissural interneurons at P14-16 (Abbinanti et al., 2012) and promotes plasticity of GABAergic transmission (Sadlaoud et al., 2010). It is therefore possible that early descending serotonergic facilitation may be involved in the maturation of both excitatory and inhibitory transmission in the dorsal horn.

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