Postnatal development of the lumbar spinal circuitry in the presence and absence of descending systems

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Submitted in accordance with the requirements for the degree of Doctor of Philosophy

The University of Leeds

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March 2016

The candidate confirms that the work submitted is his own and that appropriate credit has been given where reference has been made to the work of others.

I. Acknowledgements

I would like to dedicate this thesis to my parents Mark and Sharon Smith who have made enormous sacrifices simply to provide opportunity. Without expectation, they have gone beyond the call of duty to support me in all my endeavours. I cannot thank them enough for all they have done.

To my boets, Rayn, Nathan and Morgan, I can always rely on you oaks for good laughs and great music to take my mind away from science for a while.

To my lab mate Yazi. Thanks for all the laughs and support over the years. Most of all, thanks for putting up with my ‘slightly less than tidy’ lab practice!

I would like to thank my supervisors Drs Ronaldo Ichiyama and Samit Chakrabarty. I thank Ronaldo for giving me the opportunity to work with him and all his support and guidance over the last 5 years. I cannot begin to show my appreciation for the investments he has made in my career. Samit’s wealth of technical and subject knowledge has been invaluable. More importantly, I’ve enjoyed the many spontaneous discussions about ‘crazy ideas’ over the last three and a half years.

Finally, to my girlfriend Olivia, you have provided love, support and inspiration throughout this experience. Without question you have made the failures that much easier, and the successes that much sweeter. Thank you.

II. Abstract

Motor maturity in the rat is achieved within 3 weeks postnatally (PN). This is believed to be due to significant reorganisation and refinement of spinal and supraspinal systems; however this process is poorly understood. For example, descending innervation of the spinal circuitry is weak at birth and the extent to which developing supraspinal projections guide maturation of spinal circuits is not known. To address these questions, the present study developed a physiologically relevant preparation in which the development of motor control can be studied throughout the PN period. Using electrophysiological and immunohistochemistry techniques, we tracked the PN development of muscle afferents and their modulation in the lumbar spinal cord. Input to motoneurons (MNs) from premotor interneurons were also assessed. To establish the dependence of lumbar spinal circuit organisation on descending systems, we conducted the same assessments on rats spinalised (mid-thoracic) at postnatal day 5 (PN5 TX). Results show that Ia afferent innervation of the lumbar cord is great early PN and retracted throughout PN development. PN5 TX abolished this retraction, with greater densities of afferents found at PN14 and 21 compared to intact controls and reduced H reflex thresholds and increased H max/M max values. Presynaptic innervation of Ia afferents by GABApre neurons is low early in development and increased PN, a process which is attenuated following PN5 TX. This may have been responsible for reduced paired pulse depression of the H reflex in PN5 TX rats. Premotor inputs to MNs were significantly, but distinctly altered throughout development and PN5 TX did not significantly disrupt this profile.
Our results suggest a competition between afferent and descending systems for synaptic coverage of spinal circuits during PN development, with PN5 TX removing descending competition and preventing normal PN retraction of afferents. This, in

conjunction with reduced presynaptic inhibition leads to hyper excitability of the MSR as premotor projections are not significantly affected.

III. List of abbreviations

PN- Postnatal

PN10,14 and 21- Postnatal day 10,14 and 21

MSR- Monosynaptic reflex

TX- Transetion

CST- Corticospinal tract

MN- Motoneuron

ENG- Electroneurography

EMG- Electromyography

PP- Perfusion Pressure

ACh- Acetylcholine

VGLUT1- Vesicular glutamate transporter 1

VAChT- Vesicular Acetylcholine transporter

ChAT- Choline acetyltransferase

CB- Calbindin D28-k

GLYT2- Glycine transporter 2

GABA- Gamma-Aminobutyric acid

EM-Electron microscopy

VGLUT2- Vesicular glutamate transporter 2

RC- Renshaw cells

IN- Interneuron

PAD- Peripheral afferent depolarisation

PD- Post activation depression

NT-neurotransmitter

CNS- Central nervous system

WHBP- Working heart brainstem preparation

GABApre- GABAergic interneurone responsible for peripheral afferent depolarisation

RVLM- Rostral ventrolateral medulla

LVN- Lateral vestibular nucleus

VLF- Ventrolateral funiculus

5HT- 5 Hydroxytryptamine

HRP-Horseradish peroxidase

AVP- Arginine vasopressin

PPD- Paired pulse depression

M max- Maximal direct motor axon response

H max- Maximal synaptic response

SCI- Spinal cord injury

PFA- Paraformaldehyde

PBS- Phosphate buffered saline

OCT- Optimum cutting temperature medium

PBST- Phosphate buffered saline with Triton X

IHC- Immunohistochemistry

CPG- Central pattern generator

IPI- Interpulse interval

NMDA- N-methyl-D-adpartate

ATP- Adenosine triphosphate

GAD67- Glutamic acid decarboxylase isoform 67

GAD65- Glutamic acid decoarboxylase isoform 65

IV. Table of Contents

TABLE OF FIGURES

Chapter 1. General introduction

1.1 General introduction

Postnatal development of motor control in mammals takes place at different rates. For example, animals which are prey targets for large carnivores, such as antelope of the African planes, are capable of weight-bearing locomotion soon after birth. In contrast, other mammals such as cats, dogs, rats and humans are immature and unable to carryout weight-bearing locomotion at birth; taking weeks to years to develop mature control of movement. The precise mechanisms responsible for altered rates of maturity are not known, but it is believed to be related to development of mature connectivity between the brain and the spinal cord (Martin, 2005).

In mature animals, the brain and spinal cord constantly interact in order to produce effective, well directed movement (Lemon, 2008). This connectivity is established during pre and postnatal development. In the rat for example, much of the lumbar spinal circuitry is in place and functional at birth, but is still establishing mature circuit properties (Ozaki et al., 1996). Brainstem structures innervate the lumbar spinal cord before or shortly after birth, whilst the corticospinal tract (CST) only makes contact around postnatal day 9 (PN9) (Joosten et al., 1989; Schreyer and Jones, 1982). This suggests that the spinal circuitry relies on afferent activity early PN and gradually adapts its organisation in order to integrate progressive arrival and maturation of supraspinal projections. This has been demonstrated in studies which have shown that proprioceptive afferents are retracted and refined during development and that this process may be dependent upon arrival of the CST (Chakrabarty and Martin, 2011b; Chakrabarty and Martin, 2011a; Clowry, 2007).

This co-development of descending and afferent inputs to the spinal circuitry is of great functional significance as evidenced by the profound impact neonatal spinal

and supraspinal lesions have on motor control. Lesions to corticospinal systems for example, can result in spasticity of the upper and lower limbs making ambulation difficult. The term ‘spasticity’ is often used synonymously with others such as hypertonia and hyperreflexia. As Nielsen et al. (2007) highlighted, it is important for authors to define their use of the term in order to ensure clarity. Lance (1980) defined spasticity as ‘a motor disorder characterised by a velocity-dependent increase in tonic stretch reflexes (‘‘muscle tone‘‘) with exaggerated tendon jerks, resulting from hyperexcitability of the stretch reflex, as one component of the upper motor neurone syndrome’. Within this thesis, the term spasticity is used with reference to the chronic central changes associated with lesions to the brain and spinal cord which leads to hyperexcitability of the monosynaptic reflex (Nielsen et al., 2007). At present, spasticity following developmental lesions is thought to result from reduced developmental retraction and ectopic sprouting of afferents in the spinal cord due to a lack of competition from arriving CST during development (Gibson et al., 1999; Clowry, 2007). Developmental competition for synaptic coverage between proprioceptive and CST sources has recently been suggested to persist into adulthood but it is not known whether it is ubiquitous across spinal cord segments or what impact this has on postnatal organisation of spinal circuits (Tan et al., 2012; Jiang et al., 2016).

Spinal lesions early in development do not affect supraspinal systems directly, but act to prevent activity from these areas from reaching the spinal cord and therefore remove their influence on the development of spinal circuitry. In adults, thoracic spinal cord transections provide a unique model for establishing the role of descending systems in modulating the lumbar spinal circuitry during movement. For example, adult spinal cord transections often result in immobility and chronic

spasticity of the hind limbs, with limited recovery unless accompanied by locomotor rehabilitation and stimulation (Gerasimenko et al., 2007; Ichiyama et al., 2005; Alluin et al., 2015; Ichiyama et al., 2008). Interestingly, animals that receive the same injury early postnatally recover more function in the absence of treatment and respond much better to rehabilitation alone compared to combinatorial therapies in adults, suggesting fundamental differences in the organisation of lumbar spinal circuitry (Petruska et al., 2007; Saunders et al., 1998; Weber and Stelzner, 1977; Tillakaratne et al., 2010; Howland et al., 1995; Kunkel-Bagden et al., 1992). Adult and neonatal lesions are similar in that both remove descending control, however a striking difference between these injury models is that early neonatal transections prevent the late developing CST from ever making contact with the lumbar circuitry and other earlier descending systems from establishing mature termination profiles (Joosten et al., 1989; Leong et al., 1984).

It is clear that normal development of the spinal circuitry is dependent upon activity from descending systems, but the extent of this dependency has not been assessed in detail. This is probably due to relatively limited knowledge on how mature organisation of spinal circuitry is established during normal development, and the impact of removing descending input on this process.

The introduction to this thesis will therefore provide background on our current understanding of the maturation process of key motor control components in the spinal circuitry and how they may be disrupted by lesions to the brain and spinal cord.

1.2 Organisation of the spinal cord

The spinal cord projects from the base of the brain (brainstem) and extends caudally until it forms the cauda equina. The spinal cord can be divided into 5 main levels (cervical, thoracic, lumbar, sacral and coccygeal). The five levels can be segmented in to sub sections, the number of which varies between species. In the rat, there are 8 cervical (C1-8), 13 thoracic (T1-13), 6 lumbar (L1-6), 4 sacral (S1-4) and 3 coccygeal (Co1-3) spinal segments. The spinal cord, like the brain contains both grey and white matter. The white matter contains ascending and descending tracts, transmitting afferent and efferent information to and from the brain. Information about touch, pain and limb position amongst others are transmitted to the spinal cord and then the brain via afferent nerves and their cell bodies in the dorsal root ganglion. The grey matter of the spinal cord contains neuronal populations which are subdivided into ten laminae based on cytoarchitecture- the Rexed laminae (Rexed, 1952) . In the lumbar spinal cord, the superficial laminae (I-VI) of the dorsal horn are thought to contain mainly neurones responding to cutaneous/nociceptive inputs. Lamina VII-VIII contains many of the premotor interneurons while motoneurones (MN) are confined to lamina IX. Laminae VIII and IX reside within the ventral horn, while the intermediate region mainly comprises lamina VII, extending down into lamina IX. Lamina X, along with the lateral horns are known as the autonomic regions and thought to contain mainly autonomic neurons, although cholinergic premotor interneurons have also been identified just dorsolateral to the central canal in lamina X (Miles et al., 2007).

1.3 Postnatal development of lumbar spinal circuitry

1.3.1 Monosynaptic reflex arc

The MSR was first described by Sir Michael Foster as the ‘knee jerk reflex’ in his textbook of physiology (Foster, 1897), however the details of the central and peripheral components of this reflex were not characterised until the early 20th century. The classical monosynaptic reflex (MSR) arc involves 3 main components; the muscle, the afferent pathway from muscle spindles and the efferent pathway from the motoneurone to the muscle, ‘the final common path’ (Fig 1) (Sherrington, 1906). Sherrington (1906) also suggested that the idea of a simple reflex ‘is probably an abstract concept because all parts of the CNS are connected’. We know this to be true as proprioceptive afferents activated in the MSR have direct access to the CPG circuitry (Conway et al., 1987). Furthermore, because MN output is ‘the final common path’, their output represents the excitability of the extremely complex circuitry which innervates them. Therefore, the monosynaptic reflex can be thought of as a useful tool for assessing the excitability of the spinal circuitry as determined by its inputs from sensory and descending systems amongst other factors.

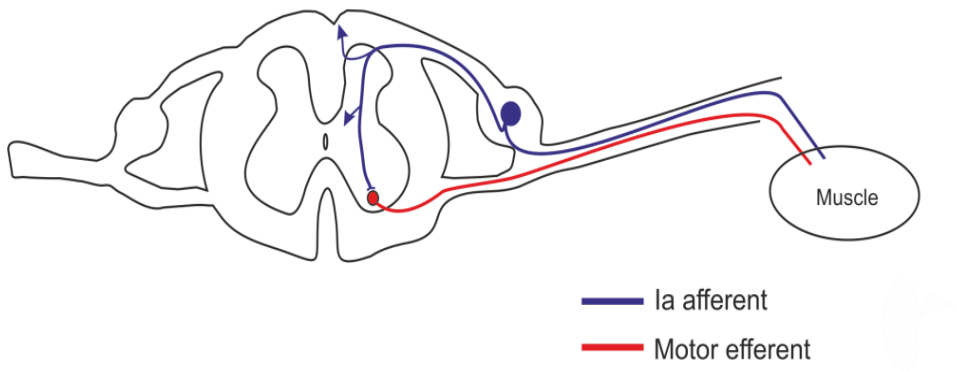

Figure 1. Schematic of the monosynaptic reflex, its components and connections.

1.3.1.1 PN development of motoneurons

Motoneurons are found in the ventral horn of the spinal cord and are organised into pools corresponding to the musculature which they innervate via their axons. Motoneurons of the lumbar spinal cord are born at E13-14 (Altman and Bayer, 1984) and undergo a period of degeneration and death up to PN5-6, after which no further decrease is observed (Bennett et al., 1983). Some work suggests there is no appreciable MN death PN, with 67% of MNs born dying pre or very early postnatally (Oppenheim, 1986). This indicates that motoneurone numbers will not change significantly with PN development. In terms of MN surface area, Westerga and Gramsbergen (1992) found significant increases in MN size between PN 8 and 12, however increases between PN12 and 20 were minimal. Other studies suggest that there is a moderate increase in MN size in the second postnatal week (Tanaka et al., 1992; Kerai et al., 1995).

Motoneurons are highly excitable at birth, becoming less so with PN development and this is thought to be due to changes in certain membrane properties. For example, although the resting potential of the MN seems to be stable from late prenatal stages, rheobase increases significantly with PN development, probably due to increasing soma size (Seebach and Mendell, 1996). In terms of permeability of the motoneurone to ions, PN changes in channel densities and properties have also been documented. Sodium currents are thought to increase in magnitude up to PN8 when they reach near mature levels. In the mouse, Jiang et al. (1999b) found that an L type calcium channel blocker was ineffective after inducing rhythmic activity in MNs aged PN2 but acted to attenuate motor output after PN7. Additionally, significant increases in expression of α1C and α1D L type calcium channel subunits were observed. The most striking change is in the effects of glycine and GABA early

postnatally. These normally inhibitory neurotransmitters are excitatory prenatally and early postnatally due to high intracellular chloride (Cl^-_i). Expression of KCC2 transporter which is responsible for Cl^- extrusion, is increased with development moving the equilibrium potential for Cl^- towards mature levels (PN9-10) and therefore inducing depolarising effects of GABA and glycine (Ben-Ari, 2002).
In general, maturity of MN and their firing characteristics, are thought to be achieved within the first 7-10 days PN.

1.3.1.2 Muscle afferents

After observing the tonic contraction of antigravity (extensor) muscles in decerebrate preparations, Sherrington (1898) postulated that the contraction of these muscles was due to a peripheral factor. When the distal tendon was detached from its insertion and pulled on, causing a stretch of the muscle, there was a resultant contraction of that muscle. This was referred to as the stretch reflex. The stretch reflex is evoked in response to stretch of the muscle, which in turn activates muscle spindles (Matthews, 1972; Matthews, 1981; Leskell, 1945). Muscle spindles are innervated by primary (Ia) and secondary (II) muscle afferents of which the former tends to be more velocity and acceleration sensitive compared to the latter which is more sensitive to static muscle stretch (Awiszus and Scha, 1993; Scott et al., 1994; Baumann and Hulliger, 1991). Ia afferents project to the spinal cord via the dorsal root ganglion and upon entering the spinal cord send projections rostral and caudal via dorsal columns to other segments and the brain via Clarke's column and the spinocerebellar pathway (Fern et al., 1988; Lloyd and McIntyre, 1950; Landgren and Silfvenius, 1971). Ia afferent terminations are widespread in the mammalian spinal cord spanning almost the entire dorso-ventral aspect of the grey matter, however terminations are meagre in the most superficial laminae (Kolmodin, 1957; Schaible

et al., 1987). Despite these widespread terminations, the identified 1st order interneurons which they activate are relatively sparse (Jankowska, 1992). Ia afferent terminations from the hind limb muscles in cats rarely project higher than L1, with volleys from large diameter afferents of the cat hind limb being greatest in lumbar regions 5-7, suggesting that most of the 1st order interneurons are located in more caudal segments (Eccles et al., 1957b). Until recently, only discrete populations of interneurons could be identified as having proprioceptive afferent terminations because it is difficult to identify interneurons based on electrophysiological properties alone. This is likely to change however, with the introduction of monosynaptic rabies virus which can selectively mark premotor interneurons specifically acting on the injected muscle up to PN14(Stepien et al., 2010; Wickersham et al., 2007).

1.3.1.3 Postnatal development of muscle afferents

The MSR can be evoked prenatally, meaning that afferent connection with target MNs is established in utero (Naka, 1964; Saito, 1979; Kudo and Yamada, 1987a). Indeed, Kudo and Yamada (1987a) showed that afferent collaterals invading the motor nuclei were present by E16 and increased throughout the prenatal period. Growth cones decreased in the same period and were down to 15% by E20.5 indicating that collateral sprouting is almost complete by birth (E21). The latency of the MSR was reduced until E18.5 and then unchanged in later stages of prenatal development. The amplitude of the MSR is increased throughout prenatal development, peaking at PN3 when it begins to decrease as the animal reaches PN8 (Kudo and Yamada, 1985; Kudo and Yamada, 1987a). Research into the postnatal development of the MSR is scarce; however some studies have assessed its

development both physiologically and anatomically. Fitzgerald et al. (1987) studied the PN development of the ventral root reflex (VRR) in rats up to PN14. The ventral root reflex is a reflex response evoked by stimulating the dorsal root and recording from the ventral root. They found that the latency of the VRR decreased between PN0 and 14 and attributed this to a reduction in central delay. This corroborates the work of Skoglund (1960) who showed that proprioceptive reflexes in the kitten reached adult latencies by PN 28. In that study, the conduction velocities of myelinating afferents were found to increase with age and contribute to decreasing reflex latencies. As well as myelination and central delay being altered with development, Chakrabarty and Martin (2011a) found that the terminal fields of proprioceptive afferents in the cervical spinal cord were refined throughout PN development of kittens. Field potentials evoked by stimulating proprioceptive afferents and recording from the intermediate and dorsal horn were wide spread early in development, becoming more localised with maturity. Similarly, Gibson and Clowry (1999) showed that cholera toxin β labelled afferents in the cervical and thoracic spinal cord of the rat were retracted from the ventral horn during PN development. This retraction of muscle afferents is believed to be dependent on arrival and maturation of CST terminations (Clowry, 2007). This was demonstrated in intact kittens by Chakrabarty and Martin (2011b), with afferent innervation of the intermediate zones of the cervical cord retracted as CST axons proliferated. In rats, lesions to the corticospinal system during development but not adulthood results in attenuated retraction of afferents from the spinal cord and even PN sprouting in some cases (Gibson et al., 2000; Clowry et al., 2004b). Interestingly, peripheral insults often result in sprouting of CST axons, suggesting that an activity dependent competition for synaptic coverage of spinal circuitry between CST and peripheral

afferents (Clowry et al., 2006). More recently, this competition has been demonstrated to persist into adulthood, with lesions to adult and peripheral loci resulting in plasticity of the uninjured system (Jiang et al., 2016; Tan et al., 2012). Much of the recent literature regarding PN development of proprioceptive afferents focusses on the cervical spinal cord and as a result we do not know if retraction and refinement of afferents or their developmental competition with descending systems is occurring in the lumbar cord as well. It will be important to identify how proprioceptive afferents in the lumbar spinal cord are altered with development as they are crucial to modulating complex behaviour such as locomotion (Pearson, 1995; Conway et al., 1987).

1.3.2 Evoking the MSR

Although the MSR can be elicited by muscle stretch, experimentally it is often necessary to bypass the muscle spindle in order to exert greater control over recruitment of afferent fibres (Fig 2). The MSR reflex can also be evoked directly by

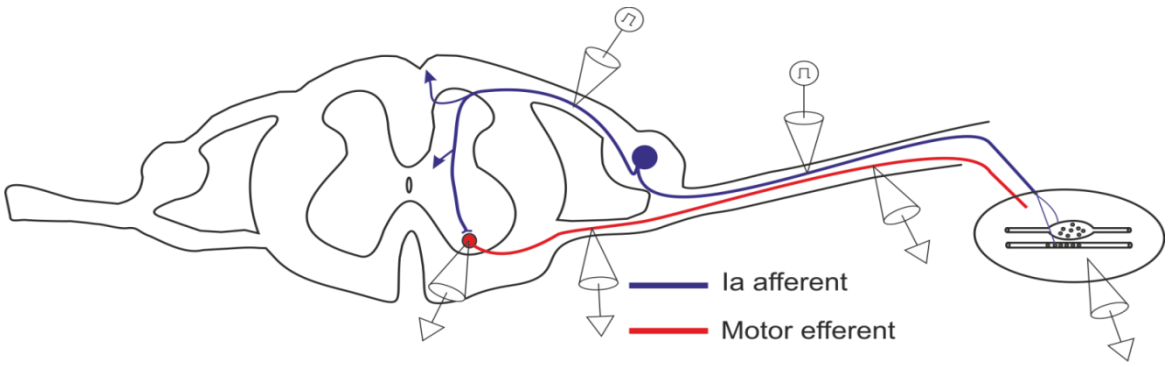

Figure 2. The monosynaptic reflex and the possible sites of stimulation and recording. The cone shapes with triangles on top represent the possible recording sites for the MSR and the shapes with a square pulse enclosed by a circle are possible stimulation sites

graded stimulation of the mixed peripheral nerve in order to specifically activate group I afferent fibres, which monosynaptically innervate their target motoneurone

(Eccles and Pritchard, 1937; Renshaw, 1940). In response to the evoked afferent volley, the motoneurone fires an action potential which can be recorded either from the motoneurone itself (Eccles, 1952), as an electroneurography (ENG) or an electromyography (EMG). Evoking these electrical analogues of the stretch reflex and the development of microelectrode recording techniques has allowed further characterisation of this circuit and its interactions (Eccles, 1952).

1.3.3 The Hoffman Reflex

Hoffmann (1918) developed a protocol for eliciting and assessing the MSR in humans by percutaneously stimulating the posterior tibial nerve to evoke a distinct response in the gastrocnemius/soleus muscle. This response was later found to be monosynaptic, termed the Hoffman reflex (H-reflex) and characterised fully in order that it may be used for studying motoneurone excitability under various conditions in humans (Magladery and McDougal Jr, 1950; Magladery et al., 1952b; Magladery et al., 1952a).

1.3.3.1 Methodology

The H-reflex was primarily designed as a non-invasive method for evoking MSRs in humans but has been adapted for use in animals as well. Some of the methodological considerations are specific to either human or animal studies while some are relevant to both.

The H reflex has well defined characteristics, which in most cases makes it easily distinguishable. Primary (Ia) muscle afferents have the largest diameter axons within the mixed nerve bundle (Hunt, 1954) and in turn, the lowest resistance. Therefore, graded stimulation of the mixed nerve should see Ia afferents activated primarily, evoking a late synaptic response which is preceded by a direct muscle response (M-

Wave) as smaller diameter motor axons are activated. Increasing stimulation intensity results in greater amplitudes of both waves, with the H wave initially exceeding the M wave. Eventually, the M wave exceeds the H wave and collisions begin to occur between M waves travelling antidromically along the motor axon and orthodromic H waves from the motoneurone traversing the same axon. This results in attenuation and eventually abolition of the H wave. Stimulation intensity is not usually increased beyond the point of H wave abolition, but some studies (mainly human) have illustrated that high intensity stimulation can result in another late response (F wave) which could be confused with the H reflex as they occur at similar latencies (Fisher, 1992). This response occurs due to excessive ‘back firing’ of the motor axons, antidromically activating the motor neurons (Trontelj, 1973).

1.3.3.2 Considerations for the H reflex in different preparations

The H-reflex was developed as a non-invasive procedure and therefore transcutaneous stimulation of muscle afferents, particularly at the popliteal fossa has been the norm in human studies. Either monopolar or bipolar electrodes can be used. The positioning of the stimulating electrodes is critical in eliciting consistent H waves, yet this depends entirely upon the preparation used. In human studies, transcutaneous stimulation is usually achieved by placing the cathode and anode in a position so that the current passes transversely through the nerve (ie cathode-popliteal fossa, anode-anterior of knee cap). In animal models, there are several different ways of stimulating the H reflex, both invasive and non-invasive. It is generally appreciated, however, that these responses are significantly more difficult to evoke in the rat, possibly due to smaller distances resulting in merging of M and H waves (Meinck, 1976).

1.3.3.2.1 Non-invasive preparations

Non-invasive techniques for evoking the H reflex have the advantage of allowing the researcher to assess how the reflex and its modulation change over time in the same animal in the absence of anaesthesia. There are, however, major drawbacks to this approach. The principles for evoking the reflex non-invasively in animals are essentially the same as in humans, with the exception of a few obvious pitfalls. The response can be evoked from many different muscles; although typically the plantar muscles of the foot are utilised when stimulating the tibial nerve, because responses in more proximal muscles (gastrocnemius/soleus-gs) often result in the M and H waves merging due to the short distance.

Changes in joint and limb position can considerably affect the amplitude of the H reflex due to changes in the sensitivity of homonymous and heteronymous muscle spindles and therefore the activation of Ia afferents (Hultborn et al., 1996; Knikou and Rymer, 2002). This is significantly harder to control in a rat. The obvious solution is to fix the limb/joint in place, but this would require light anaesthesia which is known to depress neural output (Ho and Waite, 2002). Similarly, with or without anaesthesia, it is extremely difficult to ensure that the same portion of the nerve is stimulated, exciting the same proportion of afferents each time due to variation in electrode placement and the nerve respective to the electrode. Additionally, because there is no direct contact between the nerve and the electrode, the ‘actual response threshold’ is impossible to determine because the current needs to pass through varying densities of tissue before it reaches the nerve.

In order to account for these confounds, adjustments to the stimulation parameters have been attempted. Invariably this has involved the procedure becoming more invasive. Still minimally invasive, H reflexes have been evoked using subcutaneous

stimulation of the tibial nerve at the ankle in order to evoke a response in the plantar foot muscles (Stanley, 1981; Koutsikou et al., 2015; Koutsikou et al., 2014). This might ameliorate issues described above, but certainly does not circumvent them. Limb position is still an issue and one cannot be certain that the electrodes are accessing the same portion of nerve despite increasing the proximity. Percutaneous stimulation of the proximal tibial nerve for recording the gastrocnemius/soleus H reflex has been achieved through percutaneous needle puncture but encounters the same problems as subcutaneous techniques (Bandaru et al., 2015).

1.3.3.3 Invasive preparations

Invasive approaches to H reflex experiments are used to gain greater control over the stimulation and recording conditions in animals (Hultborn et al., 1996). Specific nerve branches, innervating target muscles, can be mounted on (usually bipolar) electrodes providing more reliable thresholds (2-5 mA) for stimulating only Ia afferents than trans/percutaneous stimulation (≥ 1A) (Bandaru et al., 2015). As a result, it is easier to selectively activate Ia afferents by controlling the stimulus intensity. Although it is still difficult to ensure the exact locus on the nerve being stimulated between subjects and experiments, the control over this variable is far greater than in non-invasive techniques.

However, accessing the nerve involves incising skin and disturbing muscle and so animals needs to be rendered insentient. This is achieved by administering anaesthetic or by decerebration. Volatile (isoflurane/halothane) or injectable (ketamine, pentobarbital) anaesthesia can be used to relieve sentience, however doing so depresses neural activity. The type and level of anaesthetic has a significant impact on the H reflex and its modulation (Ho and Waite, 2002). Activity depression can be circumvented by decerebration to induce insentience but this also

removes descending modulation of the H reflex by areas rostral to the superior colliculus. Furthermore, decerebrate preparations often require artificial respiration and heart rate/blood pressure monitoring which increases set up time and makes study of these responses in smaller or younger rodents difficult.

Due to the numerous methodological difficulties described above, there have been no studies into the PN development of the H reflex. Some MSR studies have been conducted in reduced preparations however these lack presence of periphery and descending systems. There is therefore scope for development of a preparation which allows PN developmental study of the H reflex in rats.

1.3.4 Controlling variables and normalising results

1.3.4.1 Post activation depression

Activation of Ia afferents by either prior homonymous stretch, homonymous tendon tap or electrical stimulation has been shown to depress subsequent reflexes evoked from the same afferents (Katz et al., 1977; Crone and Nielsen, 1989; Hultborn et al., 1996; Magladery et al., 1952b; Magladery et al., 1952a). This depression was found to be long lasting (up to 15 seconds) and termed post activation depression (PD). It is certain that this depression is not due to decreased MN excitability, as other descending systems are capable of exciting the MNs regardless of prior activation of afferents. It has since been suggested that this depression may be due to a reduction in availability of neurotransmitter at the afferent terminal (Hultborn et al., 1996; Katz et al., 1977).

This has provided an important methodological consideration when assessing H reflexes. Experimental designs should ensure that inter-stimulus intervals are not less than 5 seconds in order to minimise the effect of PD on reflex size.

1.3.4.2 Normalisation

Due to the many different types of preparations, anaesthesia, decerebrations and variability in joint angle, etc., H reflexes can never be assessed as raw values and it is necessary to have stringent normalisation protocols. Because the maximum M wave amplitude (Mmax) is thought to represent the activation of all MNs in the pool, H waves can be normalised to this value (H/Mmax). Additionally, if conditioning pulses are being utilised, the test reflex can be normalised to the amplitude of the control reflex.

1.3.5 PN development of premotor inputs to MNs

The final stage at which motor output can be influenced (excluding Renshaw cells-RC) is at the level of the premotor interneuron. Premotor interneurons will receive and integrate input from a multitude of afferent, supra and intraspinal sources to modulate MN firing. Many interneurone populations remain elusive and have yet to be identified or characterised. Recent developments however, have allowed the identification of certain populations of interneurons based on molecular characteristics related to their progenitor domains (Jessell, 2000). Combined with the introduction of monosynaptic viruses for retrograde tracing a single synapse, targeting specific premotor interneurons for characterisation should be possible, extending our knowledge of the organisation and function of the spinal motor circuitry. Synaptic contacts on MNs can be identified and quantified using immunohistochemistry to target the specific neurotransmitter phenotypes. This can

indicate which population of interneurons the inputs may come from allowing hypotheses to be made about how motor output is being modulated.

1.3.5.1 Excitatory inputs to MNs

Cholinergic

Motoneurons utilise acetylcholine (ACh) as their neurotransmitter at the neuromuscular junction but also for inputs to Renshaw cells in a recurrent inhibitory circuit and to other MNs via axon collaterals (Lagerbäck et al., 1981; Willis, 1971). There are also many spinal interneurons which utilise ACh. Cholinergic 'C' boutons can be found on motorneurons, and although their origin has been difficult to locate, they certainly play an important role in motoneurone firing. This is demonstrated during fictive locomotion in isolated mouse spinal cord preparations, as pharmacological activation of muscarinic receptors results in increased firing of MNs and blocking these receptors significantly reduces firing (Miles et al., 2007). Huang et al. (2000) attempted to identify the location of cholinergic interneurons activated during locomotion. In their study, stimulation of the mesencephalic locomotor region (MLR) in the cat induced bouts of fictive locomotion which was used to drive cFos expression. They identified a group of $ChAT^+$ interneurons in the medial part of lamina VII, adjacent to lamina X that expressed high levels of cFos following fictive locomotion. This location corresponded to the region in which partition cells were identified by Barber et al. (1984) and so the group concluded that these cells play an important role in locomotion. Zagoraiou et al. (2009) attempted to uncover the role of these cholinergic interneurons in locomotion. They identified that a cluster of cholinergic cells in the location of partition cells can be recognised by the transcription factor PitX2 from the V0 lineage. Showing co-localisation of

fluorescence on terminals contacting MNs stained against VAChT in PitX2 green fluorescent protein (GFP) mice, they identified that PitX2 cells in lamina X are the sole source of C boutons on MNs. Additionally, they showed that activity in these neurons is phase locked with MN firing during fictive locomotion in slice preparations and genetically eliminating the enzyme used to make ACh (Choline acetyltransferase-ChAT) results in severe disruptions to the ability of mice to activate certain muscles during locomotion in vivo. This was an important finding as quantification of ChAT positive terminals on MNs under certain conditions can allow the experimenter to make inferences about the effect of that condition on cholinergic control of locomotion. It should be noted however, that these results were gathered from neonatal mice which were not yet capable of over ground locomotion and therefore developmental considerations should be taken into account.

1.3.5.2 PN development of C boutons

Conradi and Ronnevi (1975) conducted an electron microscopy (EM) study into the PN development of synaptic coverage of MNs in cats. They found that in general, coverage of MNs was high early in development and reduced significantly by adulthood. This was true for C boutons which observed a 20% reduction between neonate and adult stages. Wetts and Vaughn (2001) conducted an immunohistochemical study of cholinergic inputs to MNs and saw a slight reduction in $VAChT^{+}$ terminals on the soma between PN8 and 15, with an increase observed between PN15 and 22. These results should be treated with caution however, as they only studied 22 cells (total) analysed from 1 section (total) in 1 animal per age group (at PN8, 15 and 22) which were all from the same and only litter studied. Clearly those results hold no statistical or biological significance. Wilson et al. (2004) also suggested an increase in $VAChT^{+}$ puncta density between PN10 and 14

followed by a plateau. However, that study was limited to purely qualitative analysis of an unspecified number of animals and sections meaning their conclusions have no empirical support.

The study holding most validity regarding the PN development of C boutons on MNs is that of Conradi and Ronnevi (1975), suggesting that they are pruned during PN development of the cat. Because of the shortcomings of the aforementioned studies, there is no knowledge of the PN development of C boutons in rats. Additionally, although the net effect might be retraction in cats, there has been no study of the developmental profile of these inputs throughout PN development, so we do not know at which point they reach maturity. Considering that there are several important time points during postnatal development of rat behaviour (Altman and Sudarshan, 1975) and the importance of cholinergic premotor projections to locomotion, there is scope to study the PN development of C boutons in rats.

Glutamatergic Inputs

Glutamate is the main excitatory neurotransmitter in the CNS and many subsystems and neurons utilise it. In the spinal cord there are many interneurons and descending tracts which utilise this neurotransmitter, however in this section I will focus on identified lumbar interneurons which project directly to MNs. Locomotor-like activity can be evoked in isolated spinal cord and even slice preparations by adding agonists of NMDA and AMPA receptors, mimicking glutamatergic (probably descending/and or afferent) drive to the central pattern generators (Du Beau et al., 2012; Alvarez et al., 2004). One of the main sources of glutamatergic input to MNs is from large diameter sensory afferents which express VGLUT1 (Alvarez et al., 2004). Other sensory afferents are also glutamatergic but express VGLUT2 and do not project

directly to MNs (Alvarez et al., 2004), thus VGLUT2 would reveal either descending inputs (discussed later) or premotor interneurons (Du Beau et al., 2012). These interneurons would likely be responsible for integrating descending, afferent or intraspinal information before relaying it to MNs. One such interneuron was described in the cervical spinal cord and is believed to integrate cutaneous and to a lesser extent proprioceptive information. Bui et al. (2013) suggested that a population of VGLUT2^{+} interneurons in the deep dorsal horn contribute to processing information from cutaneous afferents, especially during grip tasks. A ‘dI3’ Interneuron was identified based on the transcription factor (*Isl1)* related to its progenitor domain. Using this molecular character, they were able to express yellow fluorescent protein (YFP) in dI3 interneurons and their axons and dendrites. This revealed that dI3 interneurons in the deep dorsal and intermediate zone projected monosynaptically to MNs and co-localised with VGLUT2. Additionally, terminals positive for Tetramethylrhodamine (TMRD), which was injected into cutaneous (sural) nerves were found on dI3 cells, indicating monosynaptic projection from cutaneous fibres. Selective knockdown of VGLUT2 expressed by this interneuron in mice resulted in severely deficient grasping abilities along with disrupted cutaneous reflexes. More recently, the same group showed that genetic ablation of these interneurons had no significant effect on locomotion in normal animals, however after a spinal cord transection they could not learn to walk with treadmill training, whereas wild type animals could (Stefani et al,. 2015). They suggested that this particular set of interneurons might play a role in processing of afferent information and contributing to reorganisation of spinal circuity following lesions.

1.3.5.3 Identified Inhibitory inputs to MNs

Ia inhibitory interneurons

Contraction of a muscle occurs in concert with the relaxation of the antagonist muscle, a phenomenon which has been described as reciprocal inhibition (Sherrington, 1897; Lloyd, 1941a; Eccles et al., 1956). Reciprocal inhibition is mediated by Ia inhibitory interneurons (IaIN) which are located in LVII, just dorsomedial to motor nuclei in LIX (Jankowska and Lindström, 1972) and project to the soma and proximal dendrites of antagonistic MNs (Burke et al., 1971). IaINs are activated primarily by Ia afferents, and so inputs from muscle spindles are dense. IaINs are difficult to locate histologically. They share the V1 lineage with Renshaw cells and are parvalbumin positive (Alvarez et al., 2005) . Recently Ia interneurons have been identified by locating neurons in the correct area, that are PV^+ and have Ia afferent (VGLUT1) and Renshaw (calbindin) terminals. Ia interneurons are thought to exert their inhibition through glycine or GABA, or a combination of both (Curtis et al., 1968; Curtis, 1959).

Wang et al. (2008) studied PN development of Ia reciprocal inhibition in the mouse and found that it was specific and functional at birth, however inhibition was weak, becoming stronger by PN3. No later assessments of this disynaptic pathway have been made.

Renshaw cell-MN recurrent inhibitory circuit

Activity of MNs was first shown to be depressed by antidromic impulses in motor axons by Renshaw (1941). This was determined to be mediated by interneurons (Renshaw cells) located in the ventral horn of the spinal cord and was termed recurrent inhibition (Renshaw, 1946; Eccles et al., 1954; Van Keulen, 1981).

Recurrent inhibition is mediated by motor axon collaterals onto RCs and therefore ACh is the active neurotransmitter, whereas the recurrent depression of the MN (or Ia IN) is mediated by glycine (Fyffe, 1991). Renshaw cells can be located mainly in lamina IX or most ventral regions of VII (Lagerbäck and Kellerth, 1985; Thomas and Wilson, 1965; Jankowska and Lindstrom, 1971). More recently, it was determined that in humans, monkeys and rats (but not cats) Renshaw cells can be identified by their strong expression of calbindin (Alvarez et al., 1999).

Although the precise function of RCs in motor control is not understood, it has recently been shown that the RC-MC recurrent inhibitory circuit may exert an extremely powerful modulatory effect on motor output, more so than was previously appreciated (Moore et al., 2015). This was done by conducting dual recordings from pairs of MNs and RCs. Using quantal analysis, the group identified that interactions in the recurrent circuit show a remarkable efficacy, exerting powerful inhibitory effects on motor output.

As well as inputs from motor axon collaterals RCs have many other inputs from descending systems and other interneurons. Interestingly, they also have been shown to have terminations from Ia afferents, but these are not thought to be functionally significant in adults as their stimulation does not activate RCs (Mentis et al., 2006a; Eccles et al., 1957a; Renshaw, 1946). Early in development (PN2-4) however, stimulation of dorsal roots does activate Renshaw cells (Mentis et al., 2006a) and VGLUT1+ terminals are relatively affluent on these cells at this point. These inputs have been shown to decrease slightly with development as motor axon collaterals are increased (Mentis et al., 2006b). Renshaw cell size is thought to increase with age, which could contribute to decreased input, however this was accounted for by normalising to cell surface area (Alvarez et al., 2013). Recently it

has been suggested that proliferation of MN axon collaterals on RCs may be dependent on Ia retraction as genetically up regulating Ia afferents in the lumbar cord decreases motor axon collaterals on RCs and knocking down Ia afferents increased collaterals (Siembab et al., 2015). As discussed above, neonatal spinal and supraspinal injuries lead to increased afferent input to ventral regions of the spinal grey. It is not known if increased termination in ventral fields results in increased contacts on neurons but it is plausible that spinal/supraspinal lesions may result in reduced retraction of Ia terminals from RCs. If the conclusions made by Siembab et al., (2015) hold true, it is expected that proliferation of motor axon collaterals on RCs with development will be significantly attenuated. This needs to be assessed, as it could have great functional implications relating to increased excitability of the neonatally injured cord or their increased prospects of functional recovery.

$GABA_{pre}$ interneurons.

The central nervous system needs to make sense of a constant barrage of afferent information from the periphery to produce well directed and efficient outputs. In order to do this it needs to select important information and dampen noise which adds variability to function, in addition to directing information to the relevant integrative centres. It therefore makes sense for the spinal cord to have neurons which can be ‘prompted’ to gate certain information from afferent sources in a way which produces efficient movements (Fink et al., 2014). This underpins the concept of practice in learning, in which repetition can provide an internal model allowing movements to be produced quickly and precisely (Perez et al., 2005). One group of neurons which has been implicated in such a function is the GABApre interneurons mediating

presynaptic inhibition of afferents (Fink et al., 2014).This has been demonstrated experimentally when relatively complex tasks are repetitively performed and H reflex responses are attenuated due to presynaptic inhibition for up to 10 minutes (Perez et al., 2005).

Presynaptic inhibition was first observed when Frank and Fuortes (1957) noticed that monosynaptic activation of MNs could be depressed in the absence of any change of the postsynaptic potential or MN excitability. Later experiments determined that the time course of the MSR was not altered and descending excitation of MNs was unaffected (Eide et al., 1968; Eccles et al., 1961). This depression was deemed to be presynaptic and mediated by depolarisation of Ia afferents (primary afferent depolarisation-PAD) leading to reduced NT release (Rudomin and Schmidt, 1999). Three types of PAD have been suggested to modulate firing of not just group I but also group II and cutaneous afferents. Type A PAD has been described as the depolarisation of spindle afferents by (mainly) flexor reflex afferents. Depolarisation of Ib afferents can be mediated by cutaneous/muscle afferents and supraspinal structures (Type B) (Rudomin et al., 1983). Stimulation of group I and supraspinal systems could also produce PAD and this is Type C PAD (Enríquez et al., 1996). This evidence suggests that activity of GABApre neurons is based upon the integration of information from multiple convergent descending and afferent inputs. Histological characterisation of P boutons has allowed their location and projections to be identified. Presynaptic contacts were first observed by Gray (1962), confirmed to be in association with Ia afferents contacting motoneurons and termed ‘P boutons’ (Conradi, 1969). Approximately 86% of Ia afferent terminals are contacted by ‘P boutons’ and have been shown to express GABA (Pierce and Mendell, 1993; Destombes et al., 1996). P boutons have also been shown to contact Ib, II and

cutaneous afferents (Lamotte d'Incamps et al., 1997; Maxwell and Riddell, 1999; Maxwell et al., 1995). GABApre neurons are thought to reside mainly in the deep medial dorsal horn and Betley et al. (2009) suggested that their projections (P boutons) are solely stimulated by the presence of proprioceptive afferents because reductions in afferents resulted in reductions in P boutons Hughes et al. (2005). GABA can be synthesised by 2 enzymes named GAD67 and 65 (based on mol weight) with the former mainly found in on GABAergic terminals on MNs and the latter in P boutons. Therefore, P boutons can be identified using antibodies against GAD65.

More recently, Fink et al. (2014) used genetic targeting of GAD65 to selectively ablate P boutons. They found that presynaptic inhibition was greatly reduced and mice displayed 'limb oscillations' when reaching for objects. Thus, presynaptic modulation of afferent firing is deemed to be crucial in gating afferent information to the spinal cord, acting to dampen noise and smooth movement. This is likely to be crucial in all movements, including gross movements such as locomotion. Indeed, rhythmic dorsal root potential are observed following induction of fictive (MLR evoked) locomotion, demonstrating that PAD may be acting to modulate the many sequential reflexes which are crucial to locomotion (Perreault et al., 1999; Dubuc et al., 1988; Rossignol et al., 1998).

Surprisingly, there have been no assessments into the PN development of P boutons. This is curious as this is when afferent and descending inputs to the spinal cord are in greatest flux. Tracking the development of GABApre neuron projections with the developmental refinement of afferents and descending systems would provide a greater understanding of how they are established and regulated.

PN development of inhibitory inputs to MNs

During PN development of kittens, Simon and Horcholle-Bossavit (1999) studied the development of glycine and GABAergic terminals apposing MNs. They showed that GABA and glycine terminals on MNs were equal early in development, but by adult hood the glycine terminals were greater in number. This was due to a significant reduction in percentage MN coverage by GABAergic terminals whilst glycine terminals remained the same. Conradi and Ronnevi (1975) suggested that all synapses apposing cat MNs undergo postnatal stripping and F boutons, which can be glycinergic, GABAergic or both, see a particularly large reduction (50%). The CNS only utilises 2 main inhibitory NTs, and there are many inhibitory interneurons and descending systems which utilise them. Therefore it is not possible to determine where all the inputs come from, but quantification does allow one to get an overall picture of how inhibitory control of motor output is changing.

1.4 Preparations for studying PN development of motor control

Furthering our understanding in anatomy and physiology of the central nervous system is often as a result of technological and technical developments. For example, a great deal of our understanding of the organisation and function of the spinal circuitry responsible for generation of locomotion comes from the development of an isolated neonatal rodent spinal cord preparation (Otsuka and Konishi, 1974). This is an excellent preparation, which has continued to produce data contributing significantly to our understanding of motor control (Cowley and Schmidt, 1997; Kjaerulff and Kiehn, 1996; Kiehn and Butt, 2003; Bouvier et al., 2015). The advantages to the preparation are that it provides easy access to the circuitry of the spinal cord with good stability for electrophysiological recording. In addition, there is

no need for anaesthesia and locomotor like outputs can be activated using neuroactive drugs such as NMDA (Kudo and Yamada, 1987b) or electrical stimulation of afferents and/or brainstem locomotor regions (Zaporozhets et al., 2004; Marchetti et al., 2001). Despite the wealth of knowledge that has been gained from this preparation, it is accompanied by caveats and questions regarding the relevance of findings. Although locomotor-like outputs and air stepping can be produced even at prenatal ages, rats are not capable of over ground locomotion until PN12-14, reflecting an immaturity of the circuitry which has been shown to be significantly reorganised with PN development. (Altman and Sudarshan, 1975; Ozaki et al., 1996; Van Hartesveldt et al., 1991). The preparation is only viable up to PN 5-7 due to increasing size of the cord which hinders oxygenation and increasing metabolic demands of more mature cells (Mitra and Brownstone, 2012). Additionally, the preparation is reduced with no periphery available.

These drawbacks have not gone unnoticed, and there have been several attempts to prolong the viability of the preparation as well as perform ‘more complete’ preparations with limbs attached in order to improve physiological relevance.

Jiang et al. (1999a) managed to extend the viability if the isolated cord preparation to PN12-14 in the mouse although the preparation has not produced much data since. Because of the smaller size of the mouse spinal cord, this would suggest that increased metabolic demands or reduced hypoxia resistance in older animals is a major contributing factor to reduced viability in superfused preparations. Furthermore, the preparation still has the caveat of not having an intact periphery.

Hayes et al. (2009) described a ‘spinal cord-hindlimb’ preparation for studying locomotion in rats and were successful in producing locomotion which could be

recorded with EMGs; however, the cord is still superfused in this preparation and so is not viable beyond neonatal ages.

Efforts have been made to develop preparations which perfuse the CNS of mice and rats with artificial cerebrospinal fluid (ACSf) via their own circulatory system instead of bathing methods seen in isolated spinal cord-brainstems, thereby circumventing hypoxic core issues. A Working heart brainstem preparation (WHBP) was developed which involves perfusing the brain and rostral spinal cord via the descending aorta in the presence of forelimbs and thorax (Paton, 1996). This preparation has been shown to be viable from ages PN2-28 suggesting it is a good candidate for studying PN development. Furthermore, the same group also showed that this preparation has superior physiological relevance to *in vitro* cord preparations, producing phrenic nerve activity cycles at >45 per minute compared to 9 per minute seen in isolated cord-brainstem preparations (Smith et al., 1990; Wang et al., 1996). A similar Trunk-hindquarters preparation was introduced by Chizh et al. (1997) which perfuses the thoracic and lumbar spinal cord of adult mice (4-8 weeks) via the descending aorta. This preparation was promising but it lacked descending systems and it was not established if it was viable at neonatal ages. More recently, Pickering and Paton (2006) described an *in-situ* perfused whole rat preparation which was entirely intact apart from a pre-collicular decerebration. Again this preparation circumvented hypoxic core issues due to tissue being transcardially perfused. The preparation was viable up to PN28, which is comfortably motor mature and was successfully used for recording from muscles, nerves and spinal cord neurons. Importantly, the group observed spontaneous rhythmic hind limb behaviour in the preparation suggesting the complex brainstem/spinal cord circuitry involved in producing locomotion is viable. However, no attempts have been made to establish viability in animals of

neonatal age and so it remains to be seen if this preparation could be used for the study of PN development of motor control. It would seem that it is very likely these studies can be performed because neonatal cells are even more tolerant of hypoxia than older animals (Mitra and Brownstone, 2012).

Although there is currently no preparation established for studying the spinal cord physiology throughout PN development, there have been some promising candidates described by certain groups. These examples will need to be adopted and further developed in order to become preparations which be routinely used in motor control. Work in this area will significantly contribute to bridging the gap between isolated neonatal spinal cords and mature intact systems.

1.5 Postnatal development of descending systems

The central nervous system as an integrated structure can be divided into several regions and sub regions which need to communicate effectively in order to produce well directed, adaptable movements of varying complexities. The spinal cord for example is capable of generating movement in the absence of supraspinal structures, yet it relies on these structures to constantly refine and adapt this movement, especially in more complex environments. Descending tracts, originating from many different supraspinal loci have been characterised anatomically and physiologically and grouped into different systems. These systems can be described initially as cortical and subcortical. Descending tracts are named based on their site of origin and termination (eg cortico-spinal tract) and can be characterised based on their role in behaviour, neurotransmitter phenotype, size of the pathway (no. of fibres) and molecular identity (Lemon, 2008; Du Beau et al., 2012).

Descending systems are involved in much complex behaviour and there have been many studies into their roles in initiation and modulation of motor output. Although this knowledge is crucial to our understanding of motor control, the scope of this thesis is limited and therefore I will focus on development of descending systems input to the lumbar spinal cord and their interaction with muscle afferents.

1.5.1 The corticospinal tract

The corticospinal tract (CST) originates in the somatosensory and motor cortices and descends via the medullary pyramids, where it decussates to send 95% of its fibres contralaterally. Axons from the CST project to many brain stem regions before decussating to send projections to the spinal grey. The location of the spinal CST projection varies amongst species, however in the rat the majority of the CST descends via a ventral portion of the dorsal columns before terminating in the spinal grey matter (Brown Jr, 1971). A relatively small portion of the CST also descends ipsilaterally via the dorsolateral funiculus and ventral funiculi (Goodman et al., 1966; Joosten et al., 1989). Once in the spinal cord, CST projects to cervical thoracic and lumbar spinal cord terminating in the dorsomedial region of the intermediate and dorsal grey, where interneurones reside, thought to be responsible for controlling muscles at the extremities of both upper and lower limbs (Kuypers et al., 1962; Brown, 1974a). In the primate there are monosynaptic projections to the motor neurons innervating the hand and digits (Bernhard and Bohm, 1954), however other species, including rodents lack direct cortico-motorneuronal connections and rely on via excitatory and inhibitory interneuronal populations to exert control of motor output (Illert et al., 1976a; Yang and Lemon, 2003; Alstermark et al., 2004).

The motor cortex is thought to play a major role in modulating the activity of motor output in the lumbar spinal cord, with terminations on widespread and diverse sets of interneurons in the dorsal and intermediate grey (Lundberg et al., 1962; Lloyd, 1941b). Carpenter et al. (1963) showed that stimulation of the motor cortex resulted in dorsal root potentials indicating PAD in the lumbosacral spinal cord of the cat. Further investigation revealed that cortical stimulation resulted in depolarisation (PAD) of Ib, II and cutaneous afferent but not Ia. In humans, voluntary contraction or transcranial magnetic stimulation (TMS) activation results in reduced presynaptic inhibition of afferents innervating homonymous (contracted) muscle, whereas it is increased to antagonistic muscles (Iles and Roberts, 1987; Nielsen and Kagamihara, 1993; Hultborn et al., 1987). These results suggest that the motor cortex plays an important role in regulating how afferent information is gated by facilitating or inhibiting specific GABApre interneurons mediating PAD. This is likely to be important when calling upon motor programs of learned skills, where the cortex can 'prime' the spinal cord to gate afferent information (via PAD) based on previous experience in order to reduce noise.

1.5.2 PN development of the CST

PN development of the CST is retarded compared to other descending systems, with axons not reaching the lumbar segment until PN9 in the rat (Donatelle, 1977). CST axons in the dorsal funiculus have reached the rostral portions of the cervical cord at birth, but the spinal grey is devoid of terminations (Donatelle, 1977). By PN5 CST in the dorsal funiculi have traversed thoracic and reached initial portions of the lumbar region with robust terminations in the cervical grey but none in the lumbar gray. Coccygeal segments are reached by PN9 and there are terminations in all segments down to sacral regions. CST termination patterns reach maturity between PN13-17

with growth cones present on CST axons in the lumbar regions until PN14 (Joosten et al., 1989; Donatelle, 1977; Hicks and D'Amato, 1975). Myelination of the CST occurs late in development. Cervical regions only start myelinating around PN14 and the process is largely complete by PN28 (Joosten et al., 1989). Interestingly, in the kitten, Bruce and Tatton (1981) were not able to evoke cortically evoked muscle potentials until PN41 and for Chakrabarty and Martin (2000) responses were only evoked at PN75 suggesting a lingering immaturity of this pathway.

There is also believed to be a period of activity dependant postnatal refinement of CST terminations in the cervical spinal cord of cats, with CST innervations of deep dorsal and intermediate regions being retracted and pruned with development (Martin et al., 2007; Chakrabarty et al., 2009; Chakrabarty and Martin, 2011b). As stated above, afferent inputs to the spinal cord are also retracted with development at about the same time, suggesting co-development of these two systems (Chakrabarty and Martin, 2011b). A theory of termination co-dependency between afferents and CST seems to persist into adulthood as several other studies have shown that peripheral lesions result in sprouting of CST axons and CS system lesions allow afferents to sprout (Tan et al., 2012; Clowry et al., 2004a; Clowry et al., 2006; Jiang et al., 2016).It is important to note however, that co-dependency of afferents and CST has only ever been demonstrated in the cervical spinal cord and mainly in cats, presumably because of the common view that CST is mainly important in control of upper limb distal musculature . This is an oversight, especially considering that CST lesions in adult and the developing system can be severely dysfunctional often inducing hyper-excitability of the lumbar spinal cord and spasticity.

1.5.3 Brainstem systems

The brainstem contains many of the areas responsible for basic survival and motivation. Functional outcomes following brainstem lesions are severe and usually fatal. Insults to cardiorespiratory centres in the rostral ventrolateral medulla (RVLM) invariably result in death and lesions to other areas can significantly reduce quality of life by affecting vital functions such as gastric motility and bladder control (Wood et al., 1985; Tang and Ruch, 1956).

In contrast to the CST, brainstem regions are thought to be more involved in initiation and control of gross movements such as locomotion and control of proximal and axial muscles (Hinsey et al., 1930; Shik and Yagodnitsyn, 1977; Grillner and Shik, 1973). Brainstem projections to the lumbar spinal cord are made up of several different tracts from different regions and often act as a site of convergence of inputs from more rostral/superior centres. For example, it is known that the CST has terminations within the brainstem. In addition, the cerebellum does not have its own specific spinally projecting tract but it has many projections to the brainstem and cortex (Armstrong, 1988; Rispal-Padel, 1979). Most brainstem region projections to the lumbar spinal cord terminate on interneurons, with some direct input to motoneurons (Holstege and Kuypers, 1987). The reticulospinal tract has 2 main tracts which originate in the reticular formation and project via the dorsolateral and ventrolateral aspects of the white matter to the lumbar spinal cord (Jones and Yang, 1985). The reticulospinal tract utilises glutamate and predominately the transporter vesicular glutamate trasporter 2 (VGLUT2) which allows its axons and terminals to be identified anatomically (Du Beau et al., 2012). The rubrospinal tract stems from the red nucleus and projects to the lumbar spinal cord via the dorsolateral funiculus and also uses glutamate as its neurotransmitter (Antal et al., 1992; Brown, 1974b;

Du Beau et al., 2012). This systems is thought to be more important in limbed animals as it is absent in non-limbed animals (Ten Donkelaar, 1988). Furthermore, its projections from the magnocellular portion of the red nucleus have been shown to be less prominent in humans and higher apes compared to quadrupeds such as cats and rats (Padel et al., 1981; Ten Donkelaar, 1988). The red nucleus receives input from the cortex and so the dorsolateral funiculus is often referred to as the corticorubrospinal tract. In fictive locomotor preparations and spinal rats and cats, 5HT or analogs have been shown to evoke locomotion or strengthen its rhythm, demonstrating the importance of the brainstem systems, especially those projecting from the red nucleus to locomotion (Barbeau & Rossignol 1991, Gerasimenko et al 2007, Ichiyama et al 2008b). The vestibulospinal tract stems from the superior, medial and lateral vestibular nuclei and projects to the lumbar spinal cord via the ventrolateral and ventral funiculi bilaterally (Peterson et al 1978). Stimulation of vestibulo, reticulo and rubrospinal tracts all result in presynaptic inhibition of Ia afferents in the lumbar spinal cord (Rudomin & Schmidt 1999).

1.5.4 PN development of brainstem systems.

In contrast to the corticospinal system, most brainstem descending tracts have reached the lumbar spinal cord prenatally or early postnatally with projections from magno and gigantocellar nuclei thought to be the latest of the projections to reach the lumbar spinal cord at PN2-3 (Lakke, 2012; Commissiong, 1983; Tanaka et al., 1992; Leong et al., 1984). Despite having reached the lumbosacral spinal cord at birth it is unlikely that all these inputs are mature because in most CNS systems, once neuronal projections reach their targets, it takes variable amounts of time for functional connections to be made (Schoenfeld et al., 1979). Indeed, in the neonatal opossum, transection of the reticulo, rubro and vestibulospinal pathways had no

detriment to hind limb motility (Martin et al., 1981). Additionally, although vestibular righting reflexes are present at birth they mature over the subsequent 2 weeks (Altman and Sudarshan, 1975). Leong et al. (1984) injected horseradish peroxidase (HRP) into the lumbosacral spinal cord and studied the stained nuclei in the brainstem. They found that although the majority of bulbospinal pathways had numbers of stained nuclei which were equivalent to mature rats, cerebello and tectospinal tracts had only few or no stained nuclei. Tectospinal nuclei were not present until PN10-12 and both tecto and cerebellospinal were not at mature levels until after the 3rd week PN (Leong et al., 1984). The lateral vestibular nucleus (LVN) projection developed gradually, with stained nuclei increasing steadily through the first 3 months of life. In the cat, rubrospinal terminations are present early PN but corticospinal inactivation (post natal week 5-7) alters its development, suggesting CST activity is crucial to rubrospinal organisation, but also suggesting that rubrospinal terminations are present but not yet mature (Williams and Martin, 2015). Most strikingly, rats are not able to produce adult like locomotion until PN15-16 suggesting that these descending structures and/or their interactions with spinal locomotor circuits are not mature (Altman and Sudarshan, 1975; Westerga and Gramsbergen, 1993; Westerga and Gramsbergen, 1992). Hindlimb elevation of the trunk in the rat is achieved at PN6-7. Brocard et al. (1999) showed that stimulation of the ventrolateral funiculus (VLF) resulted in MN and ventral root responses at birth, but the latency and strengths of these evoked potentials increased significantly up to PN6, suggesting PN maturation of brainstem control of lumbar circuitry.

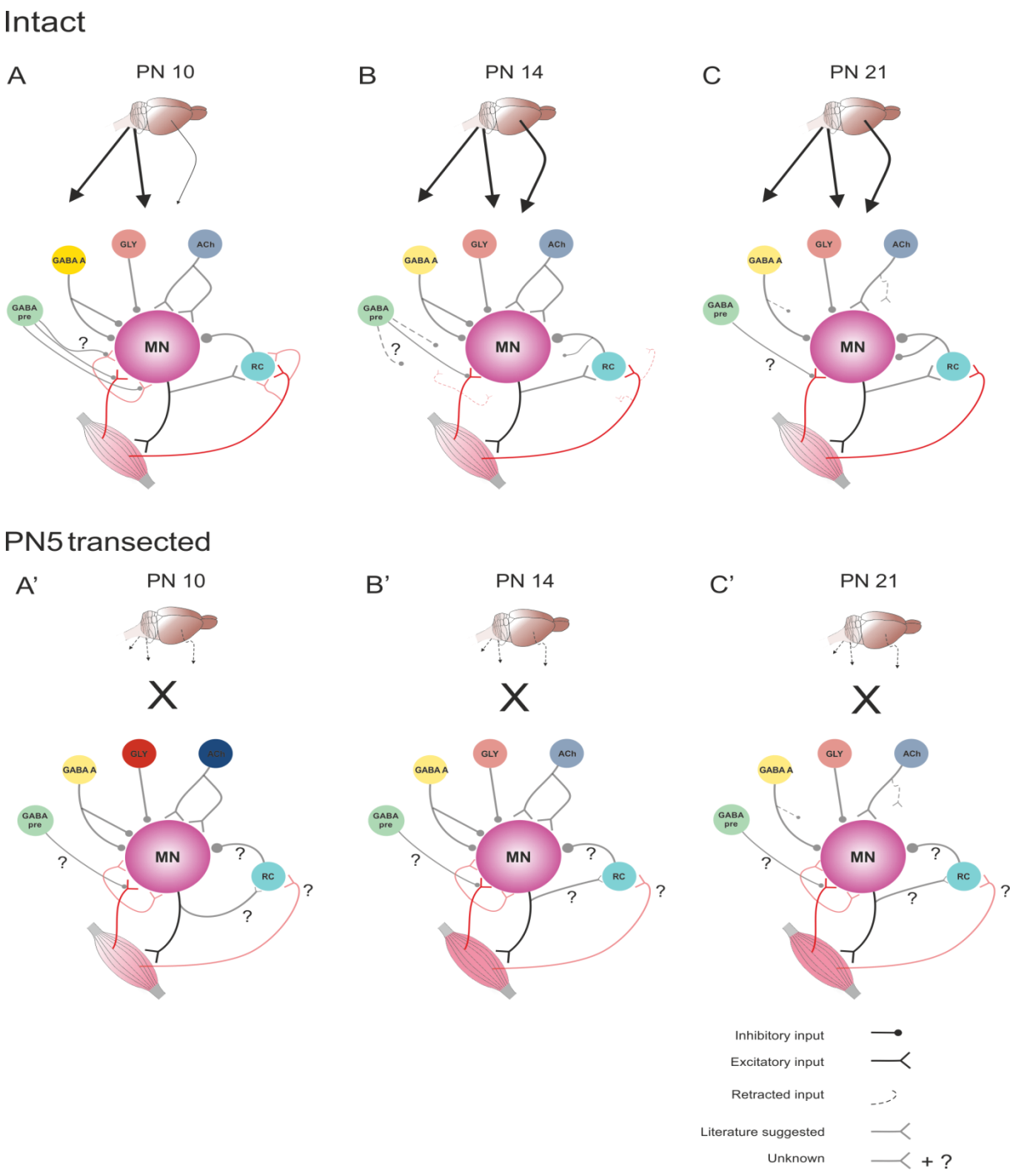

Figure 3. Schematic illustrating hypothesised lumbar spinal circuitry changes in intact and PN5 transected rats during postnatal development.
The thick arrows represent descending systems which have reached the lumbar cord at birth and thin arrow is the CST which does not arrive until PN9-10. Faded structures represent inputs which have been suggested based on the literature review. Faded structures with question marks represent inputs which have not been studied during PN development or the effect of PN5 TX is not known. MN, motoneuron; GABA, GABAergic premotor interneurone(IN); GLY, Glycinergic premotor IN; ACh, Cholinergic premotor IN;RC, Renshaw cell.

1.6 Aims and objectives

Some mammals are born with precocious motor abilities and some are naïve at birth and acquire mature behaviour as they develop postnatally. For humans, the latter is true and so it is of great interest to study how the circuitry of the CNS organises its self in order to produce mature movements. Because this process is activity dependent and the neonatal cord is highly plastic, patterns of activity during early development are likely to influence how mature circuitry is established. In the same way, insults and damage to certain structures of the nervous system during development are going to alter the organisation of other regions which normally rely on their interaction. Interestingly, equivalent injuries during adulthood and development often result in superior recovery in the case of the developmental injuries, suggesting fundamental differences in the manner in which the CNS responds to the insult. Our understanding of the process of postnatal maturation of motor circuit is limited, partly due to the lack of a preparation which allows assessments of physiology from behavioural immaturity to maturity and detailed studies of the organisation of spinal circuitry throughout PN development.

1.6.1 Aims

1) It is not currently possible to study the physiology of motor systems from neonatal ages (PN0-7) to maturity (PN21) in the same preparation. Much of the current knowledge regarding physiology and organisation of motor systems has come from isolated spinal cord preparations which are immature and lack a periphery. Therefore, we aim to establish a preparation for studying the circuitry involved in motor control throughout postnatal development. In this preparation we will

initially target evoking reflexes (such as the H reflex) in order to study their modulation throughout PN.

2) Studies in the cervical spinal cord suggest that there is considerable pruning of proprioceptive afferents with development, yet this has never been ratified in the lumbar spinal cord. Furthermore, developmental input to specific cell types such as MNs and RCs has not been assessed. Therefore we aim to study how proprioceptive afferent input to identified cell populations of the lumbar spinal cord is altered with PN development.

a) Organisation of the spinal circuitry is activity dependent. Postnatal changes to inputs from descending and afferent sources indicate altered activity patterns throughout development. This is likely to result in developmental reorganisation of spinal circuitry. To assess these changes we will use immunohistochemistry to study the development of premotor inputs to MNs.

3) Co-dependent regulation of synaptic coverage of proprioceptive afferent and descending systems has been indicated during developmental and lesion states. We aim to remove descending input to the lumbar cord at PN5, when projections are either immature or completely absent, depending on the supraspinal system. We will subsequently assess the impact on normal development of proprioceptive afferents.

a) Removing descending systems through spinalisation will alter activity patterns in the spinal circuitry, with afferent sources providing the only input to drive and modulate output. We expect that in response to this, there will be significant synaptic reorganisation of premotor inputs to MNs. Therefore we will compare the PN development of premotor inputs to MNs in the presence and absence of descending systems.

1.7 Hypotheses

1. We expect that the developmental retraction of proprioceptive afferents seen in previous studies of the cervical spinal cord will hold true in the lumbar cord. Furthermore we expect to see this retraction in relation to MNs and Renshaw cells in the ventral horn.

a. Due to altered activity patterns throughout development, with maturing inputs from peripheral and descending sources, we expect that premotor inputs to MNs will also be altered. There has not been a comprehensive study in this area; however several studies suggest retraction of inputs with development. We therefore expect that inputs will be great early in development and undergo postnatal retraction.

2. Following removal of descending systems at PN5, we expect that reduced competition from descending systems will result in proliferation and/or reduced retraction of proprioceptive afferents. This will lead to hyperexcitability of the H reflex. This is expected to be as a result of increased afferent input and reduced modulation from GABApre neurons compared to control.

a. Due to results from adult injury studies and the excitable state of the neonatal cord early in development, we expect that following PN5 TX, inhibitory inputs to MNs will be decreased and excitatory inputs increased. This is likely to contribute to the hyperexcitability of the H reflex.

Chapter 2. General Methods

2.1 Ethical approval

Experiments and procedures were performed in a manner that conformed to the UK Home Office guidelines regarding the use of animals. Approval was granted by the local ethics committee (University of Leeds).

2.2 Decerebrate, perfused whole rat preparation

2.2.1 Preparation Set up

Wistar rats aged 14-21 days postnatally(PN) were weighed then anaesthetised with Isoflurane (3% induction at 95% O^2, 5% CO^2 mix) until loss of the paw withdrawal. A midline laparotomy was performed and the stomach, spleen and free intestine were ligated and removed. Once evisceration was complete, the animal was transferred to an ice bath (5-10°C) containing artificial cerebrospinal fluid (ACSF composition listed below) bubbled with carbogen (95% oxygen, 5% Carbon dioxide) and a pre-collicular decerebration was performed. The decerebration takes < 30 seconds with a scalpel due to the thin skull; this immediately renders the animal insentient and its skin is removed. The animal was then transferred to a cold dissecting dish, where a midline sternotomy permitted access to the heart. Most of the lung parenchyma and thymus were removed for easy access to the heart. An incision was made into the apex of the heart for later insertion of the cannula (Ø 1.25 mm, DLR-4, Braintree Scientific, MA, USA) before another small incision was made into the right atrium to allow draining of the perfusate. The animal was then transferred (supine) to the recording chamber and laid on a custom sylgard (Dow Corning) bed. The cannula was then immediately inserted transcardially into the ascending aorta. The cannula was fixed in place using a single suture (no 2,

silk). Once the cannula was secured the animal was carefully transferred to the prone position and the flow rate was increased gradually over 1 minute until further increases in flow failed to increase pressure (~40mmHg). The pressure before and after turning the animal were compared to ensure the cannula had not retracted from the aorta.

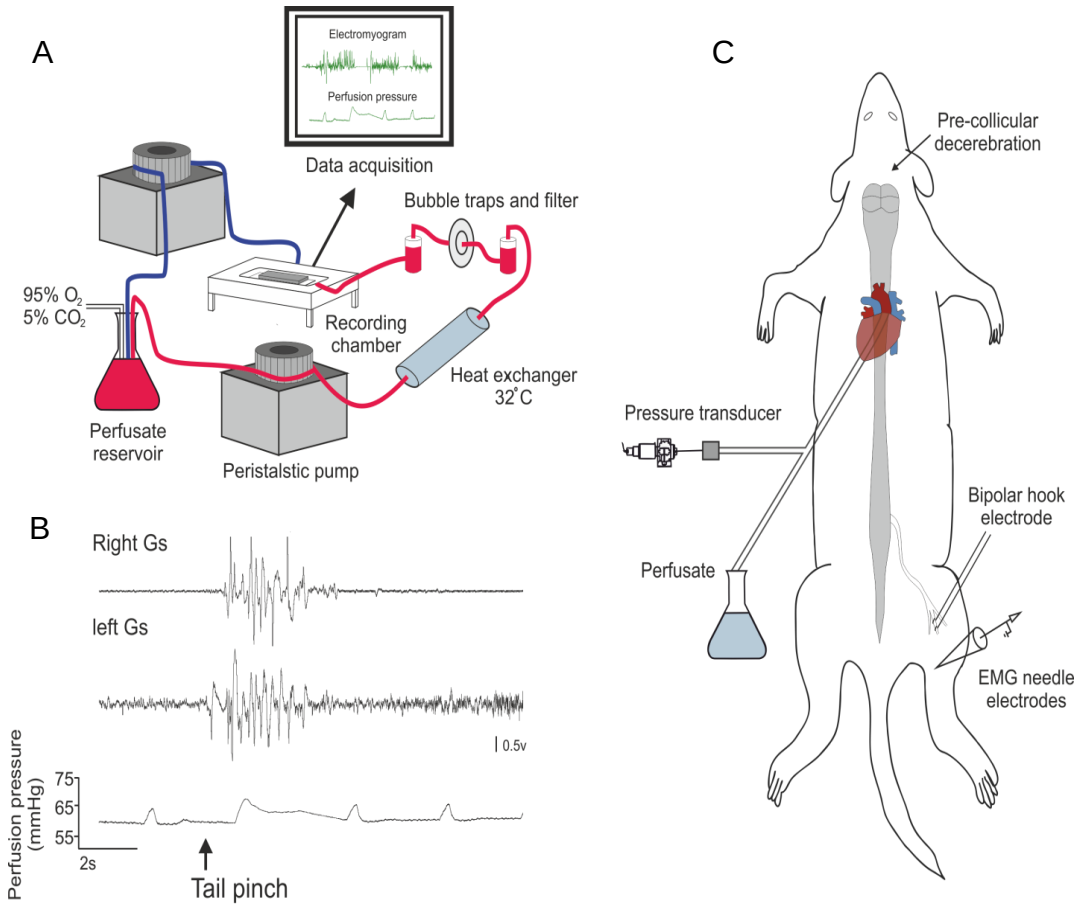

Figure 4. Preparation set up and ascertaining viability.
(A) shows the basic perfusion circuit and data acquisition set up. Red lines indicate oxygenated, efferent flow and the blue line represent afferent flow. The depiction of 2 peristaltic pumps is to better illustrate the efferent and afferent flow. In reality, flow in each direction is generated by a single pump. (B) shows how viability was ascertained. Perfusion pressure of >60mmHg resulted in pinch induced motor output. The preparation (C) is pinned to a silicone bed in the recording chamber and recordings are fed to data acquisition systems. Note the position of the cannula in the proximal ascending aorta. Schematic in (C) is adapted from Pickering and Paton (2006).

The oxygenated perfusate was pumped from a flask via a heat exchanger (32°C), bubble traps and a particle filter (25µm screen, Millipore) to the

preparation. The flow was generated by a pump (Gilson Minipuls 3) with the systemic volume being maintained at 200mL. After initiation of flow, the heart resumed beating immediately and the preparation started to warm up. Successful perfusion was indicated by liver blanching, filling of the skull cavity with perfusate and atrial distension. 100-200pM vasopressin (Arg-8-Vasopressin, AVP, AbcamBiochemicals) was added to the perfusate to increase the systemic resistance and pressure to 50-80 mmHg which has been deemed sufficient for adequate perfusion of the caudal spinal cord (Pickering and Paton, 2006). Respiratory contractions were observed once the pressure reached 40-50mmHg, indicating brainstem viability. Lumbar spinal cord viability was observed initially by spontaneous rhythmic hind limb movements and nociceptive reflexes in response to tail and hind limb pinches (Fig 4B). Increases in systemic pressure due to both nociception and muscle contractions (exercise pressor reflex) (Murphy et al., 2013; Mitchell et al., 1983) also indicated viability of the preparation. Once stimulation and recording equipment were in place, a robust H reflex confirmed viability of the lumbar cord (Fig 5C).

2.2.2 Solutions

Modified ringers solution (ACSF) was made up of: (mM) NaCl (125); $NaHCO_3$ (25); KCL (3); $CaCl_2$ (2.5); $MgSO_4$ (1.25); KH_2PO_4 (1.25); D-Glucose (10). A pH of 7.35-7.4 was attained once carbogenated. Polyethylene glycol (1.25%) was added as an oncotic agent.

2.2.3 Recording techniques

2.2.3.1 Isolation of Nerve

Once the cannula was secure and the animal was fixed in place using ear bars and hypodermic syringe needles, a small incision was made into the fascia separating the quadriceps and hamstring muscles. Using blunt dissection, the muscles were reflected, the sciatic nerve carefully freed from surrounding tissue (apart from its insertion into gastrocnemius) and the tibial branch located and mounted on a bipolar hook electrode. The nerve was kept moist by dripping aCSF intermittently.

2.2.3.2 Stimulation and Recording Methods

The nerve was stimulated via the hook electrodes with a monophasic square pulse generated by the constant current ISO-Flex isolated stimulator. Square pulses (0.3ms) were generated every 3 seconds using the A.M.P.I Master-8 to trigger the stimulator, allowing 3 s recovery between pulses. Evoked intramuscular (EMG) responses were recorded using fine bipolar, needle electrodes (SpesMedica) from the gastrocnemius muscle. For each animal, graded stimulation of the nerve produced a recruitment curve which was used to determine Ia afferent and motor axon thresholds (T, Fig 5C). H reflex threshold was determined as the intensity at which the smallest visible response occured at a frequency of ~50%. Stimulation intensity was then increased to 1.2xT, 1.4xT and 1.6xT. H reflexes on the ascending portion of the recruitment curve (small M wave, large H wave) were deemed optimal for paired pulse analysis (Fig 5B) however in some preparations robust H reflexes were only seen in the presence of relatively high amplitude M waves. During

thresholding, maximal M and H reflexes were recorded for normalisation. Care was taken to ensure that the M wave was constant throughout the experiment, however sometimes changes were unavoidable due to movement of the

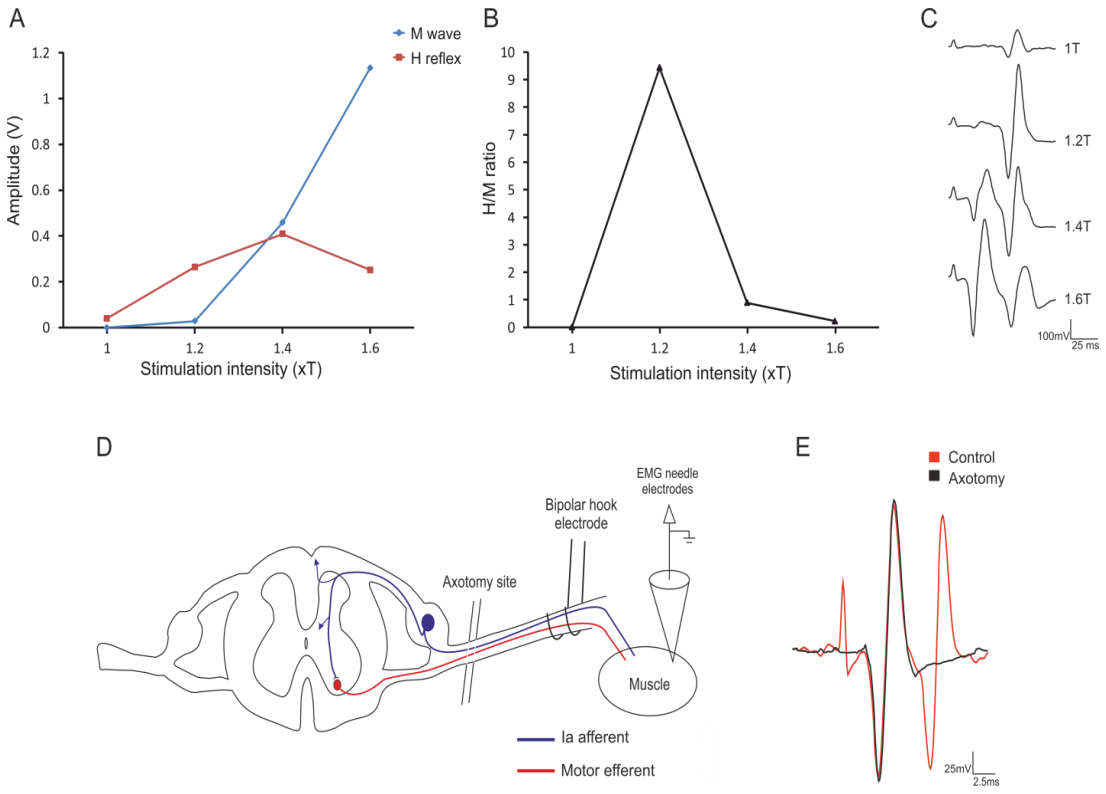

Figure 5. Characterisation and confirmation of the H reflex.
(A) Graded stimulation of the tibial nerve results in a typical H reflex and M wave recruitment curve. (B) shows the ratio of H to M and (C) illustrates the representative traces from the recruitment curves in A and B. (D) Shows the experimental set up and axotomy site. (E) post axotomy, robust M waves remains whilst the H reflex is abolished due to severance of afferent fibres.

preparation. In these instances, attempts were made to return the M wave amplitude to the same level as previous recordings. At the end of the experiment to further verify the H reflex, an axotomy was performed severing the nerve between the hook electrode and the exit of the mixed sciatic nerve from the vertebral column. This severed the sensory and motor nerves to and from the spinal cord but retained the motor axons between the stimulating hook and recording needle electrodes. Axotomy demonstrated that the H reflex was abolished but a strong M wave was retained (Fig 5E)

Paired pulse stimulation was used to assess excitability of the central circuits by evaluating the H reflex and its modulation. Dual pulses were administered to the nerve at intervals ranging from 1-700ms and the level of depression was analysed to produce a depression curve. At least 15 traces were recorded for each interval tested and there was a minimum rest period of 1 minute between trials. Control (single pulse) trials (black traces), also consisting of 15 traces, preceded each test trial for comparison and normalisation (Fig 6).

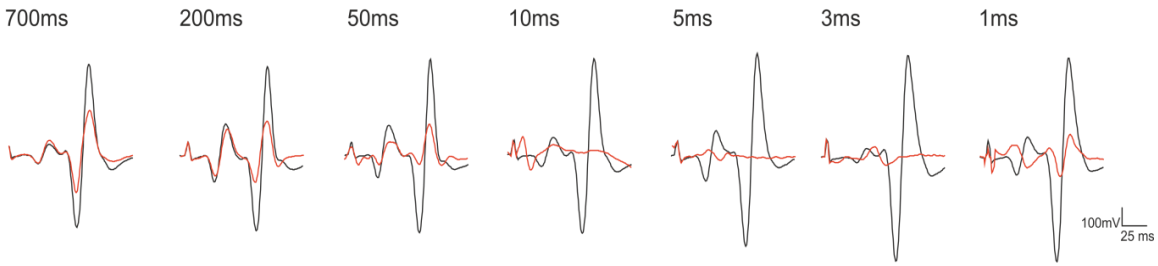

Figure 6. Inter pulse interval testing.
Black traces are control, red are test pulses. The traces are overlaid in order to show depression.

2.3 Data Analysis

EMG signals were recorded using a unit gain amplifier headstage (Digitimer, NL100) connected to a Neurolog amplifier (x 1000 amplification; Digitimer,NL900D). Signals were bandpass filtered between 50Hz and 10000 Hz and digitised using an analog to digital interface (1401micro, CED, Cambridge,UK) and the data stored on a PC. The data was visualised using Spike2 software (CED, Cambridge, UK) running on the PC, where the data was saved for offline analysis. Raw traces were analysed using Signal 2.7 (CED, Cambridge, UK). Signal 2.7 was used to generate the H reflex trace averages (n=15) at each interval. Due to the robustness of the signals, no further filtering or smoothing was needed before analysis, however, images presented here

are with a 5 point smooth applied. Latencies were measured as peak to peak and amplitudes from the baseline to its peak of response. H waves were always normalised either to M max or the amplitude of the averaged control H wave (15 averaged waves) taken immediately prior to dual pulses. A subtraction was performed for Inter pulse intervals shorter than 50ms so that the amplitude of the test reflex was not artificially amplified by 'riding' on the preceding conditioning pulse.

2.4 Surgical Procedures

2.4.1 Spinal cord transection

In order to assess the development of the MSR in the absence of descending input, neonatal spinal cord transections were performed. To correctly determine litter age with an accuracy of ± 3 hours, pregnant mothers were checked 3 times per day from gestational day 19 (E19) until the litter was born. Litters born overnight were not used as there could be error of up to 12 hours. The day of birth was called post natal day 0 (PN0) and all surgeries took place at PN5.

All the pups were separated from the mother prior to surgery. Pups were washed thoroughly with saline and then deeply anaesthetised (until loss of paw withdrawal) using isoflurane (3% induction, maintained at 1.5%). Upon loss of withdrawal reflex, pups were subcutaneously injected with warm saline (37°C, 100-200µl) and a midline, dorsal incision was made to the skin near the base of the scapulae. Saline injections were a pre-emptive measure against hypovolemia following severance of vertebral arteries and greatly improved survival rates. The paravertebral muscles were retracted laterally to reveal the

mid-thoracic vertebral laminae. A mid thoracic (T8-10) laminectomy was performed and the cord and meninges were completely severed using micro-scissors. Completion of the transection was ensured by observing the base of the vertebral canal and complete separation of the rostral and caudal stumps of the severed spinal cord. Further post mortem histological examination of the injury confirmed completeness (Fig 7). A piece of Gelfoam® was then inserted into the cavity and the wound was closed with No5 silk sutures. Pups were placed in an incubator to recover fully before being returned to the mother.

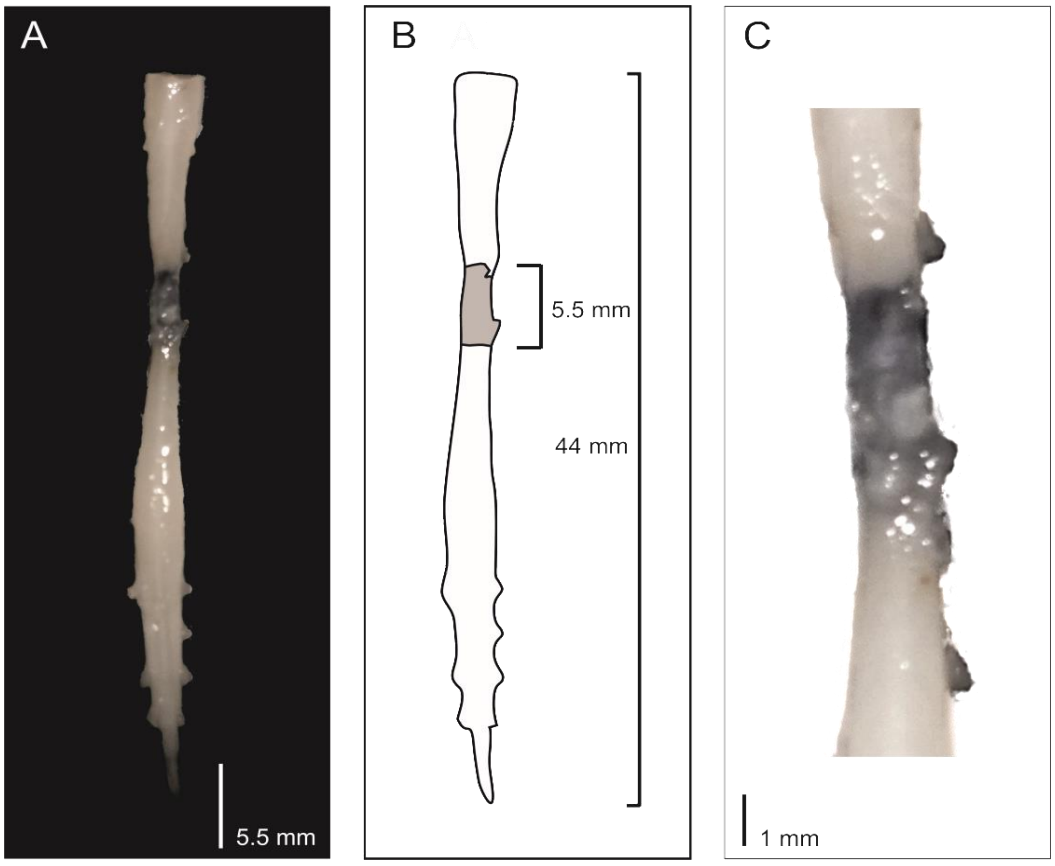

Figure 7. Photographs of a paraformaldehyde fixed, transected spinal cord 9 days after injury (PN14). (A) Dissected out transected cord, note transparency of the injury site to the black background, revealing the complete lack of spinal tissue at the injury site. (B) reconstruction of transected cord with the grey region indicating the extent of the injury as a proportion of the whole cord. (C) is a high magnification image of the injury site. Note that the dura matter has reformed but the injury site is extensive with scar tissue forming between the severed stumps.

Incidence of cannibalism was high upon the returning the pups to the mother and so several steps were taken in order to ensure pups were accepted by the mother post-surgery. Firstly, for two days preceding surgery the litter nesting was not changed and urine was collected from the mother in order to 'scent' the pups after surgery. After surgery, pups were thoroughly cleaned with saline and then rubbed with the mother's urine. Pups were not returned to the mother until they were fully recovered and once returned, the litter was monitored for 1 hour.

2.4.2 Cholera toxin β injections

During the spinal transection surgery, cholera toxin β (7.5mg/ml) was pressure injected into the gastrocnemius muscle using a glass micropipette attached to a Hamilton syringe. A small incision was made over the gastrocnemius muscle, the needle was inserted and 1 µl was slowly injected. The needle was left in the muscle for 3-5 minutes and retracted very slowly in order to prevent leakage.

2.5 Immunohistochemistry

2.5.1 Tissue Processing

Following H reflex recordings, animals were moved to a fume hood perfusion circuit and transcardially perfused with 4% paraformaldehyde (PFA) in 0.1M phosphate buffer. Vertebral columns containing the spinal cords of animals were harvested and post fixed overnight (4% PFA). Spinal cords were dissected out of the bony encasing and cryoprotected in sucrose (30% in PBS) for at least 48 hours. Segments T13-S1 of the spinal cord were individually

separated and frozen in optimal cutting temperature compound (OCT, Leica). A cryostat (1850, Leica Biosystems) was then used to cut transverse serial sections (25μm) of L4-5 which were used for all analyses in this study.

In order to visualise structures, immunohistochemical staining against certain proteins was conducted using the same principal steps. Free floating sections (3 sections per animal and 3-5 animals per group) were washed in phosphate buffered saline (0.01M PBS, 3 x 10 minutes) and then blocked in 3% normal donkey serum and PBS (0.01M) with 0.2% Triton X-100 (PBST) for 1 hour before being incubated overnight in primary antibodies for 24-48 hours (table 1). Sections were then incubated in secondary antibodies which had been raised primarily in donkey and against the immunoglobulin (IgG) in which the primary antibody had been raised. The secondary antibodies were conjugated to Alexa 488, 555 and biotinylated antibodies were revealed using avidin-Pacific Blue (Invitrogen). All antibodies were diluted in blocking solution (same concentrations as blocking step).

Table 1. All antibodies used in experiments.

Primary Antibody	Concentration	Supplier	Secondary antibody	Concentration	Supplier
CTb (Rabbit)	1:1000	Sigma	Alexa 555@rbt	1:500	Invitrogen
VGLUT-1 (Guinea Pig)	1:20000	Millipore	Biotinylated @GP	1:500	Jackson Immunoresearch
ChAT (Goat)	1:500	Millipore	Alexa 488@gt	1:500	Invitrogen
GAD67 (Mouse)	1:3000	Millipore	Alexa 488@ms	1:500	Invitrogen
GAD65 (Mouse)	1:10000	Millipore	Alexa 488@ms	1:500	Invitrogen
GLYT-2 (Rabbit)	1:10000	Synaptic Sytems	Biotinylated @rbt	1:500	Jackson Immunoresearch
VAChT (goat)	1:1000	Millipore	Alexa 555@gt	1:500	Invitrogen
Calbindin D-28k (mouse)	1:500	Swant	Alexa 488@ms	1:500	Invitrogen

2.5.2 Imaging and Analysis of IHC data

2.5.2.1 Confocal Imaging and quantification of boutons

All images were taken using a confocal LSM 700 at 63x (oil immersion). The same microscope was used for the entirety of any experiment. For most analyses the pinhole was adjusted to 1µm optical slices and images were taken as close to the centre of the cell as possible (nucleus visible). All motoneurons (n=23.22 ± 1.99 per section) on triplicate sections from L4-5 were imaged per animal (n=3-5). Synaptic boutons were only included if they were in close apposition to the neurone and the count was normalized to100µm of the neurone perimeter. For analysis of VGLUT1 puncta laminae distribution, tile scans of the entire section were taken at 20x magnification and all sections were imaged using the same settings.

2.5.2.2 Z stacks and 3D reconstruction

In some cases it was necessary to use Z stack images in order to analyse cells which received very few contacts, or if the contacts were mainly distributed on the proximal and distal dendrites (Mentis et al., 2006a). This protocol was also used for quantification of axo-axonic presynaptic contacts because it is impossible to reliably identify the centre of the VGLUT1^{+} terminal in the z plane (Betley et al., 2009). In these cases, 0.5µm steps were used with a 63x lens through the entirety of the cell and its dendrites. Because sections were cut at 25µm, it was not always possible to image the whole cell, so z stacks started in the centre of the cell and projected outwards equal distances and in opposite directions. Images were reconstructed using Imaris 8.1 (Bitplane) and 3D

rendered cells were used to quantify surface area and verify terminal contact. Dendrites were segmented from the soma and quantified separately.

2.6 Statistics

Tests for normal distribution of data were performed before comparisons were made. Most of the data did not meet normality demands for parametric tests and therefore non parametric measures were used. For analysis of differences between ages PN10,14 and 21 Kruskal-Wallis tests were used. When comparing development of intact and PN5 TX animals for a given dependent variable a Kruskal-Wallis test was used to compare between groups, followed by Bonferroni pairwise comparisons. For comparing Hmax/Mmax or threshold values between intact and PN5 TX at PN14 only, Mann-Whitney U tests were performed.

Chapter 3. Developing an in situ whole rat preparation for studying motor control throughout postnatal development

3.1 Introduction

Increases in our understanding of functions of the central nervous system often occur as a result of technological and technical developments. For example, a great deal of our understanding of the circuitry in the spinal cord responsible for locomotion (CPG) came after the development of the *in-vitro* isolated spinal cord preparation (Otsuka and Konishi, 1974; Smith and Feldman, 1987). While this preparation has many advantages, it has several drawbacks which need to be considered when interpreting results. Firstly, the isolated cord is devoid of all musculature and peripheral nervous system which are important as afferent activity is essential to normal control of behaviour such as locomotion (Pearson, 1995). Secondly, due to hypoxia of the core of the spinal cord as it becomes larger with development, this preparation is only viable at early neonatal periods when rodents are not yet capable of overground locomotion (Altman and Sudarshan, 1975; Westerga and Gramsbergen, 1993). Therefore there is scope for developing a preparation which permits motor control to be studied throughout development in animals which have an intact system, including components of the peripheral nervous system. Jiang et al. (1999a) described an isolated cord preparation which is viable up to PN14 in mice, however subsequent utilisation of this preparation has been disappointing, probably due to a lack of consistency. Moreover, it has often been questioned to what extend isolated spinal cord-brainstem preparations are physiologically relevant to *in-vivo* conditions. For example, phrenic activity cycles in the neonatal isolated spinal cord-brainstem preparation occur at only 9 per minute compared to 80-100 per minute in *in-vivo* rat preparations (Wang et al., 1996; Smith et al., 1990).

Attempts have been made to develop *in situ* preparations which have intact muscles and peripheral nervous systems. Hayes et al. (2009) described an *in-vitro* spinal cord-hind limb preparation in which the lumbar spinal cord was exposed and superfused in the same way the isolated spinal cord is, but the hind limbs remain attached. They were able to evoke locomotor activity, however this preparation lacks descending input and the hypoxic core issue persists as it is only viable at neonatal ages.

In order to overcome developmental hypoxia, *in-situ* preparations which perfuse the CNS via the animals own circulatory system have been developed. A working heart brainstem preparation (WHBP) was developed which involves perfusing the brain and spinal cord via the descending aorta (Paton, 1996). This preparation provides robust motor (phrenic) outputs which are comparable to *in-vivo* preparations (Wang et al., 1996; Dutschmann et al., 2000). In addition to the WHBP, a trunk-hindquarters preparation was developed by the same group which isolated the hindquarters from the upper body, again perfusing the CNS via the descending aorta (Chizh et al., 1997).

More recently, a perfused whole rat preparation has been developed, in which the CNS is perfused transcardially, circumventing hypoxic core issues experienced with isolated cord preparations (Pickering and Paton, 2006). This preparation is fully intact apart from a pre-collicular decerebration. Successful recording of bladder EMGs during voiding in P20-28 rats demonstrated its effectiveness in oxygenating the circuitry of the caudal spinal cord. It has not been established if this preparation is viable at younger ages, however studies by the same lab in neonatal WHBP preparations suggest it would be viable (Dutschmann et al., 2000). Interestingly, Dutschmann et al. (2000) showed that the neonatal *in-situ* WHBP produced phrenic activity cycles of >45 per minute,

suggesting motor output in this preparation has greater physiological relevance compared to isolated cord preparations (9 per minute), in which phrenic activity is greatly attenuated (Smith et al., 1990). Interestingly, fictive locomotion elicited from isolated cord preparations is very slow compared to *in-vivo* conditions, suggesting a similar attenuation of neural activity from the lumbar spinal cord as seen in the brainstem and thoracic cord in respiratory experiments. If viability of the *in-situ* perfused whole rat preparation can be achieved in neonatal animals it is possible that the wealth of knowledge on control of locomotion gained from isolated neonate cords can be tested in a more physiologically relevant preparation.

The aim of this study was to further develop the preparation described by Pickering and Paton (2006) so that it can be utilised for the study of postnatal development of motor control in rats. The 1st aim was to establish viability in rats aged PN7,10,14 and 21. Once viability was attained, we aimed to evoke monosynaptic reflexes (H reflexes) from animals of each age. We also made attempts to evoke rhythmic motor outputs from the preparation in order that it is eventually developed for studying locomotion.

3.2 Methods

Methods are described in detail in the general methods chapter.

3.3 Results

3.3.1 Establishing viability of the preparation throughout postnatal development.

Preparation viability was attained for ages PN7, 10, 14 and 21. Viability was ascertained from adequate perfusion pressure (PP, 60-80mmHg) and robust

spontaneous and evoked motor outputs. Viability of the brainstem is indicated first at 35-40 mmHg, with spontaneous rhythmic activity from phrenic MNs producing respiratory contractions which are observed as movement artefacts on the perfusion pressure trace (Fig 8A). Initial respiratory contractions are weak, shallow and relatively long in duration, occurring at a frequency of about 0.1 Hz (Fig 8B). As PP increases, respiratory contractions quadruple in frequency (~0.4Hz), indicating more robust firing of phrenic MNs (Fig 8C). This is slower than *in vivo preparations* (~1.5 Hz) which is likely due to the lower temperature (32°C) of the perfused preparation (Wang et al., 1996). Motor output from hindlimb muscles in response to mechanical stimulation (toe pinch) indicates viability of the lumbar cord (Fig 8D-E). At low PP, responses to mechanical stimulation are weak and occur at a relatively long latency (~1s, Fig 8D). When PP increases to >60 mmHg, mechanical stimulation induces robust, relatively short latency rhythmic hindlimb movements and high amplitude EMG bursts (Fig 8E).

At ages PN7-10, consistent and robust M waves were evoked following establishment of a viable preparation (Fig 9). However, H-waves could not be elicited despite the animal producing spontaneous rhythmic bursts of activity and responding to tail and toe pinches. At PN14-21, it was possible to elicit H reflexes in 92% of viable preparations, however this was heavily dependent upon systemic pressure (Fig 9). The most robust, stable responses were elicited at perfusion pressures greater than 60mmHg, however weaker responses could still be evoked at pressures between 50-60 mmHg (Fig 8). Responses at 40-50 mmHg were very weak and below 40mmHg, responses were not present.

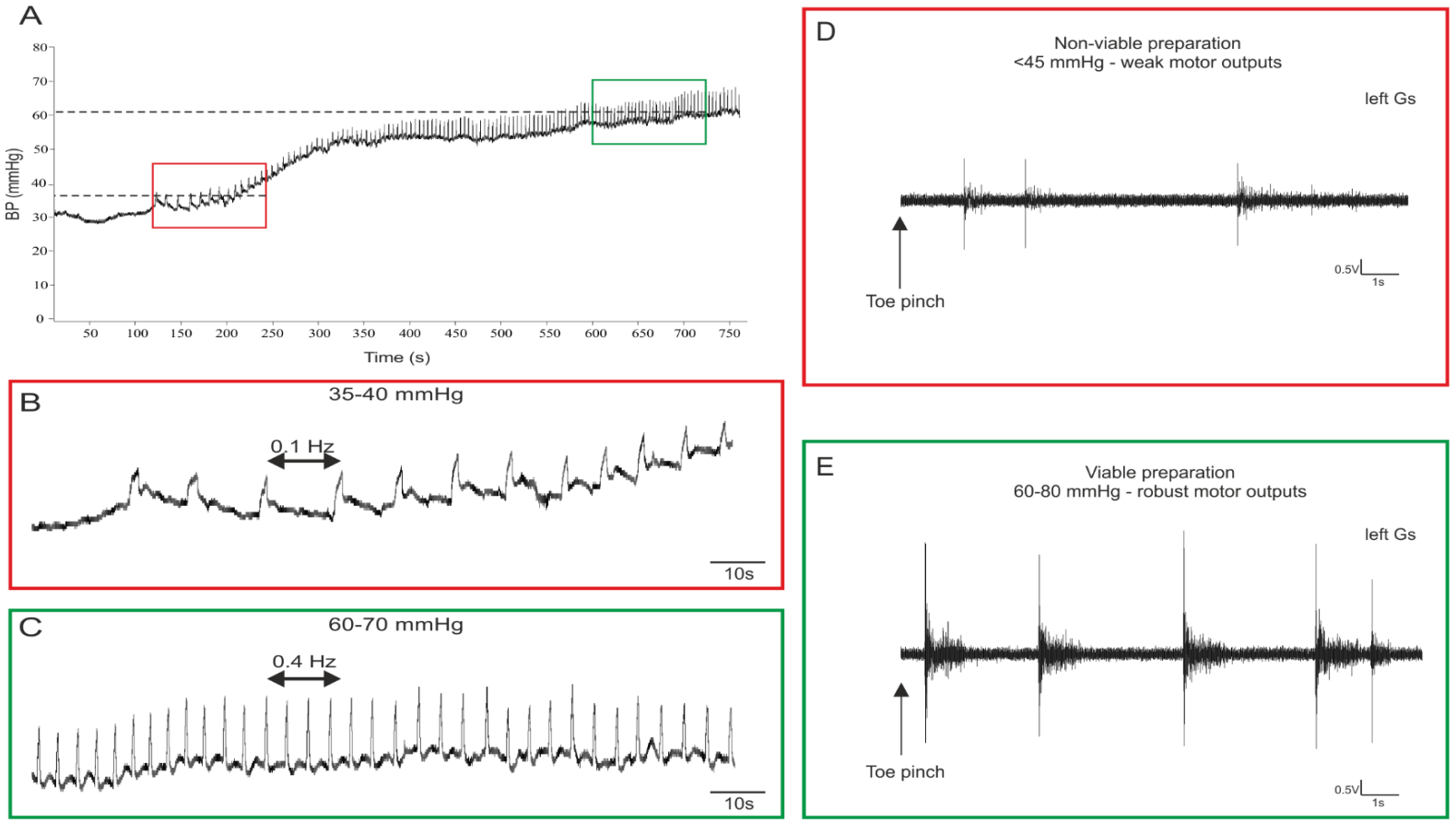

Figure 8. Preparation viability is initially ascertained by robust spontaneous and evoked motor outputs.
Respiratory contractions first seen on the pressure trace at ~35-40 mmHg demonstrate viability of brainstem. Frequency and amplitude of deflections increasing with pressure (A). Low pressure outputs are highlighted by the red box and high pressure responses by green box. (B-C) Expanded segments of trace in (A) show frequency and strength of respiratory contractions increase with pressure. (D-E) Robust responses from gastrocnemius to mechanical stimulation (pinch) of the toe on the same leg demonstrate lumbar spinal cord viability. (E) Note the robust outputs in viable preparation (60-80mmHg).

At PN14, H reflexes were most robust, being consistently greater in amplitude compared to M waves at low stimulation intensities and then displayed the classical H reflex recruitment curve during thresholding, increasing in amplitude as the M wave started to appear and decreasing in amplitude as the stimulation increased and antidromic motor axon potentials collided with H waves (Fig 10).

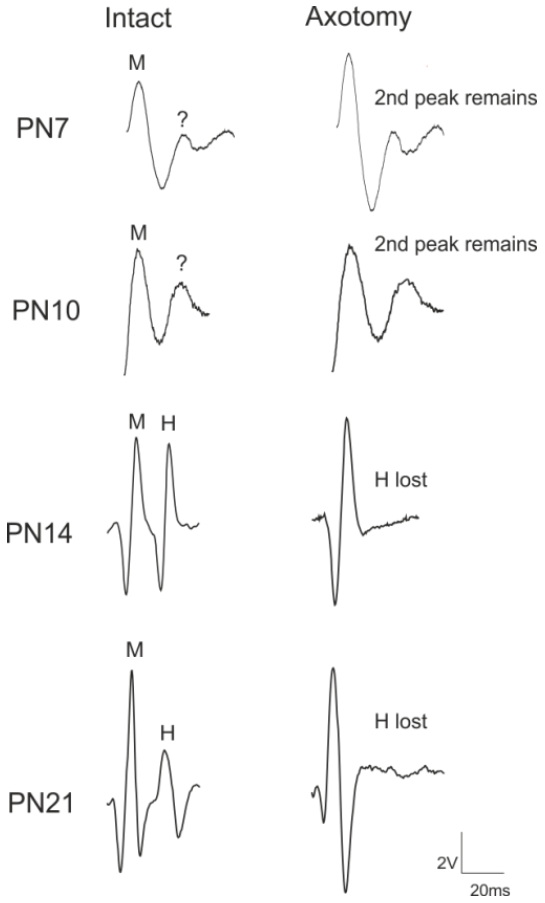

Figure 9. Evoking H reflexes in rats aged PN7-PN21.
First panel (intact) shows typical responses evoked at each age. Second panel shows responses after axotomy between the spinal cord and stimulation site. Note the loss of the second (H wave) in PN14-21 animals, but not PN7-10. (?) in PN7-10 represent unidentified late response, possibly movement artefact.

H reflexes in PN21 preparations were inconsistent and greatly decreased in magnitude shortly after viability was established. Therefore it was not possible to carry out full thresholding or quantification of H reflexes in PN21 preparations. In PN14 animals in which initial perfusion pressure of 80 mmHg was attained, robust H reflexes could be elicited for duration of 1-1.5hrs, with

responses degrading sharply after 1.5-2hrs. Adequate perfusion pressure and motor output elicited by tail and toe pinches could be evoked for up to 3 hrs however. In PN21 animals, preparation viability duration was greatly reduced with H reflex recordings seldom lasting more than 30mins and pinch evoked motor responses not lasting more than 1hr.

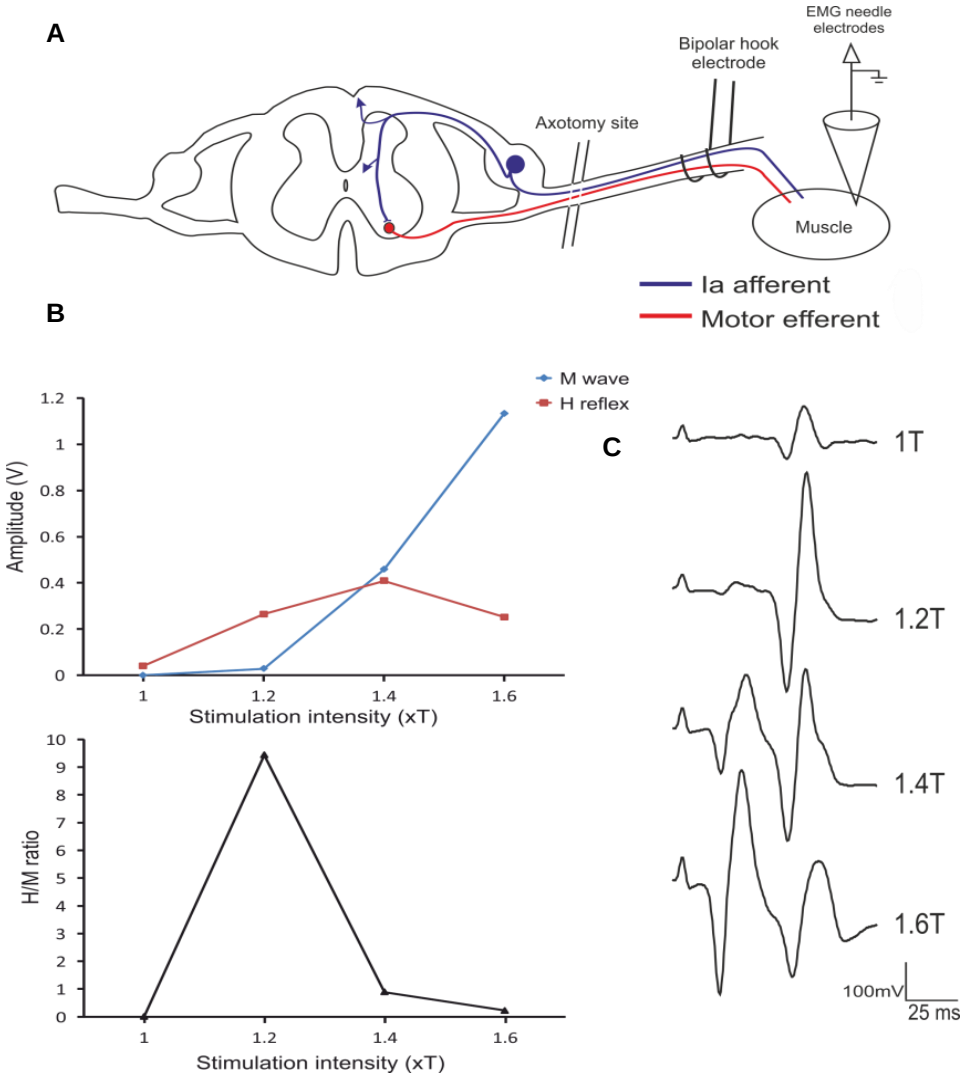

Figure 10. Typical H reflex response profiles from PN14-16 animals. (A) Schematic of the experimental set up. (B) H, M wave and H/M ratio recruitment curves. (C) Representative traces illustrating H and M wave recruitment**.**

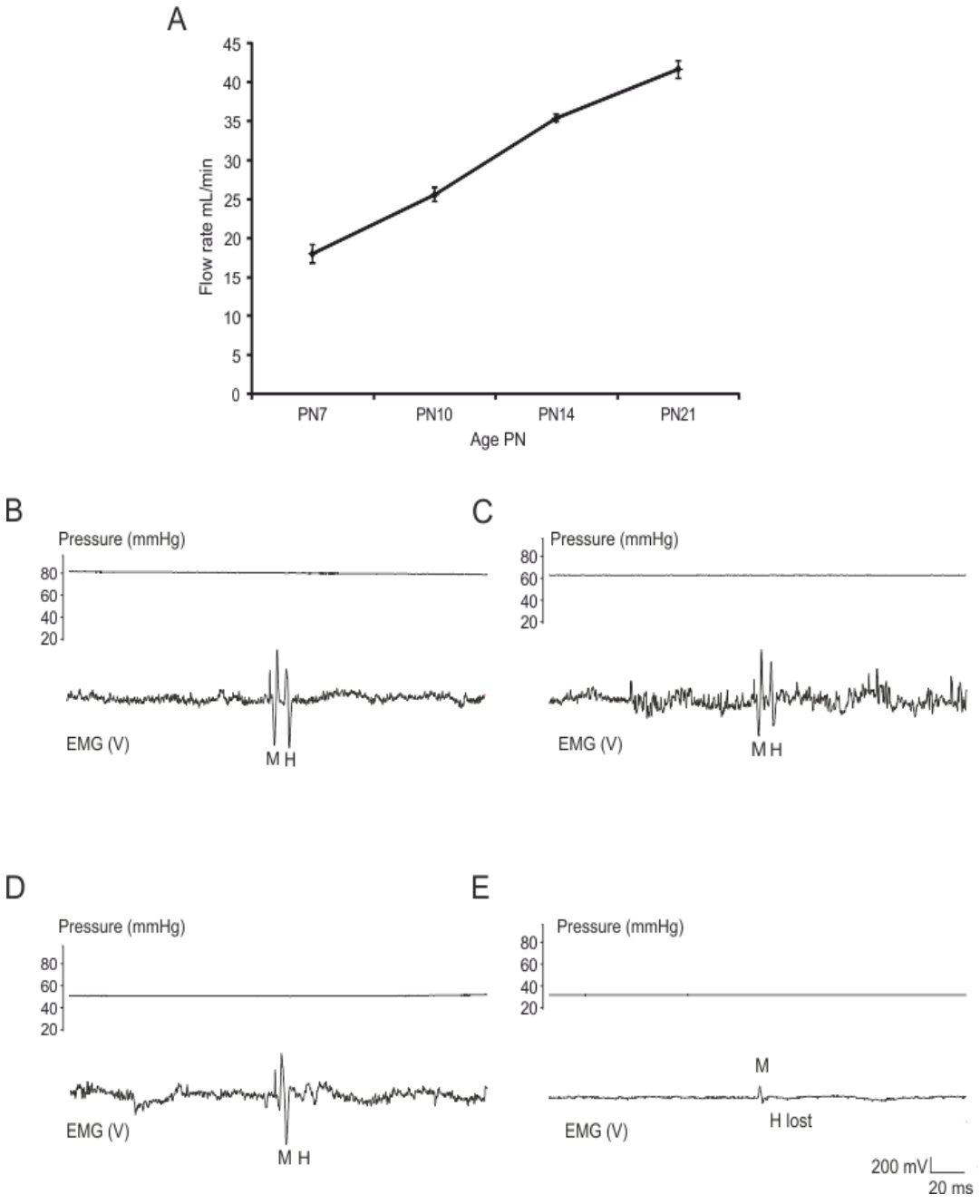

Figure 11. H reflex robustness is related to perfusion pressure.
(A) Average flow rates needed to ensure perfusion pressure reached ≥60mmHg. (B-E) Averaged EMG traces recorded at corresponding pressure above. (B) At perfusion pressures of 80mmHg+, H reflex responses were clean, crisp and of a good amplitude. (C) at ≥60mmHg, responses were still robust however amplitudes were reduced. (D) At 50-60mmHg H reflexes are very weak and cannot be used for analysis. (E) Below 50 mmHg, H reflexes and background activity are abolished.

3.3.2 Postnatal development of the M wave

3.3.2.1 Graded stimulation for recruitment curve

Although H reflexes could not be assessed at each age, thresholding was conducted in order to establish if there was any difference in motor axon recruitment with development. Results showed that the amplitude at M threshold was not significantly different between ages as there were no significant differences in M amplitude between age groups for 1-1.5T. At 2T M amplitude at PN10 (n=5, 0.10 ± 0.045, Fig 12) was significantly less than at PN21 (n=5, 0.66 ± 0.29, p= .05) but there were no significant differences

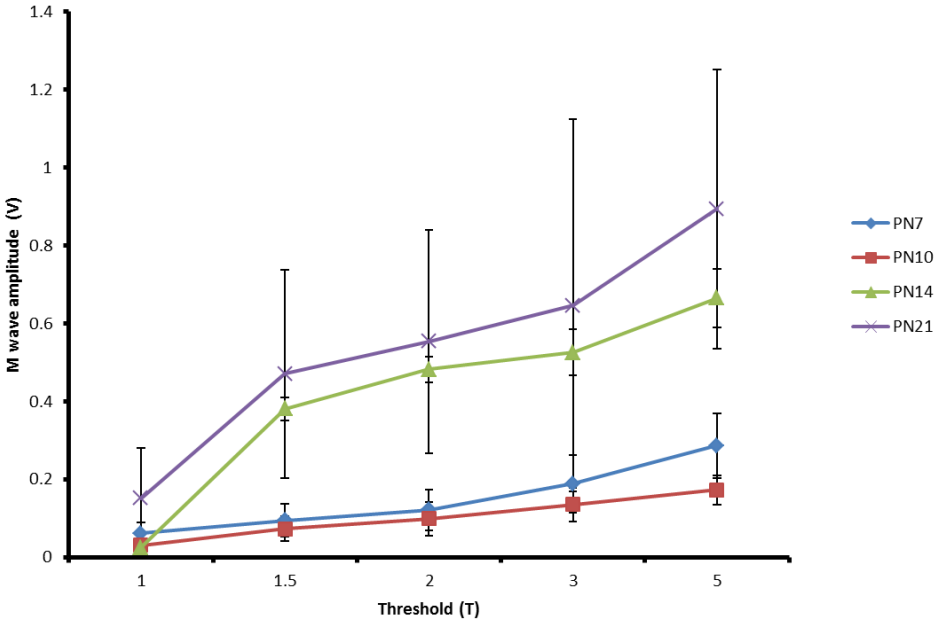

Figure 12. M wave thresholding at each age.

between the other ages. For 3T, at PN10 (0.14 ± 0.05) amplitudes were significantly less than at PN21 (0.77 ± 0.26, p=.025) but there were no differences between other age groups. At 5T the differences were most pronounced. PN14(n=5) and 21 were highest but not significantly different from each other. At PN7 (0.28 ± 0.084) and PN10 (0.182 ±0.05) M amplitudes were significantly lower than at PN21 (1.11 ± 0.46, p= .008 and .002 respectively).

Again, PN10 and 7 were not significantly different. There was a trend for PN14 to be greater than at PN7 and 10 (p=.060).

3.3.2.2 Paired pulse assessments

For paired pulse assessments, there was a significant effect of age on paired pulse depression (PPD) of M waves evoked at 2.5T (P<0.001, Fig 13). Reducing IPIs significantly reduced M amplitude at PN21 (n=4, p<.001) and PN14 (n=5, p<.001) but not at PN 7 (n=5, p=.136) or PN10 (.149). The main differences between the ages occurred at the shorter IPIs. At 1ms IPI PN14 (13.40 ± 19.89%) and 21(22.23 ± 12.79%) were not significantly different from each other, but were significantly more depressed than at PN7 (p<.001) and 10 (p<.001). At 3ms, PN14 and 21 were significantly different from PN7 (p<.001) and 10 (p<.001). At 5 ms, PN14 was significantly reduced compared to PN7 (p=.002) and 10 (p<.001) but not PN21. PN21 was less than PN10 (p<.001) and PN7 and 10 were not different. At 10ms, PN21 was significantly reduced

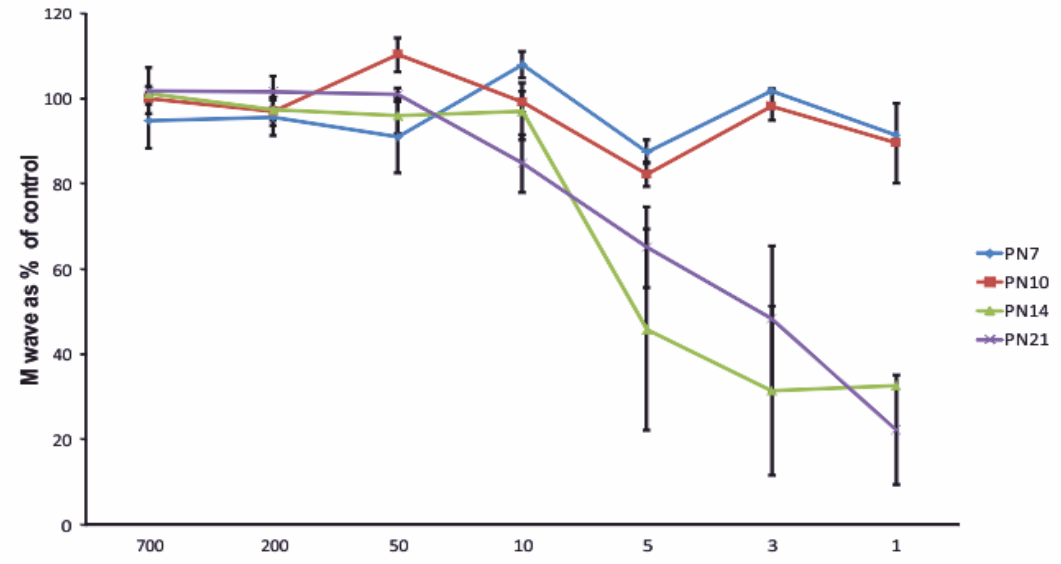

Figure 13. Paired pulse depression of the M wave is significantly greater later in development.

compared to PN7 (p=.040) but there were no other differences at this IPI or any between intervals 50-700ms (Fig 13).

3.3.3 Observations of rhythmic motor outputs

The preparation will spontaneously produce rhythmic motor outputs as perfusion pressure reaches >60mmHg (Fig 14A). These outputs were taken unilaterally from the gastrocnemius/soleus muscle prior to, or after H reflex recordings. This starts with respiratory contractions, forelimb movements and

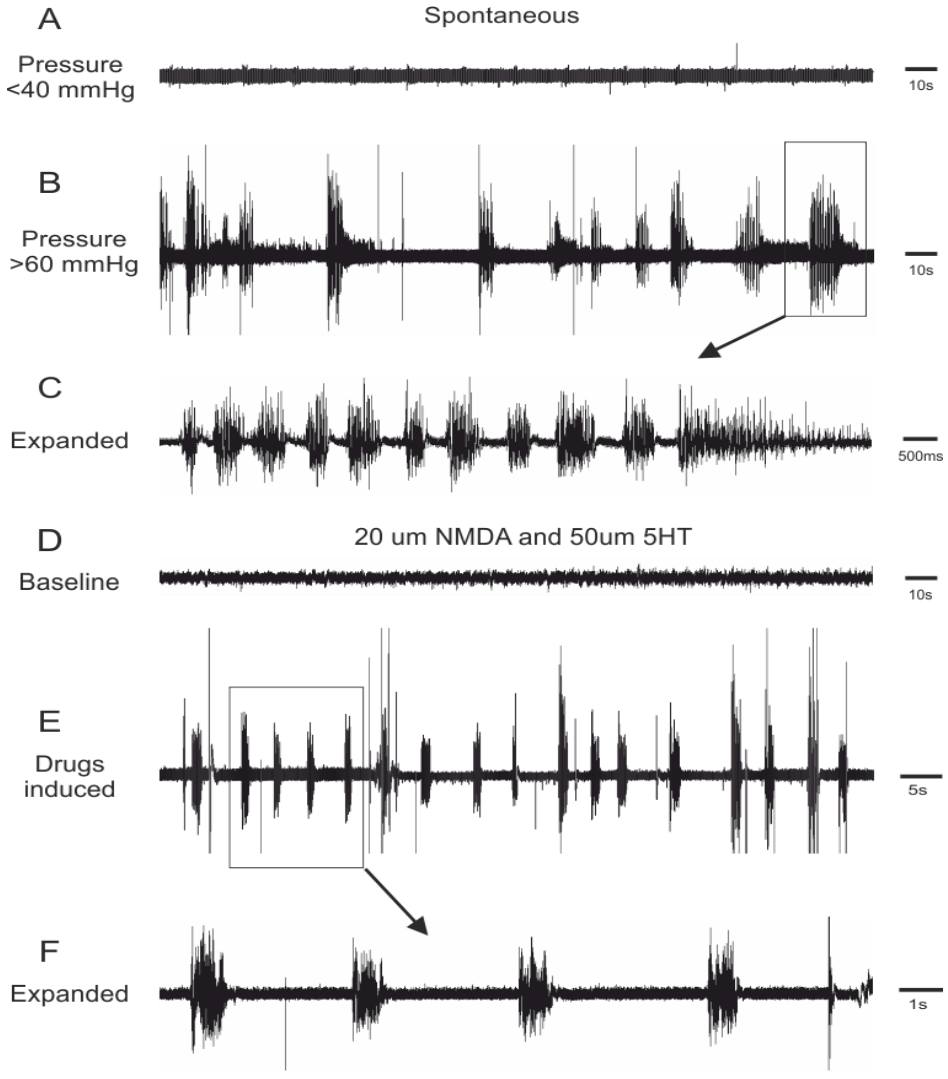

Figure 14. Spontaneous and evoked rhythmic contractions recorded in gastrocnemius/soleus muscle. (A) Spontaneous activity when pressure is < 60mmHg is minimal. (B-C) After pressure increases to >60mmHg episodes of rhythmic spontaneous activity start. (D) Baseline activity absent of spontaneous bursting. (E-F) Regular rhythmic bursting in evoked after addition of NMDA and 5HT.

then the hind limbs and tail will move. Activity in hind limbs was usually coordinated with forelimbs and resembled escape behaviour. This activity was episodic in nature and greatest early after establishing adequate perfusion pressure (>60mmHg), becoming less frequent with time. Episodes of activity in the gastrocnemius muscle would last 10-15 seconds and comprise of rhythmic contractions (Fig 14B). These spontaneous bouts of activity were recorded in animals aged PN7, 10, 14 and PN21.

Interestingly, in animals aged PN14-16, upon addition of 20μm NMDA and 50μM 5HT we observed robust, regular contractions of gastrocnemius/soleus for periods of 30s-1min (Fig 14E). Compared to the spontaneous activity, the drug induced contractions tended to be much more regular, but also slower (Fig 14B,C,E and F). These data suggest the preparation can be further developed to study locomotion in the developing rat.

3.4 Discussion

We show here that we were able to adapt and develop a transcardially perfused, in situ whole rat preparation for use in studying PN development of motor systems. The preparation is fully intact apart from a pre-collicular decerebration and is perfused via the ascending aorta, thereby oxygenating the CNS via its own circulatory system. Results here show that viability of the lumbar spinal cord in this preparation can be established at ages PN7, 10, 14 and 21 as evidenced by robust evoked and spontaneous motor outputs at all ages. Although there was spontaneous and evoked motor activity in response to mechanical pinch stimuli at all ages, it was not possible to evoke H reflexes at ages PN7-10 in this preparation. We were able to evoke H reflexes in animals aged PN14-21, but these responses were most robust at age PN14-

16. Parameters such as pressure and time after initiation of perfusion at which the H reflex responses were most reliable were determined. Assessments were conducted into the M wave throughout development and significant differences were found between PN7-10 and PN14-21. We observed spontaneous and drug induced rhythmic motor outputs from the hindlimb suggesting this preparation can be developed for studying PN development of locomotion.

It was disappointing to not be able to evoke H reflexes at ages PN7-10. The monosynaptic reflex can be evoked prenatally (E17.5-19) *in-vitro* in the form of a ventral root recordings and so failures to evoke H reflexes can only be down to peripheral (muscle) or technical reasons. Because the muscle and nerve are very small and delicate at these ages it may also be that the nerve is damaged whilst freeing it from musculature and mounting it on the bipolar hook electrode. H reflexes evoked at PN14-16 were often the most robust responses. This was likely due to the fact that at this age the nerve and muscle were larger and strong enough to cope with dissection and mounting but the animal still retained the hypoxic resistance with which neonatal animals are endowed.

The poor H reflex responses in PN21 preparations are likely due to developmental changes in metabolic demands and hypoxia sensitivity. ATP is crucial to life and is synthesised mainly by oxidative metabolism of glucose. If oxygen is lacking, oxidative phosphorylation of glucose is reduced and ATP levels drastically drop. This results in inactivation of Na^+-K^+ pump and excessive intracellular Na^+ causing osmotic swelling and eventually cytotoxic oedema (Silver and Erecińska, 1998; Rothman, 1985). At ages > PN21, the animals have matured significantly and have greater metabolic demands along with reduced resistance to anoxia meaning the unavoidable hypoxic period reduces the life span of the preparation (Kerkut and Bagust, 1995).

Alternatively, Pickering and Paton (2006) suggested that preparation viability was dependant on the mass of tissue which needed to be perfused. In this case, a logical solution would be to use mice because they don't reach weights of greater than 30g until PN80+ whereas rats can reach 30g by PN14. Recent developments have been made in the ability to identify certain interneurone populations based on their respective progenitor domains (Jessell, 2000). Subsequently, it has been possible to genetically manipulate these cells in order to assess their role in circuit function and behaviour (Bui et al., 2013; Fink et al., 2014; Bouvier et al., 2015). Most genetic manipulations have only been conducted in mice, and therefore establishment of viability of this preparation in mice could be crucial. A caveat with using mice, however, is that it has been reported that it is more difficult to evoke motor output in the isolated cord preparation. It will be interesting to see if this translates to a perfused preparation (Jiang et al., 1999a).

Assessments of the M wave revealed that there were significant differences in the stimulation intensities needed to evoke M waves and also in the depression of the M wave seen at short interpulse intervals. This is likely due to numerous immaturities in nerve myelination and the neuromuscular junction which have been previously reported at these ages (Wiggins et al., 1975; Sanes and Lichtman, 1999). Because the aim of this thesis was to examine the developmental changes occurring centrally in intact and PN5 transected animals, these peripheral developmental adaptations would have corrupted results. Therefore, H reflex assessments in subsequent chapters were only made in animals aged PN14 to compare development up to this point in the presence and absence of descending systems.

3.4.1 Advantages and scope of the preparation

These results represent the first step in establishing a preparation which could significantly increase our ability to study neural circuits responsible for motor control. At present, much of our understanding of spinal cord circuit organisation and function is from neonatal rats and mice which are not capable of overground locomotion (Altman and Sudarshan, 1975). Efforts have been made to establish or extend the viability of *in-vitro* preparations but with limited success (Jiang et al., 1999a). The preparation we describe offers a potential solution to this problem as we have established viability from an age when the animal is virtually motionless in the open field (PN7) to functionally mature in terms of its locomotor ability (PN21).

This preparation is comparable to *in-vivo* preparations but with added benefits. *In-vivo* preparations, especially in very small animals have difficulties due to the low blood volumes meaning that surgery and dissections can often lead to hypovolemic death. This preparation circumvents this issue as the experimenter has full control over the systemic volume and pressure. In the same way, the experimenter is able to control the extracellular milieu, changing ionic compositions or adding drugs to assess effects on circuit or cell function.

3.4.2 Drawbacks to the preparation

There are certain drawbacks to this preparation compared to more reduced preparations. For example, access to the ventral spinal cord and roots is more challenging and would require longer hypoxic periods during extended dissections. Despite this, Pickering and Paton (2006) suggest that with cold dissection this is would be possible with little deterioration of preparation duration. Additionally duration of preparation viability is not as great as the

neonatal isolated cord preparations, especially in the older (larger) animals and so unless it is developed so that it last longer than 2 hrs, the experimenter has limited time to perform studies at older ages.

3.5 Conclusions

We have managed to adapt an existing preparation for the study of motor control throughout PN development. With further development, this preparation could prove crucial to furthering our understanding of mature neural control of movement and how it is established throughout PN development.

Chapter 4. Postnatal development of the lumbar spinal circuitry

4.1 Introduction

Different species reach motor maturity at different rates, reflecting dissimilarities in postnatal development of the central and peripheral components necessary for producing effective movements. For example, rats acquire locomotor maturity relatively rapidly, with adult phenotypes being reached 3 weeks after birth. During this epoch, there are pertinent time points at which significant developmental changes take place. During the first 10 days PN, pups are unable to support the weight of their lower body and aside from some pivoting and paddling movements, are virtually motionless in the open field. After PN 10, pups are able to support their pelvis somewhat and engage a very immature, unsteady form of quadrupedal locomotion. Up to PN13 the predominant form of ambulation is crawling, however PN14 is marked by a very striking transition to a slow but otherwise mature quadrupedal locomotion. From PN14-21 gait becomes swift, accurate and indistinguishable from adults (Altman and Sudarshan, 1975).

PN development of central components which contribute to the acquisition of mature behaviour is poorly understood. Most of the descending systems make contact with the lumbar spinal cord shortly after birth, with the exception of the corticospinal tract which doesn't traverse the caudal portions of the lumbar enlargements until PN10-12, with termination profiles not mature until PN14 (Donatelle, 1977; Rajaofetra et al., 1992; Tanaka et al., 1992). Interestingly, there seems to be an interaction between proprioceptive muscle afferents and CST during PN development (Chakrabarty and Martin, 2011b). In the cervical spinal cord, proprioceptive afferent termination profiles have a

ventral bias at early ages and are gradually retracted and pruned throughout PN development (Gibson and Clowry, 1999; Chakrabarty and Martin, 2011a), a process which is believed to be dependent on arrival of CST (Chakrabarty and Martin, 2011b). No similar study has been conducted in the lumbar spinal cord of either the rat or cat, however proprioceptive afferent input to Renshaw cells (inhibitory interneurons residing in the most ventral portions of lamina VII and IX) has been shown to increase up to PN14 and then decrease with maturity as motor axon collaterals become the drive to these cells (Mentis et al., 2006a).

In intact adults, interactions between descending and peripheral input to the spinal circuitry is vital for normal control of movements. The spinal circuitry is highly plastic and its organisation is largely activity dependant, meaning developmental changes in weighting of descending and peripheral inputs may alter the organisation of the spinal circuitry (Bertrand and Cazalets, 2013). For example, Wilson et al. (2004) studied the development of cholinergic input to MNs in the mouse lumbar spinal cord and found a developmental increase which seemed to correlate with the acquisition of mature locomotion. In terms of inhibitory control of motor output, it has been shown that due to low intracellular chloride, GABA and Glycine can actually be depolarising early in development, a phenomenon which is reversed by PN 5 (Fulton et al., 1980; Singer and Berger, 2000).In contrast, there have been suggestions that the locomotor circuitry is near mature at birth as fictive locomotor outputs and air stepping can be evoked even pre-natally (Ozaki et al., 1996; Van Hartesveldt et al., 1991).

Assessing how the spinal circuitry is altered throughout PN development is important for furthering our understanding of how mature control of movement is established. Although there has been some work in this area, we still lack understanding of the neural components vital to acquisition of mature behaviour.

4.2 Aims

In this study we aimed to (i) establish how proprioceptive afferent input to motoneurons and Renshaw cells is altered at key behavioural time points during PN development. (ii) Determine how inhibitory and excitatory inputs to motoneurones are altered at the same time points in (i).

4.3 Hypotheses

Data from cervical cords of the cat and rat suggest postnatal retraction and refinement of cervical proprioceptive afferents and based on this we expected the same to occur in the lumbar cord, especially at time points related to arrival and maturation of the CST termination profiles (PN10-14). Furthermore, we expected the competition for synaptic coverage from peripheral and descending systems to induce changes in the lumbar spinal circuitry. Based on behavioural data, we expect that PN14 will be a key time point in development of the lumbar spinal circuitry as quadrupedal locomotion becomes dominant at this age.

4.4 Methods

Procedures here have been mainly described in the general methods (chapter 2) and so this section will only provide methods that were not described before.

4.4.1 Animals

Pregnant mothers were checked 3 times daily in late in the gestational period to ensure that date of birth (PN0) was correct to ± 3 hours. 3-5 animals at PN10, 14 and 21 were used for immunohistochemistry studies and analyses.

4.4.2 Tissue processing and Immunohistochemistry

Detailed description of these methods can be found in the general methods (Chapter 2). Briefly, 3 sections per animal (n=3-5) were processed for imaging and analyses. The antibodies used in this chapter were also displayed in table 1 (chapter 2). For quantification of lamina distribution of VGLUT1, 3 regions of interest (ROI) were defined: dorsal, intermediate and intermediate X (Fig 15). The ROIs were defined by normalised dimensions for each region at each age. The dorsal box dimensions were: height-25% of the length of a straight line from base of dorsal column to cord dorsum, width-20% of horizontal line from base of dorsal column laterally to termination of grey matter. ROI intermediate X was used to calculate density of $VGLUT1^{+}$ projections to the area of the cord where partition neurons usually reside, lateral to the central canal as defined by Miles et al. (2007). This region was defined as a box with a vertical length 10% of the total height of the grey matter and a width of 10% the total width of the grey matter from the middle of the central canal to

termination of the grey matter laterally. The medial edge of the box extended from the lateral edge of the central canal and the bottom of the box was in line with the bottom of the central canal. The intermediate ROI was the height of the central canal and extended from the edge of the Int X region of interest to the lateral termination of the grey matter. Once ROIs were established, Image J particle analysis was used in order to quantify the density of VGLUT1$^+$ terminations in the defined regions. First, images were converted to binary and thresholded. For density quantification a minimum bouton diameter of 2μm was defined based on electron microscopy studies (Ichiyama et al., 2006). Motoneurones which were selected for analysis of synaptic coverage were chosen based on size, to ensure that mainly αMNs were analysed. All motoneurones were imaged, but only cells with a diameter greater than 25μm were analysed as γMNs tend to be under 20μm in diameter (Ichiyama et al., 2006).

4.4.3 3D reconstruction and analyses

Again, this procedure is mainly described in the general methods section however the quantification of GAD65$^+$ P boutons on VGLUT1 terminals will be described here. 3D surface rendered reconstructions of motoneurons, Renshaw cells, VGLUT1 positive terminations and GAD65$^+$ P boutons were made using Imaris 8 software. All VGLUT1$^+$ terminations in close apposition to motoneurons and with a diameter greater than 2μm were used for P bouton quantification. Total coverage of P boutons was normalised to the surface area of VGLUT1 bouton, percentage of clusters of 1,2,3 and 4+ P boutons per

animal and % of VGLUT1 boutons which were not contacted by P boutons ($GAD65^{off}$) were quantified.

4.4.4 Statistics

Statistics are described in detail in the general methods section (chapter 2).

4.5 Results

4.5.1 PN development of Ia afferent input to the lumbar spinal cord

In order to assess if proprioceptive afferent input to the spinal cord is altered with postnatal development, $VGLUT1^{+}$ terminations in dorsal, intermediate, intermediate X and ventral regions were quantified (Fig 15). Quantification was density of terminations as a percentage of the total area of the respective ROI. In the dorsal ROI, there was a significant effect of age on density of $VGLUT1^{+}$ terminations (p= .008, Fig 15D'). VGLUT1 terminal density was significantly greater in PN10 animals (63.64 ± 1.46%) compared to both PN14 (39.11 ± 3.7%, p=.012) and PN21 (42.31 ± 5.2%, p=.023) but there was no difference between PN14 and 21 (p=.830, Fig 15C-C'' & D-D''). In the intermediate ROI, there was a significant effect of age on $VGLUT1^{+}$ terminal density (p=.020, Fig 15D). Similar to the dorsal region, there was a significant reduction in $VGLUT1^{+}$ terminal density between PN10 (14.31 ± 3.32%) and PN14 (3.78 ± 0.70%, p=.029) and PN10 and 21 (5.59 ± 0.65, p=.049) but there was no difference between PN14 and PN21 (p=.803). There was no

significant effect of age on VGLUT1⁺ terminal density in Int x ROI (p=.60, Fig 15D’’).

Alpha motoneurones receive monosynaptic input from Ia afferents expressing VGLUT1. Therefore, analysis of the density of VGLUT1⁺ puncta contacting MNs reflects how Ia afferent innervation of MNs is altered postnatally. Analysis showed that there was a significant effect of age (p=.005) on VGLUT1⁺ boutons in close apposition with MNs (Fig 16D). At PN10, Ia boutons were significantly greater (4.51 ± 0.24) than at PN14 (3.46 ± 0.26, p= .031) and PN21 (3.1 ± 0.26, p= .006), however there was no difference between PN14 and 21 (p= .632, Fig 16).

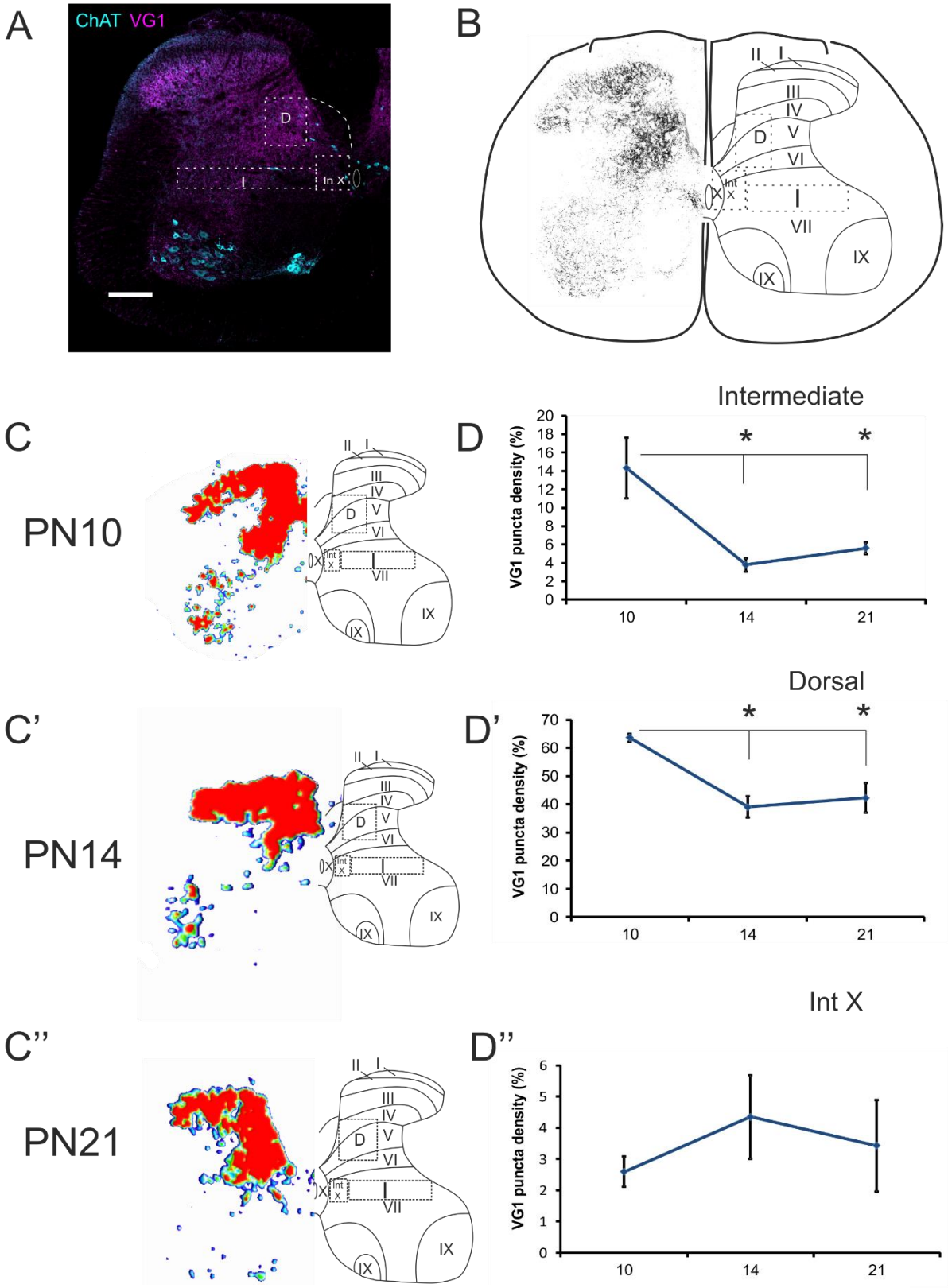

Figure 15. Postnatal development of VGLUT1⁺ puncta density in dorsal and intermediate laminae of the lumbar spinal cord. (A) representative image of transverse PN21 spinal cord section illustrating regions in which VGLUT1⁺ puncta density was analysed. (B) Binary image clearly showing lamina distribution of VGLUT1⁺ terminations. (C-C'') Heat maps illustrating the difference in lamina distribution of VGLUT1⁺ terminations at each age. (D-D'') Graphs representing puncta density in intermediate, dorsal and intermediate lamina lateral to central canal (Int X) at each age. Scale bar = 200µm.

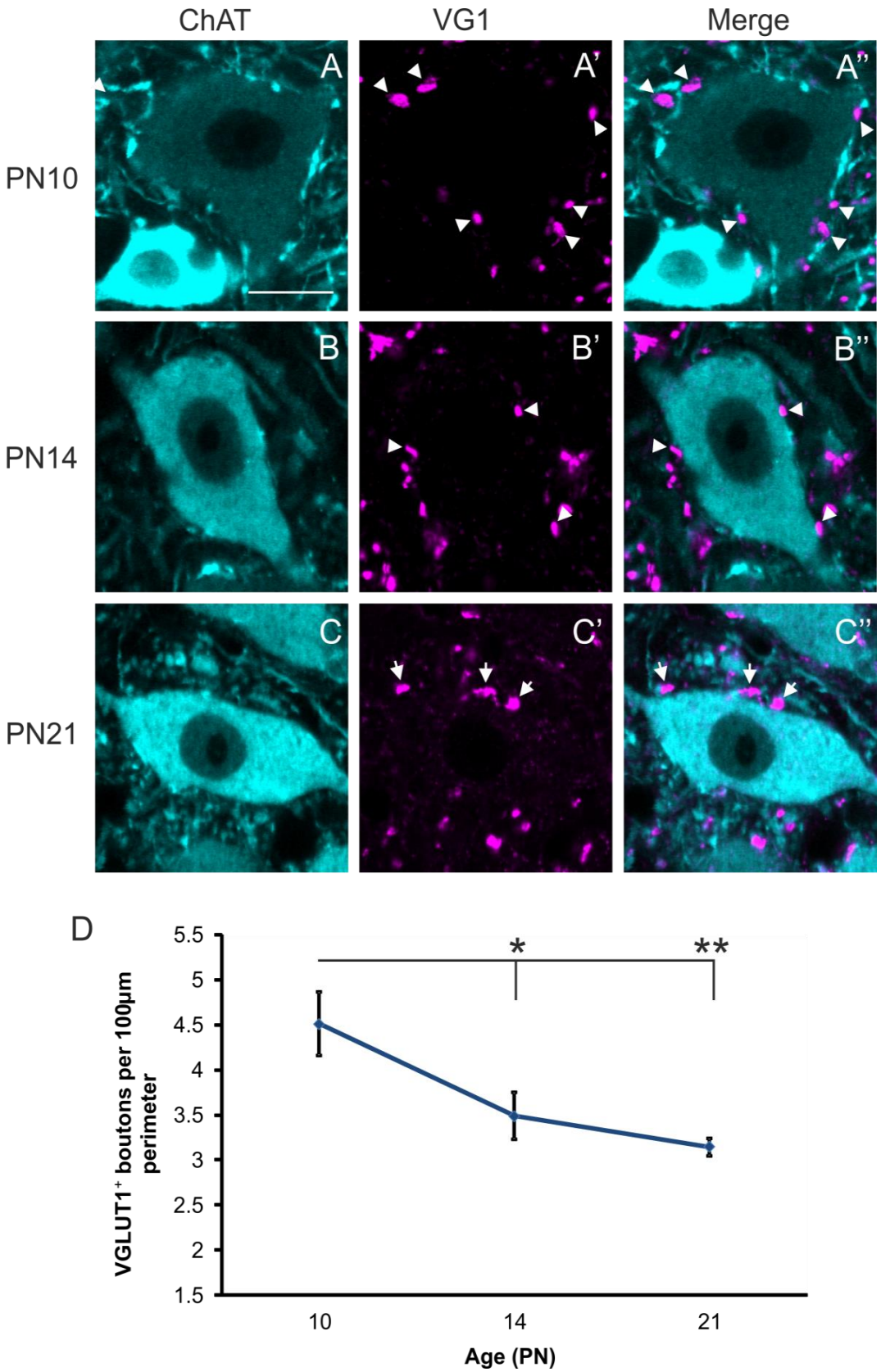

Figure 16. Postnatal development of Ia afferent boutons on Alpha motoneurones. The first panel (A-C) contains representative images of ChAT staining, marking alpha motoneurones. (A'-C') contains examples of VG1⁺ boutons with white arrows illustrating which boutons were quantified. (A''-C'') contains representative merged images.(D) contain schematics of the circuitry assessed, highlighting the pathway analysed in this figure. Scale bar =20 µm

4.5.2 PN development of Ia afferent input to Renshaw cells

Our results show a developmental retraction of Ia afferents from motoneurons and the ventral horn (Figs 15 &16). We therefore questioned whether this retraction is specific to motoneurones or if the same profile will be seen in other neurons in this area. Renshaw cells are inhibitory interneurons residing in the ventral horn which are activated primarily by motoneurones to recurrently regulate the firing of the same cell (Renshaw, 1946; Eccles et al., 1954). Although the primary input to mature RCs is from motor axon collaterals, they also receive relatively few inputs from Ia afferents (Mentis et al., 2006b). Antibodies against Calbindin D-28k revealed Renshaw cells and extensive portions of their dendrites in the ventral horn of the lumbar spinal cord (Fig 23A-G'') and VGLUT1^{+} was used to quantify proprioceptive afferent boutons contacting the soma and dendrites of Renshaw cells. Our results show that retraction of proprioceptive afferent innervation of the ventral horn is not specific to MNs as there is a significant reduction in Ia afferent boutons contacting Renshaw cells throughout PN development (p=.014, Fig 17) . This was true for the soma (p= .004) and dendrites (p= .003). For the whole cell (soma and dendrites combined), there was a significant reduction in proprioceptive afferent boutons directly apposing RCs between PN10 (1.21 ± 0.06) and PN14 (0.68 ± 0.15, p= .011) and PN10 and PN21 (0.63 ± 0.11, p= .008), however there is a plateau at PN14 as there is no significant difference between PN14 and 21 (p= .76). On the soma, there was a significant difference between PN10 (0.72 ± 0.02) and PN14 (0.52 ± 0.09, p= .043) and PN21 (0.27 ±0.02, p= .001). Additionally, there was a significant decrease between PN14 and 21 (p= .022). For the dendrites, there was a significant reduction in Ia

boutons between PN10 (1.92 ± .08) and 14 (1.17 ± .14, p= .002) and PN10 21 (1.15 ± 0.06, p= .002), however no difference between PN14 and 21 (p=.94).

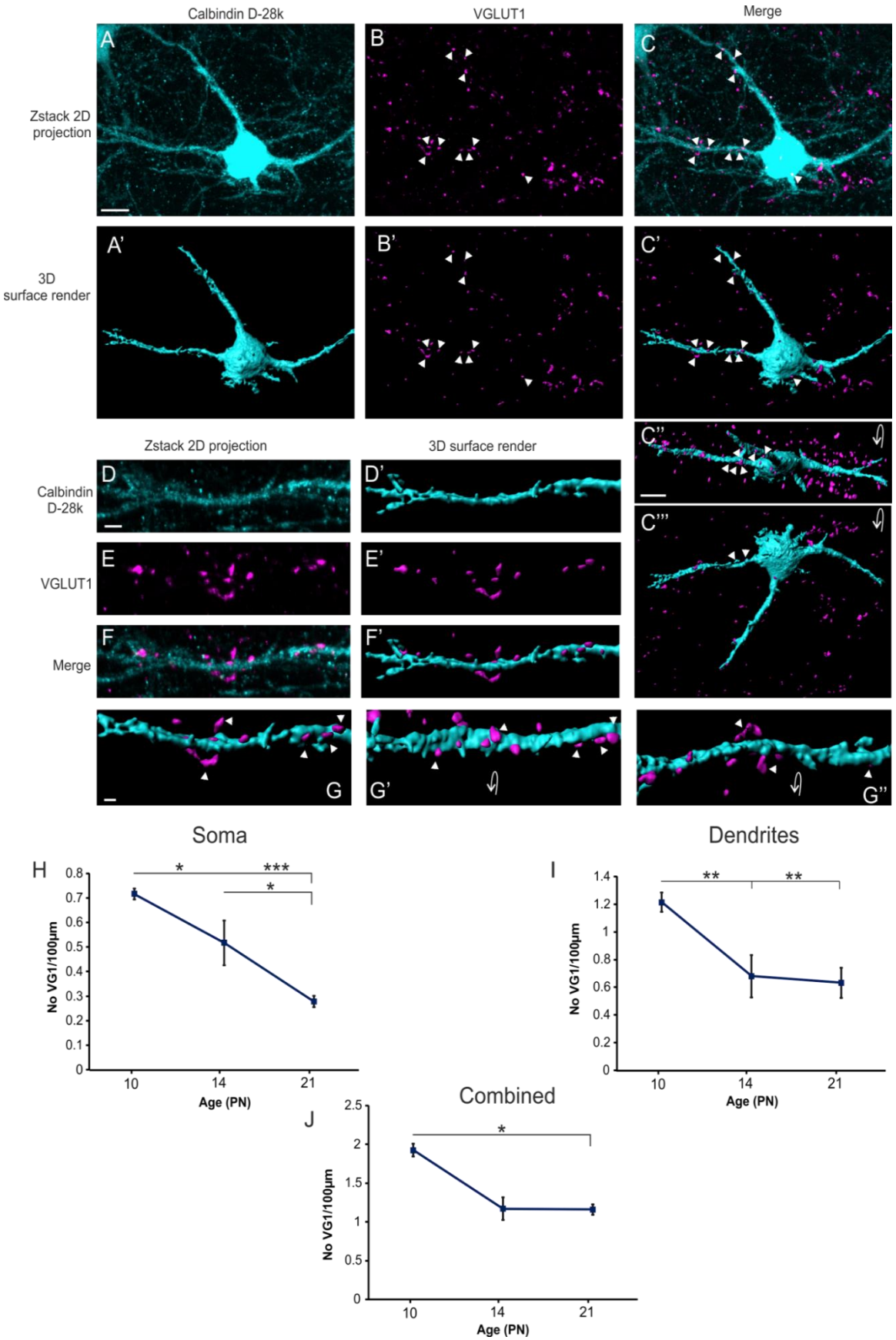

Figure 17. Postnatal development of Ia afferent input to Renshaw cells.
(A-C) 2D confocal projection of Renshaw cell soma and dendrites contacted by VGLUT1⁺ Ia afferent terminals. (A'-C') Imaris 3D reconstruction of RCs. (C''-C''') Anterior rotation of 3D reconstructed cell in C'. (D-F') illustrates 3D rendering of segmented RC dendrite. (G-G'') Rotated 3D rendered dendrite. H-I contain graphs of soma and dendrite input density. (J) is the combined values.

4.5.3 PN development of presynaptic ‘P bouton’ innervation of Ia afferents contacting αMNs

After showing retraction of VGLUT1⁺ terminals from the ventral horn of the spinal cord, we wanted to study how presynaptic control of Ia afferent axon firing might be altered with PN development. GAD65 is a selective marker for GABAergic presynaptic terminals (P boutons) making axo-axonic contacts with Ia afferent axons (Rudomin, 2009; Hughes et al., 2005). We therefore quantified GAD65⁺ boutons in close apposition to VGLUT1⁺ Ia afferent boutons terminating on αMNs.

As Ia afferent input to MNs is retracted with development, the number of P boutons per Ia bouton was significantly increased with age ($p < .0001$, Fig 18). Interestingly, this increase occurred between PN10 (0.45 ± 0.048) and PN14 (1.04 ± 0.031, $p<.0001$) with no significant difference between PN14 and PN21 (1.04 ± 0.027, $p=.997$). PN10 and 21 were also significantly different ($p<.0001$). This increase was due to a significant reduction ($p<.0001$) in the percentage of Ia afferent terminals which were devoid of P boutons (P bouton off) between PN10 (41.61 ± 2.65%) and PN14 (11.72 ± 1.9%, $p<.0001$) and PN21 (6.37 ± 0.14%, $p<.0001$, Fig 18). There was no difference between PN14 and PN 21 ($p=.192$). There was a concomitant increase ($p<.0001$) in the amount of clusters on Ia afferent terminals containing 3 or more P boutons (3+ clusters) between PN10 (7.86 ± 0.89) and PN14 (38.84 ± 3.99, $p<.0001$) and PN21 (44.47 ± 3.44, $p<.0001$) but again no increase between PN14 and 21 ($p=.060$). If Ia afferent terminal surface area (SA) was substantially increased with age, it could be argued that younger animals have less available space for P boutons to contact and this could be responsible for the reduced 3+ clusters at PN10.

Our analysis showed that there was no significant increase in Ia afferent terminal size between PN10 (21.77 ± 0.89) and PN14 (22.80 ± 0.66) however there was an increase between PN10 and PN21 (26.54 ± 0.57, p=.006). There was no effect of age on number of P bouton clusters sized 1-2 boutons (p=.880).

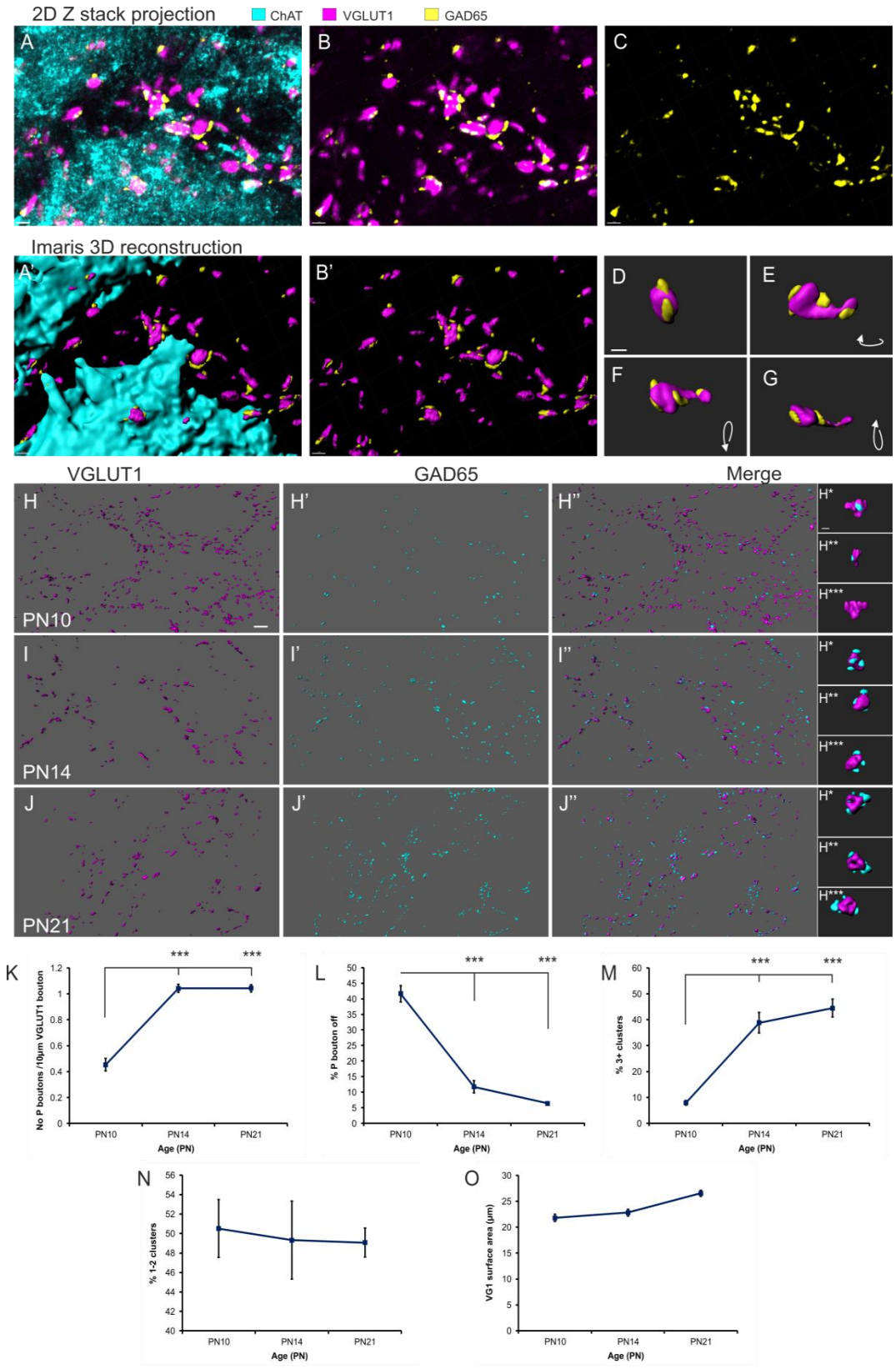

Figure 18. PN development of GAD65⁺ P boutons on motoneuronal Ia afferent terminals. (A-C) representative confocal z stack projection showing GAD65⁺ P boutons contacting motoneuronal VGLUT1⁺ Ia afferent terminal. (A'-B') 3D reconstruction on A-C. (D-G) For quantification, P boutons were rotated in order to confirm apposition and count 'hidden' contacts. (H-J'') 3D reconstruction illustrating striking increase in P boutons between PN10 and 14. (H*-H***) Rotated, high magnification representative images of P boutons apposing Ia afferents at each age.(K-O) Graphs illustrating increase in P bouton contacting Ia afferents.

4.5.4 Summary of PN development of Ia afferent innervation of the lumbar spinal cord

In summary, VGLUT1⁺ terminal densities in the dorsal, intermediate and ventral grey are relatively high early (PN10) PN and are subsequently retracted as densities are significantly reduced by PN21.This was also true for terminal densities contacting MNs and RCs. Significant reductions in density were frequent between PN10 and 14 but interestingly, differences in terminal density between PN14 and 21 were very rare.

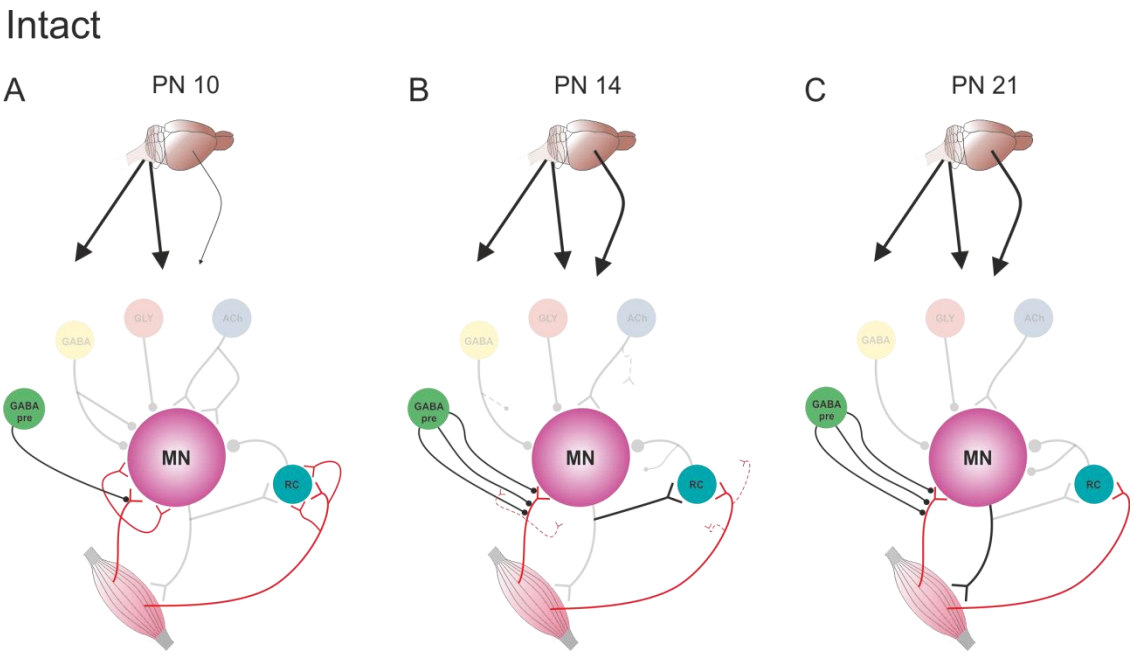

Figure 19. Schematic summary of the PN development of Ia afferent input to motoneurones and Renshaw cells. (A) At PN10, CST axons have just made contact with lumbar cord but termination patterns are weak. Ia afferent input to ventral horn neurons are many. (B) By PN14 CST termination patterns are almost mature and there is significant retraction of Ia afferents from the ventral horn. (C) Animal is functionally mature and there is some further retraction of Ia afferents.

4.5.5 PN development of cholinergic input to MNs.

Cholinergic (VAChT⁺) boutons apposing CTB labelled MNs were assessed in order to identify if there was any developmental alteration of input from

premotor, cholinergic interneuron to MNs. VAChT$^+$ terminals apposing MNs were significantly affected by development (p=.013), with a decrease in terminal density between PN10 (8.05 ± 0.11) and PN14 (5.14 ± 0.52, p=.011). There was also a moderate but non-significant decrease when comparing PN10 and PN21 (6.16 ± 1.3, p=0.66). A plateau seemed to be reached by PN14 (5.14 ±0.52) as it was not different from PN21 (p=0.351, Fig 20).

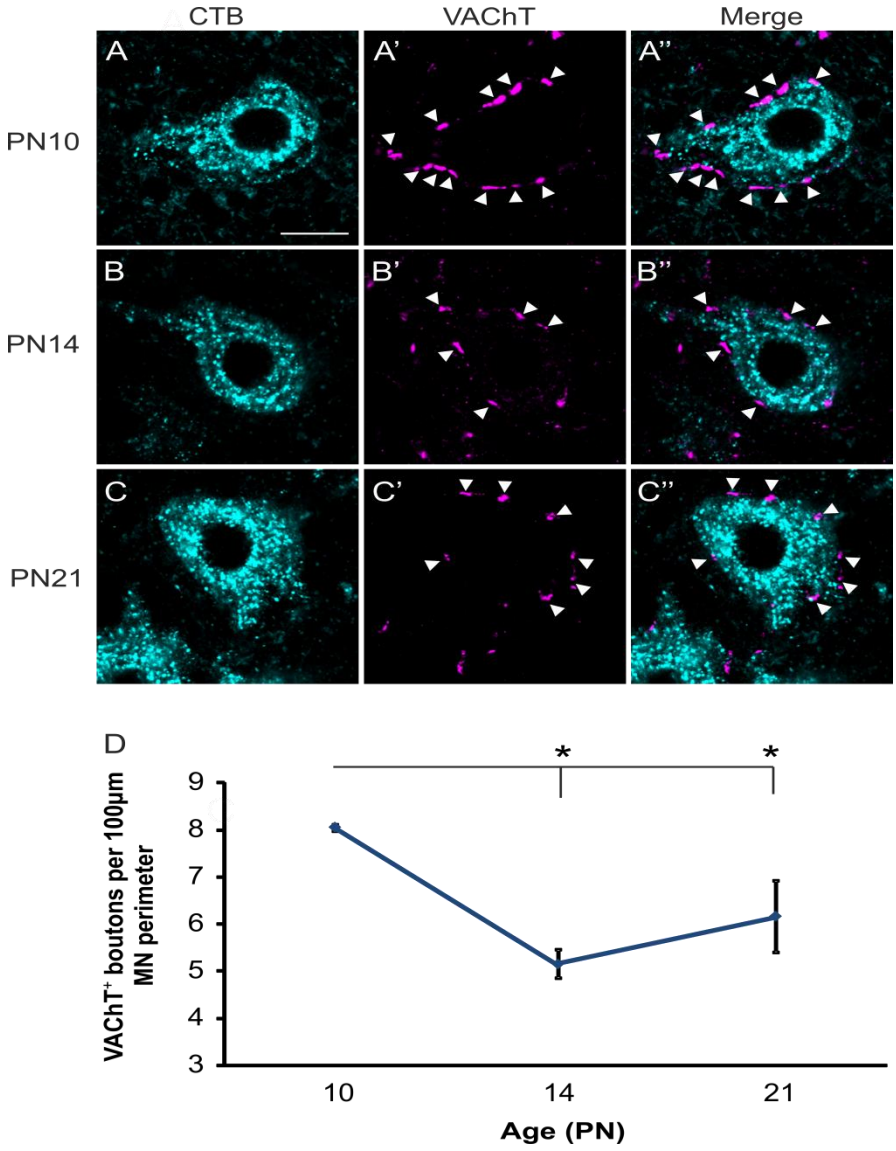

Figure 20. PN development of Cholinergic input to MNs.
(A-C) CTB labelled MNs at ages PN10, 14 and 21. (A'-C') VAChT$^+$ cholinergic terminals ages PN10-21. (A''-C'') Merged images illustrating CTB and VAChT staining. (D) Graph showing reduction in cholinergic boutons contacting MNs throughout development.

4.5.6 PN development of inhibitory inputs to αMNs

After assessing PN development of excitatory inputs to motoneurones from Ia afferents and cholinergic premotor interneurons, the main inhibitory inputs to MNs from GABAergic (GAD67) and glycinergic (GLYT2) premotor sources were assessed. Analysis revealed that there was a significant effect of age on $GAD67^{+}$ terminals contacting MNs (p=.004). There was a greater density of terminals at PN10 (18.34 ± 0.66) compared to PN14(13.58 ± 0.59, p=.010) and PN21 (12.59 ± 0.74, p=.005), however there was no difference between PN14 and PN21 (p=.975). Conversely, there was no significant effect of age on density of $GLYT2^{+}$ terminals contacting MNs (Fig 21).

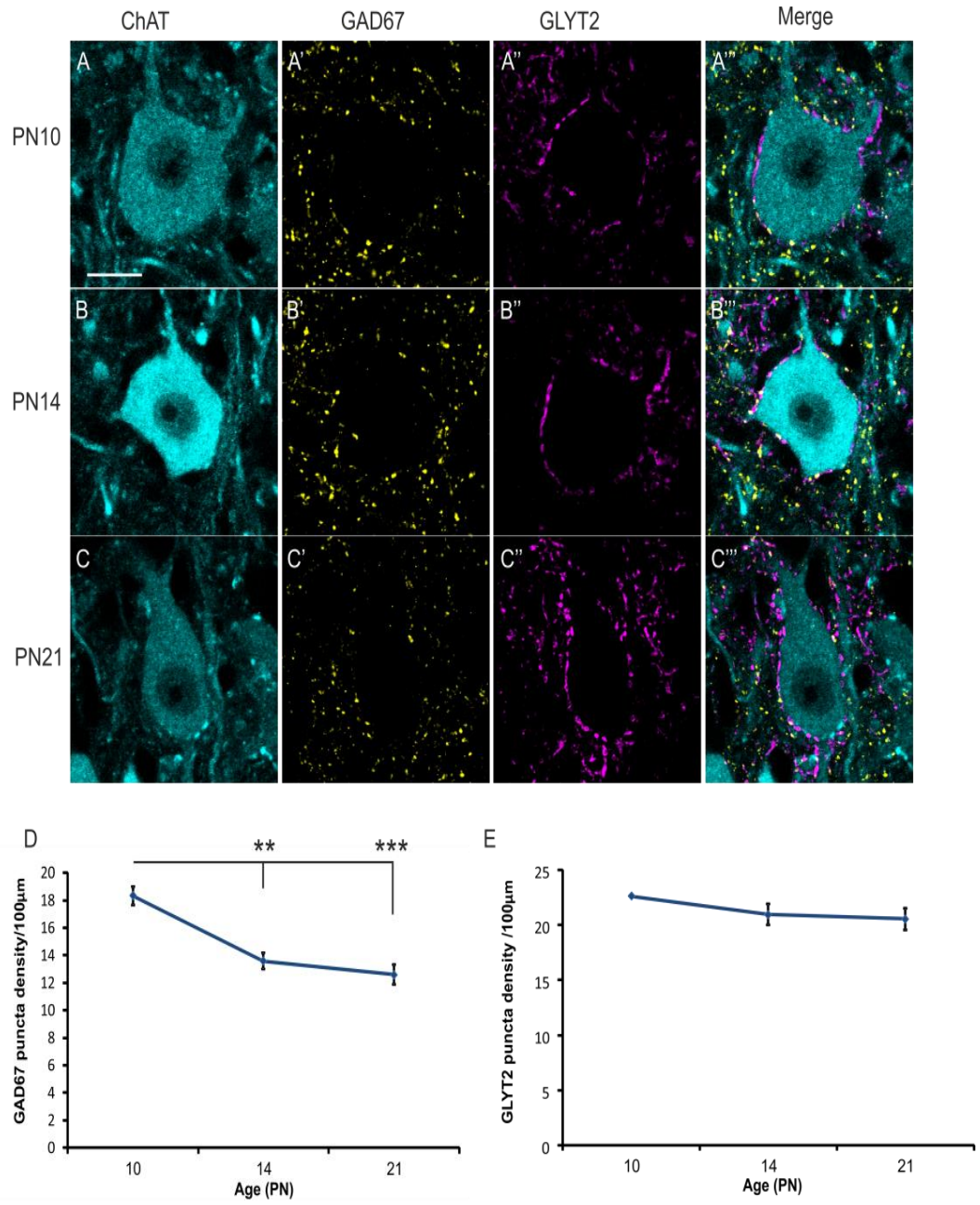

Figure 21. PN development of GAD67 and GLYT2 synaptic coverage of α MNs. (A-C) Representative examples of ChAT staining for each age. (A'-C') Representative examples of GAD67 staining for each age. (A''-C'') Representative examples of GLYT2 staining for each age. (A'''-C''') Representative merged images for each age. (D-E) Graphs illustrating PN development of GAD67 and GLYT2^{+} synaptic coverage of α MNs. Scale bar = 20µm

4.5.7 PN development of the Renshaw cell-MN recurrent inhibition circuit

4.5.7.1 Calbindin$^+$ terminations on MNs are significantly increased with postnatal development

One of the main modulators of motor output, and therefore excitability of the spinal cord is the Renshaw cell-motoneurone recurrent inhibition circuit. In mature animals, Renshaw cells are driven by motor axon collaterals (VAChT$^+$ terminals) and act to recurrently inhibit the same MNs (Calbindin$^+$ terminals) which excite them. In the ventral horn, Calbindin D-28k staining primarily labels Renshaw cells and substantial portions of their dendrites which project towards motor pools (Fig 23A') (Carr et al., 1998). Consistent with Carr et al. (1998) we found CB varicosities made 'bouton-like' terminations onto MNs (Fig 22B-B'), the majority of which are presumably from CB Renshaw cells. We quantified these terminals in order to assess if they were altered by development.

There was a significant effect of age on PN development ($p=.019$) with PN21 (3.8 ± 0.97) being greater than PN10 (1.6 ± 0.43, $p=.016$) but no difference between PN10 and 14 (2.7 ± 0.42, $p=.158$, Fig 22).

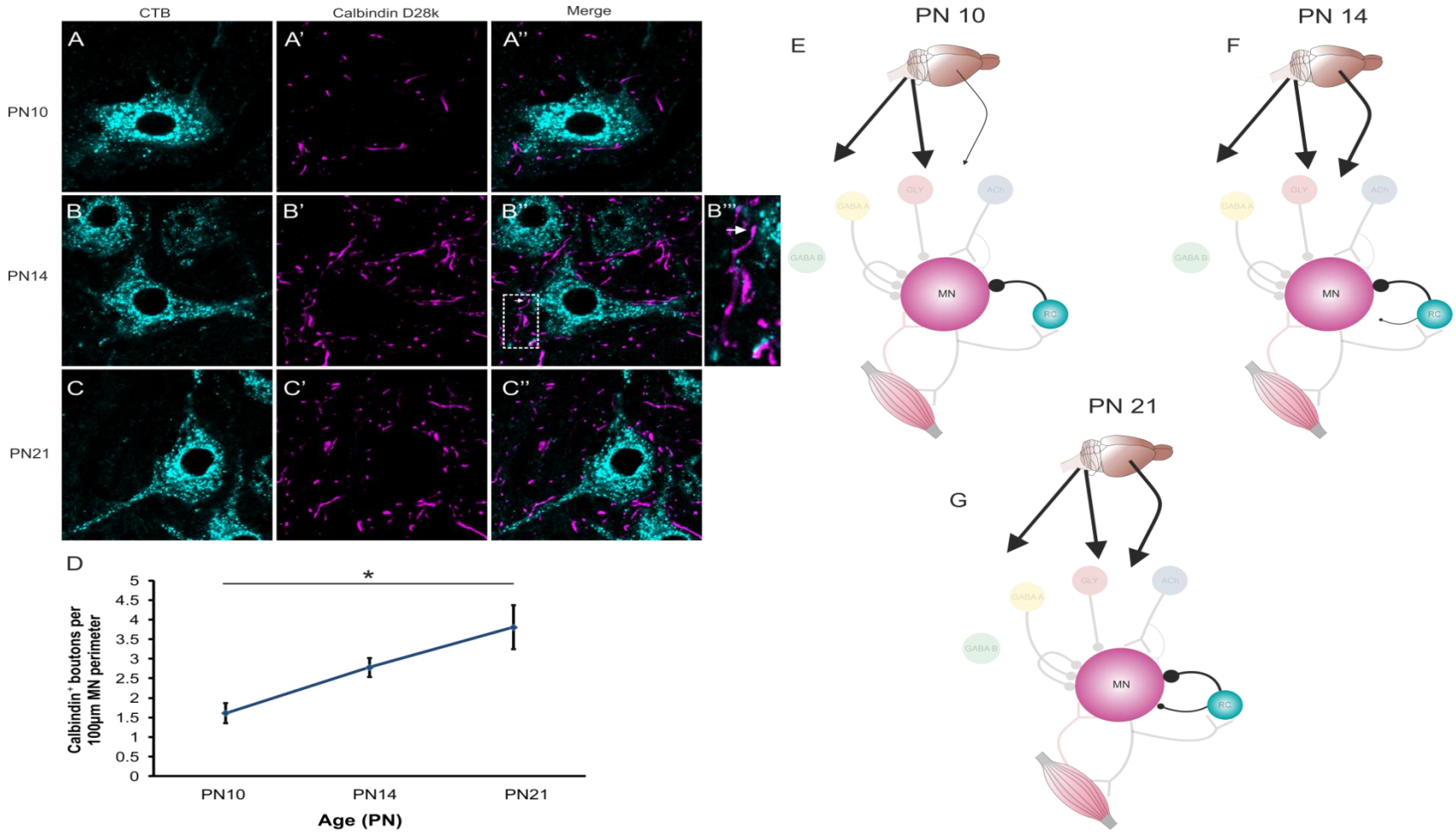

Figure 22. Postnatal development of Calbindin+ inputs to alpha motorneurones.
(A-C) Representative images CTB staining of MNs. (A'-C') Representative images of Calbindin⁺ variscosities. (A''-C'') Merged images showing Calbindin⁺ variscosities in close apposition to CTB labelled MNs. (B''') High magnification image of region outlined in B'' illustrating long Calbindin⁺ process extending towards and appearing to make contact with the CTB labelled MN.

4.5.7.2 Motor axon collateral input to RCs is not significantly altered with PN development

Next, the PN development of motor axon collaterals on Renshaw cells was assessed by quantifying VAChT$^+$ terminals on RCs. The rationale behind triple staining for CTB was that Liu et al. (2009) found that MN axon collateral terminals on Renshaw cells had co-localisation between CTB and VAChT, confirming that the terminal is from the injected motoneurone. Because CTB is both a retrograde and anterograde tracer, there was a mix between boutons which were only CTB$^+$ (presumably from Ia afferents), only VAChT$^+$ (MNs other than Gastrocnemius) and CTB/VAChT$^+$ representing inputs from Gastrocnemius MNs (Fig 23E). The majority of Renshaw cells are known to be located in the most ventral portion of lamina VII and IX (Fig 23A-A'), so our analysis was focussed on this area. There did not seem to be a significant effect of PN development on motor axon collateral input to RCs ($p= .614$) with none of the age groups being significantly different from each other (PN10 = 3.86 ± 1.1, PN14 = 4.48 ± 1.42, PN21 = 4.77 ± 0.65, $p>0.05$).

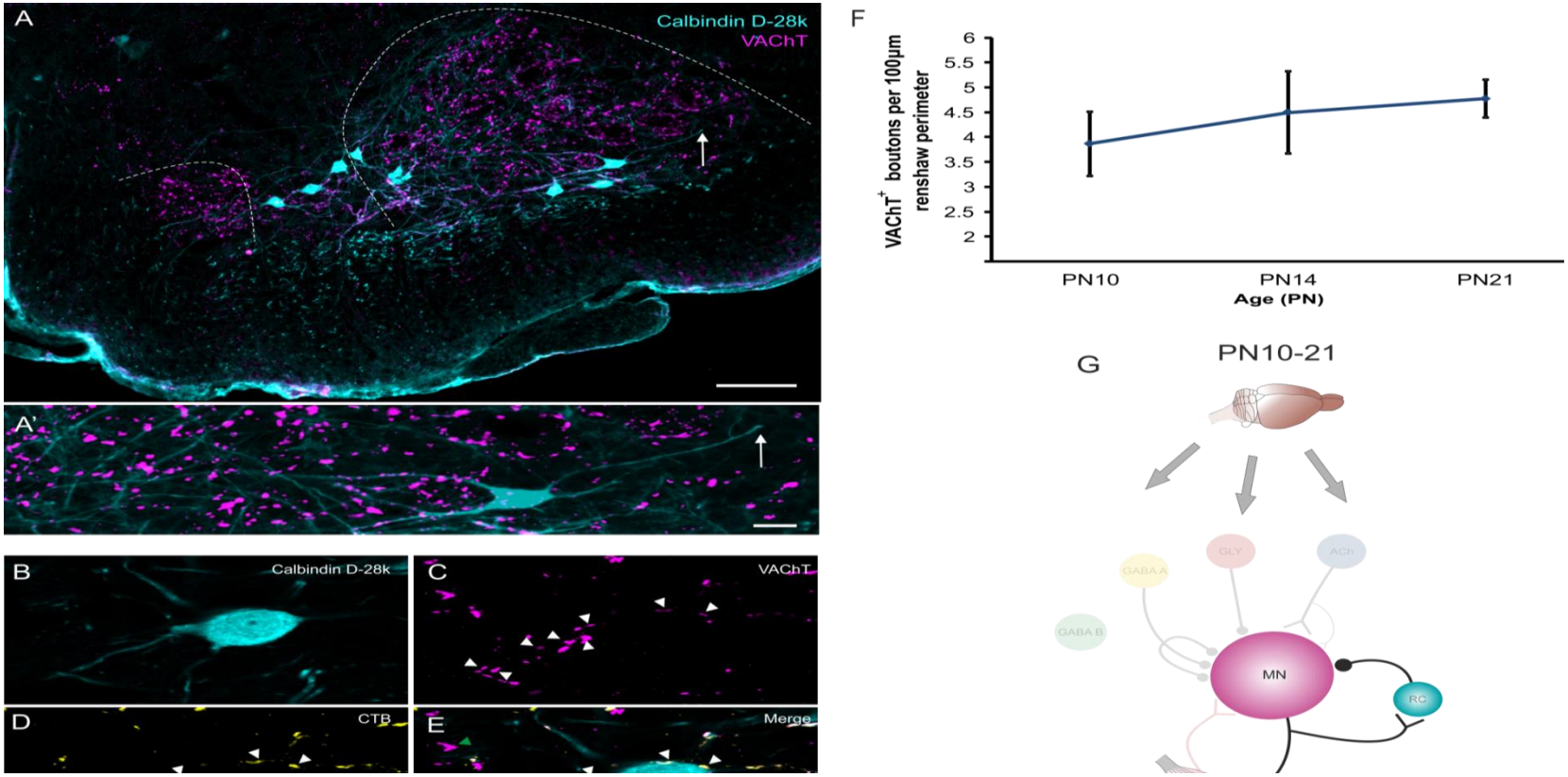

Figure 23. Postnatal development of VAChT inputs to RCs.
(A) Renshaw cells were located in the most ventral portions of lamina VII and occasionally laminae IX. (B-E) Shows typical triple labelling of Calbindin (RCs), VAChT and CTB. Note the localisation of CTB and VAChT indicating motor axon collateral termination. (E) White arrows indicate colocalised boutons and green arrows non colocalised. (F) Illustrates the developmental trend for VAChT^{+} boutons on Renshaw cells.(F) Graph of VAChT boutons per 100 µm. (G) Schematic of RC input/output. Scale bar in A= 100 µm, scale bar in B-E= 20µm

4.6 Discussion

This study demonstrates that proprioceptive afferent innervation of the lumbar spinal cord is greatest early postnatally and retracted as the animal matures. Furthermore, retraction of proprioceptive afferents from the ventral horn is not specific to MNs as innervation of Renshaw cells is also significantly reduced with postnatal development. Surprisingly, GABApre projections to Ia terminals are weak early in development and proliferate as afferents are pruned. Assessments of spinal premotor inputs to motoneurones revealed that in general, inputs to MNs were highest early in development and retracted with maturity. Interestingly, in the majority of cases, significant differences are seen between PN10 and 14, with differences between PN14 and 21 rare (1 case). This may be a pertinent finding considering the rapid acquisition of 'motor functional maturity' occurs at PN14-15 (Altman and Sudarshan, 1975).

4.6.1 Non-specific retraction of Ia afferents from the ventral horn of the lumbar cord during postnatal development.

Postnatal development of primary afferent input to the cervical spinal cord has been well studied. Gibson and Clowry (1999) demonstrated a striking retraction of CTB labelled primary afferents from the ventral horn during postnatal development of the rat cervical spinal cord, whilst Chakrabarty and Martin (2011a) showed refinement of proprioceptive afferent innervation of dorsal and intermediate regions in the cat cervical spinal cord. Although this profile is generally appreciated to also be true for the lumbar spinal cord, it has not been directly ratified. Results presented here show greater numbers of VGLUT1^{+} boutons directly apposing motoneurons and Renshaw cells at PN10, followed by synaptic stripping resulting in significantly reduced bouton density between

PN10 and 14. Because descending tracts and cutaneous afferents do not make monosynaptic contact with MNs in the rat lumbar spinal cord (Yang and Lemon, 2003; Illert et al., 1976b; Illert et al., 1978), these results suggest that this reduction is due to a retraction of proprioceptive afferents from the ventral horn. Furthermore, because this retraction was true for RCs as well as MN it is clear this process is not specific to motoneurones. There was a similar reduction in the density of VGLUT1 puncta in the intermediate and dorsal horn of the lumbar spinal cord but this cannot be attributed solely to a reduction in proprioceptive afferent fibre terminals as these areas represent terminal fields for corticospinal (CST) and low threshold cutaneous fibres which both express VGLUT1 and both undergo significant postnatal changes (Du Beau et al., 2012; Varoqui et al., 2002; Todd et al., 2003). Aδ and Aβ afferent fibres are thought to account for most non-Ia VGLUT1 positive terminals in the dorsal horn (lamina II-V) and are widely distributed early in development before becoming confined to more specific terminal fields (Fitzgerald et al., 1994; Fitzgerald, 2005). Conversely, the rat CST is the latest of the descending systems to mature, only reaching the lumbar cord in the first instance at PN9 and mature termination patterns in the intermediate grey are not seen until PN14 (Donatelle, 1977; Joosten et al., 1989). In the cat cervical spinal cord, CST fibres are thought to compete with proprioceptive afferents for synaptic coverage of premotor interneurons, which may be responsible for the retraction and refinement of proprioceptive afferents in dorsal/intermediate zones of the cat cervical spinal cord (Chakrabarty and Martin, 2011b). This process is believed to be activity dependant, demonstrated by afferent sprouting in response to neonatal CST lesions and CST sprouting with neonatal afferent lesions (Clowry et al., 2004a; Gibson et al., 1999; Gibson et al., 2000; Clowry et al., 2006). Given that

afferents (both muscle and cutaneous) make up the majority VGLUT1$^+$ terminations in the dorsal horn, and that CST terminations are proliferating here PN, one can conclude that the dorsal reduction in VGLUT1 puncta density is due to reduction in afferent (muscle and/or cutaneous) innervation. Functionally, this could explain the increased excitability of the cord at neonatal ages and postnatal refinement of exaggerated, ill-directed and lasting motor outputs to diverse afferent stimulation (Saito, 1979; Fitzgerald, 1985; Falcon et al., 1996).

Miles et al. (2007) described a group of cholinergic 'partition' interneurons lateral to the central canal which project to motoneurones and increase their excitability during locomotion. Further work identified that these interneurons are phase locked with motoneurone firing frequency and when they were

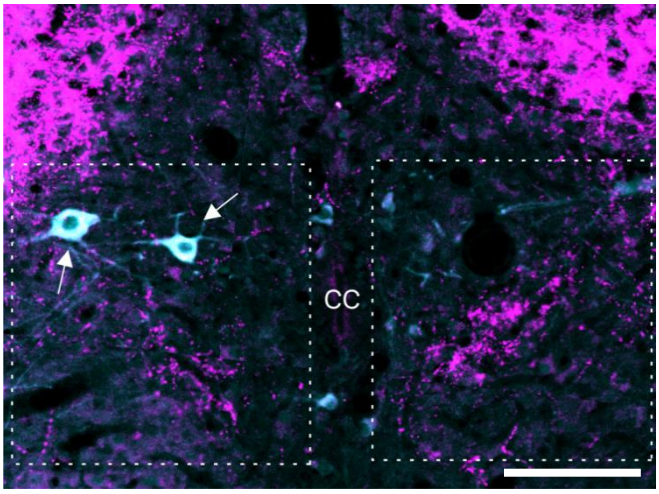

Figure 24. Image illustrating location of partition cells and the VGLUT1 puncta which were quantified as intermediate X (Int X). Scale bar=100μm

inactivated, locomotor abilities were negatively affected (Zagoraiou et al., 2009). Clearly these neurons are very important in generation of locomotion and therefore we assessed whether Ia afferent input in the intermediate zone, just lateral to the central canal and lamina X was altered with development. Results suggested that Ia innervation of this region was not significantly

affected by PN development, with no difference in VGLUT1 puncta density found.

In this study we did not consider PN development of receptor types and subtypes, which play a major role in synaptic transmission. However, in agreement with our results regarding glutamatergic boutons, previous studies have demonstrated, that NMDA and AMPA receptor subtypes are significantly down-regulated throughout the first 3 weeks postnatally (Kalb et al., 1992; Kalb and Hockfield, 1992).

Renshaw cells are the last and most potent inhibitory regulators of motor output in the ventral horn and can be identified by their expression of calcium binding protein Calbindin D28k (Renshaw, 1946; Eccles et al., 1954; Carr et al., 1998). Adult Renshaw cells are primarily driven by motor axons in a recurrent inhibitory circuit with motoneurons, but also receive relatively few inputs from Ia afferents (Mentis et al., 2006b). At birth, approximately 60% of RCs receive Ia afferent contacts and by PN10 this figure rises to 100% (Mentis et al., 2006b). Additionally, Mentis et al. (2006a) suggested that the density of VGLUT1$^+$ contacts on Renshaw cells is increased between PN10 and 15 and then drastically reduced to below PN10 levels by PN20. Our results are in agreement with these findings in that VGLUT1$^+$ terminations on RCs were reduced between PN10 and 21, however, we do not see an increase at PN14. Analyses of the soma and whole cell (dendrite+soma) reveal that there is no significant change in VGLUT1$^+$ density between PN10 and 14 but in all cases there is significant reduction between PN10 and 21. The disparity in developmental profile may be resultant of the present study using 25μm sections. This meant that in some cases it was not possible to quantify the whole cell and all its dendrites. Despite this drawback, a large number of cells

were analysed (~20/animal) and only a minority of these had incomplete cell bodies. Another explanation is that there is a sudden transient increase in VGLUT1^{+} contacts on RCs between PN14 and 15 which we do not pick up due to the lack of this time point in our analysis.

The VGLUT1 retraction profile observed on RCs matches that of MNs, suggesting that retraction of Ia afferents from the ventral horn is likely to be nonspecific to motoneurones and could be true of other premotor interneurons located in ventral and intermediate regions of the spinal grey. Monosynaptic reflexes can be evoked from MNs at birth (Kudo and Yamada, 1985) and Mentis et al. (2006a) demonstrated dorsal root stimulation was effective at evoking RC firing in neonatal rats, suggesting early contacts are functional in both cell types. In contrast, dorsal root stimulation does not activate RCs in adult animals (Renshaw, 1946; Eccles et al., 1957b) whereas MNs are still effectively activated. It is difficult to postulate as to the functional significance of early activation of RCs by Ia afferents, however it is possible that initial high innervation of the ventral horn results in non-specific neuron contact which is refined in an activity dependant manner during the PN period.

4.6.1.1 GABApre neuron projections (P boutons) to Ia afferent terminals are significantly increased with development

Presynaptic inhibition is a mechanism which allows the CNS to filter and direct afferent input in order to ensure movements are efficient and well directed (Fink et al., 2014; Rudomin and Schmidt, 1999). This is mediated by GABApre neurons in the medial deep dorsal horn which project to all afferent fibres (Hughes et al., 2005). Pierce and Mendell (1993) found that 86% of Ia afferent terminals apposing motoneurones have P boutons. To the best of our

knowledge, this study is the first to assess how GABApre innervation of Ia afferents are altered throughout postnatal development. Betley et al. (2009) suggested that GABApre projections to afferents is “soley” based upon signals from proprioceptive afferent terminals because reductions in afferents resulted in reductions in P boutons, however this would suggest that P bouton density in the ventral horn of the spinal cord should be great early in development and retract with maturity as Ia afferents do. Our results agree that P bouton density is linked to the number of afferent terminals because both undergo significant change between PN10 and 14 and then plateau. However, our results show that GABApre neuron innervation of Ia afferent terminals increased as Ia terminal density reduced. At PN21 we saw that 94.63 ± 0.14% of Ia afferents had P boutons which is slightly higher than Pierce and Mendell (1993). It is possible that this value could be reduced into adulthood, but it is more likely that the greater resolution of the 3D analysis in the present study ensured greater accuracy and Pierce and Mendell (1993) produced an underestimated figure due to 2D analysis.

4.6.1.2 PN development of cholinergic input to αMNs

Cholinergic input to MNs was significantly reduced between PN10 and 14, with no difference observed between PN14 and 21, suggesting these inputs are pruned early on before reaching near mature levels by PN21. Motoneurones receive monosynaptic input from cholinergic partition cells which act to increase excitability during locomotion (Zagoraiou et al., 2009; Miles et al., 2007; Stepien et al., 2010; Nagy et al., 1993). Although partition cells are also phasically active during locomotion in the adult cat (Huang et al., 2000), modulation of motor output by cholinergic input has largely been studied in neonate

preparations. It is therefore important to study the PN development of these inputs to understand how they mature. Electron microscopy studies by Conradi and Ronnevi (1975) found that S,F and C type boutons were reduced on cat MNs throughout PN development, with C boutons being reduced by 20%. Wetts and Vaughn (2001) conducted an immunohistological study of cholinergic inputs to MNs and saw a slight reduction in VAChT$^+$ terminals on the soma between PN8 and 15, with an increase observed between PN15 and 22 which is the opposite profile when compared to the present study and that of Conradi and Ronnevi (1975). The disparity between the studies can be easily explained by shortcomings in experimental design and lack of statistical power of the Wetts and Vaughn (2001) paper. The present study examined 13.62 ± 2.19 MNs from 3 different lumbar sections of 3-5 different animals acquired from different litters, meaning >100 cells per group were analysed. Wetts and Vaughn (2001) studied 22 cells (total) analysed from 1 section (total) in 1 animal per age group (at PN8, 15 and 22), which were all from the same and litter (only 1 litter was used). Clearly those results hold no statistical significance. Wilson et al. (2004) also suggested an increase in VAChT$^+$ puncta density between PN10 and 14 followed by a plateau. However, that study was limited to purely qualitative analysis of an unspecified number of animals and sections meaning their conclusions have no empirical support. Due to the experimental shortcomings of the aforementioned studies, and the rigor in which the present study was conducted, we provide a more complete description of PN development of cholinergic input to αMNs in rats and can conclude that there is a significant reduction of VAChT$^+$ terminals on MNs between PN10 and 14 followed by a plateau.

4.6.1.3 PN development of inhibitory input to αMNs

Gammaaminobutyric acid (GABA) and Glycine are the main inhibitory neurotransmitters in the CNS. At the motoneuronal level, two of the main glycinergic sources of inhibition are the RC and Ia inhibitory interneurons which mediate reciprocal and recurrent inhibition respectively (Eccles et al., 1954; Jankowska, 1992; Curtis et al., 1976). The addition of strychnine to neonatal isolated spinal cord preparations results in rhythmic, in phase bursts from flexor/extensors and bilateral ventral roots suggesting glycine is essential in maintaining the alternating nature of locomotion (Cazalets et al., 1996). It has also been demonstrated that bicuculline, a selective GABA-A receptor antagonist results in synchronous discharges from antagonistic muscles in the same preparation (Hinckley et al., 2005). GABA/glycine signalling to MNs is therefore likely to be very important in generation and maintenance of normal locomotor output. In the ventral laminae of the cervical spinal cord, Ma et al. (1994) found that GAD67$^+$ neurons reduced in quantity between PN7 and 21. Similarly, on rat trigeminal MNs, Paik et al. (2007) demonstrated a reduction in GABA$^+$ boutons between PN11 and PN31 whereas glycine$^+$ boutons were significantly increased in the same time period. There has not been any study of GABA or glycine$^+$ input to αMNs in the developing rat lumbar spinal cord. In the kitten however, Simon and Horcholle-Bossavit (1999) found that Glycine and GABA terminals were equivalent early in development and the adult ratio of 1.5/1 was achieved postnatally due do a significant reduction in GABAergic terminals, whilst glycine terminals were unaffected. In agreement with Simon and Horcholle-Bossavit (1999) and Paik et al (2007), the present study suggests GABA$^+$ (GAD67) postsynaptic terminals are significantly reduced between PN10 and 14 and 21 with no change in glycine synaptic coverage.

Fritschy et al. (1994) showed that in the rat brain GABA-A α1 and 2 subtypes developed in opposite directions with starting low at birth and increasing greatly with development and α2 the opposite. Interestingly in the caudal brainstem both subunits were reduced throughout development.

4.6.1.4 PN development of the recurrent inhibition circuit

Recurrent inhibition occurs in a circuit between motor neurons and Renshaw cells. Motoneurones drive RCs via axon collaterals and RCs in turn inhibit motor output. Here, we assessed the PN development of this circuit by quantifying VAChT$^+$ terminals contacting RCs and calbindin$^+$ terminals apposing MNs. Results indicate that calbindin$^+$ inputs to MNs are significantly increased with development, whilst motor axon collateral input to RCs is not significantly altered. Mentis et al. (2006a) previously showed similar results, with no increase in VAChT$^+$ contacts on the soma of RCs between PN10 and 20 but significant increases between PN5 and 15. When they assessed VAChT$^+$ input to the dendritic trees of RCs, the overall profile was similar to the soma, with a significant increase between PN10 and 20 but no significant increase between PN10 and 15 or 15 and 20. There was no assessment by this group, of calbindin$^+$ inputs to MNs but they did state that calbindin$^+$ dendrites of RCs increased in length, dendritic arbour size and branching complexity in the later stages of development, which would provide support for our findings. As described above, the same group studied PN development of Ia afferent input to RCs and showed that both motor axon collaterals and Ia afferents proliferate until PN15 when Ia afferents are reduced. Hence, Ia afferents are pruned as motor axon collaterals become dominant. Most recently, Siembab et al. (2015) showed that the proliferation of motor axon

input to RCs is dependent on afferent pruning as mlcNT3 (+/-) transgenic mice, which have strengthened afferent input, display concomitant reductions in motor axon collateral inputs. If motor axon inputs to RCs are considered as efference copies of the intended motor output and Ia afferents as feedback from the motor output, then co-development of Ia afferents and motor axon synapses on RCs becomes extremely functionally significant (Brownstone et al., 2015).

4.6.2 Summary and conclusions

This study provides the first comprehensive assessment of PN development of proprioceptive afferent innervation of the lumbar spinal cord with a focus on ventral horn neurones. Furthermore, it provides extensive immunohistochemical analysis of excitatory and inhibitory input to MNs and the recurrent inhibition circuit. In general, innervation of MNs by afferents, excitatory and inhibitory interneurones is high early in development, followed by postnatal retraction. This retraction seems to plateau by PN14. Altman and Sudarshan (1975) suggested that PN14 was key to rat locomotor development as they undergo a striking transition to near mature levels at this time point. In comparison, our results in conjunction with previous work suggest PN development of the spinal circuits provides a neural correlate of behavioural maturation. Whilst we have not shown which component of the spinal circuitry is responsible for acquisition of mature behaviour at specific time points, candidates have become apparent for manipulation or disruption of development in order to address this question. For example, descending input to the lumbar cord is week at birth, with mature termination patterns of CST not seen until PN14, when afferents and many premotor inputs to MNs mature. It is

reasonable to assume therefore, that the normal developmental profile of spinal circuits and their input from afferent sources is dependent on normal development of descending systems. This hypothesis can be tested with a complete neonatal spinal cord transection, which would allow this circuitry to develop in the complete absence of descending systems.

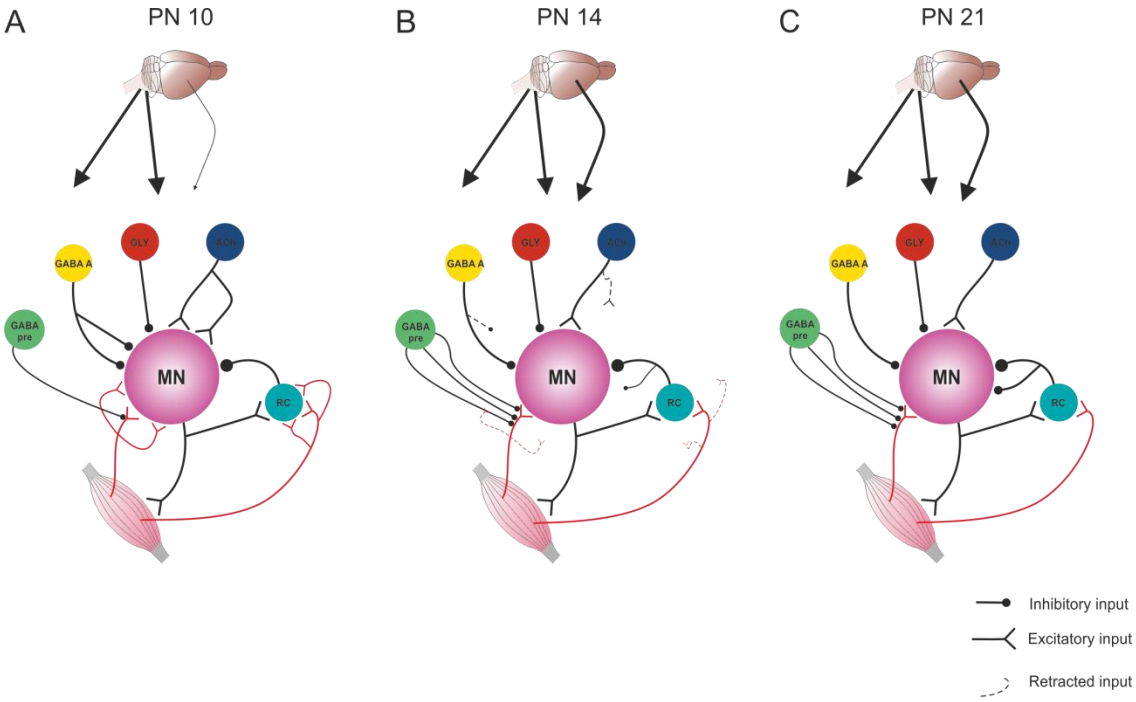

Figure 25. Schematic of PN development, PN10-21.

4.6.3 Methodological considerations

We used single optical slice images for quantification of synaptic inputs to MNs. Although this provides a widely accepted, practical way to assess changes in synaptology of MNs it is only possible to assess a fraction of the cell. Furthermore, error is introduced to quantification due to boutons being ‘cut’ at different positions in the image. Additionally, it is not possible to quantify inputs to dendrites of the cell, which in some cases are more numerous than the soma. To increase the strength of these results it would be preferable to produce z-stack images of cells for quantification of all inputs on the soma and

dendrites. In order to do this, spinal cord slices need to be cut at a greater thickness (at least 40μm) which can reduce antibody penetration to the centre of the slice. Although validity would be improved with this method, observations in our lab and comparisons with published results suggest that synaptic input profile on z stacks vs 1 optical slice are mostly comparable. Additionally, single optical images benefit the experimenter by proving them with a larger sample size due to reduced imaging time. With regards to soma vs dendritic inputs, inputs to the soma tend to have more weighting with regards to action potential generation compared to dendrites. One of the main issues which needs to be taken into consideration is level of concurrence between development of presynaptic cleft (bouton) and post synaptic (receptor).

Chapter 5 Postnatal development of the lumbar spinal circuitry in the absence of descending systems

5.1 Introduction

Postnatal development of the central nervous system is shaped by activity and therefore insults during this period can have significant consequences on its organisation and function. For example, pre and early postnatal insults to the brain can induce severe motor deficits. Loss of crucial inputs undoubtedly contributes to abnormal behaviour following neonatal insults, however, this is often subtle initially and exacerbated throughout development (Hadders-Algra, 2004). This suggests development of abnormal circuit organisation may be due to altered activity patterns. In cerebral palsy, disruption of descending input to the spinal cord is suggested to cause ectopic sprouting of afferent fibres leading to spasticity. This is reflected in current treatment approaches such as selective dorsal rhizotomies (Peacock and Staudt, 1990). Interestingly, insults to the nervous system that are incurred neonatally are often accompanied by greater functional outcomes than comparable insults in the adult CNS. For example, Hicks and D'Amato (1975) performed hemispherectomies on new-born and mature rats and observed resultant behavioural and anatomical consequences. There was a wealth of functional deficits in both groups, however neonates displayed significantly spared stride effectiveness on the side contralateral to the injury compared to adults. The authors observed crossing of CST tract fibres in the neonatally injured but not adult animals and suggested this as a mechanism. After neonatal spinal cord transections however, there is no reconnect between the brain and spinal cord (Tillakaratne et al., 2010), yet functional recovery of locomotion is often far superior to adult transections.

Because the organisation of spinal circuits is highly activity dependent and the neonatal cord is highly plastic, removing descending input should induce changes in circuit organisation. Indeed, some of these changes have been uncovered, for example Ichiyama et al. (2011) observed increased inhibitory to excitatory input ratios to MNs in neonatally transected rats compared to intact animals. Lesions to the cortex during early development result in reduced expression of parvalbumin+ cells and reduced retraction of muscle afferents which has been suggested to contribute to increased excitability of spinal reflexes (Clowry et al., 2004b; Clowry et al., 2004a; Gibson et al., 2000). Although some organisation changes have been uncovered, knowledge is still limited in this area. Similarly, many of the results related to cortical lesions only examine the cervical spinal cord.

5.2 Aims

In this study, the aims were to identify how afferent innervation of the lumbar spinal cord developed in the absence of descending systems and subsequently identify if there was how premotor interneurons organise their projections PN in the presence of only afferent input.

5.3 Hypotheses

It was expected that normal developmental retraction of afferents would be reversed or attenuated due to the lack of competition from descending projections to the lumbar cord. In the absence of descending input, it is expected that input to MNs from premotor interneurons will be significantly altered throughout development. Specifically, we expect increased excitatory

and inhibitory inputs as both are retracted during normal development (Conradi and Ronnevi, 1975).

5.4 Methods

Methods used here were identical to those used in chapter 4.

5.5 Results

5.5.1 PN development of Ia afferent input to the lumbar spinal cord in the absence of descending input

Analysis of VGLUT1^{+} puncta density in the dorsal horn region of interest revealed that there was a significant main effect of PN development ($p = .041$) however there were no significant differences between any of the ages (Fig 26, D-H). In the intermediate and lateral to X (Int X) regions of interest there was no effect of age (Fig 26, C-H).

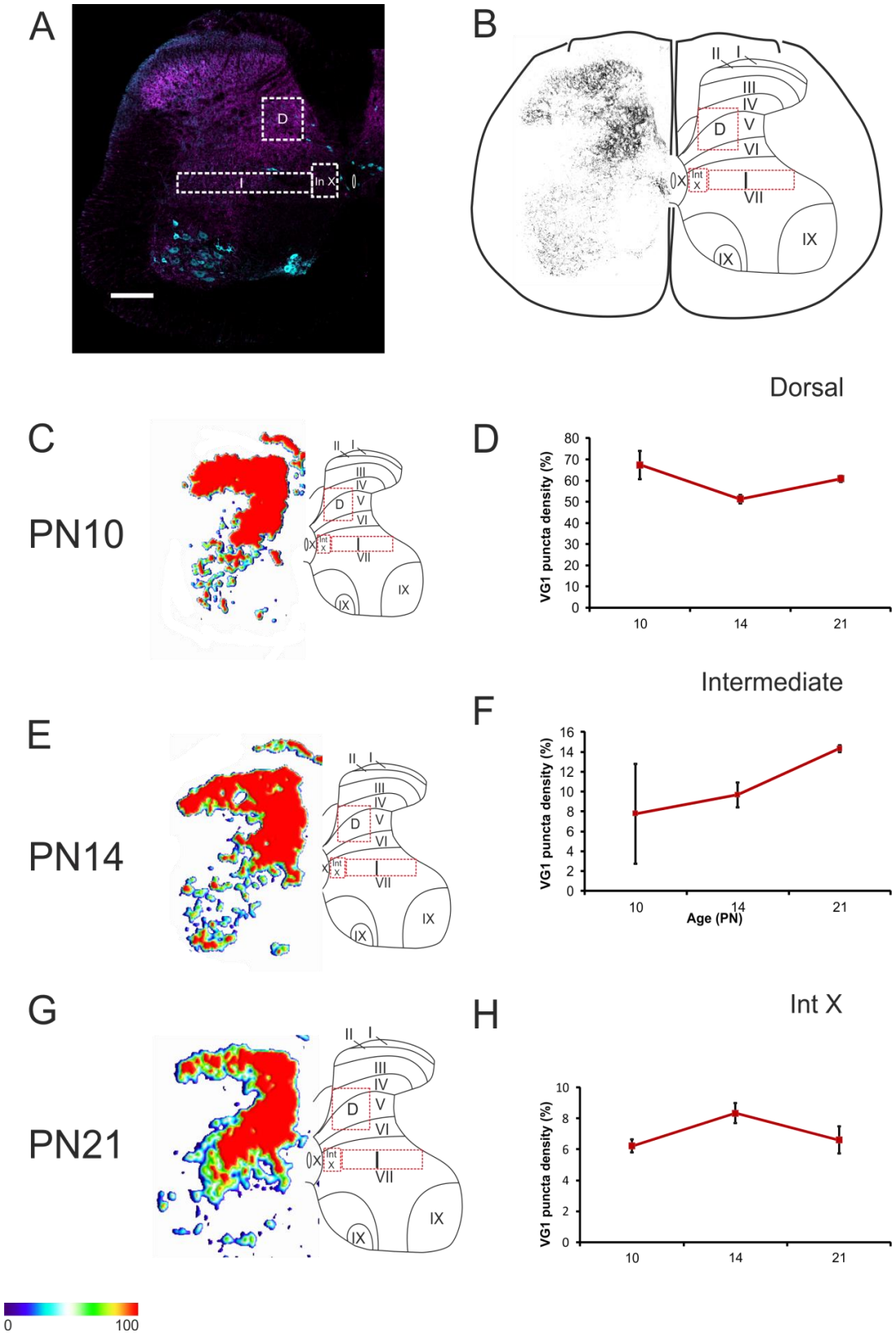

Figure 26. PN development of laminae distribution of VGLUT1⁺ puncta in PN5 transected rats. (A-B) Examples of regions of interest examined in dorsal, intermediate and Intermediate X ROI. (C,E and G) Heat maps from representative sections of PN10,14 and 21 for TX rats. (D,F and H) Graphs showing developmental profile of VGLUT1 puncta density in TX rats. Scale bar; 0-100 represent the highest and lowest densities after thresholding.

When looking at motoneurones specifically, there was no effect of age on PN development, with Ia boutons staying at the same level throughout PN development (Fig 27 A-D).

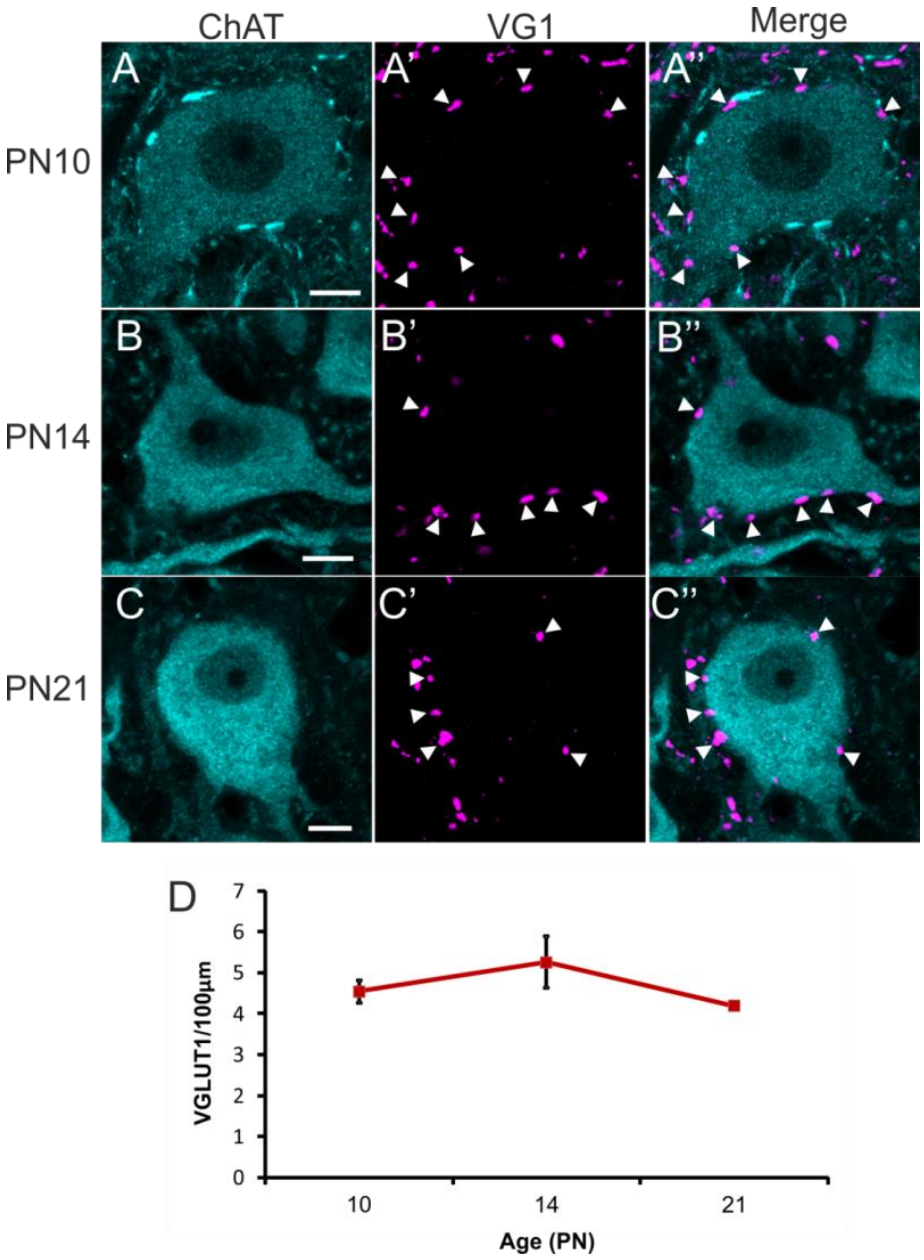

Figure 27. PN development of VGLUT1+ boutons apposing MNs in PN5 TX rats.
(A-C'') Representative images displaying VGLUT1+ boutons apposing MNs. (D) Graph illustrating developmental profile of Ia afferent innervation of MNs in the absence of descending input. Scale bar = 10µm.

5.5.2 PN development of Ia afferent input to Renshaw cells in the absence of descending input.

There was no effect of development on Ia boutons apposing RC soma (p=.144), dendrites (p=.184) or overall (p=.063). However there was a significant decrease overall in Ia boutons between PN14 (1.36 ± 0.16) and 21(0.92 ± 0.05, p=.024, Fig 28D)

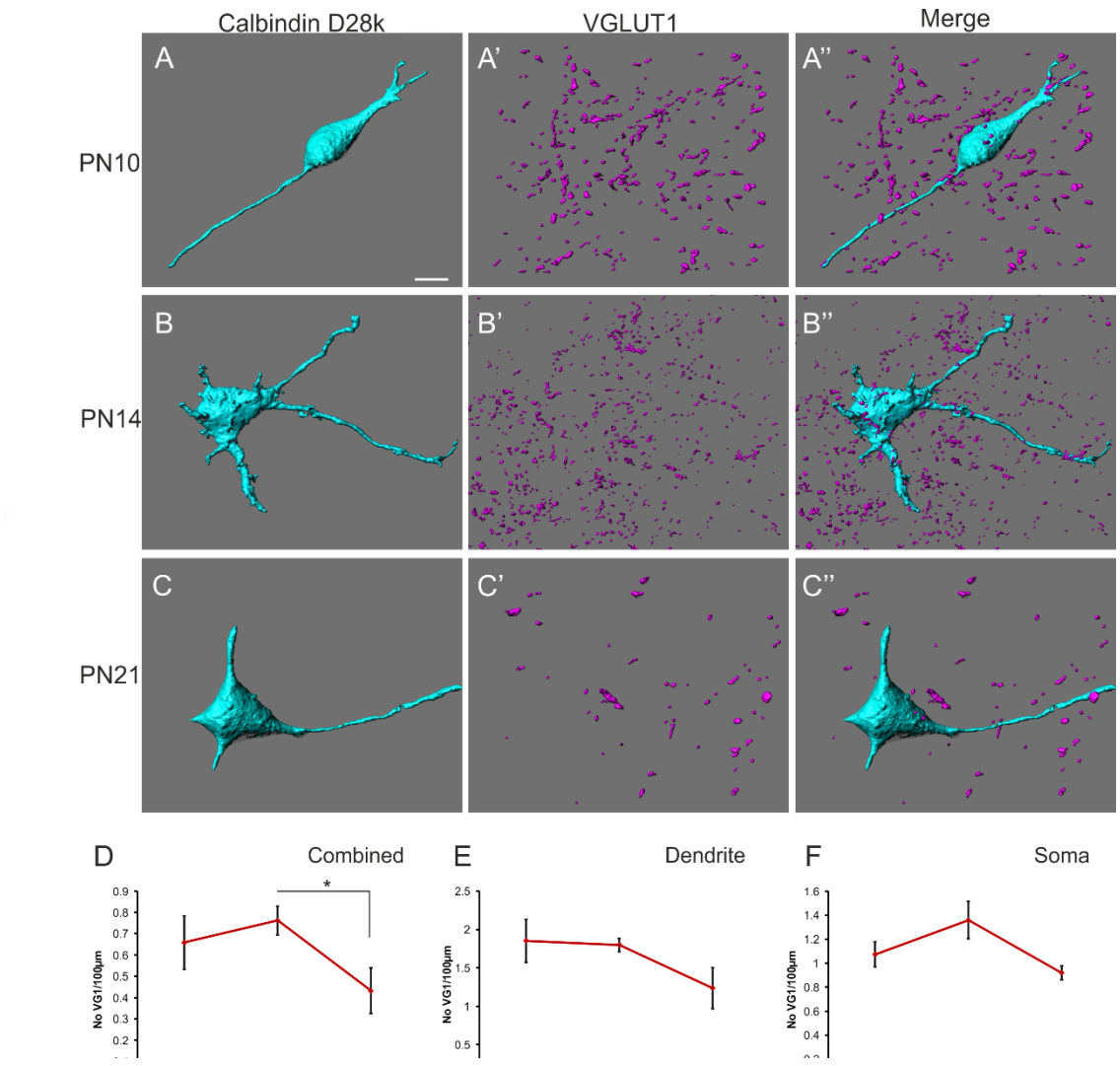

Figure 28. PN development of Ia afferent boutons apposing Renshaw cells in PN5 TX rats.
(A-C'') Imaris 3D reconstruction of Renshaw cells. Note extensive dendrite labelling allowing 3D quantification of contacts on, dendrites and soma during PN development in the absence of descending systems (D-F). Scale bar= 10µm.

5.5.3 PN development of presynaptic ‘P bouton’ innervation of Ia afferents contacting αMNs in the absence of descending input.

There was a significant effect of age on number of P boutons per 10μm Ia bouton surface area (p= .026, Fig 29D). This was largely due to a significant increase in P boutons between PN10 (0.36 ± 0.032) and PN 21 (0.75 ± 0.12, p= .022). The increase in coverage of P boutons between PN10 and 21 was likely due to significant increase in the number of Ia terminals with clusters of greater than 3 P boutons (PN10= 8.835 ± 3.46%, PN21= 27.61 ± 4.22%, p= .026, Fig 29F) rather than a decrease in the proportion which were devoid of P boutons (PN10= 45.81 ± 5.14, PN21 23.61 ± 6.91%, p=.061, Fig 29E).

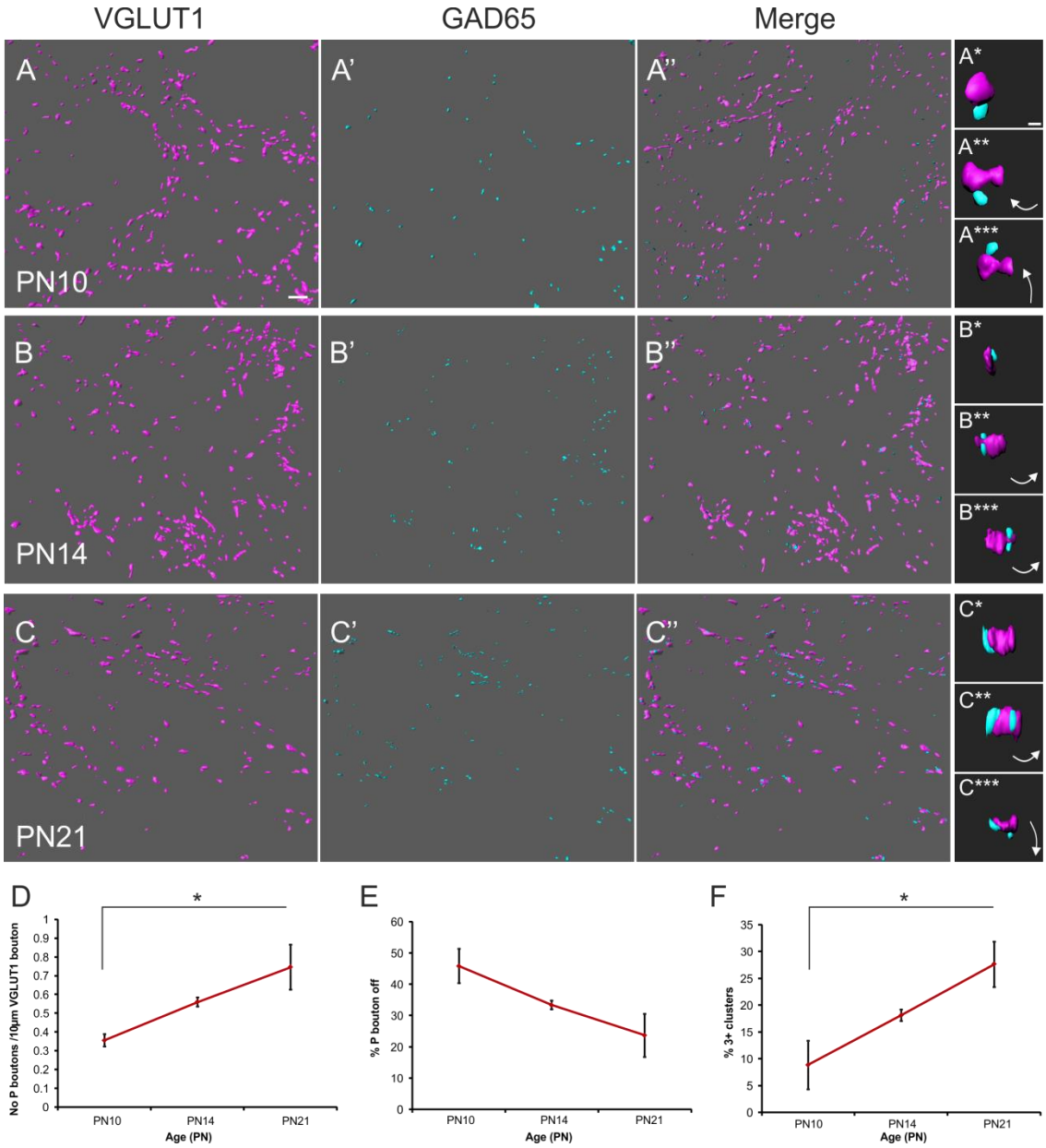

Figure 29. PN development of GAD65^{+} 'P boutons' apposing motoneuron contacting Ia afferents in PN5 TX rats. (A-C'') 3D reconstruction of P boutons apposing Ia boutons. Only boutons apposing MNs were quantified. These are representative 60x images (100µm by 100µm) taken from the motor pools. (A*-C***) High magnification images of P boutons contacting motoneuronal Ia afferent terminals. (D-F) Graphs illustrating developmental profile of P boutons innervation of Ia afferent terminals. (E) Percentage Ia terminals devoid of P boutons. (F) Percentage Ia terminals contacted by clusters of 3^{+} P boutons. Scale bars = 5µm (A-C'') and 3µm (A*-C***)

5.5.4 Summary of Ia afferent innervation of the lumbar spinal cord in the absence of descending input.

There was only a significant decrease in VGLUT1⁺ puncta in the dorsal horn; otherwise it remained stable throughout PN development in other regions including apposing MNs. This was not the case for Renshaw cells however which observed a significant retraction of VGLUT1⁺ terminals between PN 14 and 21. While there seemed to be little developmental effect on afferent terminals in the lumbar cord following PN5 TX, P boutons apposing motoneuron VGLUT1 terminals were significantly increased.

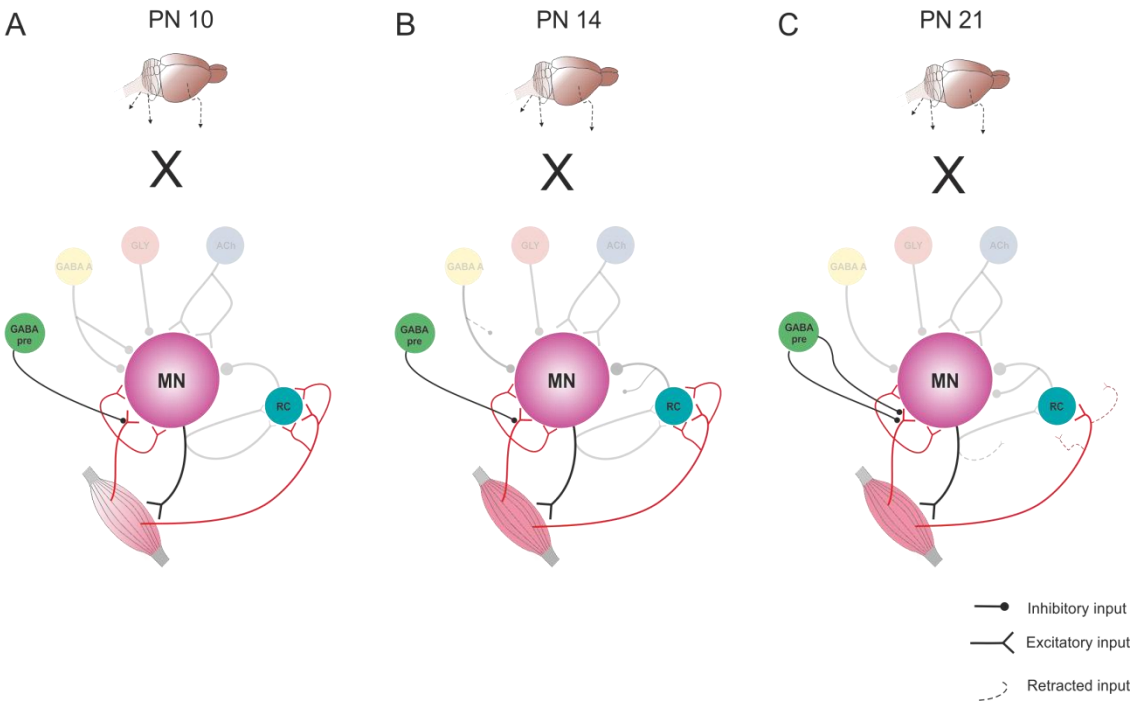

Figure 30. Schematic summarising the PN development of afferent input to the lumbar spinal circuitry in the absence of descending systems.

5.5.5 PN development of cholinergic input to MNs in the absence of descending input.

Development had no effect on the proportion of VAChT⁺ boutons apposing MNs in PN5 TX rats (p= .095, Fig 31 A-D).

5.5.6 PN development of inhibitory inputs to αMNs in the absence of descending input.

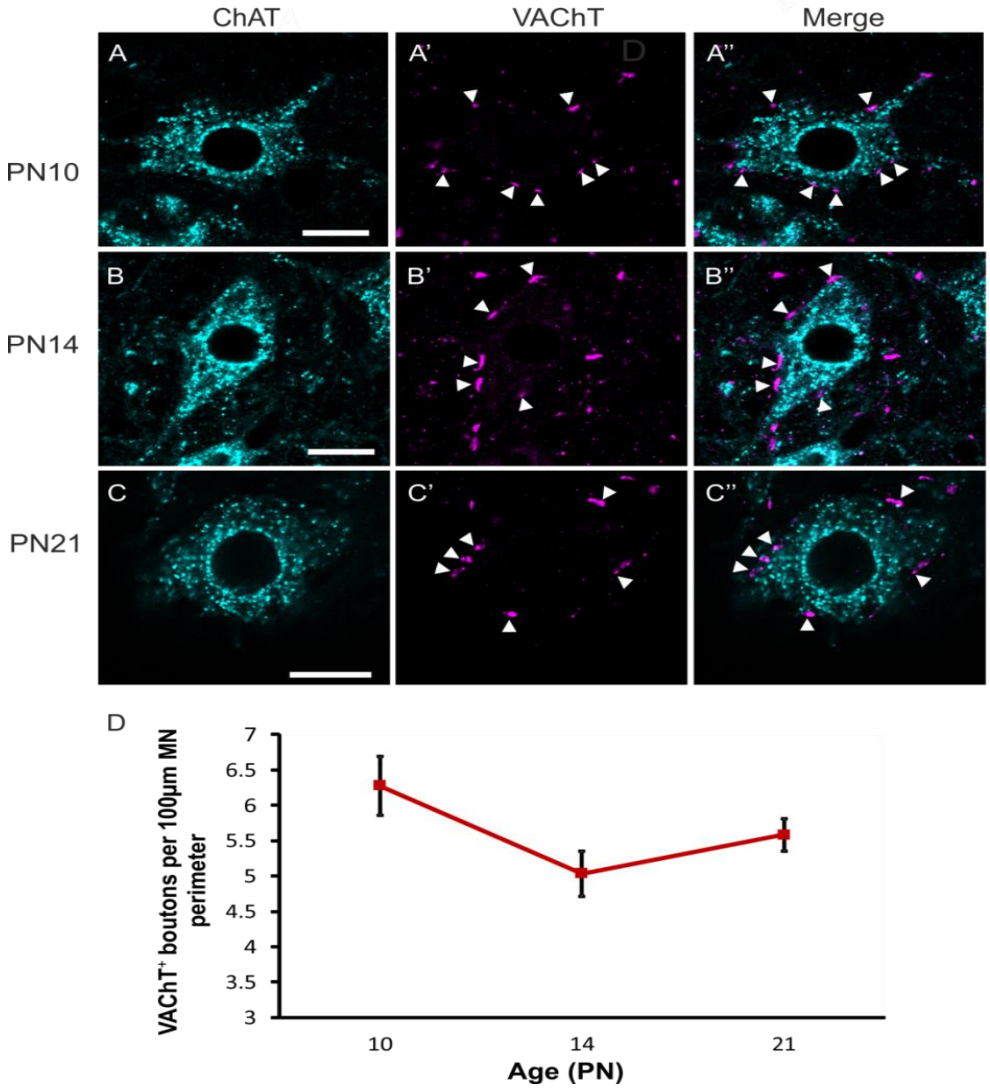

Figure 31. PN development of VAChT+ boutons apposing MNs of PN5 TX rats. (A-C'') Representative images displaying VAChT⁺ boutons apposing MNs. (D) Graph illustrating developmental profile of VAChT⁺ boutons on MNs in PN5 TX rats. Scale bars = 20µm

There was a significant effect of age on PN development of GAD67⁺ terminals after PN5 TX (p= .016) due to a reduction between PN10 (15.82 ± 0.67) and PN21 (11.10 ± 0.70, p= .018, Fig 32D). There was no effect of age PN on GLYT2⁺ terminals (p= .638, Fig 32E).

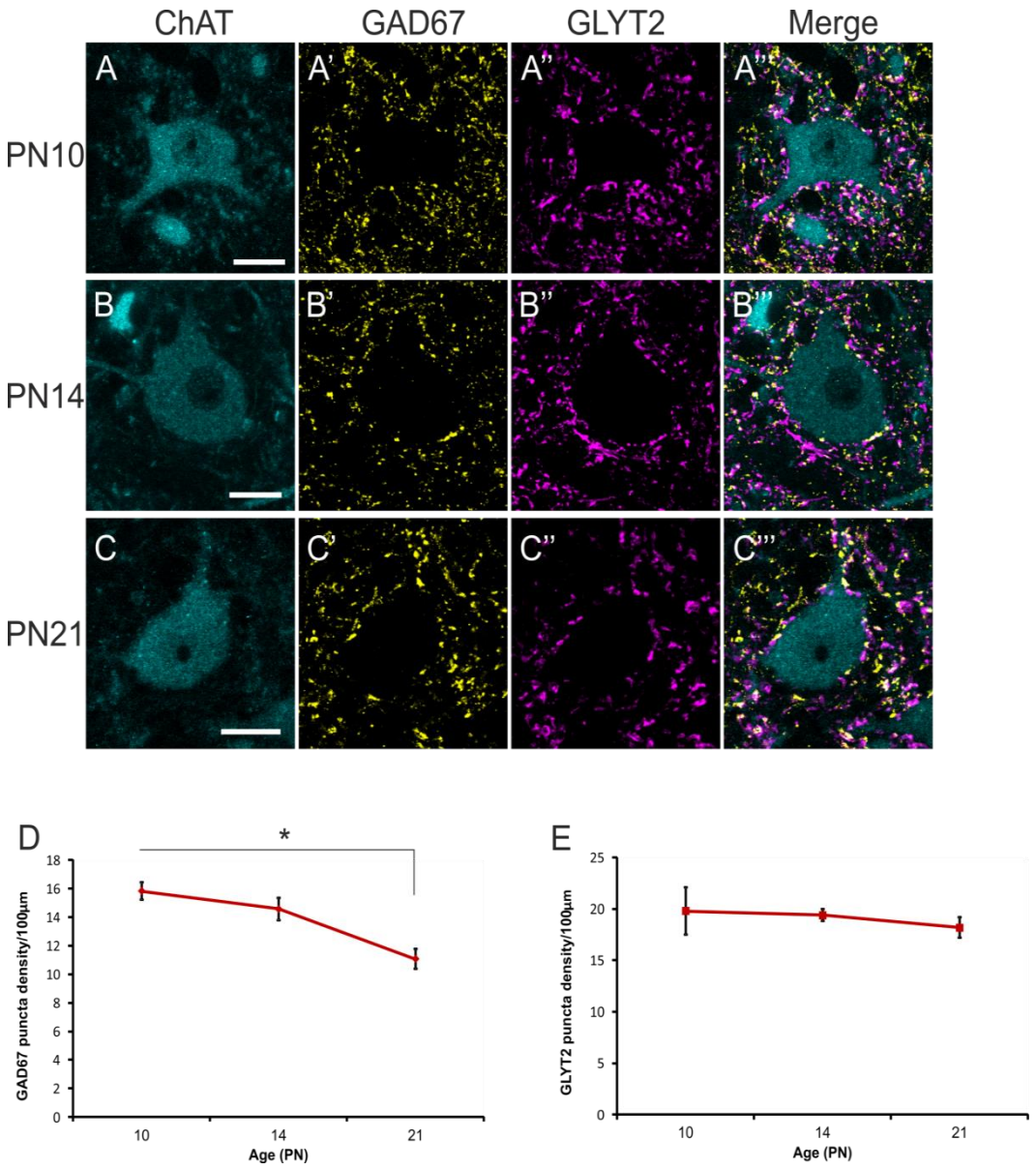

Figure 32. PN development of inhibitory boutons apposing MNs of PN5 TX rats. (A-C''') Representative images displaying inhibitory (GAD67 and GLYT2⁺) boutons apposing MNs. (D-E) Graph illustrating developmental profile of inhibitory boutons on MNs in PN5 TX rats. Scale bars = 20µm

5.5.7 Calbindin terminations on MNs are significantly increased with postnatal development in the absence of descending input.

PN development had a significant effect on calbindin D28K⁺ variscosities apposing MNs following a PN5 TX (p=.001). In this case, at PN21 (3.71 ± 0.050) there were significantly greater amounts of boutons than at PN14 (2.24 ± 0.23, p= .001) and PN10 (2.00 ± 0.16, p= .001, Fig 33A-D)

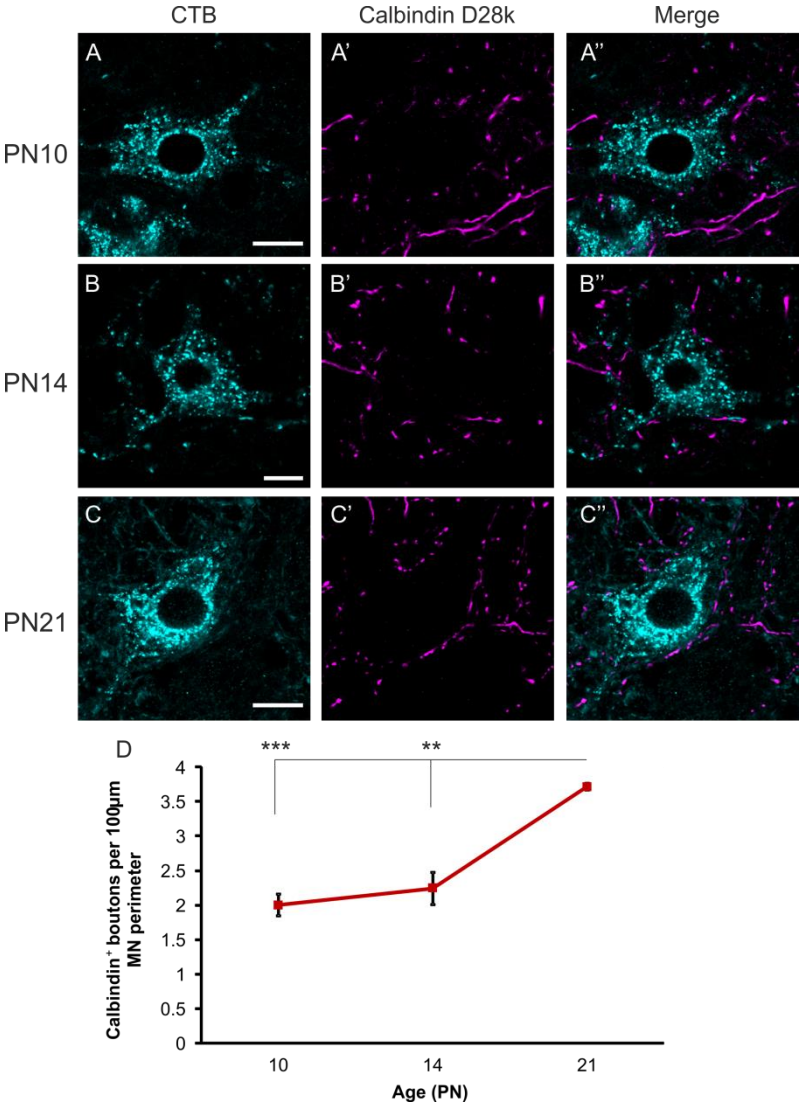

Figure 33. PN development of Calbindin+ boutons apposing MNs of PN5 TX rats.
(A-C'') Representative images displaying Calbindin⁺ boutons apposing MNs. (D) Graph illustrating developmental profile of Calbindin⁺ boutons on MNs for PN5 TX rats. Scale bars= 20µm

5.5.8 Motor axon collateral input to RCs significantly reduced following PN5 TX

There was a significant effect of age on PN development of motor axon collaterals on Renshaw cells (p= .005). At PN21 (2. 78 ± 0.18) motor axon collateral input was significantly reduced compared to both PN10 (3.94 ± 0.33, p= .027) and PN14 (4.45 ± 0.12, p= .005, Fig 34A-D).

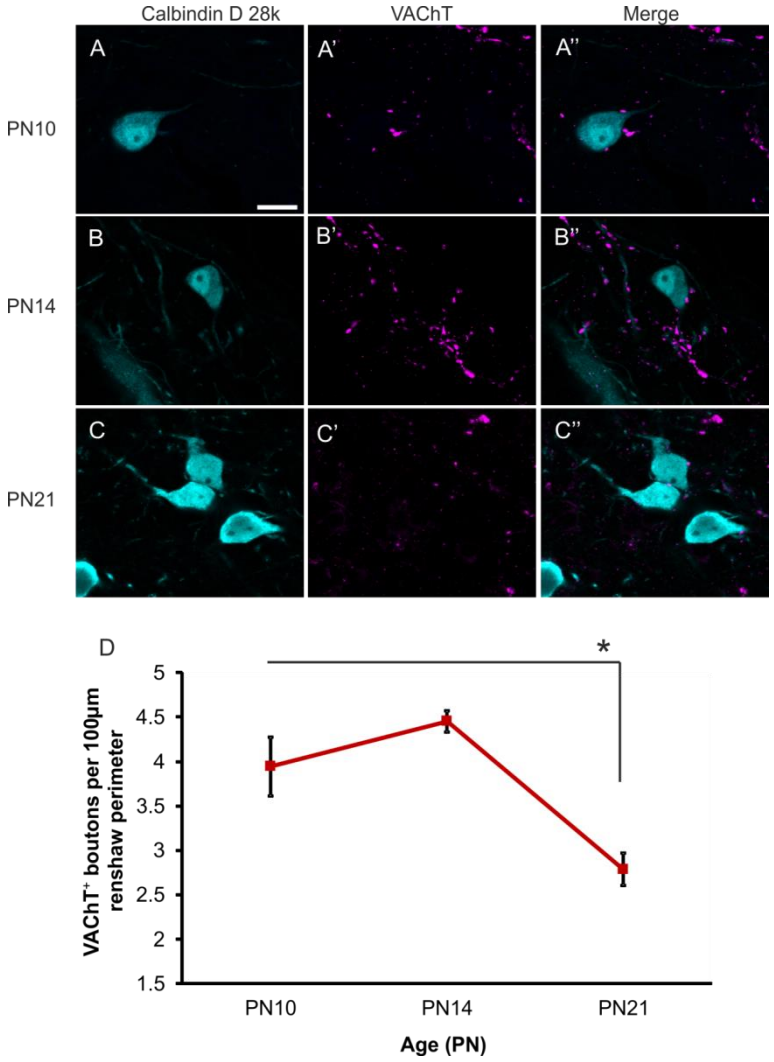

Figure 34. PN development of VACHT+ boutons apposing Renshaw cells of PN5 TX rats. (A-C'') Representative images displaying VACHT^{+} boutons apposing Renshaw cells. (D) Graph illustrating developmental profile of VAChT^{+} boutons on MNs for PN5 TX rats. Scale bar= 20µm

5.6 Discussion

The results in this chapter show that following a PN5 transection of the thoracic spinal cord, afferent innervation of the lumbar spinal cord remains relatively stable throughout PN development. This is true for dorsal, intermediate, Int X and direct Ia afferent input to motoneurons. For Renshaw cells, however, there does seem to be a retraction of Ia afferents taking place between PN14 and 21. P boutons innervating motoneuronal Ia terminals were found to increase with development despite Ia afferent input being unaltered. Apart from calbindin^{+} variscosities and GAD67^{+} terminals, there was no effect of development in the absence of descending systems on the development of other excitatory and inhibitory inputs to MNs. Interestingly; there was an effect of PN5 TX on PN development on motor axon collateral input to RCs, with a significant reduction seen between PN14 and PN21.

5.6.1 Afferent innervation of the lumbar cord remains stable following PN5 TX

Following lesions to adult spinal and supraspinal regions, sprouting of afferent fibres is observed, often leading to spasticity and hyperexcitability of spinal circuitry (Nelson and Mendell, 1979; Ondarza et al., 2003; Krenz and Weaver, 1998; Tan et al., 2012). The results in the present study suggest that afferent innervation of all regions of the spinal grey and motoneuron is unaltered during development following PN5 TX. However, studies have shown that afferent innervation of the spinal cord is high early in development and undergoes a period of retraction and refinement postnatally (Chakrabarty and Martin, 2011a; Gibson and Clowry, 1999). This would suggest that although we showed no

developmental change in afferent innervation of the lumbar cord, it is likely that retraction is abolished; but this comparison will be made in the next chapter.

If developmental retraction of afferents was abolished by PN5 TX for the areas discussed above, it was not true of Renshaw cells which did observe a significant retraction of inputs from Ia afferents between PN14 and PN21. In normal development this is expected, with motor axon collateral input becoming dominant at approximately this time point (Mentis et al., 2006a). Interestingly, we found that motor axon collateral input to Renshaw cells was significantly reduced at PN14. Siembab et al. (2015) found that genetically increasing and decreasing afferent innervation of the ventral horn resulted in decreased and increased motor axon collaterals on RCs respectively and suggested proliferation of motor axon collaterals is dependent on Ia afferent retraction. Our results support this hypothesis as we saw no retraction of afferents from the ventral horn and a significant decrease in VACHT+ boutons directly apposing RCs.

5.6.2 GABApre (P bouton) input to Ia afferents is significantly increased with development following PN5 TX

Afferent activity can be modulated presynaptically by descending and afferent drive to GABApre interneurones residing in the medial deep dorsal and intermeidiate gray (Hughes et al., 2005; Jankowska et al., 1981). Inputs from GABApre neurons have been shown to be disrupted following adult spinal cord transections and this is suggested to contribute to spasticity and hypereflexia (Kapitza et al., 2012). One would expect that with increasing innervation of GABApre neurons with normal development of descending systems, their projections (P boutons) to Ia afferents would also increase. Naturally then,

removal of descending input should significantly disrupt this process. Our results are contrary to this hypothesis as we saw significant developmental increases in P boutons contacting motoneuronal Ia afferents, despite the PN5 transection. This is not entirely surprising as GABApre neurons are also innervated by all afferents (Enríquez et al., 1996; Quevedo et al., 1993; Rudomin et al., 1983; Rudomin and Schmidt, 1999). Therefore, these interneurons still receive input from the periphery during development, which may be responsible for the increase of P boutons on afferent terminals. Our results therefore suggest that GABApre input to Ia afferent fibres is not solely dependent on input from descending systems.

Russ et al. (2013) showed that a PN12 ischemic insult to the right cortex resulted in increased GAD65^{+} staining intensity in the cervical spinal cord, relating this to increased P bouton density and therefore presynaptic inhibition of afferents. The reasons for discrepancies between the present study and that of Russ et al. (2013) are several fold. Russ et al. (2013) used a perinatal stroke model in mice, with lesions being induced at PN12. Hence, the type and level of insult are different as well as the species and age. Whilst the species type might not be expected to have such an impact on results, the type of injury and age definitely can. Their model only affected the cortex and whilst this region is important in mediating presynaptic inhibition, GABApre interneurons are also densely innervated by subcortical structures (Perreault et al., 1999; Davies and Edgley, 1994). The age of the injury is also likely to be responsible for the disparity as at PN12 the CST has already traversed the spinal cord and made terminations in the spinal grey of the cervical cord (Joosten et al., 1989). However at PN5, when the transection was made in the present study, the CST had not reached the lumbar cord.

It would be interesting to examine how P bouton innervation of afferent terminals is affected following an incomplete peripheral lesion, which would have great functional significance related to dorsal root rhizomotomy (DRR) procedures performed by the National Health Service to relieve spasticity in patients suffering with cerebral palsy (Ailon et al., 2015). Indeed, neonatal peripheral lesions have demonstrated sprouting of CST axons which may take up synaptic space normally taken up by afferents (Clowry et al., 2006). Although sprouting of CST following DRR may contribute to some recovery of function, we do not fully understand the implications of this procedure and it is not always successful. Moreover, we know that afferents can be crucial to recovery of function in CP. Daily treadmill training in children with CP significantly improves their walking ability, and this has been shown to occur in concert with increased modulation of the H reflex (Hodapp et al., 2009; Bilde et al., 2011).

5.6.2.1 No effect of development on cholinergic and glycinergic inputs to MNs, however GAD67^{+} inputs decreased.

As stated in the hypotheses, we expected that following removal of descending input to premotor interneurons in the lumbar spinal cord, there would be a reduction in the proportion of all inputs to motoneurons from excitatory and inhibitory sources. We did not observe any effect of development following PN5 TX in input to MNs from cholinergic or glycinergic premotor interneurons. If retraction of Ia afferents is disrupted, it is possible that they might take up synaptic space on premotor interneurons that is unoccupied by descending systems, leading to a maintenance of their projections to MNs. Also, specificity of synaptic contacts is thought to be dependent on signalling mechanisms at

the target rather than the source and since MN quantity or size does not change following neonatal injury there is no reason for premotor projections to be altered (Ichiyama et al., 2011; Stepanyants et al., 2004; Betley et al., 2009). We observed a significant reduction in GAD67^{+} boutons between PN10 and 21 whilst GLYT2 was unaffected. In adult rats, Tillakaratne et al. (2000) showed increased expression of GAD67 in the dorsal horn following TX, however this does not mean there would be increased innervation of MNs by premotor interneurons. Again in adult animals, De Leon et al (1999) showed that cats which were sedentary following transection could not walk unless the glycine antagonist strychnine was administered, suggesting increased glycinergic inhibition of motor output. This was in contrast to Kitzman (2007) who found no difference in the number of GLYT2^{+} boutons apposing MNs following sacral spinal cord transection in rats.

5.6.2.2 PN development of the recurrent inhibition circuit

Calbindin^{+} inputs to MNs which are presumably from Renshaw cells, but could also be from other inhibitory premotor interneurons are increased between PN14 and PN21. VAChT^{+} boutons directly apposing Renshaw cells are significantly decreased between PN14 and 21. Mentis et al. (2006a) showed that motor axon collateral input to RCs increased with development suggesting that following a PN5 TX, normal development of these input is disrupted. Calbindin+ inputs to MNs have not been previously assessed, possibly due to the lack of certainty in their origins. Observations in this study suggest that Calbindin D28k, is expressed extensively along the dendritic processes of RCs in the ventral horn and only the cell bodies Calbindin D28k expressing

interneurons which are situated more superficially in the slice. It is likely therefore, that the terminals seen in direct apposition to MNs are from RCs.

5.6.3 Summary and conclusions

Afferent innervation of the spinal cord seems to be relatively stable during development in the absence of descending inputs. This is surprising considering that descending input has been suggested to be responsible for PN retraction of afferents (Gibson and Clowry, 1999; Chakrabarty and Martin, 2011b). It is possible that the majority of change to input occurred between PN5 and 10 when our assessments started. Most postsynaptic inputs to MNs were unaffected; however the absence of descending systems significantly disrupted the development of the RC recurrent inhibition circuit. If retraction of Ia afferents during development is normal, then it can be said that a PN5 transection significantly disrupts this process. This would be in agreement with electron microscopy studies by Conradi and Ronnevi (1975) showing that F,S and C boutons were all retracted with postnatal development of kittens.

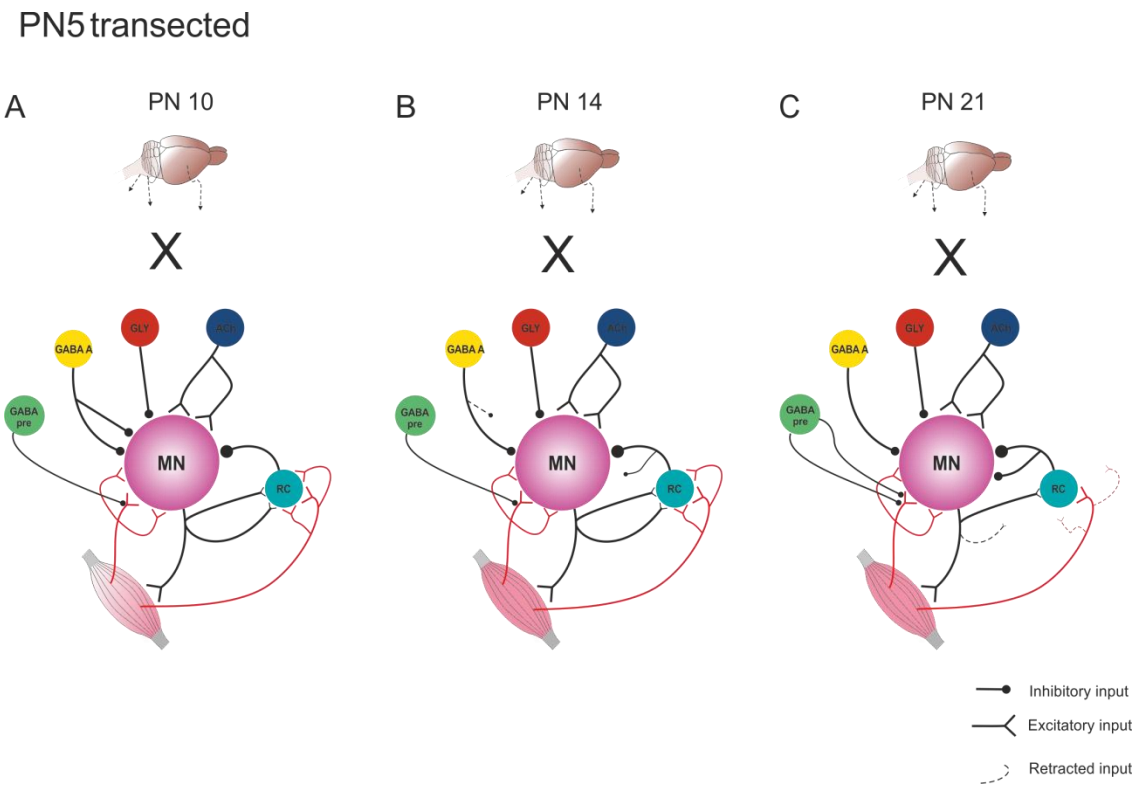

Figure 35. Schematic summary illustrating the PN development of spinal circuits in the absence of descending systems

5.6.4 Methodological considerations

Although rats are believed to reach motor maturity between PN14 and 21, it is highly likely that further PN development of the circuitry occurs beyond this time point. Also, 15 (PN21) days post injury is not considered a chronic model in adult rats, and it would be interesting to assess whether there are any alterations to these data in more chronic models of neonatal transection.

Chapter 6. Comparing development of lumbar spinal circuits in the presence and absence of descending systems.

6.1 Introduction

Normal development of the central nervous system is an activity dependent process. Although the CNS is highly plastic throughout life, the organisation of its circuitry is most sensitive to activity during early postnatal epochs known as critical periods. Alterations in normal patterns of activity during this time can result in deficits in function which usually persist the life span of the animal. This phenomenon was exquisitely demonstrated by the monocular deprivation studies by performed by Wiesel and Hubel (1963). In these studies, monocular eye closures during early postnatal development of kittens resulted in permanent reductions in the number of cells in the striate cortex which could be driven by the previously closed eye (Hubel and Wiesel, 1970). Since then, countless studies have illustrated this phenomenon in distinct regions of both the brain and spinal cord. Hind limb unloading in rats revealed a critical period for locomotor development to be between PN8 and 13, with just 1 day of air suspension during this epoch resulting in significant and permanent dysfunction of gait patterns (Walton et al., 1992). Early postnatal transection of the spinal cord also results in profound locomotor deficits (Norreel et al., 2003). Interestingly, although behavioural deficits are unavoidable with an injury at any age, animals injured early postnatally recover significantly more function than those injured during late development and adulthood (Weber and Stelzner, 1977; Saunders et al., 1998). This recovery is not due to a reconnection between brain and spinal cord but is thought to be mitigated by increased plasticity of the CNS and the absence of descending systems, allowing spinal locomotor networks to reorganise in a manner which permits recovery of walking (Tillakaratne et al., 2010). Afferent input becomes the only activity

available to drive and modulate motor activity in the spinal cord and is crucial to functional recovery. This is confirmed by studies demonstrating that following severe spinal cord injury, recovery is not possible in the absence of proprioceptive afferents (Takeoka et al., 2014) and it is enhanced by task specific activation of afferents through locomotor training (Ichiyama et al., 2011; Petruska et al., 2007; De Leon et al., 1999b; De Leon et al., 1999a). As well as afferents being crucial to recovery of motor function, it is possible that their activity drives a downstream alteration in the spinal circuitry which enables it to function with relative effectiveness in the absence of descending control. Indeed, Ichiyama et al. (2011) found that the ratio of inhibitory to excitatory inputs to MNs was increased following injury, but normalised with training. It is generally appreciated that afferent input to the locomotor circuitry is increased following any spinal cord injury as evidenced by cases of spasticity in humans and animals alike (Bennett et al., 2004; Bennett et al., 1999; Nielsen et al., 2007; Adams and Hicks, 2005). It is not known, however, to what extent this occurs following a neonatal injury, or if it is likely to be greater than after adult injuries considering the absence of competition from descending systems (Chakrabarty and Martin, 2011b; Gibson et al., 2000; Clowry et al., 2004a; Gibson et al., 1999). Furthermore, chapter 4 demonstrates that developmental retraction of afferents is not specific to motoneurons and therefore an injury induced proliferation of Ia inputs may occur on interneuronal populations. If there is a global increase in afferent input, it could be true of premotor interneurons which may be afforded with abundant synaptic space due to a lack of descending coverage. This suggests that inputs to motoneurones from premotor interneurons may be altered due to the lack of descending and affluence of afferent input. This could also be true of inputs to Ia afferents

themselves, which are modulated (presynaptic inhibition-PAD) by GABAergic axo-axonic terminals from premotor interneurones (Jankowska et al., 1981; Dudel and Kuffler, 1961; Eccles et al., 1961; Eccles et al., 1963). Adult supraspinal lesions result in spasticity that is thought to be due to increased afferent innervation in conjunction with reduced presynaptic inhibition (Kapitza et al., 2012; Bennett et al., 2004; Bennett et al., 1999; Nielsen et al., 2007). However, it is not known if this is true of neonatal injuries or if it is due to increased Ia innervation, reduced presynaptic inhibition, reduced recurrent inhibition or a combination of all 3.

In adult mammals, neural control of movement relies on interactions between the brain and spinal cord and when this is disrupted paralysis often follows. These interactions are not always present however, as connections between spinal and supraspinal regions occurs postnatally (Joosten et al., 1989; Martin et al., 1981; Lakke, 2012). It is likely then, that normal PN development of the spinal motor circuitry is dependent upon arrival of descending systems and removal of these systems at an early age will significantly alter mature organisation.

6.2 Aims

In general, the aim was to compare the development of spinal motor circuits in the presence and absence of descending systems. Specifically, we aimed to assess how PN development of Ia afferent innervation of the lumbar spinal cord is altered following PN5 transection. We aimed to identify these changes in dorsal, intermediate and ventral laminae, with a focus on MNs and Renshaw cells in the ventral horn. In addition we wanted to identify if modulation of Ia afferent firing would be altered by a lack of descending input. Finally, we aimed

to assess the transection induced impact on premotor interneuron inputs to MNs.

6.3 Hypotheses

It is expected that in the complete absence of competition from descending systems, retraction of Ia afferents will be attenuated, and therefore innervation of the lumbar cord will be greater than intact animals at the same time points of development. This loss of descending and increased afferent input will likely induce significant reorganisation of premotor interneuron input to MNs. It is expected that inputs will be increased compared to intact animals as normal development usually observes synaptic stripping (Conradi and Ronnevi, 1975).

6.4 Methods

Methods for immunohistochemistry were the same as described the general methods and previous experimental chapters. Comprehensive description of H-reflex methodology are described in the general methods.

6.4.1 Statistics

Statistics are described in the general methods section-chapter 2.

6.5 Results

6.5.1 P5 transection abolishes postnatal retraction of Ia afferents in the lumbar spinal cord

6.5.1.1 Lamina distribution of VGLUT1^{+} puncta

After assessing the development of laminae distribution of VGLUT1 in the intact cord, we wanted to determine if there was an effect of transection on this developmental trend. Analysis of the dorsal ROI revealed that the developmental effect in the intact group (.008) persisted in the tx group (.041), meaning there was no significant effect on the influence of age when comparing individual time points. There was no significant difference between intact and TX groups at PN10 (Intact= 63.64 ± 1.64 %, TX= 67.33 ± 6.63 %, p=.512) however at both PN14 (Intact= 39.10 ± 3.75 % , TX= 51.23 ± 2.10 %, p=.030) and PN21 (Intact= 42.32 ± 5.27 %, TX= 60.88 ± 1.46 %, p=.030, Fig 36F) transected groups were significantly greater. For the intermediate region (Fig 36G) in the spinal grey of intact animals, there was a significant effect of age on PN development of VGLUT1 bouton density (p=.020), however PN5 TX abolished this normal developmental trend (p=.356). Again, there was no difference between groups at PN10 (intact= 14.31 ± 3.32 %, TX= 7.88 ± 5.02 %, p=.645). Despite the TX group at PN14 seeming higher, statistics revealed that there was no difference between groups (intact= 3.78 ± 0.70 %, TX= 9.67 ± 1.26 %, p=.060). At PN21, however, TX resulted in a significantly greater

density of VGLUT1 puncta (intact= 5.59 ± 0.65 %, TX= 14.33 ± 0.34 %, p=.010). Finally, for region of interest ‘intermediate X’ (Fig 36H), which is thought to contain the premotor partition cells important in modulation of locomotion, there was no effect of age for either intact (p=.60) or TX (p=.196). Interestingly, although the same trend seemed to be followed in both groups, at each age the TX resulted in significantly higher densities of VGLUT1 puncta density (PN10 intact= 2.59 ± 0.48, TX= 6.23 ± 0.43, p=.041, PN14 intact= 4.35 ± 1.33, TX= 8.34 ± 0.64, p=.016 and PN21 intact= 3.42 ± 1.46, TX= 6.60 ± 0.88, p=.045, Fig 36 G).

6.5.1.2 The effect of transection on VGLUT1⁺ boutons in close apposition to MNs

Analysis of VGLUT1⁺ boutons apposing MNs revealed that during normal development there was a significant effect of age (p=.014) which was completely abolished by the PN5 TX (p=.514). At PN10 (Fig 37A-B’’) VGLUT1⁺ puncta densities were equivalent (Intact= 4.508 ± 0.25, TX= 4.53 ± 0.44, p= .966), whereas the TX groups were greater at both PN14 (Intact= 3.46 ± 0.28, TX= 4.99 ± 0.44, p= .009, Fig 37C-D’’) and PN21 (Intact= 3.14 ± 0.28, TX= 4.18 ± 0.36, p= .034, Fig 37E-F’’)

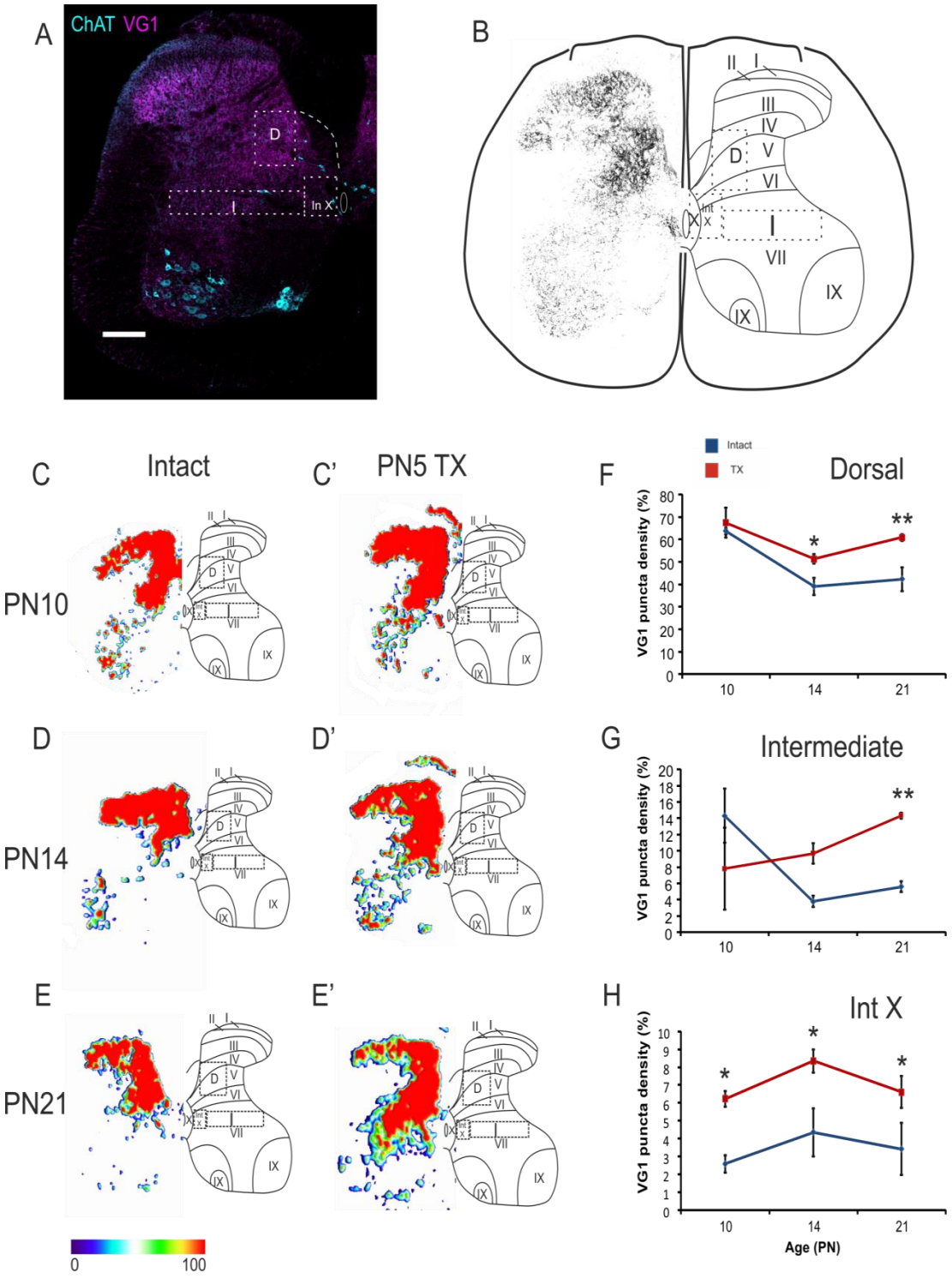

Figure 36. PN development of VGLUT1 laminae distribution in PN5 transected and intact rats. (A-B) Examples of regions of interest examined in dorsal, intermediate and Intermediate X ROI. (C-E') Heat maps from representative sections of PN10,14 and intact and TX rats. (F-H) Graphs showing development profiles of intact and TX rats. Scale bar; 0-100 represent the highest and lowest densities after thresholding.

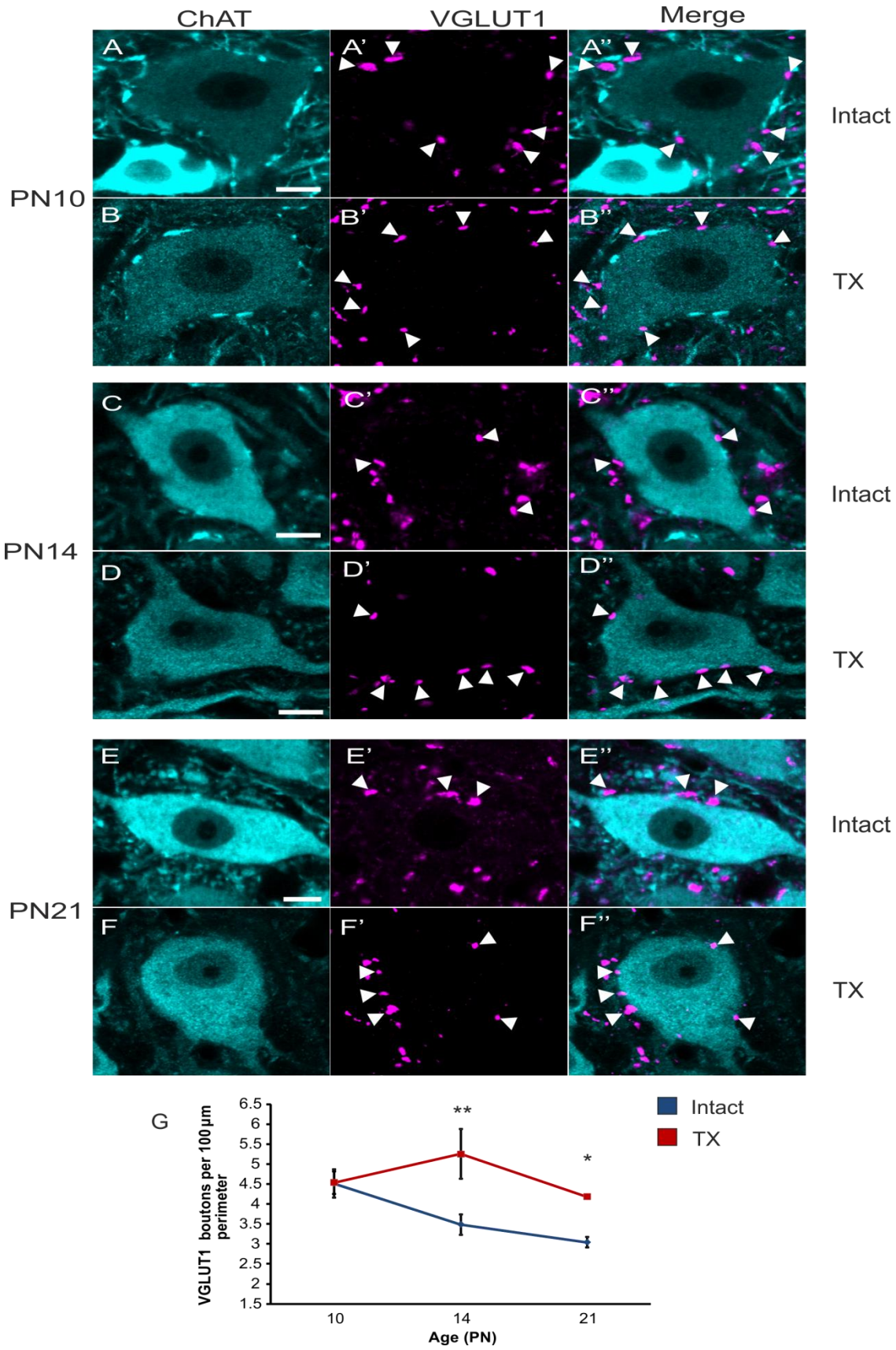

Figure 37. PN development of VGLUT1+ boutons apposing MNs in TX and Intact rats. (A-F'') Representative images displaying VGLUT1^{+} boutons apposing MNs. (G) Graph illustrating developmental profile of Ia afferent innervation of MNs in intact and TX rats.Scale bars= 10µM

6.5.2 PN5 transection induces transient increase in Ia afferent innervation of Renshaw cells during PN development

In Chapter 4, retraction of VGLUT1⁺ puncta from the ventral horn of the spinal cord was shown to be non-specific to MNs, with RCs displaying a similar profile. Above, PN5 TX results in abolition of developmental retraction from MNs (Fig 37). Analysis of VGLUT1⁺ puncta apposing RCs revealed a transient increase at PN14 followed by a return to near intact values at PN21. On RC soma of intact rats, VGLUT1⁺ boutons were significantly reduced with age ($p=.004$) and again, PN5 TX resulted in abolition of this effect ($p= .144$). Although the effect of age was abolished on the soma, there were no significant differences between intact and TX groups at any age (PN10 intact= 0.716 ± 0.02, TX= 0.66 ± 0.12, $p=.62$, PN14 intact= 0.52 ± 0.09, TX= 0.76 ± 0.06, $p=.057$ and PN21 intact= 0.27 ± 0.02, TX= 43 ± 0.11, $p=.213$, Fig 38G). On the proximal dendrites of RCs, again age had a significant effect on VGLUT1⁺ density in intact animals and again PN5 TX acted to abolish this effect. When each time point was considered, intact and TX groups were not statistically different at PN10 (intact= 1.92 ± 0.08, TX= 1.85 ± 0.27, $p=.768$, Fig 38A-B'') and PN21 (intact= 1.15 ± 0.07, TX= 1.23 ± 0.27, $p=.774$) but the TX group had greater VGLUT1⁺ puncta density at PN14 (intact= 1.17 ± 0.14, TX= 1.80 ± 0.08, $p=.027$, Fig 38C-D''). When values for dendrites and soma were combined in order to evaluate the whole cell, there was a significant effect of age onVGLUT1⁺ puncta apposing RCs ($p=0.014$), which was also abolished by PN5 TX ($p=.063$). Similar to dendritic analysis, there was no significant difference between intact and TX groups at PN10 (intact= 1.21 ± 0.07, TX= 1.07 ± 0.10, $p=.454$) or PN21 (intact= 0.63 ± 0.10, TX= 0.92 ± 0.05, $p=.454$) but at PN14 the

transection group had a greater density of VGLUT1^{+} boutons apposing motoneurones (intact= 0.67 ± 0.15, TX= 1.36 ± 0.16, p=.002).

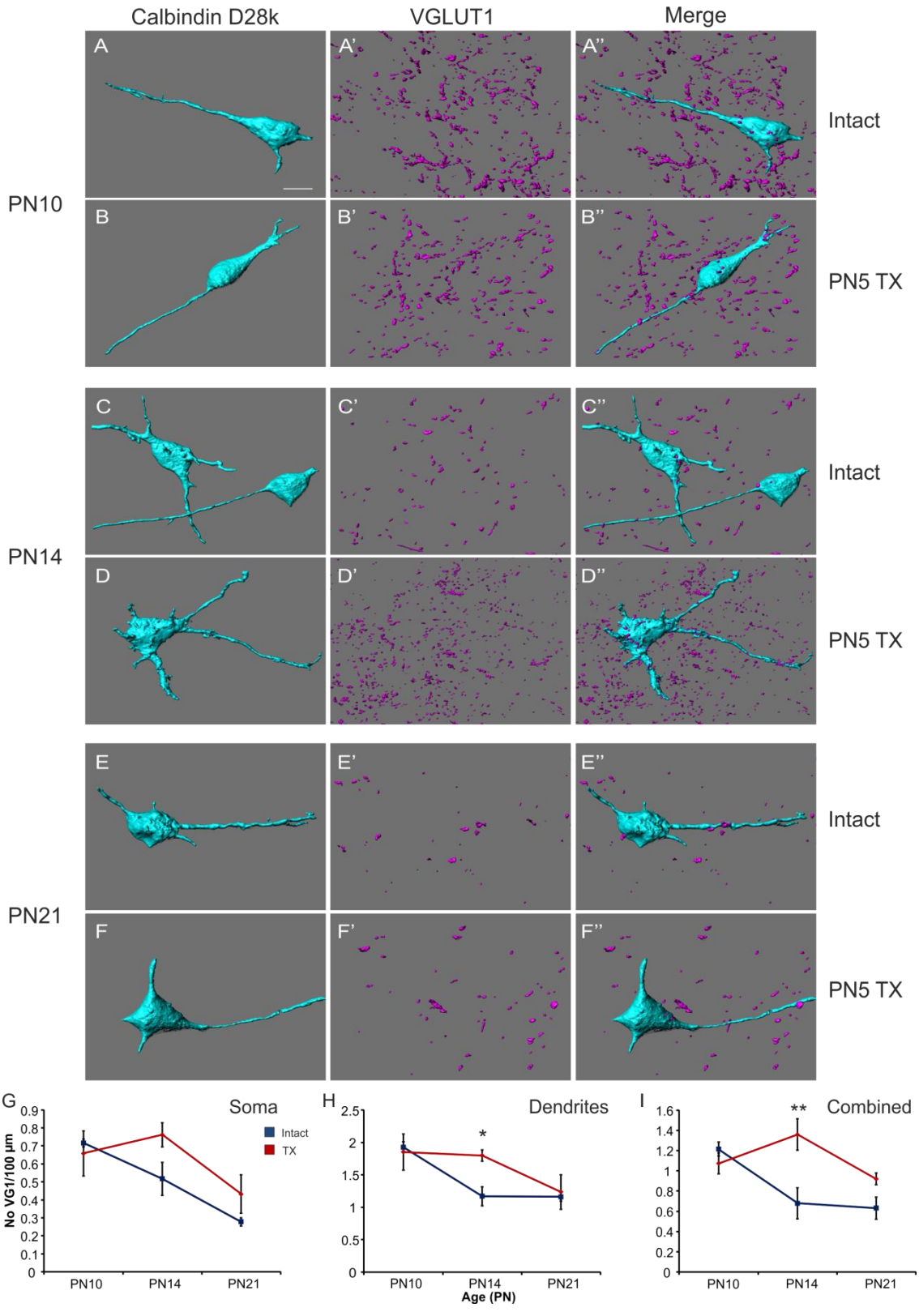

Figure 38. PN development of Ia afferent input to Renshaw cells in the presence and absence of descending systems. (A-F'') Imaris 3D reconstruction of calbindin D28k+ Renshaw cells. Note extensive dendrite labelling allowing 3D quantification of contacts on soma, dendrites and combined during PN development for intact and TX rats (G-I). Scale bar =10 µm

6.5.3 P5 transection attenuates developmental proliferation of presynaptic ‘P boutons’ on Ia afferents

Having shown that a PN5 TX severely disrupts normal Ia afferent retraction from the ventral horn, the present study aimed to assess if presynaptic P boutons, which are important modulators of afferent activity, would also be affected. Analysis of the number of P boutons per 10μm of VGLUT1^{+} bouton in intact animals revealed that there was a significant effect of age ($p>.001$) which persisted in animals which received a PN5 TX. There was no difference in P boutons in close apposition to Ia boutons between intact and PN5 TX animals at PN10 (intact= 0.45 ± 0.048, TX= 0.36 ± 0.032, p= .255, Fig 39A-B”, E); however, it was significantly greater in the TX group at PN14 (intact= 1.04 ± 0.031, TX= 0.56 ± 0.024, $p>.001$) and PN21 (intact= 1.04 ± 0.030, TX= 0.75 ± 0.12, p=.004, Fig 39 C-F’’ and C*-F***). This was due to a significant increase in the proportion of motoneuron VGLUT1^{+} boutons devoid of P boutons at PN14 (intact= 11.79 ± 1.9%, TX= 33.36 ± 1.42%, p=.002) and PN21 (intact= 6.37 ± 0.14%, TX= 23.61 ± 6.91%, p=.008, Fig 39F). Similarly, VGLUT1^{+} boutons apposed by clusters of >3 P boutons were reduced at these ages (PN14- intact= 38.84 ± 3.99%, TX= 18.10 ± 1.03%, $p<.001$, PN21- intact= 44.47 ± 3.45%, TX= 27.61 ± 4.22%, p=.00, Fig 39G).

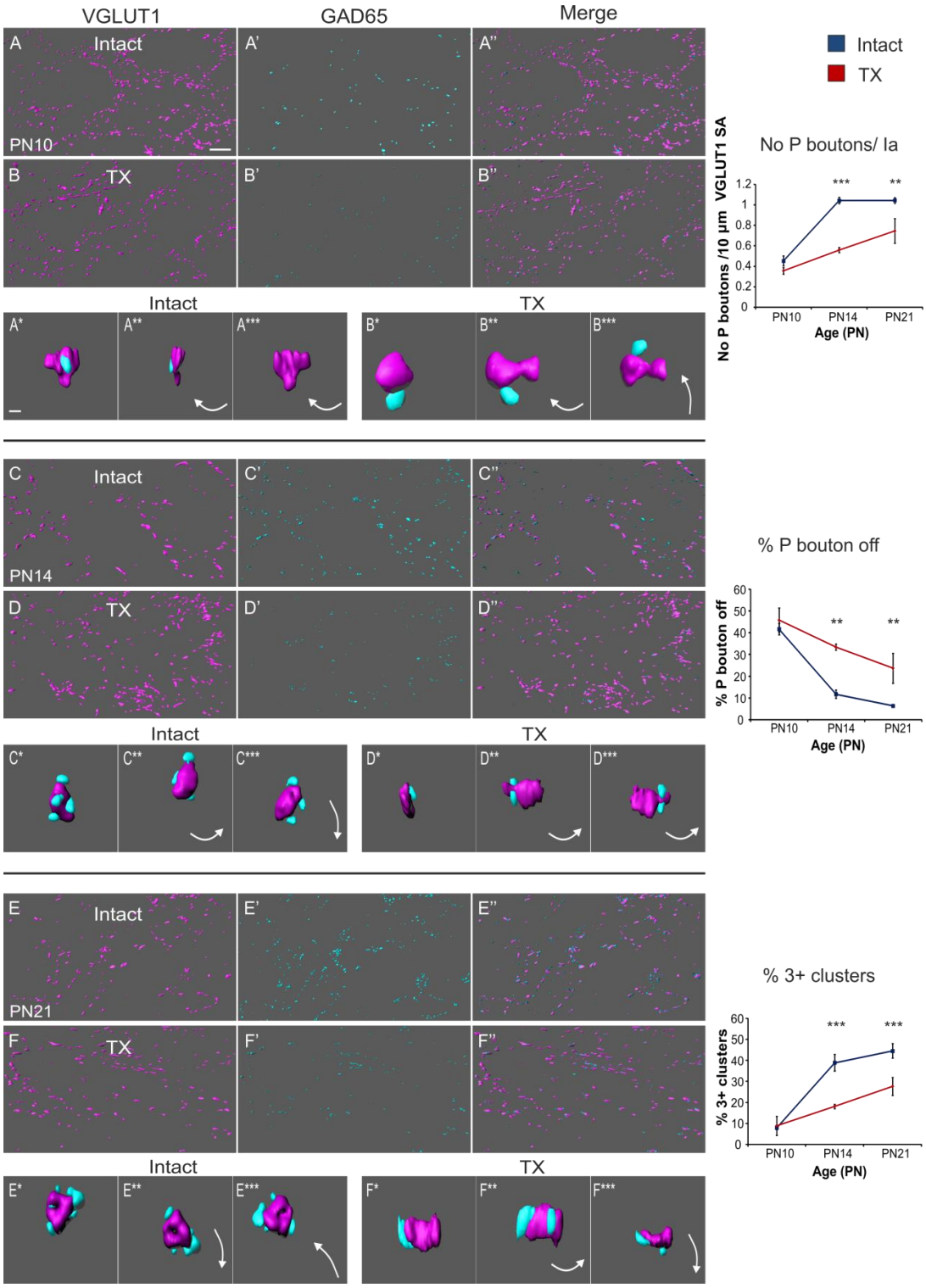

Figure 39. PN5 TX attenuates but does not abolish proliferation of P boutons during PN development.

(A-F***) 3D reconstruction of P boutons apposing Ia terminals. Only terminals apposing MNs were used for P bouton quantification. These are representative 60x images (100µm by 100µm) taken from the motor pools.(A*-F***) High magnification images of P boutons contacting Ia afferent terminals. (G) No. P boutons per 10 µm (H) Percentage Ia terminals devoid of P boutons. (I) Percentage Ia terminals contacted by clusters of 3^{+} P boutons.

6.5.4 Summary: The effect of PN5 transection on PN development of Ia afferent innervation of the lumbar spinal cord

The above results show that PN5 thoracic spinal cord transection results in the disruption of normal PN development of proprioceptive afferents from the lumbar spinal cord. This conclusion holds true for both MNs and RCs. In conjunction with a general increase in proprioceptive afferent innervation of the lumbar cord, P boutons, which have been shown to modulate afferent activity through presynaptic inhibition, are significantly reduced following a PN5 TX. Importantly, P boutons still proliferate on Ia terminals throughout development following PN5 TX (Fig 40, A-C').

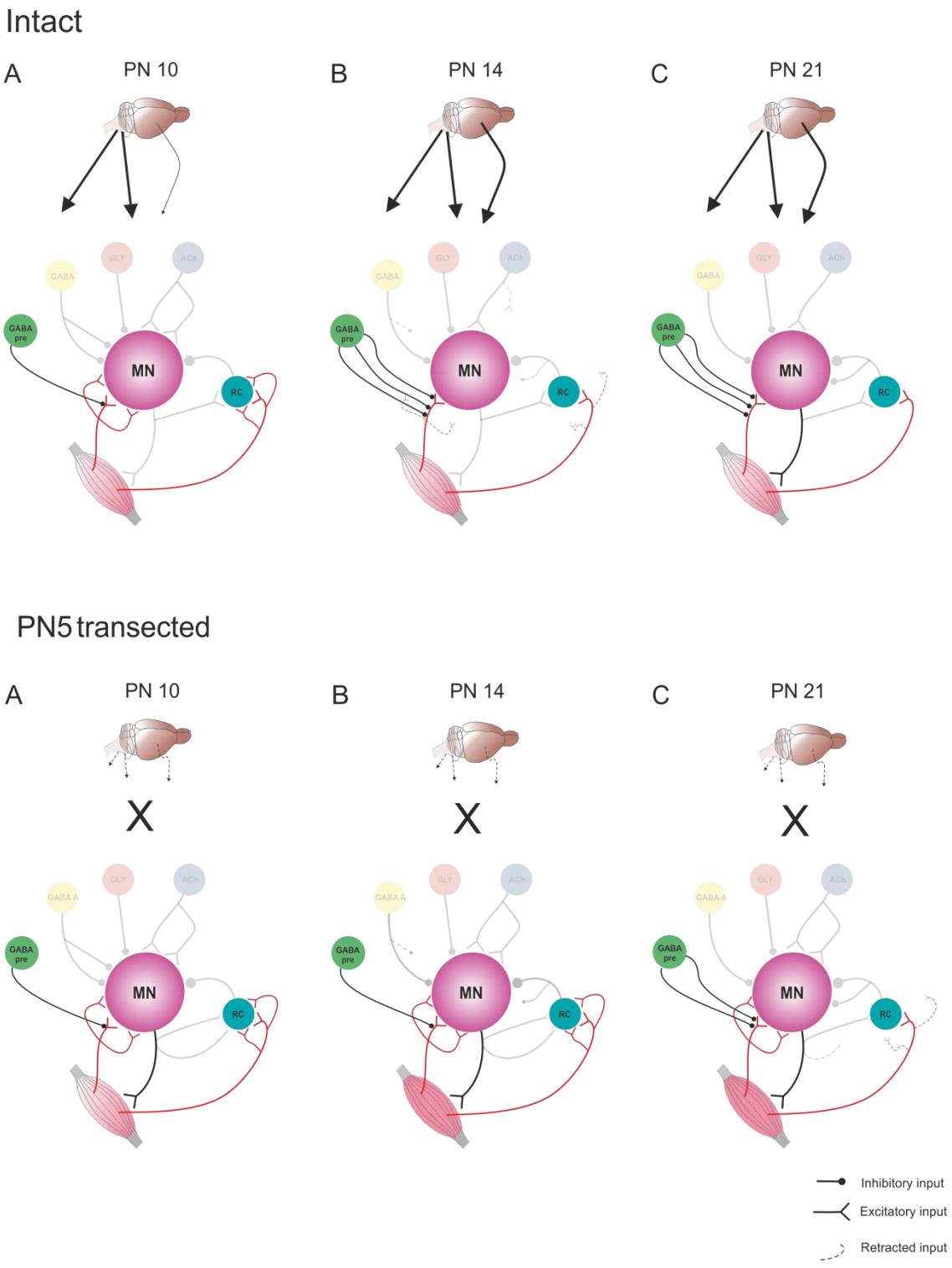

Figure 40. Schematic illustrating the differences in development of afferents and their modulation in normal and PN5 TX rats.

6.5.5 PN5 transection does not significantly affect development of cholinergic input to MNs.

For intact rats, there was a significant effect of age on density of VAChT$^+$ boutons on MNs (p=.013), however this effect was abolished by PN5 TX (p=.095). However, this was due to reduced VAChT$^+$ boutons apposing MNs at PN10 in the TX group (intact= 8.04 ± 0.06, TX= 6.27 ± 0.41, p=.010) because there was no difference between groups at PN14 (intact= 5.15 ± 0.30, TX= 5.03 ± 0.32, p=.840) or PN21 (intact= 6.15 ± 0.76, TX= 5.58 ± 0. 23, p=.339, Fig 41).

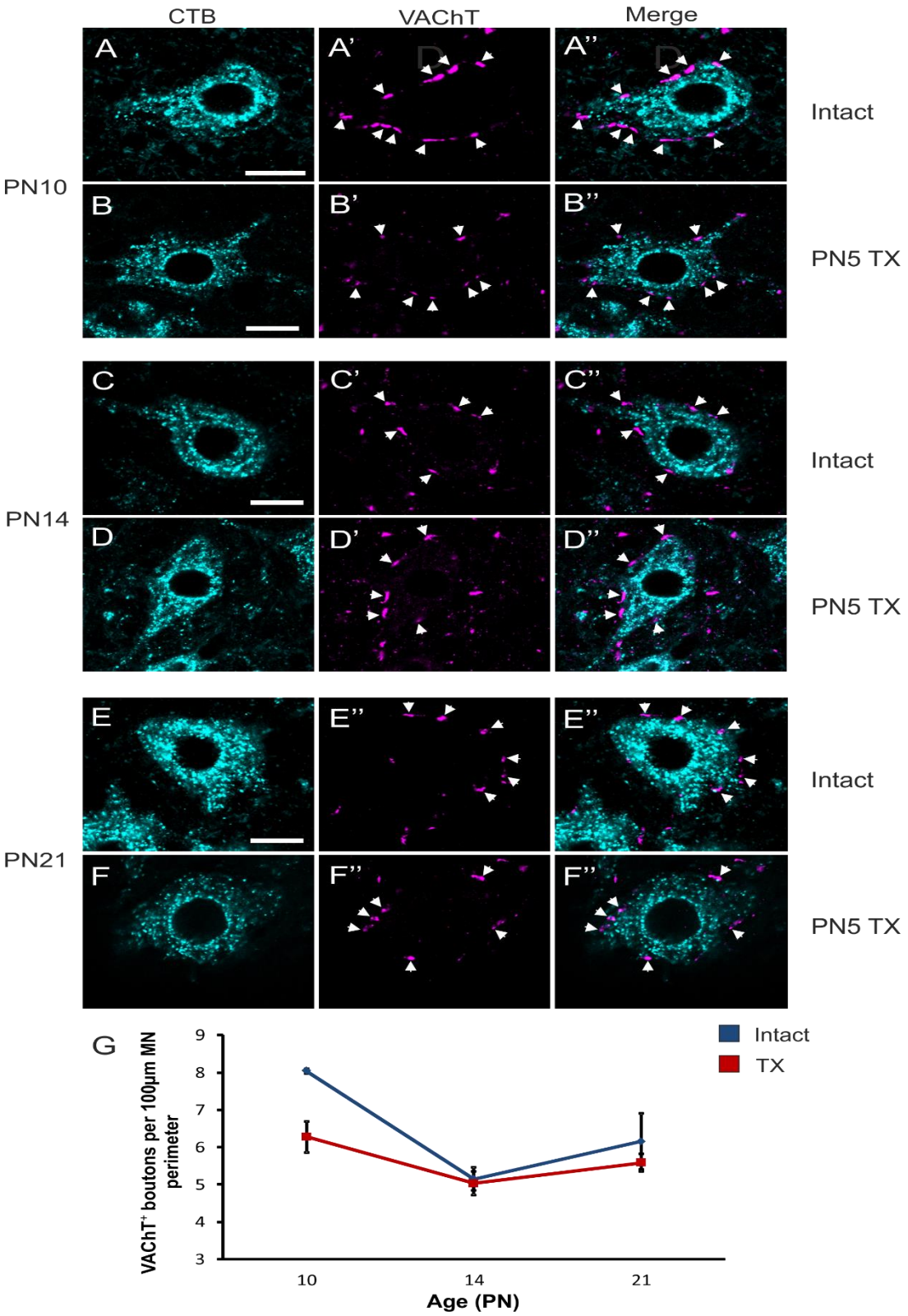

Figure 41. PN development of VAChT+ boutons apposing MNs**.** (A-F'') Representative images displaying VAChT^{+} boutons apposing MNs. (G) Graph illustrating developmental profile of VAChT^{+} boutons on MNs in intact and TX rats. Scale bar =20µm

6.5.5.1 PN5 transection does not significantly affect development of inhibitory inputs to αMNs

For intact animals, there was a significant effect of age on density of GAD67$^+$ boutons apposing MNs, with PN10 being significantly different to PN14 and 21. There was no difference between PN14 and 21 (see chapter 4 for stats). In the transected group there was still an effect of age (p= .016) however the only difference was between PN10 (15.82 ± 0.67) and PN21 (11.10 ± 0.70, p= .018). Comparing transected and intact groups at each age revealed that there were no differences at any age (**PN10**-intact= 18.34 ± 0.67, TX= 15.82 ± 0.62, p= .059, **PN14**- intact= 13.58 ± 0.60, TX= 14.61 ± 0.81, p= 0.30, **PN21**- intact= 12.59 ± 12.60 ± 0.74, TX= 14.56 ± 0.79, p= .146) suggesting TX had no effect on PN development of motoneurone GAD67$^+$ terminals (Fig 42, H).

There was no effect of age on GLYT2$^+$ terminal density on MNs for intact (see chapter 4) or TX animals (p= .638). Similarly, there were no differences between intact and TX animals at any age (**PN10**-intact= 22.60 ± 0.04, TX= 19.77 ± 2.29, p= .150, **PN14**- intact= 20.94 ± 0.95, TX= 19.39 ± 0.59, p= .292, **PN21**- intact= 20.55 ± 1.00, TX= 18.19 ± 1.00, p= .141), suggesting TX had no impact on development of these inputs (Fig 42, G).

In summary, PN5 TX did not significantly alter developmental profiles for the 2 main inhibitory inputs to MNs, GABAergic (GAD67) and glycinergic (GLYT2, Fig 42).

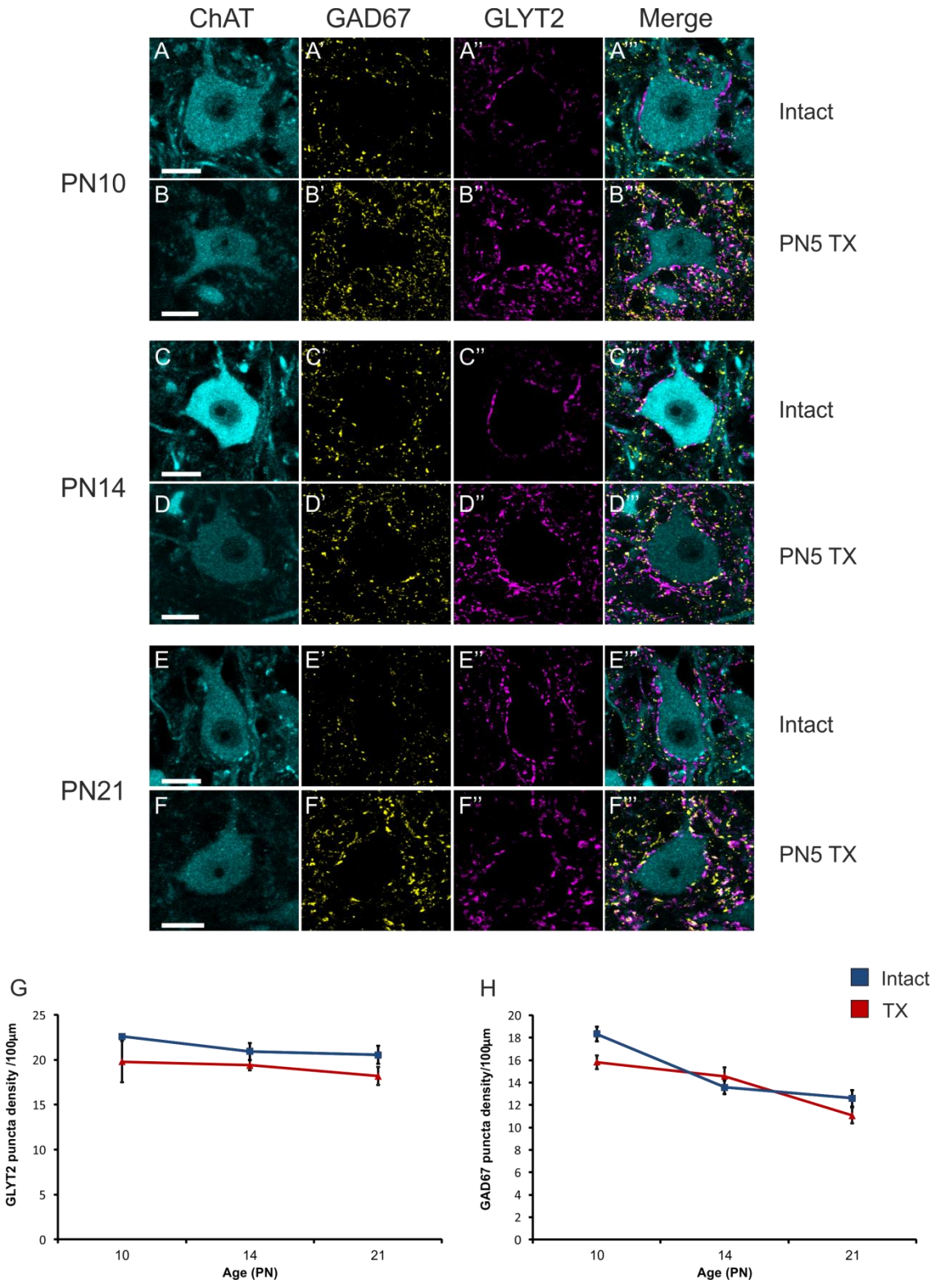

Figure 42. PN development of inhibitory boutons apposing MNs. (A-F''') Representative images displaying inhibitory (GAD67 and GLYT2^{+}) boutons apposing MNs. (G-H) Graph illustrating developmental profile of inhibitory boutons on MNs in intact and TX rats Scale bar = 20µm.

6.5.5.2 PN5 transection does not significantly affect calbindin terminations on MNs with postnatal development

There was a significant effect of age on CB^+ puncta apposing motoneurons for intact rats (p=.019), which persisted during PN development in the presence of a PN5 TX (p=.001), therefore there did not seem to be an effect of TX on PN development of CB^+ terminations on MNs. This was confirmed as there were no differences in bouton density at any age (**PN10**-intact= 1.6 ± 0.25, TX= 2.00 ± 0.16, p= .311, **PN14**-intact= 2.78 ± 0.24, TX= 2.24 ± 0.23, p= .215, **PN21**-intact= 3.80 ± 0.60, TX= 3.71 ± 0.050, p= .822, Fig 43).

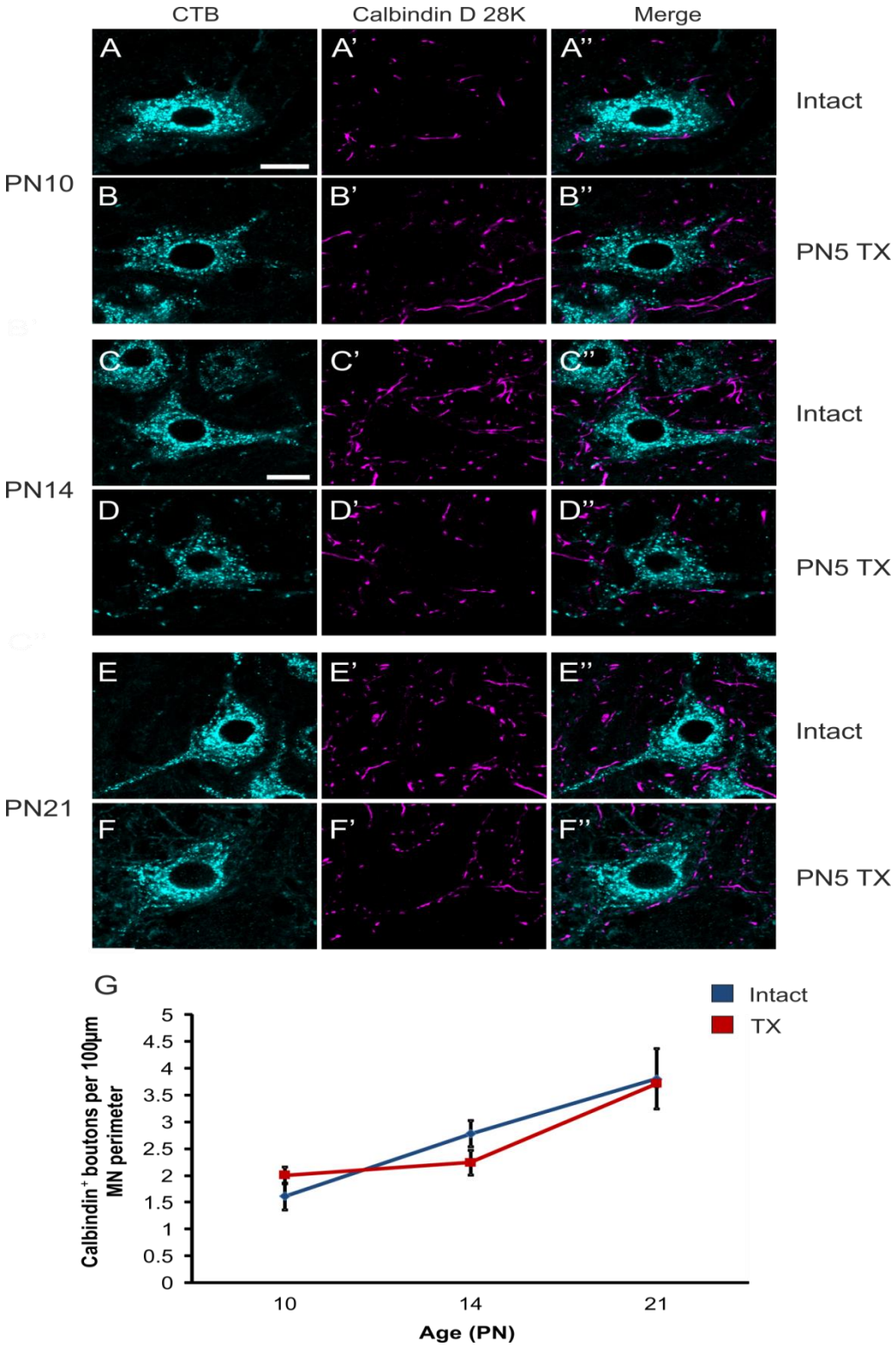

Figure 43. PN development of Calbindin⁺ boutons apposing MNs. (A-F'') Representative images displaying Calbindin⁺ boutons apposing MNs. (G) Graph illustrating developmental profile of Calbindin⁺ boutons on MNs in intact and TX rats.Scale bars=20µm

6.5.5.3 P5 transection induces significant developmental reduction in motor axon collateral input to RCs

Having assessed the effect of PN5 TX on RC input to MNs (calbindin $^{+}$ terminals), it was natural to ask whether motor axon collaterals, which are responsible for driving RCs, were affected by the TX. The study therefore looked at VAChT^{+} terminals contacting the soma and proximal dendrites of RCs labelled with calbindin D-28k (Alvarez et al., 1999). Intact animals did not observe a significant effect of age on RC VAChT terminals suggesting that these remained relatively stable through development. In the PN5 TX group, there was a significant effect of age (p=.005) suggesting that the TX significantly altered the developmental profile. Comparisons of values for the two groups at each age revealed that there were no significant differences at PN10 (Intact= 3.86 ± 0.64, TX= 3.94 ± 0.33) or 14 (Intact= 4.50 ± 0.82, TX= 4.45 ± 0.12, p= .954) however there were significantly fewer VAChT^{+} boutons on TX RCs at PN 21(intact= 4.77 ± 0.38, TX= 2.78 ± 0.18, p= .013, Fig 44 G).

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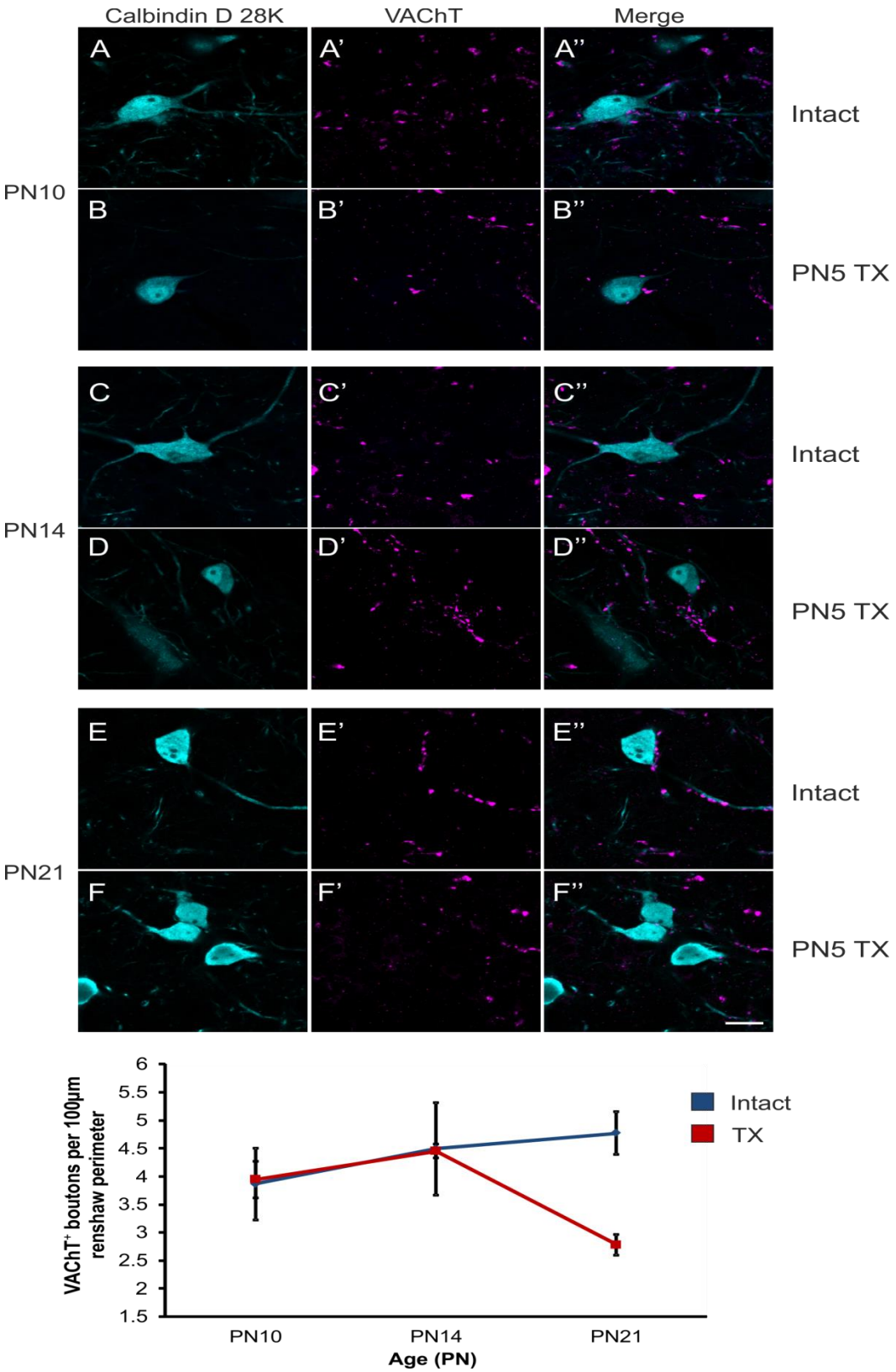

Figure 44. PN development of VACHT+ boutons apposing Renshaw cells. (A-F'') Representative images displaying VACHT^{+} boutons apposing Renshaw cells. (G) Graph illustrating developmental profile of Calbindin^{+} boutons on MNs in intact and TX rats. Scale bar= 20µm

6.5.6 Summary of the effect of PN5 TX on Renshaw cell-motoneurone recurrent inhibition circuit.

Recurrent inhibition is mediated by a simple circuit between MNs and RCs. Motoneurones drive RCs via their axon collaterals and in turn RCs inhibit MNs. Our analysis revealed that whilst PN5 TX did not significantly alter the development of Calbindin^{+} boutons in close apposition with MNs, motor axon collateral input to RCs was significantly lower in TX rats at PN21.

6.5.7 Increased H-reflex excitability in PN5 transected rats

Immunohistochemical analysis has revealed that PN5 TX resulted in disrupted PN development of Ia afferent input to the lumbar spinal cord, with input to MNs significantly greater in the TX group at PN14 and 21 (Figs 36-37). Additionally, development of GAD65^{+} P boutons which are responsible for modulating Ia afferent activity was also significantly disrupted by PN5 TX. Specifically, there were substantial reductions in P boutons apposing motoneuronal Ia afferent terminals in TX animals at PN14 and 21 (Fig 38). In order to assess the physiological manifestations of these anatomical changes, an H reflex study was conducted on intact and PN5 TX rats aged PN14. Thresholding revealed that the excitability of the monosynaptic reflex was increased in PN5 TX animals compared to intact due to lower thresholds for evoking H reflex responses (intact = 37.25 ± 6.6 mA, TX=23.92 ± 3.2 mA, p= .050). Similarly, PN5 TX resulted in significantly greater Hmax/Mmax ratio (intact= 0.25 ± 0.042, TX= 0.39 ±3.21, p= .046, Fig 45D-F). There were no significant differences between groups for H reflex latency. For assessing paired pulse inhibition between groups we assessed 7 time points ranging from 700-1ms. There were no differences between groups between 10 and 1 ms, but this was because in

the majority of cases, H and M waves were abolished, most likely as a result of axonal collisions. There were significant differences, however, at intervals 700 to 50 ms and an overall effect of PN5 TX on paired pulse depression. At each time interval, statistical analysis showed that PN5 TX significantly attenuated levels of PPD, resulting in test H waves showing greater amplitudes as a % of control traces (**700ms**-intact= 53.24 ± 2.07%, TX= 73.47 ± 7.12%, p= .026, **200ms-** intact= 46.80 ± 2.32%, TX= 74.62 ± 13.3%, p= .001, **50ms**- intact= 29.64 ± 4.14%, p= .040, Fig 45 A-C).

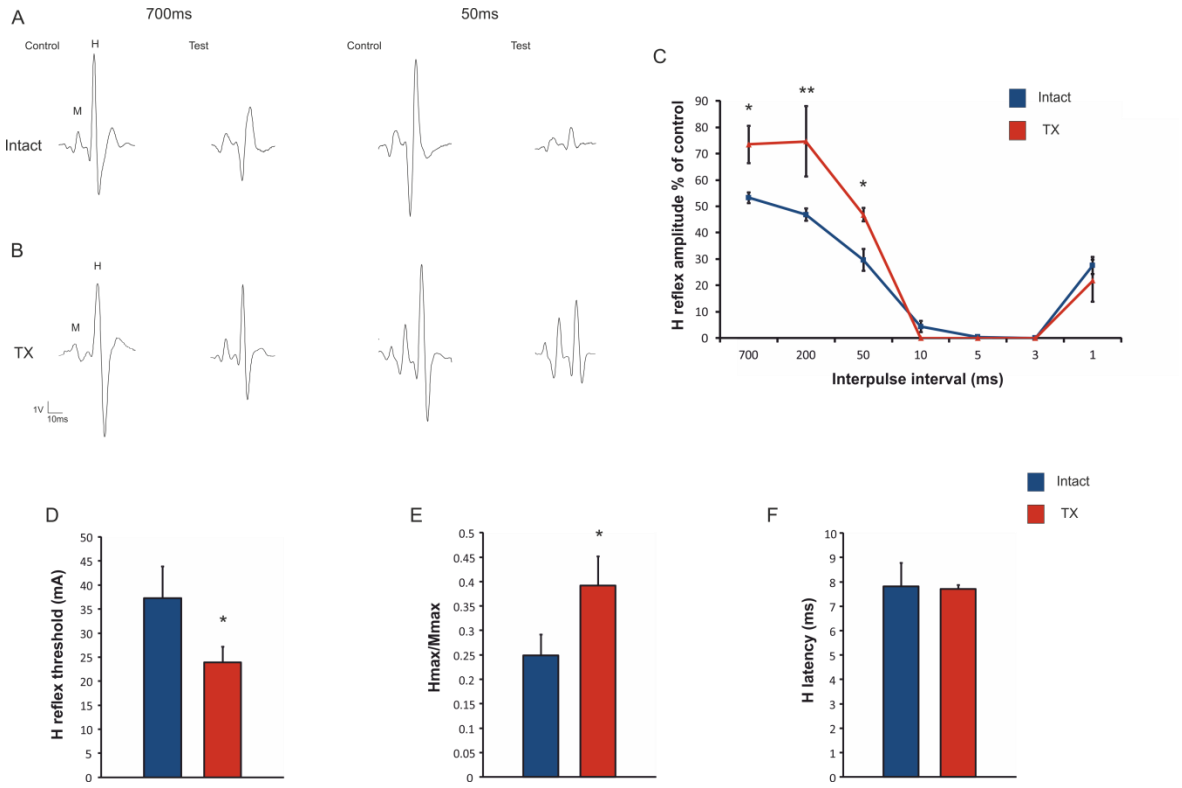

Figure 45. Assessment of H reflex excitability in intact and transected rats at PN14. (A-B) Representative traces illustrating paired pulse depression at 700 and 50ms. Note reduced depression in the PN5 TX group. (C) Graph of PPD in intact and PN5TX rats (D-F) Graphs illustrating H reflex threshold, H max/Mmax ratio and latency in intact and transected PN14 rats.

6.6 Discussion

This study demonstrates for the first time, that removing descending activity at an early PN age (PN5) significantly disrupts normal developmental retraction of proprioceptive afferents in the lumbar spinal cord. In the ventral horn, this was true of both MNs and RCs. This was likely responsible for reduced thresholds for eliciting H reflexes and the greater Hmax/Mmax ratio in PN5 TX compared to intact rats observed with electrophysiological assessments. Furthermore, we show that 'P boutons' associated with presynaptic inhibition of Ia terminals are significantly reduced following a PN5 TX which could be responsible for reduced paired pulse depression of the H reflex. Interestingly, our results suggest that a lack of descending influence combined with the subsequent increase in proprioceptive afferent activity did not significantly alter PN synaptic organisation of premotor interneurones. These results shed new light on activity dependent PN development of spinal circuitry and the role of descending input in guiding developmental organisation of afferent and intraspinal connections.

In the present study, PN5 TX resulted in greater afferent innervation of dorsal, intermediate and ventral laminae of the lumbar spinal cord compared to intact rats. Chapter 4 of this thesis, along with other studies, show that proprioceptive afferent input to the spinal cord is great early postnatally and then subjected to retraction and/or refinement during development (Gibson and Clowry, 1999; Chakrabarty and Martin, 2011a). In addition, it is well appreciated that proliferation of afferent fibres in the spinal cord can be induced by lesions to mature descending tracts or supraspinal regions, often resulting in hyperreflexia and spasticity (Tan et al., 2012; Nelson and Mendell, 1979; Liu and Chambers, 1958; Krenz and Weaver, 1998). It is likely then, that our

results showing increased proprioceptive afferent innervation of the lumbar cord following PN5 TX are resultant from either extreme proliferation of afferents, an attenuation of PN retraction and refinement, or a combination of both. Gibson et al. (2000) reported increased proprioceptive afferent innervation of the ventral horn following neonatal but not adult cortical lesions, suggesting the increase presented here is more likely resultant from attenuated retraction. Moreover, Chakrabarty and Martin (2011b) showed that developmental retraction of proprioceptive afferents from the intermediate and deep dorsal horn is dependent on the arrival of CST axons. In the rat, CST axons do not reach the lumbar spinal grey until PN9 (Donatelle, 1977), meaning our model completely removed competition for synaptic coverage from the cortex, further supporting an abolition of activity dependent retraction hypothesis. Competition from early arriving sub-cortical systems in the lumbar cord would have been released as well, but these systems have already had an influence on the development of the circuitry. The lumbar spinal circuitry, however, is completely naïve to CST influence following PN5 TX.

In PN5 TX animals, H reflex threshold was reduced in conjunction with an increased Hmax/Mmax ratio compared to intact rats. This is likely due to a physiological manifestation of the altered circuitry organisation presented above. Reduced MSR threshold means that Ia afferent excitability has increased and a superior Hmax/Mmax ratio means it is possible to recruit a greater proportion of the motor pool in the TX group for a given stimulus (Crone et al., 1990). Assessment of paired pulse depression was conducted and also suggested greater excitability of the MSR in the TX group. With these assessments, it is expected that as the conditioning-test pulse interval is reduced the amplitude of the test H wave should be reduced compared to a

single pulse control (Trimble et al., 2000; Hultborn et al., 1996; Magladery et al., 1952b). Our results in intact animals followed this profile, however PN5 transected animals exhibited severely reduced depression at each time point, again suggesting greater excitability of the MSR. There is some debate regarding the mechanism behind PPD, but reduced PPD is often seen following spinal and supraspinal lesions and therefore descending tract activation of GABApre neurons which project to Ia afferents has been suggested to be responsible for PPD (Nielsen and Hultborn, 1993; Diamantopoulos and Olsen, 1967; Tan et al., 2012; Thompson et al., 1992).

In light of this, we assessed how GABApre neuron projections to Ia afferents contacting MNs were altered throughout development in PN5 TX rats. Our results suggest that reduced presynaptic inhibition may indeed contribute to attenuated PPD as we found a profound reduction in $GAD65^{+}$ P boutons on motoneurone Ia afferent terminals in PN5 TX rats compared to intact rats. Reduced P boutons following transection in adult animals has been demonstrated before (Kapitza et al., 2012), however in that example GABApre neurons had lost supraspinal control after their synaptic organisation on afferents has developed normally. In contrast, Chapter 4 of this thesis demonstrated that low numbers of P boutons early in development (PN10, before CST maturation) are followed by an increase up to PN14 when CST termination profiles are mature (Schreyer and Jones, 1982; Jones et al., 1982; Joosten et al., 1989). This suggests that CST innervation of GABApre neurons may be responsible for maturation of their projections to Ia terminals during development, with PN5 TX severely disrupting this process.

It is important to note that P boutons still increased on motoneurone Ia terminals (but significantly less) after PN5 TX, despite the fact CST never made

contact with GABApre neurons. This may indicate that descending and afferent inputs are collectively responsible for the synaptic organisation of GABApre neurons mediating presynaptic inhibition of Ia afferents. In fact, the majority of GABApre neurons are found in the medial aspects of laminae V-VI where there is a high density of CST, proprioceptive and cutaneous afferent terminals (Todd et al., 2003; Alvarez et al., 2004; Hughes et al., 2005; Jankowska et al., 1981; Joosten et al., 1989). Additionally, it is well known that both afferent and descending systems are crucial to modulation of presynaptic inhibition (Rudomin et al., 1986; Rudomin and Schmidt, 1999). In the present study, in PN5 TX animals we showed higher densities of VGLUT1^{+} puncta in the area where GABApre neurons are found, which are likely sourced from proprioceptive or cutaneous afferents due to the complete loss of glutamatergic descending fibres such as CST. Increased P boutons on motoneurone Ia terminals throughout development despite PN5 TX could therefore be mediated by increased afferent innervation of GABApre neurons, facilitated by the lack of competition from CST axons which usually terminate in the area described. Paired pulse assessments in our study further support this hypothesis as there is still a 30-70% depression at intervals 700-50ms in PN5 TX animals, despite the lack of descending input. In contrast, adult spinal and supraspinal lesion studies often show a complete loss of PPD or even paired pulse facilitation (Tan et al., 2012).

This is of great functional significance because fundamental behaviour such as locomotion relies heavily upon sequential reflexes and therefore gating of afferent activity is crucial to ensure smooth, efficient execution (Fink et al., 2014; Pearson, 1995). For example, group I, II and cutaneous afferent evoked field potentials in deep dorsal and intermediate lamina are depressed during

MLR evoked locomotion and this is most likely mediated by presynaptic inhibition (Eccles and Lundberg, 1959; Perreault et al., 1995; Perreault et al., 1999).

Our results provide new information which will aid in our understanding of how synaptic organisation of GABApre neurons is modulated. Betley et al. (2009) suggested that GABApre synaptic organisation is ‘solely’ guided by molecular signals from Ia afferent terminals and when Ia afferent terminals in the ventral horn are genetically reduced, so are P boutons. In opposition to their hypothesis we demonstrate increased P bouton density on MN Ia terminals during development, whilst afferents are being pruned. Furthermore, P bouton density is reduced when afferent input is increased following removal of descending input to GABApre cells. We therefore provide a counter hypothesis to suggest that GABApre neuron synaptic organisation on Ia afferents is actually dependent on their convergent inputs rather than synaptic targets, shedding new light on activity dependent modulation of GABApre projections to Ia afferents.

6.6.1 Co-dependant development of Ia afferent and motor axon collaterals on Renshaw cells

During normal postnatal development, both VAChT^{+} motor axon collaterals and VGLUT1^{+} Ia afferents proliferate on Renshaw cells up to PN15, after which Ia terminals are reduced and the mature phenotype of motor axon dominant drive of RCs becomes apparent (Mentis et al., 2006a). Our results in Chapter 4 were in agreement with this hypothesis, showing a similar developmental profile. Interestingly, Siembab et al. (2015) recently showed that severely reducing Renshaw cell Ia terminals using ER81 $^{-/-}$ and Egr3 $^{-/-}$ transgenic mice resulted in

increased innervation by motor axon collaterals. Conversely, significantly up regulating Ia terminals using mlcNT3$^{+/-}$ transgenic mice resulted in a severe reduction of innervation by motor axon collaterals. This suggests development of motor axon collateral input to Renshaw cells is dependent on retraction of Ia afferents with maturation. In our study, following PN5 TX, Ia afferent innervation of the lumbar spinal cord and RCs is significantly increased whilst there is a significant reduction in VAChT^{+} motor axon collaterals on RCs, supporting a theory that the two inputs develop in a co-dependant, competitive manner. Although manipulations in both studies ultimately resulted in increased afferent innervation, the mechanisms by which this proliferation occurred and therefore the implications are distinct. In the present study, greater afferent innervation of the cord resulted from an insult, a complete thoracic spinal cord transection which cut all descending input, leaving the lumbar cord to develop solely in the presence of afferent input. In light of this, it is reasonable to assume that the decreased innervation of RCs by motor axon collaterals seen here may also be applicable to conditions such as cerebral palsy, where pre or early postnatal insults to supraspinal regions also result in greater afferent innervation of the spinal circuitry (Clowry et al., 2004a; Gibson et al., 2000). Our study also showed reduced PPD of the H-reflex after PN5 TX which, as mentioned earlier, could be due to reduced recurrent inhibition as we showed that motor axons collateral input to RCs is reduced. We also showed that calbindin^{+} inputs to MNs were not significantly affected by PN5 TX, but motor axon collateral innervation of RCs is reduced. Therefore reduced PPD following PN5 TX could be due to a reduction in motoneurone drive of RCs rather than increased inhibitory input from RCs.

6.6.2 PN5 TX does not significantly alter development of cholinergic input to αMNs.

Results here show that PN development of cholinergic inputs to MNs is not significantly affected by PN5 TX, with the only differences being observed at PN 10. This is in agreements with electron microscopy (EM) studies on the synaptology of MNs following PN5 TX which also show no difference in coverage by C-boutons between intact and TX animals (Ichiyama et al., 2011). A proportion of these C-boutons likely originate from partition cells located in the intermediate grey, just lateral to lamina X and have been shown to increase excitability of MNs during locomotion (Miles et al., 2007; Zagoraiou et al., 2009).

6.6.3 PN5 TX does not significantly alter PN development of inhibitory input to αMNs

Similar to cholinergic terminals on MNs, we found no effect of PN5 TX on the PN development of either GABAergic ($GAD67^{+}$) or glycinergic (GLYT2) boutons apposing MNs. This was also in agreement with the work of Ichiyama et al., 2011, who showed no difference between PN5 TX and intact animals when assessing inhibitory ‘F boutons’ on αMNs. Animals in the former study were killed and perfused at PN56, suggesting that ultimately, it is unlikely that there would be any difference if the animals were permitted to mature further and supporting the theory that rats are motor mature at PN21. Again, it is important to state that PN5 TX resulted in greater afferent innervation of dorsal and intermediate regions of the spinal grey, where a majority of the inhibitory premotor interneurons will reside. Due to the reduced competition from

descending systems, these afferents could assume greater synaptic coverage of these premotor interneurons, although this needs to be confirmed.

6.7 Summary and conclusions

The results presented here show that afferent innervation of the lumbar spinal cord is greatly increased following PN5 TX and this is likely due to an abolition of normal developmental retraction. Our data, in conjunction with others before, suggest abundant afferent input following PN5 TX may be due to unsuccessful growth of CST axons into the lumbar spinal cord, resulting in reduced competition for synaptic coverage of interneurons in the dorsal and intermediate laminae. We provide support for the hypothesis that motor axon collateral input to RCs is contingent upon retraction of Ia afferents and that inhibitory control of Ia afferents and motor output is greatly reduced, most likely by a combination of reduced presynaptic and recurrent inhibition respectively. In addition, we have furthered understanding of how GABApre neurons organise their synaptic projections (P boutons), suggesting that it is dependent on convergent inputs which modulate presynaptic inhibition rather than synaptic targets. Surprisingly, the absence of descending influence during PN development did not seem to affect synaptic organisation of premotor INs. This could have been due to increased afferent innervation of these cells, acting to maintain their projections to MNs in a homeostatic fashion. Alternatively, it is possible that mature projection to MNs from premotor INs is established before PN5 and so transection at this age has little effect in development.

Disruption of normal development underlines the vital importance of descending input for guiding the development of afferent systems, and may point towards potential mechanisms responsible for dysfunctional motor control

in conditions such as cerebral palsy. In the same way, it may provide insight into the mechanisms behind the greater prospects of locomotor recovery afforded to neonatally injured animals compared to adult injuries.

Chapter 7 General Discussion

7.1 Summary of findings

The spinal cord contains all the necessary components for producing complex behaviour such as locomotion, yet it is dependent upon descending systems for initiation and modulation of this behaviour. This dependence is formed throughout postnatal development as descending structures make contact with and begin to modulate in spinal circuits. Previously, it has not been known to what extent the organisation of intricate spinal circuitry depends on descending information during development. This may be partly due to the lack of a preparation which allows the physiology of the spinal cord to be studied in the presence of descending and peripheral input throughout PN development. Furthermore, studies of PN development of key intraspinal connections modulating motor output in the presence and absence of descending systems at important time points throughout PN development have not been conducted.

The thesis presented here shows that we were able develop a preparation which permits the study of motor control throughout PN development of the rat. We show for the first time, anatomical and physiological evidence that developmental retraction of proprioceptive afferent input to the lumbar spinal cord occurs and is dependent upon the presence of descending systems. Modulation of afferent activity is increased significantly with age as evidenced by increased presynaptic (P) boutons on Ia terminals contacting MNs. Interestingly, this profile is attenuated but not abolished by neonatal spinal cord transection. This anatomical data was corroborated by H reflex assessments which showed reduced paired pulse depression of the H reflex, thought to be associated with presynaptic inhibition of Ia afferents. Additionally, we provide a more in depth analysis of how premotor interneuron projections to MNs are

altered during development and demonstrate that PN synaptic organisation of these projections may be dependent on descending systems.

7.2 Discussion of key results

7.2.1 Successfully establishing a preparation for studying motor control throughout postnatal development.

Initial experiments aimed to modify an existing preparation to allow us to study the PN development of motor systems in the rat, thereby introducing a novel preparation into the field of motor control. This was an ambitious and challenging aim as the ensuing attempts would reveal, but a decerebrate, *in situ* whole rat preparation was established. Viability was attained in animals aged PN7,10,14 and 21, from an age when animals are not capable of over ground locomotion, through locomotor acquisition phases, to establishment of mature movement patterns. Many attempts have been made to extend the viability of existing *in vitro* preparations, however PN14 seems to be the limit and success has since been meagre. The introduction of our preparation could therefore help the field to bridge the gap between neonatal and adult and *in vitro* and *in vivo* preparations (Jiang et al., 1999a; Jiang et al., 1999b). Although our results could lead to a significant advancement for the field, the preparation needs further development and refinement to study more complicated aspects of motor control such as locomotion.

For example, many studies utilise the neonatal isolated spinal cord for assessing the locomotor circuitry of the spinal cord (Kiehn, 2006; Smith et al., 1988; Ozaki et al., 1996). Results from these studies form the basis of much of our current understanding of the locomotor CPG circuitry, yet some of the

findings have not been ratified in older animals and physiological relevance is questioned compared to in vivo preparations. For example, Dutschmann et al. (2000) showed that in a similar *in situ* half rat perfused preparation (working heart brainstem-WHBP), frequency of phrenic activity cycles were much closer to age matched *in vivo* values (~45 cycles/min) compared to *in vitro* isolated cord preparations (~9/min). Interestingly, fictive locomotion from isolated spinal cord preparations is much slower than *in vivo* locomotor bursts (Alluin et al., 2015; Smith and Feldman, 1987; Cazalets et al., 1992). We were able to evoke rhythmic motor outputs using afferent (cutaneous) stimulation and neuroactive drugs such as NMDA and 5HT, suggesting that similar studies can be conducted throughout development to compare the motor outputs of this preparation to both *in vivo* and *in vitro* preparations. Evoking locomotion with different combinations and/or concentrations of drugs throughout development could give greater insight into how descending systems responsible for initiation and control of locomotion are maturing.

A curious characteristic of neonatal isolated cord preparations is that fictive locomotion can be evoked by ventral root stimulation, suggesting that motor axons at this age might project to the rhythm generating centres of the CPG (O'Donovan et al., 2010). This data has stimulated the formation of theories suggesting that potential motor axon collateral activation of CPG circuits is a mechanism which is 'deselected' during development (Wenner and O'Donovan, 1999; Hanson and Landmesser, 2003). If fictive or even real locomotion can be induced in the neonatal *in situ* whole rat by ventral root stimulation, these experiments could be repeated throughout development to indicate if and when 'deselection' occurs. There are many other example of

how this preparation can be used in order to further our understanding of neural control of movement and significant time will be ensuring the preparation reaches its full potential.

7.2.2 PN retraction of proprioceptive afferents in the lumbar spinal cord is dependent on the presence of descending systems

During normal development, we have for the first time identified that proprioceptive afferent input to the lumbar spinal cord is retracted, just as it is in the cervical spinal cord. Interestingly, this retraction seems to coincide with the arrival and maturation of the CST axons in the rat which corroborates much of the cervical spinal cord work in cats and rats, suggesting proprioceptive afferents and CST compete for synaptic coverage of spinal circuitry during development (Gibson and Clowry, 1999; Chakrabarty and Martin, 2011b; Clowry et al., 2004a). Jiang et al. (2016) recently showed that competition between descending and proprioceptive afferents persists into maturity which could carry great significance for treatment of disorders such as spinal cord injury and cerebral palsy. For example, many studies utilise growth promoting treatments in order to promote regeneration and sprouting of descending axons following spinal cord injuries, resulting in significant functional recovery (Schnell and Schwab, 1990; Bregman et al., 1995; Bradbury et al., 2002; Zhao et al., 2013). However, it has also been reported that spinal cord injury results in sprouting of primary afferents, and that plasticity promoting treatments facilitate this process (Barritt et al., 2006; Nelson and Mendell, 1979; Liu and Chambers, 1958). Evidence from this thesis in conjunction with other studies suggest that growth promoting treatments which increase plasticity globally and therefore sprouting of afferents as well as descending tracts, actually act to enhance

competition against descending projections. With this, they are limiting the primary aim of their studies, which is to increase sprouting of descending tracts past or through the lesion and into the spinal circuitry caudal to the lesion. In light of this, researchers should be motivated to devise focal treatments which enhance plasticity of the target axons around the lesion site and limit afferent sprouting, at least during periods of maximal descending growth.

In cerebral palsy (CP), selective dorsal root rhizotomy (SDR) is used to decrease excitability of the MSR and has proved one of the most effective (yet somewhat crude) treatments. As well as reducing excitability of the MSR, it is possible that it may also reduce competition from the severed afferents, promoting CST sprouting. Sprouting of CST axons could in turn result in greater innervation of GABApre neurons, increasing presynaptic inhibition of Ia afferents which could lead to attenuation of spasticity. Clearly the effects of this treatment need to be studied in greater detail, especially if there is potential for developing a less invasive and destructive alternative for treatment of some symptoms of CP.

We showed that afferents were also retracted from the intermediate and dorsal regions of the spinal grey, and PN5 TX also attenuated retraction from these areas. It is important to note that we do not know the modality of the afferents in this area as VGLUT1 is expressed by proprioceptive and some cutaneous afferents. In addition, we did not assess, as we did in the ventral horn, if this retraction occurred on specific sets of interneurons. The addition of these data is needed in order to make conclusions about the significance of retraction of afferents and its abolition to control of the spinal circuitry. For example it is known that afferents drive and modulate the activity of GABApre neurons which

mediate presynaptic inhibition (Rudomin, 2009). Descending systems also exert a high degree of control over these cells and so increased afferent input to these cells could prove an important homeostatic strategy following neonatal transection. It is important to note that only 1 afferent modality was assessed in these experiments. It is likely that other PN development of other modalities, such as group II, Ib and cutaneous afferents are also affected by the transaction. It will be important to try and assess these systems as they may also contribute to changes in excitability and modulation of the spinal circuitry.

7.2.3 GABApre neuron synaptic organisation on Ia afferents is dependent on peripheral and descending inputs during development

Studying afferent input to the spinal cord alone is insufficient to draw conclusions about their impact on spinal circuitry as they are strongly modulated by presynaptic inhibition from GABApre interneurons (Rudomin and Schmidt, 1999). Ours is the first study describing the development of GABApre projections (P boutons) to Ia afferent terminals throughout PN development. We show that early in development, when afferent input is great and descending weak, there are relatively few GABApre projections (P boutons) to Ia afferent terminals. As afferents are retracted, and descending inputs are increased, P bouton coverage increases in concert, opposing conclusions made by Betley et al. (2009) suggesting that GABApre synaptic organisation is ‘soley’ based upon availability of proprioceptive afferent terminals. Instead, we propose that P bouton input to afferents is dependent upon convergent input from descending and afferent projections because of the meagre coverage of Ia afferents during early development and decreased coverage following PN5

TX. Further assessments involving identification of GABApre neurons and quantification of afferent and descending inputs will be required to test this hypothesis fully.

Removing descending input clearly impacts the density of P boutons on Ia afferent terminals, which is expected because it is known that descending systems act on GABApre neurons in the lumbar cord during tasks such as locomotion (Dubuc et al., 1988; Rossignol et al., 1998; Perreault et al., 1999). It should also be carefully noted however, that P bouton density still increases throughout development in the absence of descending input, perhaps suggesting that afferent innervation of GABApre neurons might increase in response to PN5 transection. Again identification of GABApre neurons and use of an afferent tracer such at CTβ would allow us to assess this directly. Our physiological data suggests that afferent input may compensate for loss of descending inputs to GABApre as we see 30-70% paired pulse depression of the H reflex in the TX groups, despite complete lack of descending input. Adult injuries have shown that PPD is completely lost and paired pulse facilitation is seen at times (Tan et al., 2012). Spared PPD and therefore presynaptic inhibition could contribute to greater control of locomotion in neonatally injured animals compared to adults, especially when treadmill trained, as GABApre terminals on afferents could be organised in a way which guides and gates activity to the CPG.

Kapitza et al. (2012) showed that P boutons are retracted following adult sacral spinal cord TX resulting in spasticity of tail muscles, however it is not known if afferents can compensate for this loss of descending input. H reflex studies in adult injured animals suggests this might not be the case as often there is a

complete loss of rate dependent depression or even a switch to facilitation (Tan et al., 2012). This would suggest that if there is increased afferent innervation of GABApre neurons in our model, it is due to the developmental nature of the injury; probably because of immaturity or lack of descending tract innervation of the lumbar spinal circuitry at the time of injury.

7.2.4 Development of premotor inputs to MNs in the absence of descending systems

The impact of removing descending inputs to the lumbar spinal cord at PN5 on premotor inputs to MNs was minimal. In general, there was no deviation from the trend shown in intact development, and if there was, they were assimilated by PN21. This suggests that synaptic organisation of premotor inputs to MNs is not dependent upon descending input. Alternatively, our results suggest that increased afferent innervation of interneurone populations following PN5 TX could result in a homeostatic maintenance of their projections. Afferent input to Renshaw cells was significantly increased at time points of development after PN5 TX, suggesting this may be true of other interneurons. Further studies need to be done to test this hypothesis, however this encounters difficulties in selectively identifying premotor interneurons.

A retrograde, monosynaptic pseudo-rabies virus has been developed which only travels only 1 synapse (Wickersham et al., 2007), allowing the experimenter to identify only last order premotor interneurons projecting to the MNs of a specific muscle (Stepien et al., 2010). This tool could be used to directly answer the question of whether retraction of afferent innervation to these cells occurs, and if this retraction is dependent on descending systems (Stepien et al., 2010). This technique is only reliable in animals up to PN15,

however our results indicate that retraction of afferents plateaus between PN14 and 15 and so it would be applicable to suggested experiments.

7.2.5 Developmental reorganisation of spinal circuitry in the absence of descending systems may contribute to recovery in neonatal spinal cord injuries

Animals subjected to spinal cord injuries as neonates are afforded greater prospects of functional recovery compared to adult injuries, especially when combined with locomotor training (Petruska et al., 2007; Weber and Stelzner, 1977; Saunders et al., 1998; Howland et al., 1995; Bregman et al., 1993). One might postulate that the greater plastic nature of the neonatal cord may be the underlying mechanism responsible for greater recovery. This may be a superficial truth, however plasticity of the spinal cord can be greatly enhanced in adult animals using a combination of growth factors and other treatments which can remove or inhibit inhibitory factors such as chondroitin sulphate proteoglycans (CSPGs) and Nogo (Pizzorusso et al., 2002; Bradbury et al., 2002; Wang et al., 2011; Maier et al., 2009). These treatments are effective in improving functional recovery, however this is suggested to be related to sprouting of spared descending fibres past the lesion site. In the case of complete transections, adult animals can recover some function with the help of locomotor training, but it is meagre unless treated with external stimulation and/or drugs to mimic descending drive (Ichiyama et al., 2005; Gerasimenko et al., 2007; Alluin et al., 2015) . In comparison, neonatal transected animals recover a great deal of locomotor function without training and significantly more with locomotor training alone (Petruska et al., 2007; Weber and Stelzner, 1977; Tillakaratne et al., 2010). This suggests that neonatally transected

animals respond better to afferent activity allowing reorganisation of their circuitry to allow production of relatively efficient stepping in the absence of descending control. For example, as shown in the present study, increased afferent innervation of RCs may prove beneficial because they are now receiving online feedback from the periphery as well as efference copies of intended action from MNs, allowing it to compute past, ongoing and future movements and modulate MN activity accordingly. If this holds true, corrective strategies usually the purview of descending systems may be implemented locally, allowing better control of locomotion. Increased innervation of RCs has not been demonstrated in adult injured animals and so it is not known if this adjustment is restricted to neonatal injuries. Alternatively, it is possible that because at PN5 most descending inputs are absent/immature, synaptic space on interneurons 'reserved' for descending systems is easily captured by the periphery. Put simply, it may be that it is difficult for the adult cord to change its habits, that is, to produce locomotion in the absence of descending control, whereas the cord isolated from the brain just after birth has not learned to walk and subsequently does so in the presence of afferent input alone. This would explain why neonatally transected animals respond better to treadmill training. Because the spinal cord has developed in the absence of descending control, it has been able to reorganise its circuitry in order to better utilise afferent information in isolation.

It is established that proprioceptive afferents are indeed crucial in recovery of function following spinal cord injury in adults (Takeoka et al., 2014), but this may not be the case in neonatally transected animals. Motor activity in neonatal animals is thought to be driven by cutaneous rather than proprioceptive

afferents, a phenomenon reversed throughout PN development (Weed, 1917; Windle, 1930; Skoglund, 1960; Ekholm, 1967; FitzgeraId et al., 1988). It is conceivable then, that with the absence of CST innervation, and greater afferent input displayed in this study, cutaneous fibres may retain their neonatal ability to drive and modulate motor output into adulthood.

Future studies related to this thesis should include assessments of the PN development of cutaneous afferents in the absence of descending systems. This could be done behaviourally using vonn Frey hairs, electrophysiologically by assessing EMG responses to vonn Frey hairs and direct cutaneous reflexes. Immunohistochemistry can be used to identify if developmental sprouting or reduced pruning of cutaneous fibres occurs following PN5 transection. If there is indeed greater cutaneous activation of motor output in neonatally transected animals, the relative contributions of proprioceptive and cutaneous afferents will need to be identified. This could be done using transgenic mice in which proprioceptive afferents can be acutely and selectively knocked out in neonatally transected mice and assessing locomotor ability.

7.3 Concluding remarks

This study has produced a preparation and data which contribute significantly to the field of motor control and hopefully form the foundations of future studies into the development of motor systems. This work will be extremely important to our understanding of how mature organisation of spinal circuits is established and the contributions relative components make to acquisition of mature neural control of movement. The results provide insight into mechanisms underlying functional deficits from neonatal insults to the CNS. For

example, afferent input to MNs is increased following PN5 TX but presynaptic modulation of afferents is also reduced, probably due to reduced innervation from GABApre neurons. Our H reflex results suggest that reduced P boutons along with increased afferent innervation is probably responsible for increased spasticity following neonatal lesions.

Similarly, differences in circuit organisation between adult and neonatally transected rats may be linked to enhanced functional recovery following neonatal injuries compared to adult injuries. Although we haven't isolated a mechanism responsible for enhanced recovery, we suggest some likely culprits which can be targeted with further tests. In the absence of descending control, it is likely that increased afferent innervation of the spinal circuitry contributes to enhanced recovery. As discussed above, contributions each of these pathways make towards enhanced recovery of locomotion needs to be assessed.

Although this work has uncovered interesting and pertinent findings, it needs to be extended to better understand how the spinal circuitry matures throughout development, and what contribution certain components make towards acquisition of mature behaviour. Additionally, It will be important to extend the work conducted here to injuries in adulthood in order to directly compare the changes in the spinal circuitry and identify the most important factors contributing to enhanced recovery from neonatal SCI.

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