Electrophysiological investigation of spinal cord injury and characterisation of propriospinal neurons

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B. Biomed Sci (Hons)

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Doctor of Philosophy

The School of Biomedical Sciences and Pharmacy
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Declaration of Originality

This thesis contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. I give consent to the final version of my thesis being made available worldwide when deposited in the University’s Digital Repository, subject to the provisions of the Copyright Act 1968.

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Statement of Collaboration

I hereby certify that the work embodied in this thesis has been done in collaboration with other researchers, and carried out in other institutions. I have included as part of the thesis a statement clearly outlining with whom and under what auspices.

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Statement of Authorship

I hereby certify that the work embodied in this thesis contains material from three published papers of which I am a joint author. I have included as part of the thesis a written statement, endorsed by my supervisors and co-authors, attesting to my contribution to the joint publications.


   **Contributions of JRF: Literature review and manuscript preparation.**


   **Contributions of JRF: Experimental design, data collection, data analysis, and manuscript preparation.**


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Abbreviations

ACSF: artificial cerebrospinal fluid
AMPA: α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
AHP: afterhyperpolarisation
AP: action potential
BBB: blood brain barrier
BMP: bone morphogenic protein
ChAT: choline acetyltransferase
CNQX: 6-cyano-7-nitroquinoxaline-2,3-dione
CPG: central pattern generator
CSPG: condroitin sulphate proteoglycan
CST: corticospinal tract
DCol: dorsal column
DF: delayed firer
DLF: dorsolateral funiculus
DH: dorsal horn
EMG: electromyogram
EPSC: excitatory post synaptic current
EPSP: excitatory post synaptic potential
FCIP: fluorescent calcium indicator protein
GABA: γ-aminobutyric acid
GAD67: glutamate decarboxylase 67
GDNF: glial cell derived neurotrophic factor
GEVI: genetically encoded voltage indicator
GFP: green fluorescent protein
IB: initial burster
ICR: imprinting control region
IMZ: intermediate zone
IPSC: inhibitory post synaptic current
IPSP: inhibitory post synaptic potential
LAPN: long ascending propriospinal neuron
LCN: lateral cervical nucleus
LDPN: long descending propriospinal neuron
LF: lateral funiculus
LPN: long propriospinal neuron
LRN: lateral reticular nucleus
LSN: lateral spinal nucleus
MAG: myelin associated glycoprotein
MC: multiple component
mIPSC: miniature inhibitory post synaptic current
NMDA: N-methyl-D-aspartate
OCT: optimum cutting temperature
OMgp: oligodenrocyte myelin glycoprotein
PBS: phosphate buffered saline
PBST: phosphate buffered saline with triton-X
PFA: paraformaldehyde
PIC: persistent inward current
PN: propriospinal neuron
PNS: peripheral nervous system
Q: charge
RGM: repulsive guidance molecule
Ri: input resistance
RMP: repulsive guidance molecule
RMS: root mean square
ROS: reactive oxygen species
Rs: series resistance
S-ACSF: sucrose substituted artificial cerebrospinal fluid
SC: single component
SCI: spinal cord injury
SDH: superficial dorsal horn
sEPSC: spontaneous excitatory post synaptic current
sIPSC: spontaneous inhibitory post synaptic current
SPN: short propriospinal neuron
SS: single spiker
τ: decay time constant
TC: two component
TF: tonic firer
TTX: tetrodotoxin
VF: ventral funiculus
VGAT: vesicular GABA transporter
VGlut2: vesicular glutamate transporter 2
VH: ventral horn
VIAAT: vesicular inhibitory amino acid transporter
VMF: ventromedial funiculus
YFP: yellow fluorescent protein
Thesis Summary

The work presented in this thesis had two major aims. The first was to develop an *in vitro* horizontal spinal cord slice preparation for detailed electrophysiological analysis of spinal neurons and their synaptic inputs in a mouse model of spinal cord injury (SCI). The second was to undertake a detailed anatomical and electrophysiological characterisation of a population of spinal cord neurons called long descending propriospinal neurons (LDPNs), which are an important mediator of functional recovery following SCI.

In Chapter 1, I summarize what is known about SCI pathophysiology, impediments to recovery, and the potential for electrophysiological investigation of SCI processes. I also outline our current understanding of the propriospinal system, its role in recovery from SCI and the need for more detailed anatomical and electrophysiological data on propriospinal neurons in the mouse.

In Chapter 2, I develop an *in vitro* horizontal spinal cord slice preparation to provide a way forward for SCI researchers to assess functional electrophysiological changes in the injured and recovering spinal cord. The major findings of Chapter 2 were that longitudinal axonal pathways within the spinal cord, such as the dorsal columns, can be maintained and electrically stimulated for analysis of synaptic function. In addition, this preparation allows detailed study of the intrinsic and synaptic properties of spinal neurons over long rostrocaudal distances in the spinal cord.

In Chapter 3, I use the horizontal slice preparation to study neurons in the vicinity of a spinal cord lesion both with and without a therapeutic intervention (3 weeks of exercise training). This study showed that while intrinsic properties of neurons in the vicinity on an SCI were not altered by exercise, excitatory synaptic input from dorsal column fibres was enhanced. These findings demonstrate that the horizontal spinal slice preparation allows detailed examination of intrinsic neuronal properties, and importantly, functional properties of synapses following SCI.

In Chapter 4, I undertake a detailed anatomical analysis of LDPNs in the mouse. LDPNs were identified by retrograde labelling and mapped throughout the cervical and upper thoracic spinal cord. In addition, transgenic mice were used to determine the projection pattern of inhibitory LDPNs. Ipsilaterally projecting LDPNs were spread diffusely throughout the deep dorsal horn, intermediate zone and ventral horn. In contrast,
contralaterally projecting LDPN neurons were concentrated within the ventral horn of the grey matter. Interestingly, inhibitory LDPNs (GlyT2 and GAD67 positive) projected almost exclusively to the ipsilateral lumbar spinal cord. These anatomical data provide a necessary framework for future studies on the LDPN system that aim to ascertain which LDPNs are most important for functional recovery following SCI.

In Chapter 5, I used targeted whole-cell patch-clamp recordings to study inhibitory synaptic inputs onto LDPNs, as inhibitory synapses are critical for executing motor behaviours. The main findings were that LDPNs received a higher frequency of GABA mediated inhibitory inputs compared to location matched control neurons and that 40% of labelled LDPNs exhibited a tonic inhibitory glycine-mediated current. The electrophysiological data from this study provides important baseline information for future studies that examine motor control and synaptic plasticity in LDPN populations following SCI.

Together, these two bodies of work provide a way forward for preclinical SCI models and at the same time, deliver a better understanding of the basic neural components of the spinal cord that provide a conduit for functional recovery. It is my hope that this work will drive experimental SCI studies to examine pathophysiological and recovery processes in greater functional detail, and expand our knowledge on the propriospinal system in both health and disease.
Chapter 1: Spinal cord injury and propriospinal neurons
Chapter 1: Spinal cord injury and propriospinal neurons

1.1 Spinal cord injury

Traumatic spinal cord injury (SCI) is a particularly pernicious disorder. The disruption of motor and sensory pathways after SCI leads to severe physical, psychological and social challenges that last a lifetime for both patients and their families. Even though the incidence of SCI is low relative to other chronic diseases, such as cancer and heart disease (15-40 SCI cases per million worldwide, annually), the annual financial burden of SCI is considerable. For example, SCI costs approximately $2 billion in Australia alone, with lifetime medical care costs for an individual with quadriplegia estimated to be $9.5 million (Economics-Access, 2009; Sekhon & Fehlings, 2001). Although life expectancy for SCI patients is similar to able-bodied individuals, ongoing SCI-related problems including impaired motor function and sensation, neuropathic pain, diminished bladder, bowel and sexual function; and secondary pathologies, including skin ulcers and urinary tract infections all contribute to patient suffering and a diminished quality of life (Hawryluk et al., 2008; Rossignol et al., 2007). While the cause of SCI varies widely, the most common form results from traumatic injury to the vertebral column (fracture/dislocation), and subsequent mechanical injury of the spinal cord (Rowland et al., 2008). In the developed world, motor vehicle accidents are the most common cause of traumatic SCI (50%), followed by falls and work related injuries (30%), violent crime (11%), and sports injuries (9%) (Ho et al., 2007). Given current societal trends, these risks are unlikely to change dramatically and therefore the related costs to the individual and society will persist. This means better treatment options and a greater understanding of SCI pathogenesis and recovery mechanisms is critical.

1.1.1 Pathophysiology of spinal cord injury

The pathophysiology of traumatic SCI is biphasic: first there is a primary phase that refers to the initial mechanical insult to the spinal cord; and then a secondary phase that involves both pathological and neuroprotective processes initiated by the primary injury. The mechanical insult leads to disruption of spinal tissue via compression, contusion, stretching and/or laceration, resulting in the severing of axons (Rowland et al., 2008). Processes occurring during the secondary phase are considered to be important contributors to SCI pathology as they can limit innate recovery mechanisms (neural plasticity), and persist throughout the acute (14 days), intermediate (6 months) and chronic (>6 months) stages of SCI (Lipton & Rosenberg, 1994; Norenberg et al., 2004; Rowland et al., 2008).

Immediately after the primary phase, secondary pathological mechanisms such as haemorrhage, ischaemia and oedema occur in the injured spinal cord. Subsequently, a
host of other factors alter the micro-environment in and around the lesion site and shape the outcome of the injury. These factors include the production and release of reactive oxygen species (ROS), excitotoxicity, ionic imbalance, Schwannosis, cyst/syrinx development, mesenchymal scar formation, reactive astrocystosis and inflammation (Norenberg et al., 2004; Rowland et al., 2008). Of particular importance during the secondary phase of SCI is the formation of the so-called ‘glial scar’. This structure is a chronic manifestation of the lesion and represents the end stage of reactive astrocystosis, a process involving hypertrophy and extended branching of activated astrocytes (Eddleston & Mucke, 1993; Norenberg et al., 2004). The astrogial processes arborise and extend into the damaged tissue to form an intermingled astroglial ‘web’ (Stichel & Muller, 1998). The physiological changes following reactive astrocystosis result in the secretion/expression of several cytokines, axon guidance, cell adhesion and extracellular matrix molecules within the glial scar (Stichel & Muller, 1998). Importantly, some of these sequelae have been shown to limit or inhibit neuronal plasticity and regeneration. Key pathophysiological events are summarised in Table 1.1.

Table 1.1. Key pathological events and phases of SCI

<table>
<thead>
<tr>
<th>Time After Injury</th>
<th>≤ 2 Hours (Immediate)</th>
<th>≤ 48 Hours (Early Acute)</th>
<th>≤ 14 Days (Subacute)</th>
<th>≤ 6 Months (Intermediate)</th>
<th>≥ 6 Months (Chronic/Late)</th>
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<tbody>
<tr>
<td>Injury Phase</td>
<td>1˚/2</td>
<td>2˚</td>
<td>2˚</td>
<td>2˚</td>
<td>2˚</td>
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<tr>
<td>Processes/Events</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Primary mechanical injury</td>
<td>Spinal oedema</td>
<td>Further inflammatory processes (macrophage infiltration)</td>
<td>Continued formation of glial scar</td>
<td>Prolonged Wallerian degeneration</td>
<td></td>
</tr>
<tr>
<td>Severeing of axons</td>
<td>ROS production</td>
<td>Development of glial scar (reactive astrocytes /astrocystosis)</td>
<td>Potential cyst and syrinx formation</td>
<td>Further stabilisation of lesion</td>
<td></td>
</tr>
<tr>
<td>Spinal haemorrhage</td>
<td>Excitotoxicity (glutamate)</td>
<td>BBB repair</td>
<td>Stabilisation of lesion</td>
<td>Potential cyst and syrinx formation</td>
<td></td>
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<tr>
<td>Microglial activation</td>
<td>Haemorrhage and necrosis</td>
<td>Resolution of oedema</td>
<td>Wallerian degeneration</td>
<td>Schwannosis</td>
<td></td>
</tr>
<tr>
<td>Release of inflammatory factors</td>
<td>BBB permeability (neutrophil invasion)</td>
<td>Neovascularisation</td>
<td>Mesenchymal scar formation</td>
<td></td>
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</tr>
<tr>
<td>Demyelination</td>
<td>Axonal swelling and neuronal death</td>
<td>Schwannosis</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Systemic and spinal shock</td>
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Adapted from Rowland et al., 2008 and Norenburg et al., 2004. Definitions: demyelination = death of oligodendrocytes (loss of myelin sheath surrounding axons); spinal shock = temporary loss of reflexes and sensorimotor function below the lesion site; Wallerian degeneration = anterograde disintegration of axons and their myelin sheaths following transection; mesenchymal scar = scar tissue consisting of fibrous connective tissue and collagen; glial scar = web of glial processes infiltrating the lesion site; schwannosis = aberrant proliferation of schwann cells with associated axons. Abbreviations: ROS = reactive oxygen species; BBB = blood brain barrier.
Despite the influence of these inhibitory factors, patients with incomplete SCI usually experience some level of spontaneous recovery (Burns et al., 1997; Dietz et al., 1998; Rossignol et al., 1999; Schwab & Bartholdi, 1996). The mechanisms underlying this ‘recovery’ phenomenon are thought to include: the resolution of spinal shock (temporary flaccid paralysis and loss of tendon reflexes below the level of the lesion) (Ditunno et al., 2004; Hiersemenzel et al., 2000), remyelination of surviving axons (Cao et al., 2010; Gensert & Goldman, 1997; Jeffery & Blakemore, 1997; Schwab & Bartholdi, 1996), cortical reorganisation (Bareyre et al., 2004; Fouad et al., 2001; Ghosh et al., 2009; Raineteau & Schwab, 2001) and, importantly, axonal sprouting/regeneration within the spinal cord (Bareyre et al., 2004; Courtine et al., 2008; Deumens et al., 2005; Fenrich & Rose, 2009; Fenrich et al., 2007; Filli et al., 2014; Raineteau & Schwab, 2001; Weidner et al., 2001). While these recovery mechanisms occur spontaneously, we now know they are heavily limited by the ‘growth inhibitory’ post-SCI environment.

Improved understanding of this growth-retarding environment might reveal potential therapeutic targets to improve long-term SCI outcomes.

1.1.2 Impediments to recovery following SCI

After SCI, neuronal regeneration, formation of new synapses and functional recovery are limited in adult higher vertebrates (Larner et al., 1995; Schwab & Bartholdi, 1996). Although the severed ends of most axons in the CNS reseal and begin to extend after transection, these extensions eventually adopt a ‘club-like’ morphology and cease regenerating (Cajal, 1991; Deumens et al., 2005; Norenberg et al., 2004; Schwab & Bartholdi, 1996). Factors that are intrinsic to adult CNS neurons, such as Ca\(^{2+}\) and cAMP signaling, are considered partly responsible for this phenomenon (Afshari et al., 2009; Cai et al., 2001; Fawcett, 1992; Giovanni, 2009; Goldberg et al., 2002; Plunet et al., 2002). Factors that are extrinsic to regenerating neurons (i.e. other constituents within the CNS environment) also play a role in limiting neuronal regeneration and sprouting. Albert Aguyao’s group elegantly demonstrated the importance of such extrinsic factors in the early 1980s. They showed that CNS neurons are capable of significant regeneration when allowed to grow within a peripheral nervous system (PNS) environment versus a CNS environment (David & Aguayo, 1981; Richardson et al., 1980). A later study showed CNS myelin inhibits neuronal regeneration and sprouting (Schwab & Thoenen, 1985) and subsequent work has now identified numerous molecular substrates contributing to this (Figure 1.1). These include: myelin-associated glycoprotein (MAG) (McKerracher et al., 1994; Mukhopadhyay et al., 1994), oligodendrocyte myelin glycoprotein (OMgp) (Wang et al., 2002), Nogo (Chen et al., 2000), semaphorin 4D (Moreau-Fauvarque et al., 2003), Netrin-1 (Low et al., 2008), Ephrin B3 (Benson et al., 2007).
repulsive guidance molecule (RGM) (Hata et al., 2006), and bone morphogenic protein (BMP) (Matsuura et al., 2008). As mentioned above, the glial scar also expresses factors that limit neuronal regeneration and sprouting following SCI. These inhibitory molecules include: chondroitin sulphate proteoglycans (CSPGs) (Lee et al., 2010a; McKeon et al., 1991), collagen IV (Stichel et al., 1999) and EphA4 (Goldshmit et al., 2004) (Figure 1.1).

Despite this growth inhibitory environment, some populations of mammalian CNS neurons demonstrate considerable capacity for regeneration post-SCI. These include certain ascending, long-tract sensory neurons (Inman & Steward, 2003) and, of particular importance, some types of spinal interneurons such as propriospinal neurons (Fenrich & Rose, 2009; Fenrich et al., 2007; Siebert et al., 2010a; Siebert et al., 2010b). The capacity of certain neurons to participate in SCI recovery has renewed hope that the spinal cord may actually possess the framework for more complete recovery from SCI.
1.1.3 Experimental SCI research, and an opportunity for electrophysiological ‘input’

Current preclinical SCI research is focused on either: minimizing initial spinal cord damage after trauma (surgical decompression, hypothermia, administration of anti-inflammatory agents or inhibition of excitotoxicity); alleviating the growth inhibitory micro-environment of the lesion and/or adult CNS (application of neurotrophic factors, and inhibition of myelin and glial scar associated inhibitors of neurite growth and gene therapy in growth resistant neuron populations); replacement of neurons or glia (neural grafts and stem cells); or engagement and modification of surviving spinal circuits (physical neurorehabilitation, epidural stimulation, pharmacological activation of locomotor circuits and intermittent hypoxia). For details on individual studies encompassed within these subcategories, the reader is referred to excellent reviews by Filli and Schwab (2012), and Ramer et al. (2014). Despite the vast number of preclinical therapeutic options, few have translated into successful clinical trials (Filli & Schwab, 2012; Hawryluk et al., 2008; Hug & Weidner, 2012; Ramer et al., 2014; Rowland et al., 2008). In fact, physical neurorehabilitation remains the only mainstay of care for SCI patients (Filli & Schwab, 2012; Galea, 2012).

While it is not entirely clear why most new SCI therapies have failed to translate to the clinic, lessons from stroke research demonstrate that exceptional rigor is required during the basic research phase of any potential treatment if it is to successfully translate into the clinical environment (Howells et al., 2014; Philip et al., 2009). A major component here is the tools and approaches used to study the pathophysiological and recovery mechanisms involved in the disease state. While anatomical, molecular, genetic, and behavioural analyses are commonly employed to study SCI processes, the use of intracellular neurophysiology (i.e. sharp electrode or whole-cell patch-clamp recording) is practically non-existent, except for a relatively small number of pioneering studies (Beaumont et al., 2004; Beaumont et al., 2008; D’Amico et al., 2014; Dougherty & Hochman, 2008; Fenrich & Rose, 2009; Husch et al., 2012; MacFarlane & Sontheimer, 1997; Taccola et al., 2008). This is surprising, given intracellular electrophysiology is a direct way of studying the functionality of newly formed synapses and provides unequivocally detailed information about the physiology and excitability of target neurons (Sakmann & Neher, 1984). For example, (Fenrich & Rose, 2009) performed sharp electrode recordings of cat motoneurons to study the nature of regenerated commissural spinal neurons after chronic SCI. These data not only confirmed the functional nature of these new inputs, but also their strength and post-synaptic effects on target neurons. More recently, Husch et al. (2012) demonstrated that V2a neurons (a subset of genetically defined interneurons forming part of the locomotor central pattern
generator, CPG) became hypersensitive to serotonin after SCI, while passive membrane properties and firing patterns remained largely unchanged. Importantly, this study employed whole-cell patch-clamp electrophysiology, which provides a better signal to noise ratio than sharp intracellular electrode recording. This allows high-resolution current- and voltage-clamp manipulations and analysis (Sakmann & Neher, 1984). Whole-cell patch-clamp electrophysiology is highly relevant to SCI research as it has the potential to uncover subtle changes in intrinsic neuronal membrane properties and most importantly, reveal the presence of both powerful and weak synaptic inputs onto recorded neurons.

Despite these few studies, neurophysiological data of this type has long been lacking in preclinical SCI research (Adams et al., 2007). I believe the limited work carried out to date highlights the importance of functional electrophysiological data in addition to anatomical, molecular, genetic, and behavioral information for developing a more complete picture of the pathophysiological and recovery processes that occur after SCI.

1.1.4 Conclusions and rationale for further study

SCI causes a severe and heterogeneous loss of function and is a major burden on individuals, their families and society. Over the past twenty years, a great deal has been discovered about the pathophysiological mechanisms of the primary and secondary phases of SCI, resulting in a gamut of potential therapeutic targets. Despite a range of preclinical studies showing promise, clinical translation has been slow, paralleling other translational efforts such as stroke therapies. While there are many potential reasons for this (see Howells et al. (2014)), I believe the incorporation of high-resolution, cellular neurophysiological techniques (such as whole-cell patch-clamp electrophysiology) in preclinical studies would provide an extra dimension of information in SCI research. Therefore, the aims of Chapters 2 and 3 in this thesis are to develop an acute spinal cord slice preparation that can be used to study the functional properties of neurons in both normal and injured spinal tissue.

Chapter 2: A horizontal slice preparation for examining the functional connectivity of dorsal column fibres in the mouse spinal cord. This chapter describes an acute, horizontal spinal cord slice preparation that allows visually guided, whole-cell patch-clamp electrophysiology along the rostrocaudal length of the spinal cord. Dorsal column (DCol) fibres are maintained to allow
examination of functional connectivity between axonal pathways and spinal neurons.

Chapter 3: Exercise training after spinal cord injury selectively alters synaptic properties of neurons in the adult mouse spinal cord. This chapter uses the horizontal spinal cord slice preparation developed in Chapter 2 to study the physiology and connectivity of neurons surrounding a spinal lesion. In addition, this study compares recovery from SCI with and without a therapeutic intervention. Specifically, the intrinsic and synaptic properties of spinal neurons and DC10 pathways are assessed after SCI in naïve and treadmill trained mice.

1.2 Propriospinal neurons

For over a century, the existence of an intraspinal network of interneurons connecting spinal cord segments has been acknowledged. This network, now called the propriospinal system, was first studied by Sir Charles Sherrington who demonstrated that axons “springing from the grey matter” of the spinal cord connect both proximal and distal spinal segments. He went on to argue that multiple spinal segments must communicate with each other to allow complex or “long” motor reflexes (Sherrington & Laslett, 1902, 1903). Several decades later, David Lloyd provided compelling electrophysiological evidence that lumbosacral motor pools receive descending inputs that are relayed by propriospinal neurons (PNs) located in the cervical spinal cord (Lloyd, 1942; Lloyd & McIntyre, 1948). Subsequent anatomical and physiological investigations during the second half of the 20th century confirmed that this intraspinal network of PNs plays a critical role in motor reflexes, voluntary movement, and sensory processing (Alstermark et al., 2007; Conta & Stelzner, 2009; Cowley et al., 2010; Foreman, 2000; Jankowska, 1992; Kostyuk & Vasilenko, 1979; Pierrot-Deseilligny & Burke, 2005).

Over the last decade, interest in the propriospinal system has undergone a renaissance of sorts; mostly because of an important study by Bareyre and colleagues (Bareyre et al., 2004) that identified a role for PNs in recovery from incomplete SCI in rats. They demonstrated that severed corticospinal tract (CST) axons form new contacts on surviving PNs following SCI. These new contacts are maintained on PNs that project past the lesion and effectively form an anatomical bridge that allows transmission of descending signals (for example, motor commands) to neurons below the lesion. In addition, surviving PNs that terminate caudal to the lesion increase their contacts with motoneurons. This neural plasticity, which leads to the formation of new spinal circuits,
is now considered an important substrate for functional recovery after incomplete SCI.

1.2.1 Definition of propriospinal neurons

The spinal cord contains many types of interneurons that can be assigned into various classes according to anatomical, physiological and/or molecular criteria. PNs differ from other spinal interneurons as they project to and influence activity in spinal segments outside those where their cell bodies are located. The prefix proprio (derived from the Latin, proprius) means ‘own’, and therefore the term ‘propriospinal neuron’ refers to an interneuron that is contained entirely within the spinal cord. It is widely accepted, however, that the term ‘propriospinal’ also encompasses spinal interneurons that not only project to other spinal segments, but also to supraspinal centres (Alstermark et al., 1981a; Jankowska, 1992; Nathan & Smith, 1959; Skinner et al., 1989). In such cases, the term ‘propriospinal’ is misleading as the neuron and all its processes are not confined entirely to the spinal cord. Additionally, PNs may also have intra- as well as inter-segmental projections (Matsuyama et al., 2004). For the purpose of the present work, the following definition is used: an interneuron is deemed ‘propriospinal’ if its cell body is located in the spinal cord and its axon terminals project to a different spinal segment. This may include interneurons that have supraspinal or intrasegmental projections, in addition to their inter-segmental projection(s). It is also important to note that PNs can project bidirectionally in the rostrocaudal plane (Saywell et al., 2011; Skinner et al., 1989).

1.2.2 Anatomy and organisation of propriospinal neurons

This summary is based largely on the work of Janos Szentagothai and later, Hans Kuypers and colleagues, who described the organizational principles for PNs using approaches that labeled degenerating fibres after discrete lesions within the spinal cord, or by labeling PN cell bodies via retrograde tracing.

Anatomically, PNs are classified as either ‘short’ or ‘long’ based on the length of their axon projections. Contention still exists as to what defines a short or long PN (see Cowley et al. (2010)). However, I will consider short PNs (SPNs) as those spanning over one to six spinal segments, whereas long PNs (LPNs) project over more than six spinal segments (Conta & Stelzner, 2009). While an anatomical definition which delineates SPNs and LPNs is relatively straight-forward, it does raise issues regarding the spinal interneuron subtypes they encompass. For example, many types of spinal interneurons (including several of the genetically defined interneuron populations) send axons outside their local segment for ‘short’ distances (Goulding, 2009; Lundfald et al., 2007; Pierani et al., 2001; Saueressig et al., 1999) and would therefore be regarded as SPNs. Perhaps
LPNs comprise a more discrete population of interneurons (which is reflected in their anatomy; see Table 1.2). For simplicity, the following sections describe the anatomy and organisation of SPNs and LPNs in the most general sense (for detailed morphological studies, see Matsuyama et al. (2004); Saywell et al. (2011); Szucs et al. (2010)).

**1.2.2.1 Short Propriospinal Neurons**

The cell bodies of SPNs are located in most laminae of the spinal cord grey matter throughout the rostrocaudal extent of the cord, and can project either within or between spinal regions (i.e. thoracic, lumbar, and so forth). SPNs are classified according to the direction of their projections (ascending or descending) and whether these projections cross the midline (contralateral or ipsilateral). The location of SPN cell bodies and axons, along with their projection and termination patterns within the spinal enlargements are summarised in Table 1.2 and Figure 1.2C and E.

The anatomical arrangement of SPNs in the limb enlargements (outlined in Table 1.2 and Figure 1.2C) suggests the cell bodies and axons of the short propriospinal system are organised into ‘longitudinal columns’ according to the location of their projection targets. Based on this arrangement, Hans Kuypers and colleagues proposed a somatotopic organisation of SPNs, in line with classical studies by George Romanes and James Sprague (Romanes, 1951; Sprague, 1948) which demonstrated a medio-lateral division between motoneurons and their target muscle groups, within the limb enlargements (i.e. medial motoneuron pools innervate axial muscles, whereas the lateral motoneuron pools innervate more distal muscle groups) (Figure 1.2B). Consistent with this scheme, SPNs originating in the ventro-medial region of the grey matter (lamina VIII and the medially adjoining part of lamina VII), innervate/influence motoneurons supplying axial muscles because their axons terminate within and around medial motoneuron pools. Correspondingly, SPNs with cell bodies located in lateral regions (lateral parts of laminae V-VII) innervate motoneurons supplying more distal limb muscles, as their axons terminate in the vicinity of the lateral motoneuron pools (Molenaar & Kuypers, 1978; Rustioni et al., 1971; Sterling & Kuypers, 1968) (Figure 1.2A, B and C).

**1.2.2.2 Long Propriospinal Neurons**

LPNs (PNs projecting over more than six spinal segments) which are involved in locomotor activity reciprocally connect cervical and lumbar enlargements (Figure 1.2D). The first anatomical distinction that can be made with respect to LPNs is whether their
Figure 1.2. Organisation of the spinal cord and major propriospinal circuits. (A) When viewed in cross-section, the grey matter of the spinal cord can be divided into ten regions, termed spinal lamina, denoted with Roman numerals (I-X). The white matter is divided into various funiculi: the dorsal funiculus (DF), dorsolateral funiculus (DLF), lateral funiculus (LF), ventrolateral funiculus (VLF), ventral funiculus (VF), and ventro-medial funiculus (VMF). (B) Motoneurons are organised into medial motoneuron pools (IX (m)) that innervate axial muscles and lateral motoneuron pools (IX (l)) that innervate more distal muscles. (C and D) Organization of short (SPN) and long propriospinal neurons (LPN). Only PN cell bodies with known descending projection patterns and terminations are presented (see Table 1.2 for details on cell body location). The axons of SPNs span up to six segments and project to medial and lateral motoneuronal groups and also non-motoneuronal regions corresponding to the location of their cell bodies. Their organisation matches the somatotopy described for motoneuron pools: ie, medial SPNs terminate in the medial or axial muscle motoneuron pool, whereas lateral SPNs terminate in the lateral or distal muscle motoneuron pool. LPNs involved in locomotor control connect cervical and lumbar enlargements and project predominantly to non-motoneuronal elements (often contralaterally). (E) SPN axons travel in all funiculi at the grey matter border, whereas LPN axons travel predominantly in the ventral and lateral funiculi near the outer boundaries of the white matter. Other abbreviations used: CE = cervical enlargement; LE = lumbar enlargement.
cell bodies are located rostrally (within the cervical enlargement) and project caudally, or vice-versa. These two populations are termed long descending PNs (LDPNs) and long ascending PNs (LAPNs), respectively. The anatomy of LDPNs is better understood than that of LAPNs because retrograde tracing studies can label ‘pure’ LDPN populations. In contrast, LAPN neurons cannot be distinguished, using retrograde labeling techniques, from ‘fibres of passage’ belonging to supraspinally projecting interneurons. Even so, the few degeneration studies undertaken on LAPNs show they have comparable cell body location (Molenaar & Kuypers, 1978) and termination patterns to LDPNs (Giovanelli Barilari & Kuypers, 1969; Matsushita & Ueyama, 1973), with the exception of a dense ipsilateral pathway projecting to the ventrolateral motor nucleus in caudal cervical segments (Brockett et al., 2013). The location of LPN cell bodies and axons, along with their projection and termination patterns in the spinal enlargements are summarised in Table 1.2 and Figure 1.2D and E.

Table 1.2. Organisation of SPNs and LPNs within cervical and lumbar enlargements.

<table>
<thead>
<tr>
<th>PN Type</th>
<th>Cell body location</th>
<th>Axon location</th>
<th>Projection pattern</th>
<th>Termination pattern</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Short (SPN)</td>
<td>Most laminae (excluding lamina IX). Particularly concentrated in the intermediate zone (lamina VII).</td>
<td>All funiculi. Typically in deep layers of the white matter, bordering the grey matter.</td>
<td>SPNs with medially located cell bodies often project contraterally. Laterally located SPNs project ipsilaterally. Medial SPNs project over more segments than lateral SPNs. SPNs can project bidirectionally (i.e. rostral and caudal).</td>
<td>Lamina IX and regions of the grey matter corresponding to the location of their cell bodies (e.g. SPNs with laterally located cell bodies terminate in lateral motoneuron pools and also lateral regions of the grey matter).</td>
<td>Ni et. al., 2014; Liu et. al., 2010; Burton and Loewy, 1976; Conta and Stelzner 2004; Matsushita, 1970; Menetrey et al., 1985; Molenaar and Kuypers, 1978; Petko and Antal, 2000; Rustioni et al., 1971; Saywell et al., 2011; Sterling and Kuypers, 1968; Zentagothai, 1951, 1964; Yezierski et al., 1980.</td>
</tr>
<tr>
<td>Long (LPN)</td>
<td>Majority in laminae VIII and medial lamina VII. Also located in laminae I, IV-VI and X.</td>
<td>Majority in ventral and lateral funiculi. Typically in superficial layers of the white matter.</td>
<td>High incidence of both contra- and ipsilateral projections. LPNs can project bidirectionally (i.e. rostral and caudal).</td>
<td>Laminae V-VIII. Also sparsely in lamina IX. A dense ipsilateral LAPN pathway terminates in the C7-T1 ventrolateral motor nucleus.</td>
<td>Brockett et. al., 2013; Ni et. al., 2014; Bareyre et al., 2004; Burton and Loewy, 1976; Conta and Stelzner, 2004; Giovanelli Barilari and Kuypers, 1969; Jankowska et al., 1974; Lloyd, 1942; Matsushita et al., 1979; Matsushita and Ueyama, 1973; Menetrey et al., 1985; Molenaar and Kuypers, 1978; Rustioni et al., 1971; Saywell 2010; Skinner et al., 1979.</td>
</tr>
</tbody>
</table>

Note: LPN organisation is derived from a large literature on LDPN anatomy. LAPN organisation is considered to follow a similar pattern, but based on smaller literature (see Section 1.2.2.2).

1.2.3 Neurochemistry of propriospinal neurons

Recent studies have begun to uncover the molecular phenotype of several PN populations. In terms of neurotransmitter phenotype, lumbar SPNs with descending projections to contralateral motor nuclei have mostly excitatory axons (vesicular glutamate transporter (VGLuT2) positive; 75%), with the remainder being inhibitory (γ-aminobutyric acid (GABA) and/or glycine positive; ~20%) (Liu et al., 2010). This ratio is mirrored in an LAPN pathway from L1 to the ventrolateral motor nucleus in C7/C8.
contains 85% excitatory axons (VGlurT2 positive), and 15% inhibitory axons (vesicular GABA transporter (VGAT) positive) (Brockett et al., 2013). *Premotor* LDPNs, together with descending premotor thoracic SPNs are composed of approximately equal numbers of excitatory (VGlurT2) and inhibitory (vesicular inhibitory amino acid transporter (VIAAT)) neurons (between 40-50% of each type) (Ni et al., 2014).

In addition to the use of neurotransmitters to classify various interneuron types, calcium binding proteins have been used to differentiate subpopulations of spinal neurons (Hughes et al., 2012), and have been identified various PN populations. The ascending lumbar SPN population contains 25% calretinin and/or calbindin positive neurons, while 15% and 4% of LDPNs express calretinin and calbindin, respectively (Brockett et al., 2013; Liu et al., 2010). Interestingly, almost no premotor LDPNs or descending premotor thoracic SPNs express calretinin, calbindin, or parvalbumin (Ni et al., 2014). Not surprisingly, choline acetyltransferase (ChAT) is not present in LDPNs, premotor LDPNs, or descending premotor thoracic SPNs (Brockett et al., 2013; Ni et al., 2014). There are, however, some ChAT positive ascending lumbar SPNs located in laminae VI-VII, and X (Liu et al., 2010). An important conclusion from these studies, is that the propriospinal system is comprised of a heterogeneous range of molecularly distinct neuronal subpopulations. *Thus, more work is required classify and characterise the various types of PNs.*

1.2.4 Inter-species comparisons

Most data on the organisation of PNs comes from work on cats. Other species have also been studied including monkey, rabbit, dog, and rat, using fibre degeneration and retrograde tracing techniques. Together, these studies reveal that while the general pattern of PN anatomy is conserved across species (as in Table 1.2), subtle differences exist between mammals (Burton & Loewy, 1976; Conta & Stelzner, 2004; Matsushita & Ueyama, 1973; Menetrey et al., 1985; Molenaar & Kuypers, 1978; Skinner et al., 1979). For example, in monkeys, PNs appear to have more contralateral projections (Molenaar & Kuypers, 1978), and the cell bodies of ventral LDPNs are located more medially and dorsally than in the cat (Skinner et al., 1979). Further, LAPN projections to a subset of motoneurons that innervate pectoralis major and latissimus dorsi are particularly well developed in the rabbit, compared to other mammals (Matsushita & Ueyama, 1973). While the true origin of these anatomical differences has not been conclusively resolved, they may reflect differing locomotor strategies used by each species.

An important note regarding the range of species that have contributed to our
current knowledge of the PN system is the clear absence of mice from this literature
(aside from very recent studies by Azim et al. (2014); Ni et al. (2014)). This is important,
as recent transgenic technologies can now be readily applied to the mouse to mark
various neuron types and study the cellular/molecular aspects of SCI. For these reasons, I
believe it is now crucial to assess the anatomy and organisation of PNs in this species.

1.2.5 Function of propriospinal neurons

Functionally, the propriospinal system participates in a wide variety of tasks. These include the integration/modulation of inputs from descending supraspinal pathways (transmitting, for example, motor commands from the brain) and peripheral afferents (carrying sensory information). In addition to the integration and relay of information, PNs are also important for synchronising activity in motor circuits throughout the length of the spinal cord. The following sections provide two key examples of the critical role PNs play in spinal cord function.

1.2.5.1 The C3-C4 propriospinal system

An impressive body of work from Bror Alstermark and colleagues has shown a
population of PNs, located in upper cervical segments (C3 and C4) in the cat, is critical
for certain CST dependent forelimb motor tasks (Alstermark & Isa, 2012). This
population is termed the ‘C3-C4 propriospinal system’ and its role is to transmit CST
input, as well as convergent input from the rubro-, tecto-, and reticulo-spinal tracts, to
motoneurons in segments C6 to T1 that innervate the forelimb (Alstermark et al., 1984a;
Illert et al., 1981; Illert et al., 1978; Illert et al., 1977). These PNs relay descending motor
commands (Alstermark et al., 1984a; Illert et al., 1977; Illert & Tanaka, 1978), which,
based on selective spinal lesions, are essential for ‘target-reaching’ behaviour
(Alstermark & Kummel, 1990; Alstermark et al., 1981b; Pettersson et al., 1997). These
PNs also receive significant input from peripheral afferents (mostly via inhibitory
segmental interneurons) to permit feedback from active muscles (Alstermark et al.,
1984b; Illert et al., 1978; Illert et al., 1977) (Figure 1.3). This spinal circuitry allows for
rapid correction of movement errors, while maintaining convergent supraspinal
control/influence (Illert et al., 1978; Illert et al., 1977).

In addition to their motoneuronal projections, C3-C4 PN collaterals synapse on
other important targets. In cats, an estimated 84% of C3-C4 PNs have an ascending
collateral projection to the lateral reticular nucleus (LRN) (Alstermark et al., 1981a; Illert
& Lundberg, 1978), which in turn provides mossy fibre input to the cerebellum (Apps &
Trott, 1997) (Figure 1.3A). In addition, spino-reticular and spino-cerebellar neurons
Figure 1.3. The C3 – C4 propriospinal system. A group of well-characterized PNs, located in cervical segments C3-C4, relay signals from supraspinal centres and the periphery to C6-T1 motoneurons involved in forelimb motor control. (A) C3-C4 PNs receive monosynaptic excitatory input and disynaptic inhibition (feed-forward inhibition; FFI) from CST neurons (green). They also receive monosynaptic excitatory input (brown) from other supraspinal centres such as rubro-, tecto- and reticulo-spinal neurons. In turn, C3-C4 PNs (blue) provide monosynaptic input to forelimb motoneurons (red) to mediate ‘target reaching’ behaviour. Peripheral afferents from the ‘target-reaching’ forelimb provide disynaptic inhibition to C3-C4 PNs via interposed segmental INs (feed-back inhibition; FBI). Additionally, many C3-C4 PNs send collaterals to the lateral reticular nucleus (LRN). (B) C3-C4 PNs can also inhibit forelimb motoneurons. This can occur via direct projections from inhibitory C3-C4 PNs (left panel) or via an indirect segmental IN pathway (right panel). For clarity, not all C3-C4 propriospinal system connections are illustrated. For more detailed circuit descriptions, see Alstermark et al., 2012; and Pierrot-Deseilligny and Burke, 2005.
located in C6-T1 segments receive excitatory input from C3-C4 PNs (Alstermark et al., 1990b). In combination with the direct LRN projections, these ascending projections are thought to provide supraspinal centres with a ‘copy’ of the activation pattern and output of the C3-C4 propriospinal system (Alstermark et al., 1990a; Alstermark et al., 2007; Alstermark et al., 1981a). Another target of C3-C4 PNs are LDPNs that project to more caudal spinal segments (Alstermark et al., 1991). These connections may be important for preparatory movements of the hindlimbs prior to and during targeted reaching by the forelimbs.

Regarding their molecular origins, a significant population of C3-C4 PNs was defined as a subtype of the ‘V2a’ (Chx10 positive) interneuron population in mice (Azim et al., 2014). The Azim group studied the role of C3-C4 PNs by specifically ablating them using a virally targeted diphtheria toxin receptor strategy, or optogenetic stimulation. In both cases, mice exhibited severe deficits in targeted reaching behaviour, demonstrating the previous findings in cats are conserved in mice. Clearly, a major advantage of using mice is the ability to accurately and specifically probe the structure and function of spinal cord circuits with our current knowledge of their developmental genetics (Brownstone & Wilson, 2008; Gosgnach, 2011; Goulding, 2009; Grillner & Jessell, 2009) and the vast array of virally encoded transgene delivery and optogenetics techniques (Deisseroth, 2011; Wickersham et al., 2007).

To summarise, C3-C4 PNs function as spinal processing ‘nodes’ for multiple supraspinal and peripheral pathways, allowing the precise and smooth activation of forelimb muscles. In addition, their unique intersegmental projection pattern allows the dissemination of these activation patterns to distant spinal and supraspinal targets.

### 1.2.5.2 Reciprocal connection of the cervical and lumbar motor circuits

The ability of PNs to facilitate communication between motor circuits in cervical and lumbar enlargements is well established (Alstermark et al., 1987b; Jankowska et al., 1974; Jankowska et al., 1973; Lloyd, 1942; Lloyd & McIntyre, 1948; Miller et al., 1971, 1973a; Miller et al., 1973b; Miller et al., 1975; Schomburg et al., 1978; Skinner et al., 1980; Vasilenko, 1975). Many of the earlier studies focused on facilitation and inhibition of motor reflexes. For example, Miller et al. (1973a) demonstrated that forelimb motor reflexes were facilitated or inhibited via a LAPN system that is activated by peripheral hindlimb afferents. The pattern of facilitation and inhibition, observed in these motor reflexes, mirrors forelimb/hindlimb coordination during natural stepping. The LAPN system was therefore proposed to play an important role in locomotion by coupling neural activity in cervical and lumbar enlargements.
More recent studies using isolated spinal cord preparations, have demonstrated a clear role for PNs in synchronising activity in cervical and lumbar central pattern generators (CPGs) (Ballion et al., 2001; Juvin et al., 2012; Juvin et al., 2005; Zaporozhets et al., 2006) (Figure 1.4). For example, extending on the work of Ballion et al. (2001), Juvin et al. (2005) used an in vitro, neonatal rat spinal cord preparation to demonstrate the importance of propriospinal connections in maintaining synchronised motor output between fore- and hindlimb associated ventral root bursts. Inactivation of the propriospinal circuitry between the spinal enlargements (via sucrose blockade) resulted in the previously time-locked motor output of cervical and lumbar CPGs becoming completely independent and out of phase. Furthermore, a recent in vivo study used a transgenic viral strategy to reversibly (and specifically) inhibit LAPNs with tetanus toxin (Pocratsky et al., 2014). Inactivation of LAPNs caused a breakdown in normal interlimb coordination, resulting in a symmetrical hop-like gait during volitional locomotion.

Figure 1.4. Coupling of cervical and lumbar locomotor circuits. CPGs located in the cervical and lumbar enlargements (yellow) underlie locomotor activity in forelimbs and hindlimbs, respectively. Intervening propriospinal circuitry (blue) connects cervical and lumbar CPGs and is critical for co-ordinating and maintaining appropriate forelimb/hindlimb coordination during locomotion.

Apart from shedding light on locomotor mechanisms, the above examples have important implications for functional recovery following SCI. Specifically, regeneration or re-routing of these propriospinal pathways after SCI, via short or long circuits, could provide the substrate for significant locomotor recovery.
1.2.6 The propriospinal system and functional recovery from spinal cord injury

For some time, it has been suggested that the propriospinal system plays an important role in functional recovery after SCI (Jane et al., 1964; Selzer, 1978). Importantly, several key studies have recently provided compelling evidence to this effect. These publications have reported motor improvement in SCI animals via the generation of de novo intraspinal circuits that involve PNs. More specifically, descending supraspinal signaling is re-established through new connections with intact PNs that project past the lesion site and contact neurons and circuits that can shape motor function (Bareyre et al., 2004; Courtine et al., 2008; Filli et al., 2014; Vavrek et al., 2006).

Following a study in 2001 that documented increased sprouting of CST axons after thoracic SCI in rats (Fouad et al., 2001), Bareyre and colleagues (Bareyre et al., 2004) demonstrated many of these sprouting axons terminate on PNs in the cervical enlargement. Furthermore, CST sprouts on LDPNs, which projected past the thoracic lesion to lumbar segments were maintained, suggesting they retained functional relevance for post-SCI recovery. In contrast, CST contacts on SPNs, which did not project past the lesion, retracted twelve weeks after SCI. These data suggest that new CST connections with PNs that project to targets below the lesion are preferentially maintained, possibly through activity-dependent mechanisms. In addition, the termination pattern of LDPNs projecting to lumbar motoneuron pools also expanded, resulting in a significant increase in terminal arborisations onto motoneurons (Figure 1.5A). In line with these experiments, transynaptic tracing with pseudorabies virus (PRV) into hindlimb muscles labeled cervical LDPNs and pyramidal neurons in the motor cortex of SCI animals, thus confirming the existence of a newly formed pathway. The functional integrity of this new circuit was also validated electrophysiologically by recording evoked EMG signals in hindlimb muscles after intracortical microstimulation. EMG latency was longer in lesioned versus control animals, suggesting the presence of additional synaptic linkages in the injured animals. This work was extended by Filli et al. (2014), that demonstrated reticulospinal fibres also form new contacts with PNs above the lesion, which in turn increase their number of projections both contralaterally and ipsilaterally below the lesion.

An elegant series of experiments by Courtine et al. (2008) also added weight to the hypothesis that PNs are important for SCI recovery. They severed all supraspinal axons innervating the lumbar spinal cord using rostrocaudally staggered, transverse hemisections on opposite sides of the spinal cord in mice (Figure 1.5B). Importantly, this procedure preserved an intervening zone of spinal cord between each hemisection. When hemisections were performed ten weeks apart, locomotor and hindlimb stepping ability
recovered significantly, suggesting the intrinsic spinal circuitry between and around the lesions was capable of transmitting descending supraspinal input to lumbar motor circuits. In contrast, performing both hemisections at the same time resulted in permanent hindlimb paralysis. This observation indicates that time-dependent reorganisation of descending axons and PNs, between and around the lesions, is required for recovery. Finally, infusion of a high dose of N-methyl-D-aspartate (NMDA) between the hemisections completely abolished recovery (in high concentrations, NMDA acts as an axon-sparing excitotoxin). This confirmed that the observed locomotor recovery was not due to long distance regrowth of supraspinal axons. Rather, PNs located between the lesions were critical for re-establishing descending signal pathways in SCI animals.

Using a similar multi-lesion approach, (Cowley et al., 2008) demonstrated that the linking of spinal segments by PNs could allow descending motor commands to activate the lumbar locomotor CPG, despite transection of all supraspinal axons previously innervating the CPG region. After placing two rostrocaudally staggered, lateral hemisections on opposite sides of an isolated neonatal rat spinal cord (Figure 1.5B), locomotor activity could still be elicited in the lumbar CPG following stimulation of the brainstem. This means the signals that initiate locomotor activity in the lumbar cord can be propagated through PNs within and around the inter-lesion zone and most likely involves a commissural component (Cowley et al., 2008, 2010).

A study by (Fenrich & Rose, 2009) further emphasised the importance of commissural PNs (connecting the ipsi- and contralateral spinal cord) in recovery from SCI. They demonstrated severed axons of commissural PNs can regenerate and make functional synaptic connections with spinal motoneurons. After a midsagittal, cervical transection of the cat spinal cord, Neurobiotin stained processes of commissural PNs were shown to regenerate, over a seventy-two day period, through the lesion site and into the grey matter on the contralateral side. Immunohistochemistry showed the majority of terminal boutons on the regenerated axons contained synaptophysin (a synaptic vesicle marker), suggesting these new processes had formed synapses. The functional integrity of these synapses was confirmed by recording evoked synaptic responses in contralateral motoneurons following microstimulation of regenerated commissural PNs. Apart from confirming that PNs are capable of forming new, functional synaptic connections, one of the most significant findings from this work is that commissural PNs are capable of extending axons through a spinal lesion (which is rich in molecules that inhibit growth). Such growth through a spinal lesion by supraspinal axons has rarely been observed (Deumens et al., 2005; Fenrich & Rose, 2009). This suggests the axons of PNs differ
from supraspinal neurons in their ability to penetrate the growth inhibitory micro-environment of an SCI lesion.

Figure 1.5. Plasticity in PN circuits following SCI. (A) Following dorsal hemisection in the spinal cord, axons in descending pathways (e.g. CST in green) are severed. Available data suggests these axons do not re-establish their previous connections to targets via long distance regeneration (green ‘?’). Instead, recent data suggests that CST axons sprout (dashed green lines) onto intact PNs (shown in blue) that project past the lesion, terminating in spinal regions that are important for motor function. PNs also increase their own arborisations (dashed blue line) onto lumbar motor circuits (red) in response to SCI. (B) After rostrocaudally spaced hemisections, which essentially transect all supraspinal inputs to the lumbar spinal cord, functional recovery can occur via reorganisation of descending inputs with PNs located within and around the portion of spared spinal tissue.

Based on the above lesion studies, it would appear that PNs (in particular, those with commissural projections) constitute an attractive target for therapies that encourage neural plasticity. Further, the location, varied projection pattern and sheer number of PNs mean that a significant proportion of long and short PNs can survive even severe SCI. Indeed, there is good evidence that this is the case as Conta and Stelzner (2004) and Conta Steencken and Stelzner (2010) have demonstrated that a functionally relevant number of PNs, which cross a spinal lesion/contusion site, resist postaxotomy retrograde cell death and remain viable for at least sixteen weeks post-SCI. In addition, the ability of PNs to activate and coordinate spinal CPGs (Ballion et al., 2001; Cowley et al., 2008; Juvin et al., 2005) makes them ideally suited to bring about significant locomotor recovery. Further to this point, even non-specific electrical stimulation of propriospinal networks following SCI (leading to lumbar CPG activation) results in significant
improvements in locomotor performance (Yakovenko et al., 2007).

The suggestion that PNs comprise an ideal neural substrate for motor recovery is validated further by the observation that they respond to a range of neuroregenerative strategies in animal models of SCI (Brambilla et al., 2009; Deng et al., 2013; Houle et al., 2006; Iannotti et al., 2003; Ramon-Cueto et al., 1998; Takami et al., 2002; Xu et al., 1997; Xu et al., 1995). In fact, PNs mount a greater regenerative response than supraspinal neurons to certain therapeutic approaches. These include: endogenous application/administration of either glial derived neurotrophic factor (GDNF) or neurotrophin-4/5, and Schwann cell impregnated or peripheral nerve tissue grafts (Blesch & Tuszynski, 2003; Blesch et al., 2004; Guest et al., 1997; Houle, 1991; Xu et al., 1995).

In summary, PNs appear to be a key player in the ‘re-wiring’ of spinal circuits following SCI and present an attractive target for SCI therapies.

1.2.7 Conclusions and rationale for further study

The propriospinal system is organised strategically to integrate signals from supraspinal (and intraspinal) centres with those from the periphery to ensure motoneuron pools receive up-to-date and accurate motor commands (Figure 1.3). In addition, locomotion is underpinned by coupling of cervical and lumbar spinal enlargements via a network of interposed PNs (Figure 1.4). The location, projection pattern and dynamic role of the propriospinal system in normal spinal cord physiology affords this subset of interneurons a particularly critical role in spinal plasticity following SCI. PNs are clearly capable of forming de novo spinal circuits with either severed axons from descending pathways, or by connections via their own sprouts or arborisations (Figure 1.5).

Accordingly, PNs must be considered as potential targets when developing and optimizing new or existing SCI therapies. For example, physiotherapy-based approaches could benefit from understanding the types of physical activity that ‘engage’ or ‘recruit’ particular subsets of PNs (for example, SPNs versus LPNs). This may lead to entrainment of these networks, increased plasticity, and even greater improvements in motor function for people living with SCI (Dietz, 2010; Edgerton et al., 2006; Goldshmit et al., 2008).

As noted above, our limited knowledge of PNs stands as a barrier to us being able to take full advantage of this new area of SCI research. Specifically, the anatomical, molecular and physiological properties of PNs need to be further investigated. Such work is required for the development of highly focused treatments targeting PNs (and subsets thereof) to improve the functional outcome for SCI sufferers. Of particular importance, this work would benefit from being undertaken in mice, where the full complement of
molecular/genetic tools can be utilized (Brownstone & Wilson, 2008; Deisseroth, 2011; Goulding, 2009). Thus, the aim of Chapters 4 and 5 of this thesis is to further our knowledge of the mouse propriospinal system (in particular, LDPNs) by examining the following:

**Chapter 4: Location and projection pattern of LDPNs in mice.** Knowledge on the position of LDPN cell bodies and projection patterns has accumulated over many years in several mammalian species. However, this information remains incomplete for mice. Chapter 4 provides SCI researchers with a comprehensive map of the location of LDPNs, allowing a better understanding of the populations that are likely to be engaged by specific lesions (Conta & Stelzner, 2004). This information also allows speculation on the function of LDPN populations, based on what we know about the functional specialisation of neurons in the various spinal cord laminae (Heise & Kayalioglu, 2009; Rexed, 1952).

**Chapter 5: Inhibitory synaptic inputs onto LDPNs in mice.** This chapter describes the first use of the whole-cell patch-clamp technique to study identified LDPNs. It focuses on the properties of inhibitory inputs, as the integration of inhibitory synaptic inputs within spinal circuits is critical for sensorimotor processing (Gao et al., 2001). This work serves as an exemplar for the value of employing high-resolution electrophysiology to gain detailed functional insights into neurons forming the propriospinal system. Further, the data from this study provides important baseline information for future work that examines motor control and synaptic plasticity in LDPN populations following SCI.

In summary, the experiments described in this thesis have two major aims. The first is to develop an acute, *in vitro* horizontal spinal cord slice preparation for whole-cell patch-clamp electrophysiology and employ it in a mouse model of SCI. The second is to extend our current knowledge on an important mediator of functional recovery following SCI, the propriospinal system, by way of anatomical and electrophysiological characterisation of LDPNs in mice.
Chapter 2: A horizontal slice preparation for examining the functional connectivity of dorsal column fibres in the mouse spinal cord
Chapter 2: A horizontal slice preparation for examining the functional connectivity of dorsal column fibres in the mouse spinal cord

2.1 Introduction

In the rodent spinal cord, the DCols contain longitudinally orientated ascending and descending fibres that are often deliberately damaged in various SCI models. Typically, axons within the DCols, such as those in the CST, are then examined for signs of regenerative growth primarily using anatomical methods (Bareyre et al., 2005; Carmel et al., 2010; Goldshmit et al., 2004; Iseda et al., 2004; Liu et al., 2010). An alternative to this type of analysis is intracellular recording techniques such as sharp electrode and whole-cell patch clamp electrophysiology to measure intrinsic neuronal properties (passive membrane and AP properties), thus providing valuable data on cellular responses following SCI. Most importantly, these techniques can also provide unequivocal evidence for the existence of functionally viable synapses that have regenerated across a spinal lesion (Fenrich & Rose, 2009). Even though this information is essential if functional recovery from SCI is to be attributed to regenerated axons, such experiments are rarely attempted due to their technical difficulty.

To address this shortcoming, the aim of this study was to develop an acute horizontal spinal slice preparation that preserves longitudinal fibre tracts in a mouse model, providing a new way for SCI researchers to employ electrophysiological techniques to assess the intrinsic and synaptic properties of neurons in the vicinity of a spinal lesion. Importantly, this technique permits visually guided whole-cell patch-clamp electrophysiology for high-resolution, low noise current- and voltage-clamp analysis. This enables detailed investigation of intrinsic properties of recorded neurons along with the type and efficacy of DCol synaptic inputs. In addition, a calcium imaging preparation was developed to monitor the spread of excitation evoked by DCol stimulation. Horizontal spinal cord slices have been used in previous studies where preserving the longitudinal anatomy of the spinal cord was important (Baba et al., 1994; Bentley & Gent, 1994; Chery et al., 2000; Jef tinija, 1994; Magnuson & Dickenson, 1991; Magnuson et al., 1987; Nashmi et al., 2002; Phelan & Newton, 2000; Schneider & Perl, 1988; Whyment et al., 2004). However, this work is the first to undertake DCol stimulation in combination with whole-cell patch clamp electrophysiology and calcium imaging using horizontal slices.
Chapter 2: A horizontal slice preparation for examining the functional connectivity of dorsal column fibres in the mouse spinal cord

2.2 Methods

Whole-cell patch clamp recordings were made in horizontal spinal cord slices containing caudal thoracic and upper lumbar spinal segments. The DCols were preserved and bordered by dorsal horn and intermediate zone grey matter, permitting stimulation of descending fibres during whole-cell recordings. Calcium imaging during DCol stimulation was also performed to allow the spread of excitation, evoked by DCol stimulation, to be assessed at the macrocircuit level of analysis.

2.2.1 Horizontal slice preparation

All experimental procedures were approved by the University of Newcastle Animal Care and Ethics Committee. Mice (C57Bl/6, both sexes) were anaesthetised with ketamine (100 mg/kg, i.p.) and decapitated following loss of paw withdrawal and corneal reflexes. The entire vertebral column, ribs, and surrounding soft tissue was isolated and submerged in ice-cold, oxygenated, sucrose substituted, artificial cerebrospinal fluid (S-ACSF; containing in mM: 250 sucrose, 25 NaHCO₃, 10 glucose, 2.5 KCl, 1 NaH₂PO₄, 1 MgCl₂, and 2.5 CaCl₂; pH 7.3). Using a ventral approach, the spinal cord (segments T9 – S2) was dissected free and glued ventral side down to a cutting stage using cyanoacrylate glue (Loctite 454; Loctite, Caringbah, Australia). To ensure the dorsal surface of the spinal cord was parallel to the plane of the blade, extra glue was applied underneath the thoracic and upper lumbar segments to compensate for its narrow diameter compared to the mid-lumbar region. The stage and tissue was placed in a bath containing ice-cold, oxygenated S-ACSF, and horizontal slices (250 µm thick) were cut, moving from the rostral to caudal end of the cord, using a vibrating microtome (HM 650V; Microm; Walldorf, Germany) (Figure 2.1A).

Generally, two or three horizontal slices from the dorsal spinal cord were obtained. Because of the rapid taper of the sacral spinal cord, slices did not extend far beyond the lumbar region, and thus consisted primarily of caudal thoracic and lumbar spinal segments. The first and most superficial slice contained the DCols and superficial dorsal horns (laminae I-II). The second slice contained a wide band of the DCols, flanked by dorsal horn grey matter, and the white matter of the lateral funiculus. The third slice contained a thin band of DCols, deep dorsal horn/intermediate zone grey matter, and the white matter of the lateral funiculus (Figure 2.1A, right). Slice orientation was determined by examining the width and taper of each slice. The width of the rostral (thoracic) end was less than the lumbar segments, and the caudal (sacral) end generally tapered to a point. The spinal cord segments that maintained the most consistency with respect to DCol width and depth, and therefore examined in most detail, were L2 to L5.
Chapter 2: A horizontal slice preparation for examining the functional connectivity of dorsal column fibres in the mouse spinal cord

After cutting, slices were immediately transferred to a humidified storage chamber containing oxygenated ACSF (118 mM NaCl replacing sucrose in S-ACSF) and allowed to recover for one hour at room temperature (22-24°C) before recording commenced.

Figure 2.1. Slice preparation and recording configuration. (A) The thoraco-sacral spinal cord (T9 - S2: dashed lines) was excised and glued to a cutting stage (ventral side down). Two to three slices (250 µm thick) containing the DCols were cut in the horizontal plane. (B) A bipolar stimulating electrode was positioned in the DCols and recordings were made in the grey matter caudal to the stimulation site (left panel). The rostrocaudal distance between the stimulating electrode and recording location was measured along the length of the DCols (i.e. 1 - 2, right panel). The mediolateral position of the patch pipette was measured perpendicular to the DCols (i.e. 2 - 3, right panel). (C) Location of recorded neurons relative to the stimulating electrode

2.2.2 Stimulation of the DCols in horizontal slices

A bipolar stimulating electrode fabricated from insulated tungsten microelectrodes (100 µm shank diameter, 80 µm tip separation, 1-2 MΩ impedance; FHC, ME, USA) was positioned with the aid of a micro-manipulator (LBM-7; Scientifica, Bedford, UK) so the tips were inserted in the DCols, slightly below the tissue surface at the rostral end of the slice (Figure 2.1B, left panel). In some experiments, the
stimulating electrode was placed in the grey matter (Figure 2.5A, left panel). A single channel stimulus isolator (ISO-flex; AMPI, Jerusalem, Israel) was used to stimulate either the DCols or grey matter (0.1 ms duration current pulse). For whole-cell patch clamp experiments, a single current pulse (0.2 Hz) was used to evoke synaptic inputs in caudally located spinal neurons. For calcium imaging experiments, a stimulus train (100 Hz, 1 s duration) was used to evoke a calcium signal.

2.2.3 Whole-cell patch-clamp electrophysiology

Slices were transferred to a recording chamber and continually superfused with oxygenated ACSF (chamber volume 0.4 ml; exchange rate 4 - 6 bath volumes/min). A custom-made net constructed from an outer ring of flattened platinum wire (450 µm diameter) and an inner mesh of nylon fibres was used to hold the horizontal slices in place during recording. Patch pipettes (tip resistance 2.5 - 5 MΩ) were pulled from borosilicate glass (1.5 mm OD × 1.16 mm ID; Harvard Apparatus, Kent, UK) using an upright electrode puller (PC-10; Narishige, Tokyo, Japan). Pipettes were filled with an internal solution containing (in mM): 135 KCH3SO4, 6 NaCl, 2 MgCl2, 10 HEPES, 0.1 EGTA, 2 MgATP, and 0.3 NaGTP; pH 7.3 using KOH. The position of the recording pipette was controlled with a motorised micro-manipulator (MS-285; Sutter Instrument Company, Novato, USA). Slices were visualised using infrared differential interference contrast (IR-DIC) optics on a fixed stage, upright microscope (BX50WI; Olympus, Tokyo, Japan) equipped with an infra-red sensitive CCD camera (C2400-79H; Hamamatsu, Japan).

Individual neurons in the spinal grey matter were visually targeted and the whole-cell recording configuration was established in voltage-clamp mode. A holding potential of -70 mV was used to ensure excitatory neurotransmission through NMDA receptors was inhibited via Mg2+ blockade (Hille, 2001). The current profile resulting from a 5 mV hyperpolarising step (10 ms duration, averaged over 10 repetitions, filtered at 10 kHz) was used to measure neuron input resistance, and whole-cell capacitance. Series resistance was also measured, chiefly to monitor recording conditions throughout the experiment. After establishing the whole-cell recording configuration, the DCols were stimulated to evoke synaptic responses in recorded neurons. If a response was observed, the minimum stimulus intensity required for a stable postsynaptic response, termed ‘threshold’ (1T), was determined. Ten repetitions of 1T were recorded and then repeated at 1.2 × threshold (1.2T). In some cases, the holding potential was adjusted incrementally from -70 mV to -50 mV, -30 mV and -10 mV to reveal underlying inhibitory postsynaptic currents (IPSCs) (Figure 2.3D). In some experiments, the recording
configuration was switched to current-clamp mode, where the resting membrane potential (RMP) was determined after approximately 15 s. The postsynaptic voltage response to DCol stimulation was then assessed at membrane potentials of -60 mV, -45 mV and RMP by injecting bias current into recorded neurons. In addition, the action potential (AP) discharge pattern of recorded neurons was studied by injecting a series of depolarising, rectangular step-currents (800 ms duration, 20 pA increments, delivered every 8 s). To avoid damaging recorded neurons, step-current injection was stopped when steady-state voltage deflections were more depolarised than -20 mV.

Data were acquired using either an Axopatch 200B or Multiclamp 700B amplifier (Molecular Devices, Sunnyvale, USA), digitized online (sampled at 20 kHz, filtered between 2 - 10 kHz) via a computer interface (ITC-18; Instrutech, Long Island, USA) and stored on a Mac Mini computer (Apple Macintosh, Cupertino, USA) using AxoGraph X software (AxoGraph Scientific, Sydney, Australia).

2.2.4 Pharmacology

The following drugs were used: 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX, 10 µm; AMPA-kainate receptor antagonist (Honore et al., 1988; Yamada et al., 1989)), bicuculline (10 µm; GABA_A receptor antagonist (Curtis et al., 1971; Macdonald & Olsen, 1994)), and strychnine (10 µm; glycine receptor antagonist (Callister & Graham, 2010; Larson, 1969)). Drugs were prepared as stock solutions (1000 x final concentration) in distilled water and were diluted to the required concentration in ACSF immediately prior to bath perfusion. All drugs were purchased from Sigma Aldrich, NSW, Australia.

2.2.5 Electrophysiological data analysis

Data were analysed offline using AxoGraph X software. Recordings in which series resistance was > 20 MΩ, or changed by > 30% from baseline during an experiment were not included in the analysis, along with neurons with RMPs less negative than -50 mV (note: no correction was made for liquid junction potential). In addition, only postsynaptic responses detected using stimulus intensities ≤ 75 µA at 1T were further examined for their kinetic properties (see Section 2.3.1). This analysis was performed on an averaged 1.2T trace and included: latency, conduction velocity (measured using the direct distance between the stimulating electrode and recording location, and latency corrected for one synaptic delay of 1.27 ms at 23°C; Sabatini and Regehr, 1996), peak amplitude, rise time, half-width, decay time constant (single exponential, fitted over 20-80% of the decay phase) and charge (Figure 2.3B). For responses that contained more than one component (Figure 2.3A, TC and MC), the complex waveform precluded a full
kinetic analysis. AP height was measured as the difference between AP threshold (initial inflection point of the spike) and the maximum positive peak. After each recording, images of the slice, stimulating electrode and recording pipette location were captured and imported into Adobe Photoshop (Adobe, San Jose, USA) to create a photomontage. The distance between the stimulating electrode and recording location was then measured using ImageJ (Rasband, 1997-2015) (Figure 2.1B, right panel).

2.2.6 Calcium imaging

Immediately after sectioning, the slice was bath loaded with 10 \( \mu \)M Fluo-4 AM (Invitrogen, Carlsbad, USA) in oxygenated ACSF for 20 minutes. De-esterification of the dye and washing took place in the recording chamber while the slice was superfused with oxygenated ACSF for at least 30 minutes. Calcium signals were detected at low magnification (5X) using a FITC filter set (41001; Olympus, Tokyo, Japan). Images were captured at 2 Hz (150 to 160 ms exposure) using a cooled CCD camera (ProgRes MF cool; Jenoptik, Jena, Germany) and a computer-controlled shutter to minimise photobleaching (MAC 6000; Ludl Electronic Products, Hawthorne, USA). Fluorescence intensity was measured using the Time Series Analyser plug-in for ImageJ (Balaji, 2008). Fluorescent signals were calculated using the \( \Delta F(t)/F_0 \) ratio:

\[
\Delta F(t)/F_0 = (F(t) - F_0)/ F_0
\]

where \( F_0 \) is the pre-stimulus baseline fluorescence (averaged over 10 images) and \( \Delta F(t) \) is the deviation from this baseline (Neher & Augustine, 1992). All measurements were corrected for background fluorescence.

2.3 Results

Whole-cell patch clamp recordings (\( n = 70; \) input resistance \( 337.1 \pm 24.6 \) \( \Omega \), whole-cell capacitance \( 21.8 \pm 3.7 \) pF, RMP \( -61.7 \pm 1.7 \) mV) were achieved in mice over a range of ages (P18 - 41) while the DCols were stimulated with a bipolar electrode. The recorded neurons were located caudal to the stimulating electrode within the dorsal horn and intermediate zone of the lumbar spinal cord (Figure 2.1C). Calcium imaging of DCol stimulation was performed in young mice (P9) to allow for better dye loading.

2.3.1 Establishing an upper limit for DCol stimulation intensity

To restrict current spread/stimulation to DCol fibres, an upper limit for stimulus threshold was set at \( \leq 75 \) \( \mu \)A. This was determined by comparing thresholds for
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stimulated responses from both DCol (n = 70) and grey matter stimulation (n = 15; Figure 2.2). The upper limit was designated as $\leq 75$ $\mu$A as higher intensities were more likely to elicit postsynaptic responses from the grey matter. As a result, only 42 neurons were included in further analysis of DCol stimulation.

Figure 2.2. Stimulus intensity frequency histogram. Slices were stimulated with a 0.1 ms current pulse at 0.2 Hz during whole-cell patch clamp recording of neurons caudal to the stimulating electrode. The stimulus intensity required to evoke a synaptic input from either DCol (n = 70) or grey matter stimulation (n = 15) while recording from a spinal neuron is shown in the frequency histogram (blue and red bars, respectively). The left (blue) and right (red) axes show individual counts for DCol and grey matter stimulation, respectively. Evoked synaptic inputs requiring $\geq 1000$ $\mu$A are grouped with recordings in which no postsynaptic response could be evoked. Dashed line denotes upper limit of stimulus intensities considered to evoke DCol specific postsynaptic responses ($\leq 75$ $\mu$A).

2.3.2 DCol stimulation elicits postsynaptic currents in recorded neurons

Post synaptic responses evoked from DCol stimulation were detected up to 2394 $\mu$m caudal to the stimulating electrode and 548 $\mu$m lateral to the midline of the slice (Figure 2.1C). Three types of evoked responses were observed following 1.2T stimulation (n = 42): single component (SC; 9/42), two component (TC; 13/42) and multi-component (MC; 20/42) (Figure 2.3A). The properties of these responses are summarised in Table 2.1. Application of 10 $\mu$M CNQX during DCol stimulation completely abolished the evoked postsynaptic current in 4/6 neurons. In the remaining two cases, the CNQX insensitive postsynaptic current was completely abolished with application of 10 $\mu$M strychnine (following prior application of 10 $\mu$M bicuculline, which had no effect on the CNQX insensitive currents) (Figure 2.3C). In several recordings, evoked inhibitory currents could be effectively resolved upon depolarisation of the membrane potential (Figure 2.3D).
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Figure 2.3. Synaptic currents recorded during DCol stimulation. DCols were stimulated with 0.1 ms current pulses (≤ 75 µA) at 0.2 Hz, and membrane potential was held at -70 mV unless stated otherwise. (A) Representative evoked postsynaptic currents (10 trials) recorded after DCol stimulation at 1× and 1.2× threshold. Postsynaptic currents were classified according to the number of time-locked components in the response. The incidence of each response type is presented in the bar graph (far right). (B) An averaged postsynaptic current showing parameters measured for Table 2.1. These measurements include: latency (time between stimulus onset and beginning of inward current, upper horizontal double arrow), peak current amplitude (baseline to peak, vertical double arrow), rise time (time from 10% - 90% of peak current amplitude, horizontal lines), half-width (lower horizontal double arrow), decay time constant (determined from exponential fit, green line), and charge (grey fill). (C) The majority of evoked postsynaptic currents were blocked by bath application of the AMPA-kainate receptor antagonist, CNQX (10 µM; 4/6). CNQX resistant currents (2/6) were completely blocked by the glycine receptor antagonist, strychnine (10 µM). The GABA<sub>A</sub> receptor antagonist, bicuculline (10 µM, added in the presence of 10 µM CNQX), had no effect. (D) Evoked IPSCs could be revealed by altering the holding potential of recorded neurons to more depolarised levels, away from the equilibrium potential for chloride (presumably the major charge carrying ion for evoked IPSCs), which is approximately -60 mV for this preparation. Vh = membrane potential
Table 2.1. Properties of evoked postsynaptic currents

<table>
<thead>
<tr>
<th>Response Type</th>
<th>Latency (ms)</th>
<th>Conduction Velocity (m/s)</th>
<th>Peak Amplitude (pA)</th>
<th>Rise Time (ms)</th>
<th>Half-Width (ms)</th>
<th>Decay Time Constant (ms)</th>
<th>Charge (pA ms)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SC (n = 9)</td>
<td>3.9 ± 0.4</td>
<td>0.66 ± 0.12</td>
<td>-48.3 ± 9.4</td>
<td>1.2 ± 0.2</td>
<td>5.7 ± 1.3</td>
<td>6.1 ± 2.4</td>
<td>-307.6 ± 64.1</td>
</tr>
<tr>
<td>(n = 13)</td>
<td>3.1 ± 0.2</td>
<td>0.68 ± 0.11</td>
<td>-67.4 ± 16.3</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>-396.4 ± 96.1</td>
</tr>
<tr>
<td>MC (n = 20)</td>
<td>3.6 ± 0.4</td>
<td>0.73 ± 0.13</td>
<td>-46.3 ± 11.0</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>-680.3 ± 197.4</td>
</tr>
</tbody>
</table>

Due to the complex waveform of TC and MC responses, rise time, half-width and decay time constant were not applicable. Values are reported as mean ± SEM (with range below). For details on measurements, see Figure 2.3B and Section 2.2.5.

2.3.3 Postsynaptic voltage response following DCol stimulation

To examine the functional relevance of the evoked DCol synaptic inputs, stimulated responses were also monitored in current-clamp mode (n = 12). Evoked DCol inputs were capable of eliciting large amplitude excitatory postsynaptic potentials (EPSPs; Figure 2.4A, upper trace). In some cases, the evoked EPSPs were large enough to generate APs (2/12 neurons at -60 mV and 6/12 at -45 mV; Figure 2.4A, upper inset). Inhibitory postsynaptic potentials (IPSPs) were also detected following DCol stimulation (Figure 2.4A, middle trace). IPSPs were identified in 33% of recorded neurons (4/12) and were sometimes present in combination with an EPSP (2/4; Figure 2.4A, lower trace).

2.3.4 AP discharge properties

As neurons in different spinal laminae exhibit characteristic AP discharge patterns (Ruscheweyh & Sandkuhler, 2002; Szucs et al., 2003; Theiss & Heckman, 2005; Theiss et al., 2007), the AP discharge in response to depolarising current steps was analysed (n = 40). A variety of discharge patterns were observed, including: tonic firing (TF) neurons that discharge APs for the duration of the current step (22/40); initial bursting (IB) neurons that exhibit an early volley of APs, followed by quiescence (10/40); single spiking (SS) neurons that discharge only a single AP (3/40); delayed firing (DF) neurons that have a delayed onset of AP discharge (3/40); and reluctant firing (RF) neurons that display an absence of APs (2/40) (Figure 2.4B). Mean AP height, measured from AP threshold, was 62.4 ± 2.3 mV.
A horizontal slice preparation for examining the functional connectivity of dorsal column fibres in the mouse spinal cord

![Figure 2.4](image)

**Figure 2.4.** Voltage responses recorded during DCol electrical stimulation. (A) DCols were stimulated with 0.1 ms current pulses (≤ 75 µA) at 0.2 Hz. Three types of evoked postsynaptic potential were observed: depolarisation (EPSP), hyperpolarisation (IPSP) or both (Mixed). Inset trace shows an AP evoked by DCol stimulation. Bar graph on right shows the incidence of EPSPs, IPSPs and Mixed responses. Vh = membrane potential.

**B** Discharge patterns during depolarising current injections. The incidence of each type is shown in the centre graph.

### 2.3.5 Calcium signals in response to stimulation

To allow a broader analysis of the spread of excitation through horizontal spinal slices in response to DCol stimulation, slices were bath loaded with the fluorescent calcium dye Fluo-4 AM. As a control, the grey matter was also stimulated. Grey matter stimulation (75 µA, 100 Hz, 1 s duration) resulted in a calcium signal that was restricted to the area immediately surrounding the stimulating electrode (Figure 2.5A, left panel). In contrast, DCol stimulation (75 µA, 100 Hz, 1 s duration) produced a robust increase in
fluorescence in the DCols, and the grey matter caudal to the stimulating electrode (*Figure 2.5A, right panel*). The time course of the calcium signal was also different for grey matter and DCol stimulation with peak fluorescence from stimulation of the latter occurring more rapidly and lasting longer.

![Figure 2.5. Calcium imaging during horizontal slice stimulation. Fluo-4 AM loaded slices were stimulated with 0.1 ms current pulses (75 µA) at 100 Hz over 1s. (A) The pattern of activity in response to grey matter and DCol stimulation are shown in left and right panels, respectively. The position of the stimulating electrode is shown in the bright field images (top). ‘Heat maps’ were constructed using a 16-colour lookup table (below) (B) Several ‘cellular-type’ structures (example from inset in A) showed a stimulus-locked increase in fluorescence following DCol stimulation. The change in fluorescence (ΔF(t)/F0) within the ROI (dotted white circle in top image) is shown in the lower graph as a percentage. The black arrowhead denotes onset of DCol stimulation.](image)

Finer analysis of the DCol stimulated responses revealed a number of ‘cellular-type’ structures that exhibited calcium signals over the course of the image capture period (*Figure 2.5B, upper image*). While the identity of these structures (i.e. neurons or glia) could not be confirmed at low magnification, several showed a stimulus-locked increase in fluorescence, an example of which is shown in the lower trace of *Figure 2.5B*. In this
example, fluorescence reached a peak $\Delta F(t)/F_0$ value (increase from baseline) of 19.7% and had a decay time constant of 3.3 s.

### 2.4 Discussion

This chapter describes an *in vitro*, horizontal spinal cord slice preparation using adult mice that can be used to investigate the intrinsic electrophysiological properties of spinal cord neurons, along with the presence, type and strength of stimulated DCol fibre inputs over extended rostrocaudal distances.

#### 2.4.1 Technical considerations

While characterisation of DCol inputs is possible using the horizontal slice preparation, it should be noted that the rodent DCols contain several fibre tracts including: the CST, ascending sensory, and propriospinal tracts (Brown, 1971; Kayalioglu, 2009a). Therefore, some caution is required when assigning synaptic inputs to a particular spinal cord pathway. There is evidence, however, that stimulation of the most ventral portion of the DCols selectively activates CST fibres (Hantman & Jessell, 2010) as this region is dominated by the CST in rodents (Brown, 1971). In future studies, photostimulation of specific axons in transgenic mice that selectively express light sensitive ion channels in neuronal subpopulations could be used to test this interpretation (Deisseroth, 2011).

Some caution must also be used when interpreting calcium signals generated via bolus-loaded AM ester calcium dyes as used in this study because the dye loads various cell types indiscriminately (Regehr, 2005; Tsien, 1981). While quantifiable increases in activity can clearly be detected using this technique (*Figure 2.5A*), the precise cell populations that generate the calcium signal (i.e. neurons or glia) cannot be easily defined at low magnification. To counter this problem, genetically encoded voltage indicators (GEVIs) or fluorescent calcium indicator proteins (FCIPs), which are selectively introduced using transgenic mice or targeted viral vectors, have been developed (Chen et al., 2000; Mishina et al., 2014). Alternatively, some vital dyes are available that selectively label glia and could be utilized as a counter stain to discriminate between cell types (Uckermann et al., 2004). Encouragingly, these methods are rapidly becoming more sensitive and can provide data at physiologically relevant frequencies (St-Pierre et al., 2014). In future studies, these techniques could be used, in combination with the horizontal slice preparation, to study electrophysiological changes in specific neuronal populations, as well as assessing any novel role for glia in signal processing.
2.4.2 Target neurons and their DCol inputs

Examination of AP discharge patterns is commonly used to electrophysiologically classify and identify different neuronal populations in the spinal cord (see Theiss et al. (2007)). The proportion of the various AP discharge patterns recorded in this study (Figure 2.4B) is not typical of either superficial or deep dorsal horn neurons in rodents (Graham et al., 2007; Ruscheweyh & Sandkuhler, 2002). Thus, these recordings were not confined to any particular laminae of the dorsal horn, but rather contained a mixture of neurons from both regions. In addition, based on the depth of the most ventral horizontal slice (Figure 2.1A) and previous work (Szucs et al., 2003; Theiss & Heckman, 2005; Theiss et al., 2007), these recordings would likely have included intermediate zone neurons. Neurobiotin fills and immunohistochemical analysis would be required to identify the precise location of recorded neurons.

The majority of synaptic inputs from the DCols onto spinal neurons located in the dorsal and intermediate regions of the grey matter were excitatory. The incidence of inhibitory inputs observed (33%) is similar to that shown previously (38%) in superficial dorsal horn neurons following DCol stimulation in rat (Baba et al., 1994). This result was also reflected in the pharmacological experiments, where the majority of evoked postsynaptic currents were abolished via blockade of AMPA-kainate receptor mediated glutamatergic neurotransmission using CNQX. The remaining CNQX insensitive currents were abolished via blockade of glycinergic neurotransmission using strychnine. In contrast, bicuculline (a GABA_A receptor antagonist) did not block these inhibitory currents, which is contrary to other studies that have demonstrated that DCol fibres do release GABA in the dorsal horns, causing slow, long lasting IPSPs (Baba et al., 1994). The origin of these inputs (i.e. direct DCol inputs, or inputs arriving at the recorded neuron via polysynaptic intraspinal circuits) was not tested in this study. However, the majority of the postsynaptic responses consisted of more than one component (Figure 2.3A), and the variable latency and high failure rate of some TC and MC responses (data not shown) demonstrates they are at least partially mediated by polysynaptic pathways. This implies the horizontal spinal slice preparation retains a high degree of intraspinal connectivity.

2.4.3 Applications

Despite the longstanding preference for transverse spinal cord slices in electrophysiological studies, horizontal slices have a unique advantage because longitudinally orientated fibres, including descending supraspinal and long propriospinal fibres, are preserved. Additionally, many neuronal subtypes within the spinal cord have
longitudinally orientated processes that are likely to be severed in transverse slice preparations (Goulding, 2009; Saywell et al., 2011; Szucs et al., 2010; Todd, 2010; Zhang et al., 1996). Experiments that look to examine multi-segmental processing, effects of descending and ascending inputs on spinal neurons, and in particular, the physiology and functional connectivity of neurons above and below a spinal cord lesion, would benefit from this horizontal slice preparation.

The mouse was chosen as the animal model for development of this horizontal preparation because mice are becoming increasingly important in SCI research where transgenic animals can be used to directly investigate the underlying molecular determinants of neuronal plasticity and regeneration (Cafferty et al., 2007; Goldshmit et al., 2004; Ito et al., 2010; Lee et al., 2010b). Importantly, this preparation can be used to demonstrate and assess functional neuronal regeneration across a spinal cord lesion as it permits high-resolution analysis of postsynaptic currents and potentials in response to stimulation of longitudinally orientated spinal pathways, such as the DCols. The whole-cell patch clamp technique also allows investigation of sub-threshold currents, intrinsic neuronal plasticity, and pharmacological analysis of synaptic inputs in slices from animals with spinal cord lesions. Although the whole-cell patch-clamp technique is limited in its capacity to measure population-wide neuronal activity in single animals, (which is also highly relevant to SCI research) this work has demonstrated that calcium imaging can also be applied in horizontal slices. These data can provide valuable spatial information on the extent to which regeneration restores normal DCol signaling following experimental SCI therapies.

2.4.4 Conclusions

In summary, the horizontal spinal cord slice protocol described here provides a feasible, high-resolution method for examining functional connections between DCol fibres and spinal cord neurons using the whole-cell patch clamp technique and calcium imaging. It is envisaged that adoption of this preparation for the analysis of SCI recovery will provide valuable new information that will instruct future attempts to improve post-SCI recovery and rehabilitation. In addition, this preparation will be of value in future studies on uninjured ‘normal’ animals. For example, it could be used to explore how motor commands from the cortex and other higher structures recruit the spinal machinery that mediates coordinated motor function.
Chapter 3: Exercise training after spinal cord injury selectively alters synaptic properties in neurons in the adult mouse spinal cord
3.1 Introduction

In Chapter 2, a horizontal spinal cord slice preparation was developed to permit detailed investigation of the intrinsic and synaptic properties of spinal cord neurons and their functional connectivity with DCol fibres. As neurons can be recorded over a long rostrocaudal range within a single horizontal slice, this preparation is ideally suited to SCI studies where neuronal properties and connectivity above and below a lesion are of interest. This chapter describes the application of the horizontal slice preparation to assess post-SCI functional connectivity in adult mice, and how neuronal properties are affected by exercise therapy - a commonly used intervention that is known to improve recovery after SCI.

It is well established that incomplete lesions to the spinal cord are accompanied by anatomical changes, including axonal sprouting in the vicinity of the lesion within weeks following SCI (Bareyre et al., 2004; Filli et al., 2014; Fouad et al., 2001). This post-SCI axonal sprouting can establish new intraspinal circuits that allow descending pathways to bypass the lesion site (Bareyre et al., 2004; Courtine et al., 2008; Filli et al., 2014). Importantly, recent work has shown that exercise training can enhance this process following SCI (Engesser-Cesar et al., 2007; Goldshmit et al., 2008). These studies have documented anatomical changes in the vicinity of a lesion post-injury, however the functional consequences of these changes are rarely assessed. Specifically, very few studies go on to assess how post SCI sprouting relates to the synaptic or intrinsic electrical properties of neurons that form new intraspinal circuits, especially in adult animals. Thus, a causal relationship between functional recovery from SCI and axonal sprouting remains speculative.

In this Chapter, in vitro whole-cell patch-clamp electrophysiology was used to compare the intrinsic (passive membrane and AP properties) and synaptic properties of neurons in the immediate vicinity of an incomplete spinal lesion in exercise untrained and trained adult mice. The choice of exercise training as a comparison condition was guided by the strong evidence that it increases axonal sprouting and enhances functional recovery after SCI (Battistuzzo et al., 2012b; Battistuzzo et al., 2015; Edgerton et al., 2004; Engesser-Cesar et al., 2007; Goldshmit et al., 2008; Heng & de Leon, 2009; Roy et al., 2012). By utilizing the horizontal spinal cord slice preparation, DCol pathways (that include the CST; Brown (1971)) could be activated with a stimulating electrode while recording from spinal interneurons above and below the lesion. This allowed direct assessment of the patency of signal transmission around and beyond the lesion. Together, these features of the horizontal slice preparation allowed detailed examination of the
effect of exercise training on intrinsic neuronal properties, along with their functional connectivity with longitudinal DCol pathways following SCI.

### 3.2 Methods

Adult mice received a lateral spinal cord hemisection and were randomised into untrained (no exercise) and trained (treadmill exercise for 3 weeks) groups. After 3 weeks, spinal cord slices were prepared and the intrinsic and synaptic properties of spinal neurons in the immediate vicinity of the lesion (< 2 spinal segments) were studied in both groups using whole-cell patch-clamp electrophysiology. DCol fibres were stimulated to examine functional changes in synaptic connections in recorded neurons.

#### 3.2.1 Lateral spinal hemisection and exercise training

All procedures were approved by the University of Newcastle Animal Care and Ethics Committee. Animals (C57BL/6 male mice, 9–10 weeks of age) were anaesthetised with isoflurane (5% induction and 1.5–2.5% maintenance) and medetomidine (0.03 mg/kg s.c.). Once a surgical level of anaesthesia was achieved (assessed with both hind-limb withdrawal and corneal reflexes), the skin over the thoracolumbar region was shaved and disinfected with povidine/iodine solution. The animal was placed on a heating mat (37°C) and head-fixed in a stereotaxic frame with soft ear bars (Narishige, Tokyo, Japan).

A binocular dissecting microscope (X10 magnification) was utilised throughout the surgery. First, a midsagittal incision over the thoracic and rostral lumbar region was made with a scalpel blade. The muscle and connective tissue over vertebrae T8 to T12 was reflected away with forceps, exposing the laminae and spinous processes. A T9 to T10 laminectomy was performed with micro-rongeurs and forceps to expose the underlying spinal cord; blood from the laminectomy was removed with cotton Q-tips and sterile saline. An ophthalmic knife was then used to perform a left spinal cord hemisection at the level of the T10 vertebra (i.e. between T10 and T11 spinal nerves). The overlying musculature was then sutured and the skin sealed using 7mm staples. Postsurgical analgesia was provided with buprenorphine (0.1 mg/kg s.c. every 8 h for 48 h).

After 1 week of recovery, mice exhibiting left hindlimb paralysis were randomly allocated to trained or untrained groups. Over the next 3 weeks, the trained group received enforced treadmill exercise (two 10 min sessions, 5 days/week) at speeds that matched their ability (ranging from 6 to 12 m/min). The untrained group remained in their cages during this period. To acclimate mice to the treadmill, all animals completed 2 weeks of treadmill training prior to their surgery.
3.2.2 Horizontal slice preparation, whole-cell patch-clamp electrophysiology and DCol stimulation

Following the 3 week training period post-SCI, both trained and untrained mice (~13 weeks of age) were sacrificed for in vitro whole-cell patch-clamp electrophysiology. Investigators were blinded to the training status of all animals. Full details of horizontal slice preparation, whole-cell patch-clamp electrophysiology and DCol stimulation are described in Chapter 2, Section 2.2.1, 2.2.2 and 2.2.3. In addition to these procedures, spontaneous excitatory post-synaptic currents (sEPSCs) were recorded for at least 3 minutes in voltage-clamp mode. Neurons were voltage clamped at -60 mV instead of -70 mV to ensure only excitatory post synaptic currents were detected (the reversal potential for inhibitory currents is approximately -60 mV in this preparation. See Chapter 2, Figure 2.3D). Also, data was only acquired using a Multiclamp 700B amplifier instead of an Axopatch 200B (Molecular Devices, Sunnyvale, USA).

3.2.3 Measurement of lesion extent

After electrophysiological recording, low magnification (5X) images of the horizontal spinal slices were taken and ‘stitched’ together using Photoshop (CS4; Adobe, CA, USA). The slice montages were then imported into ImageJ (Rasband, 1997-2015), along with an image of a graticule at the same magnification for scaling. The area of the lesion was measured by tracing the border of the zone of cavitation and surrounding glial scar tissue. The ‘extent’ of the lesion was measured as the distance between the medial apex of the lesion and the midline of the spinal cord. A value of 0 mm indicated that the lesion extended all the way to the midline of the cord.

3.2.4 Electrophysiological data analysis

Full details of electrophysiological data analysis are described in Chapter 2, Section 2.2.5. For the additional data in this chapter, captured sEPSCs were averaged, and peak amplitude (baseline to peak negative current), rise-time (calculated over 10–90% of peak amplitude), half-width (measured at 50% of peak amplitude), and decay time constant (calculated over 20–80% of the decay phase) were obtained. sEPSC frequency was calculated by dividing the number of events by the recording time in seconds.

3.2.5 Statistical analysis

Comparisons between data from untrained and trained groups were made using Student’s t tests or Mann–Whitney U tests, depending upon the normality of the data set. The prevalence of AP discharge patterns was compared in untrained and trained groups.
using $\chi^2$ tests. In all comparisons, the normality of data was determined using a Kolmogorov–Smirnov test. Significance was set at $p < 0.05$.

### 3.3 Results

In total, 137 neurons (70 untrained vs. 67 trained) were recorded from 28 adult mice (15 untrained vs. 12 trained), yielding ~5 neurons per animal. Recordings were limited to within 2 spinal segments of the lesion site and were located rostral, caudal, and directly contralateral to the lesion (**Figure 3.1**).

**Figure 3.1.** Location of recorded neurons on a horizontal spinal slice schematic. Light and dark gray shading represent gray and white matter, respectively, which are clearly visible under infrared differential interference contrast optics. Neurons from untrained (red circles) and trained (blue circles) mice were recorded one to two segments rostral and caudal to the hemisection (made between T10 and T11 spinal nerves).

#### 3.3.1 Lesions were similar between untrained and trained groups

To ensure any electrophysiological differences between the untrained and trained group were not attributable to variability in the spinal lesions, the size of the lesion site in slices used for recording was quantified. The area of cavitation (i.e. missing tissue) in combination with the glial/scar tissue was similar in untrained and trained animals ($0.53 \pm 0.08 \text{ mm}^2$ vs. $0.40 \pm 0.04 \text{ mm}^2$). The ‘extent’ of the lesion ($\pm SE$) was $0.15 \pm 0.03 \text{ mm}$ in both untrained and trained groups. Together, these data suggest that the hemisections were equivalent between groups.

#### 3.3.2 Intrinsic properties of recorded neurons

The passive membrane properties of neurons in untrained vs. trained groups were similar, as assessed by resting membrane potential ($-60.9 \pm 1.2$ vs. $-62.3 \pm 1.4 \text{ mV}$) and input resistance ($809 \pm 84$ vs. $881 \pm 78 \text{ M}\Omega$). Individual AP properties were also similar in untrained versus trained animals: AP amplitude ($67.2 \pm 2.4$ vs. $68.2 \pm 3.2 \text{ mV}$), AP width ($1.5 \pm 0.1$ vs. $1.6 \pm 0.1 \text{ ms}$), AHP latency ($2.6 \pm 0.2$ vs. $2.8 \pm 0.4 \text{ ms}$), and AHP peak ($46.9 \pm 1.2$ vs. $45.6 \pm 1.4 \text{ mV}$) (**Figure 3.2A**). Classification of the sustained discharge in response to square step depolarizing current injection separated neurons into
four AP discharge patterns including: tonic firing (TF) neurons that discharge APs for the duration of the current step (24% untrained, 42% trained); initial bursting (IB) neurons that exhibit an early volley of APs, followed by quiescence (31% untrained, 23% trained); single spiking (SS) neurons that discharge only a single AP (29% untrained, 26% trained); delayed firing (DF) neurons that have a delayed onset of AP discharge (16% untrained, 9% trained) (Figure 3.2B). The proportions of each discharge pattern did not differ between untrained and trained mice.

3.3.3 Synaptic properties of recorded neurons

Much like the intrinsic properties, sEPSCs recorded in neurons from the untrained and trained groups were also similar. Specifically, no significant differences were detected in frequency (2.7 ± 0.5 vs. 3.7 ± 0.6 Hz), rise time (0.71 ± 0.04 vs. 0.74 ± 0.04 ms), peak amplitude (18.7 ± 0.8 vs. 16.7 ± 0.3 pA), or decay time constant (2.6 ± 0.2 vs. 2.9 ± 0.2 ms) (Figure 3.3A and B). In contrast, DCol evoked responses from trained mice had significantly larger peak amplitudes (78.9 ± 17.5 vs. 42.2 ± 6.8 pA; p < 0.05) and greater charge (1054 ± 376 vs. 348 ± 75 pA⋅ms; p < 0.05) compared with neurons from untrained mice (Figure 3.3C and D). Despite this difference, the stimulus threshold (262 ± 64 vs. 295 ± 95 µA) and response latency (6.8 ± 1.0 vs. 10.3 ± 2.2 ms) were similar in both groups, suggesting that stimulus parameters did not account for this observation. A clear feature of DCol evoked activity was a polysynaptic EPSC profile.
that was more pronounced in the trained SCI group. This suggests the involvement of multiple synapses between the stimulation site in the DCols and the recorded neuron.

**Figure 3.3.** Properties of spontaneous and evoked excitatory postsynaptic currents (EPSCs) in untrained and trained SCI mice. (A) sEPSCs recorded in untrained (red trace) and trained (blue trace) mice (holding potential -60mV). (B) Group comparisons for sEPSC properties. These properties did not differ in untrained and trained mice. Data represented as mean ± SEM on all bar charts. (C) Overlaid DCol evoked synaptic responses from untrained and trained mice. (D) Group comparisons for DCol evoked synaptic responses. Peak amplitude and charge for evoked responses were significantly larger in the trained mice, * significant at \( p < 0.05 \).

### 3.4 Discussion

This study uses the horizontal spinal cord slice preparation developed in Chapter 2 to investigate the effects of a therapeutic intervention, exercise training, on the intrinsic and synaptic properties of neurons in the vicinity of an SCI. The major findings of this work are that 3 weeks of treadmill training following SCI does not alter the intrinsic membrane properties of recorded neurons, or local excitatory synaptic inputs (based on
sEPSC properties), but increases excitatory synaptic drive evoked by DCol stimulation.

3.4.1 Study design and technical considerations

The purpose of this study was to confirm that the horizontal spinal cord slice preparation is a valid and feasible approach to assess the intrinsic properties of neurons and their synaptic inputs in a well-characterized animal model of SCI. However, the behavioural outcomes of treadmill training in this cohort, the duration of the intervention, and some technical issues must also be considered.

A separate study by Battistuzzo et al. (2015) quantitatively and qualitatively describes the outcome of post-SCI treadmill training in the same cohort of mice used in this chapter. Videos of the untrained and trained groups running on a treadmill (prior to being sacrificed for electrophysiology) showed that after 3 weeks of treadmill training, mice had an improvement in step duration, and a trend for longer maintenance of knee flexion during the swing phase of the step cycle compared to untrained mice. However, these differences were minor, and did not clearly distinguish the locomotor ability of untrained and trained mice. This raises the question of whether a 3-week training regime was sufficient to provoke overt neural plasticity, and may explain the subtle/specific electrophysiological outcomes seen in this study (i.e. no changes in intrinsic or spontaneous synaptic properties, only DCol evoked responses). Recent work provoked by these results (Rank et al., 2014) demonstrates that some electrophysiological properties of neurons in the vicinity of an SCI are modified to a greater degree after 6 weeks of training compared to 3 weeks. While outside the scope of this thesis, a major finding in the Rank et. al. (2014) study was that sEPSC properties are significantly altered with 6 weeks training, suggesting that the limited changes in the present 3-week data represent early evidence of this plasticity. Furthermore, the locomotor capacity in the same SCI cohort is further enhanced after a 6-week training regime compared to 3-week trained and untrained controls (Battistuzzo et al., 2012b; Battistuzzo et al., 2015).

A comparison with uninjured, age-matched control mice would also provide baseline data to gauge the extent of electrophysiological recovery in untrained and trained mice. Again, while this data does not form part of the current thesis, it was collected in parallel to the presented dataset and demonstrates that SCI has a major effect on both the intrinsic and synaptic properties of spinal neurons and DCol pathways (Rank et al., 2014). Importantly, this work also showed that treadmill training restores AP discharge properties back to control values at the 3-week time point, but fails to promote recovery of voltage-gated, sub-threshold current profiles at 6 weeks. In terms of synaptic
properties, 3 weeks of treadmill training greatly enhances DCol evoked post-synaptic current amplitude relative to control values, which then return to levels resembling non-exercise trained SCI animals at 6 weeks. These results highlight the transient and complex nature of electrophysiological plasticity engaged by treadmill training. Taken together with the functional improvement seen following treadmill training (Battistuzzo et al., 2012b; Battistuzzo et al., 2015; Edgerton et al., 2004; Engesser-Cesar et al., 2007; Goldshmit et al., 2008; Heng & de Leon, 2009; Roy et al., 2012), it suggests that electrophysiological properties within the post-SCI spinal cord do not necessarily revert to pre-injury levels to effect behavioural recovery. Indeed, it may be too simplistic to expect spinal circuitry to recover to pre-SCI status. Rather, plastic changes might engage alternative physiological strategies within spinal circuitry to achieve coordinated and functionally relevant behavioural recovery in the vastly altered anatomical and physiological environment of the post-SCI spinal cord.

Unlike Chapter 2, an upper limit for threshold stimulus intensity was not used in these experiments ($\leq 75 \mu A$). This is because stimulus intensities required to evoke DCol inputs onto recorded neurons in SCI mice were higher, and more variable than those used in uninjured spinal cord slices ($262 \pm 64$ in untrained SCI mice, $295 \pm 95 \mu A$ in trained SCI mice, compared to $115 \pm 3 \mu A$ in uninjured mice from Chapter 2). This may be due to demyelination in DCol pathways following SCI (Totoiu & Keirstead, 2005) and/or physical damage of DCol fibres from the lateral hemisection. It is therefore possible that some evoked responses actually resulted from activation of propriospinal pathways within the grey matter due to current spread (Chapter 2, Section 2.3.1). Future studies would benefit from a more targeted approach, such as optogenetic stimulation of specific DCol fibres to clarify this issue (Deisseroth, 2011).

### 3.4.2 Intrinsic electrophysiological properties of neurons following SCI

Previous electrophysiological studies have used sharp microelectrode recordings to demonstrate that SCI alone can cause changes to the intrinsic properties of rat motoneurons, leading to increased excitability via a more depolarized resting membrane potential (relative to firing threshold), increased input resistance, and larger persistent inward currents (PICs; (Bennett et al., 2001; Harvey et al., 2006). Beaumont et al. (2004); (2008) extended these findings by showing exercise training ameliorates the electrophysiological changes in lumbar motoneurons that normally accompany SCI. My results differ, in that exercise training had no effect on the intrinsic properties of recorded neurons after SCI. A direct comparison is difficult to make, however, given the neurons in this study were located in the dorsal horn and intermediate zone, as opposed to lamina
IX motoneurons. These areas are important for processing tactile, proprioceptive and nociceptive information, and contain commissural neurons, along with long and short propriospinal neurons (Kayalioglu, 2009b). Importantly, the neurons recorded in these areas do not exit the spinal cord into the periphery and may therefore respond differently from motoneurons, whose axons are normally in contact with important peripheral trophic factors originating from exercising muscle (Fouad et al., 2010; Harkema et al., 1997). This same rationale also argues that the current dataset contains far greater heterogeneity than the relative homogeneity of a sample of motoneurons. Thus, the possibility that smaller, more discrete subpopulations within my sample do exhibit intrinsic plasticity cannot be excluded.

Recent studies support the notion that different neuronal subpopulations respond to SCI in a variable manner with regard to intrinsic electrophysiological properties. For example, (Husch et al., 2012) show that almost no changes in intrinsic properties (besides a lower input resistance) occur in V2a interneurons as a result of SCI, whereas Dougherty and Hochman (2008) report a more depolarized resting membrane potential, larger steady-state outward currents, and increased AP height in GAD67 neurons following SCI. Taken together, these findings demonstrate the importance of studying specific neuronal subpopulations, as their response to SCI appears to be highly variable. Future studies that combine the horizontal slice preparation with transgenic mice (in which various functionally discrete subpopulations are labeled) will be required to further elaborate on this issue.

3.4.3 Synaptic properties of neurons following SCI

It was somewhat surprising that although exercise training altered DCol evoked synaptic inputs, the properties of sEPSCs were unchanged. The simplest interpretation of this finding is that axon terminals involved in spontaneous neurotransmitter release within the spinal cord differ in their response to SCI from those recruited by DCol stimulation. Together, these data on excitatory synaptic connections suggest that plasticity induced by exercise training preferentially targets connections between DCol pathways, such as the CST, and the spinal neurons around the site of an SCI as opposed to a non-specific increase in all excitatory neurotransmission.

DCol evoked responses exhibited markedly larger peak amplitude and charge in mice that underwent 3 weeks of exercise training after SCI (Figure 3.3C). This finding is consistent with anatomical studies showing that DCol pathways exhibit significant axonal sprouting following SCI both above and below the lesion (Ballermann & Fouad, 2006;
Fouad et al., 2001; Weidner et al., 2001). Moreover, exercise training after SCI is known to enhance axonal sprouting, and increase the number of contacts on spinal neurons (Engesser-Cesar et al., 2007; Goldshmit et al., 2008). With this literature in mind, the dataset in the present chapter provides the first functional information supporting the relevance and impact of axonal sprouting evoked by exercise training in SCI animals. Thus, the horizontal slice preparation presented in Chapter 2 can be used to assess this type of neural plasticity electrophysiologically, using the high-resolution patch-clamp technique. The precise details of these new connections await further study. However, CST axons are likely contribute to this plasticity, as they lie in ventral regions of the DCols in rodents (Brown, 1971).

3.4.4 Conclusions

In summary, this study highlights a promising approach for the collection of functional data alongside current anatomical, molecular, genetic, and behavioural information on plasticity in spinal cord circuits after SCI. Achieving this in a mouse model of SCI is also important, given the increasing availability of genetic and molecular tools to identify and alter discrete neuronal populations within the spinal cord (Brownstone & Wilson, 2008; Deisseroth, 2011; Goulding, 2009; Zheng et al., 2006). In future studies, whole-cell patch-clamp electrophysiology could be used to examine how the two major determinants of neuron output; intrinsic properties and synaptic inputs (Turrigiano et al., 1994), are altered by various forms of exercise therapy and other interventions in animal models of SCI (Battistuzzo et al., 2012a).
Chapter 4: Location and Projection Pattern of Long Descending Propriospinal Neurons in Mice
4.1 Introduction

Understanding the cellular basis for functional recovery after SCI remains a critical goal for regenerative medicine (Cregg et al., 2014). As outlined in Chapter 3, exercise training leads to enhanced excitatory synaptic neurotransmission in spinal cord circuits after injury. Despite providing the first high-resolution functional evidence of synaptic remodelling after SCI, these results raise the question: “what spinal cord pathways and neuronal subtypes are involved in synaptic plasticity after injury”. As outlined in the introduction, PNs constitute an ideal neural substrate for recovery after injury because they are involved in the formation of de novo spinal circuits, by virtue of their longitudinal projection pattern and ability to bypass a SCI (Bareyre et al., 2004; Courtine et al., 2008; Deng et al., 2013; Fenrich & Rose, 2009; Filli et al., 2014; Vavrek et al., 2006). It stands to reason then, that PNs contribute, at least in part, to the altered synaptic properties outlined in Chapter 3, and more broadly, to functional recovery following SCI.

Of particular importance are long descending propriospinal neurons (LDPNs) that project from cervical to lumbar spinal circuits. These neurons receive a high number of newly formed synapses following SCI, in addition to significantly extending their own axonal arbours into regions of the spinal cord involved in motor behaviours (Bareyre et al., 2004; Vavrek et al., 2006). This, in combination with their role in directly linking cervical and lumbar motor circuits for interlimb coordination (Lloyd & McIntyre, 1948) make LDPNs an attractive target for therapeutic strategies after SCI (Conta & Stelzner, 2004; Deng et al., 2013). Remarkably, despite mice being one of the most commonly used mammals in SCI research due to their ease of genetic manipulation, anatomical data on the LDPN population in this species is lacking. To my knowledge, the most comprehensive anatomical study in mice comes from Ni et al. (2014). This group used transsynaptic viral tracing to label all descending PNs that directly innervate tibialis anterior motoneurons. Despite providing detailed data for a subset of premotor LDPNs, we know that the majority of LDPNs actually project to interneurons in laminae V-VIII and only sparsely to motoneurons in lamina IX (see Table 1.2). Thus, the Ni et al. (2014) study only included a subset of LDPNs in their data, meaning there is still a need for a broad survey of the entire LDPN population in mice.

This chapter describes experiments that used fine, hand-pulled pipettes to unilaterally inject a retrograde tracer, Fluorogold, into the mouse lumbar spinal cord to back-label cervical LDPN cell bodies. The location of LDPN cell bodies was then
mapped throughout the cervical and upper thoracic spinal cord. To address the issue of what neurotransmitter phenotypes are present in the LDPN population, I also employed genetically modified reporter mice to determine the location and projection pattern of inhibitory (glycinergic and GABAergic) LDPNs in GlyT2\textsuperscript{GFP}, and GAD67\textsuperscript{GFP} animals. Finally, the dendritic morphology of a subset of LDPNs in the ventral horn was also analysed and compared to surrounding interneurons following intracellular Neurobiotin filling and reconstruction. This work provides a basis of future studies that will characterise and manipulate subpopulations of LDPNs in mice, particularly for the development of therapeutic strategies that target the formation of de novo spinal pathways after SCI (Conta & Stelzner, 2004).

4.2 Methods

Lumbar projecting LDPNs located in segments C2 to T3 were retrogradely labelled with Fluorogold to map their cell body locations in wild-type (ICR strain), GlyT2\textsuperscript{GFP}, and GAD67\textsuperscript{GFP} mice. Fluorogold was injected into the L2 lumbar spinal cord in 3 to 5 week old animals to back-label LDPN cell bodies over a period of 5 days. Thin, transverse, serial sections of cervical and upper thoracic segments were then cut and immunolabelled for Fluorogold and GFP (transgenic animals only), and LDPN cell bodies were plotted on standardised transverse spinal cord maps. Finally, Neurobiotin filled LDPNs from targeted patch-clamp electrophysiology experiments (Chapter 5) were recovered and reconstructed with confocal microscopy to examine their dendritic length and branching patterns.

4.2.1 Intraspinal tracer injection surgery

The University of Newcastle Animal Care and Ethics Committee approved all experimental procedures. Animals (ICR, GlyT2\textsuperscript{GFP}, and GAD67\textsuperscript{GFP} mice, both sexes) were anaesthetised with isoflurane (5% induction, 1-3% maintenance). Once a surgical level of anaesthesia was achieved (assessed with both hind-limb withdrawal and corneal reflexes), the skin over the thoracolumbar region was shaved and disinfected with povidine/iodine solution. The animal was placed on a heating mat (37°C) and head-fixed in a stereotaxic frame with soft ear bars (Narishige, Tokyo, Japan). To increase the gaps between vertebral laminae, the tail was gently retracted and taped down.

A binocular dissecting microscope (X10 magnification) was utilised throughout the surgery. First, a midsagittal incision over the caudal thoracic and rostral lumbar region was made with iris scissors. Using the 13\textsuperscript{th} rib as a landmark, muscle and connective tissue over vertebrae T12 and T13 was cleared away and reflected with
forceps, exposing the laminae and spinous processes. A T12 laminectomy was performed with micro-rongeurs and forceps to expose the caudal edge of the L2 spinal segment; blood from the laminectomy was removed with cotton Q-tips and sterile saline. To prepare the L2 spinal segment for injection, the overlying dura mater was gently cleared away with a 30-gauge needle. A micropipette (0.5 µL volume, 64 mm length; Drummond Scientific, PA, USA) with volume markings (hand-drawn by the investigator) was filled with Fluorogold (4% in saline; Fluorochrome, CO, USA) via capillary action. The pipette was attached to a picospritzer (PV820; WPI, FL, USA) using polyethylene and silicone tubing, and mounted onto a stereotaxic holder. Once positioned in the midline of the L2 spinal segment, it was translated 0.4 mm mediolaterally to the right-hand side, and -0.75 mm dorsoventrally into the spinal cord (from cord surface). This placed the tip of the pipette in the intermediate grey matter of the L2 spinal segment (Figure 4.1A). Between 20-50 nL of Fluorogold was injected with several pressure pulses from the picospritzer (20 psi, 5 ms duration). The pipette was maintained in place for 5 minutes before being slowly withdrawn over another 5 minutes to minimise tracer leakage from the injection tract. Animals with injection sites that extended contralaterally (i.e. into the grey matter on the left site of the spinal cord; determined after sectioning) or did not encompass the entire dorsal horn, intermediate zone, and ventral horn of the left grey matter were excluded from the study (approximately 20%). Upon removal, the wound was flushed with saline and the overlying skin resealed with Vetbond (3M, MN, USA) and stapled. Caprofen (5 mg/kg) and Buprenorphine (0.05-0.1 mg/kg) were administered subcutaneously after surgery to reduce inflammation and provide pain relief. Animals were returned to their home cage for 5 days prior to perfusion to allow Fluorogold to be retrogradely transported back to LDPN cell bodies in the cervical cord (Figure 4.1B). Mice were then transcardially perfused with 4% paraformaldehyde (PFA). The spinal cord was dissected out and drop-fixed for a further hour (also in 4% PFA) at room temperature before cryoprotection in 0.1M PBS containing 30% sucrose.

4.2.2 Neurobiotin fills

As Fluorogold labelling was limited to the cell body and proximal dendrites, a subset of LDPNs were individually filled with Neurobiotin (0.2% w/v; Vector Labs; CA, USA) to provide a more complete assessment of their morphology. This was performed during whole-cell patch-clamp electrophysiology experiments, described in Chapter 5, Section 5.2.3. ‘Control neurons’ (i.e. unidentified spinal interneurons in the same location as recorded LDPNs) were also filled with Neurobiotin for comparison. All Neurobiotin filled neurons were sampled from the ventromedial grey matter (laminae VII and VIII), contralateral to the L2 injection site. Spinal cord slices were drop-fixed in 4% PFA.
overnight at 4°C and cryoprotected in 0.1M PBS containing 30% sucrose prior to sectioning and immunohistochemistry.

**Figure 4.1.** Labelling LDPNs using the retrograde tracer, Fluorogold. (A) Schematic summarises surgical approach and procedure to retrogradely label LDPNs. Following a T12 laminectomy, Fluorogold was pressure injected into the mouse lumbar spinal cord. *Inset upper:* the pipette tip penetrated the dorsal spinal cord at the L2 spinal segment and was lowered until positioned in intermediate zone (depth = 0.75 mm) before injecting 20-50 nL of Fluorogold. *Inset lower:* Typical spread of Fluorogold after L2 injection (grayscale and inverted fluorescence under a DAPI filter). Some injections extended rostrocaudally into L1 or L3. Animals with injection sites extending contralaterally (i.e. into the left side) were excluded. (B) Mice recovered for 5 days after surgery to allow retrograde transport of Fluorogold from the injection site in the lumbar spinal cord to cervical LDPN cell bodies.

### 4.2.3 Immunohistochemistry and imaging

Cryoprotected spinal cords from Fluorogold injected mice were mounted in OCT compound (Tissue-Tek; Sakura; Flemingweg, The Netherlands) and sectioned at 40 μm using a freezing sledge microtome. Every fifth section was mounted on electrostatically charged glass slides (Fisherbrand Superfrost Plus; Thermo Fisher Scientific; MA, USA), air-dried for 30 minutes and washed three times in 0.1M PBS containing 0.1% Triton X-100 (PBST). Sections were incubated in 10% normal donkey serum for one hour and then incubated in primary antibodies for Fluorogold (rabbit anti-Fluorogold; 1:10,000; Chemicon) and GFP (chicken anti-GFP; 1:500; Jackson ImmunoResearch) diluted in 1% normal donkey serum in PBST for twelve hours at 4°C. Sections were washed three times in PBST and incubated with AlexaFluor conjugated secondary antibodies in PBST/1% normal donkey serum for one hour at room temperature (donkey anti-rabbit 647, donkey anti-chicken 488; 1:500; Molecular Probes). Sections were then washed three times in PBST and mounted in Aqua-Poly/Mount (Polysciences; PA, USA). Photomicrographs of each section were captured at X10 magnification on an Olympus BX61 microscope (Olympus; Tokyo, Japan) with a motorised stage controlled by Metamorph software (Molecular Devices; CA, USA). Due to the size of the sections, multiple fields of view
were acquired and stitched together using Metamorph software to generate a single image of each slice.

For Neurobiotin filled neuron recovery, the original 250 µm thick transverse slices (used for electrophysiology) were incubated in Avidin conjugated to rhodamine and imaged at X20 magnification. The overall shape of the cell body and dendritic arbor (and any axonal profile) was later used for the alignment of high magnification tiles. Tissue slices were then re-sectioned at 60 µm and again labelled with Avidin conjugated to rhodamine. Initially, a low power (X20 magnification) scan of each section was taken for landmarking to further assist later reconstruction. Finally, a series of high power (X40 magnification) tiled scans were taken of the entire neuron. For each scan, the z-interval and zoom factor was set to 1. All confocal microscopy was performed with a Bio-Rad Radiance 2100 microscope (Hemel Hempstead, UK) with a krypton–argon laser.

4.2.4 Cell body location maps and reconstructions

The cell body location of Fluorogold labelled LDPNs was assessed in five wild-type ICR mice. Photomicrographs of immunostained sections from C2 to T3 (1-in-5 series) were imported into Illustrator CS4 (Adobe; CA, USA) and positioned over a transverse spinal cord slice template (adapted from Watson et al. 2009). Photos were carefully matched to the correct cervical or upper thoracic template by analysing the dorso-ventral extent of the dorsal columns, and the shape of the ventral horns. The photos were rotated and/or uniformly resized to fit the template. LDPN cell body positions were then plotted on the template. In some cases, spinal cord slices were irregularly cut and therefore difficult to fit on a single template. In these instances, the LDPN cell body was mapped by triangulating its location relative to the following structures: dorsal horn grey matter, lateral funiculus, dorsal columns, central canal, ventral horn grey matter, and the ventral funiculus. The position of LDPN cell bodies within the spinal cord templates was used to determine their lamina of origin. LDPN cell body positions for individual animals were then collated to generate ‘heat-maps’ revealing the relative density of LDPN cell bodies across each spinal cord segment as well as highlighting the overall pattern of cell body position. To estimate the total number of LDPNs within the C2-T3 region, the following calculation was applied for each spinal segment and then summed together:

\[
\text{Average number of LDPN cell bodies per 40 µm section} \times 4 \times 5 \times 2
\]

\[
\text{(average number of sections per spinal segment)} \times \text{correction for 1-in-5 series) \times \text{correction for unilateral injection)}
\]

64
The position of inhibitory LDPNs was determined in both transgenic $GlyT2^{\text{GFP}}$ and $GAD67^{\text{GFP}}$ mice and plotted using an identical strategy to that above onto standardised spinal cord templates. GFP positive LDPNs were defined by colocalisation of both fluorescent signals (Fluorogold and GFP) in a single neuronal cell body.

For Neurobiotin filled neurons, confocal image stacks were reconstructed and analysed off-line using Neurolucida for Confocal software (MicroBrightField; VT, USA). The low power (X20 magnification) images taken before and after re-sectioning were utilised to align and reconstruct the series of high power (X40 magnification) image tiles. After each neuron was reconstructed, data was collected on their dendritic length and branching patterns for comparison between LDPNs and control neurons.

4.2.5 Statistics

Group comparisons were made with an unpaired Student’s t-test when data sets were normally distributed (assessed by the Kolmogorov-Smirnov test) and had equal variance (SD of both groups different by less than a factor of 2). In cases where data sets were normally distributed but had unequal variances, Welch’s t-test was applied. Non-normally distributed data sets were compared with the Mann-Whitney U test. All statistical analysis was performed with GraphPad Prism 6 software (GraphPad Software; CA, USA). Statistical significance was set at $p < 0.05$. All values are presented as mean ± SEM.

4.3 Results

The cell body locations of lumbar projecting LDPNs were mapped throughout the cervical and upper thoracic spinal cord in five wildtype mice (ICR strain; P29-30). Inhibitory LDPNs were mapped in three $GlyT2^{\text{GFP}}$ (P32) and three $GAD67^{\text{GFP}}$ mice (P31). Neurobiotin fills of LDPNs and control cells were successfully reconstructed in seven wildtype mice (C57BL/6; P22-31).

4.3.1 Injection sites

Fluorogold injection sites were located in the L2 spinal segment on the right side, and contained within the borders of the grey matter (Figure 4.1A). Often, injection sites extended rostrocaudally into either the L1 or L3 segments, but mediolateral spread was limited to the right hand side for all animals included in the study.
4.3.2 LDPN cell body distribution

LDPNs within segments C2 to T3 that project to the lumbar spinal cord were located both ipsilateral and contralateral to the injection site (Figure 4.2A and B). A similar percentage of LDPNs were found on either side of the cord (47% ipsilateral, 42% contralateral), with the remainder (12%) located in the midline (lamina X and medial lamina IV) (Figure 4.2C). Ipsilateral LDPNs were distributed widely throughout dorsal and ventral laminae, including the deep dorsal horn (laminae IV to VI), lamina VII, and lamina VIII. Interestingly, high concentrations were also located in the ipsilateral lateral spinal nucleus (LSN) and lateral cervical nucleus (LCN; Figure 4.2A, B, and D). Contralateral LDPN cell bodies formed a dense, ventromedial population tightly packed around the border of laminae VII and VIII. Again, a population of LDPNs was identified in the LSN and LCN, however the density of these neurons was low compared to the ipsilateral side (Figure 4.2A, B, and D). LDPNs located in the midline were most commonly found dorsal to the central canal and ventral to the apex of the dorsal columns (Figure 4.2A and B).

The rostrocaudal distribution of LDPN cell bodies was consistent throughout the cervical cord, but LDPNs exhibited higher numbers per section in thoracic (versus cervical) segments (7.4 vs 14.6 neurons per 40 µm section; p = 0.02; Figure 4.2E). Based on the average number of LDPNs per 40 µm section for each segment (Figure 4.2E), it can be estimated that there are approximately 3800 LDPNs between C2 and T3 in the adult mouse spinal cord (243 in C2, 286 in C3, 319 in C4, 321 in C5, 280 in C6, 294 in C7, 339 in C8, 460 in T1, 629 in T2, and 667 in T3; see Section 4.2.4 for calculation).

4.3.3 Inhibitory LDPN cell body distribution

GlyT2 (glycine positive) and GAD67 (GABA positive) neurons were localised in discrete regions of the cervical and upper thoracic spinal cord. GlyT2 neurons were present throughout the DDH, intermediate zone, and ventral horn (excluding lamina IX), while GAD67 neurons were located in the dorsal horn (particularly within the superficial dorsal horn) and sparsely throughout the medial intermediate zone and ventral horn (Figure 4.3A and B, upper panels). Inhibitory LDPNs, identified by colocalisation of Fluorogold and GFP (Figure 4.3A, B, lower panels), are plotted on standard spinal cord schematics in Figure 4.3C and D. Overall, 15.2% of LDPNs were GlyT2 positive (LDPNGlyT2), and 10.3% were GAD67 positive (LDPN GAD67). Remarkably, only 11.9% of LDPNGlyT2, and 13.8% of LDPN GAD67 were found contralateral to the L2 injection site.
Figure 4.2. LDPN cell bodies are distributed throughout the cervical and upper thoracic spinal cord with variable density throughout Rexed’s laminae. (A) Segmental distribution of LDPNs in the cervical and upper thoracic spinal cord following a Fluorogold injection into the right side of the L2 spinal segment (n=1). ‘Ipsi’ and ‘contra’ denote the side ipsilateral and contralateral to the injection site. Each segment was reconstructed using 3 to 5 40 µm sections from a 1-in-5 series. Schematic diagrams are based on Watson et al. (2009). (B) Heat maps were generated by collating LDPN cell body locations from multiple mice to illustrate where the highest relative density of LDPNs are located (n=5). Dark red colouring denotes high LDPN density. (C) A similar proportion of LDPNs were located on both the contralateral and ipsilateral side. A small proportion was found in the midline. (D) LDPNs were identified in specific regions of the spinal cord. Contralateral to the L2 injection site, they were clustered in medial lamina VII and VIII. A population of midline LDPNs was identified in lamina IV and X. Ipsilateral to the injection site, LDPNs were distributed more dorsally through lamina of the deep dorsal horn (IV to VI) and lamina VII and VIII. A large population of LDPNs was also identified in the ipsilateral LSN and LCN. (E) Rostrocaudal distribution of LDPNs throughout the cervical and rostral thoracic spinal cord. The number of LDPNs per 40 µm section increased in more caudal segments. Each graph is comprised of data from 5 mice. Data presented as mean ± SEM, * significant at p < 0.05. LSN = lateral spinal nucleus, LCN = lateral cervical nucleus, SDH = superficial dorsal horn, DDH = deep dorsal horn.
The vast majority of LDPN<sup>GlyT2</sup> and LDPN<sup>GAD67</sup> were located in the midline (30.7% and 31.2%, respectively), or ipsilateral (57.4% and 55%, respectively) to the injection site (Figure 4.3Ei and F i). The proportion of contralateral vs. ipsilateral inhibitory LDPNs is significantly different (p < 0.01 for LDPN<sup>GlyT2</sup>, and p < 0.05 for LDPN<sup>GAD67</sup>; Figure 4.3Ei and F i).

LDPN<sup>GlyT2</sup> cell bodies were distributed within the ipsilateral ventromedial grey matter in lamina VII and VII, and the midline (lamina X, and medial lamina IV), whereas LDPN<sup>GAD67</sup> cell bodies were predominantly located in the ipsilateral LSN, and midline (Figure 4.3C, D, Eii, and Fii).

4.3.4 LDPN morphology

Neurobiotin fills were recovered in five LDPNs and four control neurons for detailed morphological analysis. To maintain consistency, all neurons analysed were located in the contralateral, ventromedial grey matter (lamina VII and VII) between segments C5 to T1 (Figure 4.4A and B). LDPNs had radial dendrites that branched widely throughout the intermediate zone and ventral horn. The dendritic arbour of LDPNs was mostly restricted within the mediolateral plane. The total dendrite length for LDPNs and control neurons ranged from 1106 to 2002 and 1409 to 2386 μm, respectively, with average dendritic length similar between the two groups (1563 ± 262 vs. 1885 ± 239 μm; p = 0.3; Figure 4.4C). The number of dendritic branches in LDPNs and control neurons ranged from 7 to 25 and 12 to 42, respectively, yielding similar values for each population (16.8 ± 4.8 vs. 23.3 ± 7.8; p = 0.4; Figure 4.4D).

4.4 Discussion

This study provides the first detailed survey of LDPN cell body locations and projection patterns, along with preliminary information on their morphology. LDPNs were found ipsilateral and contralateral to the lumbar injection site in roughly even numbers, but were localized to distinct laminae on each side. Ipsilaterally projecting LDPNs were spread diffusely throughout the deep dorsal horn, intermediate zone, and ventral horn, with a dense population in the LSN/LCN, while contralaterally projecting LDPNs were concentrated within the ventromedial quadrant of the ventral horn and LSN/LCN. A medial population was also identified in the dorsal part of lamina X and medial lamina IV. Inhibitory LDPNs projected almost exclusively ipsilaterally to the lumbar injection site, or were found in the midline of the spinal cord. Finally, Neurobiotin
Figure 4.3. Inhibitory LDPNs project predominantly ipsilaterally to the lumbar spinal cord. (A & B) Upper panels: Immunostained cervical spinal cord sections from GlyT2<sup>GFP</sup> and GAD67<sup>GFP</sup> mice (glycinergic and GABAergic neuronal subpopulations, respectively) injected with Fluorogold in the right side of the L2 spinal segment. Note the differential localisation of glycinergic and GABAergic neuron populations. Lower panels: Examples of inhibitory LDPNs depicted by Fluorogold and GFP colocalisation (white asterisk). (C & D) Typical distribution of LDPN GlyT2 (C) and LDPN GAD67 (D) in the cervical and upper thoracic spinal cord (n = 1 for each). Each segment was reconstructed using 3 to 5 40 µm sections from a 1-in-5 series. Schematic
diagrams are based on Watson et al. (2009). (Ei & Fi) LDPN$^{\text{GlyT2}}$ and LDPN$^{\text{GAD67}}$ were predominantly located either ipsilateral to the lumbar injection site or in the midline of the spinal cord. There were significantly higher numbers of LDPN$^{\text{GlyT2}}$ and LDPN$^{\text{GAD67}}$ found ipsilateral to the injection site compared to the contralateral side. * significant at $p < 0.05$, ** significant at $p < 0.005$, ($n=3$ for both groups). (Eii & Fii) LDPN$^{\text{GlyT2}}$ were commonly identified in the midline (lamina IV and X), and ipsilateral lamina VII and VIII. LDPN$^{\text{GAD67}}$ were usually found in the midline, and the ipsilateral LSN and LCN. FG = Fluorogold, LSN = lateral spinal nucleus, LCN = lateral cervical nucleus, SDH = superficial dorsal horn, DDH = deep dorsal horn.

fills of a contralateral, ventromedial subpopulation of LDPNs revealed a radial dendritic arbour branching pattern within the mediolateral plane. No differences (albeit on a small sample of neurons) were identified in dendritic length or branching patterns between LDPNs and control neurons.

### 4.4.1 LDPN cell body location and projection patterns

Knowledge of the cell body location and axonal projection pattern of LDPNs in the mouse is particularly timely and relevant for SCI research, as LDPNs represent an ideal target for therapeutic strategies. As described previously, this assertion is based on their ability to form detour pathways with distant spinal circuits (Bareyre et al., 2004; Vavrek et al., 2006), as well as their remarkable resilience following SCI (Conta Steencken & Stelzner, 2010; Siebert et al., 2010b). In fact, LDPN axons withstand both severe and moderate contusion injuries at functionally significant levels (between 7% and 23% survival after SCI, respectively), whereas the axons of other neuronal populations rarely survive (Conta & Stelzner, 2004; Conta Steencken & Stelzner, 2010). This has been attributed to the position of LDPN axons as they are located in the outermost layers of the white matter and are therefore relatively distant from secondary SCI sequelae originating within the grey matter (Conta & Stelzner, 2004; Norenberg et al., 2004).

The tight clustering of LDPN cell bodies contralateral to the injection site suggests this subpopulation forms a more discrete functional group compared to the diffusely distributed ipsilateral LDPNs. As yet, the function of these contralateral LDPNs is unknown. However, their location in the ventromedial grey matter within lamina VII and VIII means they are well positioned to integrate signals from premotor interneurons that make up the cervical CPG (Kiehn & Butt, 2003; Kjaerulff & Kiehn, 1996). In combination with their direct projections to the lumbar spinal cord, these contralateral LDPNs may form a conduit for efficient signal transmission between cervical and lumbar CPGs. In support of this, acute inactivation of direct LPN connections between the cervical and lumbar regions using a viral strategy inhibited conventional interlimb coordination in mice (Pocratsky et al., 2014). Ipsilateral LDPNs are also located in the ventromedial grey matter, but were additionally found in the deep dorsal horn. This
Figure 4.4. LDPNs have similar dendritic morphology to control neurons. (A & B) Reconstructed neurobiotin filled LDPNs and control neurons. All filled neurons were located in the ventromedial grey matter, contralateral to the L2 injection site (lower schematics). A radial dendritic arbour distributed in the mediolateral plane is typical for both LDPNs and control neurons in this region. (C & D) No difference in dendritic length or branch number was found. Scale bar = 100 μm.

suggests that as well as linking motor associated output, ipsilateral LDPNs also transmit sensory information such as nociceptive, mechanoreceptive, and proprioceptive input to the lumbar cord (Heise & Kayalioglu, 2009). When interpreting these results, it is important to note that a portion of the ‘ipsilateral’ LDPNs labeled in this study are likely to have commissural axons that re-cross the midline prior to reaching the L2 spinal segment (Reed et al., 2006).

The overall pattern of LDPN cell body labeling in mice closely mirrors that observed in other species, with the exception of the dense population found in the lateral spinal nucleus and lateral cervical nucleus (LSN/LCN). Although an LSN/LCN LDPN population has been reported by others (Brockett et al., 2013; Burton & Loewy, 1976; Menetrey et al., 1985; Molenar & Kuypers, 1978), the consistency with which they were labeled in this study is higher than previously documented. LSN/LCN LDPNs in the mouse were not reported by Ni et al. (2014); but this group only labeled a small subset of LDPNs that synapse directly onto motoneurons. This suggests LSN/LCN LDPNs do not
project directly to lamina IX but synapse in other areas of the lumbar grey matter. LDPNs arising from the LSN are known to send axons through the intermediate portion of the lateral funiculus to reach the lumbar cord (Molenaar & Kuypers, 1978). Neurons within the LSN also provide ascending projections to multiple targets throughout the spinal cord (lamina I, II, V, and VII), brainstem, midbrain, diencephalon and striatum (Heise & Kayalioglu, 2009), and respond to noxious somatic, articular and visceral stimulation (Mentrey et al., 1989). Similarly, LCN neurons provide ascending projections to supraspinal structures, including the periaqueductal grey (Mouton et al., 2004) and thalamus (Craig & Burton, 1979) and also processes noxious stimuli (Kajander & Giesler, 1987). Interestingly, the midline population of LDPNs is also localized to spinal cord nociceptive centres (i.e. lamina IV and X; De Koninck et al. (1992); Honda and Perl (1985); Lanteri-Minet et al. (1993)). Based on this, LSN/LCN and midline (lamina IV and X) LDPNs may form a nociceptive pathway connecting the cervical and lumbar cord. These direct connections could be utilized to rapidly engage hindlimb musculature in order to avoid destabilization from a forelimb withdrawal, or to prepare for avoidance/escape behaviour.

4.4.2 Inhibitory LDPNs

The number of inhibitory LDPNs labeled in this study was relatively low (15.2% of LDPNs were GlyT2 positive, and 10.3% were GAD67 positive). It is also important to note that the overall percentage of inhibitory LDPNs is not simply the sum of LDPN GlyT2 and LDPN GAD67 populations, as a significant level of glycine/GABA co-expression can occur within individual neurons in the spinal cord (Jonas et al., 1998; Todd & Spike, 1993). The low number of inhibitory LDPNs in this study is contrary to that found by Ni et al. (2014). This group used in situ hybridization to label VIAAT (vesicular inhibitory amino acid transferase; a marker for all inhibitory interneurons) and VGluT2 (vesicular glutamate transporter 2; a marker for excitatory spinal interneurons) mRNA in a subset of descending PNs that project directly to tibialis anterior motoneurons. They found this group of descending PNs consisted of ~ 50% of each subtype (i.e. equal numbers of inhibitory and excitatory PNs). It is very likely that the number of inhibitory LDPNs is underestimated in my study, as GFP is not expressed in 100% of target cell types in transgenic mouse lines. For example, in one commonly used GAD67GFP line (Oliva et al., 2000), only 63% of GABAergic neurons in the adult lumbar spinal cord are positive for GFP (Dougherty et al., 2009). However, an important point from this work is that the location and projection pattern of inhibitory LDPNs did not follow the typical projection pattern of the overall population. The vast majority of inhibitory LDPNs were found ipsilateral to the lumbar injection site, or in the midline of the spinal cord. In other words,
only a small fraction of inhibitory LDPNs exhibited contralateral projections, despite nearly half of the overall LDPN population having contralateral connections with the upper lumbar cord (compare Figure 4.3 with Figure 4.2). This demonstrates LDPNs possess anatomical specialisations based on neurotransmitter phenotype.

4.4.3 LDPN morphology

Neurobiotin fills of contralaterally projecting, ventromedial LDPNs revealed a radial dendritic tree that branched widely in the mediolateral plane. This dendritic architecture is typical of lamina VII, and some lamina VIII neurons (Schoenen & Faull, 2004). A comparison with control neurons in the same location did not reveal any significant differences in dendritic morphology, suggesting LDPNs do not have characteristic anatomical specifications apart from their long descending axons. Recently, (Saywell et al., 2011) found similar morphologies identified in this study in descending commissural PNs located in the intermediate zone and ventral horn of the thoracic spinal cord. By combining sharp, intracellular recordings with Neurobiotin fills, they were also able to correlate some anatomical features with electrophysiological behaviour, such as soma position and strength of respiratory drive. A similar approach could be used to further classify subpopulations of LDPNs. It is important to note that a larger sample size for both LDPNs and control neurons (n = 5 and n = 4, respectively) may be required to confirm these findings. In addition, LDPNs from different regions (i.e. other than those examined in the contralateral, ventromedial grey matter) should be examined in future studies.

4.4.4 Conclusions

In addition to expanding our current anatomical knowledge on LDPNs (an important component of the propriospinal system), this work serves to direct future studies that examine their contribution to functional recovery following SCI. It is a necessary first step to allow investigators to draw conclusions on how specific LDPN subpopulations contribute to different forms of functional recovery (e.g. improved locomotor ability, visceral innervation/control, or restoration of appropriate sensory pathways). Importantly, this work was performed in mice, which are the most relevant animal model for current research in motor control and SCI as the full gamut of transgenic and viral technologies can be employed (Brownstone & Wilson, 2008; Deisseroth, 2011; Goulding, 2009; Wickersham et al., 2007).
Chapter 5: Inhibitory Synaptic Inputs onto Long Descending Propriospinal Neurons in mice
5.1 Introduction

Along the same lines as Chapter 4, this study aims to shed light on the basic properties of LDPNs in order to expand our knowledge on the propriospinal system in mice, since we know that PNs play a critical role in recovery from SCI. While the anatomy of LDPNs was assessed in Chapter 4, this study employs high-resolution patch-clamp electrophysiology to examine inhibitory synaptic inputs onto LDPNs. Inhibitory inputs were examined as a first step due to their importance in sensory processing and motor control.

In the spinal cord, fast inhibitory signalling is mediated by GABAergic and glycinergic neurotransmission (Curtis et al., 1968a; Curtis et al., 1968b; Roberts & Kuriyama, 1968; Schneider & Fyffe, 1992; Werman et al., 1968; Yoshimura & Nishi, 1995). These inhibitory connections are critical for regulating incoming sensory information (Bardoni et al., 2013; Eccles et al., 1961b) and executing patterned/coordinated neural activity in spinal motor circuits (Goulding, 2009; Goulding et al., 2014; Jankowska, 1992). Even small perturbations in inhibitory signalling can cause widespread motor deficits, as documented in a number of mice with naturally occurring glycine receptor mutations. For example, the spasmodic mouse has a single point mutation in the α1-subunit of the glycine receptor (Ryan et al., 1994) that causes subtle changes to glycine current kinetics (Graham et al., 2006). Despite the modest alteration, spasmodic mice have pronounced motor deficits including rapid tremors, stiff posture and an impaired righting reflex (Lane et al., 1987). LDPNs, as discussed in Chapter 4, are anatomically positioned to both integrate sensory signals from the periphery and to also connect locomotor central pattern generator (CPG) circuitry throughout the spinal cord. It stands to reason that inhibitory inputs would play a major role in shaping LDPN output. Characterising the nature and strength of these synapses is a necessary step for understanding how inhibitory synaptic drive regulates LDPN activity. Furthermore, knowledge on synaptic integration within LDPNs in normal/uninjured spinal tissue will provide ‘benchmark’ data for future studies that examine synaptic plasticity in LDPN populations following SCI.

Despite many technical advances in recent years, electrophysiological methods remain the best approach to provide functional information on synaptic input in any given neuronal population. So far, our current understanding of LNP electrophysiology comes from a series of experiments spanning over seventy years using both sharp intracellular electrodes (A Istermark et al., 1991; A Istermark et al., 1987a, 1987b, 1987c; Brink et al.,
Chapter 5: Inhibitory Synaptic Inputs onto Long Descending Propriospinal Neurons in mice

1985; Jankowska et al., 1974; Jankowska et al., 1973; Schomburg et al., 1978; Vasilenko, 1975) and extracellular recordings (Juvin et al., 2012; Juvin et al., 2005; Lloyd, 1942; Lloyd & McIntyre, 1948; Miller et al., 1973a; Miller et al., 1973b; Skinner et al., 1980). These studies have shed light on the role of LPNs in spinal reflexes, sensory circuits, and intersegmental motor pathways. However, a detailed and systematic analysis of synaptic inputs onto LDPNs has not been attempted. This level of detail can only be obtained using the whole-cell patch-clamp technique (Sakmann & Neher, 1984) which provides a superior signal to noise ratio compared to sharp intracellular or extracellular recordings that can be used to resolve post-synaptic currents with very high fidelity. Whole-cell patch-clamp recordings have in fact been reported for lumbar SPNs, revealing a distinct set of intrinsic and synaptic properties that implicate them in a distributed CPG network (Butt et al., 2002). However, such recordings have not been attempted in LPNs. A number of related factors that have likely contributed to this omission include the reliance on intact spinal cord preparations to unambiguously identify LPNs on functional criteria, which are not amenable to visualised patch-clamp recordings. Conversely, LPNs are not easily identified in the slice preparations that are preferred for patch-clamp recordings.

This study addressed these limitations, by combining a retrograde labelling technique and whole-cell patch-clamp recordings to obtain the first high-resolution electrophysiological data on inhibitory post-synaptic currents in lumbar projecting, cervical LDPNs. LDPNs were recorded in an in vitro spinal cord slice preparation that allowed visually targeted electrophysiology and pharmacological dissection of inhibitory post-synaptic currents (IPSCs). The frequency and kinetics of synaptic events were analysed and compared to a control population of spinal interneurons.

5.2 Methods

Lumbar projecting LDPNs situated in segments C4 to T2 were retrogradely labelled with 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiI) for targeted electrophysiology. DiI was injected into the lumbar spinal cord between L1 and L3 segments in 3 to 5 week old mice to back-label cervical LDPN cell bodies over a period of 2 to 3 days. Transverse spinal cord slices of cervical and upper thoracic segments were then cut to allow visually guided in vitro whole-cell patch-clamp recordings. Several ion channel antagonists were sequentially applied to isolate specific IPSCs. The frequency and kinetics of inhibitory events were analysed and compared to non-fluorescent control neurons near LDPN cell bodies to provide a control dataset of mixed neuron identity.
5.2.1 Intraspinal tracer surgery

Details of the intraspinal tracer surgery are described in Chapter 4, Section 4.2.1. However, of difference in this study, DiI (2.5% in DMSO) was used as the retrograde tracer instead of Fluorogold in order to provide a brighter fluorescent signal for targeted patch-clamp electrophysiology and to minimise neurotoxic effects at the injection site (Honig & Hume, 1986; Schmued et al., 1993). Animals were returned to their home cage for 2 or 3 days prior to each experiment, allowing DiI to be retrogradely transported back to LDPN cell bodies in the cervical cord (Figure 5.1B). After the recovery period and immediately prior to each experiment, transverse slices of the lumbar cord (250 μm thick) were obtained to examine location and extent of the injection site (Figure 5.1A). Animals with injection sites that extended contralaterally (i.e. into the grey matter on the left site of the spinal cord) were excluded from the study.

![Figure 5.1.](image)

**Figure 5.1.** Labelling LDPNs using the retrograde tracer, DiI. (A) Schematic summarises surgical approach and procedure to retrogradely label LDPNs. Following a T12 laminectomy, DiI was pressure injected into the mouse lumbar spinal cord. Inset upper: the pipette tip penetrated the dorsal spinal cord at the L2 spinal segment and was lowered until positioned in intermediate zone (depth = 0.75 mm) before injecting 20-80 nL of DiI. Inset lower: Typical spread of DiI after L2 injection. Some injections extended rostrocaudally into L1 or L3. Animals with injection sites extending contralaterally (i.e. into the left side) were excluded. (B) Mice recovered for 2 to 3 days after surgery to allow retrograde transport of DiI from the injection site in the lumbar spinal cord to cervical LDPN cell bodies.

5.2.2 Spinal cord slice preparation

After the surgery and tracer transport time (2-3 days), animals were overdosed with ketamine (100 mg/kg i.p.) and decapitated. The torso was isolated, eviscerated, and submerged in ice-cold sACSF. Using a ventral approach, the T4 vertebral body was removed with rongeurs to expose the underlying thoracic spinal cord. Starting at this opening and moving rostrally, the remaining thoracic and cervical vertebral bodies were cut away with spring scissors. With the upper thoracic and cervical segments exposed, the spinal cord from C2 to T3 was dissected free from the vertebral canal (Figure 5.2A), mounted onto a custom made styrofoam block and glued to a cutting stage (Figure 5.2B).
The tissue stage was immediately transferred to a chamber containing ice-cold, oxygenated sACSF before 250 µm thick transverse slices were cut using a vibrating microtome (7000 SMZ; Campden Instruments, Leicestershire, UK). A small cut was made in the white matter on the right side of each slice (i.e. ipsilateral to lumbar injection site) to identify the position of recorded neurons relative to the injection (Figure 5.2D). Approximately 9 or 10 slices from mid-cervical (C4) to upper thoracic (T2) spinal cord were collected (Figure 5.2C). Full details of solutions used and equilibration times are outlined in Chapter 2, Section 2.2.1.

Figure 5.2. Preparation of transverse spinal cord slices for targeted LDPN recordings. (A) Following overdose (ketamine 100 mg/kg) and decapitation, a spinal cord block spanning between the C2 and T3 segments was ventrally excised from the vertebral canal. (B) The spinal cord was placed upright on a styrofoam block and glued to a cutting stage. (C) A vibratome was used to cut 250 µm thick transverse slices from mid-cervical (C4) to upper thoracic (T2) spinal cord (approx. 9 to 10 slices in total). (D) A small cut was made on the right side of each cervical slice to denote the side ipsilateral to the lumbar injection site. Inset: Under high magnification, LDPNs could be targeted using a TRITC filter block.

5.2.3 Whole-cell patch-clamp electrophysiology

Full details of whole-cell patch-clamp electrophysiology are outlined in Chapter 2, Section 2.2.3. However, of difference, a TRITC filter block (41002; Olympus, Tokyo,
Japan) was used to identify Dil filled LDPN cell bodies for targeted electrophysiology (Figure 5.2D), and a CsCl-based internal solution (containing [in mM]: 30 CsCl, 10 HEPES, 10 EGTA, 1 M gCl₂, 2 ATP, and 0.3 GTP, pH adjusted to 7.35 with 1 M CsOH) was used to clearly resolve IPSCs at a holding potential of -70 mV. With these recording conditions, IPSCs were represented as large inward currents. In some experiments, Neurobiotin (0.2% w/v) was added to the internal solution to allow post-hoc anatomical reconstructions of recorded neurons that were reported in Chapter 4.

5.2.4 Pharmacology

After establishing the whole-cell recording configuration, IPSCs were selectively isolated with ion-channel antagonists. Drugs were sequentially added to the bath every 6 minutes. This allowed 3 minutes for drugs to reach the tissue and take effect, and at least 3 minutes of additional recording time. CNQX (10 µM) was used to block glutamatergic AMPA/kainate receptors, isolating AP driven, mixed (GABAergic and glycinergic) spontaneous IPSCs (mixed sIPSCs; Honore et al. (1988); Yamada et al. (1989) Figure 5.3B). Under these conditions, miniature IPSCs (mixed mIPSCs) are also present. Each mIPSC results from the stochastic release of neurotransmitter at a single synapse (Bekkers & Stevens, 1989). For clarity, however, the following text and figures will refer to this condition simply as ‘mixed sIPSCs’. Tetrodotoxin (TTX; 1 µM) was then added to block voltage-gated Na⁺ channels and inhibit AP mediated neurotransmitter release, isolating mixed mIPSCs (Figure 5.3C). Bicuculline (10 µM) was then applied to block GABA_A receptors to isolate glycinergic mIPSCs (Curtis et al., 1971; MacDonald & Olsen, 1994) (Figure 5.3D). Finally, strychnine (1 µM) was applied, to block glycine receptors and inhibit all fast synaptic neurotransmission (Callister & Graham, 2010; Larson, 1969) (Figure 5.3E).

5.2.5 Data analysis

Analysis was performed offline using Axograph X software. In a semi-automated procedure, a sliding double exponential template was used to detect, capture and align post-synaptic currents within each pharmacological condition. The average frequency of post-synaptic currents was calculated by dividing the total number of events by the recording time in seconds. Instantaneous frequency was calculated by averaging the
Figure 5.3. Pharmacological dissection of inhibitory synaptic drive in LDPNs. By sequentially adding CNQX, TTX, bicuculline, and strychnine to the recording ACSF specific post-synaptic currents were isolated for analysis. All recordings were performed at a holding potential of -70 mV. (A) In normal ACSF, action potential driven sEPSCs and mixed (GABAergic and glycinergic) sIPSCs are visible. (B) The addition of CNQX (10 µM) blocks AMPA receptors, isolating mixed sIPSCs. (C) The addition of TTX (1 µM) blocks voltage-gated Na⁺ channels, inhibiting action potential mediated neurotransmitter release, leaving only mixed mIPSCs. (D) Addition of bicuculline (10 µM) blocks GABA_A receptors, isolating glycinergic mIPSCs. (E) Finally, addition of strychnine (1 µM) blocks glycine receptors, inhibiting all fast synaptic neurotransmission and resulting in a flat baseline without synaptic current activity. CNQX: 6-cyano-7-nitroquinoxaline-2,3-dione, TTX: tetrodotoxin, AMPA: α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid, GABA: γ-aminobutyric acid, sEPSC: spontaneous excitatory post-synaptic current, sIPSC: spontaneous inhibitory post-synaptic current, mIPSC: miniature inhibitory post-synaptic current.
reciprocal of all inter-event intervals. For quantitative analyses, each post-synaptic current was inspected and excluded if it had corrupted rise and decay phases, if it contained overlapping events, or if it exhibited an unstable baseline. In addition, recordings where excluded if $R_s$ was $> 20 \, \text{M}\Omega$ or if the holding current required to maintain the neuron at -70 mV was $> 400 \, \text{pA}$. Peak amplitude, rise-time (calculated over 10-90% of peak amplitude), and $\frac{1}{2}$ width were measured for each individual post-synaptic current. The decay time constant ($\tau$; determined from a single exponential fit) and charge ($Q; \text{ms} \times \text{pA}$) were obtained from the average trace of all overlayed post-synaptic currents for each neuron (Figure 5.4). Total synaptic ‘drive’ was determined by multiplying the average frequency of events by the charge ($\text{Av. freq.} \times Q$). Scatterplots of peak amplitude versus rise-time were generated with a custom MATLAB script that applied a heat map corresponding to point density (Sanchez-Barba, 2005). Rhythmic synaptic bursting was defined as visually identifiable clusters of synaptic events occurring at regular intervals (Figure 5.8A). In cases where a tonic glycine current was present (Figure 5.8B), the root mean square (RMS) noise and holding current were measured before and after strychnine application.

![Graph showing analysis of inhibitory synaptic current properties in LDPNs. Trace shows an averaged inhibitory current with the key parameters measured and compared between LDPNs and control recordings. These measurements include: peak amplitude (baseline to peak; vertical double arrow), rise-time (time from 10% to 90% of peak amplitude; horizontal lines), $\frac{1}{2}$ width (horizontal double arrow), decay time constant (determined from a single exponential fit to the decay phase), and charge (current area; grey fill).](image)

5.2.6 Statistics

Comparisons between synaptic properties of LDPN and control neurons were made with an unpaired Student’s t-test when data sets were normally distributed (assessed by the Kolmogorov-Smirnov test) and had equal variance (SD of both groups different by less than a factor of 2). In cases where data sets were normally distributed but had unequal variances, Welch’s t-test was applied. Non-normally distributed data sets were compared with the Mann-Whitney U test. All statistical analysis was performed with
GraphPad Prism 6 software (GraphPad Software; CA, USA). Statistical significance was set at \( p < 0.05 \). All values are presented as mean ±SEM.

5.2.7 Cell body location maps

Details of cell body map construction are described in Chapter 4, Section 4.2.4. However, instead of immunolabelling, LDPN locations were determined from a photograph of each slice (X5 magnification) while the recording pipette was attached to the Dil-labelled or control neuron.

5.3 Results

Whole-cell patch-clamp recordings were obtained from 53 fluorescently labelled LDPNs and 53 unlabelled control neurons. All neurons were located in the intermediate zone and ventral horn of spinal cord segments C4 to T2 (Figure 5.5A), corresponding to the spinal circuitry contributing to the cervical CPG innervating the forelimbs (Ballion et al., 2001; Tosolini et al., 2013). Importantly, the location of LDPNs and control neurons are comparable (Figure 5.5A and B), meaning any electrophysiological differences are unrelated to recording location bias.

![Figure 5.5.](image)

**Figure 5.5.** LDPN and control neuron recordings are sampled from similar, overlapping, spinal regions. (A) The position of recorded LDPNs (red) and control neurons (black) is plotted on transverse spinal cord templates from C4 to T2. The lumbar injection site is represented schematically. (B) Pie graphs compare the position of LDPN and control neuron cell bodies relative to the lumbar injection site (midline = lamina X or medial lamina IV).

5.3.1 Inhibitory post-synaptic current frequency

To examine the incidence of inhibitory synapses onto LDPNs, the frequency of mixed (GABAergic and glycineergic) sIPSCs, mixed mIPSCs and glycineergic mIPSCs
was calculated. Average frequencies in all pharmacological conditions were compared between LDPNs and control neurons (Figure 5.6A and B), with comparisons summarised in Table 5.1.

There was no significant difference in the average frequency of mixed sIPSCs (isolated with 10 µM CNQX) between LDPNs and control neurons (7.0 ± 0.5 Hz vs. 5.8 ± 0.6 Hz; p = 0.13; Figure 5.6C). However, when mixed mIPSCs were isolated by inhibiting AP dependent synaptic release with 1 µM TTX, LDPNs had a significantly higher average frequency than control neurons (3.1 ± 0.4 vs. 2.0 ± 0.2 Hz; p = 0.0052; Figure 5.6C and D). Addition of TTX to the bath strongly attenuated inhibitory synaptic neurotransmission in both groups (7.0 ± 0.5 to 3.1 ± 0.4 Hz in LDPNs and 5.8 ± 0.6 to 2.0 ± 0.2 in control neurons before versus after TTX; p < 0.0001 for both; Figure 5.6C).

When GABAergic neurotransmission was blocked with 10 µM bicuculline, isolating glycinergic mIPSCs, the difference in average frequency between LDPNs and control neurons was abolished (2.2 ± 0.3 vs. 1.8 ± 0.3 Hz; p = 0.17; Figure 5.6D). A within group comparison reveals bicuculline was only effective in reducing the average frequency of mIPSCs in LDPNs, while control neurons were unaffected (3.1 ± 0.4 to 2.2 ± 0.3 Hz in LDPNs compared to 2.0 ± 0.2 to 1.8 ± 0.3 Hz in control neurons; p = 0.047 and 0.587, respectively; Figure 5.6D). The sensitivity of LDPNs to bicuculline suggests they receive a higher proportion of pure GABAergic inputs, or that these inputs have a greater release probability, than in control neurons. Based on the average frequency before and after bicuculline, it is estimated that 29% of mixed mIPSCs in LDPNs are purely GABAergic vs. only 10% in control neurons. All remaining currents were abolished with 1 µM strychnine (Figure 5.3E), confirming they were glycinergic.

The instantaneous frequency of post-synaptic currents was also calculated in each pharmacological condition. Compared to average frequency, instantaneous frequency is particularly sensitive to clusters or bursts of post-synaptic currents. For AP driven sIPSCs, LDPNs had a higher instantaneous frequency than control neurons (14.6 ± 0.7 vs. 12.1 ± 0.9 Hz, p = 0.025; Table 5.1). Given there was no difference between LDPNs and control neurons in the average frequency in this condition (described above), it suggests that mixed sIPSCs were more clustered in LDPNs. The addition of TTX abolished any differences in instantaneous frequency between LDPNs and control neurons (Table 5.1). This finding is in line with the elevated bursting behaviour in LDPNs as TTX blocks APs, and therefore, any synchronous neural activity that underlies clustered/bursting post-synaptic currents.
Figure 5.6. The frequency and kinetics of IPSCs differ between LDPNs and control neurons. (A and B) Traces show mixed mIPSC (upper) and glycinergic mIPSC (lower) recordings from an LDPN (left, black) and control neuron (right, grey). (C) Group data plot shows the change in average frequency from mixed sIPSCs to mixed mIPSCs in LDPNs (46 and 40 neurons, respectively) and control neurons (46 and 38 neurons, respectively). The frequency was similarly reduced between sIPSC and mIPSC recordings. ** significant at p < 0.005 (intergroup comparison), ϕϕϕϕ significant at p < 0.0001 (intragroup comparison) (D) Group data plot shows the change in average frequency from mixed mIPSCs to glycinergic mIPSCs in LDPNs (40 and 34 neurons, respectively) and control neurons (38 and 31 neurons, respectively). The frequency of mIPSCs was significantly higher in LDPNs than in control neurons. Furthermore, the frequency of currents fell significantly between mixed mIPSC and glycinergic mIPSC recordings in LDPNs, but not in control neurons. ** significant at p < 0.005 (intergroup comparison), ψ significant at p < 0.05 (intragroup comparison). (E and F) Overlayed traces are averaged mixed mIPSCs (dashed lines) and glycinergic mIPSCs from an LDPN (left, black) and control neuron (right, grey). Note the slower decay phase in the average mixed mIPSC in LDPNs compared to the average glycinergic mIPSC, whereas control neurons show no differences.
Table 5.1. IPSC frequency

<table>
<thead>
<tr>
<th>Recording condition</th>
<th>Av. Frequency (Hz)</th>
<th>Inst. Frequency (Hz)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Mixed sIPSCs</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control (46)</td>
<td>5.8 ± 0.6</td>
<td>12.1 ± 0.9</td>
</tr>
<tr>
<td>LDPN (46)</td>
<td>7.0 ± 0.5</td>
<td>14.6 ± 0.7 *</td>
</tr>
<tr>
<td><strong>Mixed mIPSCs</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control (38)</td>
<td>2.0 ± 0.2</td>
<td>7.5 ± 0.7</td>
</tr>
<tr>
<td>LDPN (40)</td>
<td>3.1 ± 0.4 **</td>
<td>9.3 ± 0.7</td>
</tr>
<tr>
<td><strong>Glycinergic mIPSCs</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control (31)</td>
<td>1.8 ± 0.3</td>
<td>7.4 ± 0.8</td>
</tr>
<tr>
<td>LDPN (34)</td>
<td>2.2 ± 0.3</td>
<td>7.1 ± 0.6</td>
</tr>
</tbody>
</table>

Table 5.2. Series and input resistance

<table>
<thead>
<tr>
<th>Recording condition</th>
<th>Series Resistance (MΩ)</th>
<th>Input Resistance (MΩ)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Mixed sIPSCs</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control (37)</td>
<td>12.0 ± 0.6</td>
<td>418 ± 30</td>
</tr>
<tr>
<td>LDPN (34)</td>
<td>12.4 ± 0.6</td>
<td>348 ± 35</td>
</tr>
<tr>
<td><strong>Mixed mIPSCs</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control (23)</td>
<td>13.5 ± 0.9</td>
<td>294 ± 17</td>
</tr>
<tr>
<td>LDPN (24)</td>
<td>12.2 ± 0.6</td>
<td>299 ± 26</td>
</tr>
<tr>
<td><strong>Glycinergic mIPSCs</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control (19)</td>
<td>13.5 ± 0.8</td>
<td>298 ± 19</td>
</tr>
<tr>
<td>LDPN (20)</td>
<td>11.7 ± 0.8</td>
<td>268 ± 26</td>
</tr>
</tbody>
</table>

Table 5.3. Frequency and kinetic properties of IPSCs

<table>
<thead>
<tr>
<th></th>
<th>Peak Amplitude (pA)</th>
<th>Rise-time (ms)</th>
<th>½ Width (ms)</th>
<th>Decay Time (ms)</th>
<th>Q (pA × ms)</th>
<th>Drive x10^3 (Q × Av. Freq.)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Mixed sIPSCs</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control (37)</td>
<td>239 ± 16</td>
<td>0.63 ± 0.04</td>
<td>4.3 ± 0.2</td>
<td>4.9 ± 0.3</td>
<td>1432 ± 120</td>
<td>8.5 ± 1.5</td>
</tr>
<tr>
<td>LDPN (34)</td>
<td>197 ± 15 *</td>
<td>0.73 ± 0.03 *</td>
<td>4.8 ± 0.2</td>
<td>5.3 ± 0.3</td>
<td>1265 ± 119</td>
<td>9.3 ± 1.4</td>
</tr>
<tr>
<td><strong>Mixed mIPSCs</strong></td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Control (23)</td>
<td>192 ± 14</td>
<td>0.60 ± 0.04</td>
<td>4.0 ± 0.2</td>
<td>4.3 ± 0.3</td>
<td>1027 ± 90</td>
<td>2.2 ± 0.4</td>
</tr>
<tr>
<td>LDPN (24)</td>
<td>188 ± 14</td>
<td>0.65 ± 0.03</td>
<td>4.6 ± 0.2</td>
<td>5.3 ± 0.4 *</td>
<td>1167 ± 96</td>
<td>3.2 ± 0.6</td>
</tr>
<tr>
<td><strong>Glycinergic mIPSCs</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control (19)</td>
<td>181 ± 14</td>
<td>0.61 ± 0.04</td>
<td>4.2 ± 0.3</td>
<td>4.5 ± 0.4</td>
<td>1015 ± 120</td>
<td>1.8 ± 0.3</td>
</tr>
<tr>
<td>LDPN (20)</td>
<td>158 ± 11</td>
<td>0.63 ± 0.04</td>
<td>4.3 ± 0.2</td>
<td>4.7 ± 0.2</td>
<td>928 ± 66</td>
<td>1.2 ± 0.3</td>
</tr>
</tbody>
</table>

Brackets denote number of neurons used in analysis. * significant at p < 0.05. Values are mean ± SEM. For details on measurements, see Section 5.2.5 and Figure 5.4.
5.3.2 Post-synaptic current kinetics

To ensure recording conditions and intrinsic neuronal properties did not contribute to differences in post-synaptic current kinetics, series and input resistance were compared in both groups (Table 5.2). No differences were identified in any pharmacological condition. However, the input resistance between LDPNs and control neurons did approach a statistically significant difference while recording mixed sIPSCs (348 ± 35 vs. 418 ± 30; p = 0.06. On average, input resistance in both groups became lower throughout the recordings (Table 5.2). This is probably due to the well-known sensitivity of adult ventral horn interneurons to run down during patch-clamp recording (Husch et al., 2011).

A comparison of kinetic parameters is summarised in Table 5.3. Mixed sIPSCs in LDPNs had smaller peak amplitudes (197 ± 15 vs. 239 ± 16 ms; p < 0.05) and slower rise-times (0.73 ± 0.03 vs. 0.63 ± 0.04 ms; p = 0.01) than in control neurons (Figure 5.7A). This could be due to a higher level of electrotonic filtering of more distally located synapses, the geometry of LDPN dendritic arbours, or the ostensibly lower input resistance of LDPNs versus control neurons during mixed sIPSC recordings (described above; (Bekkers & Stevens, 1996; Rall, 1959; Spruston et al., 1994). To test this, scatterplots of amplitude versus rise-time for all mixed sIPSCs in both LDPNs and control neurons were generated to examine the point spread (Figure 5.7B and C). A broad distribution of rise-time values for low amplitude events is a hallmark of electrotonic filtering (Tian et al., 1998; Ulrich & Luscher, 1993). The overall point spread in each scatterplot demonstrates electrotonic filtering occurs in both LDPNs and control neurons. However, the high-density clusters (>30 overlapping points; green to red) reveal an asymmetric spread of mixed sIPSCs in LDPNs compared to control neurons, consistent with a higher level of electrotonic filtering (Figure 5.7B and C). In addition, histograms and cumulative probability plots of rise-times from low amplitude events (< median) show a rightward shift for LDPNs compared to control neurons (Figure 5.7D).

After isolating mixed mIPSCs with 1 µM TTX, events in LDPNs had a longer decay time constant when compared to control neurons (5.3 ± 0.4 vs. 4.3 ± 0.3 ms; p = 0.04; Table 5.3, Figure 5.6E and F). The slower decay supports the average frequency data showing that LDPNs receive a higher proportion of GABAergic events than control neurons (Table 5.1, Figure 5.5C and D), as GABAergic IPSCs have a considerably longer decay time constants than glycinergic events (Chery & de Koninck, 1999; Gonzalez-Forero & Alvarez, 2005). Consistent with this interpretation, the difference in
The decay time constant between LDPNs and control neurons was abolished once GABAergic events were blocked with 10 µM bicuculline (4.7 ± 0.2 vs. 4.5 ± 0.4 ms; p = 0.69; Table 5.3, Figure 5.6E).

**Figure 5.7.** LDPNs exhibit greater electrotonic filtering than control neurons. (A) Upper traces show overlayed averaged mixed sIPSC from an LDPN (black) and control neuron (grey). Lower traces show the same overlayed sIPSCs on an expanded time scale. Mixed sIPSCs in LDPNs had a smaller amplitudes and longer rise times compared to control neurons. (B and C) Electrotonic filtering was assessed with amplitude versus rise-time scatterplots generated using all mixed sIPSCs in LDPNs (n = 7369) and control neurons (n = 5744). High point densities are denoted with warmer colours to reveal the underlying spread of data, highlighting a difference in the amplitude/rise time relationship of low amplitude mixed sIPSCs. (D) Histogram of rise-time values for ‘low’ (< median) amplitude mixed sIPSCs demonstrates a rightward shift for LDPNs (black) compared to control neurons (light grey). Overlapping distributions are shown in dark grey. *Inset:* cumulative histogram displaying rightward shift in LDPN rise-time distribution, demonstrating the effects of greater electrotonic filtering.

### 5.3.3 Rhythmic synaptic bursting and tonic glycine currents

In addition to analyzing the frequency and kinetics of post-synaptic currents, two other noteworthy features were identified. First, a subset of LDPNs (5/53) and a single control neuron (1/52) exhibited rhythmic synaptic bursting in normal ACSF (i.e. prior to the application of ion-channel antagonists; **Figures 5.3A, and 5.8A**). The frequency of
these synaptic bursts ranged from 0.27 to 0.69 Hz and continued for between 29 and 165 seconds. After application of 10 µM CNQX, rhythmic synaptic bursts were eliminated, demonstrating that excitatory synapses form at least part of the rhythm generating circuitry. Although the incidence of rhythmic synaptic bursting was relatively low in these recordings, the prevalence is probably underestimated due to the transverse slice preparation used in this study which transects all neural circuitry above and below the slice.

**Figure 5.8.** Rhythmic synaptic bursting and tonic glycine currents were identified in LDPN and control neuron recordings. (A) Trace shows an example of rhythmic synaptic bursting from an LDPN. This form of activity was identified in normal ACSF bath conditions (i.e. prior to the addition of any ion channel antagonists) in 9% of LDPNs (5/53) and 2% of control neurons (1/52). (Bi) Trace shows a tonic glycine current that was abolished by bath application of 1 µM strychnine. These currents were detected in 40% of LDPNs (10/25) and 30% of control neurons (7/23). All point’ histograms of baseline noise on the right shows the change in RMS noise prior to and after abolition of the tonic glycine current (note the spread of the two distributions). Dashed grey lines highlight the change in holding current due to the tonic glycine current. (Bii) Group plots show the magnitude of tonic glycine currents, which were similar between LDPNs and control neurons as assessed by changes to RMS noise and holding current after strychnine application. The number of neurons in each group is denoted in brackets.

Second, tonic glycine currents were observed in 40% of LDPNs (10/25) and 30% of control neurons (7/23). A tonic glycine current is mediated by the constitutive activation of extrasynaptic glycine receptors, leading to constant level of background inhibition (Takazawa & MacDermott, 2010). Tonic glycine currents were detected by applying 1 µM strychnine to block all glycine receptors. This caused both a decrease in the RMS noise and a reduction in holding current required to voltage-clamp the neuron at -70 mV (Figure 5.8Bi). The magnitude of tonic glycine currents in LDPNs and control neurons were similar based on changes to RMS noise (-6.3 ± 1.0 vs. -6.0 ± 1.0 pA²) and holding current (-63 ± 21 vs. -61 ± 32 pA) after strychnine application (Figure 5.8Bii).
5.4 Discussion

This study provides the first high-resolution electrophysiological characterisation of inhibitory synaptic input onto LDPNs in the adult mouse spinal cord. When compared to control neurons in the same location, LDPNs received a higher proportion of GABAergic events and exhibited greater electrotonic filtering of AP mediated mixed sIPSCs. Rhythmic synaptic bursting was also detected in a subpopulation of LDPNs, along with tonic glycinergic inhibition in a significant proportion of LDPNs and control neurons. Importantly, this study can be used for comparison with future work examining synaptic plasticity of inhibitory synapses on LDPNs following SCI both with and without therapeutic interventions.

5.4.1 GABAergic inputs

Nearly 30% of mixed mIPSCs in LDPNs were purely GABAergic, compared to only 10% in control neurons. As a result, the high frequency of GABAergic inputs caused a significant increase in the average decay time constant for mixed mIPSCs in LDPNs (Tables 5.1 and 5.3 and Figure 5.6E). During embryonic development, GABA is the dominant ‘inhibitory’ neurotransmitter (note: GABA has excitatory actions during embryogenesis; Gao and Ziskind-Conhaim (1995); Ma et al. (1992); Wu et al. (1992)). After birth, however, glycinergic neurotransmission increases markedly in the spinal cord (Gao et al., 2001; Gonzalez-Forero & Alvarez, 2005; Sibilla & Ballerini, 2009). In some cases, GABAergic inputs maintain an unusually high contribution to specific classes of neurons, such as the LDPNs in this study, and Renshaw cells. Renshaw cells provide recurrent negative feedback to motoneurons, ensuring muscle activation is specific and temporally precise (Alvarez et al., 2005; Bhumbra et al., 2014; Eccles et al., 1961a; Renshaw, 1941, 1946). These neurons have low AP thresholds and receive strong, long lasting cholinergic inputs from motoneurons, necessitating powerful inhibition to modulate and limit their responses (Windhorst, 1990). This is achieved with a combination of fast glycinergic neurotransmission, and importantly, a relatively high frequency of slow decaying GABAergic events to overcome strong excitatory inputs (Gonzalez-Forero & Alvarez, 2005). The high frequency of GABAergic events in LDPNs, whether brought about by a higher number of GABAergic inputs, or greater release probability, suggests these neurons require substantial inhibition to regulate their activity. These inputs may arise from intraspinal GABA +ve neurons located in the dorsal horn and lamina X (Dougherty et al., 2009), or from descending supraspinal pathways. LDPNs receive descending inhibitory inputs from the medial and lateral vestibular nuclei, and the reticulospinal nucleus (Alstermark et al., 1987a, 1987c), all of which provide descending GABAergic projections to the spinal cord (Du Beau et al., 2012; Valla et al.,...
Chapter 5: Inhibitory Synaptic Inputs onto Long Descending Propriospinal Neurons in mice

2003). The origin of these GABAergic inputs could be elucidated using monosynaptically restricted rabies virus tracing and subsequent neurochemical identification of neurons projecting directly to LDPNs (Wickersham et al., 2007).

5.4.2 Electrotonic filtering and tonic glycine currents in LDPNs

Prior to application of TTX, AP evoked mixed sIPSCs underwent more electrotonic filtering in LDPNs than control neurons as evidenced by lower amplitudes, longer rise times, and greater point spread in the amplitude vs. rise-time scatterplot (Figure 5.7, Table 5.3). The seemingly lower (although not significantly different) input resistance during mixed sIPSC recordings between LDPNs and control neurons could contribute to this observation (Spruston et al., 1994) (Table 5.2). Specifically, once voltage-gated Na⁺ channels were blocked with TTX, the input resistance between LDPNs and control neurons equalised, coinciding with the loss of ancillary electrotonic filtering in LDPNs (Tables 5.2 and 5.3). Future experiments using the perforated patch-clamp technique could be employed to minimise neuron dilation and changes in input resistance to better assess any potential intrinsic differences between LDPNs and control neurons (Husch et al., 2011). Alternatively, AP mediated inhibitory synaptic neurotransmission could be ‘targeted’ to synapses that are susceptible to electrotonic filtering in LDPNs, such as those located on distal dendrites (Amatrudo et al., 2012; Bekkers & Stevens, 1996). The logic behind this arrangement may lie in a requirement for rapid shunting of excitatory neurotransmission in distal dendrites when required, or achieving a wide temporal window for synaptic integration at the cell body through slower inhibitory events (Branco & Hausser, 2011).

A considerable proportion of LDPNs (40%) received a tonic glycine current (Figure 5.8B), which leads to an overall decrease in neuronal excitability (Maguire et al., 2014; Mitchell et al., 2007; Salling & Harrison, 2014; Takazawa & MacDermott, 2010). Work on tonic inhibition in other CNS regions has demonstrated that persistent inhibition can modulate network oscillations, decrease input/output gain of recipient neurons and increase the dynamic range of neural circuits (Maex & De Schutter, 1998; Mitchell & Silver, 2003; Semyanov et al., 2004). The properties discussed so far would indeed be advantageous in neural networks that require fast, stable and highly dynamic computations, including those forming spinal motor circuits (Grillner, 2003; Grillner & Jessell, 2009).

5.4.3 Rhythmic synaptic input onto LDPNs

The high instantaneous frequency of sIPSCs prior to application of TTX, in addition to the rhythmic synaptic bursts preceding CNQX application (Table 5.1 and...
Figure 5.8A), is the first direct evidence that LDPNs receive coordinated, rhythmic synaptic activity. Despite only a small percentage of LDPNs receiving rhythmic synaptic bursts (9%), it is surprising that any recordings exhibited such activity in a transverse slice preparation where peripheral afferents, along with ascending and descending pathways, are completely transected. Candidate circuits capable of maintaining rhythmic activities in the absence of these inputs are spinal cord locomotor CPGs (Brown, 1911; Goulding, 2009; McCrea & Rybak, 2008). These autonomous neural networks, which are located in the intermediate zone and ventral horn of lower cervical and upper lumbar segments (Ballion et al., 2001; Kiehn & Butt, 2003), are capable of generating coordinated locomotor output, even in reduced spinal cord preparations (Butt et al., 2002; Dyck & Gosgnach, 2009; Zhong et al., 2010). It is possible that the rhythmic synaptic activity observed in my study originates from this CPG network. LDPNs are uniquely positioned to receive rhythmic input from the cervical CPG and I propose they transmit a ‘readout’ of this activity to the lumbar CPG, presumably allowing for inter-limb coordination during locomotion. This is supported by forelimb reflex facilitation/inhibition that mirrors the coordination of the forelimbs and hindlimbs during normal stepping via activation of long ascending propriospinal neurons (LAPNs; Miller et al. (1973a)). More recently, targeted inhibition of LAPNs in vivo by Pocratsky et al. (2014) resulted in the loss of normal inter-limb coordination during locomotion, causing a hopping phenotype. There are, however, many questions to be answered about this inter-enlargement pathway such as: what types of CPG neurons (both excitatory and inhibitory) drive LDPN activity? Do LDPNs have intrinsic pacemaker activity? How do LDPNs (and subsets thereof) modulate neural activity in the lumbar circuits to which they project? New genetic, viral, and optical techniques are now able to dissect these spinal circuits with much improved precision (Chung et al., 2013; Ginger et al., 2013; Hinckley & Pfaff, 2013; Kravitz & Kreitzer, 2011) and answers to these questions are now within reach. For example, Ni et al. (2014) employed transsynaptic rabies virus tracing in combination with three-dimensional imaging of the whole cord to map the projection pattern of LDPNs that make monosynaptic connections on tibialis anterior motoneurons. In addition, they used genetically modified mice to determine the developmental lineage of a subset of LDPNs.

5.4.4 Conclusions

Moving forward, the information gleaned from high-resolution patch-clamp recordings of LDPNs, the first of which are presented in this study, will allow us to build a more detailed picture of the input/output properties of these important spinal neurons. The excitatory synaptic inputs onto LDPNs provide an obvious target for future
recordings along with their intrinsic electrophysiological properties, such as AP discharge patterns and the voltage-gated currents that shape their activity. This information is particularly pertinent for computational models of spinal locomotor circuits (Gillner, 2003; Grillner and Jessell, 2006), along with SCI research that could greatly benefit by understanding the basic properties of neurons that play a role in functional recovery, especially those forming the propriospinal system. It is envisioned that the data from this study could be used to compare synaptic integration within LDPNs after SCI, either with or without therapeutic interventions, to better understand the *functional* aspects of synaptic plasticity.
Chapter 6: Summary and conclusions
6.1 Summary

This thesis had two major focuses. The first was to develop an in vitro horizontal spinal cord slice preparation that allows high-resolution, whole-cell patch-clamp electrophysiology of individual neurons and their synaptic inputs in a mouse model of SCI. The second was to extend our current knowledge on an important mediator of functional recovery following SCI, the propriospinal system, by way of anatomical and electrophysiological characterisation of LDPNs in mice.

The in vitro horizontal spinal cord slice preparation detailed in Chapter 2 was developed to provide a new way for SCI researchers to assess functional electrophysiological changes in the injured and recovering spinal cord. The major findings of Chapter 2 were:

1) Longitudinal pathways within the spinal cord, such as the DCols, can be maintained and electrically stimulated for analysis of synaptic function in horizontal spinal slices.

2) DCol inputs can be evoked in spinal cord interneurons over considerable rostrocaudal distances.

3) A range of data can be gleaned from this preparation, including intrinsic neuronal properties (such as input resistance, resting membrane potential, and AP discharge properties) and also synaptic properties (including conduction velocity, kinetics, pharmacology and effect on post-synaptic membranes).

4) The horizontal slice preparation is compatible with optical recording techniques such as calcium imaging.

This new preparation was then applied in an experimental mouse model of SCI following the therapeutic intervention of exercise therapy (3 weeks treadmill training). The major findings of Chapter 3 were:

1) Neurons in the adult mouse spinal cord remain viable for whole-cell patch-clamp electrophysiology and DCol stimulation in horizontal slices after SCI.
2) There were no changes in the intrinsic properties of spinal neurons around the lesion as a result of 3 weeks of treadmill training.

3) There were no changes in sEPSC frequency or kinetics in spinal neurons around the lesion as a result of 3 weeks of treadmill training.

4) Evoked excitatory inputs from DCol fibres onto spinal neurons around the lesion were enhanced in treadmill-trained mice.

These findings demonstrate that the horizontal spinal slice preparation allows detailed examination of intrinsic neuronal properties, and importantly, functional properties of specific synapses following SCI. Additionally, neural plasticity resulting from exercise training appears to be synaptically driven rather than by via altered intrinsic membrane properties in spinal cord neurons.

The last two experimental chapters (4 and 5) provided anatomical and electrophysiological data on LDPNs in mice. The rationale for collecting these data stems from the lack of data on LDPNs in the mouse, despite the importance of the propriospinal system in spontaneous and therapeutically induced functional recovery following SCI.

The main findings from Chapter 4 were:

1) Ipsilaterally projecting LDPNs were spread diffusely throughout the deep dorsal horn, intermediate zone and ventral horn. Contralaterally projecting LDPN cell bodies were concentrated within the ventromedial quadrant of the grey matter.

2) LDPN cell bodies were found in the LSN/LCN both ipsilaterally and contralaterally. These populations appear to be particularly dense in mice compared to other animals.

3) Inhibitory LDPNs (GlyT2 and GAD67 positive) had mostly ipsilateral or midline projections to the lumbar spinal cord.

4) Contralaterally projecting, ventromedial LDPNs had radial dendritic arbours that are typical of lamina VII and VIII neurons.
These anatomical data provide a useful framework for future studies on the LDPN system, for example, ascertaining which specific LDPN subsets underlie functional recovery following SCI, or for examining the role of LDPNs in motor control.

Chapter 5 described the first targeted, whole-cell patch-clamp recordings of LDPNs in mice. My recordings focused on inhibitory inputs onto LDPNs, as inhibitory synapses in the spinal cord are critical for processing sensory information and executing motor behaviours. The main findings from this chapter were:

1) LDPNs received a higher frequency of GABA mediated mIPSCs compared to location matched control neurons. This was reflected in a slower average decay time for mixed mIPSCs in LDPNs versus control neurons.

2) AP mediated sIPSCs underwent higher levels of electrotonic filtering in LDPNs versus control neurons.

3) Rhythmic synaptic bursting was present in a subpopulation of LDPNs.

4) 40% of LDPNs exhibit an inhibitory tonic glycine current.

The properties of the inhibitory currents detected in LDPNs are reminiscent of other, better-studied, neuronal populations involved in motor control, such as Renshaw cells and would be advantageous in neural networks that require complex and rapid computations. The electrophysiological data from this study can be used for comparison with future recordings in SCI models that examine synaptic plasticity in LDPN populations with or without therapeutic interventions.

6.2 Conclusions

The studies presented in this thesis aimed to encourage the use of whole-cell patch-clamp electrophysiology in experimental models of SCI and to improve our knowledge of the propriospinal system in mice. With regard to preclinical SCI research, I believe more routine use of electrophysiological techniques alongside current anatomical, molecular, genetic and behavioural analysis is not only necessary, but long overdue. After all, there is no better way to understand the function (or dysfunction) of a system than to study it ‘in action’. Similarly, the renewed interest in the propriospinal system comes at a time when we can apply new techniques to probe previously unanswerable questions.
regarding this system. With over a century of basic research on these fascinating neurons, we now stand in good position, thanks to transgenic technologies, to improve our understanding of the role they play in spinal cord function under both normal and pathological conditions.

These are exciting times for SCI research and hope of a cure reigns supreme. It is, however, our responsibility as researchers to maintain a sense of trepidation toward potential treatments so that we might use all the tools at our disposal to determine their validity and mechanism of action. It is a rare thing to understand a biological system in such detail that we can put it back together when it falls apart. But, it is an undoubtedly worthy pursuit.
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