# The developmental regulation of local and descending control of dorsal horn neurons in the rat

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# Declaration

The work in this thesis was conducted in the Department of Neuroscience, Physiology and Pharmacology at University College London. I, Stéphanie Colette Koch, 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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April 2010

# Abstract

Neonatal responses to peripheral cutaneous sensory stimuli appear hyperexcitable compared to those of the adult, at both behavioural and cellular levels. Little is known, however, of the mechanisms involved in the maturation of this sensory circuitry over the postnatal period. I hypothesise that the excitability of neonatal networks is due to immature local and descending inhibitory control of spinal circuits. To test this I have examined the maturation of descending and local inhibitory spinal circuitry using immunohistochemical staining in the dorsal horn, *in vivo* electrophysiological recordings of dorsal horn neurons and stimulation of brainstem descending pathways.

Firstly, I mapped the development of spinal glycinergic circuitry over the first three postnatal weeks using immunohistochemical staining of glycinergic terminals and receptors. Results show a clear shift in expression pattern from deep dorsal horn staining of both glycinergic terminals and receptors in the neonate, to selective expression in lamina III by the third postnatal week.

I then characterised the functional development of glycinergic inhibition of spinal sensory pathways at a cellular level using *in vivo* extracellular recordings of dorsal horn neurons in neonatal and adolescent rats in the presence of the glycine receptor antagonist strychnine. Results illustrate an absence of glycinergic inhibition of sensory stimuli until postnatal day 21 and a facilitatory role of glycine in the transmission of low-threshold stimuli in the neonatal spinal cord.

Finally, I examined the descending influence of the rostroventral medulla on dorsal horn neuronal activity over postnatal development. Results indicate that the influence of descending control shifts dramatically from predominantly excitatory in early development, to predominantly inhibitory at a later stage in life.

In conclusion, there is significant postnatal modulation of segmental and descending influences on spinal networks in the postnatal period, both of which are likely to contribute to the maturation of cutaneous sensory spinal processing.

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# Table of Contents

# List of Figures

## Chapter 1: General Introduction



## Chapter 2: Expression and Functional Mapping of Glycinergic Terminals and Receptors in the Spinal Dorsal Horn

## Chapter 3: The Functional Role of Glycinergic Signalling in the Immature Spinal Dorsal Horn

## Chapter 4: The Development of Descending Modulation of Dorsal Horn Neurons

## Chapter 5: General Discussion

# List of Tables

## Chapter 1: General Introduction



## Chapter 3: The Functional Role of Glycinergic Signalling in the Immature Spinal Dorsal Horn

# Abbreviations

| | |
|---|---|
| 5-HT: | 5-hydroxytryptamine, or serotonin |
| αGlyR: | α subunit of the glycine receptor |
| AMPA: | alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid |
| ANOVA: | analysis of variance |
| BL: | baseline |
| $Ca^{2+}$: | calcium ion |
| CCK: | cholecystokinin |
| CFA: | complete Freund's adjuvant |
| $Cl^-$: | chloride ion |
| CNS: | central nervous system |
| CTOP: | D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-$NH_2$; selective μ-opioid receptor antagonist |
| DA: | dopamine |
| DHEAs: | dehydro epiandrosterone sulfate |
| DLF: | dorsolateral funiculus |
| DLPT: | dorsolateral pontine tegmentum |
| DRG: | dorsal root ganglia |
| DRN: | dorsal reticular nucleus |
| EMG: | electromyography |
| ENK: | enkephalin |
| EPSC: | excitatory postsynaptic current |
| EPSP: | excitatory post synaptic potential |
| EXIN: | excitatory interneuron |
| GABA: | γ-aminobutyric acid |
| GAD: | glutamic acid decarboxylase |
| Glut: | glutamate |
| Gly: | glycine |
| GlyR: | glycine receptor |
| GlyT1: | glycine transporter 1 |
| GlyT2: | glycine transporter 2 |
| G-protein: | guanine nucleotide-binding protein |

| | |
|---|---|
| $H_2O$: | water |
| Hz: | hertz; unit events per second. |
| IB4: | isolectin B4 |
| ININ: | inhibitory interneuron |
| IPSC: | excitatory postsynaptic current |
| IPSP: | inhibitory post synaptic potential |
| $K^+$: | potassium ion |
| KCC2: | potassium chloride co-transporter |
| L4: | fourth lumbar region of the spinal cord |
| LI-LVI: | lamina I through VI of the spinal dorsal horn |
| $LII_i$: | inner lamina II |
| $LII_o$: | outer lamina II |
| LT: | low-threshold neuron |
| mEPSC: | miniature excitatory postsynaptic current |
| mIPSC: | miniature inhibitory postsynaptic current |
| mRNA: | messenger ribonucleic acid |
| NA: | noradrenaline |
| NeuN: | neuronal marker |
| NK1: | neurokinin 1 |
| NKCC1: | sodium potassium chloride co-transporter |
| NMDA: | N-methyl-D-aspartate |
| NRM: | nucleus raphe magnus |
| NS: | nociceptive specific neuron |
| NT: | neurotensin |
| P3, etc: | postnatal day 3, etc |
| PAD: | primary afferent depolarisation |
| PAF: | primary afferent fibre |
| PAG: | periaqueductal grey |
| PKCγ: | protein kinase C gamma |
| PB: | parabrachial nucleus |
| RF: | receptive field |
| RVM: | rostroventral medulla or rostral ventromedial medulla |
| s.e.m.: | standard error of the mean |
| SPA: | stimulation produced analgesia |

| | |
|---|---|
| TTX: | tetrodotoxin |
| vFh: | von Frey hair |
| VIAAT: | vesicular inhibitory amino acid transporter |
| VLF: | ventrolateral funiculus |
| VLM: | ventrolateral medulla |
| WDR: | wide dynamic range neuron |

# Chapter 1

# General Introduction

## 1.1 Introduction

The spinal cord serves as the first integrative point of primary afferent sensory information in the central nervous system and is under local segmental, as well as descending control from the brainstem. Within the spinal dorsal horn, sensory input is relayed locally before transmission to higher centres. Here it can be modulated and influenced by both excitatory and inhibitory neurotransmitters released from local interneurons; regulation of these interneurons and the balance between inhibition and excitation can therefore strongly affect the perception and discrimination of sensory information. Increasing inhibition by means of clinically utilised medicines can dampen down all afferent information from both peripheral tissues and descending from brainstem structures, resulting in anaesthesia and analgesia (Franks, 2006). In stark contrast to this, removal of spinal inhibition using pharmacological blockade of inhibitory transmitter receptor systems results in excessive neural activity and models of pathological pain states in rats (Yaksh, 1989; Sivilotti & Woolf, 1994; Sherman & Loomis, 1996; Sorkin & Puig, 1996).

Whereas this equilibrium is carefully managed in the healthy adult system, studies in both animals and humans reveal that this balance is not established at birth, and the mature balance is the result of postnatal activity-dependent strengthening of sensory synapses. Advances in the scientific understanding of biological systems have allowed us to take advantage of the versatility of the human organism in order to better treat adult pathologies, but little is known of how the neonatal system adapts to its environment and how excitatory and inhibitory neurotransmission changes over the critical learning period in early life. This gap in knowledge has led to a distinct lack of treatments directed at pain relief in newborn and premature infants. Behaviourally, neonates appear more sensitive to touch and pain, often responding with exaggerated reflexes and whole-body movements to an isolated cutaneous stimulus to the leg (Fitzgerald *et al.*, 1988; Andrews & Fitzgerald, 1994), implying excessive excitation in nociceptive networks. The mechanisms behind this sensitivity are incompletely understood, but the outdated view that infants can be treated as ‘smaller adults’ certainly no longer applies. Physiologically, premature infants can be

seen as distinct to adults and in some cases medicines commonly used in adults can have entirely opposite effects in the immature system (Koch *et al.*, 2008).

This chapter provides background on the sensory circuitry of the adult spinal dorsal horn, describing the anatomy of the dorsal horn and detailing the neurotransmitters involved in the transmission of cutaneous sensory input. Further, the influence of descending control from the brainstem upon nociceptive behaviour and the sensory coding of spinal neurons will be introduced. Finally, an overview of what is known of the development of this sensory circuitry is presented with particular emphasis on glycinergic inhibition and the role of supraspinal control in the early postnatal period. This will serve to act as a platform for the aims of this thesis, which are outlined at the end of the chapter.

## 1.2 Anatomy of the adult spinal dorsal horn

### 1.2.1 Laminar organisation adult dorsal horn neurons

The spinal dorsal horn is subdivided into parallel laminae according to cell size and packing density, named lamina I through VI ((Rexed, 1952); see Figure 1. 1). Laminae are broadly grouped into superficial (laminae I and II), and deep dorsal horn (laminae III-VI). These delineations also hold true for the functions of neurons within each band. Peripheral information, relayed through primary afferent fibres, enters the spinal cord in a highly organised fashion and each class of afferents terminates within a given lamina. This information can then be relayed locally within the spinal cord or to higher centres via projection neurons, allowing a tight control on the transmission both innocuous and noxious sensory information.

Together, laminae I and II make up the superficial dorsal horn. As the primary target for peripheral nociceptive afferents, the superficial dorsal horn plays a critical role in the processing of nociceptive information. Lamina I, also known as the marginal layer, is comprised of a thin sheet of densely packed interneurons and large projection neurons, both of which have dendrites remaining primarily within the laminar plane. Cells within the marginal layer have been classified by morphology

into fusiform, pyramidal, multipolar and flattened neurons (Lima & Coimbra, 1988; Grudt & Perl, 2002) and, more recently, by electrophysiological properties (Prescott & De Koninck, 2002). Although the percentage of projections neurons within this lamina does not extend beyond 5% (Spike *et al.*, 2003), lamina I has the highest density of projection neurons within the dorsal horn, most of which are neurokinin-1 (NK1) receptor positive and receive monosynaptic innervation from excitatory substance P-containing peripheral afferents (Todd *et al.*, 2000). Projection neurons in lamina I have been shown to be particularly important in setting the excitability of spinal circuits; they have direct connections to key brainstem areas known to be involved in nociceptive processing, such as the thalamus (Al-Khater *et al.*, 2008), periaqueductal grey (PAG; (Spike *et al.*, 2003)) and parabrachial areas (Almarestani *et al.*, 2007). These can then relay to, and receive information from, higher centres in the cerebral cortex and subsequently influence excitability of neurons in the deeper dorsal horn through descending control (Hunt & Mantyh, 2001; Suzuki *et al.*, 2002), forming both a feedback loop onto themselves and feedforward loop onto neurons in the deeper dorsal horn.

Lamina II, in contrast to lamina I, is primarily composed of small interneurons and is known as the substantia gelatinosa due to the lack of myelination within this layer. It is further subdivided into an inner layer ($II_i$) and an outer layer ($II_o$), each of which contains a subset of unmyelinated C fibre peripheral afferents allowing for neurochemical identification. Lamina $II_i$ contains non-peptidergic isolectin B4 (IB4) positive afferents, whereas peptidergic C fibres terminate in lamina I and $II_o$ and can be identified by calcitonin gene related peptide (CGRP) immunostaining (Hunt & Rossi, 1985). The high density of interneurons in lamina II and the easily distinguishable lack of myelination have allowed detailed analysis of excitatory and inhibitory interneurons. In fact, most of the electrophysiological data relating to interneurons has been obtained from recordings within this lamina. Laminae I-III is said to be comprised of around 30% inhibitory neurons (Todd & Sullivan, 1990) although a recent study has shown that the majority of interneuronal connections in the substantia gelatinosa are excitatory (Connor *et al.*, 1987; Santos *et al.*, 2007; Kato *et al.*, 2009). Interestingly, as of yet no morphological characterisation of interneurons has been fully successful. Although at least seven different neuronal subtypes have been suggested (Grudt & Perl, 2002), only two cell types can be

readily identified across species: islet cells, which have elongated dendritic trees spanning rostrocaudally and locally situated axons, and stalked cells, found in lamina $II_o$ with ventrally extending dendrites and axons projecting to lamina I (Gobel, 1978; Todd & Spike, 1993; Grudt & Perl, 2002). Immunohistochemical analysis has revealed that islet cells are GABAergic, suggesting they are inhibitory (Todd & Spike, 1993), but the morphological identification of excitatory interneurons remains unspecified and attempts at correlating these morphologies with function have been unsuccessful (Light *et al.*, 1979; Woolf & Fitzgerald, 1983; Light & Kavookjian, 1988). Lamina II is also known to contain a high number of synaptic glomeruli. These are a complex of primary afferent terminals and dendritic arbors of local interneurons and are thought to be important in the signalling of nociceptive information (Gobel *et al.*, 1980; Willis & Coggeshall, 1991; Wu *et al.*). Afferent terminals can be found both presynaptic to dendritic arbors of interneurons and postsynaptic to synaptic vesicle-containing dendrites, which are often GABAergic in origin (Barber *et al.*, 1978; Ribeiro-da-Silva *et al.*, 1985; Todd *et al.*, 1996). These glomeruli can therefore serve both as signal amplifiers, when one afferent terminal is presynaptic to several dendritic spines, and as signal integrators, when afferent terminals are found postsynaptic to local inhibitory GABAergic dendrites (see (Willis & Coggeshall, 1991; Wu *et al.*)).

Laminae III-VI form the deep dorsal horn and are composed of a heterogeneous population of both interneurons and a few projection neurons. These also contain the terminals of Aβ primary afferent fibres, which convey innocuous touch and brush. Interneurons found within these laminae possess large dendritic trees that can extend up to lamina I or ventrally to laminae IV and beyond (Gobel, 1978; Grudt & Perl, 2002; Schneider, 2008) allowing for monosynaptic input from various functional classes of primary afferents as well as information from interneurons in lamina II (Light & Kavookjian, 1988; Todd, 1989). The second largest site of spinal projection neurons after lamina I is found in lamina V. Neurons within this region send their axons to the thalamus, dorsal column nuclei, the lateral cervical nucleus as well as various regions of the spinal cord; collaterals from lamina V neurons range dorsally to lamina III to ventral lamina VII. Although primary afferent input to this region is predominantly mediated by Aβ fibres, there is also input from thinly myelinated Aδ nociceptors, allowing for multireceptive or wide dynamic range neurons (WDRs)

which respond to innocuous and noxious stimuli (see (Willis & Coggeshall, 1991; Wu *et al.*, 2010).

### 1.2.2 Anatomical organisation of peripheral terminals

Sensory information entering the spinal cord is organised by sensory modality and primary afferent terminals exhibit laminar organisation depending on their functional class ((Light & Perl, 1979; Light *et al.*, 1979; Hantman *et al.*, 2004; Heinke *et al.*, 2004); see Figure 1. 1). Cutaneous peripheral afferents can be classified into three categories, based upon their size and conduction velocities. These include:

- C fibres: thin, unmyelinated fibres that are slowly conducting (0.5-2.0 m/sec). C fibres terminals are mainly located in lamina II, but are also found in lamina I (LaMotte, 1977; Light & Perl, 1979; Cruz *et al.*, 1987; Mizumura *et al.*, 1993; Sugiura *et al.*, 1993) and primarily transmit noxious mechanical, chemical and heat stimuli.
- Aδ fibres: small, myelinated fibres, with conduction velocities that are faster than C fibres (12-30 m/sec). Terminals are found primarily in lamina I, III and lamina V (LaMotte, 1977; Light & Perl, 1979; Mizumura *et al.*, 1993) and fibres transmit noxious information such as pin-prick as well as non-noxious information from hair follicles.
- Aβ fibres: large, myelinated fibres conducting at a rate of 30-100 m/second. These distribute to deeper laminae III and IV (Cruz *et al.*, 1987; Woolf, 1987; Cruz *et al.*, 1991; Shortland & Woolf, 1993) and are sensitive to innocuous stimuli such as brush and touch.

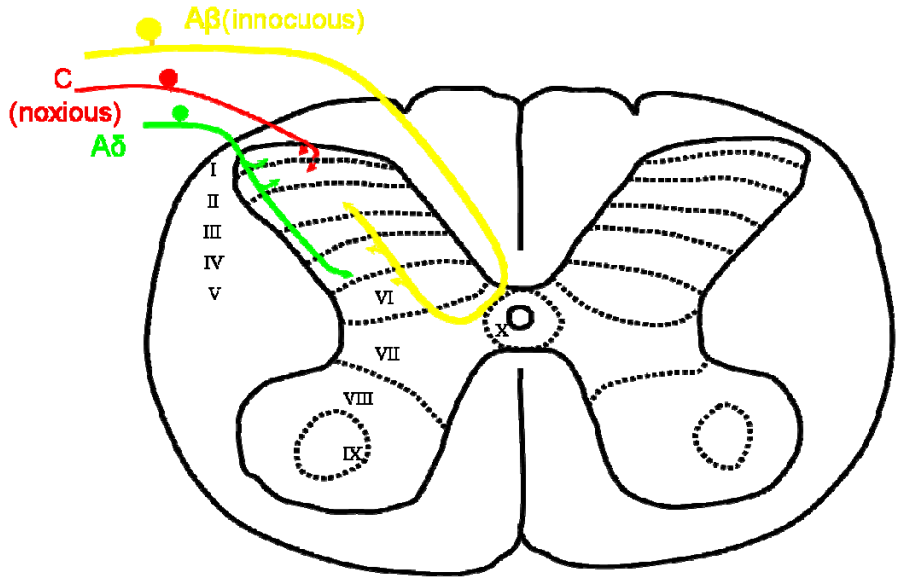



**Figure 1. 1: Schematic of afferent input into the spinal cord.**

Unmyelinated C fibres and thinly myelinated A δ fibres terminate in superficial laminae I-II, with some Aδ fibres reaching lamina V. Myelinated Aβ fibres enter the spinal cord primarily in deeper laminae III-V.

Dorsal horn neurons all possess a receptive field. In the case of those with cutaneous inputs, this corresponds to the skin region that upon stimulation alters neuronal activity beyond the resting state. Receptive fields are influenced by both monosynaptic inputs from peripheral afferents as well as by polysynaptic inputs via local excitatory or inhibitory interneurons. Stimulation of the centre of a receptive field of a neuron will generate an action potential of the target neuron, whilst stimulating the area directly surrounding the receptive field can generate excitatory or inhibitory postsynaptic potentials without reaching action potential threshold (Price *et al.*, 1978a; Brown & Fyffe, 1981; Brown *et al.*, 1987; Woolf & King, 1989). These surrounding areas have been termed "low-probability firing fringes" (Woolf & King, 1989).

Receptive fields are arranged somatotopically: specific areas of the dermatome are represented by groups of dorsal horn neurons with overlapping receptive field areas (Wall, 1967; Willis & Coggeshall, 1991; Wang *et al.*, 1997) and areas differ in size according to the laminar distribution of the cell. Recordings from both cats and rats have shown lamina II neurons to have "amoeboid" receptive fields that are dynamic in size and shape, and can change within a given recording (Dubuisson *et al.*, 1979; Woolf & Fitzgerald, 1983). Furthermore, receptive fields of deep dorsal horn

neurons are generally larger than those found in the superficial layers (Wall, 1967; Wall *et al.*, 1979; Woolf & Fitzgerald, 1983), suggesting a larger integration of input. These larger receptive field areas are thought to aid in the discrimination of stimulus area and intensity, information from which can then be transmitted to supraspinal centres by projection neurons located in lamina V.

Transient increases or decreases in receptive field area can be achieved through the application of pharmacological agents near the recorded neuron (e.g. (Zieglgansberger & Herz, 1971)) or in response to tissue injury or intense chemical stimulation near the receptive area (e.g. (McMahon & Wall, 1984; Woolf & King, 1990)). Similarly, receptive fields can be altered and even fully reorganised in response to nerve damage (Koerber & Mirnics, 1996; Wilson & Kitchener, 1996; Zhang & Rowe, 1997). This would suggest both central and peripheral changes including dorsal horn neuron sensitisation, abnormal peripheral afferent input from receptive field areas, and dysfunctional inhibitory circuits within the spinal cord and from descending supraspinal centres (Lewin *et al.*, 1994).

The range of modalities to which a cell will respond is largely determined by peripheral input and so the cell's laminar distribution, such that a large number of nociceptive specific neurons are found in lamina I of the dorsal horn, whereas a large number of wide dynamic range neurons are found in lamina V. However, laminar distribution is not necessarily an accurate gauge of cell activity and as such, dorsal horn neurons are generally classified according to their response properties, and not their laminar layer (see Figure 1. 2). Although many different forms of classification have been suggested, the most widely held classification is that set out by Menetrey (Menetrey *et al.*, 1977): (i) low-threshold neurons (LT), which respond only to innocuous brush or touch, also known as Class 1 neurons; (ii) wide dynamic range (WDR) or Class 2 neurons, respond to innocuous and noxious stimuli and are the most abundant cell type; (iii) high threshold nociceptive specific cells (NS), which response to noxious stimuli only, also known as Class 3 neurons; and (iv) Class 4 neurons, which respond to joint movement or deep pressure.

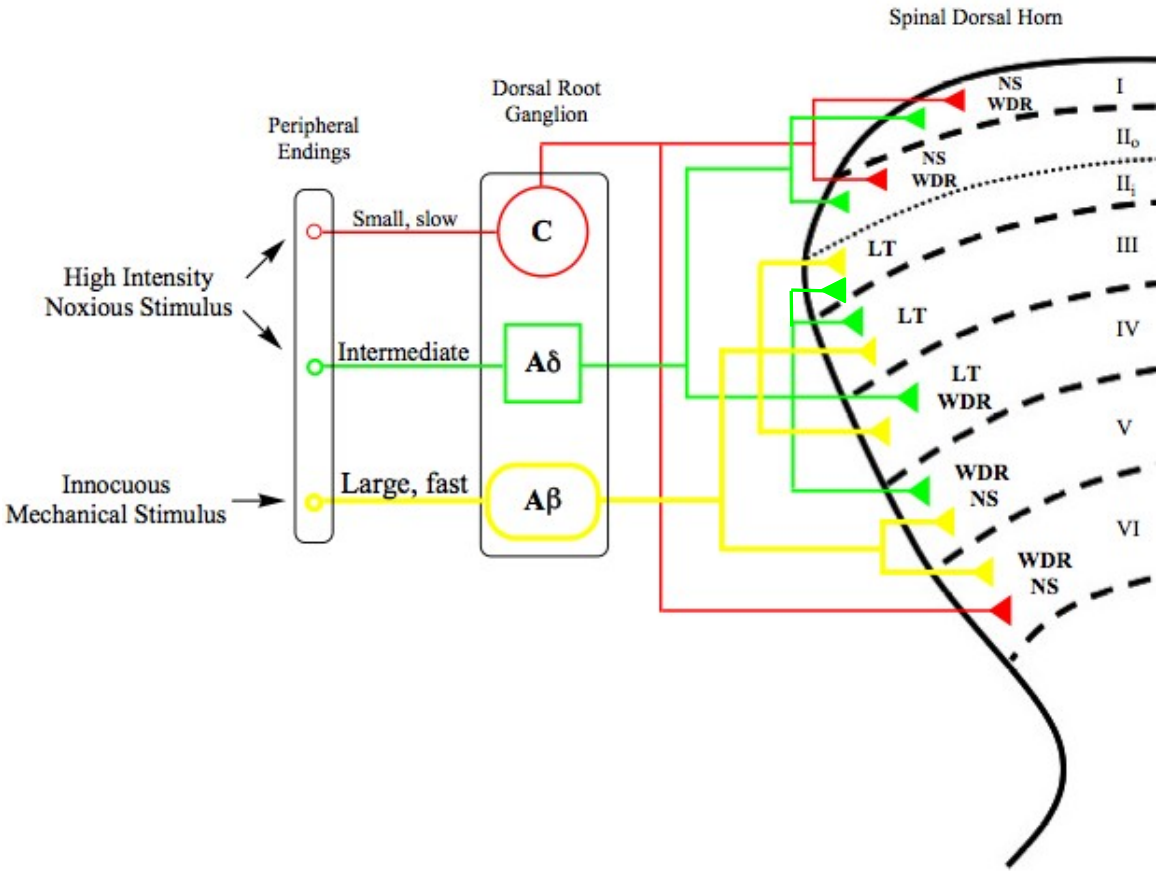



**Figure 1. 2: Schematic of neuronal populations in the dorsal horn and innervating peripheral afferents.**

Primary afferents innervate the dorsal horn in a size and modality-dependent manner, such that nociceptive afferents preferentially innervate superficial laminae and low threshold afferents terminate in the deeper dorsal horn. Cell type is not lamina-specific, and is mainly dependent on afferent input. NS: nociceptive specific neurons, which respond to Aδ and C fibre input; LT: low threshold neurons are excited by innocuous touch and brush transmitted through Aβ and Aδ fibres; WDR: wide dynamic range neurons are responsive to both noxious and innocuous stimuli applied to their cutaneous receptive fields. I, $II_o$, $II_i$ etc refer to dorsal horn laminae. Aβ thickly myelinated fibres are represented by a thick yellow line, thinly myelinated Aδ fibres by green, and unmyelinated C fibres by a thin red line. Figure modified from (Millan, 1999).

Cutaneous information from peripheral afferents is transmitted to supraspinal sites both by direct monosynaptic inputs between projection neurons and Aδ and C fibre terminals and indirect polysynaptic inputs from excitatory interneurons (Price *et al.*, 1978b; Dubner & Bennett, 1983; Ruda *et al.*, 1986; Willis & Coggeshall, 1991; Coggeshall & Carlton, 1997). Excitatory interneurons are therefore involved in the transmission of innocuous touch and brush mediated by Aβ fibres to neurons in the superficial dorsal horn. Neurons in the deeper dorsal horn receive direct monosynaptic input from Aβ fibre afferents, and wide dynamic range and

nociceptive specific neurons in lamina V are primarily thought to receive nociceptive information via excitatory interneurons found in lamina II (Dubner & Bennett, 1983; Light & Kavookjian, 1988; Todd *et al.*, 1994; Coggeshall & Carlton, 1997), although some direct monosynaptic input from C fibres has been demonstrated (De Koninck *et al.*, 1992).

Whereas excitatory interneurons and projection neurons form an integrated network involved in the transmission of nociceptive information from primary afferents to higher centres, inhibitory interneurons in conjunction with descending inhibitory influences are involved in the fine modulation and limitation of this flow of information, and are activated in parallel to excitatory interneurons by nociceptive Aδ, C fibres and by innocuous Aβ primary afferents (Zhou *et al.*, 2007, 2008). Inhibition through interneurons is achieved both presynaptically by acting directly onto primary afferents, and postsynaptically by inhibition of target wide dynamic range or projection neurons (Schmidt, 1971; Willis & Coggeshall, 1991; Todd & Spike, 1993; Malcangio & Bowery, 1996; Todd *et al.*, 1996; Wu *et al.*, 2010). This allows for tight control of incoming signals from the periphery to the spinal cord (feedback inhibition), as well as control on the transmission of information to higher centres (feedforward inhibition), both of which are crucial to dampen excitability of the spinal cord, encourage sensory discrimination and allow for fine tuned responses to noxious input.

## 1.3 Local inhibitory circuits in the adult dorsal horn

The influence of inhibitory interneurons on spinal sensory processing can be revealed experimentally by using pharmacological blockade of inhibitory receptors. Spinal application of $GABA_A$ or glycine receptor antagonists results in an expansion of the receptive field size of dorsal horn neurons and significantly increases neuronal firing to cutaneous stimuli (Sivilotti & Woolf, 1994; Sorkin & Puig, 1996; Drew *et al.*, 2004; Kawamata *et al.*, 2005). These neuronal effects are mirrored in behaving animals, which consequently display signs of pathological pain states including hyperalgesia, whereby perception of a noxious stimulus is heightened, and touch-evoked allodynia, during which a normally innocuous stimulus is perceived as

noxious (Beyer *et al.*, 1985; Yaksh, 1989; Sherman & Loomis, 1996; Sorkin & Puig, 1996).

Inhibition in the adult mammalian spinal cord is mediated primarily by the amino acid transmitters glycine and GABA, released from both intrinsic interneurons and descending fibres (Curtis *et al.*, 1967, 1968; Reichling & Basbaum, 1990; Kato *et al.*, 2006). Both GABA and glycine are expressed in neurons throughout the spinal cord (Aprison & Werman, 1965; Curtis & Watkins, 1965; Davidoff *et al.*, 1967; Aprison *et al.*, 1969). These have been shown to be extensively co-transported and co-released, resulting in a dual-component inhibitory synaptic current composed of a fast glycinergic phase and a slow $GABA_A$-receptor mediated phase (Game & Lodge, 1975; Todd & Sullivan, 1990; Baba *et al.*, 1994; Yoshimura & Nishi, 1995; Todd *et al.*, 1996). Exogenous iontophoresis of GABA or glycine onto the dorsal horn decreases the activity of interneurons throughout superficial and deep laminae across species (Curtis *et al.*, 1967, 1968; Werman *et al.*, 1968; Zieglgansberger & Herz, 1971), effects of which can be prevented through application of the $GABA_A$ receptor antagonist bicuculline or the glycine receptor antagonist strychnine (Curtis *et al.*, 1969; Game & Lodge, 1975; Sivilotti & Woolf, 1994; Yoshimura & Nishi, 1995; Sorkin & Puig, 1996; Sorkin *et al.*, 1998; Narikawa *et al.*, 2000).

Glycinergic and GABAergic inhibition in the dorsal horn is thought to occur via three main routes (see Figure 1. 3 and (Zeilhofer, 2005)): firstly, afferent fibres are thought to excite inhibitory interneurons locally in the dorsal horn as well as lead to indirect GABA and glycine release (Narikawa *et al.*, 2000; Zhou *et al.*, 2007, 2008); secondly, inhibitory descending control from the brainstem can directly inhibit target dorsal horn neurons (Antal *et al.*, 1996; Zeilhofer *et al.*, 2005); and thirdly, descending control from the brainstem can serve to excite inhibitory interneurons, resulting in polysynaptic inhibitory control (Tambeli *et al.*, 2003).

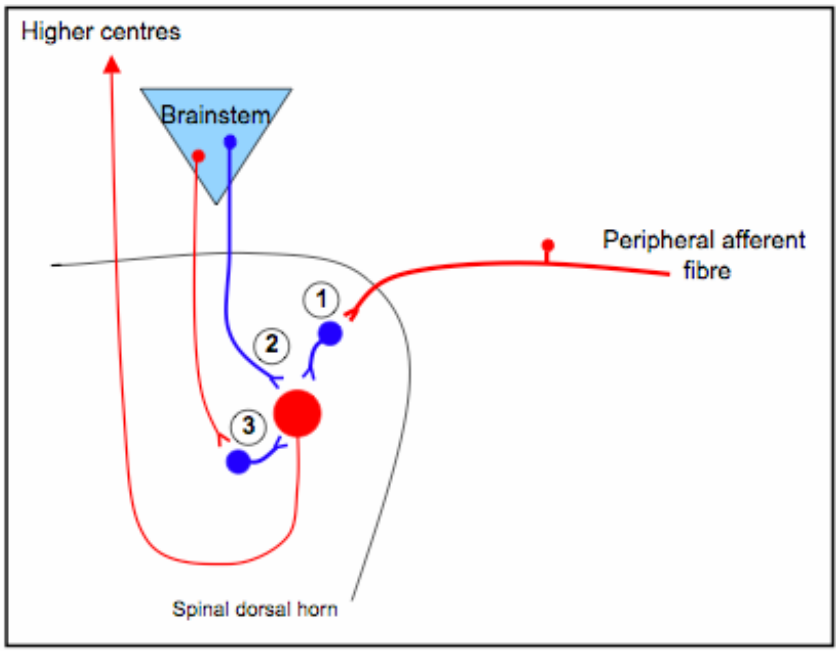



**Figure 1. 3: Three modes of GABAergic and glycinergic inhibition of dorsal horn neurons.**
Target neurons in the spinal dorsal horn can be inhibited by three means: 1) afferent primary afferent fibres excite spinal inhibitory interneurons, which synapse with, and release GABA and/or glycine onto, local target neurons; 2) descending inhibitory fibres from the brainstem synapse directly onto target neurons in the spinal cord to inhibit firing; 3) descending excitatory fibres inhibit target neurons indirectly through activation of local inhibitory interneurons. Blue indicates inhibitory signals, red indicates excitatory transmission. Modified from Zeilhofer 2005 (Zeilhofer, 2005).

### 1.3.1 GABA and its receptors

GABA has been shown to be present in approximately a third of all interneurons in laminae I, II and III of the dorsal horn (Barber *et al.*, 1982; Todd & McKenzie, 1989; Carlton & Hayes, 1990). It is found throughout the spinal cord along with its synthetic enzyme glutamic acid decarboxylase (GAD), although it is most concentrated in the superficial laminae (Curtis & Watkins, 1965; Graham & Aprison, 1969; McLaughlin *et al.*, 1975). GABA exerts its inhibitory actions through three receptor subtypes: ligand-gated anion channels $GABA_A$ and $GABA_C$ receptors and the metabotropic guanine nucleotide-binding protein (G-protein) coupled $GABA_B$ receptor.

### *1.3.1.1 $GABA_A$ receptors*

The $GABA_A$ receptor is a heteropentameric ligand-gated anion channel permeable to both chloride and bicarbonate anions (Curtis & Watkins, 1965; Bormann, 1988; Sivilotti & Nistri, 1991; Mehta & Ticku, 1999). It is known to have seven subunit subtypes (α, β, γ, δ, ε, η, and θ) each of which has multiple isoforms (Olsen & Tobin, 1990; Burt & Kamatchi, 1991; Macdonald & Olsen, 1994; De Blas, 1996). Receptor stoichiometry appears to shift according to CNS location but in the adult spinal cord is preferentially in a 2α:2β:1γ conformation (Ma *et al.*, 1993; Rudolph & Mohler, 2004).

$GABA_A$ receptors are generally categorised according to their sensitivity to benzodiazepines, conferred primarily by the α subunit of the receptor. This subunit also confers unique binding characteristics and channel kinetics (McKernan *et al.*, 1991; Rudolph & Mohler, 2004; Wafford *et al.*, 2004). Immunohistochemical and radioligand studies have demonstrated the presence of $GABA_A$ receptors throughout the dorsal horn, with particular emphasis in lamina I-III (Ribeiro-Da-Silva & Coimbra, 1980; Sur *et al.*, 1995; Alvarez *et al.*, 1996; Bohlhalter *et al.*, 1996; Todd *et al.*, 1996). Similarly, neurons under tonic GABAergic control are found in lamina I-VI, with peaks in laminae $II_o$ and V (Cronin *et al.*, 2004).

$GABA_A$ receptor-mediated inhibition occurs both on the presynaptic terminal of primary afferents and at the postsynaptic membrane of target cells. At the postsynaptic site, binding of GABA to $GABA_A$ receptors results in the opening of the anion channel. This allows the flow of chloride ions down their electrochemical gradient (Curtis & Watkins, 1965), which subsequently hyperpolarises the cell and prevents action potential firing. Presynaptically, GABA-binding to the receptor results in depolarisation known as primary afferent depolarisations (PADs) due to the high intracellular chloride concentrations of primary afferent terminals (Sung *et al.*, 2000). This depolarisation acts as a shunt by preventing further excitation and inhibiting the release of the excitatory neurotransmitters glutamate and substance P (Rudomin & Schmidt, 1999; Ishikawa *et al.*, 2000).

### *1.3.1.2 $GABA_B$ receptors*

$GABA_B$ receptors mediate slow inhibitory postsynaptic currents. These receptors are coupled to potassium and calcium ionotropic channels via G-proteins, which in turn activate second messenger systems within the cell (Sivilotti & Nistri, 1991; Bowery, 1993; Bowery *et al.*, 2002; Bettler *et al.*, 2004). $GABA_B$ receptors are composed of three known subunits ($GABA_{B1a}$, $GABA_{B1b}$ and $GABA_{B2}$), found in several brain regions including the thalamic nuclei, cerebellum and cerebral cortex, as well as in the spinal dorsal horn, where they are located on small primary afferent fibre terminals (Price *et al.*, 1987; Malcangio & Bowery, 1996; Ataka *et al.*, 2000; Towers *et al.*, 2000).

The mechanisms underlying $GABA_B$ receptor mediated presynaptic inhibition of primary afferent terminals and that of postsynaptic target neurons differs. Presynaptically, $GABA_B$ receptor activation prevents calcium influx into the cell through inhibition of calcium channels. In this way they can function as heteroreceptors by preventing release of other neurotransmitters such as glutamate and substance P and as autoreceptors, by preventing the release of GABA and so limiting their own activation (Price *et al.*, 1987; Lüscher *et al.*, 1997; Ataka *et al.*, 2000; Iyadomi *et al.*, 2000; Schuler *et al.*, 2001; Bowery *et al.*, 2002). $GABA_B$ receptor activation on the postsynaptic membrane acts to open $K^+$ channels, resulting in hyperpolarisation (Lüscher *et al.*, 1997).

### *1.3.1.3 $GABA_c$ receptors*

Much like $GABA_A$ receptors, $GABA_C$ receptors are GABA-gated chloride ion channels, however, unlike the heteromeric composition of $GABA_A$ receptors, $GABA_C$ receptors are homomeric and composed exclusively of ρ-subunits (Chebib & Johnston, 2000). The two anion channel GABA receptors can be readily distinguished on the basis of their channel properties and pharmacology: the mean channel open time for $GABA_C$ receptors is longer, and they are insensitive to the $GABA_A$ receptor antagonist bicuculline, whilst being 7 times more sensitive to GABA (Feigenspan & Bormann, 1994; Darlison & Albrecht, 1995).

$GABA_C$ receptors are found primarily in the retina, but ρ subunits have also been located in discrete areas of the brain and throughout the spinal cord, both on interneurons and motoneurons (Enz *et al.*, 1995; Rozzo *et al.*, 2002). The full functional role of these receptors has yet to be elucidated.

### 1.3.2 Glycine and its receptor

Glycine is the second major inhibitory neurotransmitter in the adult central nervous system after GABA and is involved in the processing of sensory information and motor control. Expression studies using radioactive tritiated strychnine and glycine have demonstrated the presence of glycine receptors throughout the spinal grey matter and brainstem, with decreasing expression at more rostral regions in the central nervous system (Zarbin *et al.*, 1981; Araki *et al.*, 1988). Glycine and glycine receptor immunoreactivity are found at high levels in the ventral and dorsal horns of the spinal cord. Within in the dorsal horn these are primarily located in lamina III-V with a small population of neurons in lamina I (Aprison & Werman, 1965; Aprison *et al.*, 1969; Ribeiro-Da-Silva & Coimbra, 1980; Zarbin *et al.*, 1981; Araki *et al.*, 1988; Basbaum, 1988; van den Pol & Gorcs, 1988; Todd, 1990; Todd & Sullivan, 1990; Lynch, 2004; Zeilhofer *et al.*, 2005).

#### *1.3.2.1 Glycine receptor*

The glycine receptor is a heteropentameric ion channel permeable to chloride and shares many structural characteristics of the nicotinic acetylcholine receptor. Unlike $GABA_A$ receptor-mediated inhibition, glycine receptor-mediated inhibition is primarily postsynaptic (Mitchell *et al.*, 1993; Todd *et al.*, 1996). When activated, the channel serves to increase chloride conductance in the postsynaptic membrane leading to hyperpolarisation and decreased excitability. Fos immunoreactivity following administration of the glycine receptor antagonist strychnine revealed that neurons under tonic glycine inhibition are located in the deep dorsal horn laminae III-V (Cronin *et al.*, 2004).

The glycine receptor is composed of α and β subunits, arranged around a central pore; studies have shown evidence for both a 3α:2β (Becker *et al.*, 1988; Kuhse *et al.*, 1993), and more recently 2α:3β stoichiometry (Grudzinska *et al.*, 2005). The α subunit of the glycine receptor, confers channel kinetics and pharmacology, whilst the β subunit allows anchoring of the receptor to the membrane through binding of the auxiliary structure protein gephyrin (Triller *et al.*, 1985; Schmitt *et al.*, 1987; Betz *et al.*, 2006). To date there are four known α subunit isoforms, named $\alpha_1$-$\alpha_4$ (Grenningloh *et al.*, 1990; Kuhse *et al.*, 1990; Matzenbach *et al.*, 1994) and a single β subunit isoform.

#### *1.3.2.2 Glycinergic inhibition of sensory information in the adult spinal cord*

The extent of the role of glycine in sensory transduction *in vivo* can be examined by making use of the natural alkaloid strychnine, a glycine receptor-specific antagonist (Curtis *et al.*, 1968). As mentioned above, the laminar distribution of GABA and glycine differ significantly: whereas GABA is primarily located in the superficial dorsal horn, glycine is found in deeper laminae III-V (Aprison & Werman, 1965; Aprison *et al.*, 1969; Ribeiro-Da-Silva & Coimbra, 1980; Zarbin *et al.*, 1981; Araki *et al.*, 1988; Basbaum, 1988; van den Pol & Gorcs, 1988; Todd, 1990; Todd & Sullivan, 1990; Puskár *et al.*, 2001; Lynch, 2004; Zeilhofer *et al.*, 2005). As a consequence of this, there is a certain level of modality-specificity for each inhibitory neurotransmitter: GABAergic interneurons in laminae I-II will receive predominantly nociceptive afferent input through Aδ and C fibres, whilst glycinergic interneurons in laminae III-V will receive innocuous input from Aβ fibres as well as indirect input from Aδ and C fibres (see Anatomical organisation of peripheral terminals above). Consistent with this, several studies have reported that strychnine administration results in a low threshold-specific disinhibition: treated animals show increased neuronal firing to innocuous hair deflection, mirroring responses normally only evoked by noxious stimulation in naïve animals (Yokota *et al.*, 1979; Sherman & Loomis, 1994; Sivilotti & Woolf, 1994; Sherman & Loomis, 1996; Sorkin & Puig, 1996). This is further supported by genetic studies in which the gene encoding the glycine receptor has been altered; these have shown that mice with deficits in

glycinergic transmission display extreme sensitivity to touch (White & Heller, 1982). Strychnine-induced touch-evoked allodynia is insensitive to morphine, unlike that seen following administration of $GABA_A$ receptor antagonists (Sherman & Loomis, 1994), suggesting independent mechanisms of disinhibition between both receptors.

These results have been replicated at the structural and functional cellular level in the dorsal horn. Electron microscopy and *in vivo* patch-clamp studies provide evidence of direct monosynaptic low threshold myelinated input onto glycinergic neurons in the dorsal horn (Todd, 1990; Narikawa *et al.*, 2000) and *in vivo* extracellular and *in vitro* patch clamp studies have revealed a polysynaptic protein kinase C gamma (PKC-γ)-mediated excitatory pathway between low-threshold sensitive neurons in laminae III-IV and nociceptive circuits in laminae I-II, that is normally under strong glycinergic inhibitory control in the mature spinal cord (Sorkin & Puig, 1996; Baba *et al.*, 2003; Torsney & MacDermott, 2006; Miraucourt *et al.*, 2007).

## 1.4 Descending modulation of adult dorsal horn circuits

Early studies by Sherrington were amongst the first to show the importance of supraspinal descending control onto spinal networks, where transection of the spinal cord of adult cats resulted in a significant increase in flexion reflex withdrawal (Sherrington & Sowton, 1915). This finding held true in non-motor circuits, thoracic cold block interrupting descending influences onto spinal dorsal horn neurons lead to increased excitability and larger receptive fields of spinal dorsal horn neurons, most notably of those located in the deeper dorsal horn (Wall, 1967). Although both studies revealed a tonic descending inhibitory control over spinal circuits, the true origins of this modulation were unclear. This remained the case until 1969 when Reynolds identified the periaqueductal grey (PAG) as a major site of descending control (Reynolds, 1969). Inhibition as a result of PAG stimulation was strong enough that when stimulated, would result in sufficient analgesia to enable abdominal surgery on live rats without the need for any additional anaesthesia. PAG-mediated control of spinal nociception has since been shown to act primarily via projections to the rostroventral medulla (RVM) and ablation or blocking of the RVM is sufficient to inhibit the analgesic effects of PAG stimulation (Gebhart *et al.*, 1983;

Prieto *et al.*, 1983; Sandkuhler & Gebhart, 1984; Chung *et al.*, 1987). Although these early studies focussed primarily upon descending inhibition from the brainstem, descending control from this area is in fact biphasic: high intensity electrical or chemical stimulation results in spinal inhibition or antinociception, whereas low intensity stimulation produces facilitation of nociception at the spinal level (Ren *et al.*, 1990; Zhuo & Gebhart, 1997). This adaptable system allows for heightened sensory perception and discrimination in periods of rest, whilst allowing flow of information from higher centres to influence the perception of nociceptive stimuli at the spinal levels when active or in a stressful environment.

### 1.4.1 Anatomy of descending pathways

Descending modulation of spinal nociceptive signalling has been shown to arise from stimulation of a multitude of brain centres, including the thalamus, sensory cortex, amygdala, and multiple areas in the midbrain and brainstem (Gebhart, 2004; Fields *et al.*, 2006; Heinricher *et al.*, 2009). These act primarily through polysynaptic input to the dorsal horn via the RVM and other brainstem components. The PAG integrates information from areas such as the hypothalamus, amygdala, and cerebral cortex as well as ascending information from projection neurons in the spinal dorsal horn (Menetrey *et al.*, 1982; Aggleton *et al.*, 1992; Bandler & Keay, 1996). Consequently, not only does descending modulation involve a range of brain regions, but the multitude of neurotransmitters released can also have multiple actions on dorsal horn neurons in the spinal cord. Whereas some projections and neurotransmitters can be broadly categorised into having a generally inhibitory or excitatory action at the level of the dorsal horn, the end effects of these on spinal nociceptive processing is far from straightforward. For one, descending axons can synapse onto both primary and descending afferents, as well as directly onto spinal neurons, resulting in pre and postsynaptic control (Grudt *et al.*, 1995; Almeida *et al.*, 1996; Urban *et al.*, 1996a; Urban *et al.*, 1996b; Urban & Gebhart, 1997; Zhuo & Gebhart, 1997; Millan, 1999; Baba *et al.*, 2000; Yoshimura & Furue, 2006). The phenotype of targeted spinal neurons will also influence the outcome of descending modulation. For instance, excitatory influences from the brainstem could act to inhibit nociceptive processing if acting upon inhibitory interneurons in the dorsal horn, whilst if they synapse onto

projection or excitatory interneurons, these would enhance sensory transmission. Furthermore, a single neurotransmitter can both facilitate and inhibit local transmission according to the receptor activated at the spinal level. An example of such a transmitter is serotonin, which can produce antinociception when acting on the 5-$HT_{1A}$ receptors, or algesia when acting upon of 5-$HT_3$ receptors, depending on receptor location.

Direct projections from the PAG to the spinal cord are limited and direct monosynaptic control of dorsal horn circuits arises from a few select centres; the most prominent of these are the RVM, parabrachial nucleus, dorsal reticular nucleus (DRN), ventrolateral medulla (VLM), and projections from the locus coeruleus, A5 and A7 cell groups of the dorsolateral pontine tegmentum (DLPT). Axons of spinally projecting neurons from the brainstem are located the dorsolateral funiculus (DLF) and ventrolateral funiculus (VLF) and generally project directly to spinal laminae I, II, V, VI and VII (Basbaum *et al.*, 1978; Basbaum & Fields, 1979; Bowker *et al.*, 1981; Holstege & Kuypers, 1982; Basbaum *et al.*, 1986), whereas parabrachial projections travel through the same funiculi and terminate more superficially in the dorsal horn (Yoshida *et al.*, 1997). The wider innervation of deeper neurons from the brainstem can also be observed in physiological recordings; several reports suggest wide dynamic range neurons are particularly affected by descending influences, whereas more superficially located neurons are thought to be more affected by DLF stimulation-induced descending facilitation (Fields & Basbaum, 1978; McMahon & Wall, 1988; Hudson *et al.*, 2000). Importantly, neurons within the superficial laminae also project rostrally to the brainstem, including the parabrachial nucleus and PAG. Excitation of these neurons could therefore be a result of feedforward spino-brainstem-spinal loops, or alternatively could be the result of antidromic excitation of higher brain centres in experiments in which the DLF was not cut.

### 1.4.2 Neurotransmitter networks involved in descending control

There are a wealth of neurotransmitter systems involved in descending control of spinal dorsal horn neurons. These can either be intrinsic to brainstem structures and released into the spinal cord to act upon their respective receptors, or they can act to

indirectly modulate nociception through activation or inhibition of another spinal neurotransmitter system. A brief summary of a few of those of interest is covered in the following section and shown in Figure 1. 4. For clarity, focus is primarily on neurotransmitters released in the spinal cord, which modulate nociception locally within the dorsal horn.

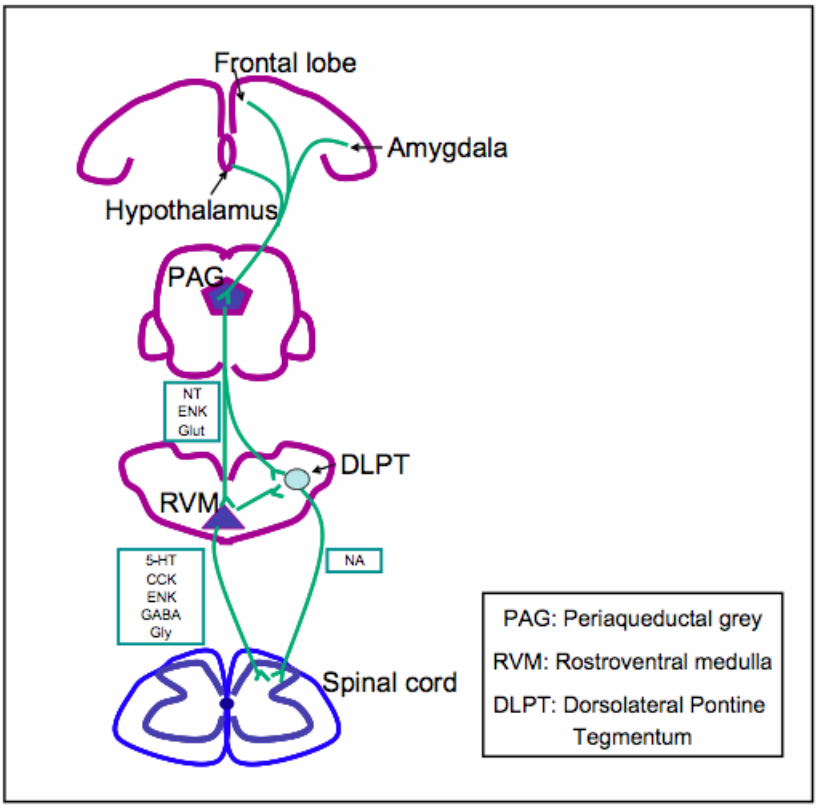



**Figure 1. 4: Simplified schematic of descending pathways that influence spinal dorsal horn neurons and neurotransmitter systems involved.**

PAG: periaqueductal grey; DLPT: dorsolateral pontine tegmentum; RVM: rostroventral medulla; NT: neurotensin; ENK: enkephalin; Glu: glutamate; 5-HT: serotonin; CCK: Cholecystokinin; Gly: glycine.

### *1.4.2.1 Opioids*

Classical opioid receptors include κ, μ, and δ opioid receptors, with recent addition of a fourth subtype, ORL1. These are present throughout the CNS, particularly in the amygdala, PAG, RVM, DLPT and spinal dorsal horn (Mansour *et al.*, 1995; Darland *et al.*, 1998). As is the case of many of the inhibitory neurotransmitters already covered in this Chapter, opioid-mediated analgesia occurs through both presynaptic

and postsynaptic mechanisms. Presynaptic inhibition, notably of glutamatergic neurons, occurs through inhibition of neurotransmitter release achieved primarily through the activation of μ and δ opioid receptors located on primary afferent and descending terminals (Glaum *et al.*, 1994; Grudt & Williams, 1994). Postsynaptically, opioids directly inhibit spinal dorsal horn neurons; this may play an especially important role in the inhibition of transmission of nociceptive primary afferent input to projection neurons in lamina I (Bennett & Mayer, 1979; Light & Kavookjian, 1988; Fields *et al.*, 2006).

Spinal iontophoresis of opioids inhibits firing of nociceptive dorsal horn neurons (Fleetwood-Walker *et al.*, 1988), a phenomenon that is particularly clear in lamina II, where μ-opioid receptors are highly concentrated (Duggan *et al.*, 1976). Although the vast majority of opioid terminals in the dorsal horn arise from local spinal interneurons (Hokfelt *et al.*, 1979), these do not account for the full analgesic effects of systemic opioids *in vivo*. In fact, the degree of neuronal inhibition in laminae I, II and V as a result of stimulation of the PAG or RVM is comparable to that seen as a result of systemic morphine administration (Gogas *et al.*, 1991; Hammond *et al.*, 1992). Further to this, microinjection of opioid antagonists into the RVM or PAG alone reduces the effects of systemically administered opioids (Kiefel *et al.*, 1993). Many of the brain regions involved in spinal analgesia appear to be linked through release of opioids, as evidenced by the reduction in stimulation produced analgesia by intrathecal administration of the opioid antagonist naloxone (Zorman *et al.*, 1982; Aimone *et al.*, 1987). Similarly, microinjection of opioid antagonists into the RVM will attenuate the antinociceptive effects of PAG-stimulation (Kiefel *et al.*, 1993; Roychowdhury & Fields, 1996).

#### *1.4.2.1.1 Cholecystokinin and Neurotensin*

There are a large number of cholecystokinin receptor-containing neurons in both the PAG and RVM, and these are often located in membranes of endogenous opioid-containing neurons (Skinner *et al.*, 1997). Cholecystokinin is therefore thought to act as an antagonist to opioid-mediated analgesia both at spinal and supraspinal levels (Mitchell *et al.*, 1998; Heinricher *et al.*, 2001) by acting through the $CCK_2$ receptor

(Crawley & Corwin, 1994). In support of this, the analgesic effects of opioids can be enhanced in the presence of $CCK_2$ antagonists (Crawley & Corwin, 1994).

The RVM receives strong neurotensinergic projections from the PAG, (Beitz, 1982) and similar to the situation described above with cholecystokinin, neurotensin has been found to be extensively co-expressed with opioids. The effects of neurotensin are heavily dose-dependent, such that low doses of neurotensin microinjected into the RVM produce hyperalgesia, whereas high doses exert analgesia (Smith *et al.*, 1997; Urban & Gebhart, 1997).

### *1.4.2.2 Serotonin*

The RVM includes the nucleus raphe magnus (NRM), nucleus gigantocellularis pars alpha and nucleus paragigantocellularis lateralis, of which the NRM contains a large number of serotonin-rich neurons (Steinbusch, 1981), comprising of around 20% of total neurons in the RVM (Moore, 1981). Electrical stimulation of the RVM has been shown to result in a spinal release of serotonin; the analgesic effects of this stimulation can be reversed by intrathecal administration of serotonin antagonists (Le Bars & Villanueva, 1988). Serotonin itself has a role in dampening of cellular responses to nociceptive stimuli in the spinal dorsal horn, and the RVM is the only source of this release. Release has been shown to occur both tonically and phasically, implicating serotonin in the maintenance of excitatory/inhibitory balance in the spinal cord (Mason, 1997; Gao *et al.*, 1998).

The complexity of the serotonergic system arises from the multiplicity of 5-HT receptors through which the neurotransmitter can act, each of which can result in either inhibition or facilitation of transmission. $5\text{-HT}_1$ and $5\text{-HT}_2$ receptors are involved in direct inhibition of nociceptive processing in the superficial dorsal horn; serotonin-mediated analgesia is thought to occur in part through $5\text{-HT}_{1A}$ and $_{1D}$ receptor subtypes located on the primary afferent terminals of C fibres (Potrebic *et al.*, 1994; Ito *et al.*, 2000). Ionotropic $5\text{-HT}_3$ receptors on the other hand, have a more complex role in nociception. They have been shown to be involved in indirect inhibition through excitation of inhibitory interneurons (Alhaider *et al.*, 1991) and in

facilitation of nociception, primarily exerted through 5-$HT_3$ receptors located on nociresponsive neurons or on primary afferent terminals (Todd & Millar, 1983). In support of this last action, 5-$HT_3$ receptor antagonism has been shown to minimise pain behaviour in rats (Oyama *et al.*, 1996; Zeitz *et al.*, 2002). Notably, descending serotonergic fibres are thought to contribute to opioid-induced analgesia, in part through direct inhibition of projection neurons via 5-$HT_{5A}$ receptors (Doly *et al.*, 2004) as well as contribute to the transmission of noxious information to higher centres via activation of projection neurons (Conte *et al.*, 2005).

#### *1.4.2.3 Noradrenaline*

The DLPT is the major source for noradrenergic projections to both the RVM and the dorsal horn (Proudfit, 1992). Spinal release of noradrenaline results in behavioural analgesia and inhibition of dorsal horn neurons through activation of $\alpha_2$ receptors. Whilst this system does not appear to be active under resting conditions, noradrenergic pathways have been shown to play a role in hyperalgesia and neuropathic pain (Xu *et al.*, 1999; Jasmin *et al.*, 2003). Noradrenergic activity can be either facilitatory or inhibitory depending on the pathology, which could be a function of receptor subtype activation as noradrenaline acting through $\alpha_1$ receptors tends to facilitate nociception, whereas noradrenaline acting upon presynaptic $\alpha_2$ receptors inhibits glutamate release from C fibres onto lamina II neurons leading to analgesia (Roudet *et al.*, 1994; Stone *et al.*, 1998; Pan *et al.*, 2002; Yoshimura & Furue, 2006). The $\alpha_2$ agonists clonidine and dexmedetomidine have been shown to significantly decrease dorsal horn firing to noxious stimuli and are also successfully used as analgesics in clinical practice (Sullivan *et al.*, 1992; Takano & Yaksh, 1992; Eisenach, 1996; Eisenach *et al.*, 1996; Millan *et al.*, 1997; Yoshimura & Furue, 2006).

Importantly, the DLPT and RVM are interconnected and the DLPT also receives direct afferents from the PAG (Bajic & Proudfit, 1999). These three brain regions can therefore all act in concert to affect nociceptive processing at the spinal level (Clark & Proudfit, 1991; Holden & Proudfit, 1998), indeed stimulation of the RVM has been shown to result in a spinal release of noradrenaline. In a similar fashion,

descending inhibition arising from the RVM or PAG can be attenuated by intrathecally administered noradrenergic antagonists (Yaksh, 1979; Barbaro *et al.*, 1985; Budai *et al.*, 1998).

### *1.4.2.4 Other neurotransmitters*

#### *1.4.2.4.1 Dopamine*

The lumbar region of the spinal cord contains only very few dopaminergic cell bodies (Mouchet *et al.*, 1986) although all three dopamine receptor subtypes ($D_1$, $D_2$, and $D_3$) have been identified within the superficial dorsal horn (Yokoyama *et al.*, 1994). Spinal dopaminergic innervation arises primarily from cerebral structures, particularly the hypothalamus (Millan, 2002) terminations from which are found throughout the dorsal horn. Although the full influence of dopamine in nociceptive processing is not yet clear, it is thought that $D_1$ receptors may be involved in pronociception, whilst $D_2$ receptors are most likely antinociceptive (Jensen & Smith, 1982; Jensen & Yaksh, 1984; Fleetwood-Walker *et al.*, 1988; Gao *et al.*, 2001b).

#### *1.4.2.4.2 GABA and Glycine*

Stimulation of the PAG does not only act to inhibit spinal neurons, it has also been shown to result in facilitation of a population of neurons in the superficial dorsal horn, a region known to contain a high number of inhibitory GABAergic and glycinergic interneurons (Millar & Williams, 1989; Todd *et al.*, 1996). Stimulation produced analgesia is therefore an effect of both direct inhibition of primary afferent fibres or projection/excitatory neurons and indirect inhibition through activation of inhibitory interneurons. Similarly, RVM stimulation results in a release of GABA and glycine in the dorsal horn (Sorkin *et al.*, 1993) and the RVM is known to contain a large number of both GABAergic and glycinergic inhibitory neurons, which synapse directly onto spinal excitatory neurons, some of which may also be projection neurons (Antal *et al.*, 1996; Kato *et al.*, 2006).

### 1.4.3 Physiology of the RVM

The biphasic response of RVM stimulation on dorsal horn neuronal firing has been shown to be a result of the physiological circuitry within its structure. The RVM contains three distinct cell types first identified by their firing responses to noxious reflex behaviour: 'ON' cells, 'OFF' cells and 'NEUTRAL' cells (Fields & Heinricher, 1985; Fields *et al.*, 1991; Heinricher *et al.*, 2009). 'On' cells begin firing just prior to a mechanical withdrawal of tail or paw from a noxious stimulus, and so are thought to be pro-nociceptive. Conversely, 'off' cells stop firing just before mechanical withdrawal, and so are thought to be anti-nociceptive. The third group of cells, 'neutral' cells, do not appear to respond to noxious stimulation of cutaneous tissue (Fields & Heinricher, 1985; Fields *et al.*, 1991; Heinricher *et al.*, 2009). A significant portion of each cell group projects to the spinal cord and both 'on' and 'off' cells respond to manipulation of the PAG.

Since the initial identification of these cell groups, their classification has been extended beyond their responses to mechanical reflex withdrawals. Thus, cells are now often discussed in terms of their general responses to noxious stimuli in the anaesthetised animal, in the absence of obvious behaviour. Ongoing 'on' cell activity has been associated with a number of persistent pain states: manipulation in order to cause activation of this cell group in the absence of pathology results in behavioural hyperalgesia and increases response to subsequent noxious stimuli (Heinricher *et al.*, 1989; Ramirez & Vanegas, 1989; Bederson *et al.*, 1990; Foo & Mason, 2003). Interestingly, some reports suggest that 'on' cells are not selective for nociception, as they respond to brisk touch or loud noises (Oliveras *et al.*, 1990; Leung & Mason, 1999). These could therefore be involved in the general enhancement of vigilance to behaviourally relevant or important stimuli (Fields *et al.*, 2006; Heinricher *et al.*, 2009).

'Off' cells have been proven to be important mediators of the analgesic effects of the RVM in that activation of this subset of neurons is sufficient to produce behavioural analgesia (Heinricher & Tortorici, 1994). Similarly, inhibition of 'off' cells leads to widespread hyperalgesia (Ramirez & Vanegas, 1989; Foo & Mason, 2003) and prevents the analgesic actions of systemic opioid administration (Roychowdhury &

Heinricher, 1997; Heinricher *et al.*, 2001). The mechanisms behind opioid analgesia appear to be in the opioidergic sensitivity of different classes of RVM neurons. Microinjection of opioids into the PAG or RVM selectively silences 'on' cells whilst rendering 'off' cells tonically active (Heinricher & Tortorici, 1994; Heinricher *et al.*, 2001; Nalwalk *et al.*, 2004). Whereas the fine detail of activation within each cell type is unclear when using the relatively large volumes of drug delivery via microinjections, the particulars of cell-selective opioidergic sensitivity have been assessed by iontophoresis of opioid agonists onto cells of each class (Heinricher *et al.*, 1992; Heinricher & Tortorici, 1994; Fields, 2004). 'On' cells are postsynaptically inhibited by opioids but surprisingly these drugs do not directly affect either 'off' or 'neutral' cells. GABA is known to preferentially inhibit 'off' cells in the RVM, thereby favouring facilitation of nociception (Heinricher & Tortorici, 1994; Thomas *et al.*, 1995; Bajic *et al.*, 2001; Gilbert & Franklin, 2001). The activation of 'off' cells in response to microinjection of opioids is therefore most probably an indirect consequence of opioid-mediated inhibition of GABAergic interneurons that normally act to silence discharging 'off' cell activity. This in turn results in tonically active 'off' cells and antinociception.

Interestingly, 'neutral' cells appear to have a different afferent input to either 'on' or 'off' cells, as they are not affected by either PAG stimulation or microinjection of opioids (Gao *et al.*, 1997; Gao *et al.*, 1998). *In vivo*, all recorded serotonergic cells within the RVM have been shown to be 'neutral' cells (Potrebic *et al.*, 1994; Gao & Mason, 2000) and although there is extensive literature on the analgesic and algesic effects of serotonin ((Suzuki *et al.*, 2002) and see above), 'neutral' cells have not as of yet been assigned to a specific role in nociceptive processing.

The balance of 'on' and 'off' cell activity at any given time is therefore a major determinant in the nociceptive thresholds and overall activity of spinal nociceptive circuits. Pharmacologically induced allodynia and hyperalgesia also result in an increase in c-fos positive neurons in the RVM (Hall *et al.*, 1999) indicating a strong feedforward loop onto spinal dorsal horn neurons.

## 1.5 The development of sensory networks in the postnatal period

The discussion so far has illustrated the critical importance for a correct equilibrium between excitation and inhibition in the mature spinal dorsal horn. This balance arises from both local excitatory and inhibitory interneurons within the dorsal horn, and descending control from supraspinal centres. Inhibition in the adult forms a crucial part of survival, yet there is growing evidence that some inhibitory processes are lacking in the neonate such that newborn rats and humans have exaggerated reflexes in response to noxious stimuli compared to mature counterparts (Fitzgerald *et al.*, 1987; Andrews & Fitzgerald, 1994; Andrews *et al.*, 2002; Fitzgerald, 2005). Experimentally, the rat is often used as a model of human development as it is born in a relatively premature state. At postnatal day 3 (P3), neonatal rats correspond developmentally to a 26-35 postconceptional week old premature human infant, whereas P21 rats are considered to be adolescents (McCutcheon & Marinelli, 2009). Rats can thus provide insight into the mechanisms behind the development of inhibitory circuitry from what would be a premature human infant to the fully independent adult. Detailed studies outlined below have provided evidence for a lack of both segmental and descending inhibition, resulting in increased sensitivity of spinal dorsal horn neurons to innocuous Aβ fibre stimulation and lower mechanical thresholds in the immature rat. The development of sensory networks in this chapter will therefore be covered in two parts: (i) the role of local spinal inhibition in the dorsal horn through development, with particular emphasis on local glycinergic inhibition; and (ii) the development of descending controls from the brainstem.

## 1.6 Spinal sensory networks and the development of spinal sensory circuitry

Over the early postnatal period, refinement of sensory networks and motor reflexes occurs through Hebbian strengthening and weakening of synapses. One major consequence of this is an inherent excitability of immature networks than is not normally found in the healthy adult. The developing spinal cord therefore has a much higher sensitivity to incoming cutaneous sensory signals. Functionally, this can be observed at three levels as outlined below.

### *1. Cutaneous reflexes are exaggerated and poorly tuned at birth*

Dorsal root stimulation evokes significant intersegmental spinal activity in the isolated neonatal spinal cord that then decrease in magnitude with increasing age (Saito, 1979). Behaviourally this is seen as synchronised whole body movements in response to a noxious stimulus to the hindpaw of a neonatal rat, as opposed to the tuned reflex withdrawal of the single affected limb seen in adults (Schouenborg, 2003). Threshold needed to elicit a reflex withdrawal are also more sensitive, such that not only is withdrawal elicited in response to a much weaker stimulus relative to the mature system, but neonatal rats can also be sensitised to innocuous stimuli, a feature absent in healthy adults (Jennings & Fitzgerald, 1998). This sensitivity has been shown to be centrally mediated, as thresholds for activating cutaneous primary afferents have been observed to be similar in both the neonate and in the adult (Fitzgerald, 1988). Tuning of withdrawal responses is slow, in the rat this occurs after the first postnatal week, whilst in the human infant this occurs at 29-35 postconceptional age (Ekholm, 1967; Fitzgerald *et al.*, 1988; Andrews & Fitzgerald, 1994, 1999; Andrews *et al.*, 2002).

### *2. Reflexes are inappropriately directed in the neonate*

In addition to the exaggerated response to noxious stimuli, there is a high incidence of misdirected reflexes consistent with disorganised receptive fields in the early postnatal period. One study showed that neonatal rats tended to move their tails towards a noxious laser more often than they would form a directed movement away from the stimulus, a feature that lasted until the second postnatal week (Waldenstrom *et al.*, 2003). Interestingly, this was found to be a selective low threshold activity-dependent process. Age-related decrease in error-rate was delayed if low-threshold tactile stimuli to the tail were blocked by local anaesthetics, yet noxious stimuli did not speed up this learning.

### *3. Activity of neonatal dorsal horn neurons mirrors behaviour*

The neonatal behavioural sensitivity described above is equally seen at the level of individual dorsal horn neurons: these are found to have larger receptive fields, lower mechanical thresholds and respond with prolonged afterdischarges in response to

noxious stimuli (Fitzgerald, 1985; Fitzgerald, 1988; Torsney & Fitzgerald, 2002). These responses mature slowly with age and are the result of a range of postnatal developmental alterations in peripheral and descending afferent input and synaptic strengthening, each of which will be covered in the following sections.

### 1.6.1 Anatomical changes in primary afferent central terminals over development

#### *1.6.1.1 C fibres*

C fibres enter the grey matter at E18-20 (Mirnics & Koerber, 1995; Jackman & Fitzgerald, 2000) and recordings from polymodal nociceptors have revealed mature firing patterns and thresholds from birth (Fitzgerald, 1985; Koltzenburg & Lewin, 1997; Koltzenburg *et al.*, 1997). Terminal fields are somatotopically arranged such that C fibres reach their adult terminations in lamina I-II from the outset (Scott, 1982; Fitzgerald, 1987a). Non-peptidergic IB4+ C fibres mature later than peptidergic C fibres and their terminals are not detectable in the dorsal horn until P5 (Fitzgerald & Swett, 1983; Fitzgerald, 1987c; Benn *et al.*, 2001).

#### *1.6.1.2 A fibres*

Low threshold myelinated A fibres penetrate the grey matter at embryonic day 15-17, several days before C fibres (Mirnics & Koerber, 1995; Jackman & Fitzgerald, 2000); these do not however settle into their adult terminal fields until later in postnatal life (Fitzgerald, 1985). A subset of neonatal A fibres, visible at the electron microscopic level, have been shown to grow beyond their adult terminations to terminate in the superficial dorsal horn (Coggeshall *et al.*, 1996; Torsney *et al.*, 2000; Beggs *et al.*, 2002; Woodbury & Koerber, 2003). These are thought to be of a subtype that is not found in the adult and are sensitive to pressure (Fitzgerald, 1987b; Woodbury *et al.*, 2001; Woodbury & Koerber, 2003). Fibres withdraw from the superficial dorsal horn by P21 to terminate in their adult laminar pattern in laminae III and IV, resulting in a significant period of overlap of both low threshold A and

nociceptive C fibre terminals in the superficial dorsal horn until the third postnatal week. The withdrawal of these A fibre terminals is activity-dependent, thus chronic blockade of dorsal horn NMDA receptors over the postnatal period prevents the withdrawal of A terminals from lamina II (Beggs *et al.*, 2002). More recently, it has been suggested that raising rat pups in a 'sensory noisy' environment, by placing the housing cage on a shaker, can also prevent the arrangement of terminals, highlighting the critical role of touch in determining adult sensory networks (Granmo *et al.*, 2008).

### 1.6.2 Development of central afferent input

#### *1.6.2.1 C fibres*

Although peripheral C fibre terminals are present from birth, the maturation of C fibre synapses takes place slowly over a number of postnatal weeks. A lower percentage of neurons in the neonate respond to noxious stimulation of the receptive field than in the adult, and C fibres input into the dorsal horn does not appear to be fully functional until the end of the first week of life (Jennings & Fitzgerald, 1998). Similarly, electrical stimulation of C fibres fails to evoke synchronised bursts of spikes in both dorsal and ventral spinal horn *in vivo* and *in vitro* (Fitzgerald *et al.*, 1987; Hori & Watanabe, 1987; Fitzgerald, 1988; Jennings & Fitzgerald, 1998; Fitzgerald & Jennings, 1999). Importantly however, *in vitro* slice work has revealed that neurons can physiologically react to topically applied capsaicin from birth (Baccei *et al.*, 2003), demonstrating the presence of functional C fibres that are presumably unable to respond in a mature synchronised manner to C fibre afferent input *in vivo*. This is possibly the result of unsynchronised presynaptic neurotransmitter release, which requires stronger and more sustained stimulation to adequately and reliably discharge in response to C fibre-mediated noxious input. The activity of C fibres in the early postnatal period is, however, critical in the organisation of sensory circuits of the dorsal horn. Chemical destruction of C fibres using the neurotoxin capsaicin in early life has been shown to prevent the withdrawal of A fibres from the superficial dorsal horn, decrease descending inhibition from the brainstem, and result in disorganised somatotopic maps in the adult (Wall *et al.*,

1982; Cervero & Plenderleith, 1985; Shortland *et al.*, 1990; Zhuo & Gebhart, 1994; Beggs *et al.*, 2002).

### *1.6.2.2 A fibres*

Myelinated A fibre evoked responses dominate until the second week of postnatal development, at which point primary afferent fibres innervating lamina II shift from a predominantly Aβ fibre to predominantly Aδ and C fibre innervation (Fitzgerald, 1985; Fitzgerald, 1988; Park *et al.*, 1999; Nakatsuka *et al.*, 2000; Daniele & MacDermott, 2009). Aβ fibre input has been shown to form a greater number of monosynaptic inputs to superficial dorsal horn neurons during the first weeks of life relative to C fibre input into the same area (Park *et al.*, 1999; Nakatsuka *et al.*, 2000). As a consequence of the overlap of noxious C fibre and innocuous Aβ fibre terminals in lamina II, electrophysiological experiments and c-fos expression studies have demonstrated that neonatal dorsal horn neurons respond to innocuous low threshold input in a manner normally expected of noxious stimuli in the adult (Fitzgerald, 1985; Jennings & Fitzgerald, 1996; Torsney & Fitzgerald, 2002). Innocuous tactile information therefore appears to be key in enabling the strengthening of synapses in the neonatal dorsal horn. Behaviourally this has been clearly shown in two experiments: (i) inhibiting the transmission of cutaneous tactile input by means of a local anaesthetic block to the tail over a critical ten day period was sufficient to prevent the development of organised withdrawal responses to a noxious stimulus (Waldenstrom *et al.*, 2003) and (ii) enhancing the transmission of tactile afferent input by means of a shaking cage was sufficient to permanently alter spinal organisation of Aβ terminals (Granmo *et al.*, 2008). The same authors have also postulated a link between spontaneous twitching of newborn infants and the strengthening of sensory pathways (Petersson *et al.*, 2003).

| Neonatal dorsal horn | Adult dorsal horn |
|---|---|
| Anatomical and electrophysiological evidence for large myelinated A fibre terminals in lamina II in the neonatal dorsal horn, leading to significant overlap of A and C fibre terminals in the superficial dorsal horn (Fitzgerald *et al.*, 1994; Mirnics & Koerber, 1995; Coggeshall *et al.*, 1996; Park *et al.*, 1999; Nakatsuka *et al.*, 2000). | Aβ fibre terminals are located in the deep dorsal horn, and lamina II contains C fibre terminals only (Willis & Coggeshall, 1991). |
| Low threshold A fibre stimulation of the peripheral cutaneous receptive field results in c-fos activation of lamina II neurons and electrophysiological responses similar to that expected of noxious stimulation in the adult (Fitzgerald, 1985; Jennings & Fitzgerald, 1996; Torsney & Fitzgerald, 2002). | C-fos expression is selectively evoked by nociceptive stimulation in the healthy adult spinal cord (Hunt *et al.*, 1987; Ma & Woolf, 1996). |
| Lack of C fibre-evoked postsynaptic activity until the second postnatal week, with merely 13% of neurons in the dorsal horn responding to C fibre stimulation at P10 (Fitzgerald, 1988; Jennings & Fitzgerald, 1998) | 32% of dorsal horn neurons respond to C fibre neurons by P21 (Jennings & Fitzgerald, 1998). |
| Repeated A fibre stimulation causes prolonged afterdischarge action potential firing *in vivo* in 33% of P6 spinal dorsal horn neurons (Jennings & Fitzgerald, 1998) | Central sensitisation is evoked by C fibres only in the healthy adult spinal cord from P21 (Mendell & Wall, 1965; Woolf *et al.*, 1988; Woolf & Thompson, 1991; Thompson *et al.*, 1993; Jennings & Fitzgerald, 1998). |

**Table 1. 1: Summary of the evidence for relative A and C fibre input in the neonatal and adult spinal dorsal horn.**

### 1.6.3 Spinal inhibition in the neonatal CNS

The sensory qualities of neonatal circuits are suggestive of an imbalance between excitation and inhibition at the spinal level. Whereas there is significant developmental tuning of excitatory neurotransmission in first week of life (Pattinson & Fitzgerald, 2004; Fitzgerald, 2005), the intrinsic excitability of dorsal horn neurons at this period does not differ from that of adult neurons (Baccei *et al.*, 2003) and the influence of late development of inhibitory synapses is becoming increasingly evident (Fitzgerald, 2005; Baccei, 2007).

The spinal cord develops along a ventrodorsal axis: motor neurons are generated first and neurons in the more superficial layers are the last to mature just prior to birth (Altman & Bayer, 1984; Kitao *et al.*, 1996). Inhibitory interneurons within the

superficial dorsal horn develop after the establishment of projection neurons in lamina I (Bice & Beal, 1997a, b). These do not fully mature until the fourth postnatal week, as *in vitro* patch clamp studies have revealed a five fold higher frequency of mini excitatory postsynaptic potentials (mEPSCs) in neonatal lamina I projection neurons compared to firing of interneurons at the same age (Dahlhaus *et al.*, 2005). Interneurons therefore undergo significant maturation in the postnatal period, including an increased axodendritic spread; a larger spread would then allow for broader spread of inhibitory transmission in later life and finer tuning of excitatory inputs into the spinal cord. This could in turn lead to fine tuned responses and tighter receptive fields in the adult as described in the sections above.

Both GABA and glycine are present in the spinal cord from the early embryonic period (Ma *et al.*, 1992; Poyatos *et al.*, 1997). They show significant co-localisation, co-release and co-transmission from this time until P23, after which time postsynaptic events are either GABAergic or glycinergic, but not both (Berki *et al.*, 1995; Keller *et al.*, 2001). *In vitro* experiments have shown that, contrary to early reports, neither GABA nor glycine are excitatory at any stage in the postnatal period (Baccei & Fitzgerald, 2004; Holmgren *et al.*, 2009; Rheims *et al.*, 2009). In fact, *in vivo* extracellular recordings of neonatal rat dorsal horn neurons in the presence of $GABA_A$ receptor antagonism have shown that GABA acts as a fully functional inhibitor of activity at this stage (Bremner *et al.*, 2006) whilst further developmental *in vitro* patch clamp studies suggest that the net inhibitory effect of $GABA_A$-receptor activation may be even stronger in the early postnatal period due to slower decay kinetics of mini inhibitory postsynaptic currents (mIPSCs; (Keller *et al.*, 2004)), leading to longer inhibitory events. Although these studies have defined a role for GABAergic signalling, no studies have as of yet determined the role of glycine at this early stage of postnatal development in the intact animal, nor is it known if it can fully function as an inhibitory neurotransmitter from birth. The lack of inhibitory control at this time could therefore be as a result of inefficient glycinergic signalling.

### *1.6.3.1 Developmental expression of glycine receptor subunits*

One striking feature of the maturation of inhibitory neurotransmission in the dorsal horn is that inhibition in the early postnatal period is mediated primarily by GABAergic networks, with little or no involvement of glycine until the end of the first postnatal week (Baccei & Fitzgerald, 2004). This is consistent with immunohistochemical analyses: the percentage of dorsal horn neurons that are positive for GABA increases steadily between the embryonic period and postnatal day 14 (P14), after which time this number decreases to reach a plateau by P21 (Schaffner *et al.*, 1993). Surprisingly, *in vitro* intracellular recordings of lamina II neurons has shown that although there is an absence of glycinergic mIPSCs at birth, exogenous glycine application onto neonatal spinal slices reveals inward currents indicative of functional glycine receptors at this age. Two possible reasons for this are (i) receptors, although functional, are not synaptically located, and so may not be able to respond to endogenous release of glycine, and/or (ii) presynaptic glycine is not released at sufficient levels to activate postsynaptic receptors. Of particular note regarding the first point therefore, is the developmental shift in glycine receptor conformation over the postnatal period.

### *1.6.3.2 $\alpha_1$ and $\alpha_2$ subunits*

Over the first two postnatal weeks, $\alpha_1$ and $\alpha_2$ subunit expression are inversely correlated: $\alpha_2$ subunit expression decreases and expression is shifted towards $\alpha_1$-containing heteromeric receptors by P20 (Becker *et al.*, 1988; Akagi *et al.*, 1991; Malosio *et al.*, 1991; Watanabe & Akagi, 1995). The immature $\alpha_2$-containing receptor is thought to be expressed as an $\alpha_2$ homomer in absence of the β subunit (Becker *et al.*, 1988; Malosio *et al.*, 1991), which is involved in binding the membrane anchoring protein gephyrin (Meyer *et al.*, 1995; Kneussel *et al.*, 1999). As a result of this, neonatal homomeric receptors display different cellular expression patterns to the adult heteromeric receptors: whereas heteromeric α/β receptors are predominantly expressed at postsynaptic sites, homomeric $\alpha_2$ receptors are primarily located extrasynaptically (Takahashi *et al.*, 1992; Kneussel & Betz, 2000a; Mangin *et al.*, 2003). The lack of anchoring of the glycine receptor to synapses could

therefore help explain the lack of glycinergic mIPSC early in development as previously mentioned (Baccei & Fitzgerald, 2004). This shift in subunit expression also has functional significance in the inherent receptor signalling; neonatal $\alpha_2$ homomeric receptors have been shown to have slower decay kinetics and longer opening times compared to adult $\alpha_1$-containing heteromer (Takahashi *et al.*, 1992; Mangin *et al.*, 2003) and the switch in subunit expression seems to correlate with the acceleration of glycinergic IPSCs. The full functional relevance of this switch on the intact nervous system is however unclear, not least due to the lack of phenotype of $\alpha_2$ knock-out mice (Young-Pearse *et al.*, 2006).

#### *1.6.3.3 $\alpha_3$ subunit*

The $\alpha_3$ subunit has been shown to be directly implicated in the development of inflammatory pain in the adult spinal cord; its expression pattern is unique in that it is found exclusively in lamina III of the superficial dorsal horn, consistent with its role in nociception (Harvey *et al.*, 2004). Although early studies of developmental regulation of $\alpha_3$ subunit expression found no postnatal change (Malosio *et al.*, 1991) a more recent study using $\alpha_3$ subunit knock-out mice found an increasing importance in the role of the $\alpha_3$ subunit in inhibitory processing over the first three weeks of postnatal life (Rajalu *et al.*, 2009), suggesting an important early role in nociceptive processing.

Thus, in the early postnatal period there is a well defined shift in inhibitory phenotype of spinal dorsal horn neurons from a primarily GABAergic driven inhibition, to a larger influence of glycinergic transmission by the second postnatal week (Baccei & Fitzgerald, 2004). Although GABA is a fully functional inhibitory neurotransmitter in the spinal dorsal horn from birth (Baccei & Fitzgerald, 2004; Bremner *et al.*, 2006) neonatal dorsal horn neurons are known to display a characteristic excitability visible both at the behavioural and cellular levels (Fitzgerald *et al.*, 1988; Jennings & Fitzgerald, 1996; Fitzgerald & Jennings, 1999; Torsney & Fitzgerald, 2002; Fitzgerald, 2005). Of particular importance is the inherent sensitivity of these neurons to A fibre stimuli, to which they can become sensitised in a manner not seen in the adult (Fitzgerald, 1988; Jennings & Fitzgerald,

1996; Fitzgerald, 2005). As glycine is selective for the transmission of Aβ fibre mediated innocuous touch stimuli in the adult ((Yokota *et al.*, 1979; Sherman & Loomis, 1994; Sivilotti & Woolf, 1994; Sherman & Loomis, 1996; Sorkin & Puig, 1996) and see 1.3.2.2) this raises the possibility that the apparent lack of inhibition within the neonatal spinal dorsal horn is due to the late maturation of glycinergic signalling and circuitry in the first weeks of life. This theory is further supported by the lack of glycinergic inhibition in the superficial dorsal horn of neonatal spinal slices (Baccei & Fitzgerald, 2004). As of yet however, the molecular correlates of this observation, and the functional significance of this lack of glycinergic inhibition on neuronal activity in the intact animal have not been investigated.

## 1.7 Postnatal development of descending modulation

It is clear that there is significant postnatal development of dorsal horn circuitry; yet several reports indicate developmental changes are also taking place at supraspinal sites. Surprisingly, whereas extracellular recordings from individual neurons revealed an inhibitory role for GABA at the level of the dorsal horn (Bremner *et al.*, 2006), intrathecal administration of the $GABA_A$ receptor antagonist gabazine in behaving neonatal rats was found to increase mechanical reflex withdrawal thresholds, suggesting GABA was acting as an excitatory neurotransmitter at this age (Hathway *et al.*, 2006). This effect was completely reversed upon removal of supraspinal control onto spinal networks by spinalisation, indicating that although GABA is inhibitory at the spinal level from birth, a tonic supraspinal facilitation at this age that is absent in the adult reverses this GABAergic inhibition in the unanaesthetised behaving animal (Hathway *et al.*, 2006).

Anatomical studies have revealed that descending serotonergic fibres innervate the spinal grey matter from birth (Rajaofetra *et al.*, 1989) but these do not reach their adult termination patterns until P21 (Bregman, 1987). As a consequence of this, spinal application of a serotonergic agonist before P10 does not significantly reduce formalin-induced pain responses in rats (Giordano, 1997). Although this implies a postnatal maturation in serotonergic receptor expression, other reports also imply delayed descending inhibition to be the result of an insufficient release of serotonin

from descending fibre terminals (Abbott & Guy, 1995). Descending noradrenergic fibre terminations, conversely, mature relatively early and $\alpha_2$ agonists are analgesic from the early postnatal period (Hughes & Barr, 1988; Kendig *et al.*, 1991; Walker *et al.*, 2005).

The influence of descending control is not apparent until the second postnatal week: whereas spinalisation in the adult results in massive spinal shock, this procedure has little influence on naïve neonatal rats until P10 (Weber & Stelzner, 1977). Similarly, stimulation produced analgesia is not functional until the third postnatal week (van Praag & Frenk, 1991) and electrical stimulation of the dorsolateral funiculus does not result in inhibition of peripherally-evoked spinal neuronal activity until the end of the second week of life (Fitzgerald & Koltzenburg, 1986). Whereas much of this previous work has focussed on the absence of inhibition, recent work using electromyographic recordings has suggested that descending control from the brainstem exerts a facilitatory effect on spinal nociceptive networks until P28 (Hathway *et al.*, 2009) suggestive of a shift in supraspinal control of spinal circuits from solely excitatory to biphasic in the fourth postnatal week. The nature of these experiments does however depend on motor function and muscle contraction, which are tightly linked to nociceptive neuronal responses such that EMG responses could be the result of non-nociceptive specific motor effects of RVM stimulation. The role of descending control on developmental nociceptive spinal networks has yet to be defined.

## 1.8 Summary

There are a significant number of postnatal modifications in the spinal processing of cutaneous sensory information, all of which lead to a defined shift in neuronal and behavioural responses to sensory stimuli. These changes can be classified into local effects within the spinal dorsal horn, and alterations in the supraspinal control of spinal sensory networks.

Within the first few weeks of life, spinal circuitry undergoes a significant shift as a result of maturing primary afferent terminal innervation, from a primarily A fibre driven excitation of lamina II dorsal horn neurons, to a largely C fibre mediated excitation. With this shift in afferent terminal patterning comes a shift in the behavioural responses of the rat: in the first week of life when A fibres dominate in the superficial dorsal horn, animals display extreme sensitivity to innocuous A fibre stimulation. Once C fibre central synapses strengthen, A fibres begin to withdraw from the superficial dorsal horn to reside in the deeper dorsal horn, at which time neuronal and behavioural responses begin to refine and sensitivity to A fibre stimuli subsides ((Coggeshall *et al.*, 1996; Jennings & Fitzgerald, 1996; Beggs *et al.*, 2002; Fitzgerald, 2005) and see Sections 1.6.1 and 1.6.2). GABAergic inhibition in the spinal cord is known to be mature by birth and the absence of tonic glycinergic activity in lamina II is suggestive of immature glycinergic circuitry and signalling (Baccei & Fitzgerald, 2004; Bremner *et al.*, 2006), which could underlie the sensitivity of neonatal spinal neurons to low-threshold stimuli. This hypothesis is further strengthened by the selective glycinergic inhibition of innocuous Aβ fibre mediated excitation of adult sensory spinal circuits ((Yokota *et al.*, 1979; Sherman & Loomis, 1994; Sivilotti & Woolf, 1994; Sherman & Loomis, 1996; Sorkin & Puig, 1996) and see Section 1.3.2.2). Importantly however, previous studies do not offer clues as to whether the lack of glycinergic activity seen in whole cell patches is due to immature presynaptic circuitry or whether there are developmental changes in postsynaptic receptor expression. Additionally, the functional role of glycine in the processing of sensory stimuli has not yet been examined in the intact animal.

Although descending fibres have reached the lumbar spinal cord before birth (Rajaofetra *et al.*, 1989), supraspinal control of spinal sensory networks is slow to

mature and has been shown in electromyographic recordings to produce a primarily facilitatory drive onto spinal networks until P28 (Hathway *et al.*, 2009), at which time brainstem control can be inhibitory or facilitatory (Urban & Gebhart, 1997; Zhuo & Gebhart, 1997; Gebhart, 2004). Interestingly, the fourth postnatal week also corresponds to the strengthening of C fibre terminals in the superficial dorsal horn, a process which is thought to lead to the maturation of descending inhibitory signals (Cervero & Plenderleith, 1985; Zhuo & Gebhart, 1994). Studies investigating the maturation of descending influences thus far have focussed on behavioural or electromyography recordings of immature and adult rats, which have the disadvantage of the involvement of motor control as outcome measures, making it difficult to assess the purely sensory and/or nociceptive role of descending control in the early postnatal period.

## 1.9 Aims of thesis

The aims of this thesis were therefore three fold:

(i) to map out the developmental expression patterns of glycinergic innervation and receptor expression in the spinal dorsal horn.

(ii) to examine the functional effects of glycinergic inhibition in individual dorsal horn neurons of P3 and P21 rats *in vivo* using the glycine receptor antagonist strychnine.

(iii) to assess the role of descending RVM control on spontaneous and peripherally-evoked activity of spinal dorsal horn neurons in the dorsal horn of P21 and P40 rats.

# Chapter 2

# Expression and Functional Mapping of Glycinergic Terminals and Receptors in the Spinal Dorsal Horn

## 2.1 Introduction

### 2.1.1 The glycine receptor

On a cellular level the actions of GABA and glycine appear similar. Although they are often co-released and co-transmitted (Todd & Sullivan, 1990; Todd *et al.*, 1996), their spinal regional expression and that of their receptors differ significantly. Immunohistochemical and electrophysiological studies have revealed dorsal horn segmentation in neurotransmitter receptor expression as well as presynaptic terminal endings. GABAergic inputs are located primarily in the superficial dorsal horn laminae I-III, whilst glycine and glycine receptor immunoreactivity appear most prominently in lamina III-V, with a small population found in lamina I (Ribeiro-Da-Silva & Coimbra, 1980; Zarbin *et al.*, 1981; Araki *et al.*, 1988; Basbaum, 1988; van den Pol & Gorcs, 1988; Todd, 1990; Todd & Sullivan, 1990; Antal *et al.*, 1996; Puskár *et al.*, 2001; Cronin *et al.*, 2004; Lynch, 2004; Zeilhofer *et al.*, 2005; Kato *et al.*, 2006).

Glycine-mediated inhibition is primarily postsynaptic, unlike $GABA_A$ receptor-mediated inhibition, which has been shown to occur both at the postsynaptic membrane of local neurons, and presynaptically onto primary afferent fibres (Mitchell *et al.*, 1993; Todd *et al.*, 1996). The glycine receptor is a heteropentameric ion channel permeable to chloride and belongs to the Cys-loop ligand-gated ion channel receptor family, sharing many structural characteristics with the nicotinic acetylcholine receptor. When activated, the channel serves to increase chloride conductance in the postsynaptic membrane leading to hyperpolarisation and decreased excitability. Expression studies using radioactive tritiated strychnine and glycine have shown glycine receptors to be located throughout the spinal grey matter and brainstem, with decreasing expression at more rostral regions in the central nervous system (Zarbin *et al.*, 1981; Araki *et al.*, 1988).

The glycine receptor is composed of α and β subunits arranged around a central pore; studies have shown evidence for both a 3α:2β (Becker *et al.*, 1988; Kuhse *et*

*al.*, 1993), and more recently 2α:3β stoichiometry (Grudzinska *et al.*, 2005). To date there are four known α subunit isoforms, named $\alpha_1$-$\alpha_4$ (Grenningloh *et al.*, 1990; Kuhse *et al.*, 1990; Matzenbach *et al.*, 1994) and a single β subunit isoform. The β subunit is thought to be responsible for receptor assembly and channel properties and allows membrane anchoring of the receptor through binding of the auxiliary structure protein gephyrin (Triller *et al.*, 1985; Schmitt *et al.*, 1987; Betz *et al.*, 2006). Primary isoforms of the α subunit in the adult CNS are $\alpha_1$ and $\alpha_3$. Expression of the $\alpha_2$ subunit decreases sharply after the first two weeks of postnatal life to minimal levels in the mature system and the $\alpha_4$ subunit is only present at low levels in discrete areas of the central nervous system.

### 2.1.2 Presynaptic glycine release

Glycine is packaged into vesicles by the vesicular inhibitory amino acid transporter VIAAT. Stored in high concentrations in presynaptic terminals of spinal interneurons, it is then released into the synaptic cleft upon cellular depolarisation. Termination of glycinergic transmission is achieved through its removal from the synaptic space by specific high affinity, high capacity transporters comprising of two subtypes: GlyT1, present in neurons and glial cells, and GlyT2, found exclusively in the plasma membrane of glycinergic neuron terminals (Burger *et al.*, 1991; Zafra *et al.*, 1995a; Zafra *et al.*, 1995b). Genetic knock-out studies have identified distinct roles for each transporter. GlyT1 appears to be involved in the clearing of glycine from the synaptic space, whereas the role of GlyT2 is primarily involved in enhancing glycinergic efficacy by transporting glycine from the synapse into the presynaptic terminal. Glycine can then be recycled and repackaged into vesicles for release (Gomeza *et al.*, 2003a; Gomeza *et al.*, 2003b; Rousseau *et al.*, 2008). In fact, expression of VIAAT and GlyT2 alone have been shown to be sufficient for adequate glycine accumulation and release in model systems, more so than co-expression of VIAAT and GlyT1 (Aubrey *et al.*, 2007). Unsurprisingly therefore, immunostaining studies have shown GlyT2 expression to overlap extensively with glycine immunoreactivity both synaptically and in terms of laminar distribution, proving it to be a reliable marker of glycinergic neurons (Luque *et al.*, 1994; Jursky & Nelson, 1995; Zafra *et al.*, 1995a; Zafra *et al.*, 1995b; Poyatos *et al.*, 1997; Spike

*et al.*, 1997; Betz *et al.*, 2006). Studies utilising GlyT2 staining in this way have therefore found its expression to be primarily restricted to laminae III in the mature spinal dorsal horn.

### 2.1.3 Postnatal development of glycinergic neurotransmission

In the motor system, $GABA_A$ receptors are present at threefold higher level than glycine receptors in the early developmental period (Gao & Ziskind-Conhaim, 1995). The amplitude of glycinergic mIPSCs then doubles from E17-P3 (Gao *et al.*, 2001a), indicating a postsynaptic increase in receptor expression and/or perhaps an increase in presynaptic quantal release. Similarly, postsynaptic glycinergic mIPSC frequency undergoes a developmental increase in the dorsal horn over the first three postnatal weeks despite the fact several studies have shown the anion reversal potential and pattern of inhibitory glycinergic innervation to be functionally mature by the second postnatal week (Rivera *et al.*, 1999; Keller *et al.*, 2001; Baccei & Fitzgerald, 2004). Both individual and mixed $GABA_A$ receptor and glycine receptor-mediated mIPSCs can be recorded within laminae I-II until P23, after which inhibitory postsynaptic events (i.e. detection) are either GABAergic or glycinergic in nature but never both although co-release continues well into adulthood, indicative of a postsynaptic shift in receptor expression from GABAergic and glycinergic synapses to glycine receptor-only postsynaptic sites (Chery & De Koninck, 1999; Keller *et al.*, 2001; Rajalu *et al.*, 2009).

Protein levels of GlyT2 are developmentally regulated, the protein is expressed from late foetal life and levels increase postnatally until P14, when they decrease to a plateau by P21 (Zafra *et al.*, 1995b). The influence of this on glycinergic activity and availability in the early postnatal period is not known, nor whether protein expression patterns are developmentally regulated in conjunction with the increase in protein level. A developmental shift in expression could potentially lead to inappropriately targeted glycine release in the dorsal horn and/or inefficient recycling of glycine for release onto postsynaptic neurons in the immature dorsal horn. Similarly, patterns of glycinergic terminals and receptor expression patterns are well established in the

adult spinal cord but little is known of how these patterns change over postnatal development or how this affects developing nervous networks.

## 2.2 Aims

The pattern of glycinergic inputs have been well described in the adult dorsal horn (Todd, 1990; Todd & Sullivan, 1990; Todd *et al.*, 1995; Zafra *et al.*, 1995b; Todd *et al.*, 1996; Spike *et al.*, 1997), yet little is known of how this pattern develops over the postnatal period, nor how this can impact on glycinergic transmission in the early stages of life. I aim to study the expression pattern of glycinergic terminals, their receptors and the pattern of neurons under tonic glycinergic inhibitory control in the spinal dorsal horn over the first three postnatal weeks in order to better understand the postnatal maturation of glycinergic circuitry over this period.

The experiments in this chapter were completed with the assistance of two BSc students: Geoffrey Brent and Azhaar Ashraf.

## 2.3 Methods

Animals aged P3, P10, P14, P21 and P40 were used for immunohistochemical studies. All sections are from lumbar regions 4 to 5 in the spinal cord (L4 and L5 respectively). Four animals per age group were used for each antibody unless otherwise stated and the sections with the clearest staining patterns were chosen for photos. Not all age groups were used for each antibody. P21 protein expression patterns matched those seen from P40 adult rat lumbar sections (see Figure 2. 2), thus P3 sections were used as a model for the neonatal spinal cord, and P21 sections were used as models of the mature spinal cord.

### 2.3.1 NeuN immunohistochemical staining

Initially, an antibody raised against the neuronal nucleic marker NeuN was used to compare relative spinal cord sizes of P21 and P40 spinal cords. NeuN is an antibody raised against a neuron-specific nuclear protein initially isolated from mouse brain lysates, and is located in most vertebrate postmitotic neurons from early in development, with the exception of few neuron cell types including cerebellar Purkinje cells (Mullen *et al.*, 1992; Wolf *et al.*, 1996; Sarnat *et al.*, 1998). Detailed studies since its discovery have shown NeuN to be located in areas of low chromatin and low DNA packing density (Lind *et al.*, 2005). Animals aged P21 and P40 were terminally anaesthetised with an intraperitoneal overdose of pentobarbital sodium (Euthatal, 200 mg/ml, Merial Animal Health Ltd, UK) and transcardially perfused with heparinised saline (0.5% heparin (Monoparin, CP Pharmaceuticals, UK) in 0.9% NaCl (Baxter, Belgium)) followed by 4% paraformaldehyde in distilled $H_2O$ (pH 7.4). The lumbar enlargement of the spinal cord (L4 and L5) was removed, postfixed for 2 hours and cryoprotected overnight at 4°C in 30% sucrose and azide solution. 40 µm transverse lumbar spinal cord sections were cut using a freezing microtome (Leitz Wezlar, Germany) and stored in a 0.1 M phosphate buffer (PB) solution containing 5% sucrose and 0.02% azide. Free floating sections were then blocked in 0.1 MPB solution containing 5% normal goat serum (NGS, Vector) and 0.4% Triton-X (BDH) for 1 hour. Following this, sections were incubated in primary

mouse polyclonal antibody anti-NeuN (NeuN; 1:5000; 48 hours at 4°C; MAB377, Chemicon, USA). Sections were then washed three times in 0.1M PB for 10 minutes before incubation in the secondary goat anti-mouse polyclonal antibody (1:500, 2 hours; Invitrogen, Eugene, Oregon, USA). After a further three washes in 0.1 M PB, sections were mounted onto gelatinized slides and left to dry for 15 minutes. Finally, slides were coverslipped in fluoromount (Sigma-Aldrich Inc.). Images were obtained using an Olympus confocal microscope with FluoView FV1000 scanning unit and IX81 inverted microscope and Olympus FluoView software version 2.0c (Olympus Optical Co. Ltd., Tokyo, Japan). Z stacks were compiled using Image J software (ImageJ 1.42q, National Institute of Health, USA, http://rsb.info.nih.gov/ij). Antibody specificity was shown by omitting the primary antibody to test for secondary antibody specificity, and by omitting the secondary antibody to check for any primary antibody fluorescence, both controls were negative in agreement with published work (Cavallaro *et al.*, 2008).

### 2.3.2 GlyT2 mapping of glycinergic terminals in the developing spinal cord

Animals aged P3, P10, P14, P21 and P40 were terminally anaesthetised with an intraperitoneal overdose of pentobarbital sodium (Euthatal, 200 mg/ml, Merial Animal Health Ltd, UK) and transcardially perfused with heparinised saline and 4% paraformaldehyde in distilled $H_2O$ (pH 7.4) as outlined above. The lumbar enlargement of the spinal cord was removed, postfixed for 2 hours and cryoprotected overnight at 4°C in 30% sucrose and azide solution. 40 µm transverse lumbar spinal cord sections were cut using a freezing microtome (Leitz GmbH, Wetzlar, Germany) and stored in a 0.1 M phosphate buffer (PB) solution containing 5% sucrose and 0.02% azide. Free floating sections were then blocked in 0.1 M PB solution containing 5% normal goat serum (NGS, Vector), 0.4% Triton-X (BDH) for 1 hour.

Three antibodies against GlyT2 were used in total, two commercially available and one offered by Professor Francisco Zafra's laboratory at the Universidad Autónoma de Madrid, Spain.

### *2.3.2.1 Santa Cruz anti-GlyT2 antibody*

After blocking, sections were incubated in primary rabbit polyclonal antibody anti-GlyT2 (1:10,000, 48 hours at 4°C; H-155, Santa Cruz antibodies, CA, USA) and washed three times in 0.1 M PB for 10 minutes before biotinylated secondary goat anti-guinea-pig antibody was added (1:500, 2 hours, Vector, CA, USA). Immunostaining for GlyT2 was visualised using an indirect tyramide signal amplification (TSA) protocol (Vectastain Elite ABC kit, Vector, CA, USA). Briefly, sections were incubated in 2.5% biotinylated goat anti-guinea pig in 0.1M PB containing 0.4% Triton-X for 90 minutes. After three 10 minute washes in 0.1 M PB, Vector ABC Elite solution consisting of 0.4% Vectastain A + 0.4% Vectastain B in TTBS was added to the sections. Following 30 minutes of incubation, sections were washed three times for 10 minutes in 0.1 M PB and left to incubate for 7 minutes in Vector kit diluent containing 1.33% biotinylated tyramide solution (Vector, CA, USA). A further series of three 10 minutes washes was followed by incubation in a 0.1 M PB solution containing 0.4% Triton-X (BDH Ltd) and 0.167% Fluoroscein Avidin D (FITC, Vector, CA, USA). The sections were washed again and mounted onto gelatinized slides before being left to dry for 15 minutes. Finally, the slides were coverslipped in fluoromount (Sigma-Aldrich Co., St Louis, MO, USA). Images were obtained using a Hamamatsu C4742-95 digital camera and digital controller (Hamamatsu Photonics K.K., Japan) attached to a Leica DMR microscope (Leica Microsystems GmbH, Wetzlar, Germany). Openlab 4.0.4 software was used to capture the image (Openlab, Improvision Ltd). Antibody specificity was shown by omitting the primary antibody to test for secondary antibody specificity, and by omitting the secondary antibody to check for any primary antibody fluorescence, both controls were negative in agreement with published work (Horiuchi *et al.*, 2001).

### *2.3.2.2 Chemicon anti-GlyT2 antibody*

After blocking, sections were incubated in primary rabbit polyclonal antibody anti-GlyT2 (1:10,000, 48 hours at 4°C; AB1773, Chemicon, USA) and washed three times in 0.1 M PB for 10 minutes before biotinylated secondary goat anti-guinea-pig

antibody was added (1:500, 2 hours, Vector, CA, USA). Immunostaining for GlyT2 was visualised using a TSA amplification protocol as outlined above (Vectastain Elite ABC kit, Vector, CA, USA). Sections were then mounted onto gelatinised slides and allowed to dry for 15 minutes before being coverslipped using fluoromount (Sigma-Aldrich Co., St Louis, MO, USA). Antibody specificity was shown by omitting the primary antibody to test for secondary antibody specificity, and by omitting the secondary antibody to check for any primary antibody fluorescence, both controls were negative in agreement with other studies (Lim *et al.*, 2004). Images obtained using a Hamamatsu C4742-95 digital camera and digital controller (Hamamatsu Photonics K.K., Japan) attached to a Leica DMR microscope (Leica Microsystems GmbH, Wetzlar, Germany). Openlab 4.0.4 software was used to capture the image (Openlab, Improvision Ltd).

#### *2.3.2.3 Anti-GlyT2 antibody from Professor F Zafra*

After blocking, sections were incubated in primary rabbit polyclonal antibody anti-GlyT2 (1:4000, 48 hours at 4°C, kind gift of Professor Francisco Zafra, Universidad Autónoma de Madrid, Spain) and washed three times in 0.1 M PB for 10 minutes before biotinylated secondary goat anti-rabbit antibody was added (1:500, 2 hours, Vector, CA). Immunostaining for GlyT2 was visualised using a TSA amplification protocol (Vectastain Elite ABC kit, Vector, CA, USA) as outlined above. Sections were then mounted onto gelatinised slides and allowed to dry for 15 minutes before being coverslipped using fluoromount (Sigma-Aldrich Co., St Louis, MO, USA). A pre-absorption study had been previously performed by the manufacturers of the antibody in a previously published peer reviewed paper (Zafra *et al.*, 1995a). An additional control omitting the primary antibody was performed and was found to be negative for immunostaining. Images were obtained using an Olympus confocal microscope with FluoView FV1000 scanning unit and IX81 inverted microscope and Olympus FluoView software version 2.0c (Olympus Optical Co. Ltd., Tokyo, Japan). Z stacks were compiled using Image J software (ImageJ 1.42q, National Institute of Health, U.S.A, http://rsb.info.nih.gov/ij).

For dual localisation of GlyT2 with Isolectin $B_4$, the fluorescently tagged primary antibody (1:500; IB4; Fluoroscein Griffonia Bandeiraea Simplicifolia; Vector, CA, USA) was added to the anti-GlyT2 antibody and incubated over 48 hours at 4°C. Sections were washed in 0.1 M PB three times for 10 minutes each as outlined above. Instead of tagging GlyT2 with FITC conjugated secondary antibody, an Alexa 594 streptavidin conjugate (1:500, Vector, CA, USA) was used to distinguish from the green fluorophore of the conjugated IB4 antibody. Antibody specificity was shown by omitting the primary antibody to test for secondary antibody specificity, and by omitting the secondary antibody to check for any primary antibody fluorescence, both controls were negative in agreement with published data (Fang *et al.*, 2006). Images were obtained using an Olympus confocal microscope with FluoView FV1000 scanning unit and IX81 inverted microscope and Olympus FluoView software version 2.0c (Olympus Optical Co. Ltd., Tokyo, Japan). Z stacks were compiled using Image J software (ImageJ 1.42q, National Institute of Health, U.S.A, http://rsb.info.nih.gov/ij).

The expression profile of GlyT2 in the dorsal horn of P3 and P21 rat spinal sections was analysed by measuring the distance from medial point above the central canal to the lateral edge of the spinal cord in order to include lamina V. The area selected and analysed included laminae I-V of the dorsal horn and was $1/8^{th}$ in width of the total distance between the medial and lateral edges of the dorsal horn (see Figure 2. 1 for details, 'selected area' is represented in a red box). The width of the selected area was chosen in order to incorporate the laminae without the distortion that occurs more laterally, where the laminae are more angled. The length was chosen to extend to beyond the dorsal surface so lamina I was included. This area remained constant within an age group to allow for grouping of data points thereafter. There were small differences within age groups, which were controlled for by ensuring the base of the selection was level with the medial edge of the dorsal horn. ImageJ software (ImageJ 1.42q, National Institute of Health, U.S.A, http://rsb.info.nih.gov/ij) was used to construct a "column average plot", where the x-axis represents the vertical distance through the selected area (shown in red in Figure 2. 1) and the y-axis the horizontally averaged pixel intensity throughout this section. Each graph represents seven sections chosen at random from four separate animals. Graphs were plotted using

graph using GraphPad Prism software (version 5.00, GraphPad Software, San Diego, CA, USA, www.graphpad.com)

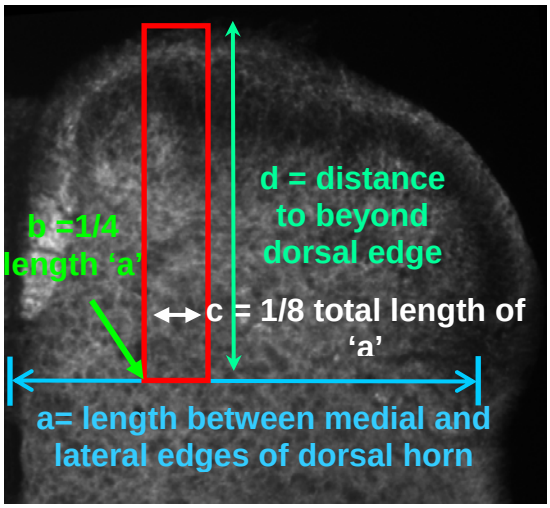



**Figure 2. 1: Diagram of measurements used to graph GlyT2 staining expression patterns.** Analysed area is shown in red, see text for details.

### 2.3.3 Developmental expression patterning of the α subunit of the glycine receptor

Animals aged P3 and P21 were terminally anaesthetised with an intraperitoneal overdose of pentobarbital sodium and transcardially perfused with heparinised saline (0.5% in NaCl) and 4% paraformaldehyde in distilled $H_2O$ as outlined above. The lumbar enlargement of the spinal cord was removed, postfixed for 2 hours and cryoprotected overnight at 4°C in 30% sucrose and azide solution. 20 µm lumbar spinal cord sections were cut using a cryostat (Bright Model OTF, 5040, Bright Instrument Company Ltd, Huntingdon, UK) and mounted directly onto SuperFrost Plus glass slides before being left to dry overnight. Slides were blocked using 10% normal goat serum solution in 0.1 M PB for 1 hour and incubated with an antibody raised against the alpha subunit of the glycine receptor (αGlyR; 1:1000 24 hours at room temperature; Santa Cruz, U.S.A). After three 10 minute washes, sections were incubated in a 0.1 M PB solution containing goat-anti rat (1:500; 2 hours; Vector, CA, USA). Slides were then washed and allowed to dry for 15 minutes before being coverslipped with fluoromount (Sigma-Aldrich Co., St Louis, MO, USA). To control for the selectivity of the antibodies used, two controls were performed: omission of

the primary antibody and omission of the secondary antibody. Both controls were found to be negative for immunofluorescence confirming the selectivity of the primary and secondary antibodies in agreement with published work (Wang *et al.*, 2009). Images were obtained using an Olympus confocal microscope with FluoView FV1000 scanning unit and IX81 inverted microscope and Olympus FluoView software version 2.0c (Olympus Optical Co. Ltd., Tokyo, Japan). Z stacks were compiled using Image J software (ImageJ 1.42q, National Institute of Health, U.S.A, http://rsb.info.nih.gov/ij).

### 2.3.4 C-fos mapping of neurons under tonic glycinergic inhibition

Animals aged 3 days (P3, n= 8) and 21 days (P21; n= 8) were anaesthetised with intraperitoneal injection of urethane (2.0-2.5 mg/kg) adjusted to allow an anaesthetic depth light enough to allow mechanical withdrawal responses to a strong pinch stimulus without full arousal from anaesthesia. Once an adequate level of anaesthesia was achieved, animals were given an intrathecal dose of either strychnine (165 ng/g; S0532, Sigma-Aldrich Co., St Louis, MO, USA; n= 4 at each age) or saline vehicle (0.9% NaCl, Baxter, Belgium; n= 4 at each age), at a volume of 2 µl for P3 rat pups and 7 µl for P21 rats. Animals were then left for 2 hours and 20 minutes in a heated recovery box. This was to account for the 20 minute time point needed for maximum strychnine response (see Chapter Three) and the 2 hours needed for maximal c-fos protein activation (see (Hunt *et al.*, 1987)). Animals were subsequently given an overdose of pentobarbital sodium (Euthatal, 200 mg/ml, Merial Animal Health Ltd, UK) and transcardially perfused with heparinised saline and 4% paraformaldehyde as outlined above. The lumbar enlargement of the spinal cord was removed, postfixed for 2 hours and cryoprotected overnight at 4°C in 30% sucrose and azide solution. 40µm transverse lumbar spinal cord sections were cut using a freezing microtome (Leitz Wetzlar, Germany) and stored in a 0.1 M PB solution containing 5% sucrose and 0.02% azide. Free floating sections were then blocked in 0.1 M PB solution containing 5% normal goat serum (NGS, Vector, CA, USA) and 0.4% Triton-X (BDH Ltd.) for 1 hour.

Sections were incubated in primary rabbit polyclonal antibody anti-c-Fos (1:10,000, 24 hours at room temperature; Calbiochem**,** La Jolla, CA) and washed three times for 10 minutes each in 0.1 M PB before a secondary goat anti-rabbit antibody was added (1:500, 2 h, Vector, CA, USA). Immunoreactivity for c-fos was visualised using the diaminobenzene amplification protocol (Vector DAB peroxidase substrate kit, Vector, CA, USA) and sections were mounted onto gelatinised slides. These were then dried overnight, dehydrated by dipping slides (2 minutes) sequentially into 70% ethanol, 95% ethanol, absolute alcohol and Histoclear (National Diagnostics, USA), before being coverslipped using DPX (VWR International Ltd, Poole, UK). To control for the selectivity of the antibodies used, two controls were performed: omission of the primary antibody and omission of the secondary antibody. Both controls were found to be negative for immunofluorescence confirming the selectivity of the primary and secondary antibodies in agreement with published work (Rodríguez & Ferrer, 2007).

The single best section from four separate animals at each age group was chosen for camera lucida drawings of cumulative cfos positive neuronal expression at both age groups. These were selected according to the level of cfos staining and quality of the tissue. Images were obtained by drawing c-fos positive neurons onto an acetate sheet using a camera lucida (Nikon Y-IDT, Nikon, Japan) and Nikon microscope (Nikon Eclipse E800, VFM, Nikon, Japan). The central canal was taken as the midway point between dorsal and ventral horns and the number of c-fos positive neurons dorsal to this were counted and plotted onto a graph using GraphPad Prism software (version 5.00, GraphPad Software, San Diego, CA, USA, www.graphpad.com). Student's t-test was performed comparing saline and strychnine treated animals within an age group using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, CA, USA, www.graphpad.com).

## 2.4 Results

### 2.4.1 NeuN staining

NeuN staining was used to compare P21 and P40 spinal cord sizes and neuronal expression patterns. These were found to be equivalent by visual examination (Figure 2. 2) and as such, P21 spinal cords were used as models for the mature spinal cord.

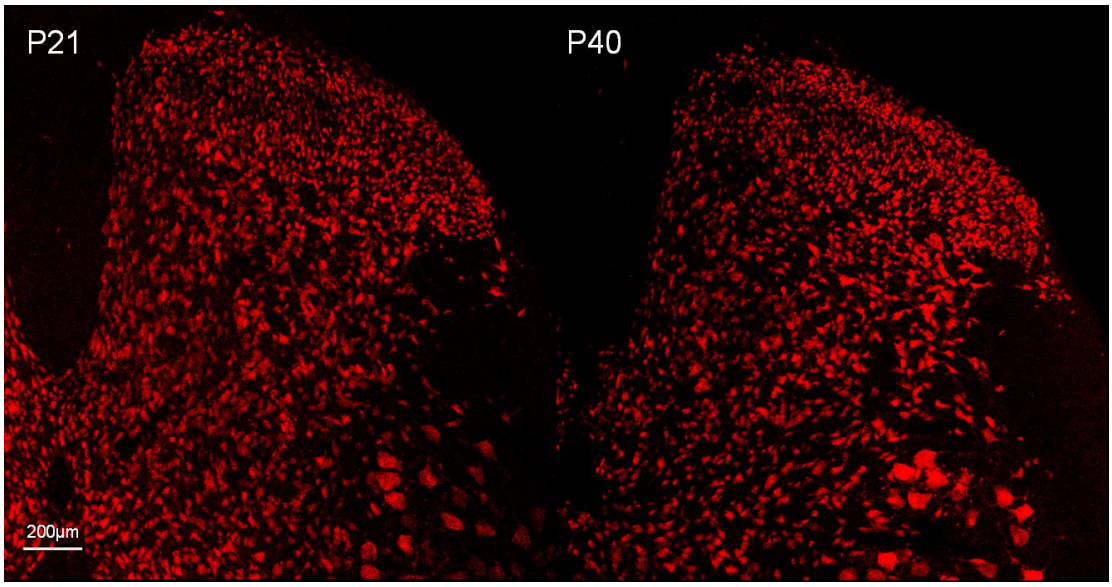



**Figure 2. 2: Representative images of NeuN staining of P21 and P40 sections.**
Pattern of neuronal staining is equivalent between the two age groups. Images were taken at 10 x magnification and scale bar is identical for both images.

### 2.4.2 GlyT2 staining

GlyT2 immunostaining was used as a marker of glycinergic terminals in order to assess the pattern of presynaptic glycinergic input in the developing spinal dorsal horn (Luque *et al.*, 1994; Jursky & Nelson, 1995; Zafra *et al.*, 1995a; Zafra *et al.*, 1995b; Poyatos *et al.*, 1997; Spike *et al.*, 1997; Betz *et al.*, 2006). Although three antibodies were tried, only the privately-acquired antibody kindly supplied by Professor Francisco Zafra's laboratory in Universidad Autónoma de Madrid, Spain offered the specificity needed and was used for the rest of the study. The antibody

raised against GlyT2 acquired from Santa Cruz appeared non-specific and resulted in immunostaining of cell bodies instead of selective staining of glycinergic terminals. Although the antibody bought through Chemicon appeared to provide the pattern expected of GlyT2 immunostaining, the background levels of fluorescence remained high (see Figure 2. 3).

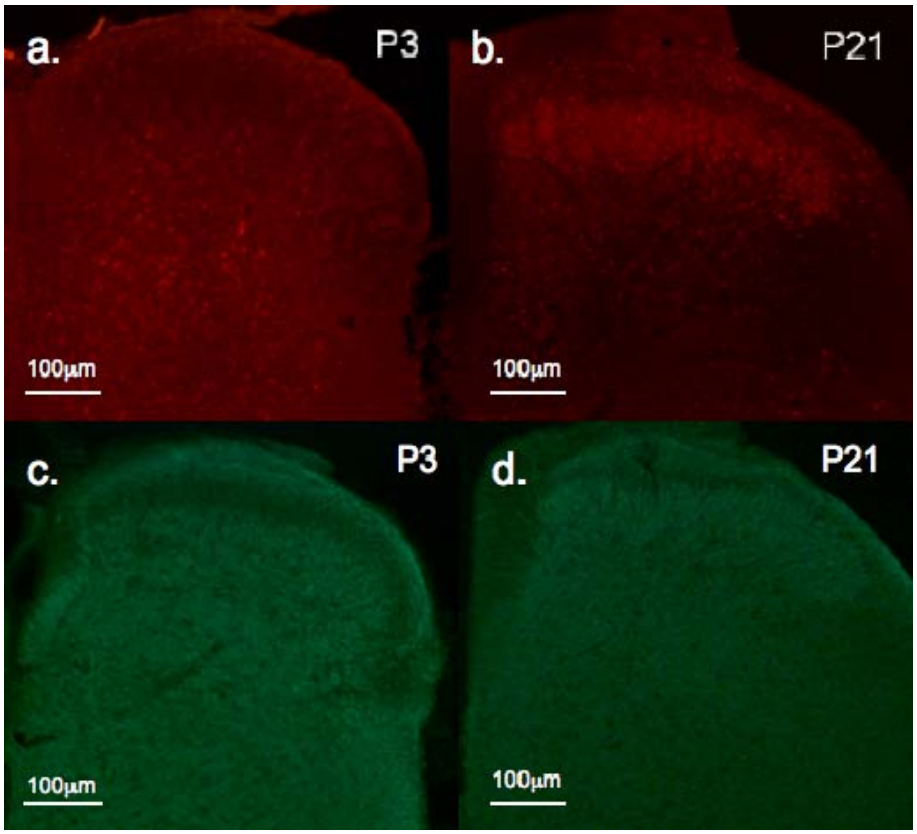



**Figure 2. 3: Representative images GlyT2 staining of spinal dorsal horn of P3 and P21 spinal cord sections.**

(left) P3 sections, (right) P21 sections. (a. and b.) Non-specific staining of cell bodies using the Santa Cruz antibody. (c. and d.) High background staining using the Chemicon antibody. Images are taken at 4x magnification; scale bar is identical for all sections.

GlyT2 staining using the privately acquired antibody offered the specificity required and clearer glycinergic terminal staining could be discriminated without high non-specific background immunofluorescence observed with the commercially available antibodies. Staining revealed a striking difference in protein expression pattern in spinal sections of the four postnatal ages tested (see Figure 2. 4). GlyT2 immunopositive staining in the P3 neonatal spinal cord slices is diffuse in the deep dorsal horn, but lacking in the substantia gelatinosa. Over the course of the next two

postnatal weeks, immunostaining gradually shifts dorsally and begins to be restricted to the lamina III from age P14. This is not fully established until P21, when a clear expression 'eyebrow' can be differentiated in lamina III.

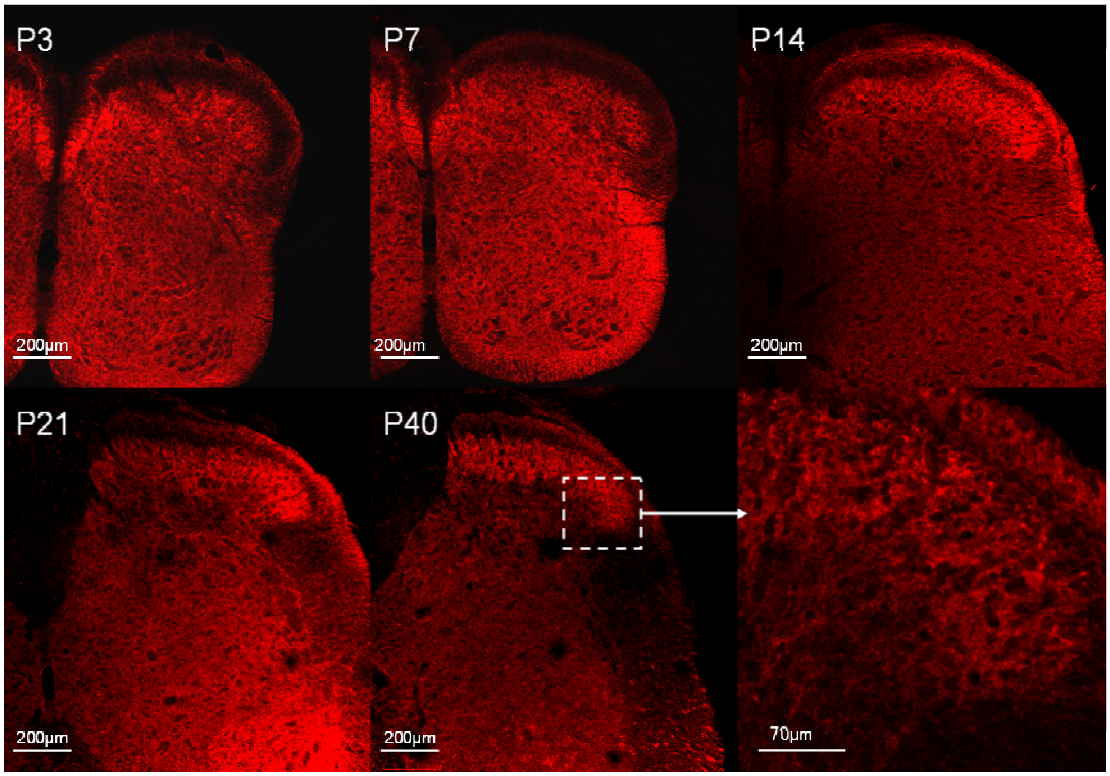



**Figure 2. 4: GlyT2 staining of representative P3, P7, P14, P21 and P40 rat lumbar spinal sections.**

Staining is diffuse and unrestricted in the neonate and becomes refined to laminae III by the third week of life. Images are taken at 10 x magnification except for the final image bottom right, which is at 40 x magnification; scale bars are as marked.

Quantification of this shift in expression was achieved through graphing a column average plot of intensity of staining versus depth from the surface of the spinal cord (Figure 2. 5). GlyT2 staining in the mature dorsal horn was found to peak at around 160 µm from the dorsal edge of the spinal cord. In agreement with previous studies examining the laminar expression of GlyT2 and the glycine receptor, the entire expression band was found to spread over 100 µm (140 µm - 220 µm) presumed to lamina III, with a smaller peak found in the very superficial layer, presumed lamina I (Mitchell *et al.*, 1993; Spike *et al.*, 1997; Harvey *et al.*, 2004).

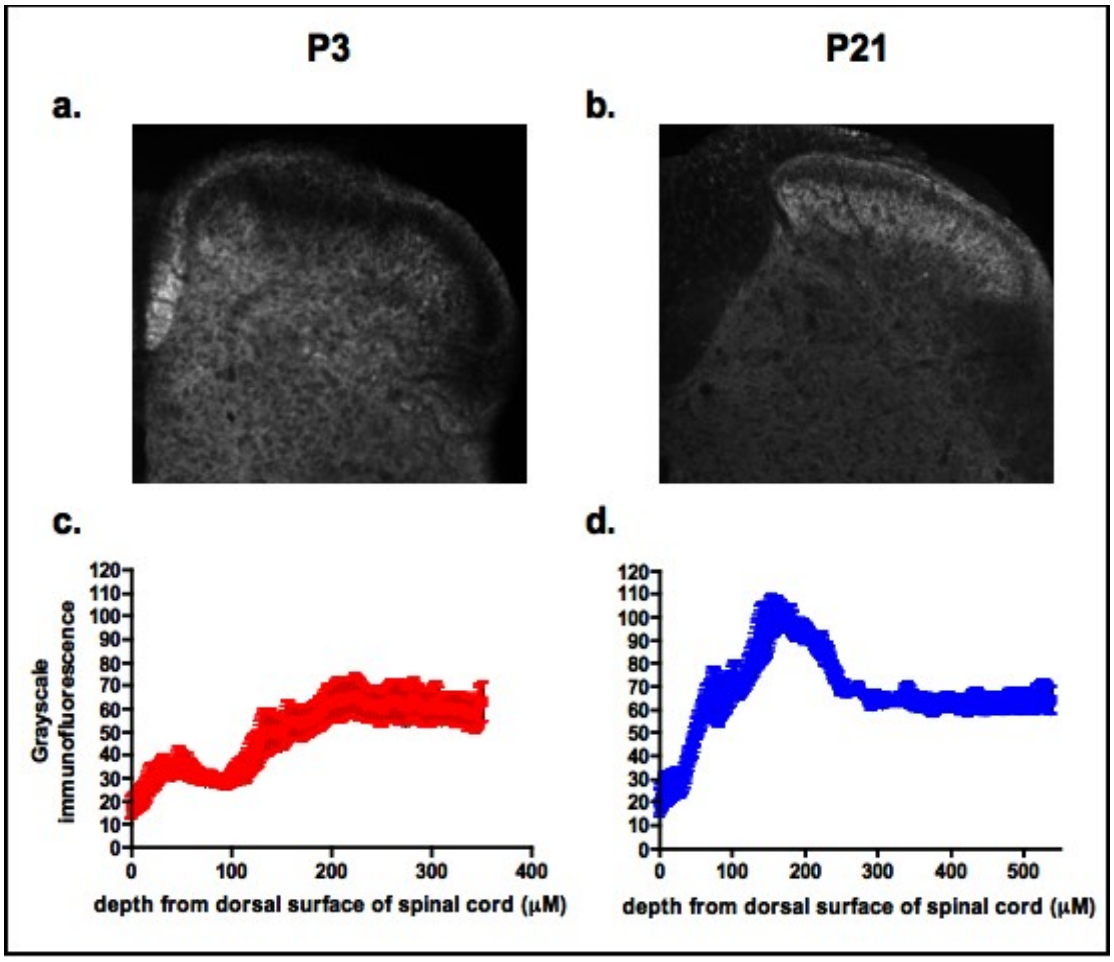



**Figure 2. 5: Graphical representations of GlyT2 immunofluorescence across the dorsal horn of P3 and P21 rat spinal cord sections.**

Example images of GlyT2 staining used for analysis of (a.) P3 (8 sections; n=4) and (b.) P21 (8 sections; n=4) spinal sections taken at 4 x magnification. (c. and d.) Graphs representing the mean and standard deviation of the intensity of GlyT2 staining at given depths across the dorsal horn. (c.) Intensity of immunofluorescence in P3 spinal sections peaks initially at 50 µm from the dorsal edge and reaches maximum intensity at 150 µm, after which intensity plateaus. (d.) Intensity of GlyT2 staining in P21 spinal sections peaks at 160 µm with the most intense staining occurring between 140 µm - 220 µm after which intensity dips and reaches a plateau.

### *2.4.2.1 GlyT2 and IB4 staining*

The predominant localization of GlyT2 in lamina III was also verified using co-staining with Isolectin B4 (IB4), a protein expressed in non-peptidergic C fibres that is restricted to inner lamina II ($LII_i$) in the spinal dorsal horn. Non-peptidergic C fibres do not to reach their adult termination points until the second postnatal week (Jennings & Fitzgerald, 1998; Park *et al.*, 1999; Nakatsuka *et al.*, 2000) and as such,

IB4 staining is faint until P14. GlyT2 was found to be expressed independently to IB4 at all ages tested (Figure 2. 6). Instead, expression was located both dorsal and ventral to lamina II in agreement with the intensity plot in Figure 2. 5.

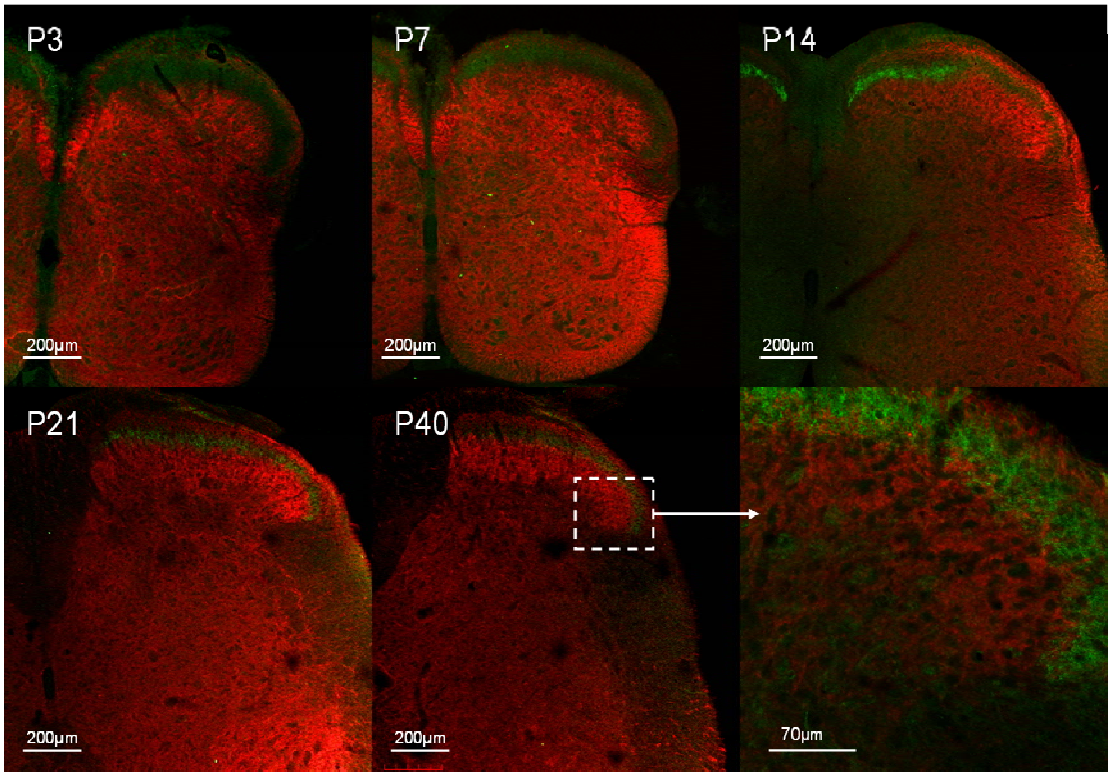



**Figure 2. 6: Representative GlyT2 (red) and IB4 (green) double-staining in P3, P7, P14, P21 and P40 rat lumbar spinal sections.**

Using IB4 as a marker for lamina II, it is apparent that GlyT2 staining is absent from lamina II at any age tested, indicating a LIII expression band in adult spinal sections. Images are taken at 10 x magnification except for bottom right image, which is taken at 40 x magnification; scale bar is as marked for each image.

### 2.4.3 αGlyR staining

Immunohistochemical staining of the α subunit of the glycine receptor was used to map the expression pattern of all α subunit-containing glycine receptors over the first forty days of life. Although staining was found to be less well-defined than that seen with the antibody raised against GlyT2, there is a distinct absence of α containing glycine receptor staining in the neonatal superficial dorsal horn (Figure 2. 7). Staining was found intracellularly and could be seen to clearly label cytoplasm and dendrites of neurons in both the dorsal and ventral horns of the spinal cord.

Maturation of this expression occurred over the same timeline as that seen for GlyT2 and in similar areas.

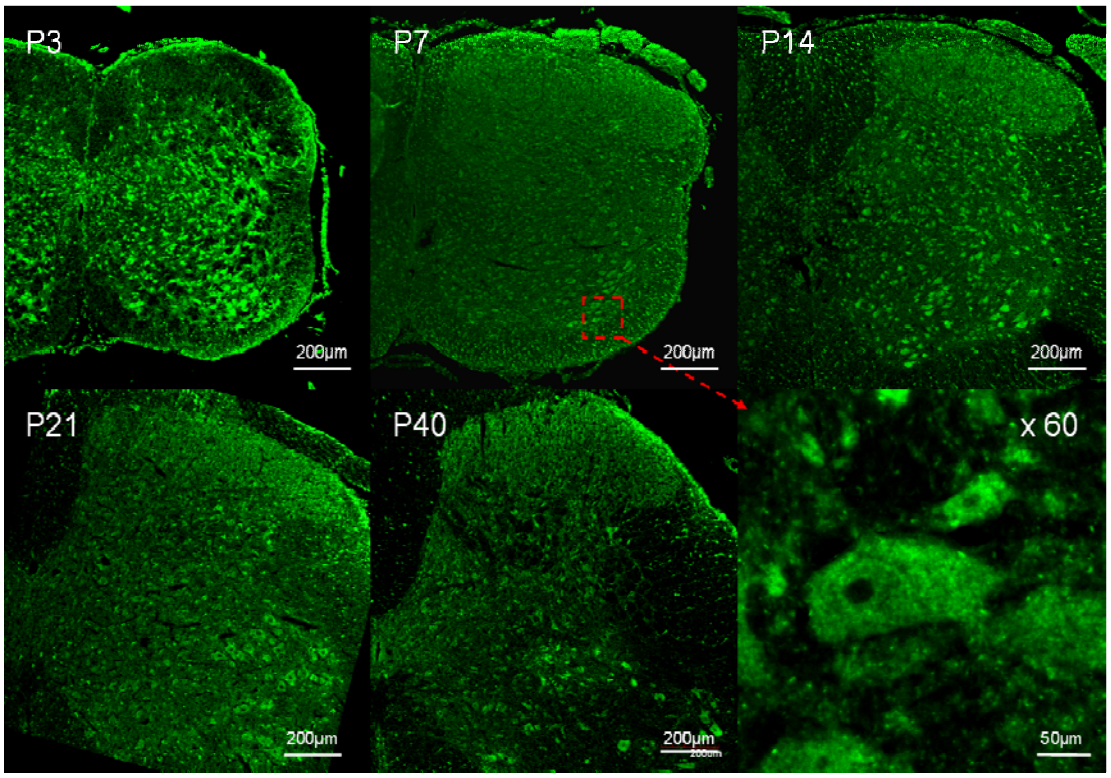



**Figure 2. 7: Immunostaining of the α subunit of the glycine receptor in representative P3, P7, P14, P21 and P40 rat lumbar spinal sections.**

Staining is diffuse and unrestricted in the neonate and becomes refined to laminae III by the third week of life. Images are taken at 10 x magnification except for the final image bottom right, which is at 60 x magnification; scale bars are as marked.

### 2.4.4 Functional mapping of postsynaptic neurons under tonic glycinergic inhibitory control

C-fos has been shown to be a reliable marker for neuronal activation in the spinal cord (Hunt *et al.*, 1987) and was used as a dynamic marker to identify the pattern of neurons normally under tonic glycinergic inhibitory control in the early postnatal period, providing an intermediate between the immunohistochemical mapping described above and functional mapping studies of glycinergic networks. Thus, neurons normally under glycinergic inhibition would be disinhibited, or facilitated, after glycine receptor antagonism using strychnine. These excited neurons will then express c-fos upon activation, and can be identified according to this protein

expression by immunohistochemistry. This has previously been used in adult rat spinal cord to show disinhibited neurons after systemic strychnine administration (Cronin *et al.*, 2004).

#### *2.4.4.1 C-fos staining*

C-fos staining was not as ubiquitous as expected, potentially due to the high level of urethane anaesthesia used. It did none-the-less reveal a significant increase in c-fos positive dorsal horn neurons in the adult following strychnine as compared to those treated with saline (4.04 ± 0.8 c-fos positive neurons in the dorsal horn of saline treated versus 6.7 ± 0.8 c-fos positive neurons in strychnine treated animals, P= 0.018; Figure 2. 9). No significant difference was found in P3 rat spinal sections, although there was a trend towards a decrease in activated neurons 2 hours following intrathecal strychnine as compared to sections of saline treated pups (4.63 ± 0.6 activated neurons in saline treated rats versus 3.5 ± 0.8 c-fos positive neurons in strychnine-treated pups). As an additional analysis, the pattern of these positive neurons was also examined using camera lucida drawings. Each lucida image consists of four sections of spinal cord from four animals (Figure 2. 9a). This was further quantified by graphing the number of c-fos positive neurons found in superficial laminae I-II, deep dorsal horn III-V and lamina X surrounding the central canal (Figure 2. 9b). Strychnine resulted in a specific pattern of neuronal activation in the adult dorsal horn, with a group of neurons activated throughout laminae I-III as well as in the regions of lamina X, thought to correspond to sympathetic activity, found to be significantly different from saline treated patterns ($P<0.02$, two-way ANOVA). In the neonatal spinal cord there was a large variability in the number of c-fos positive neurons evoked by strychnine or saline. Although not significant, glycine receptor antagonism somewhat prevented the activation of neurons in the superficial dorsal horn seen after saline treatment. The pattern of activation is broadly found in laminae III-IV and deeper lamina X, with no activation in laminae I-II.

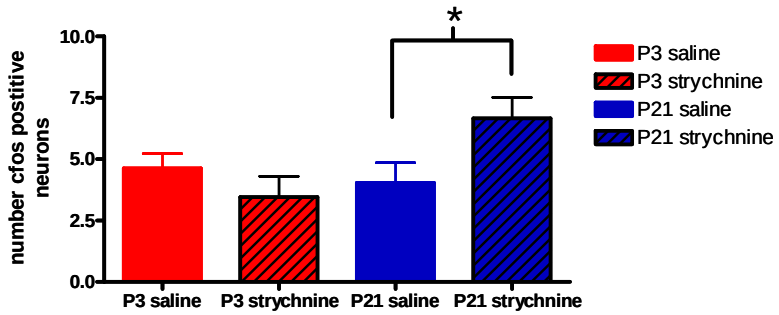



**Figure 2. 8: Graph of total number of c-fos positive dorsal horn neurons of P3 and P21 rats 2 hours following intrathecal saline or strychnine.**

There is a significantly increased activation in P21 spinal cord after strychnine treatment (n= 4; P<0.05) indicating high levels of tonic glycinergic inhibition. Strychnine had no significant effect on number of c-fos positive neurons at P3 (n=4), suggesting minimal glycinergic activity at this age.

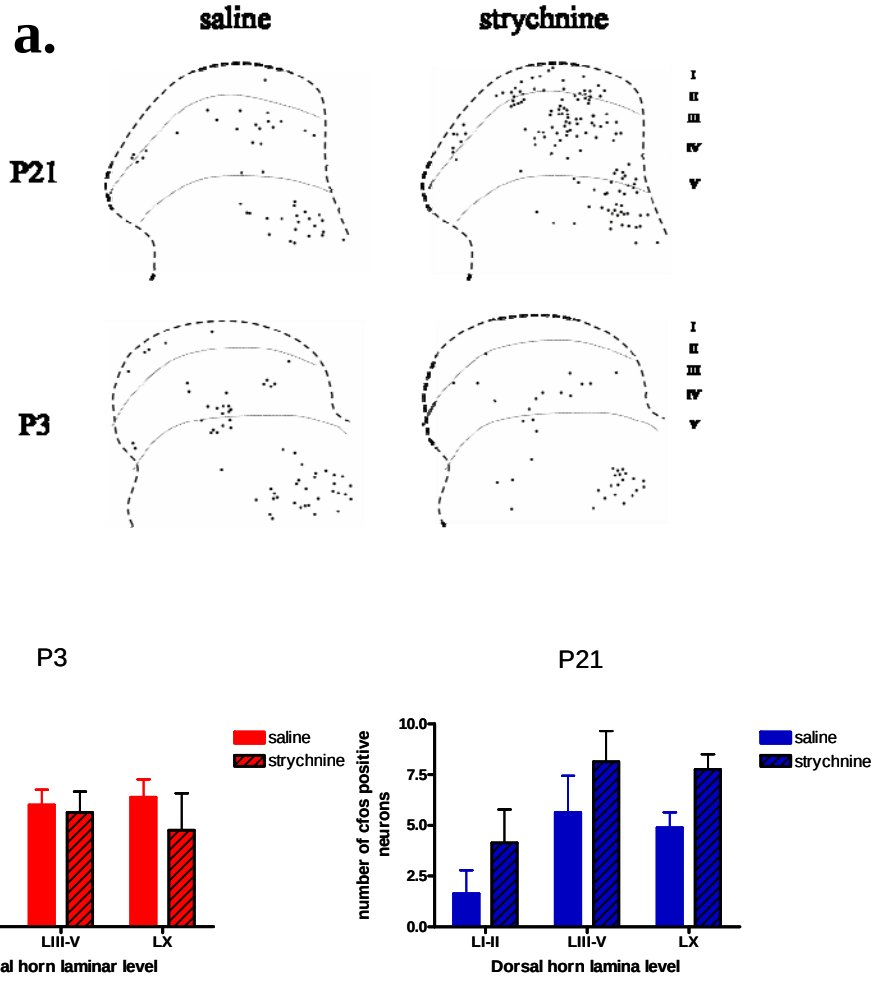



**Figure 2. 9: Mapping of neurons under tonic glycinergic inhibition in the neonatal P3 and P40 spinal dorsal horn.**

(a.) Camera Lucida images of c-fos positive neurons in the dorsal horn. Images are the summated expression patterns of four sections from four separate animals treated with (left) saline or (right) strychnine. (b.) Graph of number of c-fos positive neurons found in laminae I-II, III-V and X. Strychnine significantly increased c-fos activation at P21 (P<0.05, two-way ANOVA) but not at P3.

## 2.5 Discussion

This Chapter describes the developmental expression patterns of glycinergic inputs into the spinal cord and maps postsynaptic receptor expression over the course of the first forty postnatal days using immunohistochemical techniques. Two different methods were used to examine developmental expression changes in glycinergic pre and postsynaptic sites: (i) a study of the expression patterns of presynaptic glycinergic inputs and postsynaptic glycine receptors in the naïve neonatal and adult rats, and (ii) a functional mapping study, in which animals were challenged with intrathecal strychnine or saline to examine the effects of tonic glycinergic activity in the immature and mature dorsal spinal cord. Activated neurons were then immunostained with an antibody raised against the activity marker protein c-fos. A summary of the targets of antibodies is shown below in Figure 2. 10.

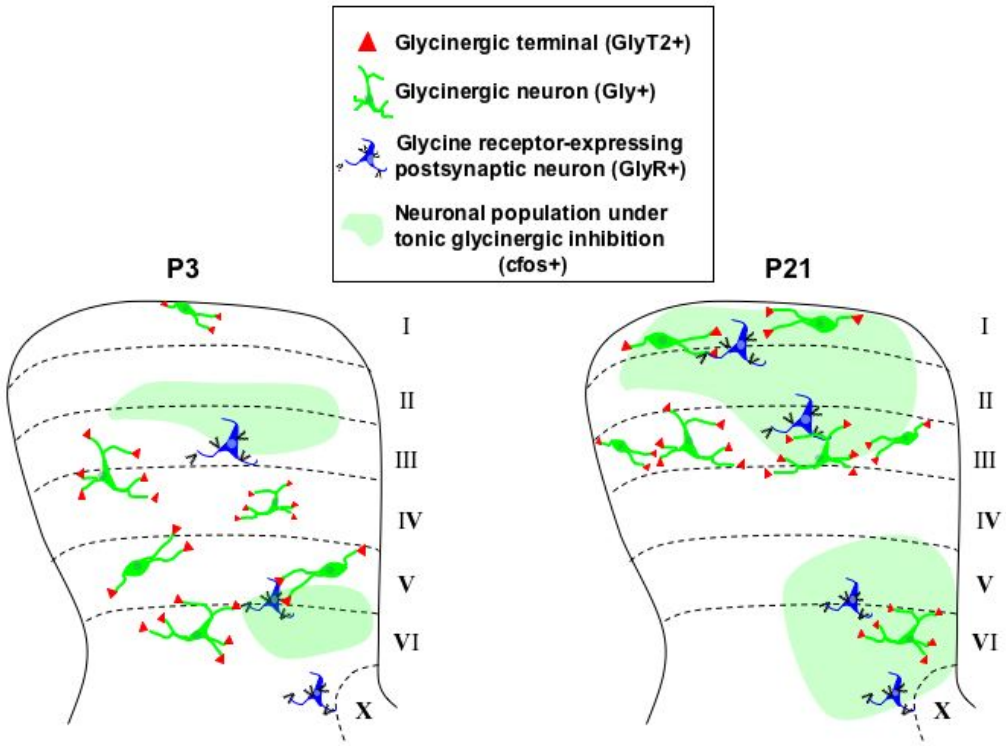



**Figure 2. 10: Summary schematic of antibodies used and results found in this study.**
Further details on the use of these antibodies and the findings of this chapter can be found in the methods and within this Chapter discussion.

GlyT2 immunostaining in the neonatal dorsal horn was diffuse and unrestricted in the deep dorsal horn and completely absent from the substantia gelatinosa until the

second week of life. After P21, GlyT2 expression became largely restricted to lamina III, consistent with previous reports (Todd, 1990; Zafra *et al.*, 1995b; Todd *et al.*, 1996; Poyatos *et al.*, 1997; Spike *et al.*, 1997; Harvey *et al.*, 2004). This pattern was also followed by glycine-receptor expressing neurons (Todd & Sullivan, 1990; Mitchell *et al.*, 1993; Todd *et al.*, 1996) suggesting a local glycinergic action from birth. Glycinergic inputs therefore do not reach their adult terminations until the third week of life, and expression of terminals is broad and ill-defined until this time. Similarly c-fos expression following strychnine suggests that cells under tonic glycinergic inhibition and so glycine receptor-containing neurons, are located deeper within the neonatal dorsal horn and absent in superficial laminae, although activation of superficial dorsal horn neurons was pronounced in the adult. Moreover, c-fos cell counts show that tonic glycinergic inhibitory control is not as widespread in the neonate as in the adult, suggesting a minor role for tonic glycinergic transmission in the first week of postnatal life, consistent with *in vitro* patch clamp analyses (Baccei & Fitzgerald, 2004).

### 2.5.1 Mapping of glycinergic terminals using GlyT2

Developmental studies have revealed GlyT2 to be expressed from the early embryonic period. Expression levels reach maximum values by postnatal day 14 and decrease to adult levels after weaning, such that GlyT2 protein levels are lower in P3 rat spinal cord than those found in P21 rats (Zafra *et al.*, 1995b). The differences in GlyT2 immunostaining between neonatal and mature spinal dorsal horn sections seen in this Chapter can therefore be attributed to both a developmental up-regulation of protein and a shift in expression pattern. The mechanisms behind this shift are still unknown, but can potentially be answered by research completed in other sensory systems. Glycinergic transmission in the auditory system has also been shown to undergo substantial functional changes in the early postnatal period at a time when GlyT2 expression begins (Sanes, 1993; Kandler & Friauf, 1995; Friauf *et al.*, 1997; Ehrlich *et al.*, 1998; Kotak *et al.*, 1998; Friauf *et al.*, 1999) indicative of a role for GlyT2 in mediating of these changes. Histochemical and *in vitro* studies support this, and have demonstrated that the developmental expression of GlyT2 in the brainstem auditory nucleus coincides with a shift of inhibitory signalling from primarily

GABAergic to glycinergic (Zafra *et al.*, 1995a; Zafra *et al.*, 1995b; Kotak *et al.*, 1998). Furthermore, pharmacological blockade of the transporter in embryonic mouse spinal cord cultures is sufficient to lead to mixed GABA/glycinergic neurons adopting a primarily GABAergic phenotype (Rousseau *et al.*, 2008).

Interestingly, GlyT2 expression in the cochlear nucleus has been found to be influenced by neuronal activity, such that increased synaptic excitation can evoke a localized increase in GlyT2 expression in cochlear neurons (Barmack *et al.*, 1999). It is therefore plausible that the maturation of sensory afferent synaptic connections in the neonatal cord evokes enough activity to promote the GlyT2 expression in lamina III. This could then allow glycinergic inhibition to function more efficiently and bring about the shift in inhibitory phenotype and the apparition of glycine receptor-only synapses found in the mature spinal cord. Indeed glycine receptor clustering in the neonate has been shown to be dependent upon glycinergic transmission, such that blocking it using the glycine receptor antagonist strychnine prevents adequate receptor clustering (Kirsch & Betz, 1998; Levi *et al.*, 1998). The sub-optimal recycling of glycine due to lack of GlyT2 in the first week of postnatal life could therefore prevent adequate receptor clustering rendering transmission ineffective.

Much of what is known of the function of glycine transporters comes from research using genetically modified mice in which GlyT2 or GlyT1 were knocked down. From these, the primary purpose of GlyT1 appears to be in clearing glycine from the synaptic space into the postsynaptic site. Conversely, GlyT2 appears to enhance glycinergic signalling by making it available for recycling in the presynaptic terminal (Gomeza *et al.*, 2003a; Gomeza *et al.*, 2003b; Rousseau *et al.*, 2008). These studies also revealed an insight into the role of glycinergic transmission early in postnatal development. GlyT1 knockout mice were terminal within a day of life, due to apparent over-inhibition that could be prevented by administration of strychnine (Gomeza *et al.*, 2003a). Interestingly however, GlyT2-null mice survived for up to two weeks. The eventual fatality was a result of a lack of glycinergic inhibition, accompanied by hyperexcitability and motor spasticity (Gomeza *et al.*, 2003b). That this would only occur after two weeks of life suggests that glycine is not released in large enough amounts to significantly affect the developing central nervous system until this time, a theory that seems heavily supported by the lack of glycinergic

terminals at the appropriate levels of the spinal cord in the neonate as shown in this Chapter.

Pharmacological blockade of GlyT2 inhibitors in adult systems seem to counteract these findings, and act to increase the extracellular concentration of glycine and prolong the duration of the glycinergic postsynaptic currents in the spinal cord (Bradaia *et al.*, 2004; Whitehead *et al.*, 2004) providing evidence of a role for GlyT2-mediated reuptake in controlling the extracellular concentration of glycine (Morita *et al.*, 2008). This could be a developmental shift in the role of GlyT2 in glycinergic signalling or potentially a factor of other pathological pain syndromes; alternatively it could reveal a dual role for GlyT2.

### 2.5.2 The influence of a developmental shift in glycine receptor subunit expression

Up until this point the discussion has focused primarily upon presynaptic changes in glycinergic signalling. However, postsynaptic changes are equally important in the transmission of glycinergic signalling. Studies completed over a century ago, and later repeated in 1921, found that behaving neonatal rats required a much larger dose of strychnine per unit weight to induce seizures and result in eventual death compared to doses needed in adult rats (Falck, 1884, 1885; Scwartze, 1921). Although this could not be explained at the time, it is now known that expression of the various glycine receptor isoforms are developmentally regulated, and these display different sensitivities (Malosio *et al.*, 1991; Rajendra *et al.*, 1997). In fact, there is a clear postnatal shift in receptor subtype over the first two weeks of postnatal life from $\alpha_2$ homomeric receptors to α/β heteromeric receptors, which has been shown to result in differing kinetics and binding properties between mature and neonatal receptors (Basbaum, 1988; Todd *et al.*, 1996). The true physiological relevance of this switch is unclear but clues can potentially be revealed in the characteristics of each receptor subtype. There are two significant differences between neonatal and adult receptors:

(i) neonatal $\alpha_2$ homomeric glycine receptors lack a gephyrin-binding site unlike $\alpha$ heteromers. The anchoring protein gephyrin is necessary for glycine receptor cluster formation by binding the $\beta$ subunit of the glycine receptor. The lack of a gephyrin-binding site on homomeric $\alpha_2$ receptors therefore implies that only $\alpha/\beta$ heteromeric glycine receptors should aggregate at inhibitory synapses (Kirsch *et al.*, 1996; Fritschy *et al.*, 2008). Evidence supporting this proposal has been derived from experiments based on antisense strategies (Kirsch *et al.*, 1993; Kirsch & Betz, 1995; Kirsch *et al.*, 1995; Meier *et al.*, 2000) and from gephyrin knockout mice in which synaptic clustering of glycine receptors is abolished (Feng *et al.*, 1998), although others have shown homomeric glycine receptor clustering at the cell surface independently of gephyrin (Meier *et al.*, 2000).

(ii) immature $\alpha_2$ homomeric receptors have slower decay kinetics and longer opening times compared to adult $\alpha_1$-containing heteromer (Takahashi *et al.*, 1992; Mangin *et al.*, 2003). Longer opening times of homomeric receptors could lead to a greater influx of chloride ions, resulting in greater hyperpolarisation and therefore more inhibition/shunting of excitatory signals. Slower kinetics would however, also lead to slow re-opening of the channel and a more 'sluggish' response to agonist binding. This could in turn provide rationale for the postnatal sharpening and tuning of glycinergic inhibition (Keller *et al.*, 2001; Baccei & Fitzgerald, 2004), which coincides with receptor subtype switch from $\alpha_2$ homomeric receptors to $\alpha/\beta$ heteromeric receptors (Malosio *et al.*, 1991). Although it would be tempting to speculate that the slower kinetics are due to a difference in number of binding sites, such that the neonatal homomeric receptor may be able stay open for longer due to a larger number of glycine molecules binding in the active site, this has not been found to be the case with $\alpha_1$ homomers (Beato *et al.*, 2004) and so would not be suspected for $\alpha_2$ homomeric receptors.

Importantly, the staining studies in this Chapter describe postnatal changes in the expression of the $\alpha$ subunit of the glycine receptor, and not of a given $\alpha$ subunit subtype. Although this provides useful insight into the areas of expression of glycine receptors in general, further immunostaining studies outlining the developmental expression profile of both the $\alpha/\beta$ heteromeric and $\alpha_2$ homomeric receptors would

provide additional detailed information concerning the postsynaptic re-organisation of glycine receptors in the spinal dorsal horn in the first three postnatal weeks.

### 2.5.3 Functional mapping of dorsal horn neurons under tonic glycinergic inhibition

In isolation, immunostaining studies are invaluable for the expression profiling of a protein of interest but cannot offer receptor functionality. To address this, c-fos immunostaining was used in conjunction with the glycine receptor antagonist strychnine to visualize dorsal horn neurons normally under postsynaptic tonic glycinergic inhibitory control in P3 and P21 rats. Neurons normally under glycinergic inhibition will be facilitated by strychnine blockade as inhibition is lifted, and will thus express the activity marker protein c-fos (Hunt *et al.*, 1987). Following strychnine treatment, the number of c-fos activated neurons in the spinal dorsal horn increased significantly in P21 spinal sections, indicating a significant number of dorsal horn neurons under tonic glycinergic control in the developmentally mature spinal cord. Conversely, the number of c-fos positive neurons was not significantly changed between spinal sections of P3 rats treated with saline or strychnine. The lack of significant increase in activated neurons in the neonatal cord following strychnine treatment is consistent with *in vitro* patch-clamp studies showing a lack of glycinergic-mediated tonic inhibition until the second postnatal week (Baccei & Fitzgerald, 2004). Although this does not offer any specific information regarding the phenotype of these neurons, it can give an indication of the influence of glycinergic inhibition in the early postnatal period. The fact that both glycine receptor immunoreactivity and c-fos staining after intrathecal strychnine treatment were found in the deeper dorsal horn of immature rats as compared to mature animals suggests that glycinergic inhibition is not appropriately targeted in the neonatal dorsal horn.

## 2.6 Conclusions

### 2.6.1 Glycinergic terminals do not reach adult spinal patterns until the end of the second postnatal week

GlyT2 staining revealed a postnatal shift in protein expression pattern in the spinal dorsal horn. Immunostaining was diffuse and unrestricted in neonatal spinal cord sections and absent from superficial laminae. Expression gradually increased over the following three weeks when the is pattern restricted to lamina III of the dorsal horn, corresponding to the area of innocuous sensory input from the periphery. This would suggest that although glycine has a specific role for local inhibition of sensory afferent information in lamina III in the mature spinal dorsal horn, this is not established until the third week of postnatal life.

### 2.6.2 Developmental receptor expression changes in the dorsal horn

Glycine receptor staining combined with functional mapping of the receptor using c-fos and intrathecal strychnine treatment mirrored results found with terminal mapping: although receptors were present in the neonatal deep dorsal horn, expression was sparse in the more superficial laminae. Further to this, no cells in laminae I or II were activated following strychnine-induced disinhibition in the neonate. The corresponding location of expression of glycinergic terminals and receptors at both ages suggests that glycinergic neurons are acting locally at both P3 and P21 but mature glycinergic circuitry is not in place until the third postnatal week. Glycinergic circuitry therefore undergoes a significant amount of postnatal adjustment over the first three weeks, both in terms of presynaptic glycinergic terminals and postsynaptic receptor expression patterns, neither of which are present in lamina III until P21.

# Chapter 3

# The Functional Role of Glycinergic Signalling in the Immature Spinal Dorsal Horn

## 3.1 Introduction

The spinal cord serves as a crucial first point of sensory integration for incoming peripheral afferent signals. Information from primary afferent fibres entering the spinal cord is not only relayed to higher centres but is also integrated and modulated locally via spinal interneurons, which can be excitatory or inhibitory in action. Regulation of these interneurons can therefore have important consequences on perception of sensory information. Glycine is one of the two major inhibitory neurotransmitters in the mature spinal cord in conjunction with GABA. Unlike GABA, glycine is mainly present in caudal areas of the central nervous system, most notably in the ventral and dorsal horns of the spinal cord where it is involved in motor control and the modulation of sensory information respectively.

### 3.1.1 The development of glycinergic inhibition in the spinal dorsal horn

GABA and glycine are present in the spinal dorsal horn from embryonic day 16 (Ma *et al.*, 1992; Poyatos *et al.*, 1997) and the proportion of spinal GABAergic neurons increases until the third postnatal week before lower adult levels are reached (Schaffner *et al.*, 1993). Both inhibitory neurotransmitters are co-transported and co-transmitted in the early postnatal period (Keller *et al.*, 2001) and are particularly important over early development as they have been shown to act as excitatory neurotransmitters through their respective receptors over the embryonic period, achieved through the presence of high intracellular chloride concentrations ((Ben-Ari *et al.*, 1989; Reichling *et al.*, 1994; Rivera *et al.*, 1999) and see (Ben-Ari, 2002)). These embryonic high intracellular levels result in a chloride reversal potential that is more positive than both the resting membrane potential and action potential threshold of neurons such that when GABA or glycine bind their receptors, chloride diffuses down its concentration gradient and the cell is depolarised, which can lead to action potential firing. Changes in chloride reversal potential have been correlated with increased expression of the potassium chloride co-transporter KCC2 (see (Ben-Ari, 2002)). As expression levels of this co-transporter increase with age, the chloride reversal potential decreases and depolarisations become less likely (Ehrlich *et al.*,

1999; Rivera *et al.*, 1999). Importantly, GABA has not been found to be excitatory in the spinal cord postnatally. *In vitro* experiments using spinal slices of neonatal rats showed that although a subset of neurons in lamina II were depolarized by GABA in the first few postnatal days, these were not sufficient to cause action potential firing and were purely hyperpolarizing by P7 (Baccei & Fitzgerald, 2004). Similarly, *in vivo* extracellular recordings of spinal dorsal horn neurons in P3 rats showed GABA to be as inhibitory in the neonate as it is in the adult (Bremner *et al.*, 2006). In fact, the longer decay time of GABAergic miniature inhibitory postsynaptic currents (mIPSCs) of neonatal spinal dorsal horn neurons could result in stronger GABAergic inhibitory control in the newborn by allowing a larger influx of chloride and so stronger hyperpolarisation (Keller *et al.*, 2001).

Chapter Two described clear changes in the expression patterns of glycinergic terminals and that of glycine receptors in the spinal dorsal horn over the course of the first three postnatal weeks. The lack of glycinergic input into lamina III of the neonatal dorsal horn is likely to have a profound effect on the functional development of sensory processing at this age yet the full implications of this expression change *in vivo* is unclear.

### 3.1.2 The functional role of glycine in modulating low threshold activity

Within the mature spinal dorsal horn, glycinergic terminals are located predominantly in lamina III, corresponding to the laminar input of low threshold Aβ fibres (Todd, 1990; Willis & Coggeshall, 1991; Mitchell *et al.*, 1993; Todd *et al.*, 1996; Spike *et al.*, 1997). Accordingly, several studies making use of the natural alkaloid strychnine, a glycine receptor-specific antagonist (Curtis *et al.*, 1968), have shown that pain as a result of glycine receptor antagonism is specific for low threshold input. In treated animals, innocuous hair deflection therefore causes an increase in dorsal horn neuron firing, mirroring responses evoked by noxious stimulation in naïve animals (Yokota *et al.*, 1979; Sherman & Loomis, 1994; Sivilotti & Woolf, 1994; Sherman & Loomis, 1996; Sorkin & Puig, 1996). This touch-allodynic behaviour is insensitive to morphine, thus seemingly unrelated to nociception as a result of activation of nociceptive fibres (Sherman & Loomis, 1994).

The selectivity of this allodynia is further confirmed by genetic studies in which mice with deficits in glycinergic transmission display extreme sensitivity to touch (White & Heller, 1982). Behavioural findings have also been extended to spinal *in vitro* patch-clamp studies, where strychnine has been shown to result in a significant increase in non-nociceptive input to superficial lamina I and II dorsal horn neurons (Baba *et al.*, 2003; Torsney & MacDermott, 2006). This has been identified as a polysynaptic protein kinase C gamma (PKC-γ)-mediated excitatory pathway between low threshold afferents terminating in laminae III-IV and nociceptive circuits in lamina II that is suppressed by inhibition in the normally functioning mature spinal dorsal horn (Yaksh, 1989; Sherman & Loomis, 1994; Sorkin & Puig, 1996; Miraucourt *et al.*, 2007). Additionally, anatomical and *in vivo* patch-clamp studies have shown monosynaptic low threshold myelinated input directly onto glycinergic neurons in laminae I-III (Todd, 1990; Narikawa *et al.*, 2000), which could be involved in the gating of flow of innocuous information to projection neurons in the more superficial dorsal horn.

The role of glycine in inhibiting the transmission of innocuous information from deep to superficial dorsal horn is clear in the mature system, yet its function in early postnatal development of sensory transduction remains to be elucidated. This is of particular interest given the apparent dominance and widespread influence of A fibres throughout the neonatal dorsal horn for the first two weeks of postnatal life, (Fitzgerald, 1985; Fitzgerald, 1988; Fitzgerald *et al.*, 1994; Coggeshall *et al.*, 1996; Jennings & Fitzgerald, 1996, 1998; Fitzgerald & Jennings, 1999; Park *et al.*, 1999; Nakatsuka *et al.*, 2000; Torsney *et al.*, 2000; Beggs *et al.*, 2002) and the neonatal sensitivity and predominance to Aβ fibre mediated sensitisation (Jennings & Fitzgerald, 1998).

### 3.1.3 The functional role of glycine in modulation of nociceptive stimuli

Although studies have primarily reported glycinergic inhibition of innocuous sensory information, others have also provided evidence for its role in nociceptive sensory transmission (Yaksh, 1989; Sivilotti & Woolf, 1994; Sorkin & Puig, 1996). Indeed studies have linked glycinergic transmission to a number of pathological pain states

in which glycinergic inhibition appears to be insufficient, most significantly of late being the involvement of $\alpha_3$ subunit containing receptors in inflammatory pain (Harvey *et al.*, 2004) and a number of neuropathic conditions, where inefficient glycinergic transmission is thought to lead to touch-induced pain or allodynia through the disinhibition of an interlaminar connection between low threshold neurons and projection neurons in the more superficial dorsal horn (see (Besson & Chaouch, 1987; Millan, 1999; Scholz & Woolf, 2002) for reviews).

Behaviourally, features of neonatal sensitivity are found across species and can be seen in behaving newborn infants, rats and kittens and include exaggerated, imprecise cutaneous reflexes and low mechanical withdrawal thresholds (Ekholm, 1967; Fitzgerald *et al.*, 1988; Andrews & Fitzgerald, 1994; Andrews *et al.*, 2002). Similarly, neonatal dorsal horn neurons have a characteristic excitability that is not normally seen in the adult: their cutaneous receptive fields are generally larger (Fitzgerald, 1985; Torsney & Fitzgerald, 2002), they sensitise to A fibre strength stimulation (Jennings & Fitzgerald, 1998) and they possess lower cutaneous mechanical thresholds (Torsney & Fitzgerald, 2002). Naïve neonatal rats therefore display sensitivity to innocuous stimuli in a manner arguably similar to those reported in neuropathic animals. This is of particular importance as neuropathic pain cannot be induced in neonatal rat pups (Howard *et al.*, 2005; Moss *et al.*, 2007; Vega-Avelaira *et al.*, 2009) suggesting that the immature glycinergic circuitry of the superficial dorsal horn outlined in the previous Chapter could underlie a lack of glycinergic control over the transmission of innocuous sensory information at this time.

## 3.2 Hypothesis

Chapter Two described the regional and developmental expression changes in glycinergic terminals and postsynaptic receptor protein patterns but the functional significance of this shift in protein expression is not yet known. This chapter addresses the functional development of glycinergic activity in the spinal dorsal horn.

I hypothesise that immature glycinergic input in the first three of postnatal weeks underlies inefficient glycinergic inhibition of sensory transduction in the neonatal spinal dorsal horn. Specifically, I propose that glycinergic control of Aβ input is lacking in young animals, allowing the transmission of innocuous information to superficial laminae that would normally be under strong glycinergic control in the adult.

To test this I performed extracellular recordings from individual spinal dorsal horn wide dynamic range neurons in *in vivo* anaesthetised intact rats of several ages. Spontaneous and evoked activity to dynamic and static mechanical stimulation of the hindpaw were recorded in the presence of the glycine receptor antagonist strychnine in order to assess the functional role of glycine in spontaneous activity of neurons, and phasic control of cutaneous sensory transduction in the developing spinal cord.

## 3.3 Methods

### 3.3.1 *In vivo* extracellular recordings

Sprague–Dawley rats of both sexes aged postnatal day 3 (P3), 10 (P10), 21 (P21) and 40 (P40) were used in these studies. Animals were allowed free access to water and food and were housed in 12-hour light/dark cycles. P3 and P10 rat pups were housed with mother and littermates, P21 and P40 rats were caged according to sex in cages of six littermates. All experiments were conducted in accordance with the United Kingdom Animals (Scientific Procedures) Act of 1986.

Rats were anaesthetised with isoflurane to achieve areflexia (induction at 3.5% isoflurane in medical $O_2$) and tracheotomised, after which the cannula was sutured in place. The animal was then mounted in a stereotaxic frame (Kopf Instruments, CA) and artificially ventilated using a ventilator pump (Small animal ventilator, model 687, Harvard Apparatus Inc.) under constant isoflurane-anaesthesia (maintenance of 1.8% in medical $O_2$, Univentor Anaesthesia Unit 400, Royem Scientific, UK) at 79 breaths per minute. The air-flow was adjusted according to the animal's size, blood perfusion and heart rate (target of 300-450 beats per minute), which was being monitored via electrocardiogram. A homeothermic blanket with probe sensor, and heating lamp were used to maintain body temperature at physiological levels (36ºC). Animals were mounted via hip and ear bars, and a laminectomy was performed to expose the lumbar spinal cord, the vertebral column secured with a clamp to the thoracic spine and the dura and pia mater removed. A subcutaneous injection of saline was given to each animal post laminectomy to maintain hydration and a thin film of mineral oil was used to cover the exposed spinal cord to prevent heat loss and excessive drying of the cord. Once stabilised, a 10 μM tipped glass-coated tungsten microelectrode was lowered onto the surface of the cord under microscopic vision, with a reference electrode inserted into the muscle near the recording area. Neuronal activity was passed through a x1 headstage amplifier and a further x 5k differential amplifier (NeuroLog, Digitimer, UK). This signal was passed through low and high pass filters, set at 1 kHz and 10 kHz respectively and onto a spike trigger, which is set manually to produce output TTL pulses for spikes above a particular voltage, an

audio amplifier as well as through an oscilloscope (TDS 2012 digital storage oscilloscope, Tektronix). Information was also fed into a PowerLab system (4SP, AD Instruments, UK), which was connected to a computer in order to allow recording and analysis of data using Chart 5 software (Chart 5 version 5.5.5, AD Instruments, UK).

In order to isolate individual neurons in the spinal cord, the microelectrode was lowered through the cord in 2- or 10 μm steps by a microdrive (Epson HX-20, Seiko Epson Co. powered by Digitimer SCAT-01 microelectrode stepper system, Digitmer, UK). Stroking of the plantar skin of the hind paw was used as a search stimulus and cells were selected once a reliable action potential spike amplitude and shape could be distinguished from background noise levels. Wide dynamic range neurons were selected such that each neuron tested responded to light touch and to noxious pinch applied to the centre of the receptive field.

The plantar paw receptive field of a cell was characterised and mapped using pinch and brush of the skin and drawn out in detail onto a representative image of the plantar paw surface (see Figure 3. 1). This image was then scanned and digitally analysed using ImageJ software (ImageJ 1.42q, National Institute of Health, U.S.A, http://rsb.info.nih.gov/ij).

Von Frey hairs were calibrated by assessing the weight needed to bend each hair in succession (see Figure 3. 2). Von Frey Hair threshold was established as the lowest hair needed to evoke spikes when applied to the centre of the receptive field (see Figure 3. 3). Subsequent to this, neuronal firing to three hairs was recorded: threshold von Frey hair, subthreshold: two hairs below threshold and suprathreshold: two hairs above the threshold. Pinch stimuli and von Frey hairs were each applied three times in succession and the brush stimulus was applied five times. The mean number of spikes evoked to each stimulus was then used for analysis. In order to try to normalise the pinch stimulus used in this study, a screw was mounted into the forcep arms, such that once the screw was tightened, forceps could only be squeezed up until a given point.

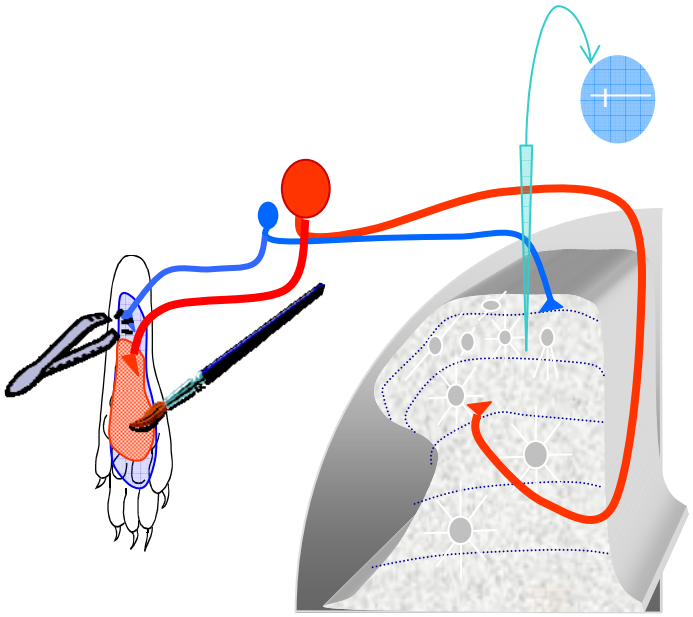


**Figure 3. 1: Schematic of *in vivo* extracellular recordings.**

An electrode is inserted into the spinal dorsal horn of an anaesthetised intact rat. Once an individual neuron is isolated, its cutaneous receptive field on the plantar hindpaw is stimulated by means of brush and pinch stimuli and neuronal responses are recorded.

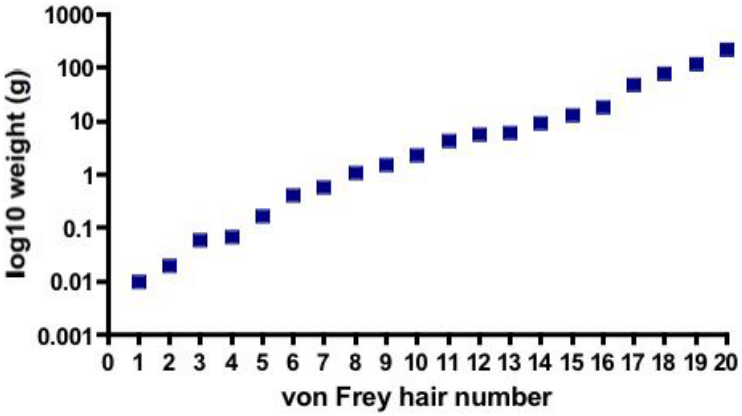



**Figure 3. 2: Calibration of von Frey hairs.**

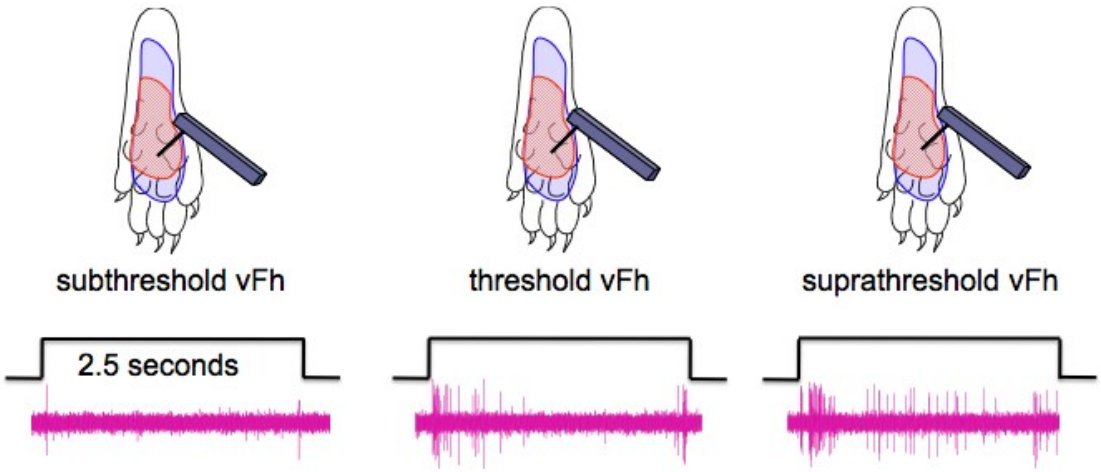



**Figure 3. 3: Example traces of dorsal horn neuron responses to subthreshold, threshold and suprathreshold von Frey hair application to the receptive field.**

Baseline responses of the cell were characterized as follows:

(i) spontaneous activity, recorded over 5 minutes

(ii) cutaneous receptive field area on the plantar paw, as mapped by brush and pinch stimuli

(iii) responses to the following stimuli applied to the cutaneous receptive field:

a. spikes fired during innocuous brush of the skin, using a fine paintbrush for 2 seconds

b. spikes fired during noxious forcep pinch of the skin for 2 seconds

c. spikes fired during application of a calibrated von Frey hair (vFh) at threshold for 1.5 seconds

d. spikes fired during subthreshold vFh application for 1.5 seconds

e. spikes fired during suprathreshold vFh application for 1.5 seconds

### 3.3.2 Pharmacology

Strychnine was applied to the surface of the cord during dorsal horn cell recording. P3 rat pups correspond approximately to the developmental stage of a 32-week old premature human infant, whilst P21 rats correspond to adolescents and P40 rats correspond developmentally to an adult (McCutcheon & Marinelli, 2009). As seen in Chapter Two, the development of a P21 spinal cord is similar to that of a P40 adult rat (see Figure 2.2), and dorsal horn neurons respond to noxious stimuli in a mature manner from P20 (Hammond & Ruda, 1991). Comparisons between P3 rat pups and P21 rats were therefore used as models of neonatal and adult spinal circuits respectively. The total amount of strychnine administered was 1.65 µg (4.9 nmol) in P3 rat pups and 8.25 µg (29.4 nmol) in P21 rats. The dose of strychnine used in this study was chosen as it is significantly lower than adult rat convulsant $ED_{50}$ dose (233 nmol; (Yaksh, 1989)) and a similar dose has been shown to result in significant changes in behaviour of awake animals ((Miraucourt *et al.*, 2007) and unpublished observations).

Once baseline responses were established, 150 µl strychnine (165 ng/g in 0.9% NaCl (Baxter, Belgium); S 0532, Sigma-Aldrich Co., St Louis, MO, USA) or saline (0.9%) was applied topically to the exposed cord. Spontaneous activity, receptive field area (measured as a percentage of the total hindpaw plantar area) and response to brush, pinch and subthreshold, threshold and suprathreshold vFhs were measured every 10 minutes for up to 60 minutes after strychnine or saline application. The 20-minute time point was used for all subsequent graphing and analysis, as this was the time point that showed the largest increase in activity from baseline. N numbers for number of animals used in the above experiments are: P21: n=14; P3: n=9. The area of the paw sensitive to brush and/or pinch was represented as a percentage of the total plantar paw surface both at baseline and 20 minutes after strychnine administration. Receptive field data is presented as maximum or total receptive field area at baseline and 20 minutes after strychnine application, and separated into pinch and brush-sensitive receptive field areas. Neuronal activity was defined for both age groups as being facilitated or inhibited if activity during stimulation increased or decreased by more than 10% of the baseline value.

Saline controls were performed in both P3 and P21 dorsal horn neurons (P3: n=11 from six animals; P21; n=4 from four animals). No changes in responses to any of the tested modalities was observed 20 minutes following saline application to the surface of the cord except for cellular responses to subthreshold von Frey hair application to the receptive field in neonatal rats, which were found to increase. The reason for this is unclear, but may be due to sensitisation of the neonatal sensory system to innocuous stimuli, which has been shown in previous studies (Fitzgerald, 1985; Torsney & Fitzgerald, 2002).

All data is presented as mean ± standard error of the mean unless otherwise stated. Number of spikes was analysed and graphed using GraphPad Prism software (version 5.00, GraphPad Software, San Diego, CA, USA, www.graphpad.com). Statistical analyses were performed using GraphPad Prism software and Wilcoxon's signed rank test within an age group or one or two-way ANOVAs between age groups and/or treatments followed by Dunnett's post hoc multiple comparisons test or Bonferroni post hoc test respectively for significant values. For all data a 95% confidence interval was used as a measure of statistical significance.

All animals were killed with an overdose of sodium pentobarbitone (i.p.) at the end of the experiment.

## 3.4 Results

### 3.4.1 General baseline electrophysiological properties of dorsal horn neurons across the first four postnatal weeks

A total of 113 dorsal horn neurons were used for single unit extracellular recordings: 18 neurons from P3 rat pups, 64 from P21 rats, and 31 neurons from P40 rats. There was no significant difference in depth of recording between age groups (average depth P3: 295 ± 42 μm; P21: 329 ± 23 μm; P40: 267 ± 26 μm; Figure 3. 4). Not all modalities could be tested in each cell and cells were only included in analysis if stable recordings could be maintained. Wide dynamic range neurons were selected over nociceptive specific or low threshold neurons.

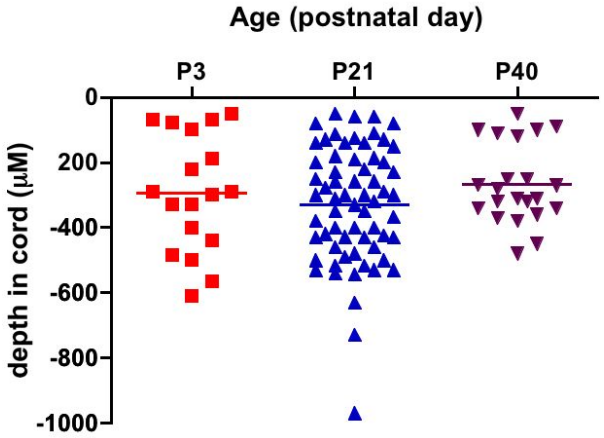



**Figure 3. 4: Depth of recorded dorsal horn cells at three postnatal ages.**
There was no significant difference in depth of recorded cells between age groups (P3: n= 18; P21: n=62; P40: n=31).

### 3.4.2 Mechanical thresholds

The gram weight force applied with von Frey hair to the skin of the receptive field required to evoke action potential firing was found to increase with age and differed significantly between P3, P21 and P40 groups in agreement with previous studies

(Torsney & Fitzgerald, 2002). The threshold at P3 was 1.1 ± 0.2 g, at P21 was 2.9 ± 0.4 g, and at P40 was 5.3 ± 1.5 g (Figure 3. 5a; one way ANOVA P<0.0001).

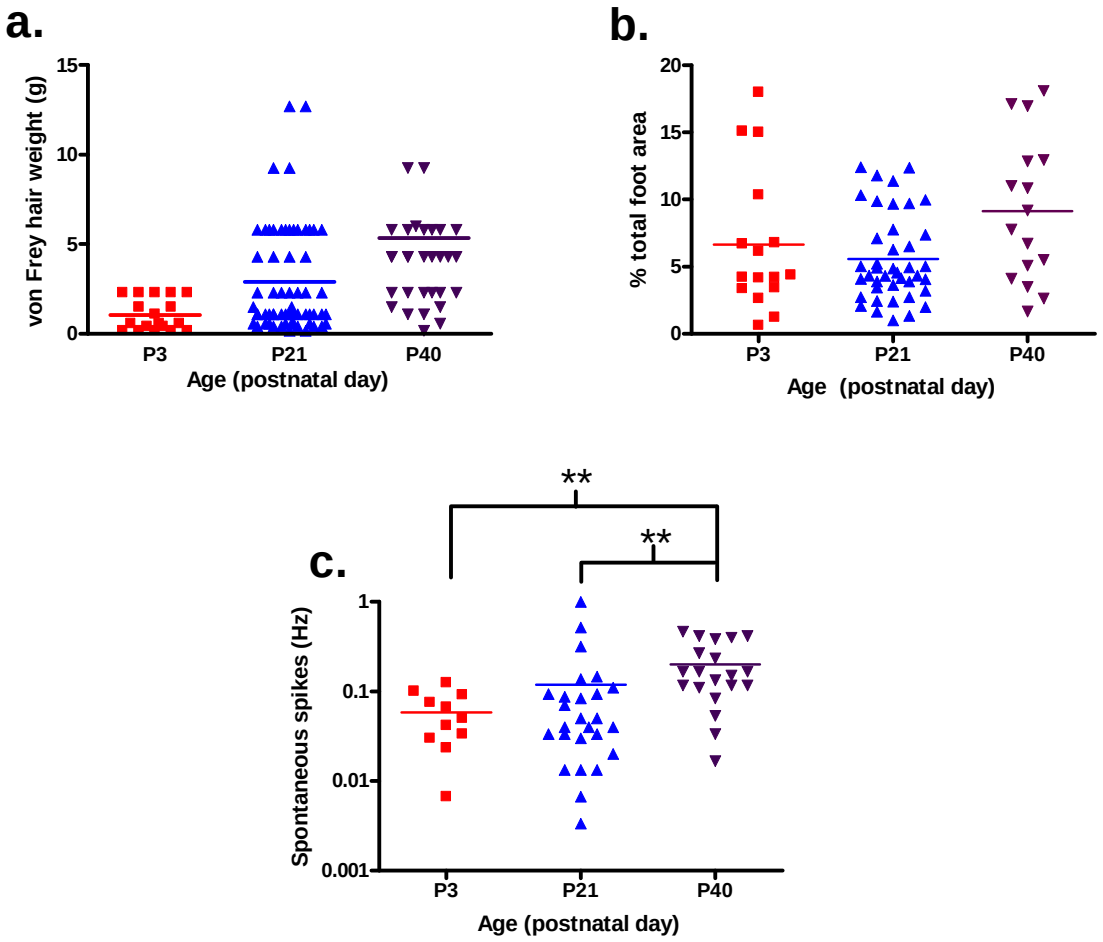



**Figure 3. 5: Baseline properties of recorded dorsal horn neurons.**

(a.) Average thresholds of P3, P21 and P40 of spinal dorsal horn neurons ages in grams (g) (P3: n= 18; P21: n=62; P40: n=31). Threshold increases significantly with age (one way ANOVA, P<0.0001). (b.) Cutaneous receptive field sizes of all recorded cells at three different age groups (P3: n= 18; P21: n=33; P40: n=18). Receptive field areas did not significantly differ between age groups. (c.) Baseline spontaneous activity of dorsal horn neurons of three different ages (P3: n= 11; P21: n=26; P40: n=20). Spontaneous activity increases significantly with age (Kruskal-Wallis across all age groups P<0.001; Dunn's multiple comparisons test: P3 vs. P40 P<0.01, P21 vs. P40 P<0.01).

### 3.4.3 Cutaneous receptive field sizes

Cutaneous receptive field size was measured as a percentage of the total foot area in order to correct for large differences in plantar foot area at the three postnatal ages tested. Average receptive field area was 6.64 ± 1.31% of the total plantar surface at

P3, 9.74 ± 1.75% at P21 and 9.12 ± 1.34% at P40 (Figure 3. 5b). Although there was a tendency towards an increase in receptive field area with increasing age, this was found to be insignificant. This could be due to the range of recording depths included in the analysis, as cells in the deeper dorsal horn of neonatal rats have previously been shown to have a tendency towards larger cutaneous receptive field sizes in comparison to their adult counterparts (Torsney & Fitzgerald, 2002).

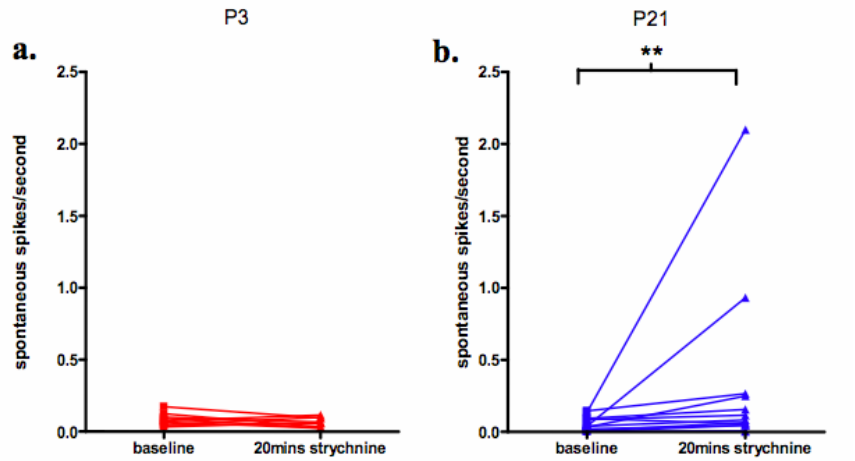
### 3.4.4 Spontaneous activity

Spontaneous activity of dorsal horn neurons was recorded continuously for 5 minutes and divided into 1-second time bins (Hz). Baseline spontaneous activity significantly increased with age from P3 to P40 (Kruskal-Wallis test across all age groups $P=0.0006$; Dunn's multiple comparisons test: P3 vs. P40 $P<0.01$, P21 vs. P40 $P<0.01$). Mean spontaneous firing rates were as follows: 0.06 ± 0.01 Hz at P3, 0.12 ± 0.04 Hz at P21 and 0.2 ± 0.03 Hz at P40 (Figure 3. 5c).

### 3.4.5 The effect of spinal strychnine upon spontaneous activity at two postnatal ages

18 neurons from P3 rats and 25 from P21 neurons were used for strychnine studies. A summary of all results is found in Table 3.1.

Strychnine applied onto the surface of the spinal cord significantly increased the number of spontaneous spikes per second of P21 spinal neurons, without significantly altering number of spontaneous spikes fired in P3 dorsal horn neurons. The baseline value of P21 spontaneous firing increased from a mean of 0.05 ± 0.01 Hz to a mean value of 0.32 ± 0.16 Hz 20 minutes post strychnine, representing a mean increase of 431% (Wilcoxon's signed rank test $P= 0.002$; Figure 3. 6a and c) and out of the 14 neurons tested, 10 were facilitated following strychnine application. However, spontaneous activity of P3 neonatal dorsal horn neurons was largely unchanged by strychnine treatment; decreasing from a mean baseline value of 0.08 ± 0.015 Hz to a mean of 0.07 ± 0.01 Hz 20 minutes post strychnine (Figure 3. 6b).




c.

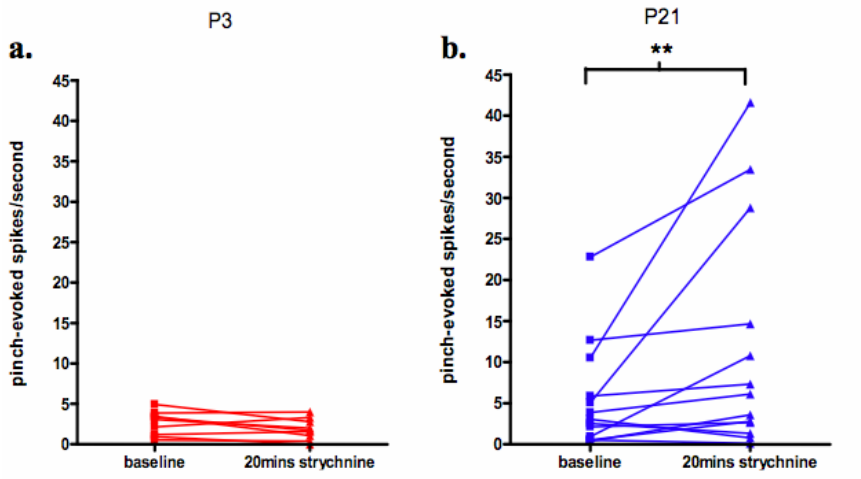
| | facilitated | inhibited | unchanged |
|---|---|---|---|
| P3 | 4 | 5 | 0 |
| P21 | 10 | 1 | 3 |

**Figure 3. 6: The effect of 165 ng/g strychnine upon spontaneous action potential firing of dorsal horn neurons of 3-day and 21-day old rats.**

(a.) Strychnine did not affect spontaneous action potential firing of P3 neurons (n=9) 20 minutes after application to the surface of the spinal cord, but (b.) significantly increased spiking in P21 neurons (n=14; Wilcoxon's signed rank test P<0.01). (c.) Table of neuronal subpopulations. Cells were characterised as facilitated or inhibited if activity was changed to a greater extent than 10% from baseline values, or unchanged.

### 3.4.6 The effect of spinal strychnine upon pinch evoked activity of spinal dorsal horn neurons at two postnatal ages

Pinch-evoked activity of dorsal horn neurons increased two fold following strychnine administration in P21 rats from a mean baseline value of 5.72 ± 1.68 Hz to 11.09 ± 3.64 Hz 20 minutes post strychnine (Wilcoxon's signed rank test P= 0.01; Figure 3. 7a and c), with a facilitation of pinch-evoked activity in 71% of cells tested (10/14). However, action potential firing of P3 neurons to pinch of the cutaneous receptive field was unchanged by glycine receptor antagonism in the neonatal spinal cord (mean baseline response of 2.61 ± 0.5 Hz to 1.88 ± 0.4 Hz post strychnine (Figure 3. 7b)).




c.

| | facilitated | inhibited | unchanged |
|---|---|---|---|
| P3 | 2 | 6 | 1 |
| P21 | 10 | 3 | 1 |

**Figure 3. 7: The effect of 165 ng/g strychnine upon pinch evoked action potential firing of dorsal horn neurons of 3-day and 21-day old rats.**

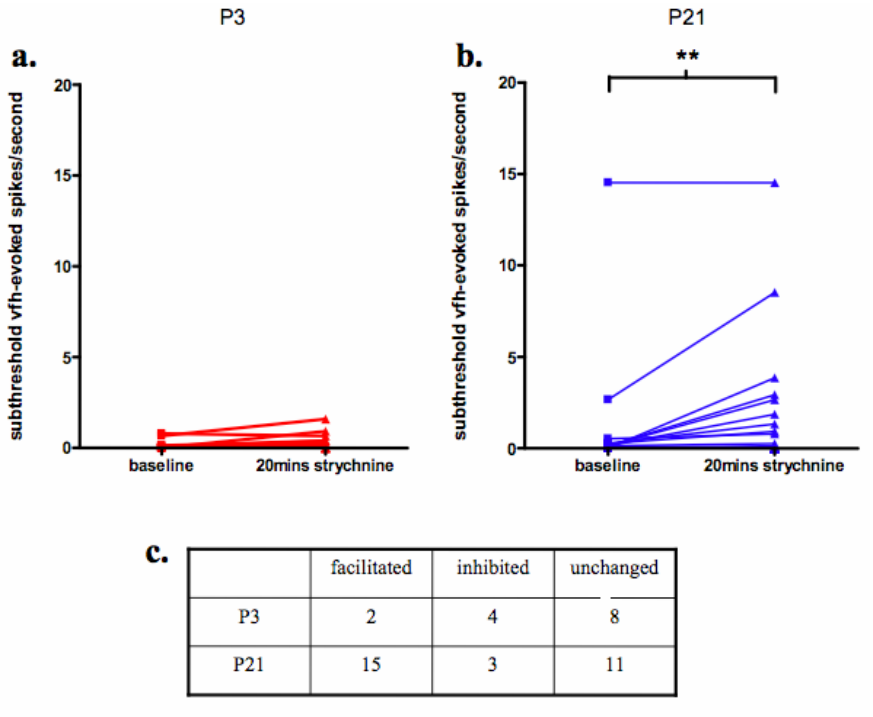
(a.) Strychnine did not affect action potential firing of P3 neurons (n=9) to pinch 20 minutes after application to the surface of the spinal cord, but (b.) significantly increased spiking in P21 neurons (n=14; Wilcoxon's signed rank test P<0.01). (c.) Table of neuronal subpopulations. Cells were characterised as facilitated or inhibited if activity was changed to a greater extent than 10% from baseline values, or unchanged.

## 3.4.7 The effect of strychnine on subthreshold, threshold and suprathreshold von Frey hair evoked spiking

### *3.4.7.1 Subthreshold von Frey hair application*

The response to a subthreshold von Frey Hair application to the cutaneous receptive field increased significantly after strychnine in P21 rats from a mean baseline value of 0.66 ± 0.5 spikes/second to 1.31 ± 0.58 spikes/second post strychnine, representing a mean increase of 245% (Wilcoxon's signed rank test P= 0.004; Figure 3. 8a and c). Firing of P3 rat dorsal horn neurons remained unaffected by strychnine (mean baseline value 0.12 ± 0.07 Hz to 0.31 ± 0.13 Hz post strychnine; Figure 3. 8a),

with the vast majority of cells (8 out of total of 14) not responding to drug application at all.




| | facilitated | inhibited | unchanged |
|---|---|---|---|
| P3 | 2 | 4 | 8 |
| P21 | 15 | 3 | 11 |

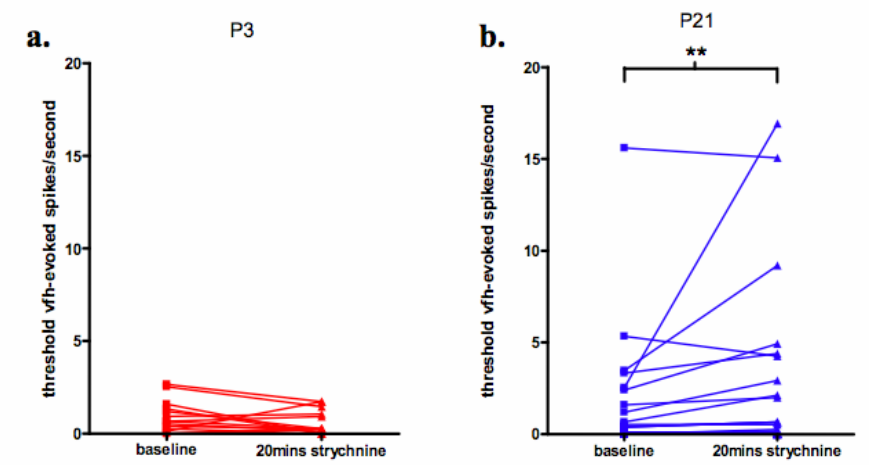
**Figure 3. 8: The effect of 165 ng/g strychnine on subthreshold von Frey hair-evoked action potential firing of dorsal horn neurons of 3-day and 21-day old rats.**

(a.) Strychnine did not affect firing of P3 neurons (n=14) to subthreshold von Frey hair application, but (b.) significantly increased spiking of P21 neurons (n=29; Wilcoxon's signed rank test P<0.01). (c.) Table of neuronal subpopulations. Cells were characterised as facilitated or inhibited if activity was changed to a greater extent than 10% from baseline values, or unchanged.

### *3.4.7.2 Threshold von Frey hair application*

The response to a threshold von Frey Hair response also significantly increased following strychnine in P21 rats (mean baseline value of 1.32 ± 0.57 to 2.26 ± 0.81 Hz post strychnine; 117% increase; Wilcoxon's signed rank test P=0.004) and was unchanged in P3 neonatal dorsal horn neurons (mean baseline of 0.92 ± 0.23 spikes/second to 0.6 ± 0.17 Hz post strychnine; Figure 3. 9a and b). Interestingly, in contrast to the neuronal response to subthreshold von Frey hairs, the threshold-evoked response of the majority of P3 neurons showed a decrease after strychnine

application relative to baseline (64%; 9 out of 14; Figure 3. 9c), whereas threshold-evoked firing of 65% of P21 neurons (19/29) was facilitated.




c.

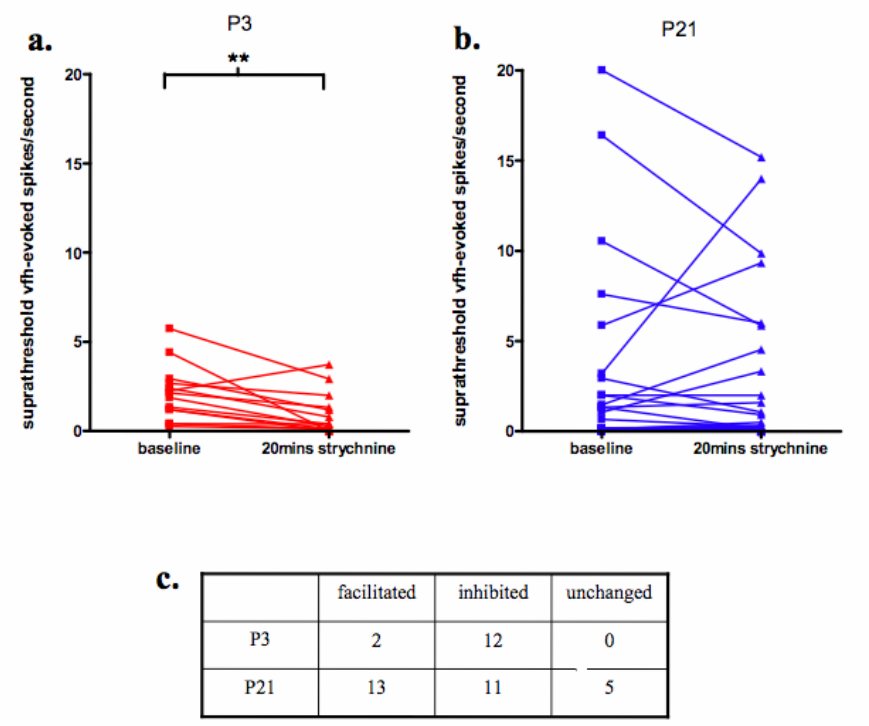
| | facilitated | inhibited | unchanged |
|---|---|---|---|
| P3 | 5 | 9 | 0 |
| P21 | 19 | 9 | 1 |

**Figure 3. 9: The effect of 165 ng/g strychnine on response to threshold von Frey hair-evoked action potential firing of dorsal horn neurons of 3-day and 21-day old rats.**

(a.) Strychnine did not affect action potential firing of P3 neurons (n=14) to threshold von Frey hair application to the receptive field 20 minutes after application to the surface of the spinal cord, but (b.) significantly increased spiking in P21 neurons (n=29; Wilcoxon's signed rank test P<0.01). (c.) Table of neuronal subpopulations. Cells were characterised as facilitated or inhibited if activity was changed to a greater extent than 10% from baseline values, or unchanged.

### *3.4.7.3 Suprathreshold von Frey hair application*

Strychnine did not affect action potential firing to suprathreshold von Frey hair stimulus in dorsal horn neurons of P21 rats (mean baseline value of 2.67 ± 0.93 to 2.62 ± 0.8 Hz post strychnine; Figure 3. 10b and c) but did cause a significant 53% decrease in action potential firing in neonatal P3 rats (mean baseline value of 2.09 ± 0.4 Hz to 0.96 ± 0.31 Hz post strychnine; Wilcoxon's signed rank test P=0.006; Figure 3. 10a and c). 86% of recorded P3 neurons exhibiting a minimum of a 10% decrease in firing relative to baseline values (12/14).




c.

| | facilitated | inhibited | unchanged |
|---|---|---|---|
| P3 | 2 | 12 | 0 |
| P21 | 13 | 11 | 5 |

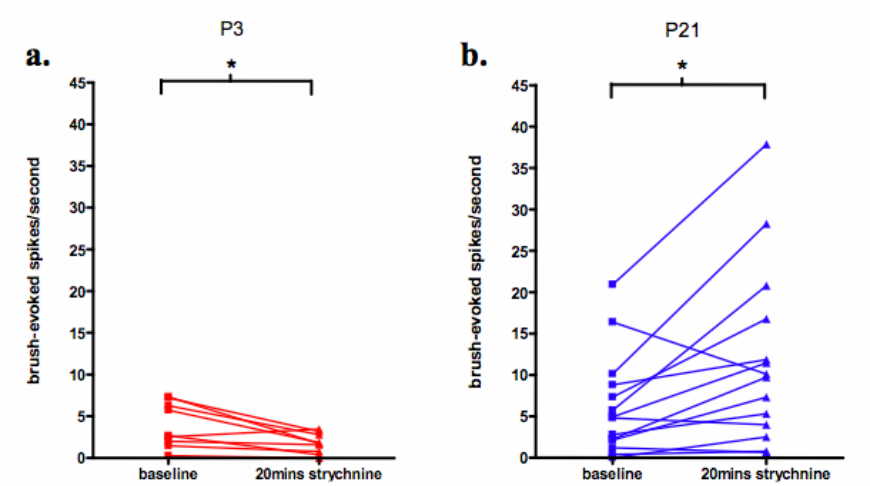
**Figure 3. 10: The effect of 165 ng/g strychnine on response to suprathreshold von Frey hair-evoked action potential firing of dorsal horn neurons of 3-day and 21-day old rats.**

(a.) Strychnine significantly increased action potential firing of P3 neurons (n=14) to suprathreshold von Frey hair application to the receptive field 20 minutes after application to the surface of the spinal cord (n=29; Wilcoxon's signed rank test P<0.01), but (b.) had no effect on that of P21 neurons. (c.) Table of neuronal subpopulations. Cells were characterised as facilitated or inhibited if activity was changed to a greater extent than 10% from baseline values, or unchanged.

### 3.4.8 The effect of strychnine on brush evoked activity of spinal dorsal horn neurons in P3 and P21 rats

A 2-second brush stimulus to the receptive field was used as an innocuous, dynamic stimulus. Brush-evoked activity of P21 adult spinal dorsal horn neurons increased significantly from baseline following application of strychnine from a mean baseline firing of 6.27 ± 1.64 spikes/second to 11.97 ± 2.89 Hz 20 minutes post strychnine, representing a 120% increase from baseline (Wilcoxon's signed rank test P=0.011; Figure 3. 11a). In sharp contrast, strychnine inhibited brush-evoked firing of P3 neurons from a mean baseline value of 3.94 ± 0.9 Hz to 1.76 ± 0.4 Hz post strychnine (53% decrease; Wilcoxon's signed rank test P=0.03; Figure 3. 11b). 79%

of P21 neurons were facilitated over 10% of baseline values (11/14) whereas 89% of P3 neurons were inhibited (8/9; Figure 3. 11c).




c.

| | facilitated | inhibited | unchanged |
|---|---|---|---|
| P3 | 1 | 8 | 0 |
| P21 | 11 | 3 | 0 |

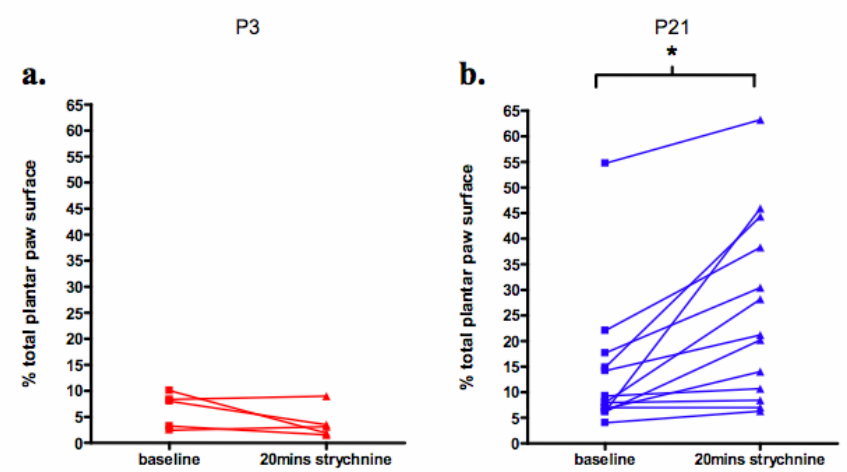
**Figure 3. 11: The effect of 165 ng/g strychnine upon brush evoked action potential firing of dorsal horn neurons of 3-day and 21-day old rats.**

(a.) Strychnine significantly decreased action potential firing of P3 neurons (n=9) to brush 20 minutes after application to the surface of the spinal cord (Wilcoxon's signed rank test $P<0.05$), but (b.) significantly increased spiking in P21 neurons (n=14; Wilcoxon's signed rank test $P<0.05$). (c.) Table of neuronal subpopulations. Cells were characterised as facilitated or inhibited if activity was changed to a greater extent than 10% from baseline values, or unchanged.

### 3.4.9 The effect of strychnine on receptive field size in the postnatal period

Cutaneous receptive field data is presented as a percentage of the total plantar paw surface area and graphed out both as a maximal or total receptive field area before and after strychnine application (Figure 3. 12), as well as separated by modality into pinch-sensitive and brush-sensitive receptive fields (Figure 3. 13 and Figure 3. 14 respectively). Maximal receptive field size was found to increase by 132% in mature dorsal horn neurons from a mean baseline size of 6.9 ± 1.3% of total plantar area to 13 ± 1.9% post strychnine (Wilcoxon's signed rank test P= 0.015; Figure 3. 12).

Receptive field areas of P3 neurons in contrast were not found to be affected by strychnine application (7.4 ± 1.5% of total foot area at baseline to 4.0 ± 1.73% after strychnine treatment).




c.

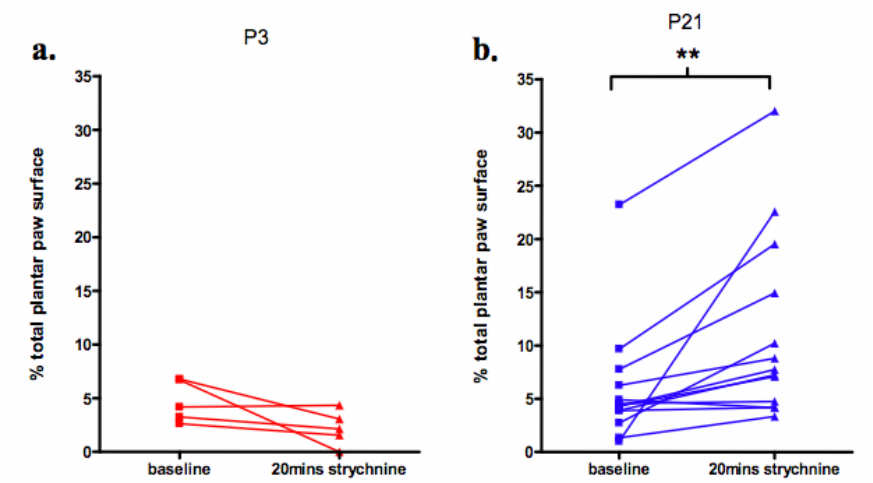
| | facilitated | inhibited | unchanged |
|---|---|---|---|
| P3 | 0 | 3 | 2 |
| P21 | 10 | 0 | 3 |

**Figure 3. 12: The effect of 165 ng/g strychnine on maximum receptive field size of spinal dorsal horn neurons in 3-day and 21-day old rats.**

(a.) Strychnine application did not significantly affect the total receptive field area of P3 dorsal horn neurons (n=5), but (b.) significantly increased the receptive field in dorsal horn neurons of P21 rats (n=13; Wilcoxon's signed rank test $P<0.01$). (c.) Table of neuronal subpopulations. Cells were characterised as facilitated or inhibited if activity was changed to a greater extent than 10% from baseline values, or unchanged.

Receptive field size was also separated into pinch and brush-sensitive modalities. Strychnine application to the spinal cord was found to increase both the pinch and brush-sensitive receptive fields of P21 neurons up to two fold, most notably, brush sensitive receptive field increased up to 240% from baseline (pinch receptive field increased from mean of 7.8 ± 2% total plantar foot surface at baseline to 14.8 ± 2.6% 20 minutes after strychnine; Figure 3. 13; Wilcoxon's signed rank test P= 0.0024; brush from baseline mean 6 ± 1.5% to 11.3 ± 2.2% after strychnine; Wilcoxon's signed rank test P= 0.0012; Figure 3. 14). P3 dorsal horn neuron brush or pinch-sensitive receptive fields did not significantly change in size after strychnine as

compared to baseline (mean baseline pinch receptive field 2.3 ± 0.7% to 1.8 ± 0.8% of total plantar paw surface post strychnine (Figure 3. 13; mean baseline brush receptive field 5.1 ± .8% to 2.5 ± 0.73% post strychnine; Figure 3. 14)

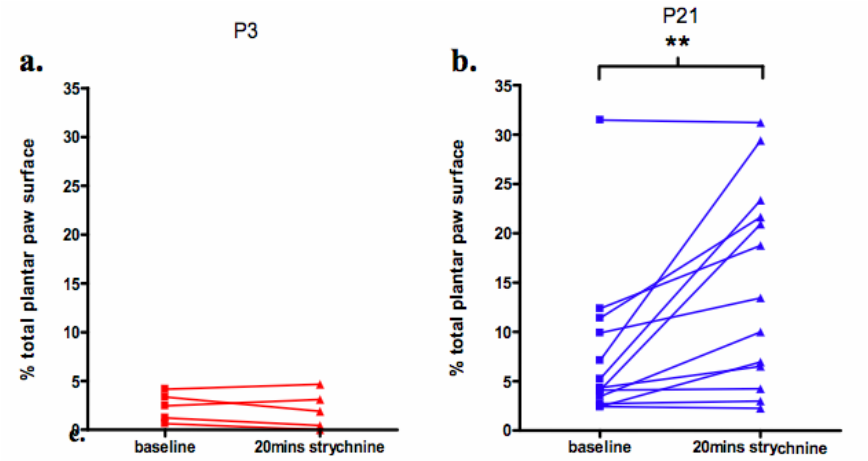



c.

| | facilitated | inhibited | unchanged |
|---|---|---|---|
| P3 | 0 | 2 | 3 |
| P21 | 10 | 0 | 3 |

**Figure 3. 13: The effect of 165 ng/g strychnine on pinch-sensitive receptive field size of spinal dorsal horn neurons in 3-day old and 21-day old rats.**

(a.) Strychnine application did not significantly affect the pinch-sensitive receptive field area size of P3 dorsal horn neurons (n=5), but (b.) significantly increased that of dorsal horn neurons in P21 rats (n=13; Wilcoxon's signed rank test $P<0.01$). (c.) Table of neuronal subpopulations. Cells were characterised as facilitated or inhibited if activity was changed to a greater extent than 10% from baseline values, or unchanged.




c.

| | facilitated | inhibited | unchanged |
|---|---|---|---|
| P3 | 0 | 2 | 3 |
| P21 | 10 | 0 | 3 |

**Figure 3. 14: The effect of 165 ng/g strychnine on brush-sensitive receptive field size of spinal dorsal horn neurons in 3-day old and 21-day old rats.**

(a.) Strychnine application did not significantly affect the brush-sensitive receptive field area size of P3 dorsal horn neurons (n=5), but (b.) significantly increased that of dorsal horn neurons in P21 rats (n=13; Wilcoxon's signed rank test $P<0.01$). (c.) Table of neuronal subpopulations. Cells were characterised as facilitated or inhibited if activity was changed to a greater extent than 10% from baseline values, or unchanged.

| | P3 | | | P21 | | |
|---|---|---|---|---|---|---|
| **stimulus** | **% change from baseline** | **N** | **significance** | **% change from baseline** | **N** | **significance** |
| **Spontaneous activity** | 1 ± 24 | 9 | **NS** | 431 ± 195 | 14 | ** |
| **Pinch** | -25 ± 16 | 9 | **NS** | 186 ± 88 | 14 | ** |
| **vFh t-2** | 142 ± 74 | 14 | **NS** | 244 ± 103 | 29 | ** |
| **vFh t** | 82 ± 92 | 14 | **NS** | 117 ± 38 | 29 | ** |
| **vFh t+2** | -52 ± 12 | 14 | ** | 55 ± 24 | 29 | **NS** |
| **Brush** | -52 ± 14 | 9 | * | 120 ± 31 | 14 | * |
| **Pinch receptive field area** | -49 ± 23 | 5 | **NS** | 130 ± 41 | 13 | ** |
| **Brush receptive field area** | -48 ± 22 | 5 | **NS** | 238 ± 161 | 13 | ** |
| **Maximum receptive field area** | -45 ± 19 | 5 | **NS** | 132 ± 48 | 13 | * |

**Table 3. 1: Summary of the effect of 165 ng/g of strychnine on dorsal horn neuronal activity of P3 and P21 rats.**

Data is presented as mean and standard error of the mean of the percentage change of spiking per second from baseline. vFh t-2 = subthreshold von Frey hair, vFh t = threshold von Frey hair, vFh t+2 = suprathreshold von Frey hair. Statistical analysis: Wilcoxon's signed rank test; NS= not significant *= $P<0.05$; **= $P<0.01$.

## 3.5 Discussion

The spinal cord serves as the first integrative point of primary afferent sensory information from the body in the central nervous system; sensory information entering the spinal cord from the periphery is transmitted to higher centres as well as relayed locally, where it can be modulated and controlled by both excitatory and inhibitory neurotransmission from local interneurons. The balance between excitation and inhibition is therefore crucial to maintaining a responsive and functional spinal circuit, both in terms of tonic activity and in response to afferent cutaneous sensory information. Previous studies have indicated that this balance is immature in the early postnatal period, leading to hyperexcitable reflexes and sensitivity to innocuous stimuli that are characteristic of young animals and humans (Fitzgerald, 1985, 1987a; Fitzgerald *et al.*, 1988; Andrews & Fitzgerald, 1994; Jennings & Fitzgerald, 1996, 1998; Fitzgerald, 2005).

Chapter Two illustrated a developmental change in the circuitry involved in glycinergic transmission over the first three postnatal weeks, but the physiological relevance of this remains unknown. In this study I aimed to examine to test the following hypotheses: (i) the protein expression shifts outlined in Chapter Two underlie a physiological change in the activity of glycine in the control of spinal sensory transduction in the first three weeks of life, and (ii) glycinergic control of dorsal horn processing of low threshold mechanical sensory information is absent in the first postnatal week. To test these I recorded from individual wide dynamic range neurons in the spinal dorsal horn of P3 and P21 rats *in vivo* in the presence of the glycine receptor antagonist strychnine. Strychnine was found to facilitate mature spinal dorsal horn neuron responses to noxious and innocuous stimuli and increase spontaneous activity, in agreement with previous findings (Yokota *et al.*, 1979; Yaksh, 1989; Sherman & Loomis, 1994; Sivilotti & Woolf, 1994; Sherman & Loomis, 1996; Sorkin & Puig, 1996). However, glycinergic inhibition was found to be absent in the intact neonatal dorsal horn. Notably, strychnine inhibited brush-evoked activity of P3 neurons implying glycine in the facilitation of low threshold mechanical sensory transduction in the early postnatal period.

### 3.5.1 Tonic glycinergic activity is not functional in the immature spinal dorsal horn

Intrinsic tonic glycinergic transmission is known to modulate activity in the adult dorsal horn, as witnessed at both the behavioural and cellular level (Yaksh, 1989; Sivilotti & Woolf, 1994; Cronin *et al.*, 2004) but whether tonic glycinergic control of neurons is present from birth has not been previously examined in the intact animal. Spontaneous firing of spinal dorsal horn neurons was monitored in the presence of strychnine in order to assess the role of glycine in tonic inhibition in the first and third weeks of postnatal life. Strychnine was found to have no effect on the spontaneous firing of immature dorsal horn neurons *in vivo*, in contrast to the five-fold increase over baseline values observed at P21. Although the level of baseline spontaneous firing is lower than in adult (see Figure 3. 5 and Figure 3. 6) - making any small changes in tonic firing of immature neurons potentially difficult to observe, the results of this chapter are in agreement with whole-cell patch clamp study of spinal slices from newborn rats aged P0 – 14. The cited study demonstrated an absence of glycinergic mIPSCs early in the postnatal period, which increase in frequency with age (Baccei & Fitzgerald, 2004), suggesting that the lack of tonic glycinergic activity found in the present study is not merely due to low firing properties of the immature spinal sensory system.

Work performed in motor and auditory systems have shown a clear shift from a primarily $GABA_A$ receptor mediated inhibitory tone at birth towards a greater role of glycinergic inhibition later in postnatal life and the late introduction of glycine-receptor only synapses by the third week (Takahashi *et al.*, 1992; Berki *et al.*, 1995; Kotak *et al.*, 1998; Baccei & Fitzgerald, 2004; Bremner *et al.*, 2006; Rajalu *et al.*, 2009). A possible physiological reason for this switch has been revealed in *in vitro* whole cell patch-clamp studies in both cultured neurons and spinal slices. In early developmental period, both GABAergic and glycinergic currents have been shown to result in calcium influx in the early postnatal period allowing for Hebbian strengthening of synapses (Wu *et al.*, 1992; Reichling *et al.*, 1994; Wang *et al.*, 1994; Gao & Ziskind-Conhaim, 1995; Serafini *et al.*, 1995; Gao *et al.*, 2001a). Even after birth when depolarisations are no longer excitatory (Baccei & Fitzgerald, 2004), the slow kinetics of depolarising GABAergic currents could allow for calcium influx and

further activity-dependent reinforcement of synaptic contacts in the first few days of life. By P7, GABAergic currents are inhibitory and hyperpolarising, but the slow kinetics of these currents would mean that, although effective, inhibition would be 'sluggish'. The appearance and increasing weight of importance of fast glycinergic inhibition later in development would then allow for fast inhibition of cutaneous afferent information in the dorsal horn and the fine-tuning of cutaneous responses seen in mature systems.

### 3.5.2 Glycinergic inhibition of cutaneous sensory processing is absent in the neonatal spinal dorsal horn

The role of glycine in transmission of evoked activity was examined using a range of natural mechanical stimuli to investigate the nature of its role in phasic evoked inhibition over the postnatal period. This ranged from noxious pinch, mechanical pressure evoked from von Frey hair application, and innocuous brush, in order to examine the role of glycinergic transmission in the processing of low threshold dynamic stimuli.

#### *3.5.2.1 Strychnine increases nociceptive-evoked dorsal horn neuronal activity in the mature dorsal horn*

Noxious pinch and suprathreshold von Frey hair application were used to examine the effects of glycinergic inhibition on the spinal processing of a suprathreshold stimulus applied to the cutaneous receptive field. As expected from previous studies, in the adult cell firing to noxious pinch was found to be significantly increased following glycine receptor antagonism (Yaksh, 1989), this did not however affect pinch-evoked activity of neonatal neurons implying glycinergic control of noxious stimuli is absent in the first week of life. Surprisingly, suprathreshold von Frey hairs did not reveal the same results: no significant change in firing to suprathreshold von Frey hair application from baseline was seen in mature neurons treated with strychnine, whilst it significantly decreased von Frey hair evoked action potential firing of immature neurons. The lack of significant effect on suprathreshold-evoked

firing in mature neurons is in agreement with recent findings by Miraucourt and colleagues (Miraucourt *et al.*, 2009). However, the enhanced neonatal response to suprathreshold von Frey hair application is less evident given the lack of effect of strychnine on pinch-evoked activity. One possible explanation is that the pinch stimulus was not as reliable as that provided by a graded suprathreshold von Frey hair. Although forceps were adapted to minimise variability in the drive applied (see Methods), it is unclear whether this was enough to control the force applied onto the foot, resulting in the large error seen in the pinch responses. Alternatively, this could reveal a surprising glycinergic role in the transmission of pressure-stimuli in the early postnatal period. Further studies will have to be conducted with a more consistent stimulus, such as noxious heat or Randall Selitto tests, to understand the underpinnings of these findings and the role of glycine in the control of noxious sensory information at this early time point.

#### *3.5.2.2 Glycine is involved in the facilitation of innocuous brush-evoked activity in the neonate*

Strychnine significantly altered brush-evoked firing of both P21 and P3 neurons: whereas the number of brush-evoked spikes fired significantly increased following glycine receptor antagonism in mature neurons, this was found to significantly decrease activity to the same stimulus in immature animal. Strychnine-induced sensitivity to dynamic innocuous stimuli is well reported (Yaksh, 1989; Sivilotti & Woolf, 1994; Miraucourt *et al.*, 2009), but the resulting decreased cellular activity at P3 implies an early life facilitatory action of glycine in the transmission of innocuous sensory information in the neonatal spinal dorsal horn. Early in development, GABAergic and glycinergic transmission result in depolarising potentials and calcium influx into the cell (Obata *et al.*, 1978; Connor *et al.*, 1987; Ben-Ari *et al.*, 1989; Ito & Cherubini, 1991), thought to be involved in increasing the activity of neurons in order to aid in the formation and strengthening of glycinergic synapses (Serafini *et al.*, 1995; Kneussel & Betz, 2000a, b). A tempting conclusion for this finding could be that glycine is acting as an excitatory neurotransmitter at this age. This is however unlikely, as such excitation has not been seen to occur in whole-cell patch-clamp studies in neonatal spinal cord slices (Baccei & Fitzgerald, 2004), where

$GABA_A$ receptor-mediated depolarisations were never sufficient to produce action potentials in the early postnatal period. Early reports of excitatory depolarising $GABA_A$- receptor mediated potentials in immature brain slices have also recently been shown to be the result of an artefact that occurs as a consequence of recording in artificial cerebrospinal fluid in which the high-energy requirements of neonatal neurons are not being adequately met (Holmgren *et al.*, 2009; Rheims *et al.*, 2009). From these results it would appear that although inhibition is functionally immature in the first week of postnatal development, GABAergic and glycinergic inputs are inhibitory from birth.

Another explanation could be that glycine-mediated facilitation of innocuous stimuli, which would enhance Aβ fibre mediated activity in early life, is critical in the functional tuning of mature nociceptive networks. Indeed it has been shown by means of *in vivo* patch-clamp experiments that Aβ fibre strength innocuous stimulation of the receptive field of a mature lamina II spinal neuron resulted in a barrage of both excitatory postsynaptic potentials (EPSPs) and inhibitory postsynaptic potentials (IPSPs) (Narikawa *et al.*, 2000). These potentials lasted throughout the stimulus, whilst noxious stimulation (pinch) of the same receptive field resulted in an increase in EPSCs during the pinch but IPSCs at the onset and offset of the stimulus alone. This could suggest an inhibitory role in the tuning of non-noxious inputs, which may in turn play a role in the transmission of noxious information to superficial dorsal horn neurons in the mature systems. The critical role for innocuous touch-mediated tuning of noxious reflexes has also been investigated in the whole behaving animal. Key experiments investigating the tuning of a reflex tail flick response to noxious laser found that neonatal rats tended to move their tails erroneously towards the noxious stimulus. This behavioural response is observed until the second postnatal week when reflexes matured and the tail would move away from the harmful stimulus. Interestingly this was found to be dependent on low threshold tactile input but not noxious input, such that mature tuning was prevented by local anaesthetic administration over the first ten days of life, but not sped up by repeated noxious stimuli (Waldenstrom *et al.*, 2003). The role of glycine could thus be to enhance innocuous stimuli in order to encourage tuning of reflex responses without the need for harmful C fibre strength stimuli.

### 3.5.3 Possible mechanisms underlying the developmental shift of glycinergic signalling

Inefficient glycinergic inhibition described in this chapter could be the result of several changes: 1) reduced inhibitory neurotransmitter release; 2) reduced availability of inhibitory receptors; 3) imprecise termination of inhibitory terminals; 4) reduced descending inhibitory control.

***1. Reduced glycinergic release in the developing dorsal horn***

Glycine has been shown to be present in the spinal cord from embryonic day 16-18 (Berki *et al.*, 1995; Allain *et al.*, 2006), but may not be present at sufficient levels to activate postsynaptic glycine receptors in the early postnatal period. The lack of mIPSCs in the superficial dorsal horn of spinal slices indicates that although glycine is present and can be released, it is not being released at levels sufficiently high to activate postsynaptic receptors in the absence of action potentials (Baccei & Fitzgerald, 2004), which could be the result of immature glycinergic input into the dorsal horn at this age. The lack of GlyT2 in the superficial dorsal horn of neonatal spinal cord described in Chapter Two and its low levels of protein expression (Zafra *et al.*, 1995b) could underlie immature neurotransmitter recycling resulting in suboptimal presynaptic glycine release for the activation of receptors.

***2. Reduced availability of glycinergic receptors***

Immunohistochemical studies performed in Chapter Two outlined the early presence of α subunit-containing receptors in the deep dorsal horn as well as sparse expression in more superficial laminae, but the functional effects of this developmental shift in expression has not been previously examined. Sciatic nerve stimulation of spinal slices at P0 resulted in both glycinergic and GABAergic IPSCs, although the majority seen were GABAergic in origin (Baccei & Fitzgerald, 2004). Interestingly, neurons that lacked glycine receptor-mediated spontaneous IPSCs responded to exogenous glycine application, indicating functional glycine receptors in postsynaptic membranes from birth. As discussed in the previous Chapter, it could be that these receptors are present but not yet located at the appropriate site for interaction with glycine. Indeed, the anchoring of glycine receptors to the postsynaptic site is reliant on the successful binding of the receptor to the auxiliary

protein gephyrin, the binding site for which is located on the β subunit of the glycine receptor (Kirsch & Betz, 1993; Meyer *et al.*, 1995; Todd *et al.*, 1995; Meier *et al.*, 2000). Although the β subunit does not undergo significant expression changes in postnatal development, the primary glycine receptor conformation in the early postnatal period is a homomeric $\alpha_2$ receptor, which lacks the β subunit and so does not possess the gephyrin binding site. This could underline an extrasynaptic location of glycine receptors, which would therefore only respond to spillover of glycine from synapses.

***3. Inefficient inhibitory control***

The dorsal horn is complex, involving disinhibitory circuits critical to the tight regulation of sensory information. Many glycinergic neurons arborise locally, leading to inhibitory control onto inhibitory interneurons within the superficial and deep dorsal horn in adult spinal networks (Todd & Sullivan, 1990; Labrakakis *et al.*, 2009). Within the first two weeks of life inhibition is highly disregulated and poorly organized; whereas inhibition in the adult spinal cord is local and tuned, the immature spinal cord may have a much wider spread of inhibition. The result of which could be a higher likelihood of 'blanket' inhibition, whereby glycine would be acting upon both excitatory and inhibitory interneurons in a non-specific fashion in the early postnatal period. This could in turn lead to a higher level of excitation as a result of un-tuned glycinergic inhibition acting upon inhibitory interneurons, which would release inhibition onto a target neuron leading to indirect glycinergic facilitation, such as was disclosed in this Chapter in response to innocuous brush.

***4. The influence of descending controls on spinal inhibitory networks.***

The role of descending signals from supraspinal centres is crucial in the tuning of spinal nociceptive reflexes and has also been shown to mediate the switch between GABAergic shunting and inhibition through KCC2 transporter expression in developing motor system (Jean-Xavier *et al.*, 2006). Descending influences could also be involved in mediating the switch between primarily GABAergic to glycinergic inhibition in the dorsal horn by an activity-dependent strengthening of glycinergic synapses in the spinal cord. Indeed, supraspinal control over inhibitory neurotransmission has been shown in neonatal rats, where spinal administration of the $GABA_A$ receptor antagonist gabazine in a neonatal rats results in a paradoxical

inhibition of nociceptive behaviour to mechanical stimulation of the hindpaw, an effect that was completely reversed on spinalisation after which gabazine affected neonates in much the same way as it did adults (Hathway *et al.*, 2006). This suggests a tonic descending facilitation onto spinal networks in the neonate that is absent in the adult. The nature of descending control on spinal dorsal horn activity will be dealt with in more detail in Chapter Four.

## 3.6 *Conclusion*

There are two main findings in this chapter. The first is that tonic glycinergic inhibition is absent in the neonatal spinal dorsal horn and is not involved in the inhibition of spontaneous activity until the third postnatal week. Secondly, glycine facilitates sensory transmission of innocuous brush in the first week of life. Interestingly, the response to noxious stimuli was modality dependent. While strychnine attenuated the neuronal response to suprathreshold von Frey hair application in the neonate, it did not alter action potential firing to pinch. This could indicate an early facilitatory role for glycinergic signalling in the fine tuning of innocuous sensory processing, which is only involved in nociceptive processing at a later stage in development.

# Chapter 4

# The Development of Descending Modulation of Dorsal Horn Neurons

## 4.1 Introduction

The first unified theory of pain which acknowledged the influence of supraspinal centres was described by Melzack and Wall in 1965 in their description of the gate theory of pain (Melzack & Wall, 1965). The importance of supraspinal modulation of spinal nociceptive networks has since been widely reported and shown to arise from a number of brain regions (Basbaum & Fields, 1984; Besson & Chaouch, 1987; Fields *et al.*, 2006; Heinricher *et al.*, 2009), but research in the area has largely focussed on projections from the midbrain periaqueductal grey (PAG) and the brainstem region of the rostroventral medulla (RVM). Although the PAG is known to affect spinal neurons it sends few direct projections to the dorsal horn, instead the PAG primarily exerts its spinal effects via projections to the RVM (Gebhart *et al.*, 1983; Prieto *et al.*, 1983; Sandkuhler & Gebhart, 1984; Chung *et al.*, 1987). The RVM also receives direct afferent input from neurons in the superficial dorsal horn (Fields & Basbaum, 1978; Dubner & Bennett, 1983). Brainstem control of spinal nociceptive networks is therefore bidirectional, allowing effective regulation of spinal nociceptive activity. Importantly, descending control from the brainstem is biphasic and dependent upon electrical stimulus strength (or concentration of microinjected pharmacological intervention), as well as ongoing supraspinal activity; although early studies mainly reported the descending inhibitory actions of the brainstem, it is now widely accepted that projections from the same areas can also result in facilitation of nociceptive reflexes depending on stimulus strength or concentration of drug used (Zhuo & Gebhart, 1997). Descending facilitation can be evoked at low stimulus intensity (e.g. 2-20 μA in the above mentioned study) in the adult rat and this has been shown to be a major contributor in neuropathic and inflammatory pain states, where the equilibrium between excitation and inhibition appears to be lacking at the level of the spinal cord (Porreca *et al.*, 2002; Ren & Dubner, 2002).

Projections from the RVM terminate predominantly in laminae I, II and V (Basbaum *et al.*, 1978; Basbaum & Fields, 1979; Ruda *et al.*, 1981; Holstege & Kuypers, 1982), which are known to contain terminals of nociceptive Aδ and C fibres (Fields & Basbaum, 1978; Light & Perl, 1979; Light *et al.*, 1979; Cervero & Iggo, 1980).

Unsurprisingly, descending inhibition of dorsal horn neurons appears to be selective, being strongest for deep dorsal horn cells that receive strong C fibre input and absent or facilitatory for cells that receive primarily Aδ fibre input (Hudson *et al.*, 2000; McMullan & Lumb, 2006a; Heinricher *et al.*, 2009), demonstrating a nociceptive-specific role in the brainstem control of dorsal horn activity.

## 4.2 Functional maturation of descending control

Descending inhibitory controls have been shown to be absent at birth and slow to mature (Fitzgerald, 1991; Hathway *et al.*, 2009). Although serotonergic descending fibres from the brainstem to the spinal cord have grown into the spinal grey matter by birth (Cabana & Martin, 1984; Rajaofetra *et al.*, 1989), these do not reach their targets in the dorsal horn for three weeks (Bregman, 1987). Few studies have investigated the physiological function of these connections in the early postnatal period, early reports provided evidence for inefficient supraspinal inhibition of spinal neuronal activity until P10, which is not fully functional until a week thereafter (Fitzgerald & Koltzenburg, 1986; Boucher *et al.*, 1998). These findings strongly suggest that although the pathways are present, descending inhibition necessary for the development of fine tuned responses is not fully functional at this point. Recently, an electromyographic study examined influence of descending RVM control on mechanical withdrawal magnitudes of intact anaesthetised rats over postnatal development (Hathway *et al.*, 2009). Results showed that not only is descending inhibition not functional until P30, descending control from the RVM is in fact facilitatory until this time, regardless of stimulus strength used, suggestive of a primarily facilitatory role of supraspinal sites onto immature spinal systems. Electromyography is of particular use in quantifying a behavioural response in a lightly anaesthetised preparation, but a complicating factor in the analysis of these results is whether descending control is affecting the motor component of the reflex or whether this control is affecting the sensory nociceptive-specific component of this withdrawal, both of which are tightly linked.

It has recently been reported that descending inhibition in mature rats selectively dampens activity in wide dynamic range neurons with strong C fibre input, whereas Aδ mediated input is facilitated (McMullan & Lumb, 2006a; McMullan & Lumb,

2006b; Koutsikou *et al.*, 2007; Parry *et al.*, 2008; Waters & Lumb, 2008; Heinricher *et al.*, 2009). This is particularly noteworthy, as C fibre inputs to the dorsal horn are known to be weak at birth and slowly strengthen over the next three to four weeks as A fibre input withdraws (Beggs *et al.*, 2002; Baccei *et al.*, 2003; Fitzgerald, 2005). In fact, this occurs much over the same timeline as the development of descending inhibition (Fitzgerald & Koltzenburg, 1986). Early studies have suggested that postnatal onset of descending inhibition is influenced by C fibre afferent input into the spinal dorsal horn as ablation of C fibres by capsaicin treatment in neonates prevents the maturation of descending inhibitory control later in life (Cervero & Plenderleith, 1985; Zhuo & Gebhart, 1994). It is therefore plausible that the weak C fibre inputs in the dorsal horn cannot excite dorsal horn neurons to a large enough degree to recruit a negative feedback loop from the brainstem onto wide dynamic range neurons in the dorsal horn. What is not yet known is whether supraspinal inhibition is selective for C fibre input-rich neurons from the first appearance of descending inhibition at P28 or whether C fibre evoked activity is in fact facilitated by supraspinal sites at this age.

## 4.3 Hypothesis

The focus of many developmental studies has been on the lack of descending inhibition from the brainstem, but a recent study has suggested that immature brainstem-spinal cord connectivity may in fact lead to an imbalance between descending inhibition and facilitation of spinal sensory networks (Hathway *et al.*, 2006). This work suggests that although descending inhibition is possible from P10 (Fitzgerald & Koltzenburg, 1986), it is not strong enough to outweigh descending facilitation of behaviour until adulthood (van Praag & Frenk, 1991; Hathway *et al.*, 2009). However, whether this effect is purely nociceptive, or alternatively whether this is only observed at the motor output level is still unclear. I hypothesise that the rostroventral medulla (RVM) exerts a primarily facilitatory role onto spinal dorsal horn neuron activity until the third week of postnatal life after which time the RVM begins to show biphasic control over dorsal horn neurons. Secondly, I suggest that C-fibre-specific modulation from the brainstem is absent in the first three postnatal weeks and only becomes functional after P21, at a time when C-fibre inputs into the dorsal horn have strengthened and A fibres have withdrawn from the superficial laminae. To test these hypotheses, I performed extracellular recordings of individual spinal dorsal horn neurons in an anaesthetised *in vivo* preparation of 21 day old and 40 day old rats in the presence of RVM stimulation at a range of stimulus intensities. The effect of descending control on both spontaneous activity and that of nociceptive-evoked dorsal horn firing was examined using a range von Frey hairs and electrical stimulation of the cutaneous receptive field.

Some of these studies have previously been published elsewhere (Hathway *et al.*, 2009).

## 4.4 Methods

### 4.4.1 *In vivo* extracellular recordings

Sprague–Dawley rats of both sexes aged postnatal day 21 (P21) and 40 (P40) were used in these studies. Animals were allowed free access to water and food and were housed in 12-hour light/dark cycles. P21 and P40 rats were caged according to sex in cages of six littermates. All experiments were conducted in accordance with the United Kingdom Animals (Scientific Procedures) Act of 1986.

Rats were anaesthetised with isoflurane to achieve areflexia (induction at 3.5% isoflurane in medical $O_2$) and tracheotomised, after which the cannula was sutured in place. The animal was then mounted in a stereotaxic frame (Kopf Instruments, CA) and artificially ventilated using a ventilator pump (Small animal ventilator, model 687, Harvard Apparatus Inc.) under constant isoflurane-anaesthesia (maintenance of 1.8% in medical $O_2$, Univentor Anaesthesia Unit 400, Royem Scientific, UK) at 79 breaths per minute. The air-flow was adjusted according to the animal's size, blood perfusion and heart rate (target of 300-450 beats per minute), which was being monitored via electrocardiogram. A homeothermic blanket with probe sensor, and heating lamp were used to maintain body temperature at physiological levels (36ºC). Animals were mounted via hip and ear bars, and a laminectomy was performed to expose the lumbar spinal cord, the vertebral column secured with a clamp to the thoracic spine and the dura and pia mater removed. A subcutaneous injection of saline was given to each animal post laminectomy to maintain hydration and a thin film of mineral oil was used to cover the exposed spinal cord to prevent heat loss and excessive drying of the cord. Once stabilised, a 10 μM tipped glass-coated tungsten microelectrode was lowered onto the surface of the cord under microscopic vision, with a reference electrode inserted into the muscle near the recording area. Neuronal activity was passed through a x1 headstage amplifier and a further x 5k differential amplifier (NeuroLog, Digitmer, UK). This signal was passed through low and high pass filters, set at 1 kHz and 10 kHz respectively and onto a spike trigger, which is set manually to produce output TTL pulses for spikes above a particular voltage, an audio amplifier as well as through an oscilloscope (TDS 2012 digital storage

oscilloscope, Tektronix). Information was also fed into a PowerLab system (4SP, AD Instruments, UK), which was connected to a computer in order to allow recording and analysis of data using Chart 5 software (Chart 5 version 5.5.5, AD Instruments, UK).

In order to isolate individual neurons in the spinal cord, the microelectrode was lowered through the cord in 2- or 10 μm steps by a microdrive (Epson HX-20, Seiko Epson Co. powered by Digitimer SCAT-01 microelectrode stepper system, Digitmer, UK). Stroking of the plantar skin of the hind paw was used as a search stimulus and cells were selected once a reliable action potential spike amplitude and shape could be distinguished from background noise levels. Wide dynamic range neurons were selected such that each neuron tested responded to light touch and to noxious pinch applied to the centre of the receptive field.

### 4.4.2 RVM stimulation

Once animals were anaesthetized, tracheotomised and mounted on a stereotaxic frame (Kopf Instruments, Tujunga, California, USA), as mentioned above, the skull was exposed and bregma located. Stereotaxic co-ordinates for RVM were calculated (adult, lateral 0 mm, antero-posterior 9.7 mm, dorso-ventral −10 mm; postnatal day 21 (P21), lateral 0 mm, antero-posterior 9.2 mm, dorso-ventral −10.0 mm (Hathway *et al.*, 2009)). A concentric bipolar stimulating electrode was lowered into the RVM using the co-ordinates above. Once an individual cell was isolated in the dorsal horn, as outlined above, and baseline responses recorded, stimulation was repeated in conjunction with electrical stimulation of the RVM, which lasted for the duration of each test stimulus. This lasted for around 2 minutes per stimulus strength with a 1 minute break between RVM stimuli to allow for afterdischarge firing (see Figure 4. 1 and

Figure 4. 2). Trains of stimuli of 500 μs pulse width were applied at 10 Hz, at amplitudes ranging from 5 to 200 μA using a stimulus isolator (Neurolog, Digitimer, Welwyn Garden City, UK). Stimulus parameters were chosen as these have been

shown to evoked reliable descending inhibition and excitation in previously published work (Zhuo & Gebhart, 1997; Hathway *et al.*, 2009).

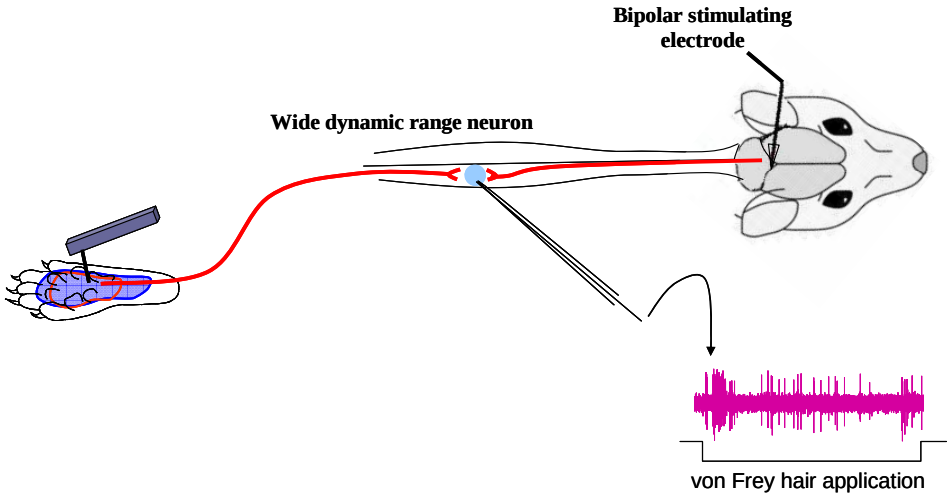



**Figure 4. 1: Schematic of in vivo preparation in which RVM stimulation can be performed whilst recording extracellularly from individual dorsal horn neurons.**

See text for details. Modified from (Keller *et al.*, 2007).

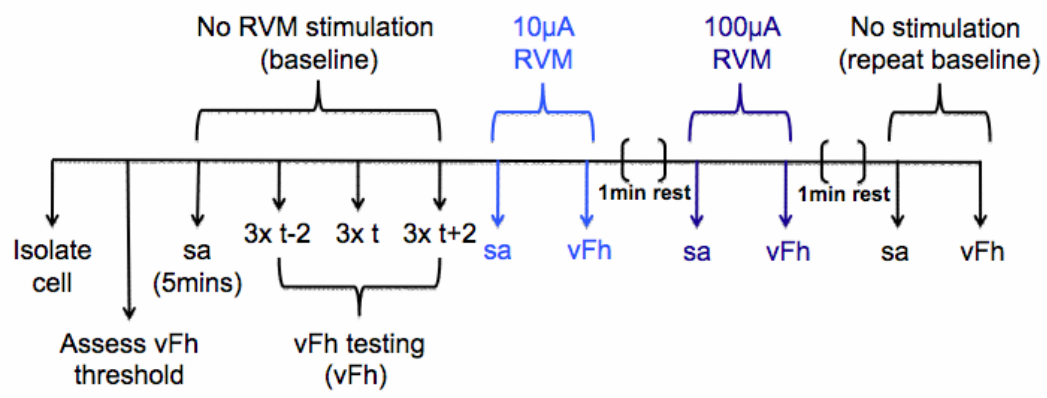



**Figure 4. 2: Schematic of the von Frey hair/RVM stimulation protocol.**

vFh: von Frey hair; t-2: subthreshold von Frey hair; t: threshold von Frey hair; t+2: suprathreshold von Frey hair; sa: spontaneous activity (1 minute); 10 µA/100 µA RVM: stimulation intensity of the RVM.

Spontaneous activity of the cell was recorded over 60 seconds and von Frey Hair threshold was established as the lowest hair needed to reliably evoke spikes (see Figure 3. 3). Neuronal firing to three hairs was recorded: threshold von Frey hair, subthreshold: two hairs below threshold, and suprathreshold: two hairs above the

threshold. Von Frey hairs were each applied three times in succession and the mean number of spikes evoked to each stimulus was then used for analysis. Neuronal activity was defined for both age groups as being facilitated or inhibited if activity during stimulation increased or decreased by more than 10% of the baseline value.

All recordings were fed into Chart software (Chart 5 version 5.5.5, AD Instruments, Chalgrove, UK), and data was analysed in GraphPad Prism 3.0 (GraphPad Software, San Diego, CA, USA, www.graphpad.com).

Statistical analysis was performed using GraphPad Prism 3.0 (GraphPad Software, San Diego, CA, USA, www.graphpad.com); data is presented as mean ± standard error of the mean (s.e.m.) unless otherwise stated. Wilcoxon's non-parametric signed rank tests were used to test for significance between treatments at a given age. One-way analysis of variance (ANOVA) was used to compare between ages followed by Dunnett's post hoc multiple comparisons test for significant values. For all data a 95% confidence interval was used as a measure of statistical significance.

Animals were killed with an overdose of sodium pentobarbitone (i.p.) at the end of the experiment and the accuracy of stimulating electrode placement assessed.

### 4.4.3 Hindpaw electrical stimulation in P21 and P40 rats

Compound action potential recordings were performed in P21 and P40 rats in order to establish which afferent groups were excited by peripheral electrical stimulation of the receptive field at the two ages tested. Rats were anaesthetised with isoflurane (induction at 3.5% isoflurane in medical $O_2$) and tracheotomised, mounted and monitored as outlined above. The hindlimb was suspended at the ankle and sciatic nerve exposed and isolated by means of a thin film of plastic at the level of the thigh. The nerve was gently pried apart using finely pulled glass forceps and mounted onto silver wire for recording through the Neurolog system as mentioned above. Filters were opened to low frequency direct current setting and 150 Hz high frequency. Two insect pins were inserted into the ankle 5 mm apart, and a current delivered via a stimulus isolator box (NL800, NeuroLog), which was connected to the pins using

crocodile clips. Trains of 1 mA, 5 mA and 10 mA at 50 μsec delay width and 500 μsec delay width were used to target A, and C fibres respectively.

#### *4.4.3.1 Experimental protocol*

Pilot experiments involving electrical stimulation of the cutaneous receptive field of the hindpaw were used to target A and C-fibres whilst stimulating descending controls in 6 P40 neurons and 9 P21 neurons in order to better dissect the specificity of descending controls on afferent-evoked activity of dorsal horn neurons throughout the postnatal period.

Laminectomy and stereotaxic placement of the bipolar concentric electrode into the RVM was performed as described above and an individual neuron isolated. Two insect pins were inserted into the hindpaw receptive field of the isolated neuron 2 mm apart, and a current delivered via a stimulus isolator box (NL800, NeuroLog) which was connected to the pins using crocodile clips. Baseline responses to a train of ten 10 mA impulses at 50 μsec delay width (A fibre-targeting electrical stimulus) and 500 μsec delay width (C fibre-targeting electrical stimulus) at 1 Hz were recorded using software mentioned previously. Stimulus strength was chosen in order to reliably target each afferent fibre at both ages. Sets of peripheral stimulation were separated by one-minute rest periods to allow for afterdischarge firing. Each stimulus was repeated with and without simultaneous 10 μA or 100 μA RVM stimulation in order to tease out the effect of descending control on different primary afferent inputs at the level of individual neurons as outlined in Figure 4. 3.

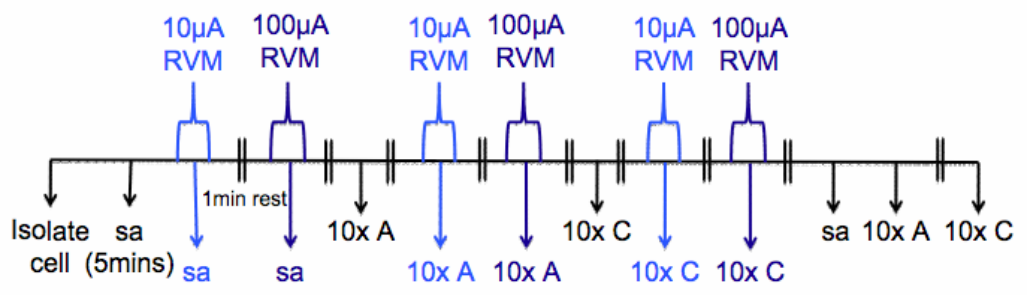



**Figure 4. 3: Schematic of experimental protocol of electrical hindpaw/RVM stimulation experiments.**

sa: spontaneous activity (1 minute); 10 μA/100 μA RVM: stimulation intensity of the RVM; 10x A: ten repeats of A-fibre strength electrical stimuli; 10x C: ten repeats of C-fibre strength electrical stimuli. See Methods text for details.

Analysis of neuronal responses was completed as follows: A fibre-mediated cellular spiking was determined as spikes generated within the first 50 milliseconds of the foot stimulus. C fibre evoked spiking was determined as action potential firing 50-130 milliseconds after the stimulus. An average of the number of action potentials fired in response to each of the ten stimuli was used as a measure of the neuronal response. Stimuli were repeated at the end of the experiment in the absence of RVM stimulation and responses not found to be significantly different from baseline.

For a single cell, neuronal responses to each of the ten individual C fibre strength stimuli was plotted in an accumulative plot in order to identify trends in cellular excitability. Number of spikes at stimulus ten therefore represents all spikes fired up to, and including, that point, i.e. total number of action potentials fired to all ten stimuli.

Statistical analysis was performed using GraphPad Prism 3.0 (GraphPad Software, San Diego, CA, USA, www.graphpad.com); data is presented as mean ± standard error of the mean (s.e.m.) unless otherwise stated. Wilcoxon's non-parametric signed rank tests were used to test for significance between treatments at a given age. One-way analysis of variance (ANOVA) was used to compare between ages followed by Dunnett's post hoc multiple comparisons test for significant values. For all data a 95% confidence interval was used as a measure of statistical significance.

Animals were killed with an overdose of sodium pentobarbitone (i.p.) at the end of the experiment, and the accuracy of stimulating electrode placement assessed.

## 4.5 Results

### 4.5.1 Electrophysiological properties of recorded cells

#### *4.5.1.1 Recording depths*

A total of 39 dorsal horn neurons from P21 rats, and 22 neurons from P40 rats were used for this group of experiments. Mean recording depths, as measured from the surface of the spinal white matter, were 340.4 ± 27.6 μm for P21 neurons, which was not significantly different from recording depths of P40 neurons (mean depth 266.73 ± 26.0 μm; Figure 4. 4). As outlined in NeuN immunohistochemical studies in Chapter Two, spinal cord size is comparable between the two ages (Figure 2.2).

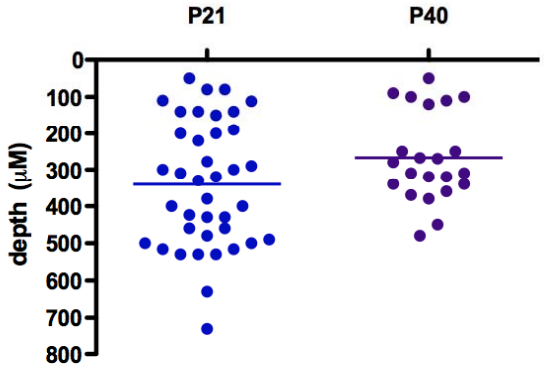



**Figure 4. 4: Depths of recorded spinal dorsal horn cells for RVM stimulation experiments.**
P21 (n= 39); P40 (n= 22).

#### *4.5.1.2 Mechanical thresholds*

Mechanical threshold was assessed using von Frey hair filaments applied to the cutaneous receptive field. The lowest force hair needed to evoke action potential firing was denoted the 'threshold hair'. Cutaneous mechanical thresholds did not

differ significantly between ages (P21: 2.26 ± 0.39 g (n = 30) versus P40: 3.74 ± 0.78 g (n = 15); Figure 4. 5a).

#### *4.5.1.3 Baseline spontaneous activity*

Baseline spontaneous firing properties of the recorded neurons were recorded over a period of 1 minute. Spontaneous firing of P21 neurons was 5.99 ± 3.15 Hz (n = 24), whilst that of P40 was 0.18 ± 0.04 Hz (n = 15). Although the significant variability in baseline firing properties of P21 neurons meant that the mean was heavily skewed; no significant difference in spontaneous activity was found between ages (Figure 4. 5b).

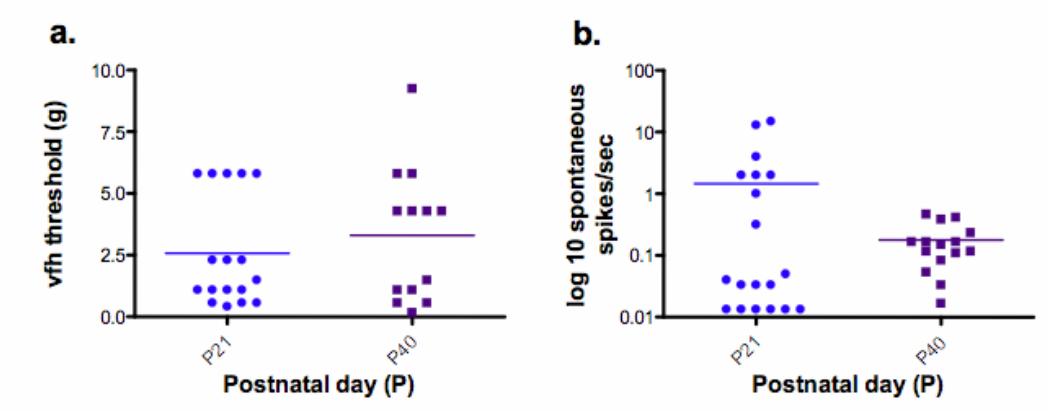



**Figure 4. 5: Baseline properties of recorded cells.**

(a.) Mechanical thresholds (P21: n= 17, P40: n = 13), and (b.) spontaneous activity (P21 : n = 24, P40 : n = 15) were not significantly different between P21 (blue) and P40 (purple) cells.

### 4.5.2 The effect of graded RVM stimulation on spontaneous activity of dorsal horn neurons from P21 and P40 rats.

Spontaneous activity of P21 and P40 spinal dorsal horn neurons was recorded over 60 seconds at baseline and then following 5, 10, 20, 50, 100 and 200 μA RVM stimulation.

Spontaneous activity during 10 μA and 100 μA electrical stimulation of the RVM was compared to baseline results as these stimulus strengths were shown to reliably

produce descending facilitation and inhibition respectively in the adult rat (Hathway *et al.*, 2009). RVM stimulation significantly increased spontaneous activity of P21 dorsal horn neurons (one way ANOVA over baseline, 10 μA and 100 μA values, P= 0.011 Figure 4. 6a and b) but did not affect spontaneous activity at P40 (Figure 4. 6a and c; two way ANOVA comparing age and RVM stimulus, P< 0.0001). Baseline values of P21 neurons increased over two fold from mean 1.47 ± 0.72 spikes/second to 3.81 ± 0.94 spikes/second during 10 μA RVM stimulation (Wilcoxon's matched pairs signed rank test between baseline and 10 μA stimulation, P= 0.012) but not during 100μA RVM stimulation (100 μA stimulation mean spontaneous activity: 2.34 ± 0.56 Hz). RVM stimulation did not have any significant influence on spontaneous firing of P40 mature neurons at either RVM stimulus intensity (baseline firing 0.179 ± 0.036 Hz; 10 μA RVM stimulation: 1.183 ± 0.53 spikes/second; 100 μA stimulation: 0.106 ± 0.04 spikes/second; Figure 4. 6a and c).

For further analysis of the effect of RVM stimulation on overall populations effects, cells were classified as excited, inhibited or unaffected (see Methods). Both 10 μA and 100 μA RVM stimulation amplitudes resulted in excitation of 60% of recorded P21 cells (16/27). Conversely, the response of P40 cells was heterogeneous at 10 μA, and primarily inhibited at 100 μA RVM stimulation (11 neurons out of 15 inhibited). RVM stimulation was therefore found to have significantly different effect upon the population responses of P21 and P40 neurons ($\chi^2$ analysis between P21 and P40 cell populations at 100 μA, P= 0.0014).

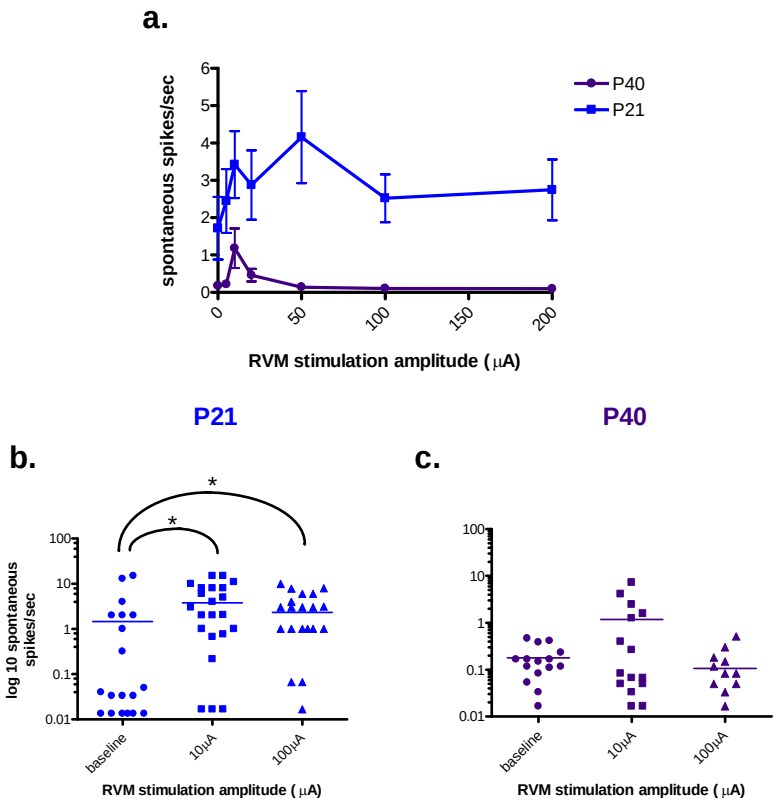



**Figure 4. 6: The effect of graded RVM stimulation on spontaneous activity of spinal dorsal horn neurons of P21 and P40 rats.**

(a.) Spontaneous activity of P21 dorsal horn neurons (n=28) in response to a range of RVM stimulation intensities was significantly different to that of P40 neurons (n=15; two way ANOVA, $P<0.0001$). (b) Activity of P21 neurons significantly increased following RVM stimulation at 100 μA ($P<0.05$) but was not significantly affected at lower intensity stimulation. (b.) Spontaneous activity of P40 neurons was unaffected by either 10 μA or 100 μA RVM stimulation as compared to baseline values. Population response of P21 and P40 neurons to 100 μA RVM stimulation was significantly different ($\chi^2$ analysis, $P<0.01$).

### 4.5.3 The effect of RVM stimulation on pooled dorsal horn population response to graded von Frey hair stimulation of the cutaneous receptive field.

Responses to subthreshold, threshold and suprathreshold von Frey hairs on the cutaneous receptive field were investigated to examine the nature of descending facilitation and inhibition on cutaneous sensory processing over the late postnatal period. A summary of these results can be found in Figure 4.7 and Figure 4. 11.

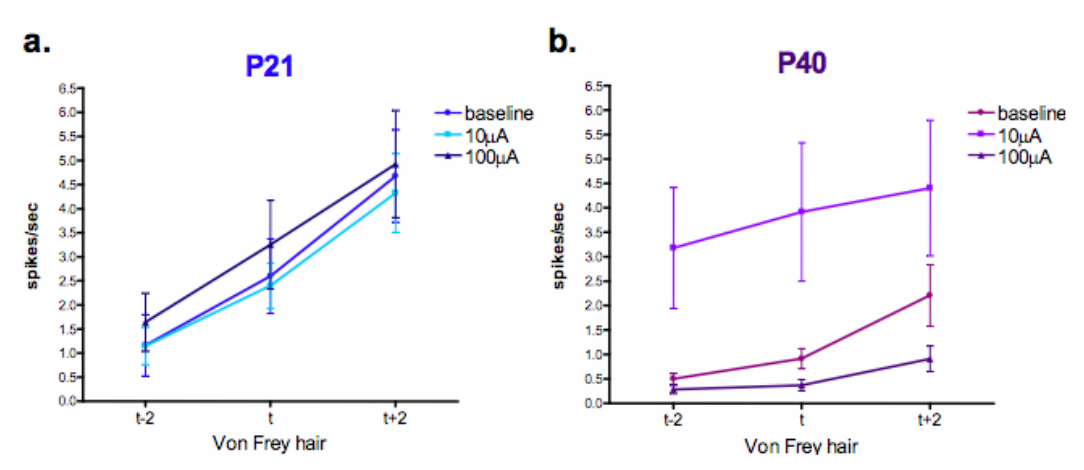



**Figure 4. 7: The effect of graded RVM stimulation on the response of P21 and P40 dorsal horn neurons to subthreshold, threshold and suprathreshold von Frey hair application to the cutaneous receptive field.**

The response of (a.) P21 (n= 30) and (b.) P40 dorsal horn neurons (n= 15) to subthreshold von Frey hair application to the receptive field in the presence of 10µA and 100µA RVM stimulation.

### *4.5.3.1 Subthreshold von Frey hair*

It could be argued that a cell which was excited by a subthreshold von Frey hair had an effectively lowered threshold in response to RVM stimulation, which could be misleading when analysing changes in neuronal sensitivity. The data here was analysed in this way in order to give an overall picture of a shift in excitability of dorsal horn neurons.

Subthreshold von Frey hair stimulation continued to evoke little or no firing of P21 neurons in the presence of RVM electrical stimulation (mean baseline firing: 1.16 ± 0.64 spikes/second; 10 µA: 1.14 ± 0.39 spikes/second; 100 µA: 1.64 ± 0.6 spikes/second; Figure 4. 7a) and subpopulation response to both 10 µA and 100 µA RVM stimulation was heterogeneous (Figure 4. 8).

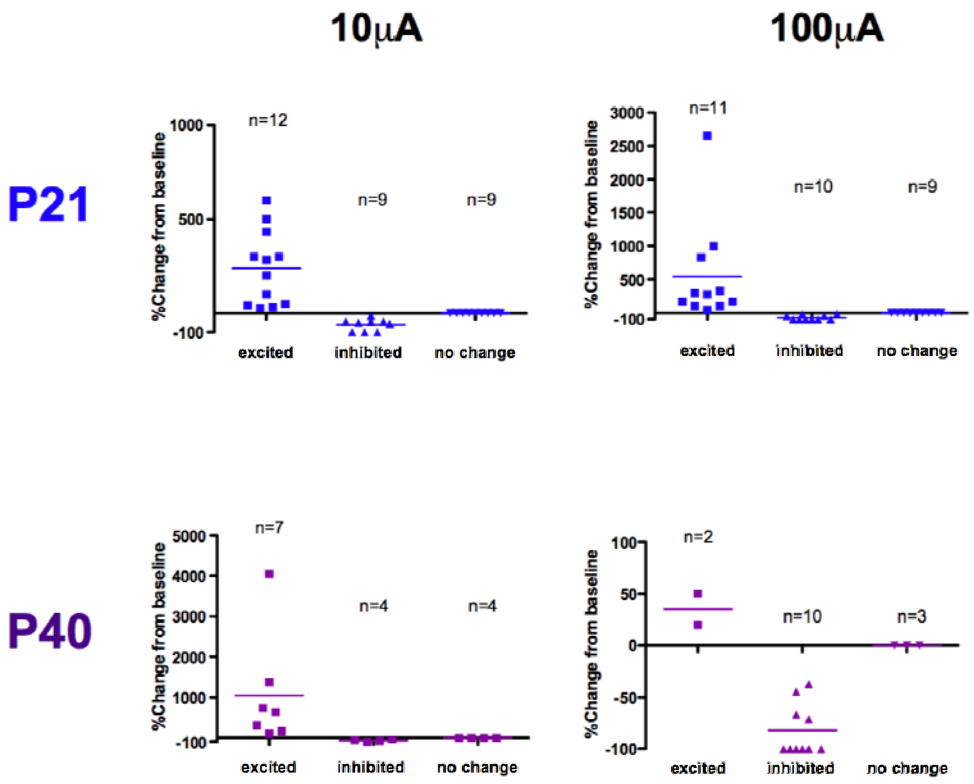



**Figure 4. 8: Subpopulations cellular response to subthreshold von Frey hair application.**
Cells were characterised as facilitated or inhibited if activity was changed to a greater extent than 10% from baseline values, or unchanged. Response of subthreshold-evoked activity of P21 neurons was heterogeneous at all RVM intensities tested, but 100μA RVM stimulation resulted in the inhibition of 66% of P40 neurons.

In contrast, although no significant change in subthreshold-evoked activity of P40 neurons was observed at low intensity 10 μA RVM stimulation (mean baseline subthreshold von Frey hair evoked activity 0.55 ± 0.12 spikes/second to 3.17 ± 1.24 spikes/second with 10 μA RVM stimulation; Figure 4. 7b), activity was significantly decreased at 100 μA stimulation by a mean percentage of 38% (mean firing of 0.24 ± 0.09 spikes/second with 100 μA stimulation; Wilcoxon's test between baseline and 100 μA stimulation value, P= 0.0398) indicating a possible increase in threshold (Figure 4. 8).

### *4.5.3.2 Threshold von Frey hair*

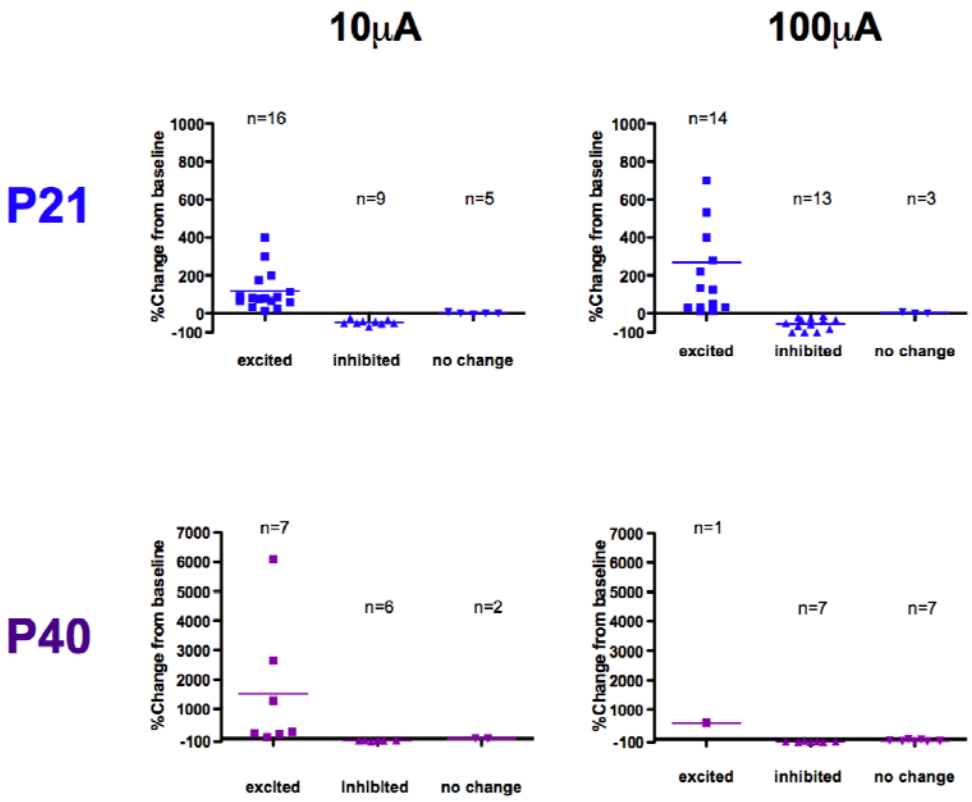



**Figure 4. 9: Subpopulations cellular response to threshold von Frey hair application.**

$\chi^2$ analysis reveals significant differences between neuronal responses of P21 and P40 cells to threshold von Frey hair application and 100 μA stimulation ($P< 0.05$). Cells were characterised as facilitated or inhibited if activity was changed to a greater extent than 10% from baseline values, or unchanged.

Threshold-evoked activity of P21 neurons was not significantly changed from baseline with either 10 μA or 100 μA RVM stimulation (baseline 2.64 ± 0.8 spikes/second; 10 μA: 2.4 ± 0.47 spikes/second; 100 μA: 3.25 ± 0.92 spikes/second; Figure 4. 7a) and both intensities resulted in highly heterogeneous neuronal responses (Figure 4. 9).

However, P40 neuronal response to threshold von Frey hair application to the receptive field significantly decreased during 100 μA RVM stimulation, with three cells ceasing to fire to the stimulus altogether (0.92 ± 0.21 spikes/second at baseline to 0.35 ± 0.12 spikes/second following 100 μA stimulation; Wilcoxon's test, $P= 0.0067$; Figure 4. 7b). $\chi^2$ analysis reveals significant differences between neuronal

responses of P21 and P40 cells to threshold von Frey hair application and 100 μA stimulation ($\chi^2$: P= 0.0168). This was not found to be the case following 10 μA low intensity RVM stimulation, which was unchanged from baseline, again seemingly due to high variance in data (3.91 ± 1.42 spikes/second; Figure 4. 9).

### *4.5.3.3 Suprathreshold von Frey hair*

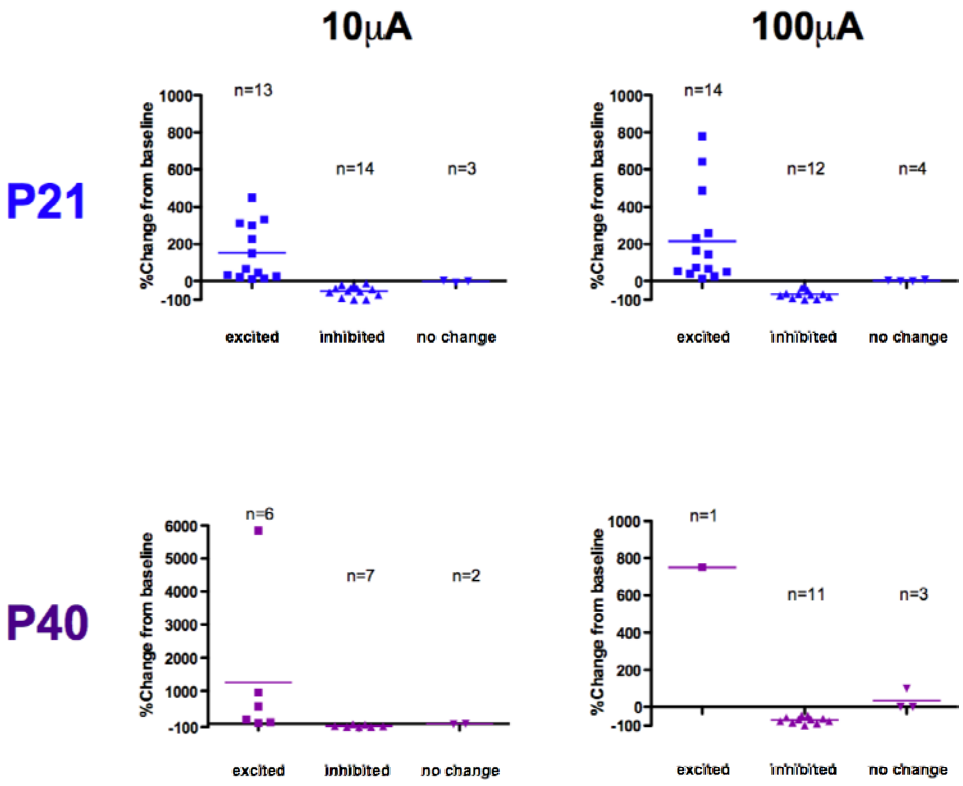



**Figure 4. 10: Subpopulations cellular response to suprathreshold von Frey hair application.**
Cells were characterised as facilitated or inhibited if activity was changed to a greater extent than 10% from baseline values, or unchanged. Response to suprathreshold von Frey hair application was highly heterogeneous, although 77% of P40 neurons had inhibited response at 100μA RVM stimulation.

P21 neuronal firing to suprathreshold von Frey hair application was unaffected by RVM stimulation at either 10 μA or 100 μA RVM electrical stimulation (baseline: 4.71 ± 0.96 spikes/second; 10 μA : 4.33 ± 0.82 spikes/second ; 100 μA : 4.93 ± 1.1 spikes/second ; Figure 4. 7a and Figure 4. 10) but P40 evoked activity was significantly decreased by 100 μA RVM stimulation (baseline : 2.21 ± 0.63 spikes/second to 0.91 ± 0.26 spikes/second in the presence of 100 μA stimulation ;

Wilcoxon's test, P= 0.0134 ; Figure 4. 7b). Again, the tendency at 10 μA stimulation was towards facilitation of von Frey-evoked firing (4.41 ± 1.39 spikes/second at 10 μA stimulation versus baseline of 2.21 ± 0.63 spikes/second) but the equal distribution of excited and inhibited cells resulted in high variability (6/15 facilitated and 7/15 inhibited; Figure 4. 10).

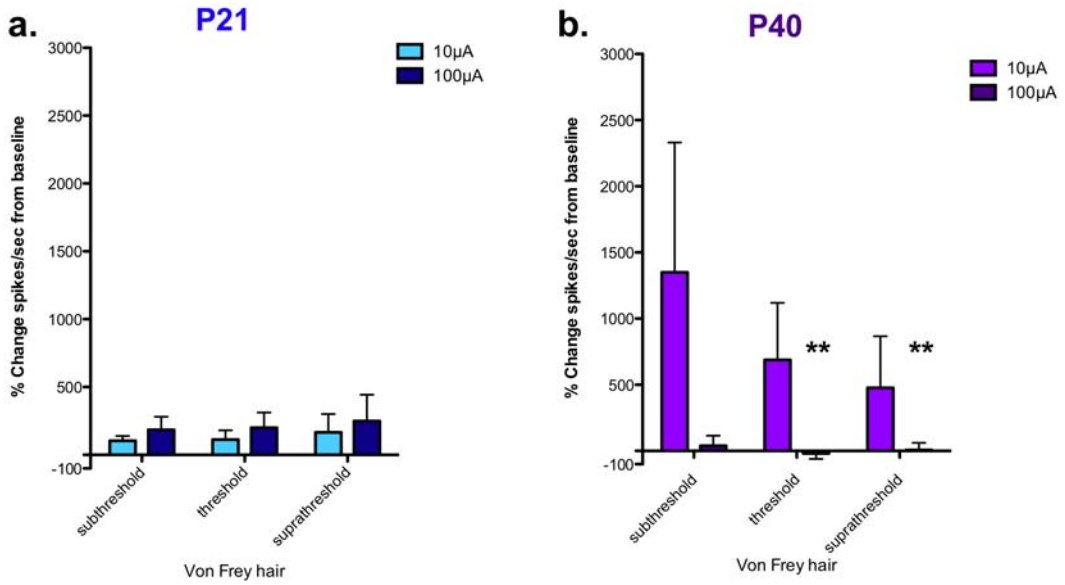



**Figure 4. 11: Summary of P21 and P40 dorsal horn responses to von Frey hair application with RVM stimulation represented as percentage change in spikes per second from baseline.**
(a.) P21 neuronal firing to subthreshold, threshold or suprathreshold von Frey hair application to the receptive field was not affected by RVM stimulation (n= 30). (b.) P40 neuronal response to both threshold and suprathreshold von Frey hair application was significantly decreased by 100 μA RVM stimulation (n= 15).

### 4.5.4 The effect of RVM stimulation on overall cutaneous evoked activity in P21 and P40 dorsal horn neurons

The influence of RVM stimulation on cutaneous evoked activity was examined by averaging responses of P21 and P40 neurons to subthreshold, threshold and suprathreshold von Frey hair application in order to provide a mean response to graded mechanical stimulation of the cutaneous receptive field and observe patterns of activity. One data point therefore represents a mean of cellular responses to all three von Frey hairs. P40 von Frey hair-evoked neuronal responses to RVM

stimulation are biphasic, low intensity stimulation elicits an increase in neuronal firing, whilst higher intensity stimulation inhibits this activity (Figure 4. 12a). The activity of P21 neurons was not significantly altered by RVM stimulation but displayed a trend towards full facilitation at all stimulation intensities.

The populations of cells differed markedly between age groups (Figure 4. 12b and d: comparing cellular response of P21 and P40 cells to vFh application during 10μA RVM stimulation; $\chi^2$ analysis, $P<0.0001$; Figure 4. 12c and e: comparing P21 and P40 cellular response to vFh application during 100μA RVM stimulation; $\chi^2$ analysis, $P<0.0001$). P21 neurons exhibited a much more heterogeneous response to RVM stimulation as compared to that of P40 neurons: both at 10 μA and 100 μA RVM stimulation there were an approximately equal number of excited and inhibited P21 neurons (10 μA: 16/30 cells facilitated, 12/30 inhibited; 100 μA: 15/30 cells facilitated, 11/30 inhibited), whereas at P40, 100 μA RVM stimulation inhibited 80% of all cells tested (12/15).

### 4.5.5 The effect of RVM stimulation on dorsal horn subpopulation response to graded von Frey hair stimulation of the cutaneous receptive field

The marked differences in cellular subpopulation responses observed raised the possibility that although when grouped P21 neurons did not appear to be affected by RVM stimulation, evoked firing of individual subpopulations was being significantly altered. The subpopulations of P21 neurons seen in Figure 4. 12 at 100 μA RVM stimulation were therefore used to plot out the effects of RVM stimulation upon subthreshold, threshold and suprathreshold von Frey hair-evoked activity. The subpopulations at 100 μA stimulation intensity were chosen as this stimulus intensity resulted in the most marked response in P40 cells.

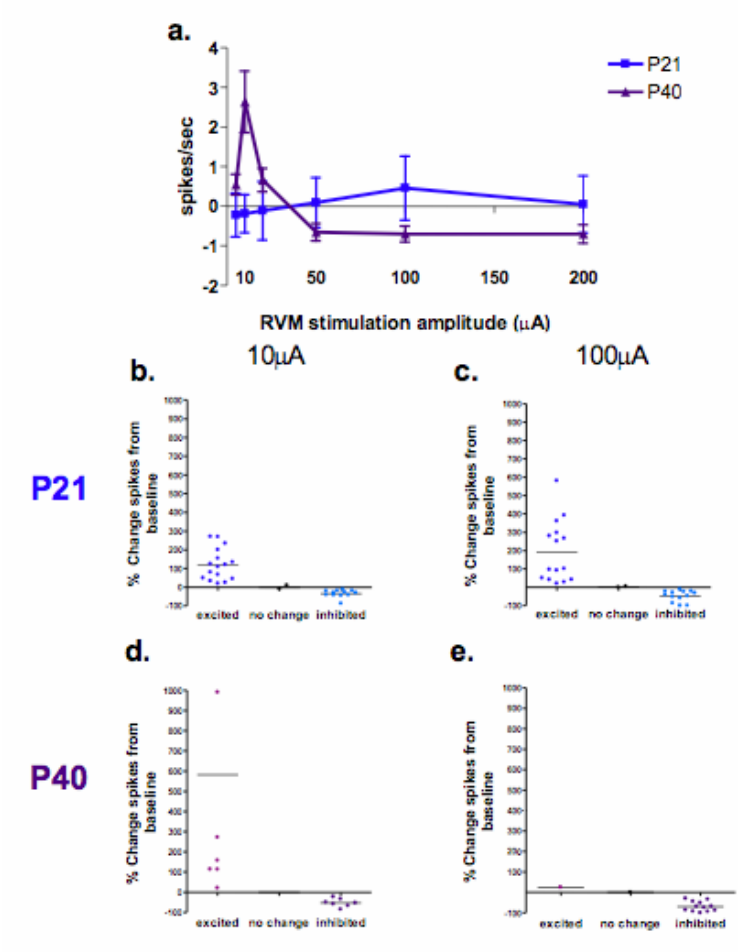



**Figure 4. 12: The effect of graded RVM stimulation on von Frey hair-evoked activity of P21 and P40 rat dorsal horn neurons.**

(a.) A total response of von Frey hair-evoked action potential firing in response to RVM stimulation elicited a biphasic response in P40 rat neurons (n=15; purple line) but a monophasic response in P21 neurons (n=30; blue line), where all stimulus intensities evoked an increase in firing compared to baseline results, response was significantly different between ages (two way ANOVA, $P<0.05$). (b. through e.) Scatter plots of populations of (b. and c.) P21 and (d. and e.) P40 neurons grouped as a function of percentage change in von Frey hair evoked firing at 10μA and 100μA RVM stimulation intensities from baseline firing values. Cells were characterised as facilitated or inhibited if activity was changed to a greater extent than 10% from baseline values, or unchanged. $\chi 2$ analysis of the subpopulations of cells at each group revealed highly significant differences in the distribution of cells at these ages at both (b. and d.) 10μA and (c. and e.) 100μA strength RVM stimulation ($P<0.0001$).

Cells characterised as excited had facilitated responses to both threshold and suprathreshold von Frey hair application at 100 μA RVM stimulation (threshold von Frey hair: baseline 2.7 ± 1.56 spikes/second to 5.48 ± 1.65 spikes/second at 100 μA stimulation; Wilcoxon's test, P= 0.0012; suprathreshold: baseline: 1.49 ± 1.28 spikes/second to 7.56 ± 1.87 spikes/second; Wilcoxon's test, P= 0.004; Figure 4. 13).

Conversely, cells characterised as inhibited had lower responses at 100 µA to both threshold (2.96 ± 1.31 spikes/second at baseline to 1.16 ± 0.31 spikes/second at 100 µA; Wilcoxon's test, P= 0.0161; Figure 4. 13) and suprathreshold von Frey hair applications (baseline: 6.38 ± 1.6 spikes/second to 1.78 ± 0.53 spikes/second in the presence of 100 µA RVM stimulation; Wilcoxon's test, P= 0.001).

Interestingly only suprathreshold von Frey hair-evoked firing of inhibited cells was significantly affected by 10µA stimulation of the RVM, and firing to subthreshold von Frey hairs was not significantly affected by either intensity in excited or inhibited cells. One-way ANOVA analysis revealed that RVM stimulation intensity had a significant effect of both threshold and suprathreshold von Frey hair evoked firing of P21 neurons classified as excited (threshold: one-way ANOVA, P= 0.0018; suprathreshold: one-way ANOVA, P= 0.0061), whereas only firing to suprathreshold von Frey hair application was significantly affected by RVM stimulation in inhibited cells (suprathreshold: one-way ANOVA, P= 0.0023).

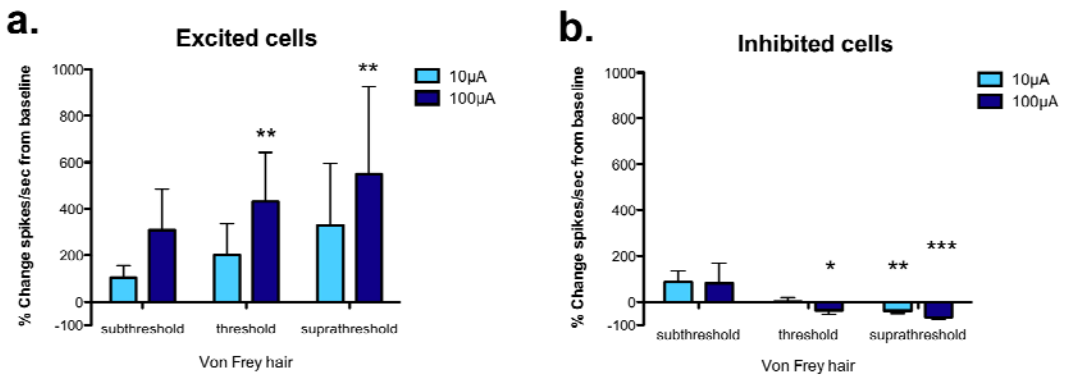



**Figure 4. 13: The effect of 10 µA and 100 µA RVM stimulation on von Frey hair-evoked firing of two separate P21 dorsal horn neuron subpopulations.**

(a.) Cells characterised as excited (n= 15), had facilitated responses to threshold and suprathreshold von Frey hairs at 100 µA RVM stimulation. (b.) Cell characterised as inhibited (n= 12) had significantly inhibited responses to both threshold and suprathreshold von Frey hair applications. Wilcoxon's t-test: *= P< 0.05, **= P<0.01, ***= P<0.0001.

### 4.5.6 The effect of graded RVM stimulation on P21 and P40 dorsal horn neuronal responses to A and C-fibre strength peripheral stimulation

In a pilot study, 6 dorsal horn neurons with both A and C fibre evoked input were recorded at P40 and compared to 9 dorsal horn neurons with A and C fibre input at P21. The effect of this selective fibre-evoked activity was then assessed in the presence of RVM stimulation.

A and C-fibre evoked compound action potential recordings could be performed from dorsal roots to establish that both P21 and P40 (Figure 4. 14). A fibre and C-fibre thresholds were 4.0 mA and 7.0 mA respectively at both ages. A suprathreshold stimulus of 10 mA was used in order to reliably recruit both afferents at each stimulus and in order to readily compare neurons of both age groups.

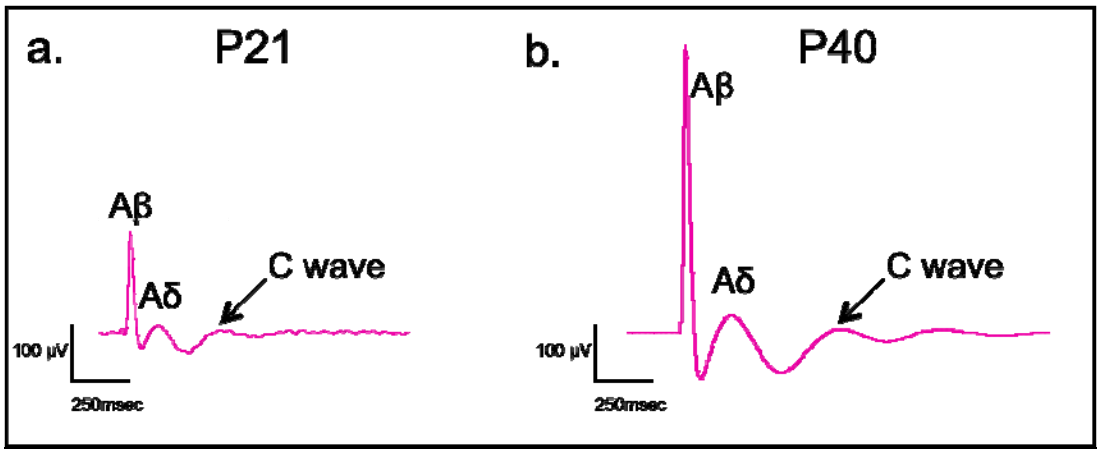



**Figure 4. 14: Compound action potential recordings from P21 and P40 dorsal roots.**
Potentials were recorded from (a.) P21 and (b.) P40 dorsal roots in response to 10 mA electrical stimulation of the cutaneous receptive field at 500 µsec pulse width. Aδ and C-fibre evoked potentials can be evoked at both ages.

When cells from each age were pooled, no significant effect of RVM stimulation could be detected upon either A or C fibre evoked responses at either age: P21: A fibre evoked spiking baseline: 13.98 ± 1.85 spikes; 10 µA: 13.93 spikes ± 1.96; 100 µA: 13.5 ± 1.88 spikes (Figure 4. 15a); C fibre evoked firing baseline: 3.84 ± 1.0 spikes; 10 µA: 3.9 ± 1.13 spikes; 100 µA: 4.45 ± 1.2 spikes (Figure 4. 15b); P40: A

fibre firing baseline: 12.2 ± 2.63 spikes; 10 μA RVM stimulation: 12.3 ± 2.85 spikes; 100 μA: 12.3 ± 2.33 spikes (Figure 4. 15c); C-fibre evoked activity: baseline 7.8 ± 2.67 spikes ; 10 μA : 8.2 ± 3.05 spikes ; 100 μA : 8.32 ± 2.45 spikes (Figure 4. 15d).

However, upon plotting individual cell responses to the ten consecutive C fibre stimuli, it was noted that only 1/9 P21 cells displayed any sign of wind-up, which is a well-described characteristic of wide dynamic range neurons (Mendell, 1984). Analysis of this cell showed a clear effect of RVM stimulation on wind-up: both 10 μA and 100 μA RVM stimulation intensities appear to facilitate C fibre mediated wind-up (Figure 4.16), suggesting that descending control from the brainstem may have a role in modulating the excitability of wide dynamic range neurons to C fibre-mediated input.

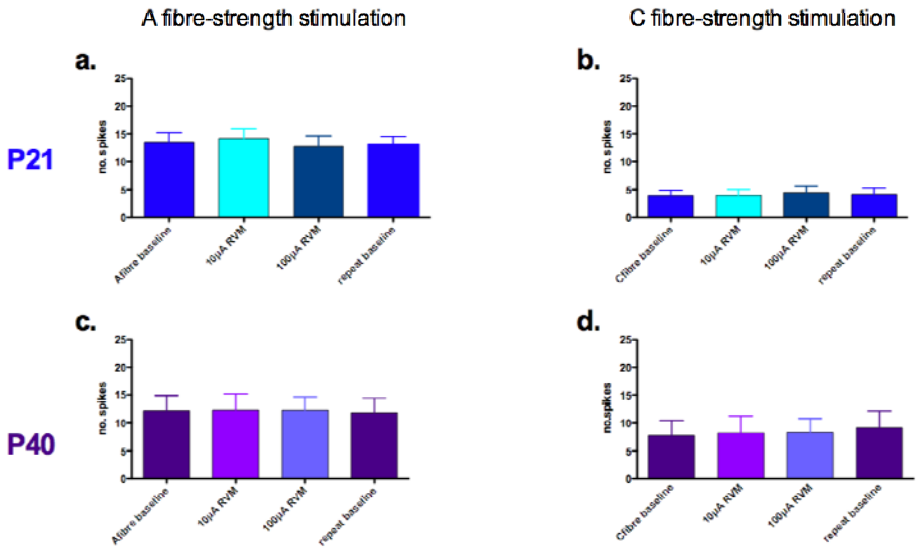



**Figure 4. 15: Low and high intensity RVM stimulation on A- and C-fibre strength stimulation of the cutaneous receptive field of P21 and P40 dorsal horn neurons.**

RVM stimulation did not significantly affect P21 (n=9) or P40 (n=6) neuronal firing to (a. and c.) A-fibre, or (b. and d.) C-fibre targeted electrical stimulation of the cutaneous receptive field.

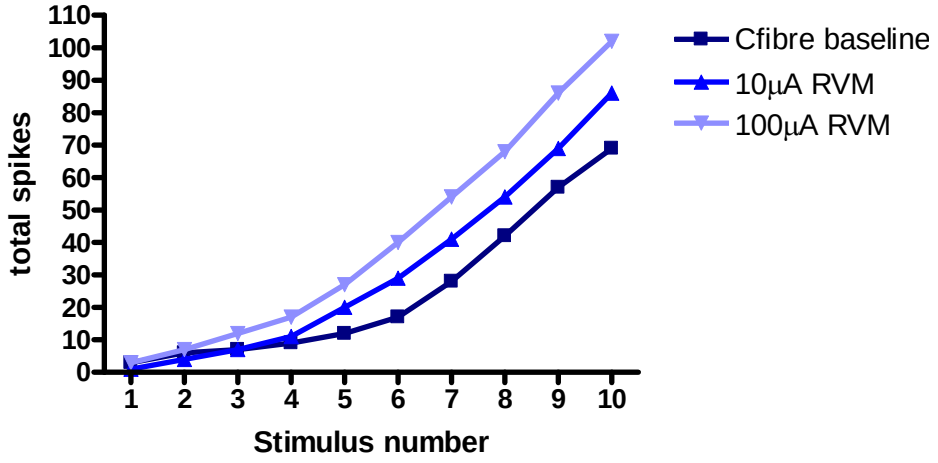



**Figure 4. 16: 10 µA and 100 µA RVM stimulation on C fibre wind-up of a single P21 dorsal horn neuron, recorded at 220 µm from the surface of the dorsal horn.**

Wind-up of a P21 neuron cell increased in the presence of both 10 and 100 µA electrical stimulation of the RVM.

## 4.6 Discussion

Descending modulation of spinal nociceptive circuits is crucial in maintaining the correct excitatory tone in the dorsal horn to enable the detection of harmful stimuli as well as to allow the correct reflex to be mounted against it. The balance between excitation and inhibition is finely tuned in the adult through descending control arising from the brainstem but this does not appear to be the case in the developing spinal cord, where descending inhibition is inefficient until the second postnatal week (Fitzgerald & Koltzenburg, 1986). Electromyographic studies have identified that descending facilitation from the RVM is predominant until the fourth postnatal week, but whether this effect is observed at the single neuron level has not been previously studied.

In this study I aimed to examine the effects of descending control from the rostroventral medulla (RVM) of the brainstem upon individual spinal dorsal horn neuron spontaneous and evoked activity over the first 40 days of postnatal life. Furthermore, I aimed to identify whether RVM selective inhibition of C-fibre input rich neurons is apparent from the third postnatal week. RVM stimulation was found to increase spontaneous activity of P21 dorsal horn neurons at both low (10 μA) and high intensity (100 μA) stimulation but had no significant effect on P40 neurons. Peripherally evoked activity of spinal neurons was significantly inhibited in the adult P40 rats in the presence of high intensity 100 μA stimulation of the RVM, whilst the effects of this same intensity stimulation on evoked firing of P21 neurons was heterogeneous, with both a population of facilitated and inhibited cells.

### 4.6.1 Technical considerations

It has been suggested that electrical stimulation may not be the most suitable method for the stimulation of brainstem structures, as it can activate fibres of passage as well as antidromically stimulate projections neurons in the spinal dorsal horn (McMullan & Lumb, 2006a). Whilst electrical stimulation of the RVM can be seen to be unnatural, these parameters have been shown to be a reliable method of recruiting descending controls and comparable to using microinjections of excitatory amino

acids (Zhuo & Gebhart, 1990; Hentall *et al.*, 1991). Importantly, electrical stimulation can be finely modulated in order to observe the effects of low and high intensity stimulation upon the same cell, without risk of biasing stimulated cells within the brainstem. This is especially important in the case of developmental studies, as populations of cells could vary over the course of the postnatal period.

Judged by the data of Ranck (Ranck, 1975) myelinated axons can be activated up to 1 mm away from the electrode with a 250 μA stimulus strength. At the stimulus intensities used here (5-200 μA) it is therefore reasonable to assume that the stimulation was restricted to the RVM. The use of concentric electrodes further allows for selective stimulation of tissue found within the barrel of the electrode. However, it is conceivable that the activation of fibres of passage would account for the lack of specific C fibre inhibition in the adult as reported in these pilot experiments as only the PAG and RVLM have been reported to selectively inhibit high intensity peripherally-evoked firing (Hudson *et al.*, 2000; McMullan & Lumb, 2006b; McMullan & Lumb, 2006a; Heinricher *et al.*, 2009). A further study including the microinjection of opioid agonists into the RVM may be able to account for this consideration.

### 4.6.2 The RVM facilitates spontaneous activity of dorsal horn neurons in adolescent P21 rats

Spontaneous activity of dorsal horn neurons in normal animals in the presence of RVM stimulation has not been fully investigated up until this point. Although c-fos studies have shown an increase in number of activated neurons following RVM stimulation (Bett & Sandkuhler, 1995), the nature of the study means that it is unclear whether these dorsal horn neurons had increased spontaneous activity or whether this was simply a matter of increased number of activated cells, nor is the identity of these neurons known. Whilst it was somewhat surprising that no significant effect on spontaneous activity was found in P40 neurons tested here, the findings of this Chapter are in agreement with a previous study performed in this laboratory, which showed that RVM injection of the selective μ-opioid receptor antagonist D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-$NH_2$ (CTOP) significantly

inhibits mechanical reflex withdrawal magnitude in P21 rats but has no effect on reflex withdrawal of P40 rats (G.Hathway and M.Fitzgerald, unpublished observations). Hence, the RVM appears to have a strong role in shaping spinal nociceptive networks in the absence of peripherally-evoked neuronal activity in the first few weeks of life, after which time descending control of spinal dorsal horn neurons specifies to the control of nociceptive sensory processing.

### 4.6.3 Supraspinal biphasic control of dorsal horn neurons is absent over the first three postnatal weeks

Both threshold and suprathreshold von Frey hair evoked firing was significantly affected by high intensity RVM stimulation in both P21 and P40 rats. Interestingly however, although there was a trend towards biphasic P40 neuronal in agreement with both previous behavioural and electrophysiology studies (Zhuo & Gebhart, 1990, 1997; Gebhart, 2004; Hathway *et al.*, 2009), this was not seen in any of the P21 cells examined; cells inhibited at 10μA were inhibited at all intensities tested. Likewise, those that were facilitated remained so regardless of the stimulation intensity. It would appear that although descending inhibition is possible by ten days of age in the rat (Fitzgerald & Koltzenburg, 1986), inhibition is not sufficiently strong or reliable to significantly affect the evoked activity of dorsal horn neurons at P21 to the same degree as is seen in the adult. This effect is also seen at larger output level, electromyography recordings in rats aged P3 to P40 demonstrated that RVM biphasic control over spinal networks was not effective until P30. Prior to this, rats displayed facilitated reflex withdrawal magnitudes at all RVM stimulation intensities (Hathway *et al.*, 2009). Interestingly, previous studies have reported that the induction of brainstem descending inhibitory influence is defined by increasing peripheral input. As such, neonatal destruction of C fibres by capsaicin treatment has been shown to result in higher levels of spontaneous spiking of spinal dorsal horn neurons in mature animals, implying ongoing descending facilitation in these animals (Zhuo & Gebhart, 1994). This apparent deficit of descending inhibition in the P21 rat may therefore be linked to weak C fibre input to dorsal horn neurons over the first three postnatal weeks (Jennings & Fitzgerald, 1998). The strengthening of C fibre input into the dorsal horn by the second postnatal week (Fitzgerald, 1985; Fitzgerald,

1988; Jennings & Fitzgerald, 1998) would allow for a stimulus strong enough to be projected to higher centres. This would then allow for strong feedback inhibition of nociceptive responses in deep dorsal horn neurons and a tighter control on nociceptive reflexes seen in the adult rat.

### 4.6.4 RVM stimulation increased C-fibre windup of an individual dorsal horn neuron at P21

In a pilot experiment, electrical stimulation was used to peripherally activate C fibres in the presence of graded RVM stimulation in order to assess the role of the RVM in selective C fibre mediated inhibition in the adolescent rat. Recent studies have shown that pharmacological stimulation of the RVM and/or PAG in the adult rat reliably inhibits deep dorsal horn neurons with strong C fibre input (McMullan & Lumb, 2006b; McMullan & Lumb, 2006a; Parry *et al.*, 2008; Waters & Lumb, 2008; Heinricher *et al.*, 2009). Whilst the study in this Chapter did not reveal any significant effect of RVM stimulation of C fibre evoked dorsal horn firing at either age tested, further analysis of the single P21 neuron that exhibited C fibre mediated wind-up revealed that firing of this neuron increased in the presence of both low intensity 10 μA or high intensity 100 μA RVM stimulation. Wind-up is a well described form of monosynaptic activity-dependent plasticity that is characterized by a progressive increase in action potential firing of dorsal horn neurons during the course of repeated low-frequency C-fibre stimuli (Mendell, 1984) and can be seen as a measure of neuronal excitability; suggesting that the RVM has a role in the regulation of the excitability of neurons to noxious stimulation. Further studies using neurons found deeper in the dorsal horn - specifically in those showing C fibre windup - would no doubt provide a clearer role for the RVM in nociceptive processing at the level of dorsal horn neurons in the early postnatal period and will help clarify whether descending facilitation at P21 is indeed specific to noxious C fibre or if it exerts a non-selective facilitation of both C and Aδ fibre inputs.

### 4.6.5 Potential mechanisms underlying the lack of descending inhibition at P21

There are a number of possible explanations for the observed lack of inhibition at P21: (i) immature neurotransmitter release from descending fibres or immature spinal postsynaptic receptor expression; (ii) a shift in phenotypic identity of RVM neurons over the first six postnatal weeks; and (iii) immature external inputs into the RVM. These will each be covered in turn in the following three sections.

#### *4.6.5.1 The development of descending fibres*

Descending modulation involves multiple neurotransmitter systems (Fields *et al.*, 2006), one of the most studied of which is serotonin (Weber & Stelzner, 1977; Fields *et al.*, 2006; Heinricher *et al.*, 2009), present in nearly half of all raphe neurons projecting to the adult spinal cord (Bowker *et al.*, 1981). Serotonin-containing neurons are among the earliest to be detected in the developing central nervous system (Lidov & Molliver, 1982; Okado *et al.*, 1992; Rubenstein, 1998). These are especially located along the midline of brainstem, where they are present from embryonic day 16 (Lidov & Molliver, 1982). Histological studies have shown the development of spinal 5-HT fibres to exhibit a ventro-dorsal gradient. As a result, although descending 5-HT fibres are present at birth in the ventral horn of the lumbar spinal cord, these do not reach the dorsal horn until the second postnatal week and final distribution of terminals is not reached until a week thereafter (Bregman, 1987; Rajaofetra *et al.*, 1989; Tanaka *et al.*, 1992; Ballion *et al.*, 2002; Tanaka *et al.*, 2006). Much as is described in the case of glycinergic terminal expression in Chapter Three, immature serotonergic terminal distribution could affect descending control by inefficient release of the neurotransmitter onto spinal postsynaptic receptors. In support of this, although *in vitro* studies have shown functional serotonergic receptors in neonatal spinal slices (Hentall & Fields, 1983), a behavioural study in neonatal rats found that systemic administration of D-amphetamine at concentrations high enough to promote serotonin release from terminals did not significantly dampen neonatal nociceptive networks indicating a stunted serotonin release in the early postnatal period (Abbott & Guy, 1995). It would therefore appear that although

receptors are functional, serotonin is not being reliably released from the presynaptic site at levels sufficient to activate postsynaptic receptors.

In addition to the presynaptic mechanisms described above, there are a number of postsynaptic modifications that could also occur at the level of the dorsal horn. Serotonin acts on a multitude of receptors and activation of different receptor subtypes can result in facilitation or inhibition of nociception (Millan, 2002); the expression of these serotonin receptor subtypes could be developmentally regulated such that dorsal horn neurons in the neonatal spinal cord preferentially express facilitatory 5-$HT_3$ receptors over inhibitory 5-$HT_1$ or 5-$HT_2$ receptors.

Although the specific development of serotonergic fibres has been most extensively studied, stimulation of the RVM is known to result in spinal release of several other neurotransmitters, including noradrenaline, GABA and glycine (Proudfit, 1992; Sorkin *et al.*, 1993). Both descending GABAergic and glycinergic projections from the RVM are known to form synapses with excitatory neurons in the superficial dorsal horn (Antal *et al.*, 1996; Kato *et al.*, 2006). This raises the possibility of a developmental change in other descending neurotransmitter systems, which could in turn lead to a predominance of facilitation in the juvenile spinal dorsal horn. The use of *in vivo* patch-clamp recordings of dorsal horn neurons in the presence of electrical stimulation of the RVM could help resolve the question of the ontogeny of descending fibres from this brain region in development: by electrical stimulating the brain and recording intracellularly from neurons in the dorsal horn in the presence of selective pharmacological agonists and antagonists, a clearer picture could emerge of the role of neurotransmitter systems in facilitation and inhibition over the first six weeks of postnatal life.

#### *4.6.5.2 The influence of 'on' and 'off' cells*

Many of the early studies exploring the effects of descending control had focussed on stimulating the dorsolateral funiculus (DLF), through which axons from the RVM travel to reach the dorsal horn. The effects of the RVM clearly go beyond what is seen by simple DLF stimulation; whilst biphasic control on dorsal horn neurons has

been widely reported by chemical and electrical stimulation of the RVM, this has not been shown to be the case of DLF stimulation, in which stimulation causes pure inhibition (albeit weakly until P10). This implicates the involvement of an RVM–mediated switch in the fourth postnatal week. Cells within the RVM have been classified into three groups: 'on' cells, 'off' cells and 'neutral' cells (Fields & Basbaum, 1978; Fields *et al.*, 1983; Heinricher *et al.*, 1989; Fields *et al.*, 2006). Selective activation of 'on' cells results in enhancement of nociception (Neubert *et al.*, 2004), whilst that of 'off' cells produces the opposite effect of analgesia (Heinricher & Tortorici, 1994). It could be that the shift in descending facilitation to inhibition in the early postnatal is due to a change in RVM neuronal phenotype; adolescent rats may have a higher relative proportion of 'on' cells as compared to 'off' cells resulting in enhanced facilitation as compared to inhibition. Alternatively, there may be potential delayed maturation of 'off' cell activity, or immature connections between the two cell groups, such that 'off' cell activity in the immature brainstem does not effectively inhibit that of 'on' cells. This could in turn account for inefficient inhibition at this age. *In vivo* recordings from the RVM at P21 and P40 would help address these questions; in addition this could be teamed with iontophoresis of selective agonists and antagonists to accurately investigate the role of these neurons in nociceptive processing over this critical time period.

#### *4.6.5.3 RVM input from the PAG*

Stimulation of the PAG is sufficient to cause behavioural analgesia in adult rats, yet this effect is not apparent until P21 (van Praag & Frenk, 1991). As the RVM is the main site through which the PAG exerts its spinal effects (Gebhart *et al.*, 1983; Prieto *et al.*, 1983; Sandkuhler & Gebhart, 1984; Chung *et al.*, 1987), it is conceivable that although supraspinal-spinal pathways are intact from birth, connections between the PAG and RVM are not yet fully functional. Retrograde tracing studies would identify the presence of connections but would not address functionality. The initial experiments of van Praag and Frenk were analysing the effects of PAG stimulation upon tail-flick behaviour of rats (van Praag & Frenk, 1991), experiments detailing the functional role of the PAG in the control of spinal neuronal activity by means of electrical or pharmacological stimulation could help

answer whether the PAG exerts any effect on spinal processing over the first three postnatal weeks.

Although P21 rats are often seen to be developmentally mature, the influence of descending control from the brainstem onto spinal dorsal horn neurons has not yet reached adult specification until a week thereafter (Hathway *et al.*, 2009). This is not to say that the control is not functional at this age, but that the influence of the RVM is markedly different from three weeks postnatal age to six weeks, and the appropriate balance between facilitation and inhibition has not been achieved until then. In the adult rat, RVM exerts a biphasic control over spinal nociceptive systems allowing for contextual response to a harmful stimulus. For example in a dangerous environment the rat may not display pain behaviour presumably due to descending influences from the amygdala and higher cortical centres. In this way, electrically stimulating the brainstem in the present study, could model increasing levels of input into the RVM: higher intensity stimulation at 100 μA would then be similar to a highly stressful or distracting situation, whilst low intensity 10 μA stimulation would mimic that of a resting animal. In the adolescent P21 rat, this biphasic control upon nociception does not appear to be as clearly defined; cells are either fully inhibited or fully facilitated in response to a range of stimulation intensities and the populations of excited and inhibited cells is much less diverse than those seen in the adult dorsal horn. This increase in activity could allow for synaptic strengthening and larger and more obvious responses to noxious stimuli. This would increase the likelihood of the mother tending to the immature rat at a time when it is just about to be weaned and protecting it from harm.

## 4.7 Conclusion

The studies in this Chapter have revealed that RVM control over spinal nociceptive circuits switches from being generally facilitatory before 3 weeks of age to predominantly inhibitory by 4 weeks. This is in line with several other studies showing the postnatal change in properties of immature spinal nociceptive networks may be due to a marked changed in the descending influence from the RVM (Hathway *et al.*, 2006; Hathway *et al.*, 2009). This effect has been observed in both spinal nociceptive reflexes and dorsal horn neuronal activity over this critical developmental period (Hathway *et al.*, 2009) and is likely the effect of maturing descending fibres and the maturation of neurons within the brainstem.

# Chapter 5

# General Discussion

## 5.1 Introduction

Spinal tactile and nociceptive processing undergo significant postnatal maturation. Immature rats display exaggerated and inappropriately directed flexion withdrawal reflexes to cutaneous mechanical stimulation. Thresholds are lower and sensory responses prolonged, effects which are observable both at the behavioural and individual neuron levels (Fitzgerald, 1985; Fitzgerald, 1988; Coggeshall *et al.*, 1996; Jennings & Fitzgerald, 1996, 1998; Beggs *et al.*, 2002). These features are indicative of sensory circuits that are more excitable compared to adults and since intrinsic excitability of spinal sensory neurons remains similar throughout postnatal development (Baccei & Fitzgerald, 2004) it has been suggested that the changes are attributable to an imbalance between excitation and inhibition in spinal sensory systems. Excitation in the spinal dorsal horn appears to be particularly directed towards sensitivity to low-threshold A fibre stimuli and developmental refinement and dampening of responses to tactile stimulation is coincident with the withdrawal of A fibres from the superficial dorsal horn and the strengthening of C fibre synaptic contacts (Fitzgerald, 1985; Fitzgerald, 1988; Coggeshall *et al.*, 1996; Jennings & Fitzgerald, 1996; Beggs *et al.*, 2002). Many studies have explored this idea by focussing on the role of GABAergic neurotransmission and its postnatal regulation. While there are changes in GABAergic signalling over this period, it is responsible for robust inhibition within sensory circuits from birth and is unlikely to be responsible for the excitability of neonatal cutaneous sensory processing (Baccei & Fitzgerald, 2004; Cordero-Erausquin *et al.*, 2005; Bremner *et al.*, 2006; Hathway *et al.*, 2006; Koch *et al.*, 2008). To date few investigators have examined the role of glycine during development. Glycine is of particular relevance in the spinal transmission of low-threshold touch information as Aβ fibres are known to synapse directly onto glycinergic interneurons in the mature dorsal horn (Todd, 1990; Narikawa *et al.*, 2000). Glycine receptor antagonism in adults results in enhanced low threshold responses in the dorsal horn and behavioural touch-evoked allodynia, whereby innocuous touch or brush is apparently perceived as noxious (Yaksh, 1989; Sivilotti & Woolf, 1994; Sherman & Loomis, 1996). Experiments described in Chapter Two of this thesis were therefore aimed at identifying the pattern of expression of glycine terminals and that of glycine receptors over the first three

postnatal weeks when tactile and nociceptive processing become less excitable and sensory responses refined. Further to this, studies in Chapter Three investigated the functional and physiological role of glycinergic neurotransmission in cutaneous sensory processing by single dorsal horn cells in the spinal cord *in vivo*.

Although local spinal networks undergo most developmental change within the first three weeks of life, there is evidence that descending controls from the brainstem take longer to mature. Thus an overriding descending facilitatory drive onto spinal networks persists until the fourth postnatal week before shifting to the predominant resting descending inhibition observed in the adult (Hathway *et al.*, 2009). The maturation of these inhibitory controls has been shown to be dependent upon the development of C-fibre central synapses (Cervero & Plenderleith, 1985; Zhuo & Gebhart, 1994) and interestingly in the adult, descending inhibitory signals from the brainstem are selective for wide dynamic range neurons with a strong C-fibre input (Hudson *et al.*, 2000; McMullan & Lumb, 2006b; McMullan & Lumb, 2006a; Koutsikou *et al.*, 2007). Experiments in Chapter Four of this thesis were therefore aimed at analysing the functional maturation of descending influences from the brainstem upon spontaneous and peripherally evoked activity of spinal dorsal horn neurons over the critical 3-6 week postnatal period when descending inhibition matures.

## 5.2 Summary of findings

Immunohistochemical studies in Chapter Two sought to map markers of spinal glycinergic circuitry in the dorsal horn over the first three postnatal weeks. To this end, three antibodies were used: (i) an antibody mounted against the glycine transporter GlyT2, (ii) an antibody raised against the alpha subunit of the glycine receptor, and (iii) c-fos immunostaining of cells that were activated by strychnine and were therefore concluded to be normally under tonic glycinergic inhibitory control. GlyT2 has been shown to be a reliable marker of glycinergic terminals and is expressed predominantly in lamina III and deeper laminae in the adult spinal cord in agreement with glycine expression (Jursky & Nelson, 1995; Zafra *et al.*, 1995b; Poyatos *et al.*, 1997; Spike *et al.*, 1997). A developmental increase in GlyT2 protein

expression has been reported using Western Blot (Zafra *et al.*, 1995b), but the immunostaining performed here additionally revealed a shift in expression pattern that could significantly alter the availability of synaptic glycine in the dorsal horn over postnatal development. My results show that GlyT2 and glycine receptor expression undergo a significant shift over the first three postnatal weeks, from peak expression in the deep dorsal horn at P3 to a gradual dorsally directed shift reaching adult termination patterns in lamina III by P21. These findings were echoed in c-fos studies, which revealed an absence of superficially located neurons under tonic inhibitory glycinergic control at P3.

The postnatal maturation of glycinergic activity in spinal sensory processing was further investigated using *in vivo* extracellular recordings from rats aged 3 days postnatal (P3) and 21 days (P21). Whole cell patch recording from lamina II neurons in spinal cord slices has shown that inhibitory transmission in the spinal dorsal horn in early postnatal life is dominated by GABA-mediated currents, the majority of which are inhibitory from birth (Baccei & Fitzgerald, 2004; Bremner *et al.*, 2006), but the maturation of glycinergic signalling in intact dorsal horn circuits had not been fully investigated. Chapter Three of this thesis describes the first set of *in vivo* recordings investigating the influence of glycinergic transmission on cutaneous sensory processing in the first three weeks of life using the glycine-receptor antagonist strychnine. While strychnine unmasked a strong tonic glycinergic inhibition of spontaneous activity in adolescent P21 wide dynamic range dorsal horn neurons, it had no effect on spontaneous firing of neonatal P3 neurons. Furthermore, while strychnine substantially increased low threshold brush evoked responses in P21 rats, in line with its allodynic action in adult rats (Yaksh, 1989; Sivilotti & Woolf, 1994; Sherman & Loomis, 1996; Sorkin & Puig, 1996), it significantly decreased neuronal firing to innocuous brush in P3 rats. Thus, glycinergic control facilitates the transmission of low threshold sensory information in the early postnatal period and only matures to its adult inhibitory role by the third postnatal week. As in the adult, this effect was highly modality-specific and neonatal neuronal activity to pinch or von Frey hair application was unchanged by strychnine, indicating that glycinergic activity, including the shift over the postnatal period, is selective for innocuous stimuli.

The experiments in Chapter Two and Three concentrated on the development of segmental inhibition in the dorsal horn, but there is considerable evidence for a critical role of supraspinal control in the maturation of spinal networks, notably on inhibitory transmission (Levinsson *et al.*, 1999; Branchereau *et al.*, 2002; Hathway *et al.*, 2006; Koch *et al.*, 2008). Maturation of descending supraspinal activity occurs over a longer timeframe than that of local spinal networks (Fitzgerald & Koltzenburg, 1986; Hathway *et al.*, 2009) and although functional from early in life, brainstem control of spinal circuitry is not fully mature until the fourth postnatal week (Hathway *et al.*, 2009). The experiments outlined in Chapter Four are the first to show the changing effects of descending control from the brainstem on individual dorsal horn neurons in P21 and P40 rats, allowing detailed analysis of brainstem control of cutaneous sensory-evoked activity over this developmental period. While descending inhibition of flexion reflexes is completely absent at P21 (Hathway *et al.*, 2009), descending inhibition of single dorsal horn neurons could be evoked by RVM stimulation in P21 rats, but was not observed in all cells tested and 50% were facilitated. In fact the effect of RVM stimulation on wide dynamic range neurons of P21 rats was found to be significantly more heterogeneous than the responses of dorsal horn neurons in P40 rats suggesting that although the appropriate networks are in place, they are not sufficiently synchronised to reliably produce biphasic inhibition and facilitation of nociceptive-evoked motor reflexes as observed in the adult (Hathway *et al.*, 2009).

### 5.2.1 Experimental considerations

As with any *in vivo* preparation, there are a few considerations when it comes to the interpretation of data. The advantage of *in vivo* recordings from individual neurons is that a more detailed analysis of cutaneous tactile or nociceptive responses of spinal neurons is possible when compared to reflex responses, which are the result of considerable sensory/motor integration. This is especially important when investigating developmental changes in nociceptive behaviour; the development of motor circuitry alone could confound the results behavioural or electromyographical studies without alterations in nociceptive circuitry. Secondly, the animal is intact and descending and peripheral influences can be investigated together, which is not

possible in *in vitro* slice preparations. Two disadvantages accompany these benefits: firstly, responses of individual neurons will rarely be as uniform as those found in behavioural or electromyographic studies, leading to a significant variability in response. Secondly, the data is subject to sampling variation and, the possibility of identifying the phenotype of recorded neurons in *in vivo* extracellular recordings is limited. Thus, recordings could have been made from projection neurons or excitatory/inhibitory interneurons, the activity of each of which would lead different outcomes. The data is therefore better viewed as a population of cells all contributing to the full functioning of the spinal cord.

The cells used for data analysis in this thesis were characterised and chosen according to their responses to cutaneous sensory stimulation of the receptive field, and only those responding to both noxious and innocuous stimuli (wide dynamic range neurons) were included. This criterion in itself limits the variability of response and to a certain degree limits variation in laminar distribution of these cells. Although no significant differences in recording depth between ages was noted, it is difficult to infer exact laminar position of recorded neurons. Future experiments to overcome this could involve the use of juxtacellular labelling of neurons in which extracellular recordings are performed using a glass micro-pipettes filled with biocityn or Neurobiotin. After recording is complete, low intensity positive-current pulses are passed through the pipette and so through the membrane of the recorded neuron located nearby. This is turn allows the iontophoresis of the tracer into the neuron of interest (Pinault, 1996). The animal can then be perfused, spinal cord removed, sectioned and stained in order to identify its laminar location and neuronal phenotype, for example by combining immunostaining of neuronal marker NeuN with antibodies against glycine, GABA or glutamate. Precise identification of the neuronal phenotype of the recorded cell could also be achieved using *in vivo* whole-cell patch clamp analysis in conjunction with selective receptor antagonists and agonists. Alternatively, intracellular recordings could be performed in genetically modified mice in which green fluorescent protein is expressed under the promoter of a protein of interest to allow for detailed analysis of a given subset of neurons of interest under visual guidance. This has successfully been achieved in *in vitro* slices (e.g. (Harvey *et al.*, 2004)) and could be used in two-photon visually directed *in vivo* patch-clamp studies, which would help answer many unanswered questions

concerning the detailed function of selected neurons over the developmental period. Furthermore, projection neurons could be identified by means of stimulation of long axons or brainstem nuclei (Keller *et al.*, 2007), this has however proved difficult and was beyond the scope of this thesis.

Another consideration is the use of anaesthetics in *in vivo* electrophysiology, which exert some of their effects by interfering inhibitory neurotransmission (Leite & Cascio, 2001; Lynch, 2004). Although the full impact of this on neuronal responses is not clear, the level of isoflurane used in Chapters Two and Four were identical between ages, and so comparisons are still valid. Nevertheless it is important to take anaesthetic regime into account in the analysis of any *in vivo* recordings.

## 5.3 Developmental changes in glycinergic synaptic function

It is important in any developmental study to consider the wealth of changes that occur over the postnatal period, the influence of a few of these on pre- and postsynaptic glycinergic transmission will be covered in this section, and have been summarised in Figure 5. 1.

### 5.3.1 The influence of glycine transporters on synaptic transmission

#### *5.3.1.1 Glycine transporters in controlling spillover activation of NMDA receptors*

In addition to its role as an inhibitory neurotransmitter, glycine is also an obligatory co-agonist at the NMDA receptor (Johnson & Ascher, 1987; Kemp *et al.*, 1988; Kleckner & Dingledine, 1988). In fact, strong presynaptic activity is sufficient to result in a glycinergic release large enough to cause spillover activation of NMDA receptors located outside of glycinergic synapses, and so potentiate excitatory neurotransmission (Supplisson & Bergman, 1997; Berger *et al.*, 1998; Bergeron *et al.*, 1998; Ahmadi *et al.*, 2003; Aubrey *et al.*, 2005). The concentration of synaptic glycine, and so the likelihood of spillover activation of NMDA receptors, is controlled by the sodium chloride-dependent transporters GlyT1 and GlyT2 (Berger

*et al.*, 1998; Bergeron *et al.*, 1998; Roux & Supplisson, 2000; Zafra & Giménez, 2008). It is therefore tempting to speculate that a lack of GlyT2 in the superficial laminae of the neonatal dorsal horn observed in immunohistochemical studies of chapter two could lead to NMDA receptor activation by glycine spillover in response to low-threshold stimulation, due to decreased clearance of the neurotransmitter. However, this specific scenario is unlikely, given that the role of regulation of glycine availability to NMDA receptors has been specifically attributed to neuronal GlyT1, whereas glial GlyT1 and neuronal GlyT2 are more geared to clearance of glycine from glycinergic synapses (Cubelos *et al.*, 2005a; Cubelos *et al.*, 2005b; Stevens *et al.*, 2010).

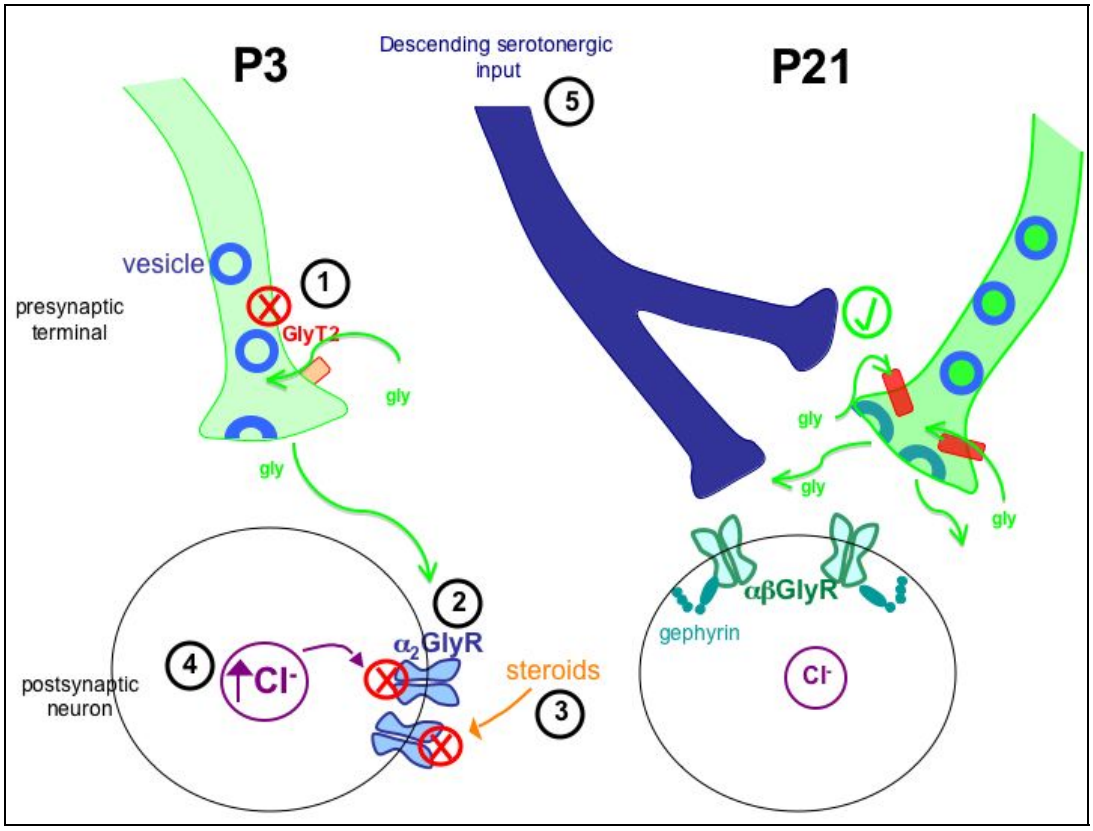



**Figure 5. 1: Schematic of developmental factors that could affect glycinergic signalling.**
(1) Immature levels of GlyT2 could lead to decreased presynaptic glycinergic recycling in the early postnatal period. This could in turn decrease the synaptic availability of glycine. (2) Neonatal $\alpha_2$ homomeric receptors cannot bind the anchoring protein gephyrin. These are therefore thought to be located extrasynaptically and be less available for activation by glycine. (3) Circulating neurosteroids selectively inhibit neonatal homomeric receptors over adult heteromeric receptors. (4) High intracellular chloride levels in developing neurons can slow glycine receptor kinetics, resulting in more 'sluggish' inhibition. (5) Descending serotonergic fibres from the brainstem have been shown to indirectly result in increased glycinergic transmission. See text for further details.

### *5.3.1.2 Effects of GlyT2 on presynaptic and postsynaptic terminals*

The transmembrane gradient maintained by GlyT2 is much steeper than that maintained by GlyT1 transporters due to the dependence of an extra sodium ion in the actions underlying GlyT2 transport. This has important implications on the function of these transporters. Immediately apparent is the fact that lower thermodynamic coupling of GlyT1 (two sodium ions to transport one molecule glycine) enables this transporter to theoretically function in reverse, allowing glycine to be pumped out of the cell (Roux *et al.*, 2001; Aubrey *et al.*, 2005). This could be particularly important in pathological pain situations, given that glycine spillover could activate excitatory NMDA receptors as mentioned above (Ahmadi *et al.*, 2003) and could cause excessive excitation of postsynaptic neurons. Secondly, the high thermodynamic coupling of GlyT2 (three sodium ions to one molecule of glycine) preferentially leads to the presynaptic accumulation of glycine. This has indeed been observed in several reports including whole-cell patch clamp studies on GlyT2 knock-out mice, which showed a marked reduction in glycinergic IPSCs compared to wildtype mice, consistent with decreased presynaptic glycine release (Poyatos *et al.*, 1997; Roux *et al.*, 2001; Gomeza *et al.*, 2003b). High levels of intracellular glycine mediated by GlyT2 function have been shown to be necessary for the functioning of the low-affinity vesicular glycine transporter VIAAT (Rousseau *et al.*, 2008), and so are critical in both the reuptake of glycine and its packaging for re-release, by allowing terminal concentrations of glycine to reach a level high enough to activate VIAAT. Consistent with this, blocking the function of GlyT2 has been shown to decrease glycinergic transmission (Bradaia *et al.*, 2004).

That GlyT2 regulates the presynaptic accumulation and release of glycine is of particular relevance to the studies in Chapters Two and Three of this thesis, given that tonic and peripherally-evoked glycinergic inhibition was absent in the neonatal dorsal horn at a time when GlyT2 immunostaining was diffuse and unrestricted. Lower levels of GlyT2 in the neonatal dorsal horn could therefore result in insufficient glycine release, which could in turn explain the lack of mIPSCs in neonatal spinal slices seen in *in vitro* whole-cell recordings (Baccei & Fitzgerald, 2004). Interestingly, blocking of GlyT2 has been shown to be sufficient to prevent the switch in inhibitory phenotype from GABAergic to glycinergic in auditory and

motor networks (Kotak *et al.*, 1998; Friauf *et al.*, 1999; Gao *et al.*, 2001a; Nabekura *et al.*, 2004; Awatramani *et al.*, 2005). As the apparition of GlyT2 in lamina III by P14 outlined in immunohistochemical studies of Chapter Two coincides well with the greater weight of glycinergic transmission in the spinal dorsal horn, this could therefore underlie the phenotypic switch from GABA to glycine in the spinal dorsal horn (Baccei & Fitzgerald, 2004).

Studies have also suggested that postsynaptic receptor clustering requires the presence of functional glycinergic neurotransmission, and blockade of glycine receptors with strychnine is sufficient to prevent this (Kirsch & Betz, 1998; Levi *et al.*, 1998). This could suggest a crucial role for GlyT2 in the functional maturation of postsynaptic terminals, through presynaptic glycine accumulation and release. The use selective blockade of GlyT2 in the presence of strychnine in the *in vivo* experiments in Chapter Three would help identify the influence of low levels of GlyT2 expression on neuronal activity in the neonatal cord. It is logical to predict that neonatal neuronal response to brush in the presence of strychnine and GlyT2 blockade would be identical to that of strychnine alone, whereas the combination of strychnine and GlyT2 blockade in the adult would reveal facilitation of brush-evoked activity as seen in the neonate.

#### *5.3.1.3 Other glycinergic modulators*

- Neurosteroids

These are of particular relevance in the maturation of the central nervous system, and 3α5α reduced neurosteroids have been shown to significantly affect the kinetics of GABAergic signalling in the neonatal dorsal horn (Keller *et al.*, 2001; Keller *et al.*, 2004). There is little evidence for the functional influence of steroids on glycinergic inhibition in the developmental period, but neonatal $\alpha_2$ homomeric receptors are known to be selectively inhibited by both dehydro epiandrosterone sulfates (DHEAs) and progesterone over adult heteromeric receptors (Maksay *et al.*, 2001; Lynch, 2004). This could have a significant relevance in the development of neuronal connections depending on the resting levels of these neurosteroids in the developing spinal cord. If levels of circulating steroids are high enough under resting conditions,

selective blockade of neonatal homomeric receptors could result in the inefficient glycinergic inhibition that was observed in the studies of this thesis.

- High intracellular chloride

A recent *in vitro* study in P10 spinal slices revealed the surprising role of intracellular chloride concentration in the control of glycinergic transmission (Pitt *et al.*, 2008). Using recordings from gramicidin-perforated vesicles, the detailed kinetics of glycine receptors were examined without artificially altering intracellular chloride levels. These studies found that high intracellular chloride significantly slows the decay kinetics of glycinergic currents, recently also shown to affect cerebellar $GABA_A$ receptors (Houston *et al.*, 2009). This is of particular importance when interpreting developmental differences in inhibitory neurotransmission given the fact that neonatal neurons have higher intracellular chloride levels than in the adult (Ben-Ari, 2002). Although developmental levels of chloride are not as high as in the experimental conditions tested in the studies above (Pitt *et al.*, 2008; Houston *et al.*, 2009), higher intracellular chloride levels in neonatal dorsal horn neurons could result in longer decay kinetics of glycinergic currents at this stage of development and would allow for longer inhibitory currents and "broader", or less precise, inhibition of fast sensory input. In support of this, glycinergic mIPSCs in the neonatal dorsal horn have been shown to display slower kinetics than in the adult, which decrease in rise time and duration with increasing age (Keller *et al.*, 2001). This is especially relevant in the control of sensory information from fast conducting myelinated Aβ fibres transmitting innocuous touch. The slow kinetics of neonatal glycinergic currents could therefore account for differential effects of strychnine on brush-evoked activity seen in Chapter Three, through high intracellular chloride levels.

### 5.3.2 The influence of maturing descending influences on inhibitory signalling

Several studies have shown a crucial link between the development of functional descending connections onto spinal neurons and the maturation of spinal inhibition, generally focussing on the postnatal maturation of descending serotonergic fibres and

their influence on inhibitory signalling in the ventral horn (Branchereau *et al.*, 2002; Jean-Xavier *et al.*, 2006; Sadlaoud *et al.*, 2010). These studies could therefore have important consequences on the findings of this thesis, which outline the postnatal maturation of both local and descending inhibition in the dorsal horn. Interestingly, it would appear that serotonergic signalling specifically inhibits GABAergic inhibition in the early postnatal period: spinal cord transection in the first week of life does not affect the development of glycinergic inhibition, but does prevent the postnatal down-regulation of GABAergic receptors (Branchereau *et al.*, 2002; Sadlaoud *et al.*, 2010). Additionally, studies have shown that severing descending fibres from birth prevents the hyperpolarising shift in chloride reversal potential that normally occurs over the first week of life (Jean-Xavier *et al.*, 2006). It would therefore appear that the serotonergic inhibition of GABAergic inhibition in the spinal cord is sufficient to remove inhibitory competition between GABA and developing glycinergic transmission and therefore indirectly allows the maturation of glycinergic receptors and their postsynaptic clustering.

### 5.3.3 Potential mechanisms for glycinergic facilitation of brush-evoked activity in the neonatal spinal cord

The finding that glycine antagonism in the neonate resulted in facilitation of brush-evoked neuronal activity is somewhat surprising given the inhibitory action of both GABA and glycine from birth (Baccei & Fitzgerald, 2004; Bremner *et al.*, 2006). One mechanism that could underlie this effect is outlined in Figure 5. 2. In this figure I propose that the broad and unrestricted expression of GlyT2, and so of glycine terminals, in the first week of postnatal week could result in the non-selective presynaptic release of glycine onto local neurons within the deep dorsal horn. In the adult dorsal horn, incoming low-threshold afferents directly facilitate glycinergic inhibitory interneurons in deeper laminae, which in turn prevent excessive excitation of target neurons by selective inhibition of excitatory interneurons. This inhibition is well directed in the adult, and results in selective inhibition of low-threshold tactile stimuli. Importantly, GlyT2 immunostaining and c-fos experiments in Chapter Three suggest that this targeted inhibition of lamina III neurons is absent in the immature dorsal horn. Firstly, glycinergic terminals were diffusely expressed in the deep dorsal

horn in the neonatal spinal cord, and secondly, strychnine was found to activate (or disinhibit) neurons located in deeper dorsal horn of neonatal rats. I suggest that in the neonatal spinal cord, unrestricted inhibition leads to larger probability of glycine-mediated inhibition of inhibitory interneurons than in the mature system, which would remove inhibitory control onto target neurons and lead to glycine-mediated facilitation of brush activity. Removal of glycinergic inhibitory block of this inhibitory interneuron by administration of strychnine would in turn allow inhibition of the target neurons. Within the context of the electrophysiological experiments of Chapter Three, this could be seen as a means of strychnine-induced inhibition of brush-evoked neuronal activity in the immature spinal cord. This theory could be investigated by performing the c-fos experiments outlined in Chapter Three in conjunction with a co-stain using an antibody raised against glycine, GABA or glutamate to highlight inhibitory or excitatory interneurons. Based on current knowledge, the predicted observation would be increased c-fos activation of inhibitory interneurons in the neonatal dorsal horn.

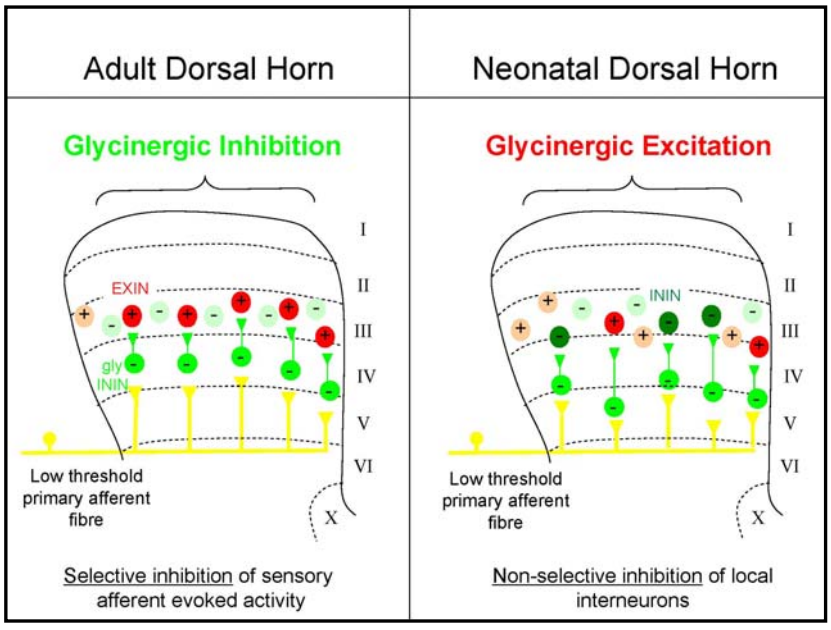



**Figure 5. 2: Potential mechanism underlying glycinergic facilitation of low-threshold stimulation in the neonatal spinal dorsal horn.**

(Left) In the adult, low threshold afferent input glycinergic interneurons (gly ININ; light green), which inhibit excess activity by selective inhibition of excitatory interneurons (EXIN; red) in lamina III. (Right) In the neonatal dorsal horn, glycinergic inhibition may less precisely directed leading to non-selective inhibition of inhibitory interneurons (ININ; dark green). This could result in disinhibition of target neurons, leading to excessive excitation and glycine-mediated facilitation of low-threshold input.

## 5.4 Integration of sensory inputs in the developing dorsal horn

Inhibition is clearly functional to some degree in the neonatal spinal cord (Baccei & Fitzgerald, 2004; Bremner *et al.*, 2006; Bremner & Fitzgerald, 2008) and although outside the remits of this thesis, it is important to also note the marked changes in excitatory neurotransmission that occur over the postnatal period, not limited to changes in both glutamatergic and substance P driven transmission (see (Pattinson & Fitzgerald, 2004; Fitzgerald, 2005) for reviews). Thus, the real difference between the neonatal and adult dorsal horn neurons is likely to be both in the balance between excitation and inhibition and the ability of neuron to integrate information from local interneurons, afferent fibres from the periphery and from supraspinal centres. The recent advancement in analytical tools has allowed a greater understanding of the impact of immature integration on sensory circuits and neuronal activity.

Although single channel kinetics are not direct predictors of neuronal activity within a working circuit they can provide important insight into the workings of a single cell, and so offer information on how a network of neurons may respond to incoming stimuli. The efficacy of an inhibitory signal is dependent on both the amplitude and the time course of an IPSC, and so developmental alterations in kinetics and presynaptic and postsynaptic receptor function can therefore have profound consequences on a neuron's integrative ability. Indeed, many studies have shown an age-dependent decrease in kinetics and increase in amplitude of response of both glycinergic and GABAergic mIPSCs and IPSCs (Keller *et al.*, 2001; Baccei & Fitzgerald, 2004; Keller *et al.*, 2004; Ingram *et al.*, 2008). Slower kinetics would lead to a longer channel opening time, which in turn could mean that inhibition is not as precisely tuned to the fast conduction velocities of Aβ fibre innocuous stimuli, offering further insight into the brush-specific effects of strychnine outlined in Chapter Three.

Studies have also started to shed light on the detailed local anatomical wiring of inhibitory and excitatory inputs onto neurons within the dorsal horn. Inhibitory and excitatory inputs onto islet cells within lamina II are precisely arranged such that inhibitory interneurons synapse in the peri-somatic region of the postsynaptic cell, whereas excitatory inputs are primarily focussed to the dendritic tree (Yasaka *et al.*,

2007), implying a wider input of excitatory signals and local inhibition (Kato *et al.*, 2009). The somatic location of inhibitory inputs also suggests an absolute clamp over incoming excitation. Conversely, the spread of excitation throughout the dendritic tree lends itself to neuronal summation of subthreshold excitatory inputs from intralaminar and translaminar cells. The precise arrangement of these synaptic associations raises the possibility that imbalanced excitation in the neonate is due to a lack of appropriately directed synapses at this stage, especially given the late development of inhibitory interneurons (Bicknell & Beal, 1984).

This has been characterised at the postsynaptic level using outside-out patch recordings from ventral horn motoneurons of P12-16 (Beato & Sivilotti, 2007). Juvenile rat motoneurons were shown to contain a low density of somatic glycine receptors, which could result in inefficient glycinergic inhibition and therefore a lower probability of coincidental inhibition occurring to outweigh incoming excitatory signals. Consistent with this, detailed analysis in neonatal motoneurons has shown that the specific location of inhibitory inputs onto a target neurons has a profound effect on the neuronal integration of excitatory signals (Jean-Xavier *et al.*, 2007). The ability of an IPSP to suppress excitatory inputs was largely dependent on the location of the inhibitory input and the chloride reversal potential, which has again been shown to vary between the soma and dendritic tree of a single neuron (Duebel *et al.*, 2006; Jean-Xavier *et al.*, 2007; Glickfeld *et al.*, 2009). Furthermore, axo-dendritic inhibitory synapses as described in the immature ventral horn are known to preferentially result in local shunting of excitatory signals (Rall, 1959). Immature synaptic arrangements in the neonatal dorsal horn could therefore lead to glycinergic shunting of afferent input without full hyperpolarisation of the postsynaptic neuron. Interestingly, the dendritic localisation of glycinergic receptors would in itself account for lower amplitudes and slower decay times of glycinergic mIPSCs in the second postnatal week (Lim *et al.*, 1999; Keller *et al.*, 2001; Baccei & Fitzgerald, 2004; Keller *et al.*, 2004), at a time when the postsynaptic switch from slow $\alpha_2$ homomeric receptors to faster heteromeric glycine receptors has already occurred (Malosio *et al.*, 1991).

Several studies have added another level of complexity to spinal networks by examining interlaminar differences in glycinergic inhibitory currents. The overriding

view appears to follow immunohistochemical studies, whereby glycinergic inhibition plays a larger role in the deeper dorsal horn than in superficial laminae (Todd, 1990; Poyatos *et al.*, 1997; Spike *et al.*, 1997; Inquimbert *et al.*, 2007; Anderson *et al.*, 2009). Kinetics of glycinergic currents in the deep dorsal horn are also faster and occur at a higher frequency in agreement with its role in the fast inhibition of Aβ fibre afferent signals (Inquimbert *et al.*, 2007; Anderson *et al.*, 2009).

## 5.5 Conclusion

Immature spinal inhibition and dysfunctional descending inhibition have a significant influence on the processing of nociceptive stimuli at the level of the dorsal horn. Maturation of inhibitory dorsal horn circuitry does not occur for at least two weeks after birth, whilst that of descending control upon nociceptive processing appears to occur over a much later period. This lack of inhibitory transmission, both from local interneurons and from descending fibres of supraspinal sites, leads to an imbalance between excitation and inhibition and could be the cause for the characteristic sensitivity of neonatal systems. This sensitivity is necessary for the appropriate strengthening of synapses within sensory circuits and allows for the appropriate reflexes to be mounted in response to noxious stimuli later in life.

Descending facilitation in the early postnatal period may be a means for increasing dorsal horn activity to reinforce strengthening synapses within the spinal cord. This could also provoke an increase in protein expression over the first three postnatal weeks, including that of GlyT2 in lamina III of the dorsal horn. That the maturation of descending serotonergic inputs has been shown to promote the phenotypic shift from predominantly GABAergic to glycinergic inhibition in other sensory systems reinforces the idea of descending control being a key factor in the maturation of glycinergic inhibition in spinal sensory circuits (Branchereau *et al.*, 2002; Jean-Xavier *et al.*, 2006; Sadlaoud *et al.*, 2010). Interestingly, the same has been shown in the reverse: strong afferent input into the dorsal horn by means of strengthening C fibre mediated input in the neonatal superficial dorsal horn has been shown to be integral in the development of descending inhibition. Two separate studies have shown that preventing C fibre input by means of neonatal ablation prevents the

normal development of descending inhibition to noxious stimuli (Cervero & Plenderleith, 1985; Zhuo & Gebhart, 1994). Together, the general facilitatory role of glycine in neonatal sensory transduction and the trend towards full facilitation of dorsal horn neurons throughout the dorsal horn in the presence of brainstem stimulation appear to support the idea of a bimodally-dependent maturation of spinal-supraspinal connectivity.

An interesting point to consider is the differing roles of spinal and descending facilitation. Whereas the role of glycine appears to be in facilitating the transduction of innocuous brush - through immature inhibitory networks and perhaps in the lack of appropriate synapses - descending facilitation is more tuned towards facilitating the transmission of noxious information. Within the first week spinal networks are set up to increase activity to non-noxious stimuli in order to strengthen synapses in the absence of harmful noxious stimuli at a time when neonates are fragile and unable to fend for themselves. Later in the third postnatal week, descending facilitation serves as a protector to the young adolescent to draw attention to noxious harmful stimuli in order to encourage future avoidance of this stimulus whilst strengthening synaptic contacts in the dorsal horn involved in nociceptive transmission.

Crucially, in clinics the understanding of neonatal nociceptive processing is still lacking and until recently, premature infants were treated as ‘small adults’ as opposed to physiologically distinct organisms. Understanding of the details of the postnatal maturation of this circuitry will help improve the quality of pharmacological treatment of neonatal infants. These could also offer insight into the intrinsic plasticity of sensory and nociceptive systems, leading to a greater understanding of network changes underlying pain pathologies in the adult.

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