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THE ROLE OF INTER-ENLARGEMENT PROPRIOSPINAL NEURONS IN
LOCOMOTION FOLLOWING SPINAL CORD INJURY

By

Courtney Therese Shepard
B.A., University of Colorado Boulder, 2014
M.S., University of Louisville, 2017

A Dissertation
Submitted to the Faculty of the
Graduate School at the University of Louisville
In Partial Fulfillment of the Requirements
For the Degree of

Doctor of Philosophy in
Interdisciplinary Studies: Specialization in Translational Neuroscience

Interdisciplinary Studies
University of Louisville
Louisville, Kentucky

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DEDICATION

This dissertation is dedicated to

My guardian angels, Ralph “Papa” D. Groff, Virginia “Grandma GiGi” Groff,
Ernestine “Grandma Ernie” Shepard, and Coach Rey Reyes

My parents, Ronald N. and Patricia A. Shepard;

My godmother, Therese G. Shoop;

And my husband, Ryan E.B. Shepard.

Your support, love, and dedication to me and my success made this work possible.
Thank you.

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ABSTRACT

THE ROLE OF INTER-ENLARGEMENT PROPRIOSPINAL NEURONS IN LOCOMOTION FOLLOWING SPINAL CORD INJURY

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May 4, 2020

The focus of this dissertation is to explore the functional role of two anatomically-defined pathways in the adult rat spinal cord before and after spinal cord injury (SCI). To do this, a Tet^{On} dual virus system was used to selectively and reversibly silence neurons with cell bodies at spinal segment L2 and projections to spinal segment C6 (long ascending propriospinal neurons, LAPNs) and neurons that originate in the C6 spinal segment and terminate at L2 spinal segment (long descending propriospinal neurons, LDPNs).

This dissertation is divided into five chapters. Chapter One is a brief introduction to locomotion and the central pattern generator (CPG), as well as a description of other influences upon CPG circuitry in the spinal cord. It also provides evidence for the presence of inter-enlargement propriospinal neurons and their interactions between the cervical and lumbar enlargements in the spinal cord. These propriospinal populations are then discussed with reference to progenitor domains, anatomical characteristics, and their potential importance in recovery following spinal cord injury. A brief section is included to address discrepancies that are present when defining populations of long propriospinal neurons. The chapter concludes with a summary of the dual virus silencing system, how

it has been used in prior studies, and how it will be used in the subsequent chapters in this dissertation.

Chapter Two details the functional consequences of silencing LAPNs and LDPNs in uninjured animals, with specific regard to sensory context during overground locomotion. The focus of this chapter is on the disruptions seen in hindlimb interlimb coordination for each pathway (LAPNs and LDPNs) and how these disruptions vary based upon the pathway silenced and the sensory context of the stepping surface. Left-right alternation of the hindlimbs was equally perturbed for both LAPN and LDPN silencing on a rubbery surface, but was differentially affected on an acrylic, smooth surface. Neither resulted in changes to the fundamental features of the step cycle. Left-right alternation during a swimming task was unperturbed in both cases. It is proposed that LAPNs and LDPNs distribute temporal information essential for left-right alternation at the shoulder and pelvic girdles, with LAPNs doing so in a context-dependent manner and LDPNs doing so in a context independent manner.

Chapter Three describes the consequences of silencing LAPNs following a mild/moderate spinal cord contusion injury. Spinal cord injury (SCI) fundamentally affects the ability to maintain patterned weight-supported stepping. Silencing LAPNs resulted in drastically improved stepping capability, including improved intralimb coordination, interlimb coordination, and fundamental features of the step cycle. Silencing restored the coupling of the hindlimb-hindlimb pair and prevented large shifts in coordination on a step-by-step basis. These results demonstrate that LAPNs are a maladaptive pathway after SCI and removing them from the remaining circuitry reduces

the aberrant “noise” within the system, thus permitting improved function of hindlimb locomotor circuitry.

Chapter Four focuses on the functional outcomes of silencing the reciprocal descending inter-enlargement pathway, LDPNs, after mild/moderate spinal cord contusion injury. Silencing LDPNs post-SCI modestly improves some facets of locomotor function, such as hindlimb interlimb coordination and postural stability, but has little effect on others, like intralimb coordination and hindlimb-forelimb coordination. Ultimately, these findings suggest that LDPNs are acting deleteriously on animals’ postural stability and their role in recovered locomotion of the hindlimbs is less influential than that of the reciprocal ascending pathway.

Finally, Chapter Five compares the differential roles of LAPNs and LDPNs in left-right coordination prior to injury, especially in a sensory context-dependent manner. A section of this chapter is devoted to a recap of injured data for both LAPN and LDPN silencing post-injury and attempts to place this work in context with other studies whose focus is on propriospinal pathways after SCI. The concept of maladaptive neuroplasticity is addressed and mechanisms to mitigate the negative effects of this plasticity should it be occurring. In the context of SCI, the concept of neural network noise is discussed, as it is a potential explanation to why we saw improvements after SCI. We further explore the current weaknesses plaguing SCI research with specific regard to locomotor outcomes and how anatomical studies could improve our understanding of these pathways in the future. Finally, the clinical significance of this work is addressed, with focus on how propriospinal populations can be considered in future clinical experiments.

TABLE OF CONTENTS

	PAGE
SIGNATURE PAGE	ii
DEDICATION.....	iii
ACKNOWLEDGEMENTS	iv
ABSTRACT.....	vi
LIST OF FIGURES.....	xv
LIST OF TABLES.....	xviii
CHAPTER I. A BRIEF INTRODUCTION TO LOCOMOTION AND SPINAL CORD INJURY.....	1
Central pattern generators: a brief introduction.....	1
Supraspinal and afferent influences of CPG circuitry.....	3
Evidence of forelimb, hindlimb, and inter-enlargement interactions.....	5
Genetic and anatomical characteristics of propriospinal populations.....	7
Differential roles of propriospinal neuron populations following SCI.....	12
Important discrepancies in terminology.....	13
Reversible silencing of intraspinal neurons in uninjured adult rats.....	17
CHAPTER II. DIFFERENTIAL CONTEXT DEPENDENCE OF ASCENDING AND DESCENDING PROPRIOSPINAL PROJECTIONS DURING LOCOMOTION.....	26
Introduction.....	26
Materials and Methods	29

Viral vector production.....	29
Intraspinal injections of viral vectors to double infect LAPNs and LDPNs.....	29
Experimental design.....	31
Overground gait analyses.....	32
Context-specific overground stepping behavioral analyses.....	33
Analyses for swim phase.....	34
Statistical analyses.....	35
Results.....	36
A brief overview of locomotor measures.....	36
Silencing-induced perturbations as a result of sensory surface variations differ for LAPNs and LDPNs.....	38
Silencing LAPNs and LDPNs preserves the stability of the stepping pattern despite disrupted coupling at the shoulder and hip girdles.....	41
Coefficient of friction differences: “slippy” vs “grippy”.....	43
Interlimb coordination persists during swimming.....	43
Discussion.....	44
CHAPTER III. SILENCING LONG ASCENDING INTER-ENLARGEMENT PROPRIOSPINAL NEURONS AFTER SPINAL CORD INJURY RESTORES HINDLIMB STEPPING ABILITY IN ADULT RATS.....	63
Introduction.....	63
Materials and Methods.....	65
Viral vector production.....	66
Intraspinal injections of viral vectors to doubly infect LAPNs.....	66
Spinal cord injury.....	67
Experimental timeline.....	67

Identification of hindlimb joints.....	68
Hindlimb kinematics and intralimb coordination analysis.....	68
Overground gait analyses.....	70
BBB assessments.....	72
Coordination indices.....	73
Spatiotemporal gait indices.....	73
Histological analyses.....	74
Statistical analyses.....	76
Results.....	78
Silencing alters hindlimb interlimb coordination while maintaining intralimb coordination and key locomotor features in uninjured animals.....	78
Presence of putatively eTeNT-positive synapses in the cervical spinal cord and cell bodies in the lumbar spinal cord.....	81
Silencing LAPNs post-SCI restores coordination indices and improves gross locomotor outcomes.....	83
LAPN silencing leads to modest improvements in intralimb coordination after SCI.....	84
Hindlimb-hindlimb, but not hindlimb-forelimb, coupling relationships were restored during post-SCI silencing.....	86
Hindlimb-hindlimb coordination stability was restored during post-SCI silencing.....	89
Key features of locomotion are restored during post-SCI LAPN silencing.....	90
Discussion.....	91

CHAPTER IV. SILENCING LONG DESCENDING INTER-ENLARGEMENT PROPRIOSPINAL NEURONS AFTER SPINAL CORD INJURY MODESTLY IMPROVES HINDLIMB STEPPING ABILITY AND POSTURAL CONTROL IN ADULT RATS.....	120
Introduction.....	120
Materials and Methods.....	123
Viral vector production.....	124
Intraspinal injections of viral vectors to doubly infect LDPNs.....	124
Spinal cord injury.....	125
Experimental timeline.....	125
Identification of hindlimb joints, hindlimb kinematics, and intralimb coordination analysis.....	125
Overground gait analyses.....	126
BBB assessments.....	127
Coordination indices.....	127
Postural Stability.....	129
Ladder.....	129
Spatiotemporal gait indices.....	129
Histological analyses.....	129
Statistical analyses.....	129
Results.....	131
Silencing LDPNs alters hindlimb interlimb coordination while maintaining intralimb coordination and key locomotor features in uninjured animals.....	131
Presence of putatively eTeNT-positive synapses in the lumbar spinal cord and cell bodies in the cervical spinal cord.....	133

Silencing LDPNs post-SCI restores coordination indices and improves gross locomotor outcomes.....	135
LDPN silencing leads to modest improvements in intralimb coordination after SCI.....	137
Hindlimb-hindlimb, but not hindlimb-forelimb, coupling relationships were restored during post-SCI silencing.....	138
Silencing LDPNs modestly improves balance and posture post-SCI.....	140
Key features of locomotion are restored during post-SCI LAPN silencing.....	142
Discussion.....	143
CHAPTER V. GENERAL DISCUSSION AND FUTURE DIRECTIONS.....	168
A comparison of propriospinal pathways: differential roles in left-right coordination.....	168
Quadrupedal locomotion: top-down or bottom-up control?	170
Swimming versus stepping: left-right alternation driven by muscle loading.....	172
The importance of sensory context: exploring friction and surface changes during locomotion.....	174
A perplexing finding: improvements in stepping during post-SCI silencing.....	178
Disparate influences of LAPNs and LDPNs on CPG circuitry after SCI.....	179
The “V” neuron story in a post-injury context.....	182
Post-injury neuroplasticity: a gift or a curse?.....	184
Mitigating negative effects of maladaptive plasticity post-SCI using activity-based training.....	187
The field’s approach to analyzing locomotion after SCI: significant weaknesses that plague interpretation.....	190

Neural network noise: a potential explanation for improvements in a fine-tuned system.....	193
The importance of propriospinal neurons in other motor behaviors: clues to interpret complex findings post-SCI.....	196
The new frontier: exploring the anatomy and synaptic inputs of propriospinal populations.....	199
Clinical significance of inter-enlargement propriospinal neurons.....	201
Spinal cord stimulation: the be-all and end-all locomotor recovery?.....	203
REFERENCES.....	205
LIST OF ABBREVIATIONS AND SYMBOLS.....	235
CURRICULUM VITA.....	239

LIST OF FIGURES

FIGURE	PAGE
Figure 1. Proposed location of lumbar and cervical locomotor CPGs.....	20
Figure 2. Classical definitions of various neuron populations.....	21
Figure 3. Locations of long and short propriospinal neurons based on experimental focus.....	22
Figure 4. Tet ^{ON} dual virus approach to silencing long spinal pathways.....	24
Figure 5. Examples of overground gait analyses.....	48
Figure 6. Silencing-induced perturbations for LAPNs are context-dependent.....	50
Figure 7. Silencing-induced perturbations for LDPNs are not context-dependent.....	52
Figure 8. Relationships between limbs pairs are maintained during LAPN and LDPN silencing.....	54
Figure 9. Silencing LAPNs and LDPNs does not affect key features of locomotion.....	56
Figure 10. Coefficient of friction differences on acrylic and Sylgard surfaces.....	58
Figure 11. Hindlimb coordination during swimming is maintained during LAPN and LDPN silencing.....	59
Figure 12. Experimental timeline and viral schematic for LAPN silencing.....	97
Figure 13. Intralimb coordination during LAPN silencing in the uninjured rat.....	98
Figure 14. Interlimb coordination during LAPN silencing in the uninjured rat.....	100

Figure 15. Histological analysis of LAPN-silenced spared white matter at the injury epicenter.....	102
Figure 16. Immunohistochemical analysis of LAPN putative synaptic terminals and cell bodies.....	103
Figure 17. Gross motor improvement during post-injury LAPN silencing.....	105
Figure 18. Modest improvements in intralimb coordination during post-SCI LAPN silencing.....	107
Figure 19. Precision of stepping improvements during post-SCI LAPN silencing.....	109
Figure 20. Behavioral analyses of animals excluded from main dataset.....	112
Figure 21. Improvements in step-by-step phase change and gait stability.....	114
Figure 22. Improved spatiotemporal measures during post-injury LAPN silencing.....	116
Figure 23. Experimental timeline and viral schematic for LDPN silencing.....	149
Figure 24. Interlimb coordination and spatiotemporal measures during LDPN silencing in the uninjured rat.....	150
Figure 25. Histological analysis of LDPN-silenced spared white matter at the injury epicenter.....	152
Figure 26. Immunohistochemical analysis of LAPN putative synaptic terminals and cell bodies.....	153
Figure 27. Gross motor improvement during post-injury LAPN silencing.....	155
Figure 28. Unchanged intralimb coordination during post-SCI LDPN silencing.....	157
Figure 29. Modest improvements in precision of stepping during post-SCI LDPN silencing.....	159
Figure 30. Behavioral analyses of animals excluded from main dataset.....	161

Figure 31. Improvement in postural stability during post-injury LDPN silencing.....	163
Figure 32. Modestly improved spatiotemporal measures during post-injury LAPN silencing.....	165

LIST OF TABLES

TABLE	PAGE
Table 1. Euclidian distance for LAPN silencing	60
Table 2. Euclidian distance for LDPN silencing	61
Table 3. Watson's U^2 circular statistics calculations for hindlimb and forelimb pairs during LAPN and LDPN silencing	62
Table 4. Immunohistochemistry antibodies	118
Table 5. Euclidian distance for uninjured spatiotemporal measures during pre-injury LAPN silencing	119
Table 6. Euclidian distance for uninjured spatiotemporal measures during pre-injury LDPN silencing	167

CHAPTER I

A BRIEF INTRODUCTION TO LOCOMOTION AND SPINAL CORD INJURY

Central pattern generators: a brief introduction

Locomotion is a fundamental behavior shared by almost all animals. Mammals rely on different gait patterns that vary based on their speed to effectively navigate their environment (Hildebrand 1976, Grillner 1981, Grillner 1975). Traditional overground gaits – walk, trot, gallop, and bounding – are often measured as a function of speed and can be quantified using defined spatiotemporal measures (Hildebrand 1989). The gait patterns of quadrupedal animals are also characterized by distinct relationships between the limbs and the limb joints, which are tightly regulated by their own set of extensive intraspinal circuitry (Hildebrand 1989, Danner et al 2017).

The importance of these intraspinal networks of interneurons was established over a century ago by Sir Charles Sherrington, who demonstrated that isolated activity could occur within the spinal cord, which he then termed “long motor reflexes” (Sherrington 1903, Sherrington 1910). Thomas Graham Brown later showed that stepping movements could be elicited from the spinal cord of the guinea pig without descending command signals or sensory reflex feedback, a concept now known as central pattern generators (CPGs, Graham Brown 1911; Graham Brown 1916). CPGs are neuronal networks that

generate the coordinated, rhythmic muscle activities associated with patterned behaviors without requiring afferent input from the periphery or supraspinal centers (Kiehn 2006, Stein 2010). In addition, the rhythmic activities generated by the circuits are often involved in control of vital functions, such as breathing swallowing, swimming, and stepping (Van Emmerick et al 1998; Marder et al 2001; MacKay-Lyons 2002). Often, CPG circuitry is described as producing these complex movements. Experimentally, the locomotor CPG is most commonly studied in *in vitro* isolated spinal cord preparations and is known as fictive locomotion. *In vitro* preparations span a variety of animal models including lamprey (Cohen AH & Wallén P 1980; Benthall et al 2017; Buchanan & McPherson 1995; Grillner 1988; Grillner 1995), neonatal rodent (Suzue 1984; Cazalets et al 1992; Eide et al 1999; Cazalets & Bertrand 2000; Cowley et al 2010; Ballion et al 2002; Hanson & Landmesser 2003), and decerebrate cat (Villablanca et al 1993; Forssberg & Grillner 1973; Forssberg et al 1980). Evidence for the presence of locomotor CPGs is well-established, especially in regards to hindlimb locomotor function. Following complete transection of the thoracic spine in adult cats, alternating, coordinated movements of the hindlimbs can be achieved using a treadmill (Grillner & Zangger 1984; Coggeshall 1980). These locomotor patterns persist even if afferent input from the involved limbs has been abolished (Shik & Orlovsky 1976). Moreover, when eliminating movement by applying paralytic agents to block receptors at the neuromuscular junction, locomotor patterns can still be recorded in ventral roots or motoneurons (Shik et al 1966). Research on CPGs has persisted and continues to be studied in many labs due to its importance in uninjured and injured locomotion. However, the locomotor CPG circuitry only comprises a piece of the motor control

system as a number of other components provide critical input, namely supraspinal pathways and peripheral afferent feedback.

Supraspinal and afferent influences on CPG circuitry

Aside from intraspinal neuronal networks, normal locomotion involves feedback from supraspinal centers and peripheral sensory systems onto spinal cord locomotor circuitry. It is important to consider that motor behavior occurs on a continuum, in which some motor movements are voluntary where others are automatic. Therefore, the mix of supraspinal oversight and intraspinal control is greatly dependent upon context (MacKay-Lyons 2002). Supraspinal oversight is classically thought of as areas such as primary motor cortex, which are essential for motor planning and execution of voluntary locomotion (Shenoy et al 2013).

However, it is known that the commands for initiation and termination of rhythm generators come from lower areas of the brainstem, like the mesencephalic locomotor region (MLR) (Armstrong 1986; Armstrong 1988; Whelan 1996; Jordan 1998; Noga et al 2009; Drew & Marigold 2015). In a decerebrated cat, complete quadrupedal stepping can be evoked by electrical stimulation of the MLR, which was found to project to the nearby reticular formation (Shik et al 1966). Depending on the strength of the stimulus, different gait patterns could be produced (walking, trotting, galloping), and termination of locomotion could be achieved by simply removing the excitatory input to this region (Shik & Orlovsky 1966; Armstrong 1986; Shik & Orlovsky 1968). Reticulospinal neurons involved in the control of locomotion originate in the pontine (PRF) and medullary reticular formations (MRF) and project ipsilaterally and contralaterally to both

cervical and lumbar enlargements, branching extensively throughout the mammalian spinal cord (Martin et al 1981, Matsuyama et al 2004, Sivertsen et al. 2014, 2016).

After gait initiation, afferents deliver movement-related feedback and input to spinal and supraspinal levels. The CNS is informed of the position of the body and its interactions with the environment through sophisticated sensors located in joints, muscles, and skin (Frigon 2017). Some of this feedback acts directly on the CPG to aid the phase transitions (flexion/swing to extension/stance) during the step cycle thus allowing incorporation of gait variations to meet environmental demands (Markin et al 2010). Afferent inputs from mechanoreceptors exert powerful effects on the locomotor pattern by regulating phase transitions, reinforcing ongoing activity and by correcting movement trajectory in response to perturbations (Duysens et al. 2000; Frigon and Rossignol 2006; Pearson 2004, 2008; Rossignol et al. 2006; Zehr and Stein 1999). Grillner and Zangger claimed that interlimb coordination during hindlimb walking deteriorated following deafferentation in the decerebrate cat, suggesting that afferent input is vital to patterned hindlimb locomotor output (Grillner & Zangger 1984).

Importantly, characterizations of the CPG have repeatedly shown rhythmic movements in *in vitro* preparations devoid of supraspinal inputs (Bjursten et al 1976; Douglas et al 1993; Miller et al 1975) and afferent inputs (Knapp et al 1963; Rothwell 1982; Marsden et al 1984). However, this does not mean that these inputs are not important to locomotion in the intact animal. Their involvement is critical to adapt to the environment and it is critical to consider the potential role of both afferent feedback and supraspinal influence to the CPG in the recovery of locomotor function following spinal cord injury (SCI) (Fig 1a).

Evidence of forelimb, hindlimb, and inter-enlargement interactions

In recent years, combined electrophysiological and molecular genetic approaches in the developing mouse spinal cord have allowed deletion of genetically identified classes of spinal interneurons and delineated their effects on circuit function and locomotor behavior (Kiehn 2011; Kiehn 2016). These studies have led to a deeper understanding of the organization of mammalian CPGs, but are often difficult to interpret given the wide range of neuronal populations they encompass. Genetic progenitor populations, discussed in greater detail later in this chapter, are made up of a number of anatomical classes of neurons, many with essential functions to the CPG circuitry. Within the spinal cord, quadrupedal locomotion is controlled by a separate CPG for each limb located in left and right quadrants within the lumbar and cervical enlargements (Danner et al 2017; Kiehn 2016; Kato 1990; Ballion et al 2001; Juvin et al 2005; Juvin et al 2012)(Fig 1b,c). Given the exquisitely controlled temporal arrangement of interlimb coordination and gait execution, it is critical to consider the circuits mediating the interactions between each limb. Within each girdle, the most likely candidates are short commissural propriospinal projections, which are considered regulators of left-right interlimb pattern generation at the lumbar and cervical CPG levels (Talpalar et al 2013; Bellardita & Kiehn 2015). Critical elements of the forelimb CPGs are primarily located in caudal cervical (C6-C8) spinal segments with some rhythmogenicity capabilities found in the upper thoracic spinal segments (T1) (Ballion et al 2001; Yamaguchi et al 2004)(Fig 1b). This was further demonstrated by transecting the lateral funiculus just rostral to C6, which abolishes supraspinal-evoked fictive locomotion at the forelimb level in the decerebrate cat, whereas a transection below C7 does not (Hishinuma & Yamaguchi

1990). The localized projection of the command implies that short, localized interneurons of the forelimb CPG receive the initiation command from brainstem centers and are likely important for forelimb coordination.

Contrary to the forelimb CPG, the location of the hindlimb CPG is more extensively studied. Most studies propose the location of the hindlimb CPGs in the rostral lumbar segments, specifically L1 and L2 in rodent models (Cazalets et al 1995; Cazalets et al 1996) and L2 and L3 in cat models (Langlet et al 2005; Marcoux & Rossignol 2000)(Fig 1c). The importance of the L2 spinal cord segment to CPG circuitry was further solidified in a series of studies where kainic acid injections were delivered to the intermediate gray matter at L2, which resulted in profound effects on locomotor function, while injections in the caudal lumbar enlargement produced less severe deficits (Hadi et al 2000; Magnuson et al 1999). Other studies have utilized a neonatal rat *ex vivo* spinal cord preparation to demonstrate that the lumbar enlargement holds rhythmogenic potential (Cazalets & Bertrand 2000; Cowley et al 2010; Cazalets et al 1995, Jordan & Schmidt 2002, Hägglund et al 2013). However, a majority of investigators in the field have concluded that the rostral lumbar segments are most critical for the production of hindlimb stepping rhythm and these segments are still the focus of hindlimb CPG research (Cazalets et al 1995; Cazalets et al 1996; Langlet et al 2005; Marcoux & Rossignol 2000; Hadi et al 2000; Magnuson et al 1999).

While cervical and lumbar CPGs are capable of functioning independently, several studies have provided electrophysiological evidence that afferent stimulation in the cervical spinal cord results in efferent output by the lumbar spinal cord, suggesting that intraspinal or propriospinal connections may be relaying descending functional

information from the cervical to the lumbar enlargement (Drew & Marigold 2015; McKenna et al 2000). These findings are likely due to populations of ipsilateral and commissural long propriospinal projections that control homolateral and heterolateral interactions between the hindlimbs and the forelimbs (Eide et al 1999; Matsushita et al 1979; Skinner et al 1979; Skinner et al 1980; Bannatyne et al 2003; Stokke et al 2002; Nissen et al 2005). Early studies found that electrical stimulation of hindlimb afferent nerves was responsible for the discharge in forelimb motoneurons and that the pattern of discharge between the hindlimbs and the forelimbs remained after deafferentation in the decerebrate cat (Miller et al 1975; Miller et al 1973). These results provided evidence towards the presence of an ascending anatomical pathway that links the forelimb and hindlimb locomotor CPGs. Juvin et al concluded that the cervical enlargement is able to maintain an independent rhythm (Juvin et al 2005). However, it can be entrained to the lumbar rhythm, suggesting a strong influence of the lumbar circuitry over the cervical enlargement. Many authors have suggested long propriospinal neurons as likely candidates to functionally and reciprocally couple the neural activity of the lumbar and cervical CPGs (Miller et al 1975; Cazalets et al 1995; Miller et al 1973; English et al 1985; Vasilenko 1975). As the field has continued to advance, many have sought to characterize subsets of long propriospinal neurons based on anatomical tracing and easily identifiable progenitor domains.

Genetic and anatomical characteristics of propriospinal populations

Many experimental models utilize developmental genetic deletions to study the functions of these neuronal pathways *in vivo* (Crone et al 2009; Kiehn 2006; Kullander et al 2003). For behavioral analysis of components of the hindlimb CPG, propriospinal

neurons have been extensively studied in the context of their transcription factor expression to identify their developmental origin in the mouse spinal cord (Goulding 2009). Specifically, manipulations of ventrally-derived class of neurons (“V” neurons) have now provided important insight into the roles of various populations for locomotor output in the genetically modified mouse model. For instance, glutamatergic V2a interneurons form a group of descending thoracic premotor neurons that express the transcription factor Chx10 and are essential for left-right alternation *in vivo* and alternating burst activity in the *ex vivo* spinal cord preparation (Crone et al 2009; Dougherty et al 2010; Ni et al 2014). Other populations of genetically identified commissural interneurons, known as V0_v and V0_D, are involved in the expression of multiple gaits, including alternation, in a speed-dependent fashion (Talpalar et al 2013; Bellardita & Kiehn 2015). Specifically, genetic ablation of commissural V0_v neurons removed the transitional gait, trot, but maintained walk, gallop and bound. Ablation of both neuronal populations led to a bounding gait at all speeds (Zhang et al 2014). V3 commissural interneurons are not essential for left-right alternation, but are needed to establish the regularity of the locomotor rhythm (Zhang et al 2008). In other words, their role is non-essential for patterned stepping but is vital in maintaining the locomotor clock. In addition, intralimb coordination is modulated and controlled via two separate but synergistic ipsilaterally projecting V neuron populations: V1 and V2b (Zhang et al 2008; Britz et al 2015). While genetic-based manipulations provide exceptional insight into locomotor behaviors, it cannot account for the involvement of neuronal pathways that do not fall within defined progenitor domains. Further, developmental genetic deletion may not be the best method with which to study interneuron populations as the

products of many genes are essential to normal function and unexpected compensatory developmental and functional mechanisms may arise (Goulding 2009; Sigmund 2000). Additionally, functional differences between anatomical subpopulations within identified progenitor domains are documented, making some genetic ablation studies difficult to interpret (Crone et al 2009; Gogsnach 2011; Lanuza et al 2004).

The disparity between genetic and anatomical classifications is evident when describing anatomically distinct CPG spinal interneurons such as long ascending (LAPNs) and long descending (LDPNs) propriospinal neurons. These pathways are not currently characterized by specific transcription factor expression or developmental origin and a number of them fall into multiple progenitor domain categories. Despite this, they comprise significant functional pathways that are essential for communication between the hindlimbs and the forelimbs during locomotion. To successfully coordinate activity between the CPG centers, anatomically defined ascending and descending projections are necessary to connect the cervical and lumbar enlargements. In the mammalian spinal cord, LAPNs and LDPNs (sometimes called long ascending and descending propriospinal tracts, respectively, in the literature), are components of the lumbar interlimb pattern generator that form direct connections between the cervical and lumbar enlargements (Reed et al 2006).

Mainly using retrograde tracing techniques, early studies anatomically defined several populations of long propriospinal neurons in cat, rat, and monkey (McKenna et al 2000; English et al 1985; Dutton et al 2006; Miller et al 1998; Molenaar & Kuypers 1978; Skinner et al 1979). More recent studies have identified specific inter-enlargement propriospinal pathways that involve both ascending and descending projections between

L1-L3 and C6-C8 spinal cord segments, with extensive axonal projections travelling in the ventrolateral funiculus (VLF) (Reed et al 2006; Brockett et al 2013). These studies utilize a variety of anatomical tracing and mapping techniques have been used to define these populations, but each comes with its own set of benefits and drawbacks. Briefly, the classic retrograde tracer horseradish peroxidase (HRP) is capable of being taken up non-selectively by neurons, but is primarily limited to the cell body and the primary dendrites (Matsushita et al 1979; Molenaar & Kuypers 1978; Alstermark et al 1987; Köbbert et al 2000). While more effective once conjugated to wheat germ agglutinin (WGA), it still does not completely label neurons (Menétrey et al 1985; Schwab et al 1978; Levy et al 2017). Cholera toxin subunit (B) is taken up similarly and was first introduced to improve the sensitivity of HRP (Vercelli et al 2000). Its high signal strength, low toxicity, and quick transport makes it a far more suitable option for tracing than HRP and improves the ability to identify neurons *in vitro* (Korim et al 2014; Bou Farah et al 2016). However, this technique has not been extensively applied to many populations of intraspinal propriospinal neurons. FluorogoldTM and similar fluorescent inorganic compounds, which provide extensive and strong retrograde labeling of neurons have been widely used to trace propriospinal neurons (Miller et al 1998; Flynn et al 2017; Siebert et al 2010). Unfortunately, they provide no insight into the function of selected pathways and are non-specific, labeling anything with a cell body in the area of interest. More recent studies have used two types of viral tracers, which drive the expression of fluorescent reporter proteins: (1) static viral tracers that target a specific cell population based on anatomical location and (2) trans-synaptic viral tracers which allow for tracing of a selected population of neurons and their synaptic connections (Saleeba et al 2019; Brink

et al 1985). These techniques require the presence of multiple viruses, often one at the level of the selected cell body and the other at the cell terminal. However, these techniques have not yet been applied to long ascending or descending inter-enlargement neuronal populations and will provide critical insight for future studies.

Anatomically defined descending propriospinal populations have been the focus of recent literature due to their potential as functional mediators after SCI. The anatomical location of their cell bodies and axons in the ventrolateral funiculus (Reed et al 2006; Brockett et al 2013) makes them ideally suited to transmit supraspinal signals past the injury site. Electrophysiological data demonstrate that several supraspinal centers located in the cortex, cerebellum, and brainstem, as well as primary afferents, provide monosynaptic input onto LDPNs (Alstermark et al 2000; Brink et al 1985). Ni et al confirmed the existence of corticospinal, serotonergic, and intraspinal inputs onto LDPN populations (Ni et al 2014). Immunohistochemical labelling also revealed that contralateral LDPNs received putative synaptic input from both excitatory and inhibitory pathways, including vesicular GABA transporter/parvalbumin (VGAT/PV) positive inhibitory premotor interneurons such as Renshaw cells and group Ia afferent input as well as VGLUT1 positive excitatory corticospinal and group Ia proprioceptive muscle afferent input (Ni et al 2014). Takeoka et al utilized a mouse model that lacked functional muscle spindle feedback and found that muscle spindle feedback was essential for precision locomotor tasks and involved projections onto multiple descending neuronal populations in the ventrolateral quadrant (Takeoka et al 2014). These studies provide evidence for many different forms of synaptic input onto LAPN/LDPN populations. Based upon these convergent inputs, LAPNs/LDPNs are ideally placed to integrate

temporal information between the cervical and lumbar enlargements in response to both sensory feedback from the limbs and supraspinal input.

Differential roles of propriospinal neuron populations following SCI.

Most clinical patients with SCI have some sparing of white matter at the lesion epicenter, indicating that LAPNs/LDPNs axons are potentially left intact following SCI to form an anatomical bridge across the injury site (Basso 2000). Based on their axonal length and resiliency to cell death following injury, LAPNs/LDPNs may be well suited for aiding in recovery following SCI (Siebert et al 2010, Conta Steencken & Stelzner 2010). Their anatomical preservation may be indicative of their potential functional implications after SCI (Magnuson et al 1999).

Fouad et al initially described an increase in sprouting of the corticospinal axons after thoracic SCI (Fouad et al 2001). Bareyre et al expanded on this finding and determined that many of the sprouting corticospinal tract (CST) axons terminate on propriospinal neurons in the cervical enlargement (Bareyre et al 2004). Furthermore, CST axons that contact inter-enlargement LDPNs, which project past the level of injury in the thoracic cord, were maintained, while connections with short propriospinal neurons were not. A significant increase in the LDPNs terminal arborizations onto motoneurons was also observed, all of which indicates the importance of propriospinal neurons that cross the level of lesion (Bareyre et al 2004). Vavrek et al affirmed these findings by showing that administration of brain-derived neurotrophic factor (BDNF) promoted formation of corticospinal detour circuits onto LDPNs in spinal cord injured rats (Vavrek et al 2006). Using spatially and temporally separated lateral hemisections with and without cytotoxic ablation, Courtine et al demonstrated that functional recovery can occur after SCI without

direct supraspinal input and may be mediated by the reorganization of propriospinal connections (Courtine et al 2008). Severed reticulospinal projections also appear to bypass the lesion by forming similar spontaneous contacts onto propriospinal relay neurons after spinal hemisection (Filli et al 2014, Filli & Schwab 2015). In the lamprey, sensory stimulation elicited normal muscle burst activity, despite an interruption of reticulospinal projections via spinal cord transection (Benthall et al 2013). Collectively, these data strongly suggest an important role of LDPNs in functional recovery after SCI, especially in the absence of supraspinal contributions. There is some evidence of a distinct ventral subset of corticospinal neurons extending throughout the length of the mouse spinal cord (Bareyre et al 2005). However, it is unknown whether LAPNs receive any of that direct supraspinal input, making their function after SCI and their role in functional recovery unclear.

Important discrepancies in terminology

Given the plethora of research attention propriospinal neurons receive, it is important to state that similar vernacular is applied to a variety of ascending and descending populations of interneurons, despite distinct functional differences. In the most general sense, an interneuron is any neuron synaptically positioned between sensory neurons and motoneurons or that connects other neurons within the CNS (Duysens et al 1992; Crone et al 2009). In the anatomical and genetic spinal cord literature, however, finer distinctions are usually made, and certain categories, such as those described above, have become conventions. With regards to axon projection, the broad term “spinal interneuron” applies to any neurons that is completely contained within the spinal cord that is not a primary motor neuron, primary afferent, or projection neurons to supraspinal

levels. This category can be further identified as propriospinal neurons whose axonal projections are wholly limited to the spinal cord. Many researchers, however, restrict the term “interneuron” to local interneurons whose axons are limited to the same segment as their cell bodies (Kiehn 2006; Kullander et al 2003; Goulding 2009). Current literature provides an even broader definition of propriospinal neuron length classes, which are said to project intersegmentally over short or long distances (Crone et al 2009; Dougherty & Kiehn 2010). In this convention, the term “short” propriospinal neuron is limited to those neurons with axon projections spanning three or less segments but restricted to spinal levels, consequently “long” projection or tract neurons would be considered anything greater (Markin et al 2010; Duysens et al 1992; Grillner & Zangger 1984; Ni et al 2014; Zhang et al 2008). As an all-encompassing definition, this does not aid in determining a specific role for certain populations of propriospinal neurons and generally lumps them under a single over-arching term. During development, the distinctions between local interneurons (Fig 2a), propriospinal neurons (Fig 2b,c), and projection neurons (Fig 2d) can be even more difficult to make. Genetic knockout models likely remove subsets of neurons that belong to each category (Kiehn 2016; Crone et al 2009; Pierani et al 2001), an inherent risk when removing neuronal populations based solely upon progenitor domains. Moreover, older anatomical techniques such as retrograde axonal tracing and immunohistochemical staining might not fully reveal differences in the correct length of axons (Saleeba et al 2019) and might be limited in their interpretation based upon the researcher’s focus (i.e. lumbar cord, brainstem, etc.).

The issues in defining populations of propriospinal neurons become increasingly apparent when reviewing the published literature. For instance, inter-enlargement LDPN

populations are referenced extensively for their “role in inter-enlargement coordination”. Yet, each paper refers to a distinct subset of these neurons, often without specifying any difference (Fig 3). Some anatomical papers define all neurons extending from cervical spinal segments and upper thoracic segments down to rostral lumbar segments as “LDPNs” (Matsushita et al 1979; Ni et al 2014; Reed et al 2006; Brockett et al 2013; Flynn et al 2017; Jankowska et al 1974). The opposite is true as well, as several authors deem the cell body locations in the cervical cord to be finite while specifying only the lumbosacral enlargement as the place of termination (Siebert et al 2010; Doperalski et al 2019; Bareyre et al 2004; Cowley et al 1997). Others suggest that LDPNs extend from C1/C2 spinal segments down to L5/S1 segments (Miller et al 1998), and still others call LDPNs extensions from C3-C6 to L1/L2 (Conta Steencken & Stelzner 2010; Mitchell et al 2016; Sheikh et al 2018; Conta Steencken et al 2011; Swieck et al 2019). Some papers also reference “short thoracic propriospinal neurons”, which have been suggested to have a role in trunk control. Interestingly, this subset of literature shares the same discrepancies in language as their long descending cousins. Short thoracic propriospinals have been said to travel from T5/T7 to T13/L1 (Conta Steencken et al 2011), T6/T8 to the lumbar enlargement (Doperalski et al 2019; Swieck et al 2019), and T8/T11 segments to the lumbar enlargement (Deng et al 2013). Courtine et al, considered a ground-breaking study for the preservation of propriospinal neuronal connections following SCI, defines descending propriospinal neurons as originating at T8-T10 without specifying a location for cell terminals (Courtine et al 2008).

Unlike LDPNs, LAPNs have a more compact definition with less disparity in the anatomical location of cell bodies and cell terminals. Despite this, we still see

discrepancies in how we refer to “LAPNs” as a population. Some have focused on *in vitro* classification of ascending propriospinal pathways. Primarily, ascending projections are merely referred to as the “reciprocal” pathway to long descending populations (Miller et al 1975; Miller et al 1973). Anatomically, few studies have sought to characterize these projections. English et al described an anatomical pathway that “links the hindlimb and forelimb motor centres” and projects from rostral lumbar segments to C5-C8 spinal cord (English et al 1985). More recent studies have attempted to classify the terminal locations of ascending neurons originating L1/L2 rostral lumbar segments, with most settling on the C6-C8 range (Reed et al 2006; Brockett et al 2013; Reed et al 2009; Pocratsky et al 2020). However, several other studies have focused their attention on other aspects of the ascending pathways, including pathways from L4-L5 to C5 (Deng et al 2013) and L3-S4 to C3 (Dutton et al 2006).

The evident disconnect in language used to describe both ascending and descending populations of propriospinal neurons creates significant confusion that must be clarified moving forward. Both long and short propriospinal neurons require a name “makeover” to improve the clarity and understanding of the specific subpopulations of propriospinal neurons to which are being referred. To truly uncover the functions of these proposed ascending and descending pathways before and after SCI, it is critical that we begin to parse out the disparities amongst the various subsets of propriospinal neurons, especially inter-enlargement populations. Furthermore, and especially pertaining to this dissertation work, determining the relevance and impact of the proposed populations of propriospinal neurons by other works in relation to the pathways proposed here remains

difficult, as many may be suggested as having similar function without having a basis with which to compare to anatomically.

For the remainder of this dissertation, the term “LDPNs” will refer to descending intraspinal neurons with cell bodies ranging from C6-C7 and terminations in L1-L2 spinal cord. “LAPNs” will refer to ascending propriospinal neurons with cell bodies in L1-L2 spinal segments and terminations in C6-C7 spinal segments.

Reversible silencing of intraspinal neurons in uninjured adult rats.

As detailed above, permanent developmental genetic deletion may not be the best method with which to study interneuron populations. Conversely, the use of anatomical techniques, such as those that have defined the anatomical layout of LAPNs/LDPNs, leaves the function of the pathway open for speculation. In an ideal situation, spinal cord circuitry could be functionally studied using manipulations that can reversibly and acutely inactivate the pathway in question. To address questions of function, Tadashi Isa and colleagues developed a two viral-vector silencing technique to conditionally and reversibly silence anatomically defined pathways in the nervous system, specifically hand dexterity in macaque monkeys and visual orientation in mice (Kinoshita et al 2012; Sooksawate et al 2013). Hi-Ret-TRE-EGFP.eTeNT (Fig 4a) is a lentiviral vector that is designed to be retrogradely transported from the level of the neuronal cell terminals to the level of the cell bodies with high efficiency. A second viral vector, AAV2-CMV-rtTAV16 (Fig 4b), is injected at the level of the cell bodies and expresses the doxycycline (Dox)-activated Tet^{On} sequence, a variant of the reverse tetracycline transactivator (Zhou et al 2006). Without Dox, neurons constitutively express rtTAV, but it is not active. Upon administration of Dox in the animals' drinking water, rtTAV16 becomes active and binds

to the tetracycline response element (TRE) promoter on Hi-Ret-TRE-EGFP.eTeNT, leading to EGFP.eTeNT expression only in doubly infected neurons. EGFP.eTeNT is then anterogradely transported to neuronal cell terminals, where it cleaves vesicle-associated membrane protein 2 (VAMP2) which impairs vesicle binding to the cell membrane, vesicle exocytosis, and neurotransmission (Fig 4c). Yamamoto et al demonstrated that expression of eTeNT does not affect neuronal survival (Yamamoto et al 2003). This method eliminates the potential side effects of genetic deletions and allows for functional dissection of neural pathways that are anatomically defined by the location of their cell bodies and terminal fields. Therefore, conditional silencing can be used to functionally dissect LAPNs/LDPNs both before and after SCI in the adult rat model, independent of cell-specific promoters.

We have successfully used this method to effectively silence both short propriospinal neurons that project from L2-L5 and LAPNs (Pocratsky et al 2017, 2020). That previous work revealed that silencing L2-L5 interneurons uncoupled the hindlimbs, leading to synchronous hindlimb stepping (Pocratsky et al 2017). The alternation of the forelimbs was unimpaired. Conditional silencing of LAPNs in the rat results in disruptions of left-right alternation in both the hindlimbs and forelimbs, as well as the contralateral hindlimb-forelimb pair, in a context-dependent manner (Pocratsky et al 2020). The changes in interlimb coupling were independent of fundamental locomotor relationships including speed and spatiotemporal gait indices. From these findings, we conclude that inter-enlargement LAPNs and L2-L5 interneurons are responsible for relaying temporal information about left-right alternation at both the pelvic and shoulder girdles.

Here, we will use the same dual-virus Tet^{On} system to reversibly silence two intraspinal pathways: (1) LAPNs that project from L2 to C6 post-SCI (Fig 4d) and (2) LDPNs that project from C6 to L2 before and after SCI (Fig 4e). The remainder of this dissertation will focus on the consequences of silencing on locomotor function and the implications these findings have on the fields of spinal interneurons and locomotor recovery following SCI.

Figure 1. Proposed location of lumbar and cervical locomotor CPGs.

The suggested location of the cervical locomotor CPG can be found within the cervical enlargement, while the lumbar locomotor CPG can be found in the lumbar enlargement (a). Supraspinal input and peripheral afferent input provide feedback and act as modulators for the CPG motor output. Green ovals indicate the approximate location of the CPG within the corresponding enlargement. Based on previous findings^{5,48,53}, the forelimb CPG is located within the C6-C8 spinal segments with an independent CPG for the front right (FR) and front left (FL) limbs (b). Similarly, the location of the hindlimb CPG is proposed at L1 and L2 spinal segments⁵⁵⁻⁵⁸, with independent CPGs for the rear right (RR) and rear left (RL) limbs (c). The flexor (F) and extensor (E) components of each limb's CPG are indicated by green circles and labeled with the limb they represent.

Figure 2. Classical definitions of various neuron populations.

Examples of each of the defined spinal populations of neurons are shown. Local interneurons are commissural, found within the same spinal segment, and are thought to coordinate the functions of independent limb CPGs found within the cervical and lumbar enlargements (**a**, purple circles). Short propriospinal neurons are defined as traveling several segments only within the spinal cord before their termination (**b**, yellow circles), while long propriospinal neurons are defined as ascending and descending extensions between the cervical and lumbar enlargements (**c**, blue circles). Projection neurons are said to originate from supraspinal centers and to project onto either cervical or lumbar CPG neurons to modulate their output (**d**, pink circles).

Figure 3. Locations of long and short propriospinal neurons based on experimental focus.

Examples of long descending, short descending, and long ascending propriospinal neurons are shown based upon experimental studies that define them. For LDPNs (a), populations are defined by the following sets of spinal segments: brainstem to T11/T13 & L4 (1, Jordan & Schmidt 2002), cervical enlargement to caudal to L4 (2, Matsushita et al 1979), cervical enlargement to rostral lumbar segments (3, Ni et al 2014; Reed et al 2006; Brockett et al 2013; Flynn et al 2017), C3-C6 spinal segments to L1 and L2 spinal segments (4, Conta Steencken & Stelzner 2010; Mitchell et al 2016; Sheikh et al 2018; Conta Steencken et al 2011; Swieck et al 2019), C1-C3 spinal segments to T11 (5, Jankowska et al 1974), and C1 spinal segment to L5-S1 spinal segments (6, Miller et al 1998). For short propriospinal neurons (b), populations are defined by the following sets

of spinal segments: T5/T7 spinal segments to T13/L1 spinal segments (**9**, Conta Steencken et al 2011), T6/T8 to the lumbar enlargement (**7**, Doperalski et al 2019; Swieck et al 2019), and T8/T11 spinal segments to the lumbar enlargement (**8**, Courtine et al 2008; Deng et al 2013). For those specified “lumbar enlargement” only, no lumbar or sacral levels were identified for termination and are therefore assumed for the purposes of the figure. Finally, for LAPNs (**c**), populations are defined by the following sets of spinal segments: rostral lumbar segments to C5-C8 spinal segments (**11**, English et al 1985; Pocratsky et al 2020), L4-L5 spinal segments to C5 spinal segment (**10**, Deng et al 2013), L1-L2 spinal segments to cervical enlargement (**12**, Reed et al 2006; Brockett et al 2013; Reed et al 2009), and L3-S4 spinal segments to C3 (**13**, Dutton et al 2006).

Figure 4. Tet^{On} dual virus approach to silencing long spinal pathways in an adult rat model.

The AAV2 viral vector construct constitutively expresses the rtTAV16, or reverse tetracycline transactivator, VP16 (**a**). The lentiviral vector construct expresses eTeNT.EGFP in the presence of an active Tetracycline Response Element promoter (**b**, TRE). When each viral vector is injected into the location of anatomically defined neuronal cell bodies (AAV2, yellow) and cell terminals (EGFP.eTeNT, green), the

neuron becomes doubly-infected. rtTAV16 is activated in the presence of doxycycline (Dox, red) and can bind to the TRE promotor, which induces eTeNT.EGFP expression. It is anterogradely transported down to the cell bodies, where it cleaves vesicle associated membrane protein 2 (VAMP2) and prevents the release of vesicular contents into the synapse (**c**, silenced neurotransmission). This system will be utilized in the bilateral injection protocols specified for LAPNs (**d**) and LDPNs (**e**).

CHAPTER II

DIFFERENTIAL CONTEXT DEPENDENCE OF ASCENDING AND DESCENDING PROPRIOSPINAL PROJECTIONS DURING LOCOMOTION

Introduction

Successful locomotion depends upon the precise coordination of multiple muscles across numerous joints and limbs, as well as the simultaneous engagement of multiple trunk and stabilizing muscles (Frigon 2017; Laliberte et al 2019). This patterned motor output, part of the central pattern generator (CPG), must be adjusted dynamically at differing speeds and in response to various obstacles and perturbations and requires constant integration of sensory information. The integration of proprioceptive afferent information and cutaneous sensory cues is critical for guiding appropriate locomotor output. Experiments using chronic spinal kittens showed that the mammalian CPG was able to modify the locomotor pattern based on sensory cues, such as instantaneous adjustments to speed (Forssberg et al 1980; Frigon et al 2013, Laliberte et al 2019). Within this system of complex interactions, propriospinal neurons are essential for communicating this information over short and long distances within the spinal cord. Specifically, long propriospinal neuron populations involved with coordination of locomotor activity have an essential function of integrating sensory context from the periphery and relaying it between the hindlimb and forelimb CPGs to modulate patterned output (Pocratsky et al 2020). To do this successfully, both ascending

and descending projections are necessary to anatomically and functionally connect the cervical and lumbar enlargements (Reed et al 2006; Brockett et al 2013).

Long descending propriospinal neurons (LDPNs) have cell bodies that reside within the cervical enlargement and axons that project caudally to innervate the rhythmogenic centers in the lumbar enlargement (Reed et al 2006; Ni et al 2014; Flynn et al 2017; Alstermark et al 1987). Initial electrophysiological studies performed in the cat suggested that this descending system is critically involved in postural control as it relays proprioceptive inputs from the head and neck down to the hindlimb motor pools (Alstermark et al 1987). A more recent study elaborated on these findings and showed that the selective ablation of inter-enlargement projections resulted in postural instability, a reduction in the maximum speed during overground stepping, and impaired interlimb coordination at high velocities on the treadmill (Ruder et al 2016). These studies suggest that the descending, inter-enlargement system has some role in maintaining postural control, and to some extent interlimb coordination at higher step speeds.

Immunohistochemical labelling revealed that contralateral LDPNs received putative synaptic input from both excitatory and inhibitory pathways, including vesicular GABA transporter/ parvalbumin (VGAT/PV) positive inhibitory premotor interneurons such as Renshaw cells and group Ia afferent input as well as VGLUT1 positive excitatory corticospinal and group Ia proprioceptive muscle afferent input (Ni et al 2014). Takeoka et al (2014) utilized a mouse model that lacked functional muscle spindle feedback and found that muscle spindle feedback was essential for precision locomotor tasks and involved projections onto multiple descending neuronal populations in the ventrolateral quadrant. Collectively, these findings further indicate that LDPNs play a key role in

interpreting sensory information from the limbs and transmitting that information to CPG circuitry.

Relatively less is known about inter-enlargement ascending projections. Long ascending propriospinal neurons (LAPNs) are the “reciprocal” pathway to LDPNs with their cell bodies residing in the lumbar enlargement and their terminal field throughout the cervical enlargement (English et al 1985; Brockett et al 2013; Reed et al 2006, 2009). LAPNs are heterogeneous and are comprised of ipsilateral and commissural projections that provide excitatory as well as inhibitory inputs onto neurons throughout the cervical gray matter, including motor neurons (Giovanelli and Kuypers 1969; Brockett et al 2013; Miller 1970). Previous work in our laboratory hypothesized that, given their lumbar-to-cervical connectivity, silencing LAPNs would disrupt hindlimb-forelimb coordination during locomotion. However, we showed that LAPNs are key distributors of temporal information at the shoulder and pelvic girdles and are essential for securing left-right alternation during overground locomotion at the lumbar and cervical CPGs independently. Interestingly, these results also indicated that silencing-induced disruptions to interlimb coordination were not present during treadmill tasks, exploratory locomotion, or swimming, suggesting that long propriospinals relay context-based information to hindlimb and forelimb girdles (Pocratsky et al 2020).

Here, we provide a brief comparison of the consequences of silencing LAPNs/LDPNs in three separate sensory contexts in uninjured animals: swimming, overground locomotion on a smooth surface, and overground locomotion on a coated surface. Based upon our previous work, we hypothesized that silencing LAPNs and reciprocal LDPNs would result in equal disruptions of the hindlimbs and forelimbs

regardless of stepping surface, while left-right alternation during swimming would be preserved in both experiments.

Materials and Methods

Experiments were performed in accordance with the Public Health Service Policy on Humane Care and Use of Laboratory Animals, and with the approval of the Institutional Animal Care and Use and Institutional Biosafety Committees at the University of Louisville.

Viral vector production

Plasmids used to generate virus were a generous gift from Tadashi Isa and colleagues (Kinoshita *et al*, 2012). HiRet-TRE-EGFP.eTeNT (1.68×10^7 vp/mL) and AAV2-CMV-rtTAV16 (2.66×10^{12} vp/mL) were grown following previously described methods (Pocratsky *et al.*, 2017, 2020).

Intraspinal injections of viral vectors to doubly infect LAPNs and LDPNs.

Intraspinal injections (Fig 4c-e) and power analyses for kinematic measures are based on previous literature (Pocratsky *et al.*, 2017). The procedural details are described in the *Nature Protocol Exchange* (<http://dx.doi.org/10.1038/protex.2017.125>). Power analysis for gait measures revealed that N=6-10 could detect a true significant difference with power of 85-95%.

For LAPNs, N=8 rats were anesthetized (ketamine:xylazine:acepromazine, 40 mg/kg:2.5 mg/kg:1mg/kg; I.P., Henry Schein Animal Health, Dublin, OH; Akorn Animal Health, Lake Forest, IL) and placed into spinal stabilization units³³. Animals received a C6/C7 laminectomy to expose the C6 spinal cord segment. Four bilateral injections of HiRet-TRE-EGFP.eTeNT (0.5 μ L/injection site) were made into the intermediate gray

matter (0.6 mm mediolateral, 1.3 mm dorsoventral, 1.5 mm rostrocaudal). Virus was delivered in two 0.25 μ L boluses over three minutes. Pre-operative glycopyrrolate (0.02 mg/kg, I.M.; Butler Schein, Dublin, OH) was given. After injection, incision sites were sutured by layer and closed with surgical staples. Topical antibiotics (Bacitracin; Perrigo; Allegan, MI) were applied to the incision site to prevent infection. Upon waking, animals were given buprenorphine (0.1 mg/kg, S.Q.; Par Pharmaceuticals, Chestnut Ridge, NY) every 12 hrs for 48-72 hrs for post-surgical pain management. Post-operative gentamicin (Gentafuse, 20 mg/kg, S.Q.; Henry Schein Animal Health, Dublin, OH). Five mL of lactated Ringer's (10 c.c.; Hospira, Lake Forest, IL) was given twice daily for two days, followed by administration as needed for hydration for five additional days. Prophylactic doses of gentamicin were administered once daily for one week following surgery.

The above surgical procedure was repeated one week later at the level of the lumbar spinal cord. Animals were reanesthetized (ketamine:xylazine:acepromazine, 40 mg/kg:2.5 mg/kg:1mg/kg; I.P., Henry Schein Animal Health, Dublin, OH; Akorn Animal Health, Lake Forest, IL) and received a T13 laminectomy to expose the L2 spinal cord segment. Isoflurane (Isothesia; 1-3% inhalant; Henry Schein Animal Health; Dublin, OH, USA) was used as needed for any animals that required additional assistance for anesthesia induction. AAV2-CMV-rtTAV16 (0.5 μ L/injection site) was injected bilaterally using coordinates of 0.6 mm mediolateral, 1.5 mm dorsoventral, and 1.5 mm rostrocaudal (four injections sites total). Post-surgical procedures follow those described above. Yohimbine (0.1 mg/kg; I.M., Lloyd Laboratories, Shenandoah, IA), a xylazine reversal agent, was administered after surgical procedures were complete to counteract xylazine's effects.

Experimental design

The influence of the stepping surface was discovered in two separate silencing studies: LAPN silencing (N=8) and LDPN silencing (N=19). For LAPN silencing, N=8 adult female Sprague Dawley rats (215-225 grams) received the aforementioned viral vector injections with behavioral testing performed prior to injection (Baseline, BL), before Dox administration (Pre-Dox1, PD1), during Dox (Dox1^{On}-D5, D1D5; Dox1^{On}-D8, D1D8) and after 2 weeks of Dox washout (Dox^{Off}, PostD1).

Doxycycline hydrochloride (Dox, 20 mg/ml; Fisher Scientific BP26531; Portsmouth, NH) was dissolved in 3% sucrose water and provided *ad libitum* for 5-8 days. Approximate volumes of consumption were recorded and replenished daily. Functional testing was performed prior to injections (Baseline), before Dox (Pre-DOX1), during Dox (D1D5, D1D8), and one week post-Dox (PostD1). Before Baseline, animals were acclimated to the stepping/swimming chamber. Stepping was spontaneous and volitional. Animals did not receive task-specific or positive/ negative reinforcement training. The order of animal testing was random. Due to the overt change in behavior during silencing, it was impossible to blind the experimenters to control versus treated time points. However, raters were blinded before beginning any assessments as to time point and condition. Each animal served as its own control based on the following: (1) the total number of interneurons that are doubly-infected is unique between animals, (2) the inherent variability in transgene expression across animals, and (3) the variability of normal behavior between animals. Control versus experimental time point comparisons were made on an individual and group basis. Group data are shown.

In a separate study for LDPN silencing, adult female Sprague Dawley rats (225-245 grams, N=19) received the previously described injections and were tested at the same time intervals as previously listed. After the first completed round of behavioral testing following Dox administration, animals were split into two groups: (1) animals that would undergo an additional round of Dox administration and behavioral assessments (N=8) and (2) animals that would receive a 12.5 g/cm spinal cord NYU contusion before undergoing additional behavioral assessments (N=11) (results discussed in Chapter IV). For animals that remained uninjured, further behavioral testing was performed again prior to Dox administration (Pre-Dox2, PD2) and during Dox (Dox2^{On}-D5, D2D5; Dox2^{On}-D8, D2D8). A final terminal assessment was performed on Dox2^{On}-D13 (D2D13).

Overground gait analyses

During stepping, 3 high-speed cameras recorded their movements at 100 frames/s from one ventral and two sagittal viewpoints (Basler Ace ACA640 cameras; Basler, Inc; Exton, PA). In this chapter, only ventral view gait kinematics will be discussed. Videos were analyzed using MaxTraQ software (Innovision Systems, Columbiaville, MI). A minimum of six passes were chosen using the following criteria: 1) the animals walked continuously across at least three-quarters of the tank, 2) the pass contained a minimum of 3 continuous step cycles, and 3) the pass was representative of the animal's locomotor behavior as a whole. The six chosen passes were representative of all gait patterns that the animal produced during that given time point. A total of 10-15 step cycles were analyzed for each animal across all time points.

The digitized steps from the single ventral camera were used to analyze paw contacts and lift offs of each of the four paws. Phase was represented as circular polar

plots to demonstrate interlimb coordination regardless of lead limb or was converted to a linear scale (0.5-1.0) to eliminate variable lead limb preferences (Gorska et al 1998, 1999). When plotted linearly, blue boxes on graphs represented >2 standard deviations (S.D.) as calculated from uninjured control average and S.D. Values found within the blue box are considered outside of normal variability observed at control time points and were graphically represented using pie charts to indicate the percent of total steps that existed outside of that range. For phase versus phase plots, each step was plotted against the corresponding phase values for two limb pairs.

For each individual step, temporal and spatial measures were plotted against their instantaneous speed (centimeters/second): swing time (the amount of time the limb is in the air from lift off to initial contact, seconds), stance time (the amount of time the limb is in contact with the ground from initial contact to lift off, seconds), stride time (stance time + swing time, seconds), and stride distance (distance traveled per step, centimeters). The relationships were analyzed using lines of best and 95% prediction intervals, followed by regression analyses to determine the closeness of fit (R^2). Average speed was calculated for each time point using individual steps (bar) with the average speed of each animal included (circles).

Context-specific overground stepping behavioral analyses

For the LAPN and LDPN silencing studies, animals were tested in two acrylic walkway chambers with different stepping surfaces. One walkway was coated with Sylgard, a clear, silicone substance (“coated”; coefficient of friction = 1.41) (Sylgard-184 Silicone Elastomer Kit; Dow Corning; Midland, MI, USA) while the other walkway was uncoated acrylic (coefficient of friction = 0.47). The control threshold (average + 2 S.D.)

was calculated for each stepping surface, respectively, from data generated at Baseline, Pre-Dox1, and Dox^{Off} for LAPNs and Baseline, Pre-Dox1, Dox^{Off}, and Pre-Dox2. No significant differences were detected between the stepping surfaces at control time points. Before baseline behavioral assessments, animals were allowed to acclimate to the walking tank. Animals did not receive any positive or negative reinforcement training for any behavioral tasks.

The coefficients of friction reported for each stepping surface were calculated using the following approach. First, an alert adult female Sprague Dawley rat (229 grams) was positioned into one side of the stepping chamber. While the animal calmly rested, the tank was slowly raised until paw traction was lost. This angle was measured in three separate trials for both the Sylgard-coated and uncoated acrylic tanks, respectively. The coefficient of friction was then calculated based on the average of the tangent of the three measured angles. This process was repeated with an object that closely approximates the texture of the paw surface (e.g. smooth wooden block), yielding similar coefficients for each surface (uncoated acrylic: 0.44, Sylgard coated: 1.73).

Analyses for swim phase

For swimming tasks, the acrylic walking tank was filled with 7-8 inches of 25-28°C water and a neoprene-covered exit ramp was attached to one end via single chain to the external wall of the tank. A high-speed camera set up approximately 13-16 inches from the tank recorded 3-5 complete stroke cycles per pass. A maximum of 3 passes (or 8 stroke cycles) were analyzed per animal in each direction (to the left and to the right) following the previously defined criteria. The peak downward extension of the toe was digitized for both hindlimbs to determine the phase relationship during swimming. To

quantify this relationship, the time of peak extension of the facing toe (toe closest to the camera) was divided into the length of time for one complete stroke cycle of the reference opposite hindlimb (toe furthest from the camera). Values were transformed as described above and the proportion of phases >2 S.D. from transformed control mean were compared across time (LAPN mean=0.54; 2 S.D. >0.64 ; LDPN mean=0.54; 2 S.D. >0.64).

Statistical analyses

Statistical analyses were performed using the SPSS v22 software package from IBM and custom-built Excel macros. Differences between groups were deemed statistically significant at $p \leq 0.05$. Two-tail p values are reported.

Circular statistics were performed on the non-transformed interlimb coordination datasets (Pocratsky *et al.*, 2017; Zar, 1974). We primarily used the non-parametric two-sample U^2 test based on a previously described rationale (Pocratsky *et al.*, 2017, 2020). The null hypothesis tested is whether two time points have the same concentration in coupling pattern expression, i.e. values are concentrated around 0.5 and not equally distributed amongst phasic values.

The Binomial Proportion Test was used to detect significant differences in the proportion of coordination values beyond control threshold for the transformed interlimb coordination data taken from overground gait analyses on both sensory surfaces and from swimming. Correction for extreme values (0 and 1) was used when appropriate.

Regression analyses to compare the slopes for the lines of best fit were performed on the speed versus spatiotemporal gait indices datasets, including speed vs. swing time, stance time, stride time, stride frequency, and stride distance. Analyses were performed

for hindlimb-hindlimb relationships in all sensory contexts. To further analyze the differences in spatiotemporal measures, Euclidian distance was calculated for each measure. Euclidean distance measures how much 2 variables plotted against one another (one on the x-axis, one on the y-axis) change relative to one another. Euclidian distance was used to calculate the relationship between the kinematic assessment and speed for the Dox and Control groups. Euclidean distance calculations determined the distance between the two variables for each animal, and those distances were compared between the Control and Dox groups to show if the relationship was the same for the two measures. Paired t-tests were used for each variable's (i.e. swing time vs speed) average Euclidian distance.

Mixed model analysis of variance (ANOVA) followed by Bonferroni *post hoc* t-tests (where appropriate) were used to detect a significant difference in the average speed based on pathway silenced and sensory context (i.e. Control vs Dox; Acrylic vs Sylgard).

Results

A brief overview of locomotor measures

To look at locomotion in a variety of sensory contexts, we must first quantify the pattern and rhythm relationships that are essential to producing patterned locomotion. The step cycle itself can be broken into two main components: stance and swing. The stance portion of the step cycle is the amount of time the limb is in contact with the surface, beginning at initial toe contact and ending with toe liftoff (Fig 5a, stance time). Swing refers to the time in which the limb is in the air (Fig 5a, swing time). Together, these make up stride time, or the time in which it takes to complete an entire step cycle, and stride distance, or the distance travelled during a complete step cycle, which both

begin when the toe contacts the surface and ends when the same toe reinitiates contact on the subsequent step. Finally, step or stride frequency, or the inverse of time for a single step cycle, is also essential to examine. Stride duration, in combination with stride frequency, is a key component of locomotor rhythm. Specifically, as stepping transitions from slower gaits (walk, trot) to faster gaits (gallop, bound), there is a simultaneous increase in step frequency and decrease in stride duration (Bellardita and Kiehn 2015).

The above measures also have stereotypical relationships with speed. Typically, stance time and stride time exponentially decrease as speed increases (Fig 5b), while stride distance and step frequency linearly increase as speed increases (Fig 5c). Swing time demonstrates a modest decrease in duration as speed increases (Fig 5d); however, this relationship is less apparent than those previously described (Gorska et al 1998,1999; Hruska et al 1979). Together, these spatiotemporal relationships comprise the essential features that govern locomotion.

Finally, patterned locomotion is classified, in part, by the relationships of one limb to another limb, or interlimb coordination. Interlimb coordination can be measured using phase, a ratio calculated by dividing the initial contact time of one limb by the stride time of the other reference limb (Fig 5e; Bellardita and Kiehn 2015). This measure can be used to quantify the relationship of any of the limb pairs, including hindlimb-hindlimb, hindlimb-forelimb, or forelimb-forelimb (Fig 5f). Initially, the phase value will range from 0 to 1 and can be plotted on a circular graph to illustrate the relationship between the chosen limb pair (Fig 5g). In slower gaits, such as walk and trot, the hindlimb pair and the forelimb pair are out-of-phase or alternating (Fig 5e,g; values concentrated around 0.5). Further, the contralateral, or opposite-sided, hindlimb-forelimb

pair will be in-phase or synchronous (values concentrated around 0 and 1), while the ipsilateral, or same-sided, hindlimb-forelimb pair will also be in alternation. As the speed increases, the girdle relationships (hindlimb-hindlimb and forelimb-forelimb) will undergo a phase shift in which alternation gaits will move towards in-phase synchronous movements and contralateral intra-girdle relationships will shift out-of-phase. The ipsilateral relationships, however, will remain out-of-phase.

This chapter will focus primarily on the functional outcomes of silencing LAPNs and LDPNs during overground locomotion. During overground stepping tasks, animals volitionally commute from one end of an acrylic tank to the other. Consequently, animals select the speed at which they locomote and will typically demonstrate a preferred lead limb (Gorksa et al 1999). To eliminate lead limb variability, raw phase values were transformed from a circular scale of 0-1 to a linear scale of 0.5-1 (Fig 5h). Once on a linear scale, the average phase value for the control time points was calculated for each of the respective limb pairs. Two standard deviations above the means was utilized as a threshold for normal phase variability for a given limb pair, meaning that any value above the control threshold was a deviation from normal stepping behavior. This method was used for all subsequent testing in various sensory contexts.

Silencing-induced perturbations as a result of sensory surface variations differ for LAPNs and LDPNs

To determine the context-dependence of LAPNs and LDPNs and their effects on locomotion, we used the dual-virus Tet^{ON} system previously described in Chapter I (Fig 4C). Briefly, Hi-Ret-TRE-EGFP.eTeNT, a lentiviral vector (Fig 4A), is injected into the cell terminal field. A second viral vector, AAV2-CMV-rtTAV16 (Fig 4B), is injected at

the level of the cell bodies and expresses the doxycycline (Dox)-activated Tet-On sequence, a variant of the reverse tetracycline transactivator. In the presence of doxycycline or a tetracycline derivative, a doubly-infected neuron will actively produce enhanced tetanus neurotoxin (eTeNT) conjugated to EGFP, resulting in silenced neurotransmission. Removal of doxycycline from the drinking water will halt production of the virus, subsequently restoring any altered behavior to normal. This conditional and reversible system allows for the functional dissection of an anatomically-defined pathway in the adult animal without permanent genetic modifications or *in vitro* preparations.

In this case, LAPNs and LDPNs were examined in two separate studies (N=8 and N=19, respectively). We first examined the functional consequences of silencing the inter-enlargement LAPNs. Our previous study utilized the same conditional silencing system and found that LAPNs were essential for securing left-right alternation of the hindlimb pair and the forelimb pair (Pocratsky et al 2020). Initially, we sought to recreate these findings in a different study intended to examine the role of LAPNs following SCI. Interestingly, we were only able to reproduce the findings on Sylgard. To further parse out this surprising result, another study was performed. Animals were placed in two overground tanks: one with an uncoated, acrylic stepping surface and the other with a coated, Sylgard stepping surface. During control behavioral testing, left-right alternation was the primary gait pattern for the shoulder (Fig 6a, forelimbs, top small grey circles) and pelvic (Fig 6a, hindlimbs, bottom small grey circles) girdles, indicated by strong concentrations of steps around 0.5 on circular plots. Silencing LAPNs significantly disrupted the alternating hindlimb and forelimb step pattern only on the coated surface

(Fig 6a,d,e; small red circles), with alternation continuing to predominate on the acrylic surface (Fig 6a,b,c; small yellow circles).

Given these interesting findings during LAPN silencing, we attempted another study in which we examined the same behavioral outcomes in the LDPN population. Of note, LDPN control time points demonstrated some increase in variability of steps as compared to LAPN controls, possibly an unintended result of more frequent exposure to the walking tanks during the LDPN study (Fig 7a-e; small grey circles). Contrary to the LAPN findings, LDPN silencing resulted in perturbations to left-right alternation of the hindlimb pair and the forelimb pair on both the acrylic surface (Fig 7a-c; small yellow circles) and the Sylgard-coated surface (Fig 7a,d,e; small red circles). Interestingly, a second round of Dox administration led to greater disruptions in alternation of the hindlimbs, such that there are a greater number of steps above normal variability. Disruptions in forelimb coupling were similar between pathways on the Sylgard surface, but were only present during one time point of LDPN silencing on the acrylic surface. Together, these findings suggest LAPNs secure left-right alternation in a manner that is more context-dependent than the reciprocal LDPN pathway. Further, silencing of both LDPNs and LAPNs resulted in disruptions to the hindlimb pair and the forelimb pair, implying that each pathway selectively affects left-right alternation at the shoulder and pelvic girdles. No differences in the base-of-support were detected between the acrylic and Sylgard during silencing for either pathway, suggesting that balance/postural changes likely do not account for these fascinating results (LAPN Dox: $18.36 \pm 2.97^\circ$ vs $21.44 \pm 4.48^\circ$, $p > .05$, n.s., paired t-test; LDPN Dox: 18.95 ± 7.41 vs 20.11 ± 11.91 , $p > .05$, n.s., paired t-test).

Silencing LAPNs and LDPNs preserves the stability of the stepping pattern despite disrupted coupling at the shoulder and hip girdles.

Next, we plotted the phases of each limb pair against each other to examine the relationships between limb pairs on each surface. Typically, gait switches occur in a linear fashion as speed increases. Therefore, as forelimb coordination shifts towards synchrony, hindlimb coordination should do the same. Likewise, as contralateral hindlimb-forelimb coordination moves towards alternation, hindlimb coordination should approach synchrony. During control time points, steps were clustered in the center of the plot for the forelimb and hindlimb comparison, indicating that alternating forelimb steps were concomitant with alternating hindlimb steps. Similarly, alternating hindlimb steps were clustered with synchronous hindlimb-forelimb steps. This held true for control testing on both the acrylic and Sylgard surfaces (Fig 8a,b,e,f). Largely, LAPN and LDPN silencing on the Sylgard surface maintained the expected linear interactions between the forelimb and hindlimb limb pairs and the hindlimb-forelimb and hindlimb limb pairs (Fig 8c,d,g,h; small red circles). Interestingly, there were specific regions for the phase versus phase comparisons that did not appear during acrylic LAPN silencing. Differences became apparent when the two populations were compared on the acrylic surface. There were no perturbations in left-right alternation on the acrylic surface for the LAPNs, which is clearly manifested in the lingering concentration of steps near alternation (Fig 8c, small yellow circles) and synchrony (Fig 8d, small yellow circles). Notably, left-right disruptions during LDPN silencing followed the same linear patterns as disruptions seen on Sylgard (Fig 8g,h; small yellow circles), suggesting that silencing is not disturbing the interactions between limb coupling patterns.

As previously described, several other key features of the step cycle are fundamental for producing stereotypical locomotion. During LAPN silencing, the relationships between speed and swing time and stance time remained unaffected on both acrylic and Sylgard (Fig 9a,b,g,h). Further, the fundamental relationship between speed and stride duration, frequency, and length remained intact, indicative of preservation of the underlying stepping rhythm (Fig 9c-e,i-k). Interestingly, the speed between the surfaces remained unchanged during control time points, but was increased during Dox on both surfaces (Fig 9f). Silencing LDPNs resulted in similar findings, as the key features of locomotion were unchanged (Fig 9l-p, r-v). The speed, however, was significantly different between Control and Dox on Sylgard and acrylic, suggesting that speed plays a greater role in the phase disruptions than during LAPN silencing and that animals are able to reach speed of $>150\text{cm/s}$ (Fig 9q). Euclidian distance calculations bolster these findings, as there is no significant difference between Control and Dox measures during LAPN silencing on either surface (Table 1). Significant differences were seen during LDPN silencing only on Sylgard, likely as a result of increased speed and thus the expression of a wider range of gait patterns (Table 2). However, there are some steps that occur at speeds not typically associated with bounding behaviors ($>180\text{cm/s}$), meaning that speed could not be the only variable that is changed as a result of silencing. Furthermore, animals left-right disruptions seen on acrylic and Sylgard are independent of speed, despite being able to attain higher speeds on Sylgard (data not shown). These data illustrate that silencing LAPNs and LDPNs reversibly disrupts interlimb coordination independent of speed-dependent spatiotemporal indices and the underlying locomotor rhythm despite increases in speed, while still maintaining interactions

associated with classically identified gait patterns. It is also important to note that animals may not experience the full range of speeds on either surface, given that the length of the walking tanks are short and do not allow them to reach maximum speed. Further exploration of these phenotypes in a longer tank may provide more clues as to the differences in phenotype between pathways.

Coefficient of friction differences: “slippy” vs “grippy”

To better quantify the differences between the acrylic and Sylgard-coated surfaces, we calculated the coefficient of friction for each surface. An alert adult female Sprague Dawley rat (229 grams) was positioned on one side of the stepping chamber. While the animal calmly rested, the tank was slowly raised until paw traction was lost (Fig 10a). This angle was measured in three separate trials for both the uncoated acrylic tank (Fig 10b) and Sylgard-coated tank (Fig 10c), respectively. The coefficient of friction was then calculated based on the average of the tangent of the three measured angles (Fig 10a; uncoated acrylic: 0.47, Sylgard-coated: >1.0). This process was repeated with an object that closely approximates the texture of the paw surface (e.g. smooth wooden block), yielding similar coefficients for each surface (uncoated acrylic: 0.44, Sylgard-coated: >1.0).

Interlimb coordination persists during swimming

Finally, we examined the effects of LAPN and LDPN silencing on left-right hindlimb coordination in water (Fig 11a). Swimming is a bipedal task where the hindlimbs provide the major propulsive force while the forelimbs steer (Gruner & Altman 1980). During swimming, the limbs are unloaded and the proprioceptive and cutaneous feedback associated with stepping is altered (Miller & van der Burg 1973, Duysens &

Stein 1978, Akay et al 2014). In contrast to our overground findings, silencing LAPNs and LDPNs had no effect on left-right hindlimb alternation during swimming (Fig 11b,c). This was especially interesting given the differential effect of the sensory surface between LAPNs and LDPNs. Had LDPNs been completely context-independent, we would have expected to see disruptions in alternation during swimming. However, the circuitry responsible for hindlimb alternation during swimming is unperturbed by LAPN and LDPN silencing, suggesting it may be completely lumbar autonomous and does not rely on sensory input integrated by LAPNs and LDPNs to perform its essential task.

Discussion

Contrary to our hypothesis, disruptions to left-right alternation of the hindlimb pair and the forelimb pair were distinctive for (1) each pathway silenced and for (2) the sensory context of the stepping surface. During LAPN silencing, patterned stepping perturbations were only seen on the Sylgard-coated surface, leaving alternation intact on the acrylic surface. Similarly, LDPN silencing resulted in an analogous stepping phenotype on Sylgard. However, stepping disruptions were seen equally on acrylic during LDPN silencing, especially during repeat administrations of Dox, unlike during LAPN silencing. Swimming was unaffected during silencing in either the ascending or descending pathway.

To understand the complexity behind these findings, it is essential to break it down the layers into individual concepts. Silencing both LAPN and LDPN pathways resulted in perturbations in left-right coordination of the hindlimbs. In combination with our previous findings, the hindlimbs and the forelimbs are equally disrupted during LAPN silencing (Pocratsky et al 2020). That is to say, the same number of forelimb steps

reached synchrony during overground locomotion as did the number of hindlimb steps. However, few forelimb steps reached synchrony during LDPN silencing, suggesting dominance of the ascending pathway in the regulation of intra-girdle coupling. *In vitro* work by Juvin and colleagues (2005) supports this concept. They found that propriospinal coupling between the lumbar and cervical CPGs was mediated to a greater extent by ascending influences from the lumbar CPG to its forelimb counterpart in the cervical cord, suggesting that interlimb coordination between the hindlimb pair and the forelimb pair relies on a caudorostral hierarchy (Juvin et al 2005). In some ways, our LDPN findings resemble the disruptions to left-right alternation described by Ruder et al (2016). The extent of decoupling of the hindlimb-hindlimb pair closely parallels their described gait impairments post-ablation of cervico-lumbar projection neurons, as did the less severe forelimb-forelimb perturbations (Ruder et al 2016). Taken together, these results suggest that these two pathways may be anatomically reciprocal but not functionally reciprocal. The targeted ascending projections clearly have greater influence over both cervical and lumbar CPGs. However, we did not find that silencing LDPNs or LAPNs resulted in significant differences in postural stability or reduced locomotor speeds as was described by Ruder et al (2016) (data not shown, Pocratsky et al 2020).

The next layer to consider is the distinct differences in silencing-induced perturbations in different sensory contexts. One interpretation would be that LAPNs are necessary for securing interlimb coordination in select conditions, such as directed overground stepping (Pocratsky et al 2020) or on a surface with good traction such as Sylgard. Conversely, in other conditions such as stepping overground on uncoated acrylic or swimming, LAPNs are non-essential to the CPG circuitry. LDPNs do not share this

indispensability, as they still act to interpret incoming sensory information from the periphery and are not overridden based upon sensory context. Again, *ex vivo* findings suggest that fictive quadruped patterned locomotion could be driven by low-threshold afferent input from any forelimb or hindlimb. Interestingly, lumbar afferent stimulation in the absence of lumbar CPG activity can elicit cervical CPG rhythmicity, whereas stimulation of cervical afferent inputs is unable to drive the lumbar generators without concomitant cervical CPG activation (Juvin et al 2012). This indicates that the essential activating influence of lumbar sensory inputs on the cervical generators is conveyed via direct ascending pathways, such as LAPNs, that communicate information between the hindlimb and forelimb CPG centers. The asymmetric influence by peripheral sensory inputs onto LAPNs over LDPNs may serve as a stabilizer during locomotion that transmits information to rapidly adapt to environmental changes, as the incoming sensory information may be greater onto LAPNs rather than LDPNs.

Based upon these findings, it is likely that a balance exists between intraspinal circuitry, peripheral input, and supraspinal command. In a more “spinal autonomous” context (e.g. a Sylgard-coated surface), LAPNs are critical for limb coupling at each girdle such that their conditional silencing disrupts intra-girdle alternation and this disruption is not “corrected” by supraspinal input. As circumstances demand increased input from supraspinal centers (e.g. slippery surface), functionally parallel pathways may be engaged that would ensure stability of locomotion as the sensory environment changes, subsequently concealing the functional consequences of silencing LAPNs. LDPNs do not have the same functional influence on the locomotor output and likely summate more information from supraspinal centers, such as the reticulospinal tract

(Mitchell et al 2016). When this is lost during silencing, LDPNs cannot convey changes in the environment to the hindlimbs to the same extent that LAPNs can, thus leaving the functional disruptions unchanged on varied sensory surfaces. Thus, in certain behavioral contexts or under certain environmental conditions, the functional importance of LAPNs for securing interlimb coordination would rise or fall in balance with decreased or increased supraspinal influence. In similar contextual situations, the importance of LDPNs may remain unchanged, as they receive more supraspinal oversight and may be more important in maintaining other functions rather than left-right alternation.

Swimming, a primarily bipedal hindlimb-driven task, may be a “lumbar autonomous” activity. The locomotor output during this task, then, may not rely on input from LAPNs or LDPNs at all, given its preservation regardless of propriospinal silencing influences.

Ultimately, the mechanisms underlying these remarkable findings are unknown. In the future, it would be interesting to compare the results of silencing on different sensory surfaces as well as sensory surfaces that change over the course of a single locomotor bout to determine if this effects silencing-induced disruptions. An additional administration of Dox to LAPN-silenced animals would also prove interesting as it would give some indication as to whether learning and repeat exposure plays a role in functional outcomes.

Figure 5. Examples of overground gait analyses. An example of a series of steps is shown in (a) to demonstrate how stride time, stride distance, stance time, and swing time are determined. Stance time and swing time are exemplified using the left hindlimb (LH, yellow) while stride time and stride distance are demonstrated using the right hindlimb (RH, blue). Light blue/light yellow strips indicate when the respective limbs are not in contact with the surface (swing time). Dark blue/dark yellow indicate contact with the surface (stance time). The stereotypical relationships of stance time and stride time with speed follow an exponential decay relationship (b), with faster gaits having higher speed and lower stance/stride times. Stride or step frequency and stride distance have a positive linear relationship with speed (c), while swing time shows a modest linear decay as speed increase (d). Example footfall graphs are shown for relationship of the hindlimbs for each of the stereotypically classified gaits (e; walk-trot, gallop, and bound). This plot can be used to plot the relationship of all limbs, known as a duty cycle graph (f; RH: right hindlimb, blue; LH: left hindlimb, yellow; LF: left forelimb, green; RF: right forelimb, red). The relationships are graphed circularly as phase values with relationships between

the hindlimbs, the forelimbs, and the homolateral hindlimb-forelimb pair concentrated around 0.5 and synchrony between the heterolateral hindlimb-forelimb pair concentrated are 0 or 1 (**g**) for alternating gait pattern. Finally, these phase values are transformed to a linear scale of 0.5 to 1.0 to eliminate lead limb preferences (**h**).

Figure 6. Silencing-induced perturbations for LAPNs are context-dependent. During

Control and Dox time points, individual hindlimb and forelimb steps are graphed using circular phase plots (a; see Table 3 for statistics, Watson's Non-Parametric U^2 circular statistics). All control steps are indicated by grey circles. Acrylic Dox steps are indicated by yellow circles and Sylgard Dox steps are indicated by red circles. Hindlimb phase relationships and forelimb phase relationships were transformed and graphed linearly for each time point (b,c). No significance was seen between any of the time points on the acrylic surface (b_i, PD1 hindlimbs $n=2/89$ [2.24%] vs D1D5 hindlimbs $n=3/82$ [3.65%]; $n.s.$, $z=.547$; PD1 hindlimbs $n=2/89$ [2.24%] vs D1D8 hindlimbs $n=5/84$ [5.95%]; $n.s.$, $z=1.24$). Hindlimb stepping was significantly altered during Dox time points only on the Sylgard surface (b_{ii}, PD1 hindlimbs $n=2/87$ [2.30%] vs D1D5 hindlimbs $n=23/89$ [25.84%]; $p<.001$, $z=4.47$; PD1 hindlimbs $n=2/87$ [2.30%] vs D1D8 hindlimbs $n=37/81$

[45.68%]; $p < .001$, $z = 6.65$). Forelimb phase relationships are shown in (c) for each time point, with similar indications of no coordination disruptions on acrylic (c_i, PD1 forelimbs $n = 6/89$ [6.74%] vs D1D5 hindlimbs $n = 2/82$ [2.44%]; n.s., $z = 1.33$; PD1 forelimbs $n = 6/89$ [6.74%] vs D1D8 hindlimbs $n = 9/84$ [10.71%]; n.s., $z = .928$) and dramatic coordination disruptions on Sylgard (c_{ii}, PD1 forelimbs $n = 3/87$ [3.45%] vs D1D5 hindlimbs $n = 19/89$ [21.35%]; $p < .001$, $z = 3.59$; PD1 forelimbs $n = 3/87$ [3.45%] vs D1D8 hindlimbs $n = 20/81$ [24.69%]; $p < .001$, $z = 4.0025$).

Figure 7. Silencing-induced perturbations for LDPNs are not context-dependent.

During Control and Dox time points, individual hindlimb and forelimb steps are graphed using circular phase plots (**a**; see Table 3 for statistics, Watson's Non-Parametric U^2 circular statistics). All control steps are indicated by grey circles. Acrylic Dox steps are indicated by yellow circles and Sylgard Dox steps are indicated by red circles. Hindlimb phase relationships and forelimb phase relationships were transformed and graphed linearly for each time point (**b,c**). No significance was seen between the time points on the acrylic surface for the first Dox administration, but was significantly altered during the subsequent Dox administration (**b_i**, PD1 hindlimbs $n=3/238$ [1.26%] vs D1D5 hindlimbs $n=12/218$ [5.50%]; $p<.05$, $z=2.49$; PD1 hindlimbs $n=3/238$ [1.26%] vs D1D8 hindlimbs $n=11/218$ [5.05%]; $p<.05$, $z=2.29$; PD2 hindlimbs $n=4/82$ [4.88%] vs D2D5 hindlimbs $n=38/82$ [46.34%]; $p<.001$, $z=6.91$; PD2 hindlimbs $n=4/82$ [4.88%] vs D2D8 hindlimbs $n=25/88$ [28.41%]; $p<.001$, $z=4.39$). Hindlimb stepping was significantly

altered during all Dox time points on the Sylgard surface (**b_{ii}**, PD1 hindlimbs n=2/191 [1.05%] vs D1D5 hindlimbs n=37/177 [20.90%]; $p<.001$, $z=6.32$; PD1 hindlimbs n=2/191 [1.05%] vs D1D8 hindlimbs n=51/171 [29.82%]; $p<.001$, $z=8.05$; PD2 hindlimbs n=12/81 [30.86%] vs D2D5 hindlimbs n=44/76 [57.89%]; $p<.001$, $z=6.24$; PD2 hindlimbs n=12/81 [30.86%] vs D2D8 hindlimbs n=39/80 [48.75%]; $p<.001$, $z=4.96$). Forelimb phase relationships are shown in (**c**) for each time point, with less severe coordination disruptions on acrylic (**c_i**, PD1 forelimbs n=5/238 [2.10%] vs D1D5 forelimbs n=7/218 [3.21%]; n.s., $z=0.73$; PD1 forelimbs n=5/238 [2.10%] vs D1D8 forelimbs n=7/218 [3.21%]; n.s., $z=0.73$; PD2 forelimbs n=6/82 [7.32%] vs D2D5 forelimbs n=14/82 [17.07%]; n.s., $z=1.93$; PD2 forelimbs n=6/82 [7.32%] vs D2D8 forelimbs n=17/88 [19.32%]; $p<.005$, $z=2.35$) and dramatic coordination disruptions on Sylgard (**c_{ii}**, PD1 forelimbs n=8/191 [4.19%] vs D1D5 forelimbs n=23/177 [12.99%]; $p<.005$, $z=3.02$; PD1 forelimbs n=8/191 [4.19%] vs D1D8 forelimbs n=33/171 [19.30%]; $p<.005$, $z=4.51$; PD2 forelimbs n=3/81 [3.70%] vs D2D5 forelimbs n=34/76 [44.74%]; $p<.001$, $z=6.75$; PD2 forelimbs n=3/81 [3.70%] vs D2D8 forelimbs n=24/80 [30.00%]; $p<.001$, $z=4.75$).

Figure 8. Relationships between limb pairs are maintained during LAPN and LDPN silencing. Control relationships between limb pair coordination are shown for hindlimb-hindlimb vs heterolateral hindlimb-forelimb (**a,g**), homolateral hindlimb-forelimb (**b,h**), and the forelimb-forelimb pairs (**c,i**). Control acrylic steps are indicated by light grey circles and control Sylgard steps are indicated by dark grey circles. Values are clustered, likely due to the overwhelming presence of alternating gait at control time points. The relationships for the previously described limb pairs are demonstrated for LAPN silencing (**d-f**) and LDPN silencing (**j-l**). Dox acrylic steps are indicated by yellow circles and Dox Sylgard steps are indicated by red circles. During LAPN silencing, acrylic steps remain clustered, also likely due to the large concentration of alternating steps. The patterns are similar for the limb pairs during LAPN silencing on the Sylgard surface and during LDPN silencing on both surfaces.

Figure 9. Silencing LAPNs and LDPNs does not affect key features of locomotion.

Spatiotemporal measures (swing time, stance time, stride time, stride distance) were plotted against speed for Control (LAPN: **a-e**; LDPN: **i-p**) and Dox (LAPN: **g-k**; LDPN: **r-v**) time points. Lines of best are not displayed on graphs for clarity. Stance time and stride time display exponential decay relationships during silencing of both pathways, regardless of surface (**b-c,h-i,m-n,s-t**; LAPN acrylic stance time: Control $R^2 = 0.751$ vs Dox $R^2 = 0.837$; LAPN Sylgard stance time: Control $R^2 = 0.696$ vs Dox $R^2 = 0.820$; LDPN acrylic stance time: Control $R^2 = 0.809$ vs Dox $R^2 = 0.896$; LDPN Sylgard stance time: Control $R^2 = 0.764$ vs Dox $R^2 = 0.811$; LAPN acrylic stride time: Control $R^2 = 0.749$ vs Dox $R^2 = 0.759$; LAPN Sylgard stride time: Control $R^2 = 0.791$ vs Dox $R^2 = 0.714$; LDPN acrylic stride time: Control $R^2 = 0.801$ vs Dox $R^2 = 0.881$; LDPN Sylgard stride time: Control $R^2 = 0.840$ vs Dox $R^2 = 0.842$), while a linear relationships are indicated for stride

distance (**d,m,o,u**; LAPN acrylic stride distance: Control $R^2=0.813$ vs Dox $R^2=0.698$; LAPN Sylgard stride distance: Control $R^2=0.708$ vs Dox $R^2=0.656$; LDPN acrylic stride distance: Control $R^2=0.799$ vs Dox $R^2=0.745$; LDPN Sylgard stride distance: Control $R^2=0.780$ vs Dox $R^2=0.719$) and stride frequency (**e,k,p,v**; LAPN acrylic stride frequency: Control $R^2=0.763$ vs Dox $R^2=0.772$; LAPN Sylgard stride frequency: Control $R^2=0.699$ vs Dox $R^2=0.751$; LDPN acrylic stride frequency: Control $R^2=0.864$ vs Dox $R^2=0.879$; LDPN Sylgard stride frequency: Control $R^2=0.793$ vs Dox $R^2=0.788$). The average instantaneous speed is shown for LAPN silencing (**f**, Average speed acrylic Control 62.13 ± 14.65 vs Dox: 82.16 ± 10.23 , $p < .001$; Mixed-Model ANOVA; Average speed Sylgard Control 75.26 ± 12.07 vs Dox: 91.90 ± 15.80 , $p < .001$, Mixed-Model ANOVA) and LDPN silencing (**q**, , Average speed acrylic Control 62.13 ± 14.65 vs Dox: 82.16 ± 10.23 , $p < .05$; Mixed-Model ANOVA; Average speed Sylgard Control 75.26 ± 12.07 vs Dox: 91.90 ± 15.80 , $p < .05$, Mixed-Model ANOVA).

Figure 10. Coefficient of friction differences on acrylic and Sylgard surfaces. An example of the experimental setup to determine coefficient of friction is shown in (a). To determine coefficient of friction on different surfaces, an alert adult female Sprague Dawley rat (229 grams) was positioned on one side of the stepping chamber. While the animal calmly rested, the tank was slowly raised until paw traction was lost. The coefficient of friction (μ) was calculated using the measured angle at which traction was lost (a). The calculated angle was found using the Pythagorean Theorem. For the acrylic tank, the coefficient of friction was determined to be .466 using a rat and .438 using a block of wood (b, wood was used as a well-established comparison). For the Sylgard-coated tanks, the coefficient of friction was determined to be 1.412 using a rat and 1.732 using the wood block (c). With a coefficient of friction greater than 1, there would be no slip while locomoting on the Sylgard surface.

Figure 11. Hindlimb coordination during swimming is maintained during LAPN and LDPN silencing. Similar to hindlimb coordination during overground locomotion, phase can be calculated between the hindlimbs during a swimming task (a). Hindlimb alternation was maintained during silencing of LAPNs (b, PD1 hindlimbs $n=4/294$ [1.36%] vs D1D5 hindlimbs $n=8/302$ [2.65%]; n.s., $z=1.13$; PD1 hindlimbs $n=4/294$ [1.36%] vs D1D8 hindlimbs $n=8/278$ [2.88%]; n.s., $z=1.25$) and LDPNs (c, PD1 hindlimbs $n=9/408$ [2.21%] vs D1D5 hindlimbs $n=23/416$ [5.53%]; $p<.05$, $z=2.5$; PD1 hindlimbs $n=9/408$ [2.21%] vs D1D8 hindlimbs $n=24/468$ [5.13%]; $p<.05$, $z=2.33$).

	Acrylic				Sylgard			
Variable	Control	Dox	p-value	Sig	Control	Dox	p-value	Sig
Swing time vs speed	362.9 ± 42.6	375.9 ± 27.9	.339	n.s.	416.5 ± 31.5	430.7 ± 63.7	.285	n.s.
Stance time vs speed	362.8 ± 42.7	375.9 ± 28.0	.339	n.s.	416.6 ± 31.5	430.8 ± 63.8	.284	n.s.
Stride time vs speed	362.1 ± 42.6	375.3 ± 28.0	.390	n.s.	415.8 ± 31.6	430.2 ± 63.8	.279	n.s.
Stride frequency vs speed	345.7 ± 41.4	360.0 ± 27.5	.325	n.s.	397.2 ± 30.6	411.6 ± 61.3	.260	n.s.
Stride distance vs speed	257.4 ± 35.6	275.3 ± 26.4	.160	n.s.	306.8 ± 28.8	333.7 ± 60.0	.061	n.s.

Table 1. Euclidian distances for LAPN silencing. Euclidian distances calculations are shown for LAPN spatiotemporal measures. Averages and one S.D. are shown for each variable. Paired t-tests were used to calculate statistical differences in the averages, indicated by p-values.

	Acrylic				Sylgard			
Variable	Control	Dox	p-value	Sig	Control	Dox	p-value	Sig
Swing time vs speed	435.5 ± 75.3	464.8 ± 158.6	.273	n.s.	444.5 ± 68.0	534.2 ± 197.6	.014	p<.05
Stance time vs speed	435.3 ± 75.4	464.7 ± 158.6	.271	n.s.	444.6 ± 68.0	534.3 ± 197.6	.013	p<.05
Stride time vs speed	434.5 ± 75.4	463.9 ± 158.5	.270	n.s.	443.8 ± 68.1	533.6 ± 197.5	.013	p<.05
Stride frequency vs speed	395.6 ± 68.6	421.8 ± 144.7	.280	n.s.	399.7 ± 62.7	512.0 ± 190.9	.003	p<.005
Stride distance vs speed	313.9 ± 65.4	344.7 ± 130.7	.163	n.s.	327.3 ± 59.0	414.3 ± 163.4	.006	p<.01

Table 2. Euclidian distances for LDPN silencing. Euclidian distances calculations are shown for LDPN spatiotemporal measures. Averages and one S.D. are shown for each variable. Paired t-tests were used to calculate statistical differences in the averages, indicated by p-values.

Behavioral context	Watson's test	Left-right FL	Left-right HL
LAPN overground locomotion on Sylgard-coated surface	U ²	0.2791	0.6128
	p-value	**p<0.01	****p<0.001
LAPN overground locomotion on acrylic surface	U ²	-0.0655	0.0914
	p-value	p>0.5	0.2<p<0.5
LAPN overground locomotion, Sylgard-coated vs acrylic (comparing control time points)	U ²	-0.5710	0.0600
	p-value	p>0.5	p>0.5
LAPN overground locomotion, Sylgard-coated vs acrylic (comparing Dox ^{On} time points)	U ²	0.4412	0.4918
	p-value	****p<0.001	****p<0.001
LDPN overground locomotion on Sylgard-coated surface	U ²	1.9012	2.0597
	p-value	****p<.001	****p<.001
LDPN overground locomotion on acrylic surface	U ²	.1549	-0.5407
	p-value	n.s.	n.s.
LDPN overground locomotion, Sylgard-coated vs acrylic (comparing control time points)	U ²	0.1689	0.0599
	p-value	n.s.	n.s.
LDPN overground locomotion, Sylgard coated vs acrylic (comparing Dox ^{On} time points)	U ²	0.3649	0.7102
	p-value	***p<.005	****p<.001

Table 3. Watson's U² circular statistics calculations for hindlimb and forelimb pairs during LAPN and LDPN silencing. We performed Watson's non-parametric two-sample U² circular statistics to determine function uncoupling in the hindlimb pair and forelimb pair. The null hypothesis tested was that two samples were from two populations with the same direction. Silencing LAPNs significantly disrupted left-right forelimb and left-right hindlimb phase data such that it reduced clustering at 0.5, indicating uncoupling of the limb pair only on the Sylgard surface. Silencing LDPNs also only affected the directionality of the data on the Sylgard surface. (Critical value of Watson's U² (0.05,∞,∞) = 0.1869; Appendix D, Table D.44)

CHAPTER III

SILENCING LONG ASCENDING INTER-ENLARGEMENT PROPRIOSPINAL NEURONS AFTER SPINAL CORD INJURY RESTORES HINDLIMB STEPPING ABILITY IN ADULT RATS

Introduction.

Locomotion is a fundamental behavior shared by almost all animals. In mammals, locomotion involves descending commands from supraspinal centers and peripheral input from sensory systems converging on spinal locomotor circuitry. The spinal locomotor circuitry includes central pattern generators (CPGs), first described by Sir Charles Sherrington and Thomas Graham Brown, as the intrinsic spinal networks capable of generating the coordinated muscle activity associated with stereotypic fore and hindlimb movements during stepping (Sherrington and Laslett, 1903; Sherrington, 1910; Graham Brown, 1911). It is now believed that each limb has its own CPG, and that the lumbar and cervical pattern generators are interconnected by long-ascending (LAPNs) and long-descending (LDPNs) propriospinal neurons that provide the functional coupling of the two enlargements allowing precise temporal information to be passed between and among the hindlimb and forelimb CPGs (Giovanelli and Kuypers, 1969; Miller *et al.*, 1975; English, 1979; Rossignol *et al.*, 1993; Juven *et al.*, 2005; Juven *et al.*, 2012). LAPNs soma reside in the intermediate gray matter, primarily in laminae VII and VIII, with approximately 40-60% having commissural axons (Reed *et al.*, 2006, 2009; Brockett

et al., 2013) before traversing through thoracic segments in the outermost regions of the lateral and ventrolateral funiculi (Reed *et al.*, 2006, 2009; Basso *et al.*, 2002).

Spinal cord injury (SCI) disrupts the communication between the brain and spinal cord, resulting in an immediate inability to initiate and maintain patterned weight-supported locomotion at or below the level of lesion (Dietz and Harkema, 2004; Fong *et al.*, 2009; Côté *et al.*, 2017). Even if classified as neurologically complete, most SCIs are anatomically incomplete as there is some sparing of white matter at the lesion epicenter (Bunge *et al.* 1993), most often the outermost rim of the lateral and ventrolateral funiculi where the LAPN axons reside. Therefore, these neurons and their axons may comprise a percentage of the anatomically spared circuitry, thus providing a potential functional bridge across the injury site. Due to these anatomical characteristics, their resiliency to cell death following incomplete SCI, and their known function in intact animals, these neurons are well-suited to participate in locomotor recovery after incomplete SCI (Conta and Stelzner, 2004; Conta Steencken and Stelzner, 2010, 2011; Siebert *et al.*, 2010).

Many studies have reinforced this notion of anatomical preservation and have suggested that propriospinals, albeit descending, participate in locomotor recovery directly or indirectly via serving as a *de novo* bridge to bypass lesion epicenter (Bareyre *et al.*, 2004; Vavrek *et al.*, 2006; Flynn *et al.*, 2011; Filli *et al.*, 2014; Benthall *et al.*, 2017). Previous work revealed that LAPNs secure left-right coordination of the hindlimbs and forelimbs during locomotion. This functional role was revealed through the use of a dual virus Tet^{ON} neuronal silencing system (Pocratsky *et al.*, 2020). Their anatomical location suggests they are partially spared post-SCI and could serve as a neural substrate for functional recovery (Reed *et al.* 2006, Brockett *et al.* 2013, Pocratsky *et al.* 2020).

Importantly, the extent of LAPN sparing is dependent on injury severity, such that a milder injury will result in more spared LAPN axons at the injury epicenter. Here, we use a mild-moderate injury to both mimic mild/moderate clinical injuries and to ensure sparing of LAPNs for analysis. Since a significant portion of this pathway should be spared, we hypothesize that LAPNs will contribute to recovery of function post-SCI, such that any recovered patterned stepping ability will be diminished and gross motor outcomes will be drastically reduced.

To test this hypothesis, we conditionally silenced spared LAPNs post SCI reasoning that if they contributed to recovery then their silencing would result in locomotor deficits. Unexpectedly, we found that silencing this spared pathway post-SCI improved locomotor function, suggesting that spared pathways may deleteriously influence locomotor recovery. Unexpectedly, silencing spared LAPNs post-thoracic SCI improved locomotor performance, including inter- and intralimb coordination. These findings suggest that spared LAPNs post-SCI negatively impacts recovered function, potentially through the transmission of aberrant afferent processing below the level of injury through mechanisms of maladaptive plasticity, ultimately deleteriously influencing the role LAPNs play in the production of locomotion. Removing or reducing the maladaptive “noise” within the system may permit the locomotor circuitry caudal to the injury to function freely and to respond to appropriate afferent cues unimpeded.

Materials and Methods.

Experiments were performed in accordance with the Public Health Service Policy on Humane Care and Use of Laboratory Animals, and with the approval of the

Institutional Animal Care and Use (IACUC) and the Institutional Biosafety (IBC) Committees at the University of Louisville.

A total of 16 adult female Sprague-Dawley rats (215-230 g) were used in this study. Animals were housed two per cage under 12-hour light/dark cycle with *ad libitum* food and water throughout the course of the study. Power analysis for gait measures revealed that N=6-10 could detect a true significant difference with power of 85-95%. Additional animals were used to mitigate animal mortality following additional surgical procedures. Twelve of sixteen animals displayed behavioral changes pre-SCI; the four animals that did not display behavioral changes were removed from the main data set to be analyzed separately as a post-injury control. To be excluded, animals had <10% of their steps outside of normal variability during Dox testing. A total of N=3 died after SCI surgery (N=2/12 with phenotype, N=1/4 without phenotype), leaving N=10 with phenotype that were used for the main pre- and post-injury data set. Animals that lacked behavioral outcomes pre-injury (N=3) did not show improvements in locomotion post-SCI.

Viral vector production.

Viruses were grown and titered following previously described methods (Pocratsky et al 2017, 2020).

Intraspinal injections of viral vectors to doubly infect LAPNs.

Intraspinal injections and power analyses for kinematic measures are based on previous literature. The procedural details are described in the *Nature Protocol Exchange* (<http://dx.doi.org/10.1038/protex.2017.125>). Intraspinal injections were performed (N=16) using the same LAPN injection protocol described in Chapter II methods.

Spinal cord injury.

Animals received a moderate spinal cord contusion at T9/T10 spinal cord approximately 2 weeks after conclusion of uninjured Dox^{ON} assessments. For SCI surgery, animals were reanesthetized (ketamine:xylazine, 80mg/kg:4mg/kg; Henry Schein Animal Health; Akorn Animal Health) and a dorsal midline incision was made through the skin and musculature overlying the mid-thoracic spinal segments. A laminectomy was performed at T9 to expose the T9/T10 spinal cord segments. The spine was immobilized using custom-built spine stabilizers (Hill et al 2009; Zhang et al 2008). Moderately severe weight-drop contusion injuries (12.5 g/cm) were delivered using the NYU impactor (Young 2002). After injury, the incision site was closed in layers using sutures and closed with surgical staples. Topical antibiotics were applied to the incision to prevent infection at the surgical site. Bladders were expressed manually for seven days or until they emptied spontaneously. Injured animals were housed individually in recovery cages placed on heating pads that contained a single layer of blue surgical paper (Argyle Surgical Drape Material; Medtronic, MN, USA) for 24 hours. After 24 hours, animals were put back into double housing with Alpha Dri bedding. Weekly BBB assessments were performed to ensure locomotor recovery was progressing similarly in all animals and to determine when animals reached behavioral plateau post-SCI. Animals were allowed to recover for ~6 weeks before any additional pre-DOX assessments.

Experimental timeline.

Doxycycline hydrochloride (Dox, 20 mg/ml; Fisher Scientific BP2653-5, Pittsburgh, NH) was dissolved in 3% sucrose and provided *ad libitum* for 8 days pre-injury and 8-21 days post-injury. Dox water was made fresh and replenished daily and

monitored for consumption. All behavioral assessments were performed during the light cycle portion of the day and concluded several hours before the dark cycle began.

Prior to SCI, behavioral assessments were performed prior to viral injections (BL), prior to Dox (pre-Dox), during Dox (Dox1^{ON}D5-D8), 10 days post-Dox (PostD1). Following SCI, pre-Dox and Dox^{ON} time point assessments were reproduced twice (Dox2 and Dox3) following SCI to assess the reproducibility of any behavioral changes that were seen. Data shown are compiled from pre-injury control and Dox timepoints (“uninjured”) and post-injury control and Dox timepoints (“injured”). Control vs Dox uninjured and Control vs Dox injured time point comparisons were made both on an individual and group basis. Behavioral analyses began on day 5 of Dox (DoxD5) administration and were repeated on Dox day 8 (DoxD8). For terminal assessments after injury, behavioral assessments occurred on DoxD8 as well as DoxD21.

Identification of hindlimb joints.

To maintain consistency of joint locations during kinematics testing, the bony landmarks of the anterior iliac crest (“I”) and the head of the greater trochanter of the hip (H) were manually probed, marked with black marker, and permanently demarcated using green tattoo ink (Ketchum Manufacturing, Inc., Animal Tattoo Ink Green, Brockville, Ontario, CA). The lateral malleolus of the ankle (ankle, “A”) and the MTP (metatarsophalangeal) joint of the 5th metatarsal (toe, “T”) were manually identified at the start of each behavioral acquisition session and were marked with black ink.

Hindlimb kinematics and intralimb coordination analysis.

Acquisition of hindlimb kinematics data was performed as previously described (Pocratsky et al 2017, 2020). Animals were placed in a clear acrylic walking tank

(length×width×height: 150 cm, 30 cm, 14 cm) with a Sylgard-coated (Sylgard 184 Silicone Elastomer Kit, Dow Corning, MI, USA) walking surface. Before baseline behavioral assessments, animals were allowed to acclimate to the walking tank. Animals did not receive any positive or negative reinforcement training for any behavioral tasks. Animals were allowed to locomote freely within the tank and were not positively or negatively encouraged to move. As animals walked in the tank, 3 high-speed cameras recorded their movements at 100 frames/s from one ventral and two sagittal viewpoints. Videos were analyzed using MaxTraQ software (Innovision Systems, Columbiaville, MI, USA). A minimum of three passes for each hindlimb were analyzed if: 1) the animals walked continuously across three-quarters of the tank, 2) the pass contained a minimum of 3 continuous step cycles, and 3) the pass was representative of the animal's locomotor behavior as a whole. All analyzed passes from sagittal frame numbers were matched to analyzed passes from ventral frame numbers to ensure consistency in selected passes and to assist with paw touchdown and liftoff (start and end of stance phase). This ensures each peak-trough occurs during an actual step. All values shown were graphed as separate left and right hindlimb data.

Two-dimensional stick figures were generated as examples of control and silenced hindlimb stepping. Pre-injury stick figures represent alternating hindlimb stepping during control and synchronous hindlimb stepping during silencing. Post-injury stick figures shown are representative of a control pass with instances of dorsal stepping and discoordination, as well as a silenced pass with improved coordination and reduced dorsal stepping. The proximal and distal angles generated during these

passes were plotted against each other in an angle-angle plot to further demonstrate the above points.

The analyzed points from the two sagittal cameras were exported to a Microsoft Excel workbook and 2D average angles were calculated for each digitized frame. Maximum and minimum angles (maximum extension and flexion, respectively) were manually identified and were used to calculate average excursion (Maximum Angle – Minimum Angle) for the proximal and distal hindlimb joint angles. The temporal relationship between the proximal and distal hindlimb joint angles was calculated using the peak-to-peak duration of the lead angle during a single step cycle. Within this duration, the maximum excursion of the first angle was determined and depended on which angle peaked first (proximal or distal angles). The time of onset of the second angle was divided by the peak-to-peak distal angle duration to determine the temporal relationship between intralimb angles. A coordination value of 0/1 indicates in-phase coordination of the proximal and distal intralimb joints, while a phase value of 0.5 indicates anti-phase, uncoordinated joint movements. Intralimb joint phase was calculated for each step cycle of the left and right hindlimbs independently.

Overground gait analyses.

Overground gait analyses were performed as previously described in Chapter II Methods. During injured recordings (control and silenced), a dorsal step was classified by the dorsum of the foot coming into contact with the ground during the stance portion of the step cycle and is considered a step if it maintains contact with the surface and completes the swing portion of the step cycle. Any hindlimb-centric steps used in the data set were left-lead limb dominant (i.e. RLRR, RLFR, RLFL) to maintain consistency

between uninjured and injured data and to avoid excessive data. As left limb was used for primary data analysis, dorsal steps were identified in the data set if they fell on the left side (i.e. a right-side dorsal step would not have been marked a dorsal step in the shown data).

Interlimb phase was calculated by dividing the initial contact time of the trailing limb by the stride time (initial contact to initial contact) of the leading limb. Alternating or “anti-phase” gaits were defined by phase values concentrated around 0.5 (Lemieux et al 2016) (defined as 0.375-0.625). Synchronous, “in-phase” gaits were likewise defined by phase values near 0 or 1 when plotted circularly (defined as 0.875-0.125). All other phase values concentrated around 0.25 or 90° (0.126-0.374) and 0.75 or 270° (0.626-0.874) represented a wide range of phase shifts and were defined as asynchronous or “out-of-phase” gaits. Frequency of steps per defined gait category was plotted using a frequency histogram. Individual steps ranged in value from 0-1 and were binned in intervals of 0.125 (0-0.125; 0.126-0.375; etc.). The frequency of anti-phase steps centered around alternation during both control and silenced timepoints is indicated by pink bars. Out-of-phase values associated with transitioning steps are graphed in orange and in-phase synchronous steps are graphed in green. Frequency data were also plotted as a pie chart to view the percentage of each gait classification and to eliminate lead limb preferences.

Phase was represented as circular polar plots to demonstrate interlimb coordination regardless of lead limb or was converted to a linear scale (0.5-1.0) to eliminate any lead limb preferences. When plotted linearly, blue boxes on graphs represented >2 standard deviations (S.D.) as calculated from uninjured control average

and S.D.. Values found within the blue box are considered outside of normal variability and were quantified using pie charts to indicate the percent of total steps that existed outside of that range. Plantar steps are indicated by grey circles during control and red circles during silenced time points. Post-injury dorsal steps were indicated on both circular and linear phase plots as teal circles.

For change in phase calculations, raw interlimb phase (values of 0-1) were calculated for individual steps within a pass. The difference between a step and the subsequent step was deemed to be the change in phase (i.e. phase value of step 2 – phase value of step 1 = phase change). Uninjured control phase change averages and two S.D. were calculated for each limb pair and were used to identify steps that fell outside normal variability post-injury.

BBB assessments.

BBB assessments were performed by trained individuals blinded to experimental time points. Raters were aware that overground stepping was assessed using the BBB Open Field Locomotor Scale as previously described (Basso et al 2002; Caudle et al 2015). Prior to injury, BBB assessments occurred at baseline, pre-Dox, and Dox time points. After SCI, BBB scores were acquired weekly beginning at 7 days post-injury until scores reached plateau (~6 weeks post-SCI). During Dox assessment weeks, BBB testing occurred prior to Dox administration (pre-Dox) and on any days of kinematic testing. The low end of the BBB scale (0–7) is characterized by individual hindlimb joint movements, whereas the intermediate (8–13) and high (14–21) parts of the scale are characterized by weight support, coordination, and paw position. BBB assessments were performed on all kinematic assessment days.

Coordination indices.

Regularity index (RI), central pattern index (CPI), and plantar stepping index (PSI) were used to evaluate gross motor coordination. RI is the number of normal step sequence patterns (NSSP) x4 divided by the number of total cycles. RI excludes any dorsal steps or irregularly patterned steps, indicating a more sensitive measure of irregularity after SCI. CPI is calculated as the number of correctly patterned step cycles divided by the total number of step cycles (Hamers et al 2001, Koopmans et al 2005). CPI includes both dorsal and plantar steps. PSI is the number of hindlimb plantar steps divided by the number of forelimb plantar steps. In an uninjured animal, the ratio of hindlimb to forelimb steps is 1:1, or a PSI score of 1.0 (Magnuson et al 2009). This provides a measure for how well animals can achieve plantar hindlimb stepping in comparison to forelimb stepping after SCI. Dorsal stepping index (DSI) measures the number of dorsal steps in relation to the number of total hindlimb steps taken (both plantar and dorsal).

Spatiotemporal gait indices.

For each individual plantar and dorsal step, temporal and spatial measures were plotted against their instantaneous speed (centimeters/second): swing time (the amount of time the limb is in the air from lift off to initial contact, seconds), stance time (the amount of time the limb is in contact with the ground from initial contact to lift off, seconds), stride time (stance time + swing time, seconds), stride/step frequency (1/stride time), and stride distance (distance traveled per step, centimeters). The relationships were analyzed using line of best and 95% prediction intervals, followed by regression analysis to determine the closeness of fit (R^2). Average swing time, stance time, stride time, and

stride distance were determined using the individual step values independent of speed. The average instantaneous speed was calculated from the instantaneous speeds of each step analyzed. Averages were generated for each animal and were plotted with the combined animal average. To further evaluate the relationships between speed and spatiotemporal measures, Euclidian distance was calculated for each animal (described in detail below). Average swing time, stance time, stride time, and stride distance were determined using the individual step values independent of speed. The average instantaneous speed was calculated from the instantaneous speeds of each step analyzed. Averages were generated for each animal and were plotted with the combined animal average.

Histological analyses.

Animals were killed at two terminal behavioral assessment time points: D3D8 (N=2) to compare with previous studies or D3D20 (N=11) to determine if viral expression and behavioral changes would persist after one week of Dox administration. Animals were overdosed with sodium pentobarbital, followed by pneumothorax and transcardial perfusion with 0.1 M phosphate-buffered saline (PBS) (pH 7.4) followed by 4% paraformaldehyde diluted in PBS. Spinal cords were dissected, post-fixed for 1.5 hours, and transferred to 30% sucrose for a minimum of 4 days at 4C. Spinal segments C5-C8, T8-T12, and T13-L3/L4 were dissected, embedded in tissue freezing medium, and stored at -20C until they were cryosectioned at 30 μ m.

The presence of EGFP.eTeNT in the cervical spinal cord was confirmed immunohistochemically. Milk solution was made using 0.75 mL of 5% Bovine Serum Albumin (BSA), 0.75 g of powdered skim milk, and 14.25 mL of phosphate buffered

saline with Tween 20 (PBST). During this time, slides are heated at 37°C for 30 minutes. This was followed by 10 minutes of hydration in PBS (pH 7.4, room temperature, 60 minutes of blocking solution (90% milk solution and 10% of 10% Normal Donkey Serum (NDS)), another 10 minute hydration in PBS, and overnight incubation in a milk solution containing primary antibodies. The primary antibody milk solution contained a combination of rabbit anti-GFP and either guinea pig anti-synaptophysin, guinea pig anti-vesicular glutamate transporter 1 (VGlut1), guinea pig anti-vesicular glutamate transporter 2 (VGlut2), or goat anti-vesicular GABA transporter (VGAT). For information on primary antibodies, see Table 5.

On Day 2, tissue sections were washed several times, alternating between PBS and 0.1M PBST, followed by a 1 hr incubation with the following secondary antibodies in a dark room: donkey anti-rabbit Alexa-Fluor Plus 488 (1:500), donkey anti-guinea pig Alexa-Fluor 594 (1:200), and donkey anti-goat Alexa-Fluor 594 (1:200) (Table 3). Tissue sections were washed for 10 minutes and fluorescent Nissl (NeuroTrace 640/660 Deep Red, ThermoFisher N21483, dilution of 1:50) was added to the tissue to stain neuronal cell bodies. After a 1-hour fluorescent Nissl incubation and final 2-hour wash, tissue was coverslipped using Fluoromount (Southern Biotechnology Associates, Inc.; Birmingham, AL, USA). The above procedure was repeated on lumbar spinal cord sections. For a negative GFP control, isotype matched IgG with identical protein concentration was used (donkey anti-rabbit IgG; Jackson ImmunoResearch #711-005-152).

To capture fluorescent images of lumbar and cervical sections, an Olympus Fluoview 1000 confocal microscope was used. Tissue sections were viewed with an oil

immersion 100x objective using 488, 543, and 647 lasers (Olympus; PA, USA). Z-stacks ranged from 55-64 slices at 0.45 μm optical steps. Neurons within the intermediate grey matter were imaged for both cervical and lumbar sections. Images were analyzed using Amira software as previously described (Pocratsky et al 2017, 2020).

To examine the spared white matter at the injury epicenter in the thoracic spinal cord, sections were stained with eriochrome cyanine (EC, Magnuson et al 2005). Briefly, slides were allowed to warm at room temperature for 60 minutes before being placed in a hydration gradient consisting of xylenes, ethanol, and distilled water. EC stain was applied to the slides for 10 minutes, followed by 2 short applications of distilled water to remove excess stain (10-15 seconds). Slides were placed in differentiating solution for 30 seconds before air drying overnight in a hood. On Day 2, slides were placed in xylenes solution for 10 minutes and were coverslipped with Permount. Sections were imaged using a Nikon Eclipse E400 light microscope at 10x magnification. Spot Software (v.5.1) was used to format images.

Statistical analyses.

Statistical analyses were performed using the SPSS v22 software package from IBM. Additional references for parametric and non-parametric testing were used in complementation to SPSS (Hays, 1981; Siegel & Castellan, 1988; Ott, L, 1977). Differences between groups were deemed statistically significant at $p \leq 0.05$. Two-tail p-values are reported.

The Binomial Proportion Test was used to detect significant differences in the proportion of coordination values beyond control threshold for the raw and transformed

interlimb coordination data of various limb pairs prior to and post-SCI. It was also used to determine statistical significance for per-step changes in left-right coordination and change in interlimb phase, raw BBB score differences, intralimb phase and per-step changes in intralimb phase, dorsal steps as a percentage of total steps, and percentage of categorically organized steps (anti-phase, out of phase, in phase). Correction for extreme values was used when appropriate.

Circular statistics were performed on the stepping inter- and intralimb coordination datasets, as well as the swimming hindlimb coordination data (Pocratsky et al 2017; Zar 1974). We primarily used the non-parametric two-sample U^2 test based on a previously described rationale (Pocratsky et al 2017, 2020). The null hypothesis tested is whether two time points have the same concentration in couple pattern expression, i.e. values are concentrated around 0.5 and not equally distributed amongst phasic values.

Regression analyses to compare the slopes for the lines of best fit were performed on the speed versus spatiotemporal gait indices datasets, including speed vs. swing time, stance time, stride time, and stride distance. Analyses were performed for hindlimb-hindlimb relationships as well as forelimb-forelimb relationships prior to and after SCI. For regression analyses post-SCI, plantar and dorsal steps were included in the analysis and dorsal steps are shown in blue on graphs for identification. 95% prediction intervals are indicated on the graphs by dashed lines. Dorsal steps were not present for the forelimbs and all forelimb regression analyses were performed on plantar steps only. To further analyze the differences in spatiotemporal measures, Euclidian distance was calculated for each measure. Euclidean distance measures how much 2 variables plotted against one another (one on the x-axis, one on the y-axis) change relative to one another.

Euclidian distance was used to calculate the relationship between the kinematic assessment and speed for the Dox and Control groups. Euclidean distance calculations determined the distance between the two variables for each animal, and those distances were compared between the Control and Dox groups to show if the relationship was the same for the two measures.

Mixed model analysis of variance (ANOVA) followed by Bonferroni *post hoc* t-tests (where appropriate) were used to detect a significant difference in the BBB scores based on sidedness and condition (i.e. Control vs Dox; graph not shown). Repeated measures ANOVA analyses were used to compare number of average steps per animal during uninjured and injured time points.

Paired t-tests were used to detect significant differences in proximal and distal angle excursion for uninjured and injured intralimb coordination, average gross stepping measures including RI, CPI, PSI, and BBB scores, average intralimb phase, percentage of dorsal step sidedness, average swing time, average stance time, average duty cycle, and overall average speed at Control and Dox combined time points.

Two-Sample Kolmogorov-Smirnov test was used to determine the normality of the distribution of steps within gait categories (anti-phase, out-of-phase, in-phase) following SCI.

Results.

Silencing alters hindlimb interlimb coordination while maintaining intralimb coordination and key locomotor features in uninjured animals.

Figure 12 shows a schematic of the experimental design. We performed bilateral injections at C6 and L2 spinal cord segments to doubly infect LAPNs at their terminals

and cell bodies, respectively (Fig 12a). In the presence of Dox, doubly-infected neurons that constitutively express rtTAV16 induce expression of eTeNT. At the level of the cell terminal, active eTeNT prevents synaptic vesicle release, leading to “silenced” neurotransmission (Fig 4c). Removing Dox restores functional neurotransmission, allowing for reversible silencing of LAPNs both before and after SCI (Fig 12b).

To understand how SCI affects locomotion and how silencing modifies these effects, we marked the skin overlying the iliac crest, hip, ankle, and toe (Fig 13a,e) to assess intralimb coordination of the hip (proximal) and ankle (distal) joint angles. In uninjured animals, we observed normal rhythmic excursions of the proximal and distal limb segments (Fig 13b,d,h), as well as coordinated flexor-extensor movements of the proximal and distal joint angles during normal walking passes (Fig 13c,i,j; Video 9). Conditional silencing of LAPNs did not affect the excursion of the proximal joint angle and had slight, but significant effect on the peak-trough excursion of the distal joint angle (Fig 13f,d,h), consistent with Pocratsky et al (2020). Onset times of peak proximal excursion (blue) relative to duration of peak-to-peak distal excursions (purple) were used to calculate intralimb coordination (Fig 13i). Values were plotted on circular graphs on which 0 denotes normal, in-phase coordination of the maximum extension of proximal and distal limb segments (Fig 13j). Silencing LAPNs did not disrupt intralimb coordination of the left or right hindlimbs during stepping (Fig 13k,l), which was maintained bilaterally (Fig 13m, Video 10).

Next, we evaluated whether we could reproduce our previous findings in which silencing LAPNs caused disruptions to the left-right alternation of the hindlimbs, the forelimbs, and the heterolateral hindlimb-forelimb pair. We examined the coupling

patterns of various limb pairs by comparing the temporal relationship, which can be expressed as a phase value. As previously described, phase is determined by dividing the initial contact time of the trailing limb by the stride time of the leading limb. Phase values are typically plotted circularly, with values of 0 or 1 indicating synchrony depending on the lead limb and values of 0.5 indicating alternation of the limb pair. For typically slower gaits such as walk and trot, the phase values of the hindlimbs, forelimbs, and homolateral hindlimb-forelimb pair would be concentrated around 0.5, indicating alternation, while the heterolateral hindlimb-forelimb pair would center around 0 or 1.0, indicating synchrony (Fig 14a_i). These values are reversed for bounding behavior, with synchrony of the hindlimb, forelimb, and homolateral limb pairs and alternation of the heterolateral limb pairs (Fig 14a_{ii}). To eliminate discrepancies between lead limb selection in the animals, phase values were converted from a circular scale (0-1) to a linear scale (0.5-1) (Fig 14b, Pocratsky et al 2017, 2020).

During control timepoints, the hindlimbs maintained phase values concentrated around 0.5, as did the forelimbs. Further, the heterolateral and homolateral hindlimb-forelimb phase relationships trended toward synchrony and alternation, respectively, although with greater variability (Fig 14c-f, grey). We determined the mean phase value of the limb pairs. Any value >2 S.D. from this mean was considered “irregular”, as defined by the blue boxes. Silencing LAPNs led to disruptions in the left-right alternation of the hindlimb and forelimb pairs, with modest changes to the heterolateral hindlimb-forelimb pair (Fig 14c,d,f, red). The homolateral limb pair remained unaffected (Fig 14e, red). The silencing effects on limb pair relationships were restored when Dox was removed from the drinking water (Fig 14c-f, “PostD1”). We

further examined whether silencing uninjured animals would adversely affect the coordination of the limbs in time (temporal measures) and space (spatial measures). Swing time, stance time, stride time, and stride distance comprise well-described gait indices that have been shown to have specific relationships with speed (Gillis & Biewener 2001). Silencing did not affect the fundamental gait relationships of swing time (Fig 14g,l), stance time (Fig 14h,m), stride time (Fig 14i,n), stride frequency (Fig 14j,o), or stride distance (Fig 14k,p). Using Euclidian distance, we see that any differences that manifest between Control and Dox spatiotemporal measures are likely due to higher concentrations of steps at higher speeds (Table 6). Importantly, these findings are similar to previous observations (Pocratsky et al 2020) and suggest that LAPNs secure interlimb coordination with little to no change in intralimb coordination or fundamental gait characteristics in an otherwise intact rat.

Presence of putatively eTeNT-positive synapses in the cervical spinal cord and cell bodies in the lumbar spinal cord.

Neuroanatomical evidence has confirmed the presence of LAPNs from rostral lumbar spinal cord to the cervical region along the ventrolateral funiculus (VLF) (Molenaar and Kuypers, 1978; English et al., 1985; Reed et al., 2006) Lesions to the thoracic VLF in the cat disrupt forelimb–hindlimb coupling (Brustein and Rossignol, 1998), further confirming the location of these ascending propriospinal projections. Given this location in the VLF, it was essential to determine whether LAPNs in the VLF were preserved after SCI. To examine this, animals were euthanized during Dox administration. Their spinal cords were harvested and were blocked in three sections: caudal cervical spinal cord (cell terminals), caudal thoracic spinal cord (injury epicenter

and penumbra), and rostral lumbar spinal cord (cell bodies). Thoracic tissue sections were stained at the injury epicenter using EC to quantify to amount of spared white matter and imaged to determine where that spared white matter was located. Amongst all animals, the average spared white matter was approximately 19% (Fig 15a) and the location of LAPN axons within the VLF was spared (Fig 15b-k). Thus, a proportion of LAPN axons are likely spared after SCI, a critical finding for interpretation of any behavioral assessments moving forward.

Knowing that LAPN axons were likely preserved at the level of injury, we next confirmed that eTeNT.EGFP-expressing putative LAPN axons were present in the caudal cervical spinal cord, as well as eTeNT.EGFP-expressing putative LAPN cell bodies in the intermediate grey matter of the rostral lumbar cord. Histological analysis for EGFP immunoreactivity showed that putatively positive eTeNT.EGFP fibers were found surrounding neuronal processes in the caudal cervical enlargement (Fig 16a,b). Similar to uninjured histology (Pocratsky et al 2020), eTeNT.EGFP co-localized with synaptophysin (Fig 16d,e; synaptic marker), vesicular GABA transporter (Fig 16g, VGAT, inhibitory neurotransmitter), and vesicular glutamate transporter 2 (Fig 16h, VGlut2, excitatory neurotransmitter). Isotype control revealed minimal-to-no immunoreactivity (Fig 16c,f).

Using the same immunohistological protocol as described for cervical spinal tissue, rostral lumbar spinal cord segments were assessed for the presence of eTeNT.EGFP within LAPN soma. Putatively eTeNT.EGFP positive LAPN cell bodies co-localized with fluorescent Nissl stained (NeuroTrace) in the intermediate gray matter (Fig 16j-l, Reed et al 2006; Pocratsky et al 2020). Isotype controls showed no immunoreactivity (Fig 16m-o). Taken together with the cervical spinal cord histology,

these data suggest that double-infected LAPNs maintained expression of eTeNT at the level of cell terminal and the level of the cell soma following SCI. Further, LAPNs were largely spared post-SCI and were functionally silenced, indicating that any behavioral changes seen during Dox administration were concomitant with active eTeNT.EGFP expression.

Silencing LAPNs post-SCI restores coordination indices and improves gross locomotor outcomes.

Having validated that (1) the viral-based silencing system is active post-SCI and (2) LAPN axons are likely intact post-SCI, we explored the effects of LAPN silencing on locomotion after injury. Based on the perturbations to patterned stepping in uninjured animals, we hypothesized that silencing LAPNs after recovery from SCI would result in diminished stepping capacity, such that any spontaneously recovered stepping ability after SCI would be reduced to hindlimb dragging. To assess the overall locomotor function during control and silencing post-SCI, we evaluated the step sequence patterns to determine whether gross coordination indices, regularity index (RI) and coordinated pattern index (CPI), were improved. The RI scores plantar stepping and forelimb-hindlimb coordination and is an excellent measure for animals with mild to moderate injuries where treatment or training may bring about improvements in coordination (Hamers et al 2001; Koopmans et al 2005). CPI takes into account all steps, including plantar and dorsal steps, while RI only accounts for correctly patterned steps and excludes dorsal steps, making it a more sensitive measure with which to quantify coordination after SCI. Fig 17a,b show example step sequence pattern graphs demonstrating large shifts between defined gait patterns of alternation and cruciate

stepping during control stepping and consistent alternating gait patterns during silenced stepping. RI and CPI were both modestly improved during Dox administration, an indicator that stepping was improved as a result of silencing (Fig 17c,d). We also examined the plantar stepping index (PSI), which simply represents a ratio of the number of hindlimb to forelimb plantar steps, was also modestly improved during silencing, further signifying improvements in stepping (Fig 17e). It is important to consider that RI and CPI account for the step sequence pattern of all 4 limbs, while PSI relies on the plantar stepping ratio of the hindlimbs to the forelimbs.

As an added measure, the Basso, Beattie, Bresnahan (BBB) Locomotor Rating Scale was used to evaluate locomotor recovery (Basso et al 2002). Control BBB scores were concentrated between 11-13, consistent with previously described literature for this injury severity (Basso et al 2002; Smith et al 2006). Surprisingly, average BBB scores were modestly increased during silencing (Fig 17f), with a greater proportion of raw scores for each limb between 13-18 (Fig 17g). The increased BBB scores can be attributed to improved weight support of the hindlimbs as well as better coordination, further validating potentially improved stepping during silencing. Important to note, some BBB scores remained concentrated around 13, indicating that not all animals' stepping improved at all Dox time points. However, a significant proportion of BBB scores were greatly increased, suggesting better stepping occurred as a result of silencing. To better assess whether these improvements in coordination indices involved the hindlimb and forelimb girdles together or the hindlimbs alone, we examined more specific intralimb kinematics and interlimb gait analyses.

LAPN silencing leads to modest improvements in intralimb coordination after SCI.

BBB, in particular, includes improvements in both intralimb joint movements and interlimb coordination, with lower scores being linked to joint movements and more precise measures (coordination, paw rotation, etc.) added as the scale increases. Given the improvements seen in gross motor assessments, especially BBB, we next determined whether changes occurred in intralimb coordination, as these changes would be essential to reach higher BBB scores. During control timepoints, proximal and distal joint angle excursions were reduced and intralimb coordination was disrupted such that peaks and troughs were no longer occurring simultaneously (Fig 18a-c). Important to note, some passes were able to maintain intralimb coordination (18ac-). Conditional silencing normalized coordination of the proximal and distal hindlimbs joints during volitional overground locomotion, such that the cyclic properties of each joint were restored (Fig 18d-f). We examined the peak-trough excursion of the proximal and distal angles of the hindlimb as well as the temporal relationship between them during overground locomotion. Peak-trough excursions of both the distal and proximal joint angles only demonstrated modest, though significant, improvements (Fig 18g,h), suggesting that silencing may not be primarily affecting the biomechanics of the hindlimb joints. Next, we explored the temporal relationship between the proximal and distal angles. In uninjured animals, the peak extension of the distal angle typically occurs in-phase with the peak extension of the proximal angle during the stance phase of the step cycle, resulting in a coordination value ranging from 0.9-0.1 on a circular scale, depending on the lead angle. Coordination values from 0.0-0.5 indicate the distal, or ankle, angle peaks first, while phase values from 0.5-1.0 indicate a lead proximal, or hip, angle. These values can be converted to a linear scale, ranging from 0.5-1.0 to eliminate any lead angle

differences (Fig 18i). Following injury, the temporal coordination of these two angles is reduced at control timepoints (Fig 18j,k, grey), indicating that the distal and proximal angles are not reaching peak extension simultaneously and are highly variable in regards to the lead angle. Interestingly, silencing shifted the phase relationship towards values of 1.0 (Fig 18j,k, red), indicating a return to temporal coordination of the limb joints. Taken together, these results suggest that improvements seen during LAPN silencing after SCI are primarily acting on the temporal coordination and temporal stability of the hindlimb joints, while minorly impacting the biomechanics of the hindlimb joints.

Hindlimb-hindlimb, but not hindlimb-forelimb, coupling relationships were restored during post-SCI silencing.

After SCI, the components of the step cycle are altered in a way that prevents each step from achieving plantar placement, resulting in a dorsal step. A dorsal step is classified by the dorsum of the foot coming into contact with the ground during the stance portion of the step cycle and is considered a step if it maintains contact with the surface and completes the swing portion of the step cycle. From the sagittal perspective, a dorsal step does not follow the same biomechanical relationship that a normal plantar step does (Fig 19a). From the ventral view, a dorsal step can be quantified based on the appearance of one or more toes curled or the paw itself oriented such that dorsal surface was observable (Fig 19b, Keller et al 2017). We compared the dorsal stepping index (DSI) during control and silenced timepoints. DSI is a measure of the number of dorsal steps in relation to the number of total hindlimb steps taken (both plantar and dorsal). The DSI accounts for both right and left hindlimb dorsal steps, so it is a useful measure for determining whether the overall number of non-plantar steps is reduced. DSI during

control timepoints was 24.41 ± 16.10 , but was drastically reduced during silencing to 11.21 ± 7.97 indicating a significant reduction in the overall number of dorsal steps (Fig 19c). The sidedness of dorsal steps did not change between control and silencing, with ~60% of dorsal steps occurring in the right hindlimb and ~40% of dorsal steps occurring in the left hindlimb during both control and silenced time points (Fig 19d). This is an important observation, as it indicates that one limb did not improve to a greater extent than the other. For the purposes of simplicity and ease of comparison with pre-injury data, we will focus on left lead limb steps to demonstrate the principle behavioral changes post-SCI.

After SCI, the hindlimbs and the hindlimb-forelimb limb pairs are partially uncoupled, leaving the temporal relationship between limbs highly unstable such that they are unable to maintain their phase relationship on a step-by-step basis. This variability is accounted for, in part, by the presence of dorsal steps. However, many plantar steps also fall into irregular gait patterns after SCI. We included both plantar and dorsal steps for the purposes of quantifying interlimb coordination, with dorsal steps identified as teal throughout the remaining figures. Further, these irregularly patterned steps are present during multiple control and silenced timepoints. Interlimb phase values for plantar and dorsal steps were converted to a linear scale as described in previous figures (Fig 19e). When compared to pre-injury control variability (uninjured control hindlimb 2 S.D.; blue box), hindlimb and heterolateral hindlimb-forelimb phase values were highly variable, with ~33% and ~41% of abnormal steps, respectively (Fig 19f,g,i,j; grey). Silencing drastically improved the left-right hindlimb coupling, reducing the

number of abnormal steps to ~12% (Fig 19g, pink). However, the heterolateral hindlimb-forelimb pair saw no coupling improvement (Fig 19j,k,m,n; red/pink).

We also removed any passes with left dorsal steps and re-examined the interlimb coordination. Interestingly, there was still a significant improvement in hindlimb interlimb coordination in plantar only passes, despite a reduction in the number of abnormally coordinated steps for both control and Dox time points (Fig 19h). The coordination of the heterolateral hindlimb-forelimb pair remained unaltered (Fig 19k). However, it is important to consider that the right dorsal hindlimb steps are still present and may still be affecting the interlimb coordination even after the removal of left dorsal hindlimb steps from the data set. The forelimbs were unaffected and maintained left-right alternation through all control and Dox administrations (Fig 19l).

Three animals were removed from the data set prior to injury as they showed no perturbations to left-right alternation at any pre-injury Dox time point (Fig 20a-i; see Methods for exclusion criteria). Interestingly, these animals did not show improvements in coordinated stepping post-SCI, suggesting that recovery of hindlimb stepping coordination is a silencing-induced perturbation and not improvement by random chance (Fig 20j-l).

Together, these results suggest that any functional gains as a result of LAPN silencing are concentrated below the injury epicenter only at the level of the hindlimb girdle. Silencing had minimal effect on the hindlimb-forelimb relationship and had no effect on the intra-girdle forelimb relationship. Further, the reduced prevalence of dorsal steps during silencing accounted for some, but not all, of the improvement in hindlimb interlimb coordination. This indicates that LAPNs are having a functional impact on

hindlimb coordination without improving the coordination of other limb pairs. Further, they may play a role in hindlimb interlimb coordination regardless of the ability to achieve plantar stepping.

Hindlimb-hindlimb coordination stability was restored during post-SCI silencing.

To further examine the degree of uncoupling and the dynamic coordination of the interlimb temporal relationships, we quantified the raw phase change between steps during single bouts of locomotion. Examples of the per-step changes or transitions are shown during control (Fig 21a_i) and Dox overground locomotion (Fig 21a_{ii}). The direction of shift is irrelevant for interpretation as the primary goal of this analysis was to determine whether there were large shifts towards other gait patterns (i.e. alternation to gallop or synchrony) over the course of a single pass. Interestingly, large shifts were present in hindlimb step-by-step coordination prior to silencing that were alleviated as a result of silencing (Fig 21b), with the percentage of transitions outside of normal variability reduced by ~17% (Fig 21c). When similarly plotted for the homolateral and heterolateral limb pairs, there was minimal reduction in the change of phase (Fig 21d,e). These data largely support improvements in hindlimb-centric interlimb coordination stability during silencing that is independent of the perturbed hindlimb-forelimb relationship.

We also examined whether alternation was the largely preferred gait during silencing post-SCI. For this analysis, coupling was identified as in-phase (phase = 0 ± 0.125), anti-phase (0.5 ± 0.125), or out-of-phase (low coupling: $0.125\text{--}0.375$, high coupling: $0.625\text{--}0.875$). We chose ± 0.125 (or 45°) to equally distribute coupling values among quadrants (Fig 21f, Lemieux et al 2016). There was an increase in anti-phase steps

for both right and left lead limb preferences and decreased out-of-phase and in-phase steps, suggesting a shift towards alternation as the preferred locomotor pattern (Fig 21g). When plotted as percentage of total steps regardless of lead limb, the number of in-phase steps was reduced in the hindlimbs (Fig 21h_i) while variability remained in the hindlimb-forelimb pair (Fig 21h_{ii}). Together, these data suggest that hindlimb-hindlimb coordination and hindlimb-forelimb coordination are fundamentally disrupted after SCI, and removing the LAPNs from the inter-enlargement circuitry has a profound positive effect on patterned stepping as well as the stability of that coordination on a step-by-step basis.

Key features of locomotion are restored during post-SCI LAPN silencing.

In the uninjured animal, silencing did not affect the fundamental relationships between speed and spatiotemporal features of limb movements (e.g. swing time, stance time, etc.). As previously described in Chapter II, these parameters change in relation to speed in a stereotypic and well-characterized manner (Gorska et al 1998, 1999; Hruska et al 1979). To determine whether the silencing effected these relationships, we plotted swing time, stance time, and stride distance in relation to speed. These relationships are disrupted as a result of SCI, with a number of individual plantar and dorsal steps falling outside the typically described relationships (Fig 22a-c). Interestingly, measures that are typically associated with locomotor rhythm (stride time and stride frequency) were also disrupted (Fig 22d,e), though the abnormal steps were typically associated with a dorsal step, which we have already shown to fundamentally alter interlimb coordination. Silencing LAPNs restored these relationships and resulted in a dramatic reduction in the variability of abnormal steps, regardless of dorsal or plantar stepping (Fig 22f-j).

Calculated Euclidian distance supports this improvement, as Euclidian distance is significantly improved and approaches pre-injury Control and Dox values for each spatiotemporal measure (Fig 22k-o). Average swing time (Fig 22p) and average stance time (Fig 22q) are improved, with swing time showing the greater improvement. Interestingly, the ratio of stance time to total stride time, or duty cycle, was unchanged after silencing (Fig 22r), suggesting that the improvements in the salient features of locomotion were not as a result of changes in one aspect of the step cycle. Rather, the ratio between stance time and swing time are maintained. Finally, the average speed amongst animals was unchanged during post-injury silencing (Fig 22s) indicating that increasing speed was not a primary factor in the improved spatiotemporal relationships. Together with the previous findings, these data suggest that silencing LAPNs after SCI is largely impacting the temporal coordination of multiple aspects of locomotion, primarily interlimb coordination. Importantly, intralimb coordination and step cycle spatiotemporal relationships were also improved, leading to an overall improved ability to step during silencing after SCI.

Discussion.

Current results are consistent with our previous findings that, in the otherwise intact system, LAPNs play an important role in securing interlimb coordination of both the forelimbs and hindlimbs in a context specific manner (Pocratsky et al 2020). Unexpectedly, we found that silencing this spared pathway post-SCI improved locomotor function, suggesting that spared pathways may deleteriously influence recovered locomotor function. Our results demonstrate modest but meaningful improvements in intralimb coordination of the hindlimbs when LAPNs are silenced, concomitant with

robust improvements in hindlimb interlimb coordination, including paw placement order and timing, and speed-dependent gait indices. These improvements occurred in the absence of improvements in hindlimb-forelimb coordination.

Taken together, these findings suggest that LAPNs interfere with the afferent feedback of stepping following a thoracic SCI, perhaps through maladaptive gain from propriospinal feedback that leads to inaccurate interpretation by lumbar pattern circuitry. Post-injury, the information carried by the LAPNs between the girdles is no longer capable of reliably communicating temporal information at both the hindlimb and forelimb girdles. Rather than securing alternation of the hindlimbs and forelimbs, spared LAPNs hinder the capability of intralimb and interlimb lumbar circuitry below the level of lesion to function appropriately, contributing to diminished stepping capacity at chronic time points. The mechanism through which this is occurring remains unclear. Silencing LAPNs may be acting to remove erroneous error signal within the locomotor system, thereby increasing the ability of intrinsic lumbar circuitry to function independently. Alternatively, maladaptive plasticity may be occurring below the contusion site, leading to detrimental interactions between LAPN input and CPG circuitry.

These results are distinctly counter-intuitive. An anatomically-spared inter-enlargement pathway should aid in recovery and participate in recovered locomotor function. In fact, these results potentially contradict a number of studies in the field of locomotor recovery in which various populations of descending propriospinal relays of both thoracic and cervical origin are seen as an essential component of recovered stepping ability (Bareyre et al 2004; Vavrek et al 2006; Flynn et al 2011; Courtine et al

2008; Cowley et al 2008). However, the descending populations of neurons described in those studies are likely better suited anatomically to relay descending motor commands from higher cortical structures to lumbar circuitry than LAPNs. However, these data raise important questions as to whether select spared pathways post-SCI are beneficial for recovery of function.

To better understand these perplexing observations, we must look outside the current literature available for propriospinal involvement after SCI. Interpreting current results requires a thorough examination of spinal cord transection. Essentially, a transection eliminates all descending and ascending information between intrinsic lumbar circuitry and descending cortical and brainstem centers. Conditional silencing LAPNs “removes” circuitry from the spinal cord in a manner similar to a transection in the sense that information that would typically cross the level of lesion is no longer able to do so. Interestingly, animals are able to regain weight-supported stepping after full spinal cord transection, indicating the presence of functional autonomous circuitry below the level of lesion that is capable of governing locomotion without interference from inter-enlargement CPG pathways (Lovely et al 1986). Recovery of locomotion following a partial spinal cord lesion often requires longer periods of time, with less success than stepping that follows full transections alone (Cohen-Adad et al 2014). Animals that receive a partial contusive spinal cord lesion with locomotor training followed by a complete transection are more likely to recover bilateral, symmetrical locomotion than those with a partial lesion only, suggesting that the recovery of the hindlimb locomotor pattern likely results from changes to the intrinsic CPG circuitry and its interpretation of afferent input at the lumbar level (Singh et al 2011; Barriere et al 2008). The change in

the role of LAPNs after SCI may be due, in part, to this functional remodeling below the level of lesion.

Another interesting aspect to consider is the loss of influence of LAPNs on forelimb circuitry post-injury, suggesting that their primary role is now acting solely upon intact lumbar circuitry, a substantial departure from their pre-injury role. The influence of ascending circuitry after SCI may be drastically reduced in the presence of increased supraspinal drive. When hindlimb function is diminished as a result of injury, forelimbs become the primary means by which to navigate the environment. Essentially, there is inherent risk in the forelimbs transitioning from alternation to other gait patterns, as instability would result in loss of body posture and balance. Computational modeling could provide some insight into this conundrum as it would contribute to improved understanding of the drive between the hindlimbs and the forelimbs post-SCI.

Current literature has only provided a basic understanding of LAPN anatomy in the intact spinal cord. Therefore, it is unclear whether anatomical changes to LAPNs at either the level of the cell bodies in the lumbar cord or at the level of the cell terminals in the cervical cord are contributing to altered behavior after SCI. Further exploration of the anatomical make-up of this population of neurons will be essential moving forward to understand improvements seen during LAPN silencing. Another point of interest will be the continued exploration of plasticity as a result of silencing techniques as improvements in locomotor recovery during silencing were continuous for 20 days. Determining whether plasticity or any anatomical changes in the LAPN population play a role in improved stepping behaviors during or after silencing will further contribute to our understanding of these perplexing outcomes.

Alternatively, LAPNs may be unaffected by this remodeling and any behavioral changes as a result of silencing could be due to the misinterpretation of incoming afferent information from the hindlimbs, leading to detriments in locomotor pattern expression. *In vitro* studies suggest that the lumbar and cervical enlargements are capable of producing locomotor-like rhythms in the absence of inter-enlargement circuitry and that the lumbar circuitry has a greater drive on cervical circuitry than its reciprocal pathway (Juvin et al 2005, 2012; Ballion et al 2001). If LAPNs are misinterpreting sensory information from the hindlimb or are communicating disruptive information at the level of the lumbar locomotor CPG, removing them from the CPG circuitry could allow autonomous correction of the intrinsic patterning within the lumbar cord.

Clinically, the use of epidural stimulation is a widespread technique to improve locomotor outcomes in chronic SCI patients (Angeli et al 2014, 2018; Harkema et al 2011). In large part, the mechanism that governs this recovery is unknown. It would seem that our findings directly contradict the improvements described in clinical literature. However, only some patients ever regain the ability to volitionally walk overground, suggesting that there are fundamental differences from patient to patient in regards to remaining spared pathways (Angeli et al 2018). It is critical to consider the presence of some spared pathways, such as this distinct population of LAPNs, as maladaptive to locomotor improvement in humans as it may be creating a ceiling effect for human locomotor recovery. Alternatively, a prosthetic that provides descending drive that could specifically and adaptively attune to propriospinal activity might dramatically improve locomotion in the human condition. Stimulating beneficial pathways while selectively silencing other dysfunctional pathways may act to heighten the ceiling producing greater

locomotor recovery in clinical cases. Continued work into the importance of ascending propriospinal pathways will be essential to our continued growth in the field of locomotor recovery post-SCI.

Figure 12. Experimental design to reversibly silence LAPNs in the adult rat

following SCI. (a) Bilateral injections of eTeNT (green) and rtTAV16 (yellow) were performed at C6 and L2 spinal cord levels, respectively, followed by a SCI (black star). Administration of doxycycline (Dox, red boxes) induces eTeNT expression in doubly-infected neurons. eTeNT is transported to the cell terminals where it prevents synaptic vesicle release into the synaptic cleft, effectively silencing neurotransmission in the targeted neuronal population. Following viral injections (i) and (ii), pre-injury behavioral assessments were taken at three control time points and a single round of Dox administration. Following SCI (iii, black star), behavioral assessments were repeated at two control time points and during two rounds of Dox administration (grey boxes) (b).

Figure 13. LAPNs do not contribute to intralimb locomotor coordination of uninjured animals. Representative images of intralimb joint angles of the proximal (iliac crest–hip–ankle) and distal (hip–ankle–toe) joints during Control (a) and Dox (e) timepoints. Example images are taken from 3 moments within a step cycle: peak extension, peak flexion, and mid stance. Two-dimensional stick figures of hindlimb stepping (b,f) and angle-angle plots (c,g) from the same passes shown in (a,e). The average excursions of the proximal angles (d, Control right: 58.79 ± 4.94 vs Dox right: 56.56 ± 3.12 , $t=2.08$, $df=11$, $p=.062$; Control left: 60.40 ± 4.04 vs Dox left: 58.65 ± 4.23 , $t=2.17$, $df=11$, $p=.053$; paired t-tests) and distal angles (h, Control right: 55.84 ± 4.93 vs Dox right: 65.57 ± 8.46 , $t=4.55$, $df=11$, $p=.001$; Control left: 56.19 ± 4.24 vs Dox left: 66.64 ± 7.16 , $t=6.63$, $df=11$, $p<.001$; paired t-tests; center bars indicate mean with extending bars indicating range of data) for control and silenced timepoints are shown.

We examined the temporal relationship between the peak excursion of each angle at control and Dox timepoints using excursion traces of the proximal and distal joint angles, indicated by the blue and purple lines, respectively (**I,k**). Grey boxes indicate stance portions of the step cycle. Phase between angles was calculated and plotted on a scale from 0/1 with 1 indicating in-phase coordination of the angles (**j,I**). Average phase values were calculated for the left and right rear limbs at all uninjured Control (grey) and Dox (red) timepoints and were found to have slightly decreased (**m**, Control right: $.956 \pm .012$ vs Dox right: $.945 \pm .009$, $t=2.73$, $df=11$, $p=.019$; Control left: $.947 \pm .013$ vs Dox left: $.933 \pm .018$, $t=3.31$, $df=11$, $p=.007$; paired t-tests; bars=group mean $\pm$ S.D.).

Figure 14. Silencing LAPNs disrupts interlimb coordination without affecting key features of locomotion. Representative foot fall graphs are shown with corresponding coordination phase values for both Control and Dox timepoints (**a**). Phase value were converted to a scale of 0-1 to 0.5-1 for the hindlimb, forelimb, and homolateral hindlimb-forelimb limb pairs and 0-0.5 for the heterolateral hindlimb-forelimb pair so they could be further examined on a linear scale (**b**). The hindlimb and forelimb limb pairs were

significantly altered during LAPN silencing, while the heterolateral and homolateral hindlimb-forelimb limb pairs remained unaffected (**c-f**, # steps beyond control variability: PD1 hindlimbs $n=7/168$ [4.17%] vs D1D5 hindlimbs $n=14/166$ [8.43%]; n.s., $z=1.62$; PD1 hindlimbs $n=7/168$ [4.17%] vs D1D8 hindlimbs $n=72/161$ [44.72%]; $p<.001$, $z=9.63$; PD1 heterolateral $n=8/168$ [4.76%] vs D1D5 heterolateral $n=1/166$ [0.60%]; $p<.05$, $z=2.38$; PD1 heterolateral $n=8/168$ [4.76%] vs D1D8 heterolateral $n=21/161$ [13.04%]; $p<.01$, $z=2.65$; PD1 homolateral $n=7/168$ [4.17%] vs D1D5 homolateral $n=3/161$ [1.80%]; n.s., $z=1.27$; PD1 homolateral $n=7/168$ [4.17%] vs D1D8 homolateral $n=4/161$ [2.48%]; n.s., $z=0.85$; PD1 forelimbs $n=14/168$ [8.33%] vs D1D5 forelimbs $n=12/166$ [7.23%]; n.s., $z=0.38$; PD1 forelimbs $n=14/168$ [8.33%] vs D1D8 forelimbs $n=35/161$ [21.74%]; $p=.001$, $z=3.45$; Binomial Proportion Test; circles=individual step cycles; shaded region=values beyond control variability). Spatiotemporal measures (swing time, stance time, stride time, stride distance) were plotted against speed for Control (**g-j**) and Dox (**k-n**) timepoints. An exponential decay line of best fit is displayed for stance time and stride time graphs (stance time: Control $R^2=.785$ vs Dox $R^2=.735$; stride time: Control $R^2=.708$ vs Dox $R^2=.667$), while a linear line of best fit is displayed for stride distance (stride distance: Control $R^2=.584$ vs Dox $R^2=.513$; line of best fit indicated by dotted line). 95% prediction intervals are also shown as solid lines.

Figure 15. White matter sparing at injury epicenter. The percentage of spared white matter at the epicenter ranges from 13.01% – 34.64% (a). White matter damage at the spinal cord injury epicenter as confirmed by histology (b-j). Individual images represent the injury epicenter of each animal used in the main data set (N=10; average white matter percentage: 19.02%, S.D. 6.44%).

Figure 16. Presence of eTeNT-EGFP in putatively silenced LAPNs across the level of injury. High magnification, volume-rendered images demonstrating eTeNT.EGFP putatively positive fibers (green) surrounding NeuN stained neurons (red) with Hoechst nuclear counterstain (blue) in cervical spinal cord segments of interest (**a,b**) (100x magnification, C6-C7 spinal cord). White arrows indicate areas of colocalization. Isotype control reveals minimal immunoreactivity (**c**, IgG controls for eTeNT.EGFP shown).

eTeNT.EGFP (green) signal co-localizes with neuronal processes (blue) and synaptophysin (red) (**d,e**). Colocalizations are shown for specific positions and are indicated by numbers. Isotype controls further show minimal reactivity (**f**, IgG controls of synaptophysin and eTeNT.EGFP shown). eTeNT.EGFP also colocalizes with the inhibitory neurotransmitter marker vesicular GABA transporter (**g**, VGAT, red), and the excitatory neurotransmitter vesicular glutamate transporter 2 (**h**, VGlut2, red) and vesicular glutamate transporter 1 (**i**, VGlut1, red). eTeNT.EGFP putative cell bodies (green) in the lumbar spinal cord colocalized with NeuroTrace fluorescent Nissl stained neurons (blue) (**j-l**, L1-L2 spinal cord). Minimal presence of eTeNT.EGFP signal in isotype controls (**m-o**, L1-L2 spinal cord).

Figure 17. Silencing LAPNs post-SCI restores coordination indices and improves gross locomotor outcomes. Footfall patterns are shown from example stepping passes to demonstrate how gait indices such as regularity index (RI) and central pattern index (CPI) differed between Control (a) and Dox (b) stepping. The average RI scores (c, Control RI: 65.74 ± 18.52 vs Dox RI: 76.97 ± 7.70 , $t=2.88$, $df=9$, $p=.018$; paired t-test), CPI scores (d, Control CPI: 78.37 ± 8.39 vs Dox CPI: 84.84 ± 3.39 , $t=2.94$, $df=9$, $p=.016$; paired t-test), and PSI scores (e, Control PSI: 82.72 ± 9.97 vs Dox PSI: 89.07 ± 4.87 , $t=2.76$, $df=9$, $p=.022$; paired t-test) are demonstrated with individual animal averages (grey circles and red circles for Control and Dox, respectively). Average BBB scores for Control and Dox timepoints are shown (f, group average $\pm$ S.D. [Control to Dox]; $p=.663$, mixed model ANOVA, Bonferroni *post hoc*). No significant difference was found between right and left BBB scores so they were combined for average and raw score ([Left vs Right];

p=.001, mixed model ANOVA, Bonferroni *post hoc*; data not shown). Right and left hindlimb raw BBB scores are shown in (g, Control: n=1/40 [0.025%] vs Dox: n=18/76 [23.68%]; p<.001, z=4.23; Binomial Proportion Test; circles=individual left or right BBB scores; shaded region=values beyond control variability).

Figure 18. LAPN silencing leads to modest improvements in intralimb coordination after SCI. Excursion traces of the proximal (purple) and distal (blue) joint angles during Control (a) and Dox (d) timepoints are shown. Two-dimensional stick figures of hindlimb stepping (b,e) and angle-angle plots (c,f) from the same passes shown in (a,d). Toe height throughout the pass is indicated as a blue trace on the bottom of the two-dimensional stick figures in b and e. Minor improvements in distal joint angle excursion (g, Control right: 75.29 ± 8.73 vs Dox right: 83.40 ± 6.99 , $t=2.70$, $df=9$, $p=.024$; Control left: 74.62 ± 11.96 vs Dox left: 85.74 ± 7.24 , $t=3.35$, $df=9$, $p=.009$; paired t-tests) and proximal joint angle excursion (h, Control right: 46.45 ± 5.58 vs Dox right: 49.37 ± 3.80 , $t=2.93$, $df=9$, $p=.017$; Control left: 47.64 ± 7.33 vs Dox left: 51.47 ± 4.33 , $t=2.65$, $df=9$, $p=.027$;

paired t-tests; middle bar indicates group average with extension bars indicating range of raw data) are demonstrated as a result of silencing. Intralimb phase values relating the peak of one angle to the peak of the other are converted from a scale of 0-1 to 0.5-1.0 to be viewed linearly (**i**). The intralimb coordination values for the right hindlimb and the left hindlimb (**j,k**: Control right: n=78/182 [42.86%] vs Dox right: n=38/155 [24.52%]; $p < .001$, $z = 3.64$; B.P. test; Control left: n=62/189 [32.80%] vs Dox left: n=31/244 [12.70%]; $p < .001$, $z = 4.99$; Binomial Proportion Test; circles=individual step cycles; shaded region=values beyond control variability) are plotted for Control and Dox timepoints.

Figure 19. Hindlimb, but not hindlimb-forelimb, coupling relationships are restored during post-SCI silencing. Example plantar steps and dorsal steps are seen from the sagittal view (a) with an example pass with multiple dorsal and plantar steps shown in (b). Dorsal stepping index (DSI) is shown in (c, Control DSI: 24.41 ± 14.69 vs Dox DSI: 11.75 ± 5.79 ; $t=3.383$, $df=7$, $p=.012$). Dorsal stepping index accounts for total dorsal steps for both left and right hindlimbs. The total dorsal steps from (c) were separated based on sidedness: right hindlimb (RHL) and left hindlimb (LHL) (d, Control right: 0.568 ± 0.247 vs Dox right: 0.605 ± 0.238 , $t=.554$, $df=7$, $p=.597$; Control left: 0.432 ± 0.247 vs Dox left: 0.395 ± 0.238 , $t=.554$, $df=7$, $p=.597$; paired t-tests). The right hindlimb showed more

dorsal steps overall; however, it maintained that percentage during Dox. No significant differences were seen in sidedness. As with uninjured data, circular phase was transformed from a scale of 0-1 to 0.5-1.0 to eliminate lead limb preferences (**e**). Transformed phase values for hindlimbs (**f**, Control hindlimbs with dorsal steps: $n=91/288$ [31.60%] vs Dox hindlimbs with dorsal steps: $n=62/514$ [12.06%]; $p<.001$, $z=7.05$) and heterolateral hindlimb-forelimb pairs (**i**, Control heterolateral limbs with dorsal steps: $n=94/288$ [32.64%] vs Dox heterolateral limbs with dorsal steps: $n=160/514$ [31.13%]; n.s., $z=.44$, Binomial Proportions tests) are shown. Plantar steps are indicated by grey circles (Control) and red circles (Dox), while dorsal steps are indicated by teal circles for both Control and Dox datasets. The blue boxes indicate values outside of normal variability for the specified uninjured limb pair mean. The percentage of abnormal steps found above normal variability is calculated for their respective limb pairs (**g,j**: statistics as shown above, B.P test). Passes with any left hindlimb dorsal steps were removed and plotted for each of the aforementioned limb pairs (**h,k**: Control hindlimbs without dorsal steps: $n=33/157$ [21.02%] vs Dox hindlimbs without dorsal steps: $n=39/397$ [9.82%]; $p<.005$, $z=3.13$; Control heterolateral limbs without dorsal steps: $n=59/157$ [37.58%] vs Dox heterolateral limbs without dorsal steps: $n=142/397$ [35.77%]; n.s., $z=0.4$; B.P. tests). For the forelimb pair, no significant differences were seen between Control and Dox phase during the first post-injury Dox administration (**l**; PD2 forelimbs: $n=7/143$ [4.90%] vs D2D5 forelimbs: $n=4/137$ [2.92%]; n.s., $z=.86$; PD2 forelimbs: $n=7/143$ [4.90%] vs D2D8 forelimbs: $n=4/133$ [3.00%]; n.s., $z=.81$, B.P. tests). Significance was detected between Control and Dox at the D1D8 time point during the second Dox administration, but no significance was found at the extended Dox time point

(D3D20; PD3 forelimbs: $n=14/145$ [9.66%] vs D3D8 forelimbs: $n=2/135$ [1.48%]; $p<.05$, $z=2.94$; PD3 forelimbs: $n=14/145$ [9.66%] vs D3D20 forelimbs: $n=4/109$ [3.67%]; n.s., $z=1.84$, B.P. tests).

Figure 20. Animals excluded based on lack of behavioral outcomes pre-injury show no improvements in hindlimb coupling post-injury.

N=3 animals were excluded from post-injury group data based on lack of behavioral phenotype at any time point during pre-injury silencing. No disruptions were seen in hindlimb-hindlimb (**a**, Control hindlimbs: n=5/160 [3.13%] vs Dox hindlimbs: n=0/110 [0%]; n.s., z=1.87), heterolateral hindlimb-forelimb (**b**: Control heterolateral: n=3/160 [1.88%] vs Dox heterolateral: n=3/110 [2.72%]; n.s., z=.45), homolateral hindlimb-forelimb (**c**; Control homolateral: n=2/160 [1.25%] vs Dox homolateral: n=3/110 [2.72%]; n.s., z=.83), or forelimb-forelimb (data not shown) phase values during silencing. No changes were seen in relationships between swing time (**d,g**), stance time (**e,h**), or stride distance (**f,i**) and swing time prior to injury. After SCI, improvements were not seen in hindlimb-hindlimb phase (**j**: Control hindlimbs: n=30/87 [34.48%] vs Dox hindlimbs: n=24/87 [27.57%]; n.s., z=.99) and similar disruptions in heterolateral hindlimb-forelimb (**k**: Control heterolateral: n=43/87 [49.42%] vs Dox heterolateral: n=38/87 [43.68%]; n.s., z=.76) and homolateral hindlimb-forelimb (**l**, Control homolateral: n=41/87 [47.12%] vs Dox homolateral: n=33/87 [37.93%]; n.s., z=1.23) pairs were unchanged during silencing. Lack of improvement was seen in spatiotemporal measures during silencing (**m-o**, **p-r**). Control plantar steps = grey circles, Dox plantar steps = red circles. All dorsal steps are shown as teal circles.

Figure 21. Hindlimb-hindlimb coordination stability was restored during post-SCI silencing.

The step-by-step change in hindlimb coordination was calculated using the difference between the raw phase value within a given pass. Examples of Control (**a_i**) and Dox (**a_{ii}**) hindlimb interlimb coordination are shown. The change in raw phase values is shown for the hindlimbs and the heterolateral hindlimb-forelimb pairs (**b,d**: Control hindlimb phase change: $n=67/182$ [36.81%] vs Dox hindlimb phase change: $n=60/301$ [19.93%]; $p<.001$, $z=3.97$; Control heterolateral limb pair phase change: $n=83/182$ [45.60%] vs Dox heterolateral limb pair phase change: $n=113/301$ [37.54%]; $n.s.$, $z=1.74$, B.P. tests). Each raw phase change value is defined as a transition with the percentage of abnormal transitions or phase changes shown as the yellow portion of the pie chart. The percentage of transitions outside normal variability are shown for each of the limb pairs previously described (**c,e**: statistics based on percentages described for b, d). To look at lead-limb differences, raw phase values were categorized as in-phase (green, 0.875-0.125), out-of-phase (orange; 0.125-0.375, 0.625-0.875), and anti-phase (pink, 0.375-

0.625) with values ranging from 0-0.5 indicating right lead limb and values from 0.5-1.0 indicating left lead limb (**f**). The relative frequency of hindlimb steps for both Control and Dox are shown and the percentage of steps within each gait category for each of the aforementioned limb pairs is demonstrated regardless of lead limb (**g,h**, Control hindlimb non-anti-phase proportion: $n=91/288$ [31.60%] vs Dox hindlimb non-anti-phase proportion: $n=67/514$ [13.03%]; $p<.001$, $z=6.03$; Control homolateral limb pair non-anti-phase proportion: $n=187/288$ [64.93%] vs Dox homolateral limb pair non-anti-phase proportion: $n=310/514$ [60.31%]; n.s., $z=1.3$; Control heterolateral limb pair non-anti-phase proportion: $n=243/288$ [84.37%] vs Dox heterolateral limb pair non-anti-phase proportion: $n=446/514$ [86.77%]; n.s., $z=0.92$, B.P. tests).

Figure 22. Key features of locomotion are restored following post-SCI LAPN silencing.

Relationships between swing time, stance time, stride time, and stride distance are plotted against speed for Control (a-e) and Dox (f-j) time points. Dorsal steps are indicated with teal circles for both Control and Dox, while plantar steps are indicated with either grey or red circles for Control and Dox, respectively. An exponential decay line of best fit is displayed for stance time and stride time graphs, while a linear line of best fit is displayed for stride distance (dotted line indicates line of best fit; Stance time:

Control $R^2=0.433$ vs Dox $R^2=0.656$; Stride time: Control $R^2=0.351$ vs Dox $R^2=0.516$; Stride distance: Control $R^2=0.124$ vs Dox $R^2=0.367$). 95% prediction intervals are shown for lines of best fit as solid lines. To calculate Euclidian Distance, each step's speed was multiplied by its spatiotemporal measure (i.e. speed multiplied by swing time) to create a single variable. Euclidean distance calculations measured that distance between the two variables for each animal. Those differences were compared between Control and Dox groups to show if the relationship was the same for the 2 measures (**k-o**; Euclidian Distance swing time vs speed: Control 305.6 ± 47.2 vs Dox 388.8 ± 69.4 , $t=6.074$, $df=9$, $p<.001$; Euclidian Distance stance time vs speed: Control 305.1 ± 47.3 vs Dox 388.1 ± 69.5 , $t=6.057$, $df=9$, $p<.001$; Euclidian Distance stride time vs speed: Control 304.6 ± 47.4 vs Dox 387.4 ± 69.5 , $t=6.043$, $df=9$, $p<.001$; Euclidian Distance stride distance vs speed: Control 230.7 ± 43.3 vs Dox 275.3 ± 59.4 , $t=6.074$, $df=9$, $p<.005$; Euclidian Distance stride frequency vs speed: Control 237.6 ± 43.0 vs Dox 319.8 ± 64.2 , $t=6.074$, $df=9$, $p<.001$; paired t-test). Average swing time (**p**, Control swing time 0.087 ± 0.016 vs Dox swing time: 0.106 ± 0.012 , $t=7.062$, $df=9$, $p<.001$, paired t-test) and average stance time (**q**, Control stance time $.196 \pm .038$ vs Dox stance time: 0.213 ± 0.031 , $t=4.994$, $df=9$, $p=.001$, paired t-test) are indicated with circles representing individual animal averages. The average duty cycle (stance time/stride time) (**r**, Control duty cycle 0.679 ± 0.028 vs Dox duty cycle: 0.663 ± 0.030 , $t=2.678$, $df=9$, $p=.025$, paired t-test, likely significant due to tightness of data) and average speed (**s**, Control speed 55.17 ± 9.20 vs Dox speed: 52.70 ± 7.47 , $t=1.789$, $df=9$, $p=.107$, paired t-test) are plotted for Control (grey) and Dox (red) time points with averages indicated by bars. 1 S.D. is shown.

Antibody	Species	Dilution	Company (Catalog #)
GFP	Rabbit	1:3500	abcam (ab290)
VGAT	Goat	1:100	Frontier Institute (VGAT-Go-Af620)
VGlut1	Guinea Pig	1:500	Synaptic Systems (135-304)
VGlut2	Guinea Pig	1:250	Synaptic Systems (135-404)
Synaptophysin	Guinea Pig	1:750	Synaptic Systems (101-004)
Neurotrace 640/660 Fluorescent Nissl	None	1:50	Fisher Scientific (2047616)
Anti-Rabbit Alexa Fluor Plus 488	Donkey	1:400	Invitrogen (A32790)
Anti-Guinea Pig Alexa Fluor 594	Donkey	1:200	Jackson Immuno Research (AB_2337442)
Anti-Goat Alexa Fluor 594	Donkey	1:200	Jackson Immuno Research (AB_2340432)

Table 4. Immunohistochemistry antibodies. Antibodies used during IHC analysis for Chapter III and Chapter IV staining.

	Uninjured				Injured			
Variable	Control	Dox	p-value	Sig	Control	Dox	p-value	Sig
Swing time vs speed	461.9 ± 63.4	395.3 ± 52.7	.0014	p<.005	305.6 ± 47.2	388.8 ± 69.4	.0002	p<.001
Stance time vs speed	461.8 ± 63.5	395.4 ± 52.8	.0014	p<.005	305.6 ± 47.3	388.1 ± 69.5	.0002	p<.001
Stride time vs speed	461.1 ± 63.5	394.6 ± 52.8	.0014	p<.005	304.6 ± 47.3	387.4 ± 69.5	.0002	p<.001
Stride frequency vs speed	414.2 ± 57.6	354.2 ± 48.5	.0013	p<.005	237.6 ± 43.0	319.8 ± 64.2	.0003	p<.001
Stride distance vs speed	350.9 ± 59.0	293.4 ± 49.3	.0018	p<.005	230.7 ± 43.3	275.3 ± 59.4	.0028	p<.005

Table 5. Euclidian distance for uninjured spatiotemporal measures during pre-injury LAPN silencing. Euclidian distances calculations are shown for LAPN pre-injury and post-injury spatiotemporal measures. Averages and one S.D. are shown for each variable. Paired t-tests were used to calculate statistical differences in the averages, indicated by p-values.

CHAPTER IV

SILENCING LONG DESCENDING INTER-ENLARGEMENT PROPRIOSPINAL NEURONS AFTER SPINAL CORD INJURY MODESTLY IMPROVES HINDLIMB STEPPING ABILITY IN ADULT RATS

Introduction.

Locomotion is a universal and robust behavior shared by almost all animals. In mammals, locomotion involves descending commands from supraspinal centers and peripheral input from sensory systems converging on spinal locomotor circuitry. The spinal locomotor circuitry includes central pattern generators or CPGs, first described by Thomas Graham Brown, as the intrinsic spinal networks capable of generating the coordinated muscle activity associated with stereotypic fore and hindlimb movements during stepping (Graham Brown, 1911). It is now believed that each limb has its own CPG, and that the lumbar and cervical pattern generators are interconnected by a propriospinal system comprised of subsets of spinal interneurons with axonal projections that extend between the cervical and lumbar enlargements (Graham Brown, 1911; Reed et al 2006; Brockett et al 2013). Of interest are long ascending propriospinal neurons (LAPNs) and long descending propriospinal neurons (LDPNs) that provide the functional coupling of the two enlargements allowing precise temporal information to be passed between and among the hindlimb and forelimb CPGs (Giovannelli and Kuypers, 1969; Miller *et al.*, 1975; English, 1979; Rossignol *et al.*

1993; Juven *et al.*, 2005; Juven *et al.*, 2012). This intraspinal network is also important for propagation of supraspinal signals (Cowley *et al* 2008), interlimb coordination of the hindlimbs and forelimbs (Lloyd & McIntyre 1948; Miller *et al* 1973; Ballion *et al* 2001; Pocratsky *et al* 2020), and sensorimotor integration (Alstermark & Isa 2012) in uninjured system.

LDPNs, a large subset of the spinal inter-enlargement circuitry with cell bodies in the cervical enlargement and projections to the lumbar enlargement, are the current subject of anatomical and molecular investigation due to their potential involvement in coordinated patterned behaviors such as locomotion (Flynn *et al* 2017; Reed *et al* 2006; Brockett *et al* 2013; Ni *et al* 2014), as well as their potential for promoting functional recovery from spinal cord injury (SCI) via collateral sprouting and formation of a de novo bridge to bypass the lesion epicenter (Bareyre *et al.*, 2004; Vavrek *et al.*, 2006; Flynn *et al.*, 2011; Filli *et al.*, 2014; Benthall *et al.*, 2017). It is well-known that SCI disrupts the communication between the brain and spinal cord, resulting in an immediate inability to initiate and maintain patterned weight-supported locomotion at or below the level of lesion (Dietz and Harkema, 2004; Fong *et al.*, 2009; Côté *et al.*, 2017). Even if classified as neurologically complete, most SCIs are anatomically incomplete as there is some sparing of white matter at the lesion epicenter, most often the outermost rim of the lateral and ventrolateral funiculi where the LDPN axons reside. Therefore, these neurons and their axons may comprise a percentage of the anatomically spared circuitry, thus providing a potential functional bridge across the injury site. Due to these anatomical characteristics, their resiliency to cell death following incomplete SCI, and their known function in intact animals, these neurons are well-suited to participate in locomotor

recovery after incomplete SCI (Conta and Stelzner, 2004; Conta Steencken and Stelzner, 2010, 2011; Siebert et al., 2010).

Previous work targeted LAPNs before and after injury. This work revealed that the LAPNs secure left-right coordination of the hindlimbs and forelimbs in a context-dependent manner during locomotion. Their functional role was revealed through the use of a dual virus Tet^{ON} neuronal silencing system (Pocratsky *et al.*, 2020). Post-SCI, silencing LAPNs restores overground stepping ability, suggesting they have a maladaptive role in recovered locomotion after injury. Further work explored the role of LDPNs in uninjured rat locomotion and found that LDPNs secure left-right alternation of the hindlimbs and forelimbs in a similar fashion to LAPNs; however, these findings are less context-dependent than those observed during LAPN silencing. As with LAPNs, the anatomical location of LDPNs suggests some are spared post-SCI and could serve as a neural substrate for functional recovery (Reed et al 2006, Brockett et al 2013, Pocratsky et al 2020). We hypothesize that LDPNs will contribute to recovery of function post-SCI and that the role of LDPNs will be less influential on locomotor outcomes than that of LAPNs.

To test this hypothesis, we conditionally silenced spared LDPNs post-SCI, reasoning that if they contributed to recovery then their silencing would result in locomotor deficits. Unexpectedly, we found that silencing this spared pathway post-SCI improved some facets of locomotor function while having no effect on others, suggesting that spared pathways may deleteriously influence certain aspects locomotor recovery without influencing others. Unexpectedly, silencing spared LDPNs post-thoracic SCI improved gross motor behavior and postural stability, while having a mild effect on

interlimb coordination and no effect on intralimb coordination of the hindlimbs. These findings suggest that spared LDPNs post-SCI negatively impacts some recovered function, but is not having the same remarkable effect on locomotor circuitry below the level of injury that was seen during LAPN silencing. Ultimately, these findings suggest that LDPNs are negatively influencing the animals' postural stability and that their role in recovered locomotion of the hindlimbs is less influential than that of LAPNs after injury. However, improvements were still seen, suggesting that removing or reducing some of the maladaptive “noise” within the system may permit fine tuning of the entire locomotor system, including postural stability, thereby leading to overall improved locomotor ability.

Materials and Methods.

Experiments were performed in accordance with the Public Health Service Policy on Humane Care and Use of Laboratory Animals, and with the approval of the Institutional Animal Care and Use (IACUC) and the Institutional Biosafety (IBC) Committees at the University of Louisville.

A total of N=12 adult female Sprague-Dawley rats (215-230 g) were used in this study. Animals were housed two per cage under 12-hour light/dark cycle with *ad libitum* food and water throughout the course of the study. Power analysis for gait measures revealed that N=6-10 could detect a true significant difference with power of 85-95%. Additional animals were used to mitigate animal mortality following repeat surgical exposure. N=10 out of 12 animals displayed behavioral changes pre-SCI; the two animals that did not display behavioral changes were removed from the main data set to be analyzed separately as a post-injury control. In order to be excluded, animals had to have

less than 10% of their steps outside of normal variability during Dox testing. A total of N=1 died after first viral injection from respiratory complications and N=1 died after SCI surgery, leaving N=10 remaining for post-injury analysis. N=8 animals of the remaining 10 animals showed behavioral perturbations prior to injury and were used for the main pre- and post-injury data set. Animals that lacked behavioral outcomes pre-injury (N=2) did not show improvements in locomotion post-SCI; however, both animals had very mild control injury phenotypes, clouding deeper interpretations of post-injury silencing data for these animals.

Viral vector production.

Viruses were constructed and titered following previously described methods (Pocratsky et al 2017, 2020).

Intraspinal injections of viral vectors to doubly infect LDPNs.

Intraspinal injections and power analyses for kinematic measures are based on previous literature. The procedural details are described in the *Nature Protocol Exchange* (<http://dx.doi.org/10.1038/protex.2017.125>). Intraspinal injections were performed on N=12 animals using the same LDPN injection protocol described in Chapter II methods.

Spinal cord injury.

Animals received a moderate spinal cord contusion at T9/T10 spinal cord approximately 2 weeks after conclusion of uninjured Dox^{ON} assessments. For SCI surgery, animals were re-anesthetized (ketamine:xylazine:acepromazine, 40 mg/kg:2.5 mg/kg:1mg/kg; I.P., Henry Schein Animal Health, Dublin, OH; Akorn Animal Health, Lake Forest, IL). Spinal cord contusion injuries were performed using the injury protocol described in Chapter III.

Experimental timeline.

Doxycycline hydrochloride (Dox, 20 mg/ml; Fisher Scientific BP2653-5, Pittsburgh, NH) was dissolved in 3% sucrose and provided ad libitum for 8 days pre-injury and 8-14 days post-injury. Dox water was made fresh and replenished daily and monitored for consumption. All behavioral assessments were performed during the light cycle portion of the day and concluded several hours before the dark cycle began.

Prior to SCI, behavioral assessments were performed prior to viral injections (BL), prior to Dox (pre-Dox), during Dox (Dox1^{ON}D5-D8), 14 days post-Dox (PostD1). Following SCI, pre-Dox and Dox^{ON} time point assessments were reproduced twice (Dox2 and Dox3) following SCI to assess the reproducibility of any behavioral changes that were seen. Post-injury control time points included assessments prior to Dox (PD2, PD3), during Dox (D2D5, D2D8, D3D5, D3D8, and D3D14), and after the second Dox administration (PostD2). Some data shown are compiled from pre-injury Control and Dox timepoints (“uninjured”) and post-injury Control and Dox timepoints (“injured”). Control vs Dox uninjured and Control vs Dox injured time point comparisons were made both on an individual and group basis. Behavioral analyses began on day 5 of Dox (DoxD5) administration and were repeated on Dox day 8 (DoxD8). For terminal assessments after injury, behavioral assessments occurred on Dox Day 14 (D3D14).

Identification of hindlimb joints, hindlimb kinematics, and intralimb coordination analysis.

Hindlimb joints identification and hindlimb kinematics was acquired as previously described in Chapter III. The analyzed points from the two sagittal cameras were exported to a Microsoft Excel workbook and 2D average angles were calculated

for each digitized frame. Maximum and minimum angles (maximum extension and flexion, respectively) were identified using a custom Microsoft Excel macro. Excursion for the proximal and distal hindlimb joint angles (Maximum Angle – Minimum Angle) were also calculated using the same macro. The temporal relationship between the proximal and distal hindlimb joint angles was calculated using the peak-to-peak duration of the lead angle during a single step cycle. Within this duration, the maximum excursion of the first angle was determined and depended on which angle peaked first (proximal or distal angles). The time of onset of the second angle was divided by the peak-to-peak distal angle duration to determine the temporal relationship between intralimb angles. A coordination value of 1 indicates in-phase coordination of the proximal and distal intralimb joints, while a phase value of 0.5 indicates anti-phase, uncoordinated joint movements. Intralimb joint phase was calculated for each step cycle of the left and right hindlimbs independently.

Overground gait analyses.

Overground gait analyses were performed as previously described in Chapter II Methods. Locomotor passes were chosen based upon the same criteria previously listed.

During injured recordings (control and silenced), a step was classified as dorsal when the dorsum of the foot came into contact with the ground during the stance portion of the step cycle and is considered a step if it maintains contact with the surface and completes the swing portion of the step cycle. Any hindlimb-centric steps used in the data set were left-lead limb dominant (i.e. RLRR and RLFR) to maintain consistency between uninjured and injured data for consistency and to avoid excessive data. As left limb was used for primary data analysis, dorsal steps were identified in the data set if they fell on

the left side (i.e. a right-side dorsal step would not have been marked a dorsal step in the shown data).

Interlimb phase was calculated by dividing the initial contact time of the trailing limb by the stride time (initial contact to initial contact) of the leading limb. Alternating gaits were defined by phase values concentrated around 0.5 (Lemieux et al 2016).

Phase for individual steps were transformed from a circular scale and were plotted linearly. Blue boxes on graphs represent >2 standard deviations (S.D.) as calculated from uninjured control average and S.D. for the specified limb pair. Values found within the blue box are considered outside of normal variability and were quantified using pie charts to indicate the percent of total steps that existed outside of that range. Plantar steps are indicated by grey circles during control and red circles during silenced time points. Post-injury dorsal steps were indicated on both circular and linear phase plots as teal circles.

BBB assessments.

BBB assessments were performed by individuals blinded to experimental time points. All raters were aware that assessments were being performed post-injury, but were blind to Control and Dox. Overground stepping was assessed using the BBB Open Field Locomotor Scale as previously described (Chapter III methods, Basso et al 2002; Caudle et al 2015).

Coordination indices.

Regularity index (RI), central pattern index (CPI), plantar stepping index (PSI), and dorsal stepping index (DSI) were calculated as previously described in Chapter III.

Postural stability.

Several measures were used to determine the postural stability of animals during locomotor bouts. Gait angle is the angle between two consecutive initial contacts of a rear hindlimb in reference to the other hindlimb. Typically, a smaller gait angle would indicate higher stability during locomotion, as the hindlimbs are in the line with the body. Larger gait angles would suggest diminished stability as the hindlimbs would be set wider. Gait angle is calculated using the Pythagorean theorem and the X,Y coordinates of the foot while it is in contact with the ground. Statistics were performed on the group means for the left and right hindlimbs (bars: average $\pm$ S.D.; circles: individual means).

The diagonal length is the distance between initial contacts of diagonal paws. For this calculation, the diagonal pair used was left hindlimb–right forelimb. This pair was chosen to remain consistent with hindlimb phase data in which left hindlimb is the lead limb. The diagonal length is calculated using the Pythagorean Theorem and the X,Y coordinates of each foot.

Rear track width is the distance between the hindlimbs during consecutive initial contacts. Track width is calculated by taking the absolute value of the Y-coordinate of the left hindlimb initial contact subtracted from the Y-coordinate of the subsequent right hindlimb initial contact. Larger track width indicates a wider stance during stepping, while smaller track width indicates stance phases more in line with the body.

Ladder.

We quantified the animals' ability to effectively traverse a ladder with fixed-spacing rungs (Columbus Instruments, Burke & Magnuson 2012; Metz & Whishaw 2002). Behavioral testing was performed on the same time points as BBB testing (PD2, D2D5, D2D8, PostD2, PD3, D3D5, D3D8, and D3D13). Each animal received five

stepping trials in each direction. The total number of footfalls were calculated for the left and right hindlimbs, respectively, for each animal across the time points. We then calculated each animal's average number of foot slips during the Control and Dox time points listed above. Left and right limbs were not combined to demonstrate that the number of foot slips decrease by a similar amount for each hindlimb (i.e one foot is not different from the other in terms of recovery). Statistics were performed on the group means (bars: average $\pm$ S.D.; circles: individual means).

Spatiotemporal gait indices.

Spatiotemporal gait analyses were performed as previously described in Chapter III methods.

Histological analyses.

Animals were killed on D3D14 (N=10) following terminal BBB and kinematic behavioral assessments. Animals were overdosed with sodium pentobarbital, followed by pneumothorax and transcardially perfusion with 0.1 M phosphate-buffered saline (PBS) (pH 7.4) followed by 4% paraformaldehyde diluted in PBS solution. Spinal cords were dissected, post-fixed for 1.5 hours, and transferred to 30% sucrose for a minimum of 4 days at 4C. Spinal segments C5-C8, T8-T12, and T13-L3/L4 were dissected, embedded in tissue freezing medium, and stored at -20C until they were cryosectioned at 30 μ m.

Histological analysis was performed using the immunohistochemistry protocol described in Chapter III.

Statistical analyses.

Statistical analyses were performed using the SPSS v22 software package from IBM. Additional references for parametric and non-parametric testing were used in complementation to SPSS (Hays, 1981; Siegel & Castellan, 1988; Ott, L, 1977). Differences between groups were deemed statistically significant at $p \leq 0.05$. Two-tail p-values are reported.

The Binomial Proportion Test was used to detect significant differences in the proportion of coordination values beyond control threshold for the raw and transformed interlimb coordination data of various limb pairs prior to and post-SCI. It was also used to determine statistical significance for per-step changes in left-right coordination and change in interlimb phase, raw BBB score differences, intralimb phase and per-step changes in intralimb phase, dorsal steps as a percentage of total steps, and percentage of categorically organized steps (anti-phase, out of phase, in phase).

Regression analyses to compare speed versus spatiotemporal gait indices datasets, including speed vs. swing time, stance time, stride time, and stride distance. Analyses were performed for hindlimb-hindlimb relationships prior to and after SCI. For regression analyses post-SCI, plantar and dorsal steps were included in the analysis and dorsal steps are shown in blue on graphs for identification. Trend lines are shown on graphs with 95% prediction intervals indicated by dashed lines. To further analyze the differences in spatiotemporal measures, Euclidian distance was calculated for each measure. Euclidean distance measures how much 2 variables plotted against one another (one on the x-axis, one on the y-axis) change relative to one another. Euclidian distance was used to calculate the relationship between the kinematic assessment and speed for the Dox and Control groups. Euclidean distance calculations determined the distance between the two

variables for each animal, and those distances were compared between the Control and Dox groups to show if the relationship was the same for the two measures.

Mixed model analysis of variance (ANOVA) followed by Bonferroni *post hoc* t-tests (where appropriate) were used to detect a significant difference in the BBB scores based on sidedness and condition (i.e. Control vs Dox; graph not shown). Repeated measures ANOVA analyses were used to compare number of average steps per animal during uninjured and injured time points.

Paired t-tests were used to detect significant differences in proximal and distal angle excursion for uninjured and injured intralimb coordination, average gross stepping measures including RI, CPI, PSI, DSI, and combined BBB scores, average intralimb phase, percentage of dorsal step sidedness, average swing time, average stance time, average duty cycle, average number of ladder errors, average gait angle, average diagonal length, average rear track width, and overall average speed at Control and Dox combined time points.

Results.

Silencing LDPNs alters hindlimb interlimb coordination while maintaining intralimb coordination and key locomotor features in uninjured animals.

Figure 12 shows a schematic of the experimental design. We performed bilateral injections at L2 and C6 spinal cord segments to doubly infect LDPNs at their terminals and cell bodies, respectively (Fig 23a). As previously indicated, in the presence of doxycycline (Dox), doubly-infected neurons that constitutively express rtTAV16 induce expression of enhanced tetanus neurotoxin (eTeNT). At the level of the cell terminal, active eTeNT prevents synaptic vesicle release, leading to “silenced” neurotransmission

(Fig 4c). Removing Dox restores functional neurotransmission, allowing for reversible silencing of inter-enlargement LDPNs both before and after SCI (Fig 23b).

Given the effects of silencing LDPNs in the uninjured rat (Chapter II) and the effects on the reciprocal ascending pathway post-SCI (Chapter III), we first evaluated whether we could reproduce our previous findings as proof of principle. Previously, silencing LDPNs caused significant disruptions to the left-right alternation of the hindlimbs, with milder disruptions to the forelimb pair and the heterolateral hindlimb-forelimb pair. We examined the coupling patterns of these limb pairs by comparing the temporal relationship, which can be expressed as a phase value. As previously described, phase is determined by dividing the initial contact time of the trailing limb by the stride time of the leading limb. Phase values of 1 indicate synchrony and values of 0.5 indicating alternation of the limb pair. During control timepoints, the hindlimbs maintained phase values concentrated around 0.5, as did the forelimbs (grey circles; Fig 24a,b). Further, the heterolateral hindlimb-forelimb phase relationships trended toward synchrony (grey circles; Fig 24c). We determined the mean phase value of the limb pairs and any value >2 S.D. from this mean was considered “irregular”, as defined by the blue boxes. Silencing LDPNs led to disruptions in the left-right alternation of the hindlimb pair, with modest changes to the forelimb pair and the heterolateral hindlimb-forelimb pair (red circles; Fig 24a-c). The silencing effects on limb pair relationships were restored when Dox was removed from the drinking water (Fig 24a-c, “PostD1”).

We further examined whether silencing uninjured animals would adversely affect the coordination of the limbs in time (temporal measures) and space (spatial measures). Swing time, stance time, stride time, and stride distance comprise well-described gait

indices that have been shown to have specific relationships with speed (Gillis & Biewener 2001). Silencing did not affect the fundamental gait relationships of swing time (Fig 24d,i), stance time (Fig 24e,j), stride time (Fig 24f,k), stride frequency (Fig 24g,l), and stride distance (Fig 24h,m). Importantly, these findings are consistent with previous observations and suggest that LDPNs secure interlimb coordination without affecting fundamental step cycle characteristics or the locomotor rhythm in an otherwise intact rat.

Presence of putatively eTeNT-positive synapses in the lumbar spinal cord and cell bodies in the cervical spinal cord.

Neuroanatomical evidence has confirmed the presence of inter-enlargement LDPNs that originate in the cervical enlargement, terminate in the rostral lumbar cord, and project through the ventrolateral funiculus (VLF) (Menetrey et al 1985, Reed et al., 2006, Flynn et al 2017) Lesions to the thoracic VLF in the cat disrupt forelimb–hindlimb coupling (Brustein and Rossignol, 1998), further confirming the location of these long descending propriospinal projections. Given this described location, it was essential to determine whether the VLF, the area of white matter in which LDPN axons project, was preserved after SCI. To examine this, animals were euthanized during post-injury Dox administration. Their spinal cords were harvested and were blocked in three sections: caudal cervical spinal cord (cell bodies), caudal thoracic spinal cord (injury epicenter and penumbra), and rostral lumbar spinal cord (cell terminals). Thoracic tissue sections were stained at the injury epicenter using eriochrome cyanine (EC) to quantify to amount of spared white matter and imaged to determine where the spared white matter was located. Amongst all animals, the average spared white matter percentage (SWM %) was 26%, slightly higher than that previously described in the LAPN SCI study (Fig 25a) The

proposed location of LDPN axons within the ventrolateral funiculus was spared, as evidenced by the remaining spared white matter in each animal (Fig 25b-i). Thus, a proportion of LDPN axons are likely spared after SCI, a critical finding for interpretation of any behavioral assessments moving forward.

Knowing that LDPN axons were likely preserved at the level of injury, we next confirmed that eTeNT.EGFP-expressing putative LDPN axons were present in the rostral lumbar spinal cord, as well as eTeNT.EGFP-expressing putative LDPN cell bodies in the intermediate grey matter of the caudal cervical segments. Histological analysis for EGFP immunoreactivity showed that putatively positive eTeNT.EGFP fibers were found surrounding neuronal processes in the rostral lumbar enlargement (Fig 26a).

eTeNT.EGFP co-localized with synaptophysin (Fig 26b; synaptic marker), vesicular GABA transporter (Fig 26c, VGAT, inhibitory neurotransmitter), and vesicular glutamate transporter 1/2 (Fig 26d,e; VGlut1/VGlut2, excitatory neurotransmitters). Isotype control revealed minimal-to-no immunoreactivity (Fig 26f).

Using the same immunohistological protocol as described for lumbar spinal tissue, caudal cervical spinal cord segments were assessed for the presence of eTeNT.EGFP within LDPN somata. Putatively eTeNT.EGFP positive LDPN cell bodies co-localized with fluorescent Nissl stained (NeuroTrace) in the intermediate gray matter (Fig 26g-i, Reed et al 2006). Isotype controls showed no immunoreactivity (Fig 26j-l). Taken together with the lumbar spinal cord histology, these data suggest that double-infected LDPNs maintained expression of eTeNT at the level of cell terminal and the level of the cell soma following SCI. Further, any post-SCI spared LDPNs axons were

functionally silenced, indicating that any behavioral changes seen during Dox administration were concomitant with active eTeNT.EGFP expression.

Silencing LDPNs post-SCI restores coordination indices and improves gross locomotor outcomes.

Having validated that (1) the viral-based silencing system is active post-SCI and (2) LDPN axons are likely intact post-SCI, we began to explore the effects of LDPN silencing on locomotion after injury. In our previous LAPN study, silencing LAPNs post-SCI unexpectedly resulted in improved stepping. In conjunction with perturbations to hindlimb patterned stepping in uninjured animals, we hypothesized that silenced LDPNs would play a similar role in recovered locomotion post-SCI.

The Basso, Beattie, Bresnahan (BBB) Locomotor Rating Scale was used to evaluate locomotor recovery (Basso et al 2002). Control BBB scores were concentrated between 11-13, consistent with previously described literature for this injury severity and with our previous LAPN silencing study (Fig 17, Basso et al 2002; Smith et al 2006). In similar fashion to LAPN post-SCI findings, average BBB scores were modestly increased during silencing (Fig 27a), with a greater proportion of raw scores for each limb between 13-18 (Fig 27b). The increased BBB scores can be attributed to improved weight support of the hindlimbs as well as better coordination, validating potentially improved stepping during silencing. Some BBB scores still remained concentrated around 13, indicating that not all animals' stepping improved at all Dox time points. However, a significant proportion of BBB scores were greatly increased, suggesting better stepping occurred in multiple animals at multiple time points as a result of silencing.

To further assess the overall locomotor function during control and silencing post-SCI, we evaluated the step sequence patterns to determine whether gross coordination indices, such as regularity index (RI) and coordinated pattern index (CPI), were improved. As previously described, RI scores plantar steps according to paw placement order and represents the gait as the ratio of plantar steps that are in order (one of four different orders that normal rats exhibit) over the total number of steps, while CPI represents the number of correctly patterned step cycles (dorsal and plantar) to the total number of cycles (dorsal and plantar) (Hamers et al 2001, Caudle et al 2015). CPI accounts for whether or not the animal can achieve coordination with regardless of ability to attain plantar stance, while RI only accounts for correctly patterned plantar steps. RI and CPI were both modestly improved during Dox administration, an indicator that stepping was improved as a result of silencing (Fig 27c,d). We also examined the plantar stepping index (PSI), which was also modestly improved during silencing, further signifying improvements in stepping (Fig 27e). It is important to consider that RI and CPI account for the step sequence pattern of all 4 limbs, while PSI relies on the plantar stepping ratio of the hindlimbs to the forelimbs.

When using RI and CPI, an important differentiator is the presence of dorsal steps. After SCI, the components of the step cycle are altered in a way that prevents each step from achieving plantar placement, resulting in a dorsal step. Dorsal stepping index (DSI) allows for comparison of the number of dorsal steps to the number of total hindlimb steps. We compared DSI during control and silenced time points. DSI accounts for both right and left hindlimb dorsal steps, so it is a useful measure for determining whether the overall number of non-plantar steps is reduced. DSI during control

timepoints was 24.31 ± 21.02 , but was drastically reduced during silencing to 9.64 ± 8.70 indicating a significant reduction in the overall number of dorsal steps (Fig 27f). This decrease closely resembles the decrease seen in dorsal stepping indices during LAPN post-SCI silencing.

We also compared the sidedness of dorsal steps. Dorsal step sidedness refers to the percentage of dorsal steps that occur on the right and the percentage that occur on the left. The sidedness did not change between control and silencing, with ~55% of dorsal steps occurring in the right hindlimb and ~45% of dorsal steps occurring in the left hindlimb during both control and silenced time points (Fig 27g). This is an important observation, as it indicates that one limb did not improve to a greater extent than the other. For the purposes of simplicity and ease of comparison with pre-injury data, we will focus on left lead limb steps to demonstrate the principle behavioral changes post- SCI as we did with LAPN SCI data. To further elucidate these improvements in coordination indices, we examined more specific intralimb kinematics and interlimb gait analyses.

LDPN silencing leads to modest improvements in intralimb coordination after SCI.

BBB, in particular, includes improvements in both intralimb joint movements and interlimb coordination, with lower scores being linked to joint movements and more precise measures (coordination, paw rotation, etc.) added as the scale increases. Given the improvements seen in gross motor assessments, especially BBB, we next sought to determine whether changes occurred in intralimb coordination as these changes would be essential to reach higher scores on the scale. During control timepoints, proximal and distal joint angle excursions were reduced and intralimb coordination was disrupted during a large number of passes such that peaks and troughs were no longer occurring

simultaneously (Fig 28a), while other passes maintained intralimb relationships (Fig 28b). Conditional silencing normalized coordination of the proximal and distal hindlimb joints during volitional overground locomotion in a great majority of passes, such that the cyclic properties of each joint were restored for (Fig 28c,d). We next examined the peak-trough excursion of the proximal and distal angles of the hindlimb as well as the temporal relationship between them during overground locomotion. Interestingly, the peak-trough excursions of both the distal and proximal joint angles were not significantly improved during silencing (Fig 28e,f), suggesting that silencing LDPNs may not be primarily affecting the hindlimbs themselves. Next, we explored the temporal relationship between the proximal and distal angles. In uninjured animals, the peak extension of the distal angle typically occurs in-phase with the peak extension of the proximal angle during the stance phase of the step cycle, resulting in a coordination value ranging from 0.9-0.1 on a linear scale. Following injury, the average coordination value of these two angles is unchanged between Control and Dox (Fig 28g_{i,ii}), indicating that LDPNs are having minimal effect on intralimb biomechanics and temporal coordination post-SCI.

Hindlimb-hindlimb, but not hindlimb-forelimb, coupling relationships were restored during post-SCI silencing.

After SCI, the hindlimbs and the hindlimb-forelimb limb pairs are partially uncoupled, leaving the temporal relationship between limbs highly unstable such that they are unable to maintain their phase relationship on a step-by-step basis. This variability is accounted for, in part, by the presence of dorsal steps; however, many plantar steps also fall into irregular gait patterns after SCI. We included both plantar and dorsal steps for the purposes of quantifying interlimb coordination, with left dorsal steps

identified as teal throughout the remaining figures. Further, these irregularly patterned steps are present during multiple control and silenced timepoints. As was seen during LAPN silencing, the hindlimb-hindlimb pair and the hindlimb-forelimb pair are partially decoupled at all Control time points (Fig 29a_i, grey circles). However, coupling of the hindlimbs is only restored during the initial round of post-injury silencing, with minimal restoration occurring during the second Dox administration (Fig 29a_i, red circles). Interestingly, hindlimb coupling is significantly improved when Control and Dox time points are collapsed, suggesting that the total number of steps that fall outside normal variability are wholly reduced (Fig 29a_{ii}) from ~23% to approximately ~12% (Fig 29a_{iii}). In addition, the heterolateral hindlimb-forelimb pair only saw coupling improvement during a single Dox time point (D2D8), but the reduction in abnormally phased steps is not as apparent as any changes to the hindlimbs (Fig 29b_{i,ii}), with a small reduction in abnormal steps from ~43% to ~37% (Fig 29b_{iii}).

Several animals (N=2) were removed from the data set prior to injury as they showed no perturbations to left-right alternation at any pre-injury Dox time point (Fig 30a-f; see Methods for exclusion criteria). Interestingly, these animals did not show improvements in coordinated stepping post-SCI, suggesting that recovery of hindlimb stepping coordination is a silencing-induced perturbation (Fig 30g-l). It is important to note that these two animals showed minimal disruption during Control testing with very few dorsal steps during either Control or Dox testing, so lack of improvement in these animals is not definitive. For reference, the percentage of spared white matter was similar to animals in the main data set, though the percentages fall on the high side of the average.

Together, these results suggest that any functional gains as a result of LDPN silencing are concentrated below the injury epicenter only at the level of the hindlimb girdle. Silencing had minimal effect on the hindlimb-forelimb relationship and had no effect on the intra-girdle forelimb relationship. Further, the reduced prevalence of dorsal steps during silencing accounted for some, but not all, of the improvement in hindlimb interlimb coordination. This indicates that LAPNs are having a functional impact on hindlimb coordination without improving the coordination of other limb pairs. Further, they may play a role in hindlimb interlimb coordination regardless of the ability to achieve plantar stepping.

Silencing LDPNs modestly improves balance and posture post-SCI.

Populations of descending cervico-lumbar projections have been previously implicated as essential for postural stability during overground locomotion in the uninjured mouse (Ruder et al 2016). To determine if the same principle is true for these LDPNs post-SCI, we examined measures that are associated with maintaining balance and postural control.

First, we examined the base of support during overground locomotion by measuring the external rotation of the hind paws on a step-by-step basis (see methods for detail). Uninjured animals typically have a relatively narrow base of support. In conditions with increased postural instability, such as spinal cord injury, the paws become externally rotated and the base of support widens (Basso et al 1996). Several measures allow us to explore the changes in postural stability. First, gait angle determines the angle between each initial contact of the hindlimbs (Fig 31a_i). A large angle would represent footfalls that have a greater distance between each other and are rotated

outward; while a smaller angle would indicate a narrower footbase and decreased external rotation. A slight, but significant, decrease in gait angle occurs only for the left foot, with no significant improvement in gait angle of the right foot (Fig 31a_{ii}).

Diagonal length can also be an important measure of postural stability during locomotion, as it demonstrates the ability to maintain consistent distance between one hindlimb and the contralateral forelimb (Fig 31b_i). A higher value usually indicates that the hindlimb is extending further than normal, a phenomenon that is commonly seen post-SCI in order to keep multiple limbs on the ground for stability. Interestingly, diagonal length is reduced during silencing (Fig 31b_{ii}). Taken together with the lack of improvement in hindlimb-forelimb coordination that was described in Fig 30, the reduction in diagonal length indicates that animals may be adopting a different strategy for maintaining postural support post-SCI as the forelimb and hindlimb are asynchronous and are closer to one another during the step cycle. Rear track width, or the calculated distance between the initial contact of each hindlimb, goes hand-in-hand with gait angle (Fig 31c_i). Rear track width also showed mild improvements (Fig 31c_{ii}) similar to those seen in gait angle. The reduction in track width indicates that animals are likely adopting a narrower foot base during locomotion. Given the size of the animals in width, small reductions in foot base are highly meaningful to locomotor success.

We next challenged the animals' ability to maintain balance and posture by testing them on a ladder task (Fig 31d_i). In order to successfully traverse the ladder apparatus, animals must maintain postural control as they cross in order to accurately place their feet on the fixed-space ladder rungs. The number of foot slips, or times that the foot doesn't maintain contact with a rung, was reduced for both the left and right

hindlimbs (Fig 31d_{ii}). Silencing modestly improves indicators of postural stability and balance, which supports the notion that LDPNs post-SCI may be obstructing postural control.

Key features of locomotion are restored during post-SCI LAPN silencing.

In the uninjured animal, silencing did not affect the fundamental relationships between speed and spatiotemporal features of limb movements (e.g. swing time, stance time, etc.). As previously described in Chapter II, these parameters change in relation to speed in a stereotypic and well-characterized manner (Gorska et al 1998, 1999; Hruska et al 1979). To determine whether the silencing effected these relationships, we plotted swing time, stance time, and stride distance in relation to speed. These relationships are disrupted as a result of SCI, with a number of individual plantar and dorsal steps falling outside the typically described relationships (Fig 32a-c). Interestingly, measures that are typically associated with locomotor rhythm (stride time and stride frequency) were also disrupted (Fig 32d,e), though the abnormal steps were typically associated with a dorsal step, which we have already shown to fundamentally alter interlimb coordination. Silencing LDPNs restored these relationships and resulted in a dramatic reduction in the variability of abnormal steps, regardless of dorsal or plantar stepping (Fig 32f-j). Calculated Euclidian distance supports this improvement, as Euclidian distance is significantly improved and approaches pre-injury Control and Dox values for each spatiotemporal measure (Fig 32k-o).

Average swing time (Fig 32p) and average stance time (Fig 32q) were unchanged during post-SCI silencing. Interestingly, the ratio of stance time to total stride time, or duty cycle, was also unchanged after silencing (Fig 32r). Finally, the average speed

amongst animals was increased during post-injury silencing (Fig 32s) indicating that speed may be a primary factor in the improved spatiotemporal relationships and increased Euclidian distance calculations. Together with the previous findings, these data suggest that silencing LDPNs after SCI is impacting the temporal coordination of multiple aspects of locomotion, primarily interlimb coordination. Importantly, LDPN silencing post-SCI demonstrated a much weaker phenotype than that of LAPNs, suggesting that LAPNs may be exerting greater influence on CPG circuitry post-injury. intralimb coordination and step cycle spatiotemporal relationships were unchanged, suggesting that the extent of their influence is not extending to the hindlimbs.

Discussion.

Considerable evidence suggests that LDPNs play a role in interlimb coordination in uninjured animals (Chapter II, Matsushita et al., 1979; Skinner et al., 1980; Menétrey et al., 1985; Alstermark et al., 1987; Nathan et al., 1996). Supporting those data, current results indicate that inter-enlargement LDPNs are an essential pathway in securing interlimb coordination of both the forelimbs and hindlimbs in non-context specific manner. Moreover, the influence they exert upon the hindlimbs in the uninjured animal is greater than that exerted upon the forelimbs, as evidenced by the number of affected steps during stepping. Ruder et al (2016) reported that in the adult mouse, ablation of subsets of LDPNs reduced spontaneous locomotor speed and decreased the duration of locomotor bouts. In contrast, our data indicate that rats are still able to achieve high rates of speed and that locomotor bouts are sustained and unperturbed when LDPNs are silenced. These differences may arise from several discrepancies between the two studies. For one, the specific pathway they chose to target is unclear. It appears that they examined a wide

range of descending pathways from multiple cervical segments. Given that we have chosen to focus specifically on LDPNs that originate from C6, this may provide a partial explanation for the differences in function as they may only represent a subset of those targeted in their studies. Further, we perform overground behavioral assessments in a short tank, which may not allow animals to express the higher rates of speed that were described (Ruder et al 2016).

It is possible that the LDPNs of interest in this study are partially made of other populations of genetically-defined long spinal projection neurons that also contribute to aspects of the described phenotypes. Notably, V0v and V2a, but not V0d, neurons were implicated in securing left-right limb alternation at high speeds (Bellardita and Kiehn 2015; Crone et al 2009; Talpalar et al 2013). V0v are composed of commissurally projecting inhibitory interneurons and are recruited to maintain left-right alternation as the speed of movement increases (Lanuza et al 2004; Crone et al 2009). The V2a population of neurons are also classified as ipsilateral excitatory glutamatergic interneurons that are involved in left-right alternation (Crone et al 2008; 2009) and minimal involvement in rhythm generation. There is potential that our LDPN population is partially comprised of both populations, given the perturbations to left-right alternation during silencing. However, it is important to note that LDPNs have characteristics that fall in line with both progenitor domains, as they are both ipsilaterally and commissurally projecting and co-localize with both glutamatergic and GABAergic synapses. Therefore, it is difficult to place these anatomically-defined neurons within the previously described context of genetically-defined V-neuron populations.

No current literature has examined the role of LDPNs after SCI. After injury, our results demonstrate modest but meaningful improvements in postural stability measures when LDPNs are silenced. This was concomitant with mild improvements in hindlimb-hindlimb coordination, including paw placement order and timing, and speed-dependent gait indices. These improvements occurred in the absence of robust changes in hindlimb-forelimb coordination. Collectively, these data suggest that rather than securing alternation of the hindlimbs and forelimbs, spared LDPNs hinder the capability of interlimb lumbar circuitry below the level of lesion to function appropriately, contributing to diminished stepping capacity at chronic control time points. In addition, these data provide evidence that LDPNs may also be acting on lower thoracic circuitry associated with maintaining trunk stability during locomotor bouts. These results may seem counter-intuitive as a potentially spared pathway should aid in recovery after injury, though given our previous findings of improvement in stepping ability in the reciprocal ascending LAPN pathway, not unexpected (Chapter III). Specifically, silencing LAPNs resulted in the restoration of interlimb and intralimb coordination, a result that is shared to some extent by LDPNs during post-SCI silencing. The mechanism through which this is occurring remains unclear.

By virtue of their role in communicating higher motor commands to spinal circuits, a major focus of spinal cord repair has been the regeneration of descending tracts across the lesion site to restore lost motor input (Tuszynski & Steward, 2012). As a consequence of the LDPN cell body position within the spinal cord and their central role in the generation of patterned locomotor output prior to injury, long descending pathways are theoretically well-situated to propagate supraspinal commands to motor systems

below the level of injury and evidence continues to point to these descending propriospinals as potential targets for functional recovery (Taccola et al 2018; Loy & Bareyre 2019). Our targeted population of LDPNs represents an anatomically-spared inter-enlargement pathway that should continue to relay reliable temporal information across the level of injury. At first glance, our results contradict a number of studies in the field of locomotor recovery in which various populations of descending propriospinal relays of both thoracic and cervical origin are seen as an essential component of recovered stepping ability (Bareyre et al 2004; Vavrek et al 2006; Flynn et al 2011; Courtine et al 2008; Cowley et al 2008). However, the descending populations in these studies are inconsistent in terms of their defined anatomical location of their cell bodies and terminals (Fig 3a). This is a critical consideration, as even minor differences in the anatomical location of spared pathways may drastically alter their influence on spared circuitry and propensity to enhance recovery after SCI. The extensive differences in how LDPNs are defined offer some clue to the described differences we see in recovered function in this chapter. The descending pathways with more rostral cell bodies within the cervical cord (Bareyre et al 2004) may have an entirely different function than those located more caudally. Others refer to LDPNs without specifying defining characteristics. (Vavrek et al 2006; Courtine et al 2008, Filli et al 2014). This is problematic for elucidation of our results, as the ambiguity surrounding long propriospinals without providing the distinct spinal segments they are targeting remains.

Some of these studies have also focused specifically on the potential of supraspinal pathway regeneration onto LDPNs (Vavrek et al 2006; Courtine et al 2008), but the question remains whether regeneration leads to positive improvements in

functional outcomes. Our data suggest otherwise, as the locomotor functional outcomes only showed modest improvements, especially when considering the extent of improvement seen during LAPN silencing (Chapter III). Supraspinal-propriospinal reorganization has been shown previously to form an anatomical ‘bridge’ allowing transmission of descending signals below the lesion to activate the lumbar locomotor central pattern generator (CPG) (Bareyre et al., 2004; Courtine et al., 2008; Cowley et al., 2008; Vavrek et al., 2006). However, these studies did not extensively test functional outcome measures and pursue anatomical regeneration without determining whether that regeneration is functionally meaningful. Our results provide evidence that LDPNs should not be the primary target of regenerative efforts if the primary goal is improved locomotion as: (1) they are likely detrimental to recovered locomotion and (2) their primary outputs may not be directly affecting lumbar motor circuitry but but may have some collaterals that target thoracic postural circuitry. Further anatomical analysis may provide more insight into these findings, as it is essential to explore the extent of LDPN plasticity and the breadth of LDPN terminal fields after SCI. Additionally, definitive identification of those descending pathways that do facilitate functional recovery after SCI is essential to understand therapeutically functional plasticity.

Another important consideration is the extent of remaining intact circuitry that is still providing input to this propriospinal population that may be leading to the perplexing findings seen below the level of injury. Early electrophysiological data showed that supraspinal motor centers in the cerebellum, cerebral cortex, and brainstem, in conjunction with primary afferents, provide monosynaptic input onto LDPN populations in the cervical spinal cord (Brink et al 1985; Alstermark et al 1987). More recent work

has confirmed the presence of corticospinal and descending serotonergic pathways onto LDPNs (Ni et al 2014). Flynn et al (2017) showed that contralateral LDPNs received putative synaptic inputs from both excitatory and inhibitory pathways, such as other populations of inhibitory pre-motor interneurons, excitatory corticospinal or myelinated afferents, and proprioceptive afferent input from group Ia muscle afferents. Our data showing IHC colocalization with GABA and glutamate (Fig 26) are consistent with those data, but the specific pathways with which inter-enlargement LDPNs interact is not apparent and requires further investigation.

Given the variety of inputs onto LDPNs, it is unsurprising that silencing post-SCI does not result in dramatic improvements. Inter-enlargement LDPNs are a major node of integration for a variety of supraspinal and afferent inputs. A majority of this input is still intact and it is likely that LDPNs still properly integrate this information after injury, suggesting that other pathways, such as LAPNs below the level of injury, may be taking on a larger role in the recovery of hindlimb function post-SCI (see Chapter III). There is potential, given their location within the cervical cord, that their essential role may be in maintaining the security of the forelimbs and postural stability during post-SCI locomotion. A better understanding of their anatomical makeup after injury, as well as inputs onto this population of LDPNs, will be critical in further interpreting these results and determining whether LDPNs merit further investigation as therapeutic targets for locomotor recovery.

Figure 23. Experimental design to reversibly silence LAPNs in the adult rat following SCI.

(a) Bilateral injections of eTeNT (green) and rtTAV16 (yellow) were performed at L2 and C6 spinal cord levels, respectively, followed by a spinal cord injury (black star). Administration of doxycycline (Dox, red boxes) induces eTeNT expression in doubly-infected neurons. eTeNT is transported to the cell terminals where it prevents synaptic vesicle release into the synaptic cleft, effectively silencing neurotransmission in the targeted neuronal population. Following viral injections (i) and (ii), pre-injury behavioral assessments were taken at three control time points and a single round of DOX administration. Following SCI (iii, black star), behavioral assessments were repeated at two control time points and during two rounds of DOX administration (grey boxes) (b).

Figure 24. Silencing LDPNs disrupts interlimb coordination without affecting key features of locomotion.

Phase value were shown on a linear scale of 0.5-1 for the hindlimb and forelimb limb pairs and 0-0.5 for the heterolateral hindlimb-forelimb pair. The hindlimb and forelimb limb pairs were significantly altered during LAPN silencing, while the heterolateral and homolateral hindlimb-forelimb limb pairs remained unaffected (**a-c**, # steps beyond control variability: PD1 hindlimbs $n=3/79$ [3.79%] vs D1D5 hindlimbs $n=17/81$ [20.99%]; $p=.001$, $z=3.29$; PD1 hindlimbs $n=3/79$ [3.79%] vs D1D8 hindlimbs $n=25/79$ [31.64%]; $p<.001$, $z=4.58$; PD1 heterolateral $n=6/79$ [7.59%] vs D1D5 heterolateral $n=11/81$ [13.58%]; n.s., $z=1.23$; PD1 heterolateral $n=6/79$ [7.59%] vs D1D8

heterolateral n=19/79 [24.05%]; $p < .005$, $z = 2.84$; PD1 forelimbs n=2/79 [2.53%] vs D1D5
forelimbs n=14/81 [17.28%]; $p < .005$, $z = 3.11$; PD1 forelimbs n=2/79 [2.53%] vs D1D8
forelimbs n=16/79 [20.25%]; $p < .001$, $z = 3.51$; Binomial Proportion Test;
circles=individual step cycles; shaded region=values beyond control variability).
Spatiotemporal measures (swing time, stance time, stride time, stride frequency, stride
distance) were plotted against speed for Control (**d-h**) and Dox (**i-m**) timepoints. An
exponential decay line of best fit is displayed for stance time and stride time graphs
(stance time: Control $R^2 = .728$ vs Dox $R^2 = .851$; stride time: Control $R^2 = .773$ vs Dox
 $R^2 = .787$), while a linear line of best fit is displayed for stride distance (stride frequency:
Control $R^2 = .744$ vs Dox $R^2 = .794$ stride distance: Control $R^2 = .793$ vs Dox $R^2 = .665$;
trendline indicated by dotted line). 95% prediction intervals are also shown as solid lines.

Figure 25. White matter sparing present at injury epicenter.

Spared white matter percentage at the epicenter ranges from 17% – 39% (a).

White matter damage at the spinal cord injury epicenter as confirmed by histology (b-i).

Individual images represent the injury epicenter of each animal used in the main data set

(N=8; average white matter percentage: 26%, S.D. 7.76%).

Figure 26. Presence of eTeNT-EGFP in putatively silenced LDPNs across the level of injury.

High magnification, volume-rendered images demonstrating eTeNT-EGFP putatively positive fibers (green) surrounding fluorescent Nissl-stained neuronal

processes (blue) at synaptophysin-labeled putative synapses (red) in lumbar spinal cord segments of interest (**a-b**, 100x magnification, L1-L2 spinal cord). White arrows indicate areas of colocalization, which are further marked by numbers and xz, yz planes. Isotype control reveals minimal immunoreactivity (**c**, IgG controls for eTeNT.EGFP shown). eTeNT.EGFP (green) signal co-localizes with neuronal processes (blue) and with inhibitory neurotransmitter marker vesicular GABA transporter (**d**, VGAT, red), and excitatory neurotransmitter vesicular glutamate transporter 2 (**e**, VGlut2, red) and vesicular glutamate transporter 1 (**f**, VGlut1, red). eTeNT.EGFP putative cell bodies (green) in the cervical spinal cord colocalized with NeuroTrace fluorescent nissl stained neurons (blue) (**g-i**, C6-C7 spinal cord). Minimal presence of eTeNT.EGFP signal in isotype controls (**j-l**, C6-C7 spinal cord).

Figure 27. Silencing LDPNs post-SCI restores some coordination indices and improves gross locomotor outcomes.

Average BBB scores for Control and Dox timepoints are shown for each hindlimb (**a**, group average $\pm$ S.D.; Control Left to Dox Left, $p=.021$; Control Right to Dox Right, $p=.019$; mixed model ANOVA, Bonferroni *post hoc*). No significant difference was found between right and left BBB scores so they were combined for average and raw score (mixed model ANOVA, Bonferroni *post hoc*; statistics not shown on graph). Right and left hindlimb raw BBB scores are shown in (**b**, Control: $n=0/48$ [0.0%] vs Dox: $n=28/80$ [35.0%]; $p<.001$, $z=4.64$; Binomial Proportion Test; circles=individual left or right BBB scores; shaded region=values beyond control variability). Average CPI scores (**c**, Control

CPI: 84.47 ± 8.61 vs Dox CPI: 88.28 ± 3.07 , $t=1.66$, $df=7$, $p=.141$; paired t-test), RI scores (**d**, Control RI: 60.92 ± 3.61 vs Dox RI: 78.81 ± 8.97 , $t=7.69$, $df=7$, $p>.001$; paired t-test), PSI scores (**e**, Control PSI: 79.32 ± 15.97 vs Dox PSI: 91.71 ± 6.45 , $t=3.22$, $df=7$, $p=.015$; paired t-test), and DSI scores (**f**, Control DSI: 91.71 ± 18.49 vs Dox DSI: 9.64 ± 6.66 , $t=3.33$, $df=7$, $p=.013$; paired t-test) are demonstrated with individual animal scores from each Control and Dox time point to show variability (grey circles and red circles for Control and Dox, respectively). Dorsal stepping index accounts for total dorsal steps for both left and right hindlimbs. The total dorsal steps were separated based on sidedness: right hindlimb (RHL) and left hindlimb (LHL) (**d**, Control right: 0.546 ± 0.183 vs Dox right: 0.553 ± 0.162 , $df=6$, $p=.942$; Control left: 0.454 ± 0.183 vs Dox left: 0.447 ± 0.162 , $df=6$, $p=.942$; mixed model ANOVA). The right hindlimb showed more dorsal steps overall; however, it maintained that percentage during Dox. No significant differences were seen in sidedness (mixed model ANOVA, statistics not shown on graph).

Figure 28. LDPN silencing has no effect on intralimb coordination after spinal cord injury.

Angle-angle plots and excursion traces of the proximal (hip, purple) and distal (ankle, blue) joint angles during control (a,b) and Dox (c,d) timepoints are shown. Distal joint angle excursion (e, Control right average: 90.90 vs Dox right average: 93.45, $df=7$, n.s., R.M. ANOVA; Control left average: 91.49 vs Dox left average: 93.81, $df=7$, n.s., R.M. ANOVA) and proximal joint angle excursion (h, Control right average: 42.67 vs Dox right average: 45.93, $df=7$, n.s., R.M. ANOVA; Control left average: 45.08 vs Dox left average: 47.45, $df=7$, n.s., R.M. ANOVA; middle bar indicates group average with

extension bars indicating range of raw data) were unchanged as a result of silencing.

Intralimb phase values relating the peak of one angle to the peak of the other are

converted from a scale of 0-1 to 0.5-1.0 to be viewed linearly. The average intralimb

coordination values for the right hindlimb (g_i) and the left hindlimb (g_{ii}) are plotted for

Control and Dox timepoints (Control right: 0.87 ± 0.04 vs Dox right: 0.89 ± 0.02 , $t=2.44$,

$df=7$, $p=.045$, paired t-test; Control left: 0.88 ± 0.03 vs Dox left: 0.90 ± 0.02 ; $t=1.16$, $df=7$,

$p=.286$, paired t-test; circles=individual step cycles; shaded region=values beyond control variability).

Figure 29. Hindlimb-hindlimb and hindlimb-forelimb, coupling relationships are marginally improved during post-SCI silencing.

Transformed phase values for hindlimbs at each post-injury time point are separated in **a_i** (# steps beyond control variability: PD2 hindlimbs n=33/114 [28.94%] vs D2D5 hindlimbs n=8/107 [7.48%]; $p < .001$, $z = 4.10$; PD2 hindlimbs n=33/114 [28.94%] vs D2D8 hindlimbs n=17/108 [15.74%]; $p < .05$, $z = 2.35$; PD3 hindlimbs n=18/107 [16.82%] vs D3D5 hindlimbs n=13/107 [12.15%]; n.s., $z = 0.97$; PD3 hindlimbs n=18/107 [16.82%] vs D3D8 hindlimbs n=12/102 [11.76%]; n.s., $z = 1.04$; PD3 hindlimbs n=18/107

[16.82%] vs D3D13 hindlimbs n=12/107 [11.21%]; n.s., z=1.18) and are collapsed into Control and Dox in **a_{ii}** (Control hindlimbs with dorsal steps: n=75/332 [22.59 %] vs Dox hindlimbs with dorsal steps: n= 62/531 [11.67 %]; p <.001, z= 4.23, B.P. test).

Transformed phase values are also shown for the heterolateral hindlimb-forelimb pairs (**b_i**, (# steps beyond control variability: PD2 heterolateral n=47/114 [41.23%] vs D2D5 heterolateral n=42/107 [39.25%]; n.s., z=0.3; PD2 heterolateral n=47/114 [41.23%] vs D2D8 heterolateral n=28/108 [25.92%]; p<.05, z=2.41; PD3 heterolateral n=44/107 [41.12%] vs D3D5 heterolateral n=49/107 [45.79%]; n.s., z=0.47; PD3 heterolateral n=44/107 [41.12%] vs D3D8 heterolateral n=37/102 [36.27%]; n.s., z=0.72; PD3 heterolateral n=44/107 [41.12%] vs D3D13 heterolateral n=36/107 [33.64%]; n.s., z=1.13, B.P. tests) are shown. Dorsal steps were similarly collapsed into Control and Dox in **b_{ii}** (Control heterolateral limbs with dorsal steps: n= 143/332 [43.07 %] vs Dox heterolateral limbs with dorsal steps: n= 192/531 [36.16 %]; p<.01., z=2.7, B.P. tests). Plantar steps are indicated by grey circles (Control) and red circles (Dox), while dorsal steps are indicated by teal circles for both Control and Dox datasets. The blue boxes indicate values outside of normal variability for the specified uninjured limb pair mean. The percentage of abnormal steps found above normal variability is calculated for their respective limb pairs (**a_{iii}**, **b_{iii}**; statistics as shown above, B.P test).

Figure 30. Animals excluded based on lack of behavioral outcomes pre-injury show no improvements in hindlimb coupling post-injury.

N=2 animals were excluded from post-injury group data based on lack of behavioral phenotype at any time point during pre-injury silencing. No disruptions were seen in hindlimb-hindlimb (**a**, Control hindlimbs: $n = 1/67$ [1.49 %] vs Dox hindlimbs: $n = 0/38$ [0.00 %]; n.s., $z = 0.76$) or heterolateral hindlimb-forelimb (**b**; Control heterolateral: $n = 1/67$ [1.49 %] vs Dox heterolateral: $n = 0/38$ [0.00 %]; n.s., $z = 0.76$). No changes were

seen in relationships between swing time (**c,e**) or stride time (**d,f**) to speed prior to injury. All other spatiotemporal measures were graphed and relationships were maintained (data not shown). After SCI, improvements were not seen in hindlimb-hindlimb phase (**g**; Control hindlimbs: $n=1/131$ [0.76 %] vs Dox hindlimbs: $n=5/79$ [6.33 %]; $p<.05$, $z=2.35$) and similar disruptions in heterolateral hindlimb-forelimb (**h**; Control heterolateral: $n=58/131$ [44.27%] vs Dox heterolateral: $n=28/79$ [35.44%]; n.s., $z=1.26$) pairs were unchanged during silencing. Lack of improvement was seen in spatiotemporal measures during silencing (swing time: **i,k**; stride time: **j,l**). Control plantar steps = grey circles, Dox plantar steps = red circles. All dorsal steps are shown as teal circles. Example spared white matter for the removed animals is shown in (**m,n**) with 35% and 24% spared white matter percentage, respectively.

Figure 31. Indicators of postural stability were mildly improved during post-SCI LDPN silencing.

Throughout this figure, the right hindlimb (RH) will be shown in blue, the left hindlimb (LH) will be shown in yellow, the right forelimb (RF) will be shown in red, and the left forelimb (LF) will be shown in green. Control values are indicated by grey bars and Dox values are indicated by pink bars. Individual animal averages are shown for each postural stability variable are indicated by grey circles (Control) and red circles (Dox). An example of gait angle from a single set of initial paw contacts for the right hindlimb in reference to the left hindlimb is demonstrated in **a_i**. The average gait angle was calculated for both the left and right hindlimbs separately (**a_{ii}**; Control left 34.81 ± 4.26 vs Dox left 29.95 ± 2.82 , $df=7$, $p < .05$; Control right 33.37 ± 4.21 vs Dox right 30.35 ± 2.54 , $df=7$, n.s.; RM ANOVA). An example of diagonal length, calculated from the initial contact of each hindlimb to its contralateral forelimb, is also shown between the right hindlimb and the left forelimb (**b_i**). Averages for Control and Dox are demonstrated in (**b_{ii}**; Control diagonal length: 8.70 ± 1.02 vs Dox diagonal length: 6.00 ± 1.16 , $t=6.96$, $df=7$,

$p < .001$; paired t-test). Rear track width, calculated as the distance between each hindlimb's initial contact, is exemplified in (c_i), with averages seen in (c_{ii}; Control rear track width: 4.55 ± 0.47 vs Dox rear track width: 4.22 ± 0.42 , $t = 3.99$, $df = 7$, $p = .005$; paired t-test). Finally, the fixed-rung setup for ladder testing is shown in (d_i). The black box indicates a dark box found at the end of the ladder, which is used as incentive for rats to traverse the apparatus. The average foot falls were calculated for both the left and right hindlimbs separately (d_{ii}; Control left 8.58 ± 1.19 vs Dox left 6.34 ± 0.92 , $df = 7$, $p < .001$; Control right 8.33 ± 1.88 vs Dox right 6.24 ± 1.43 , $df = 7$, $p < .01$; RM ANOVA).

Figure 32. LDPN silencing restores key features of locomotion are restored following post-SCI.

Relationships between swing time, stance time, stride time, and stride distance are plotted against speed for Control (a-e) and Dox (f-j) time points. Dorsal steps are indicated with teal circles for both Control and Dox, while plantar steps are indicated with either grey or red circles for Control and Dox, respectively. An exponential decay trendline is displayed for stance time and stride time graphs, while a linear trendline is displayed for stride frequency and stride distance (dotted line indicates trendline; Stance

time: Control $R^2 = 0.476$ vs Dox $R^2 = 0.638$; Stride time: Control $R^2 = 0.406$ vs Dox $R^2 = 0.556$; Stride distance: Control $R^2 = 0.194$ vs Dox $R^2 = 0.458$; Stride Frequency: Control $R^2 = 0.259$ vs Dox $R^2 = 0.495$). 95% prediction intervals are shown for lines of best fit as solid lines. To calculate Euclidian Distance, each step's speed was multiplied by its spatiotemporal measure (i.e. speed multiplied by swing time) to create a single variable. Euclidean distance calculations measured that distance between the two variables for each animal. Those differences were compared between Control and Dox groups to show if the relationship was the same for the 2 measures (**k-o**; Euclidian Distance swing time vs speed: Control 357.1 ± 57.8 vs Dox 522.8 ± 82.9 , $t = 10.24$, $df = 7$, $p < .001$; Euclidian Distance stance time vs speed: Control 356.6 ± 58.0 vs Dox 522.2 ± 83.1 , $t = 10.22$, $df = 7$, $p < .001$; Euclidian Distance stride time vs speed: Control 356.0 ± 58.0 vs Dox 521.5 ± 83.1 , $t = 10.20$, $df = 7$, $p < .001$; Euclidian Distance stride distance vs speed: Control 270.6 ± 50.9 vs Dox 397.5 ± 76.4 , $t = 7.93$, $df = 7$, $p < .001$; Euclidian Distance stride frequency vs speed: Control 330.5 ± 56.0 vs Dox 490.9 ± 80.0 , $t = 10.53$, $df = 7$, $p < .001$; paired t-test). Average swing time (**p**, Control swing time 0.094 ± 0.011 vs Dox swing time: 0.093 ± 0.010 , $t = 0.17$, $df = 7$, n.s., paired t-test) and average stance time (**q**, Control stance time 0.189 ± 0.034 vs Dox stance time: 0.174 ± 0.027 , $t = 1.94$, $df = 7$, n.s., paired t-test) are indicated with circles representing individual animal averages. The average duty cycle (stance time/stride time) (**r**, Control duty cycle 0.651 ± 0.020 vs Dox duty cycle: 0.641 ± 0.019 , $t = 1.83$, $df = 7$, n.s., paired t-test) and average speed (**s**, Control speed 53.33 ± 9.67 vs Dox speed: 62.31 ± 11.05 , $t = 4.177$, $df = 7$, $p < .005$, paired t-test) are plotted for Control (grey) and Dox (red) time points with averages indicated by bars. 1 S.D. is indicated by the bar above the average.

	Uninjured				Injured			
Variable	Control	Dox	p-value	Sig	Control	Dox	p-value	Sig
Swing time vs speed	417.9±50.5	407.4±49.3	0.329	n.s.	357.1±57.8	522.8±82.9	.000	p<.001
Stance time vs speed	417.4±51.3	407.6±49.3	0.547	n.s.	356.6±58.0	522.2±83.1	.000	p<.001
Stride time vs speed	417.2±50.6	407.0±49.4	0.348	n.s.	356.0±58.0	521.5±83.1	.000	p<.001
Stride frequency vs speed	398.8±49.4	389.9±48.0	0.295	n.s.	330.5±56.0	490.9±80.0	.000	p<.001
Stride distance vs speed	390.0±48.0	314.4±46.8	0.282	n.s.	270.6±50.9	397.5±76.4	.000	p<.001

Table 6. Euclidian distances calculations for LDPN uninjured and injured spatiotemporal measures.

Euclidian distances calculations are shown for LDPN pre-injury and post-injury spatiotemporal measures. Averages and one S.D. are shown for each variable. Paired t-tests were used to calculate statistical differences in the averages, indicated by p-values.

CHAPTER V

GENERAL DISCUSSION AND FUTURE DIRECTIONS

A comparison of propriospinal pathways: differential roles in left-right coordination

The importance of inter-enlargement intraspinal pathways in limb coordination has long been discussed. Previous studies have suggested that LAPNs and LDPNs likely mediate hindlimb-forelimb coordination during locomotion (Juvin et al 2005; Cowley et al 2010; Brockett et al 2013; Miller 1970; Miller et al 1973). More recent work found that LAPNs are key distributors of left-right interlimb coordination of the hindlimb pair and forelimb pair (Pocratsky et al 2020). The present work has expanded on these findings and has determined that both the ascending and descending components of the inter-enlargement propriospinal system are both critical for distributing left-right temporal information at the hindlimb and forelimb pairs, with a few critical differences.

In the case of LAPNs, our results were largely consistent with those described by Pocratsky et al (2020) in terms of interlimb coordination. During LAPN silencing, patterned stepping perturbations were only seen on the ‘grippy’ Sylgard-coated surface, leaving alternation intact on the hard, acrylic surface. Similarly, LDPN silencing resulted in an analogous stepping phenotype on Sylgard. However, unlike LAPN silencing, LDPN silencing resulted in left-right disruptions on both Sylgard and acrylic equally. LDPN silencing induced similar disruption to limbs at the shoulder and pelvic girdles, but

interestingly, had less of an effect on forelimb coordination in comparison to LAPN silencing. This is interesting as it suggests that LDPNs exert less influence on forelimb locomotor circuitry, a concept we will address in later chapters. Finally, hindlimb-forelimb coordination, which was predicted in previous literature to be the major outcome affected, was minimally perturbed, suggesting that neither pathway is essential for coordination across the neuraxis.

Given that these pathways have reciprocal intraspinal projection patterns, their similarities in function are unsurprising. Namely, their involvement in left-right alternation at the hindlimb and forelimb girdles was expected. It is clear, however, that each has specific function within the CPG, even if there is some overlap. Silencing both LAPN and LDPN pathways resulted in perturbations in left-right coordination of the hindlimbs. Consistent with our previous findings, the hindlimbs and the forelimbs are equally disrupted during LAPN silencing (Pocratsky et al 2020). That is to say, the forelimbs and hindlimbs exhibited approximately the same number of steps that reached synchrony during overground locomotion. However, few forelimb steps reached synchrony during LDPN silencing, suggesting dominance of the ascending pathway in the regulation of intra-girdle coupling. *In vitro* work by Juvin and colleagues supports this concept. They found that propriospinal coupling between the lumbar and cervical CPGs was mediated to a greater extent by ascending influences from the lumbar CPG to its forelimb counterpart in the cervical cord, suggesting that interlimb coordination between the hindlimb pair and the forelimb pair relies on a caudorostral hierarchy (Juvin et al 2005). As was previously discussed in Chapter I, CPG researchers agree that the primary rhythmogenic core of the hindlimb CPG is confined to the rostral segments of

the lumbar spinal cord (Cazalets et al 1995; Bertrand & Cazalets 2002; Pocratsky 2017).

We hypothesized that LDPNs would likely have a less profound effect on hindlimb behavioral outcomes in comparison to LAPNs, and our data largely suggest this to be the case. In short, the rostral lumbar cord is capable of driving the patterned output of not just the hindlimbs, but the entire CPG.

Quadrupedal locomotion: top-down or bottom-up control?

These findings raise a critical conundrum in the field of motor control: is locomotion largely reliant upon “top-down” control, in which the cortex is responsible for driving and modulating locomotor output from the spinal cord, or “bottom-up” control, in which the spinal cord, in conjunction with sensory input, drives and modulates its own output? This has long been a controversial topic within the CPG field, given that there has been evidence provided for each school of thought (Thibaudier & Hurteau 2012). For instance, Juvin et al (2012) showed that stimulating L2 and C8 dorsal roots induced locomotor-like activity at the cervical and lumbar levels, respectively. Interestingly, stimulating the L2 dorsal root could still activate cervical CPG while synaptic transmission was blocked at the L2 spinal segment. The same result could not be produced reciprocally. The importance of this work is two-fold: (1) ascending propriospinal pathways clearly play a critical part in conveying and entraining the cervical CPG during quadrupedal locomotion and (2) this drive provides evidence for the concept of “bottom-up” control for locomotion.

Studies in the cat model provide evidence to the contrary. Akay et al (2006) used a split-belt treadmill that allowed for the forelimbs and the hindlimbs to walk at different speeds. Decreasing the speed of locomotion of the forelimbs reduced the hindlimb

locomotor cycle so that a 1:1 stepping ratio was maintained between the forelimbs and hindlimbs, but modulating hindlimb speed did not alter forelimb cycle duration, thus altering the fore-hindlimb stepping ratio (Akay et al 2006). This suggests that forelimb activity had a greater impact on the regulation of quadrupedal locomotion, or “top-down” control. It’s important to note, here, that “top-down” refers to forelimb control over hindlimbs, and not necessarily cerebral control over spinal output. This discrepancy in definition is important to understand, as it suggests that even within the field of locomotor control, terminology remains an issue.

Obviously, there are still many questions that remain unanswered in regards to locomotor control. The role of propriospinal neurons within that system cannot be understated, as the coordination of these systems is extremely complex in nature. Our findings shed some light on this puzzle given that we have looked at and examined both the ascending and descending components of inter-enlargement circuitry. Our data show that LAPNs exert a greater influence over the forelimbs and the hindlimbs jointly, while LDPNs have some influence on both but have action primarily focused at the level of the hindlimbs. While this suggests that the lumbar cord exerts a greater drive on cervical circuitry, it is likely that both “bottom-up” and “top-down” control systems exist and that the dominance of one over another reflects the specificity of the task and alterations in the environment.

In truth, the core of the locomotor system is a careful balance between spinal networks, supraspinal inputs, and sensory feedback. Our data do little to clarify the complexity underlying the traditional definition of “top-down” motor control, as we largely still do not have a full grasp of the inputs and outputs of these neurons from/onto

other neuronal population. However, they offer potential affirmations of *ex vivo* work and hint at a greater influence of sensory inputs for their functional output. Understanding their anatomical complexity, a topic which will be discussed further in a subsequent section, is essential moving forward as it will provide more pieces to the locomotor control puzzle.

Swimming vs stepping: left-right alternation driven by muscle loading

The next layer to consider is the distinct differences in silencing-induced perturbations in different sensory contexts. Throughout our studies, we saw that the “behavioral context” exerted a profound influence on the expression of the silencing phenotype. Specifically, it appears as though certain tasks overrode the silencing effects. In the next two sections, I will discuss two conditions where we saw apparent context-driven modulation of the silencing phenotype.

The first sensory context we examined was swimming. Regardless of the pathway that was silenced, the disruptions to interlimb coordination during stepping were abolished when the animals performed a swimming task. Given that swimming is a primarily hindlimb-driven behavior, what is it about swimming that results in the profound behavioral differences we observed in both studies? We must first consider the disparities between overground stepping and swimming. The most obvious difference is the environment. During stepping, the limbs are loaded throughout the stance phase of the step cycle (Kiehn 2016). Hip extension and reduced load are important sensory signals that promote the initiation of the swing phase during forward locomotion (Whelan et al 1995; Pang & Yang 2001). Swimming reduces the load applied to all four limbs (Gruner & Altman 1980), likely due to the inherent buoyancy in water. Sensory and

proprioceptive feedback from load sensors like Golgi tendon organs and group Ib afferents, are the key players in this difference (Akay et al 2014). Their contributions are essential for producing patterned limb movement during overground locomotion, a role which is drastically reduced during swimming.

In a direct comparison, the swim cycle is comprised of two phases known as the power stroke and the return stroke, akin to the stance and swing phases of the step cycle, respectively (Gruner & Altman 1980). The stance phase is typically classified by the activation of limb extensor muscles, a phenomenon that is also true during swimming. However, differences exist in the activation of these muscle groups, as unlike stepping, the hip, knee, and ankle extensors are activated simultaneously (Gruner & Altman 1980). The return stroke requires muscle activation sequentially, a feature that is distinctly different from patterns of activation seen during overground locomotion. Interestingly, the return stroke shares a similar pattern of flexor burst latency to that of stepping swing phase, indicated that the sequential activation during the return stroke is necessary to the lift the toe and reduce drag (Gruner & Altman 1980). Clearly, there are fundamental differences between stepping and swimming, despite the manifestation of rhythmic interlimb coordination of the hindlimbs in each behavior.

It is also interesting that silencing of neither LDPNs nor LAPNs was able to perturb alternation of the hindlimbs during swimming, despite considerable evidence that they influence interlimb circuitry of the hindlimbs during overground stepping. This suggests that inter-enlargement circuitry is dispensable during hindlimb-driven tasks such as swimming. Further, swimming may rely on separate circuitry that requires minimal input from muscle load afferents, responsible only for left-right alternation when the

limbs are unloaded and body weight support is lessened. We previously postulated that swimming was a largely driven by cortical input, like a safety mechanism required to keep the animal from drowning. While we do not know whether supraspinal pathways have direct connections with any swim circuitry, we can now say that indirect supraspinal influences do not affect the behavioral outcome during swimming as this would require input and modulation from LDPNs, a phenotype that was not seen during LDPN silencing experiments. Needless to say, this remains a perplexing outcome that leaves the question: what does it take to fundamentally perturb alternation during swimming?

The importance of sensory context: exploring friction and surface changes during locomotion

As previously outlined (Chapter 2), the locomotor system requires fine-tuned balance of supraspinal drive, intraspinal communication, and sensory input to produce what we consider stereotypical patterned locomotion. The execution of locomotor commands from supraspinal structures, such as the brainstem, likely occurs through interactions with distinct circuits at the level of the spinal cord (Ruder & Arber 2019). However, the locomotor command is not the only component that regulates the action of locomotion or changes due to the environment. Importantly, Ji et al (2020) demonstrated that bi-directional communication between sensorimotor and neuro-modulatory circuits was necessary for animals to flexibly select behavioral states appropriate for their sensory context. Clearly, the state of the environment can profoundly influence the behavioral state of the animal, especially in a highly dynamic behavior like locomotion. Animals can choose their behavioral state based upon sensory context and is flexible enough to switch between states (i.e. walk to gallop to bound) when the context changes (Andalman et al

2019; Ji et al 2020). Alterations in context may be as simple as changing the sensory context of the stepping surface, as we saw in both LAPN and LDPN silencing. During LAPN silencing, animals showed no disruption in left-right alternation on a smooth, slick surface, but significant perturbation in left-right coordination on a thicker, rubbery surface (Sylgard). Conversely, LDPNs silencing led to disruptions on both surfaces, suggesting that the context of the stepping surface was less influential on the descending circuitry. This presents an interesting dichotomy in which context is key for ascending inter-enlargement circuitry and dispensable for descending inter-enlargement circuitry. What could be responsible for these differences? There are a number of levels to consider, including joint loading, surface friction, and behavioral adaption or learning when repeatedly exposed to the sensory condition.

Sensory feedback from muscle and skin afferents, as well as other sensory modalities, such as the visual and vestibular systems, dynamically adapt the locomotion pattern to the requirements of the environment (Rossignol et al 2006). More specifically, actual sensory feedback can be integrated with predicted sensory feedback for subsequent steps to accurately monitor and correct ongoing motor output (Frost et al 2015). Breaking this down by each component, mere comparison of the two stepping tanks reveals an immediate difference as Sylgard is only partially transparent while acrylic is entirely transparent. Given that rats are traversing these tanks, it is possible that visual feedback signals the system to be more cautious on a see-through surface as it would pose a greater risk to the animal while walking. However, this theory fails when comparing silencing effects of LAPNs and LDPNs. If this were the case, LAPN and LDPN phenotypes would have manifested the same for both surfaces (i.e. no phenotype on acrylic and phenotype

on Sylgard). We must also consider that LDPN animals were exposed to the acrylic walking surface more times than LAPN animals were. Repeat exposure may play a role here, as it is possible that animals may learn that acrylic is a stable walking surface over time and become less hesitant upon that surface. It is also entirely possible that the vestibular system contributes to this behavioral phenotype given that balance may be affected based on joint loading and surface friction as we will consider next.

The concept of joint loading has been extensively studied in the pig model, as pigs are prone to developing locomotor disorders and joint diseases as result of abnormal joint loading on slippery surfaces. Pigs adapt their gait to potentially slippery floors by lowering their walking speed and prolonging the stance phase of their step cycle (Thorup et al 2007). Further, joint loading differed on a slippery surface as the pigs place more of the body weight on the forelimbs rather than the hindlimbs (Thorup et al 2007, 2008). In the case of silencing, we do not see extensive changes in average stance phase or walking speed for either silenced pathway, suggesting that joint loading may not be significantly altered in either situation. The concept of shifting weight-bearing to the forelimbs is interesting here, as it may relate back to the fewer perturbed forelimb-forelimb steps we see during silencing. Alternation is the more stable gait during locomotion. Therefore, forelimb disruption may be secondary to hindlimb disruption as a means of maintaining stability in a higher risk environment.

At a given moment during travel, a quadrupedal animal must use a particular gait, such as walking or bounding. Gait characteristics prior to and at limb contact play crucial roles in slip severity (Moyer et al 2006). A limb usually begins to slide once contact is made with a low-friction surface and the sliding distance, or slip displacement, is

proposed to dictate the probability of a fall (Clark & Higham 2011). Floor friction also has a considerable impact on walking pattern in another quadrupedal species, cows (Phillips & Morris 2001). Similarly, cows increase their step length and reduced the number of steps on surfaces that have a higher coefficient of friction. Interestingly, cows maintained their speed and moved toward their target with frequent, short steps (Phillip & Morris 2001). The coefficient of friction for acrylic was around 0.4 (akin to a slippery tile floor), while the coefficient of friction for the Sylgard-coated surface was above 1 (think, hot rubber tire to asphalt). Once again, this conveys stability to the locomotor system as animals are more likely to adopt a risky gait (bounding) when there is less likely to be slippage during the subsequent steps. It appears that LDPNs are less sensitive to this tactile input, as these animals largely ignore the slipperiness of the surface and proceed with riskier gaits regardless of the sensory surface. Moving forward, altering the sensory surface at unpredictable intervals across a single locomotor pass could provide some insight into how the system adapts to changes in the sensory environment in both the silenced and unsilenced conditions.

In the bipedal guinea fowl, postural control strategies, such as a forward shift in center of mass over the limb's base of support, were employed to avoid slips during locomotor bouts (Clark & Higham 2011). Similar findings have been described in humans (Strandberg & Lanshammar 1981; Cham & Redfern 2002; Moyer et al 2006). Whether these findings extend to animals that rely on quadrupedal locomotion remains unclear. Further exploration into measures of postural stability in uninjured, silenced animals may shed more light on these findings as it is currently unclear whether animals adopt any distinct postural changes to adapt to either sensory surface.

A perplexing finding: improvements in stepping during post-SCI silencing

In humans, SCI is a devastating neurological disorder that affects thousands of individuals each year. Within the field of SCI, a major focus has been on restoring body systems to their once fine-tuned, highly functional state. Enormous progress has been made in understanding the molecular and cellular events generated by SCI, providing insight into crucial mechanisms that contribute to tissue damage and regenerative failure of injured neurons. The treatment of SCI in clinical patients remains predominantly palliative, largely instituting measures to handle muscle spasticity, autonomic dysreflexia, and neuropathic pain, amongst others.

Propriospinal neurons have emerged as a potential target for recovery of locomotion post-SCI. The descending propriospinal components have received a large part of this focus, given their potential sparing across the injury site and their role in integrating neural signals from descending supraspinal pathways (Cowley et al 2008). A number of studies have implicated LDPNs, specifically, as they have been shown to lead to functional recovery via collateral sprouting and formation of “detour circuits” (Bareyre et al 2004; Vavrek et al 2006; Ballermann & Fouad 2006; Courtine et al 2008; Fenrich and Rose 2009; Rosenzweig et al 2010; Flynn et al 2011; Filli et al 2014; Benthall et al 2017). Despite this, the extent of function recovery is still largely unexplored and is often classified differently between studies. Over the course of this dissertation, we have attempted to further understand the role of long propriospinal neurons in recovered locomotion after SCI and have unearthed some perplexing findings as a result. Before diving deeper into potential explanations for these findings, I will recap those findings.

Both LAPNs and LDPNs represent intraspinal pathways whose axons reside within the rim of spared white matter that remains after SCI. Immunohistochemistry and white matter staining confirmed this for both neuronal populations (Fig 15,16,25,26), going so far as to prove that EGFP.eTeNT was actively transported across the level of injury as it is found in the terminal fields for both pathways. This is essential, as our results cannot be conclusively interpreted without evidence of viral transport across the injury epicenter. LAPNs are the lesser explored pathway after SCI. We first silenced this pathway, predicting that silencing would be detrimental to recovered locomotor function as it represents spared functional neural circuitry. What we found was at best surprising and at worst, controversial and difficult to explain. LAPN silencing resulted in substantial improvements in interlimb coordination and intralimb coordination, both key temporal components to the production of better stepping after injury. As we continued to digest these findings, we turned to the LDPNs with the same predictions and were once again shocked that silencing LDPNs not only had minimal effect on recovered hindlimb locomotion, but any effects that were seen were positive. Interestingly, forelimb coordination remained unaffected during silencing for either pathway. Together, these findings suggest that after SCI, these specific inter-enlargement propriospinal neurons are deleteriously influencing hindlimb motor function such that when they are functionally silenced, locomotor outcomes improve in a pathway-specific manner. In the next few sections, we will further discuss these results and attempt to reconcile them with other findings in the field of SCI research and beyond.

Disparate influences of LAPNs and LDPNs on CPG circuitry after SCI.

Takeoka & Arber (2019) noted that after SCI, spinal circuits caudal to the lesion retain the ability to generate basic motor patterns and are often adequate to re-establish basic motor functions. Our data are consistent with this statement. When we remove propriospinal influences on lumbar motor circuitry, those spinal circuits are still able to maintain patterned motor function. In fact, they perform this function better without propriospinal interference. Prior to injury, the LDPNs exerted less influence on the overall circuitry of the spinal cord in comparison to LAPNs, leading to a phenotype that was primarily hindlimb-focused rather than globally-focused. Based on the data presented here, LAPNs wield greater influence on hindlimb and forelimb circuitry prior to injury, which translates to greatly increased influence on hindlimb circuitry post-injury. Is it possible that pathways with cell bodies below the level of injury are now unbalanced in their respective input onto basic pattern generating hindlimb circuitry that remains intact and unperturbed? And further, two pathways that once interacted with both forelimbs and hindlimbs now only interact with hindlimb circuitry. Therefore, is forelimb circuitry the safety net of the locomotor system after injury such that it cannot be perturbed as it is required to maintain any movement? Several concepts are likely at play here in a complex system that we don't fully understand.

To parse this out, let's examine the decreased impact that LDPNs have on hindlimb circuitry after injury in comparison to LAPNs. Three distinct differences can be immediately identified: (1) cell bodies are located above the level of lesion with (2) likely unperturbed connections with supraspinal centers and peripheral inputs that are (3) a much greater distance from the injury site than LAPNs. These features are all important to consider for a number of reasons. As pointed out in previous sections, descending

propriospinal neurons have been implicated in establishment of descending detour circuits to innervate circuitry below the level of lesion (Bareyre et al 2004; Vavrek et al 2006; Courtine et al 2008). These circuits have been described as a functional bridge between supraspinal centers and hindlimb motor circuitry that is well-suited to pass the injury site (Ballermann & Fouad 2006; Courtine et al 2008; Rosenzweig et al 2010). Yet, many of these studies fail to address changes in LDPNs themselves; rather, they address new supraspinal connections onto descending propriospinal populations. The concept of neuroplasticity is critical here, which is why there is an entire section devoted to the topic later in this chapter. Making connections after SCI is essential, but making functionally meaningful connections is far more important. There is the potential that LDPN populations are now receiving information from supraspinal centers that they are unable to interpret or that they may be misinterpreting. Therefore, they cannot effectively carry out their role and are potentially contributing to functional locomotor deficits that we see after chronic SCI. It is also important to note that the long descending pathways discussed in this literature often originate from different locations throughout cervical and rostral thoracic spinal segments and terminate throughout the lumbar cord. Our specific subset of LDPNS may be carrying out a different or diminished function in comparison to other LDPN pathways with cell bodies located in higher cervical segments.

However, if we consider the same principle for LAPNs, the picture is even muddier. Any supraspinal pathways would need to anatomically and functionally cross the level of injury to be able to connect with LAPN cell bodies in the lumbar cord. Importantly, incoming sensory information from the hindlimbs would likely maintain its

contact with LAPNs, as it would for LDPNs with respect to the forelimbs. It seems plausible that misinterpretations are happening on both sides of the injury, though this mechanism remains unclear. Could it be that cell body proximity to the injury is directly related to the negative influence it has upon spared hindlimb circuitry? Our studies indicated that LAPNs exert a much greater influence on lumbar circuitry after SCI than LDPNs, providing evidence for this case. Perhaps maladaptive plasticity is occurring for both LDPNs and LAPNs and the degree to which this affects lumbar circuits is directly related to location. Or perhaps LDPNs, that retain a majority of unperturbed connections, are responsible for maintaining posture and body orientation, leaving unbalanced input on the part of LAPNs for modulating locomotor output.

The “V-neuron” story in a post-injury context.

In the uninjured spinal cord, spinal interneurons can be classified into distinct populations based on the transcription factors they express during embryonic development, also known as progenitor domains. Most relevant to motor control are those that are derived from ventral domains or “V” neuron classes. In Chapter IV, we briefly addressed the concept of V-neurons and how LDPNs relate to these classes prior to injury. Here, we will review the functional roles of V-neuron populations prior to and post-SCI and how the long propriospinal populations we presented here fit within this context after injury.

V-neurons can be further broken down into 4 sub-categories: V0, V1, V2, and V3. V0 interneurons derive from Dbx-1 expressing progenitor cells and are mainly composed of commissural interneurons projecting axons to the contralateral side of the spinal cord (Cote et al 2018). They can be further divided into two subgroups: a ventrally derived

population (V0v) comprised of mostly Evx1-expressing excitatory interneurons and a dorsally derived population (V0D) comprised non-Evx1 expressing inhibitory interneurons (Arber 2012). Functionally, the dorsal population contributes to left-right alternation, while the ventral population is recruited for a similar function at higher speeds (Lanuza et al 2004; Crone et al 2009; Talpalar et al 2013). V3 neurons are also mainly commissural and are defined by the expression of Sim1 transcription factor. It is highly heterogenous in terms of its synaptic targets and its distribution throughout the cord (Zhang et al 2008). V3 interneurons are also thought to play a critical role in interlimb coordination (Zhang et al 2008).

In contrast to V0 and V3, V1 and V2 classes of interneurons project almost exclusively ipsilaterally (Cote et al 2018). V1 interneurons are primarily inhibitory interneurons expressing the transcription factor, EN1. The function of V1 interneurons is largely unknown, as it has been shown that selectively silencing all V1 interneurons decreases the speed of locomotor bursts, but it does not impair ipsilateral coordination (Gosgnach et al 2006). V2 interneurons are broken up into two subclasses: V2a neurons, Chx10-expressing ipsilaterally-projecting excitatory neurons, and V2b neurons, Gata2-expressing ipsilaterally-projecting inhibitory neurons (Jessell 2000; Flynn et al 2017). V2a neurons are believed to be involved in left-right alternation (Crone et al 2008, 2009). V2b interneurons are thought to have some level of functional redundancy with V1 interneurons, as removing both pathways impairs ipsilateral flexor-extensor coordination (Zhang et al 2014).

Despite the extensive amount of attention V-neuron populations receive, their roles after SCI are largely unexplored. Glutamatergic V2a interneurons have been looked

at in the context of respiratory deficits after SCI (Zholudeva et al 2018). Lin and colleagues also found that sensory activation of V3 neurons post-SCI led to the production of muscle spasms (Lin et al 2019). Other than these, the information on ventrally-derived neurons after SCI is sparse.

We have been unable to reconcile our silencing data with current genetic models. To better address this issue, we would first have to explicitly demonstrate which genetically-encoded V-class series the LAPNs and LDPNs represent in the adult mouse spinal cord. While this idea is simple in theory, it poses significant technical challenges. Silencing LAPNs and LDPNs in the uninjured cord results are consistent with functional roles of some V-class neurons, including the V1, V2a, and V3 populations, but the populations of LAPNs and LDPNs on interest here are heterogenous in both their anatomical properties and their neurotransmitter phenotypes. Lumbo-cervical neurons with similar anatomical layouts to both LAPNs and LDPNs are not derived from one genetically-encoded domain (Ruder et al 2016). Moreover, there are clear differences in developmental versus functional modules of select, genetically-encoded neurons (Borowska et al 2013; Borowska et al 2015). Post-injury, it is even more difficult to reconcile our findings with genetic correlates as few studies exist that explore V-neuron classes after SCI, likely due to the fact that the genes that mark V-class subtypes are downregulated soon after neurogenesis (Alaynick et al 2011). There is a high likelihood that these anatomically-defined pathways are derived from multiple progenitor domains with a range of transcription factor expression profiles, a problem which creates a potentially permanent void between anatomical and genetic literature.

Post-injury neuroplasticity: a gift or a curse?

One finding that is interesting to note is the effects of silencing in regards to forelimb alternation: silencing either LAPNs or LDPNs did not perturb forelimb alternation at any point post-injury, despite significant disruptions in the otherwise intact animal. Clearly, there is some level of hierarchy in which the forelimbs are deemed essential. The risk of losing coordination of any kind could pose too high a risk for the animal's ability to navigate its environment. Hindlimb-forelimb coordination, which was perturbed during control time points post-SCI, remained unaltered during silencing, suggesting that the only circuitry that can be influenced is that of the hindlimbs. Do these findings suggest the presence of maladaptive plasticity below the injury site?

First, it is critical to consider that there are many forms of plasticity and not all plasticity is maladaptive. Neuroplasticity can range from changes in gene expression following SCI (Gris et al 2009) to physical growth of new dendritic synaptic connections. Regeneration of intraspinal axons after SCI may allow them to restore connections that were lost, strengthen existing synapses, or make new connections altogether (Brown & Weaver 2012; Ferguson et al 2012). Some recovery of motor control after spinal cord hemisection in cats has been attributed to collateral sprouting of primary afferent axons (Goldberger et al 1993; Helgren & Goldberger 1993). In chronic spinal cats, increased excitatory post-synaptic potentials were attributed to an increase in sprouting of primary afferent fibers (Nelson & Mendell 1979). More recent studies have demonstrated that among sensory feedback populations, proprioceptive afferents innervating muscle spindles were essential for spontaneous locomotor recovery after SCI (Takeoka et al 2014; Takeoka & Arber 2019). Therefore, neuroplasticity can be considered a key feature

of spontaneous recovery from SCI, regardless of whether that plasticity has a positive or negative influence on remaining spinal circuitry.

Maladaptive plasticity is not exclusive to SCI. After stroke, maladaptive plasticity contributes to the pathogenesis of phantom pain and dystonia (Flor 2008), weakens motor function and limits motor recovery (Takeuchi & Izumi 2012; Murase et al 2004; Takeuchi et al 2007). Interestingly, there are few discussions of maladaptive plasticity in locomotor recovery following SCI, but it is a concept that has been addressed in other areas of SCI. In terms of post-SCI outcomes, maladaptive neuroplasticity is thought to manifest as neuropathic pain (Christensen & Hulsebosch 1997; Grau et al 2017), urinary tract dysfunction (deGroat & Yashimura 2006), autonomic dysreflexia (Michael et al 2019), cardiac arrhythmias (Collins et al 2006), and sexual dysfunction (Nout et al 2006; Johnson 2006).

Some of these dysfunctions are caused, in part, by an imbalance of inhibitory and excitatory synaptic inputs to spinal neurons, while others relate to a loss of coordination of autonomic and somatic control (Brown & Weaver 2012). This imbalance of excitatory to inhibitory terminals may aid in explaining the perplexing findings here. We have shown that LAPNs and LDPNs colocalize with GABAergic and glutamatergic markers, indicating a heterogeneous population that is both excitatory and inhibitory in nature after injury. However, there is no indication whether the proportion of these markers changes post-SCI. Flynn et al (2017) found that 85% of LDPNs were excitatory (15% inhibitory) which is supported by the dominance of excitatory projections in LAPNs (Brockett et al 2013). In contrast, a population of premotor LDPNs and descending thoracic PNs that directly innervate tibialis anterior motoneurons contain roughly *equal* numbers of

excitatory and inhibitory interneurons (Ni et al 2014). Unfortunately, each of these studies focuses on a different subset of propriospinal neurons, making unequivocal interpretation impossible. However, these studies do agree on the heterogeneity of inter-enlargement pathways, which if altered due to maladaptive plasticity, may change their function and the effectiveness of communication with other neuronal populations.

After SCI, supraspinal and spinal sources of control of movement differ substantially from that which existed prior to the injury, thus resulting in an altered spinal cord. This concept implies that the spinal cord processes input and generates motor output in a different manner as a result of injury-related adaptations (Edgerton et al 1997, Edgerton et al 2001). Interestingly, our data did not indicate that any functional plasticity is occurring, as in both studies, animals returned to poor locomotor function between silencing bouts and the effects were maintained for up to three weeks during silencing. However, in this altered state, it would be naïve to think that LDPNs and LAPNs were not undergoing some forms of plasticity themselves. It could be that the length of Dox administration was not long enough to induce extensive plasticity, or perhaps it is not a great enough insult to produce drastic plastic changes in the cord. However, it is safe to assume that, given the presence of plasticity in other facets of the entire motor system, LDPNs and LAPNs are not singled out from the phenomenon which begs the question: can we control their plasticity after injury? Some have attempted to answer this question with activity-based training, which we will address in the next section.

Mitigating negative effects of maladaptive plasticity post-SCI using activity-based training

Rehabilitative therapies often target circuitry both rostral and caudal to injury in the spinal cord by activating components of the nervous system specific to the behavior being trained (Lynskey et al 2008). In a series of studies, spontaneous partial functional recovery was improved by systematically training spinalized cats to step on a moving treadmill. Trained spinalized cats showed functional recovery three times greater than untrained animals after two months of training (Lovely et al 1986; de Leon et al 1998). These findings were critical to the understanding of motor circuitry, as De Leon and colleagues demonstrated that spinal cord circuits disconnected from their supraspinal inputs are able to ‘learn’ a motor task (De Leon et al 1998). This leads us to the concept of activity-based plasticity, in which behaviors such as stepping can be produced and refined by utilizing training paradigms that specifically train the behavior of interest.

Activity-dependent training may be one rehabilitative approach to address the potential maladaptive plasticity occurring in LAPNs and LDPNs after SCI. Clinically and in animal models, the benefits of increased physical activity and rehabilitative training post-SCI are well established, and include improved motor function, reduced cardio-metabolic syndrome, and attenuated neuropathic pain (Sandrow-Feinburg & Houle 2015; van den Brand et al 2012; Behrman & Harkema 2000; Kuerzi et al 2010). In fact, engaging somatosensory and proprioceptive feedback specifically plays a key role in retraining the system toward improvement rather than aberrant detour circuits (Ballermann and Fouad, 2006; Courtine et al., 2008; Rosenzweig et al., 2010; Takeoka et al., 2014; Tohyama et al., 2017; Zorner et al., 2014). Recently, Takeoka & Arber (2019) looked at somatosensory feedback from proprioceptive afferents caudal to the level of injury and found these afferents are indispensable for maintaining regained motor

function of the hindlimbs and are necessary to disseminate proprioceptive information to remaining intact circuitry. These results implied that task-specific rehabilitative training with an emphasis on sensory feedback would maximize functional outcomes (Takeoka & Arber 2019). Further studies support these findings as Goldshmit et al (2008) found that proprioceptive and cutaneous inputs were essential to induce plasticity in spinal cord neurons. Swimming, a task which effectively eliminates the limb-loading phase of the step cycle, was beneficial only when cutaneous feedback was provided (Kuerzi et al 2010; Smith et al 2009). Swimming-intensive rehabilitative training brought about significant improvements in hindlimb performance during swimming, but did not alter the normal course of recovery during overground stepping (Smith et al 2006; Smith et al 2009). Taken together, these findings suggest that sensory input to the CPG circuitry requires refinement and retraining in a task-specific manner to produce patterned locomotion after spinal cord injury.

Our behavioral data show that LAPNs are heavily involved in temporal coordination of individual limb CPGs. It is conceivable that local interneurons (Renshaw cells and short propriospinals) provide inputs to LAPNs. Further, our data showed that VGlut1 terminals make contacts on LAPNs, providing more evidence that sensory afferents will also provide input to LAPNs. Based upon this anatomy, it is possible that LAPNs are misinterpreting incoming sensory information and descending drive, which translates to a diminished capacity of the hindlimb CPG to produce appropriate temporal output. Perhaps the relationship between sensory input, LAPNs, and CPG circuitry could be improved through activity-based refinement of their synaptic connections. Very little is known about the mechanism of plasticity that drives motor improvement in the

presence of activity-based therapies. If their anatomical or molecular plasticity could be refined, LAPNs could be key players in restoring the balance necessary to push patterned locomotion towards normalcy. If they are interfering with essential communications between proprioceptive afferents and their CPG targets, removing LAPNs from the system would lead to the improvements we see in our data.

This theory makes sense in the context of LDPNs as well. LDPN populations co-localize with a diverse range of convergent inputs including putative premotor inhibitory interneurons, corticospinal fibers, and proprioceptive afferents (Flynn et al 2017). Unlike LAPNs, LDPNs are more likely to maintain these relationships given their distance from the lesion epicenter, suggesting most of their communication ability remains intact post-SCI. LDPNs do not appear to be the primary source of locomotor disarray for the hindlimbs, since silencing only modestly restores stepping ability.

Additionally, we have shown that both pathways demonstrate different responses in regards to the sensory context of the walking surface. In conjunction with the sensory findings described in Chapter II, further exploration is warranted to determine the effect of sensory surface context on silencing outcomes after SCI, especially given the extensive evidence provided here as to the importance of sensory feedback on adaptive plasticity in the locomotor system. Furthermore, if we are going to expect to flip the switch on these perplexing findings, extensive task-specific overground locomotor training may be necessary to refine the connections of these neuronal populations after injury.

The field's approach to analyzing locomotion after SCI: significant weaknesses that plague interpretation

Activity-based rehabilitation may be a promising therapeutic approach in recovering lost function after SCI, but the components necessary for optimal locomotor retraining have not yet been determined. SCI research often uses the term “recovery”, yet the wide range of techniques employed to improve locomotor outcomes leads to highly varied definitions, making actual recovery difficult to classify. Different exercise training strategies have been applied to improve locomotion following SCI, including forced exercise regiments like treadmill training or bicycling and volitional retraining via in-cage activity or overground locomotion. Measures like overground stepping, treadmill stepping, BBB scores, swimming, and cycling are all behavioral outcomes that are also utilized to determine functional recovery after SCI (Battistuzzo et al 2012).

The enormous amount of variability in both the definitions of exercise/training and the behavioral tests used to evaluate functional outcomes post-injury presents an innate problem in the field of locomotor recovery post-SCI. In a methodological analysis, Battistuzzo *et al* (2012) found that exercise training was reported to improve some aspect of locomotor recovery in 30 of 41 SCI studies. Yet, a more concerning problem still remains. Battistuzzo *et al* also found that positive outcomes were observed in 55% of rat studies that had anatomically complete SCIs and 75% of rat studies with anatomically incomplete SCIs (Battistuzzo et al 2012). Amongst all 41 studies that were examined, functional scales such as the BBB scale were the most widely used method to assess recovery of locomotion. Of 28 studies using observational scales in addition to other functional outcomes, only 13 (46%) used blinded observers, and of the 16 studies that used observational scales alone, only 3 showed negative results (Battistuzzo et al 2012; Erschbamer et al 2006, Ung et al 2010). The validity of these studies is questionable, as

they rely on subjective outcomes rather than objective outcomes. This is concerning on a grander scale, as it is not only counter-productive to the field of post-SCI locomotor research, but it brings into question the reliability of a great many studies not mentioned here that may fall victim to the same plight. It is critical to keep this in mind as we interpret the improvements we find during LAPN and LDPN silencing post-injury. A number of studies describe extensive training protocols but use BBB as the primary outcome of improvement, hardly convincing evidence for a behavior as complex as locomotion.

Randomized clinical trials comparing treadmill training with conventional overground locomotor training have shown only small or no differences between these two approaches in outcomes related to walking in the human condition (Dietz et al., 1995, Dobkin et al., 2006, 2007; Field-Fote and Roach, 2011). It can be argued that over-ground training offers an environment more similar to functional walking than the treadmill. As it has been established that the behavior of interest requires task-specific training, exercise may only improve performance in the trained activity itself, and may even reduce the capacity to perform other motor tasks (De Leon et al., 1998; Magnuson et al., 2009).

This is not to say that there are no studies that have explored volitional overground locomotion post-SCI. Several studies were found that assessed overground locomotion using objective methods similar to those described in Chapters III and IV. Interestingly, though, there were still disparities amongst these papers, as some reported a positive outcome in only a few of the test parameters, while others reported improvements in all outcome measures (Van Meeteren et al 2003; Lankhorst et al 2001;

Singh et al 2011, Takeoka et al 2014; Takeoka & Arber 2019). From this perspective, the improvements we found as a result of silencing LAPNs and LDPNs after injury are even more difficult to interpret. Moreover and more importantly, the lack of clarity in these findings may be the root of the problem plaguing clinical progress in the field of locomotion. Animal studies have rarely led to substantial developments in the clinical setting, which may be due, in part, to the issues presented here. Ultimately, the lack of consistency plagues the field of functional recovery and reconciling our findings in a sea of inconsistent results may only be possible with significant improvement in the rigorosity of behavioral outcomes.

Neural network noise: a potential explanation for improvements in a fine-tuned system

In a seminar at the University of Louisville in the spring of 2020, Dr. Shawn Hochman said, “There’s a lot of ways to get to a solution in a system of intense complexity.” As spinal cord scientists, this concept of “intense complexity” is one we know exists but we don’t necessarily like to consider. Obviously, we cannot study every aspect of the spinal cord in individual studies, as doing so would be impossible. At its core, the spinal cord environment is highly intricate and finely tuned. Spinal cord injury adds a layer of complexity that fundamentally changes this environment in ways that create chaos and misinterpretation at multiple levels.

We have speculated thus far that L2-C6 interneurons are detrimental due to maladaptive plasticity or lack of refinement of this plasticity in the context of CPG circuitry, such that removing them results in improvement. In the midst of these speculations, the question still remains: why did we see improvement at all? Could it also

be that both pathways represent aberrant “noise” within the system such that removal leads to improved communication and function for other neural circuitry? It seems as though a synergy exists between these pathways prior to injury in which they balanced “good” and “bad” signal with incredible precision, like a fine-tuned humming machine. For these two systems to work in synergy, each system must have intrinsic activation and inhibition patterns that can generate coordinated motor outputs and the system itself must be able to recognize and interpret these signals as such. The temporal patterns of peripheral inputs must be matched with the intrinsic CPG activity for locomotion to continue effectively. After SCI, it appears that the “good” signal may be lost amongst excessive noise in the system, resulting in an inability to differentiate between signals. Removing any of this additional noise may result in improved communication. This is an interesting concept, given that LAPNs specifically were shown to be detrimental to the temporal components of the step cycle when un-silenced.

Excess variability within the nervous system poses a fundamental problem for information processing from perception of sensory signals to the generation of motor output (Faisal et al 2008). The concept of synaptic noise has mainly been explored in the context of cortically driven behaviors such as audition and sleep (Steriade et al 1993; Cowan & Wilson 1994; Wehr & Zador 2003). However, the principle components of synaptic noise still ring true in a spinal context. Synaptic noise results from the noisy biochemical processes that underlie synaptic transmission and is present even in the uninjured spinal cord (Faisal et al 2008). The spinal cord is built to interpret these signals, quickly integrating those that are essential to the current behavioral context and ruling out others (Christakos et al 2006). For our purposes, LAPNs and LDPNs represent noise

inputs to the hindlimb CPG. In the uninjured cord, certain contexts, such as that of the sensory surface, determine whether the input of propriospinals is relevant to the production of motor output at any given moment. On the acrylic surface, the input of LAPNs is required to secure left-right alternation. That same input is dispensable on the Sylgard surface, indicating that the system is now interpreting this input differently. LDPNs are required on either surface, suggesting that their input is not context dependent and is needed in multiple behavioral contexts. Alternatively, the inputs of LAPNs is required to maintain alternation on the Sylgard surface and their removal results in an inability to maintain alternation in that sensory context, consequently indicating that LDPNs are essential in alternation maintenance on both acrylic and Sylgard surfaces. This may be due to redundancy in the system that renders LAPNs dispensable on the slippery surface and essential on the grippy surfaces.

If we carry these principles to the injured cord, the baseline level of noise is now fundamentally altered, though whether it is increased or decreased as a whole is unknown. The neural networks that were in careful balance prior to injury are now in chaos, with some systems exerting metaphorical noise levels that are too quiet to be heard and others that are too loud to be silenced. In a way, these signals are not “bad” noise; they are just the wrong frequency. In this case, LAPNs and LDPNs would represent the latter. LDPN influences may take a backseat to LAPNs, though, as LAPN noise may be so loud, the system is disregarding other, less bothersome inputs. If we put this entire picture together, LAPNs and LDPNs represent an anatomically spared population of neurons after injury that are maladaptively contributing to the locomotor system, through unknown mechanisms of plasticity or neural misinterpretation. The spinal cord, a system

of “intense complexity” has become more complex as a result of injury and in addition, we add maladaptive plasticity as yet another layer of complexity. As we take away the aberrant noise produced by these pathways, we remove complexity, thereby restoring function.

The importance of propriospinal neurons in other motor behaviors: clues to interpret complex findings post-SCI

Thus far, we have only referred to long propriospinal populations in the context of locomotion generated by the spinal cord. However, we previously mentioned that long propriospinal pathways, especially descending populations, are strategically placed in the cord to receive supraspinal input that influences their effect on motor output. In the next two sections, we will discuss what is known about different populations of propriospinals in relation to their inputs and in the context of other diseases, as well as address what is currently understood about propriospinal anatomy after SCI and how that affects our path moving forward.

Other than locomotion, propriospinals are also implicated in skilled forelimb behaviors. A recent study performed in monkeys and using a mix of retrograde and anterograde viral tools showed that the silencing of neurons located at C3-C5 and projecting to C6-T1, or short descending propriospinal neurons, induces impairments in forelimb reaching and grasping behaviors (Kinoshita et al. 2012). The deficits seen during silencing reversed a few days later, suggesting that compensation of function developed via unaffected descending supraspinal projections, such as corticospinal, reticulospinal or rubrospinal pathways (Kinoshita et al 2012). A series of studies by Isa and colleagues found that propriospinal neurons originating from C3-C4 could mediate

disynaptic pyramidal tract excitation to the forelimb motor neurons in the macaque monkey (Isa et al 2006, Isa et al 2013). Early experiments in cats looked at another populations of intersegmental C3-C4 interneurons, and suggested they were involved in forelimb-specific behaviors such as reaching (Alstermark et al. 1981). Interestingly, a fraction of these propriospinal neurons also send ascending projections to the lateral reticular nucleus (LRN) in the brainstem, which in turn interact with cerebellar mossy fibers (Alstermark & Ekerot 2013, Ruder & Arber 2019). Obviously, these are very different populations of spinal interneurons, as they originate from higher cervical segments and maintain influences on behaviors not related to locomotion. Despite this, it is interesting to consider the idea that our population of LDPNs may also be adapting to ongoing behavior through both descending motor command integration as well as through other pathways, such as cerebellar circuitry. We will further discuss the importance of exploring the potential inputs and outputs of LDPNs in the subsequent section, but for now, it is critical that we understand that propriospinals are involved in complex behaviors aside from locomotion.

For instance, studies in stroke patients revealed that facilitation of descending drive to forearm flexor muscles was being transmitted via C3-C4 propriospinals as a post-stroke compensatory mechanism (Stinear & Byblow 2004). Propriospinal neurons have also been implicated as key players in generating essential tremor, characterized as postural or kinetic tremors that accompany any motor action (Louis 2005; Zhao et al 2011). Hao and colleagues postulated that cortical commands of both single and double tremor frequencies are processed at the level of propriospinal neurons and are necessary to compute the alternating burst pattern between flexor and extensor muscles, suggesting

that propriospinals relay essential corticospinal transmission of oscillatory signals in Parkinsonian tremors (Hao et al 2013). Using computational models, Qu *et al* further confirmed this finding by concluding that propriospinal neurons with strong inhibitory cortical connections acted as a gate in the generation of essential tremor as they were essential for relaying the central oscillation signal (Qu et al 2017). This is intriguing, as essential tremor oscillations are characterized by the quick oscillations between flexion and extension, a behavior that is slower and heavily controlled during locomotion.

This provides evidence for another instance in which propriospinals are regulating the temporal characteristics of a behavior. It stands to reason that similar assumptions could be made of LAPNs and LDPNs in this case, as it seems entirely plausible that inter-enlargement propriospinals could be acting as a similar gating mechanism for locomotor outputs. Perhaps acting as a “gate” for gait? From our data, we know that LAPNs and LDPNs are involved in the temporal coordination of certain aspects of stepping in a context-dependent manner. It’s possible that upon receiving certain inputs from the periphery, inter-enlargement propriospinals will either leave the gate in place, effectively maintaining alternation of the hindlimbs and forelimbs during uninjured stepping, or will lift the gate, resulting in greater flexibility of the system to choose an appropriate gait for the context. After injury, perhaps this gating mechanism is ineffective, largely preventing lumbar autonomous circuitry from generating temporal coordination conducive to “good” stepping, thus suggesting that removing them during silencing results in the gate being opened and restraint on the lumbar cord being lifted, resulting in improved function of autonomous pattern-generating circuitry below the level of lesion.

The new frontier: exploring the anatomy and synaptic inputs of propriospinal populations

Much of the speculation discussed in this chapter could be explained with further exploration of propriospinal anatomy and the changes in that anatomy after SCI. Conta Steencken and colleagues devoted several studies to addressing some of these questions (Conta Steencken et al 2009; Conta Steencken & Stelzner 2010). Initially, they found that proportionally more long descending tract neurons remained after spinal contusion in comparison to short thoracic propriospinal neurons (Conta Steencken & Stelzner 2004). They later explored the extent to which spared long descending tract neurons (C3-C6 to lumbar segments) sprouted after injury, describing descending fiber terminals as “small abnormal-appearing putative terminal boutons or reactive endings in the intermediate grey matter of the lumbosacral cord, with little axonal arborization and no evidence of injury induced sprouting” (Conta Steencken et al 2009). At first glance, these findings may seem counter-intuitive to the ideas we have postulated previously; however, when we further dissect the above statement, there are several key concepts to address. Despite suggesting that extensive anatomical branching did not occur, the concept of small abnormal terminal boutons is highly relevant to our silenced findings. This could suggest that even in the absence of anatomical plasticity, propriospinal neural transmission may be significantly altered in a way that compromises the integrity of the signal as it passes to the post-synaptic connection. Additionally, they suggest that these endings are highly reactive, indicating that they may be over-excitabile, further supporting our idea of noise-related improvements when LAPNs and LDPNs are removed from the system. Finally, the pathway they have chosen to examine is extensive and less specific than which we

have described, originating from higher cervical segments and terminating throughout the lumbar enlargement. These neurons could be involved in a wide variety of other tasks outside of lumbar temporal coordination, as our neurons of interest may represent only a subset of their described population.

No studies have attempted to address the anatomical characteristics or the functional role of LAPNs after SCI. It is difficult to extrapolate our results to those of LDPNs, given that we know they have different influences on lumbar CPG circuitry. Work in our lab has suggested that LAPNs receive few 5-HT inputs, as these putative terminals are found mostly in laminae 1 and 2 and surrounding motor neurons, with only modest amounts in the intermediate grey matter (unpublished work, Brown). Immunohistochemical (IHC) staining in Chapters III and IV suggested that both LAPNs and LDPNs co-localize glutamatergic and GABAergic putative synaptic terminals, suggesting both are heterogenous populations. But these indications are not definitive, as IHC can only provide so much information about synaptic details.

To further parse out these perplexing findings, future explorations of the anatomical makeup of post-injury LAPNs and LDPNs will be crucial. In addition to anatomical changes, defining the inputs and outputs of both pathways will also be essential, as we need a better understanding of which pathways are talking to them and which pathways they are talking to. New techniques, such as the development of double-deletion-mutant rabies viral vectors, are emerging to better speak to this conundrum, as they will allow us to directly address these questions (Chatterjee et al 2018). Specifically, the use of recombinant rabies would allow us to explore a wide range on inputs onto LAPN and LDPN cell bodies. The number of dendritic connections may not be

physically changing, but knowing pathways that supply input would be an excellent step towards making sense of our functional data.

Clinical significance of inter-enlargement propriospinal neurons

Animal models have shown that CPGs are essential for rhythmic generation of stepping movements, a finding that carries to human locomotion (van Hedel & Dietz 2010; Dietz et al 1995; Duysens & Van de Crommert, 1998). Though there is no substantial anatomical evidence that directly identifies the presence of inter-enlargement propriospinal pathways in people, several observations strongly argue for their presence. For one, stimulation of some spinal networks underlying locomotion resulted in the activation of flexor afferents in motor complete SCI patients (Roby-Brami & Bussel 1995). Sleep-related rhythmic movements have also been observed in motor complete SCI subjects and coordinated leg movements can be observed in experimental conditions such as spinal cord stimulation and vibration-induced air stepping (Lee et al 1996, Rosenfield et al 1995, Selionov et al 1997, Gerasimenko et al 2010). Taken together, these findings present a clear case for the presence of the locomotor CPG in humans, especially after spinal cord injury.

The potential for LAPNs and LDPNs to be spared after injury is the main component that drove the studies described in Chapters III and IV, so establishing the clinical relevance of these studies is key. Yet, the amount of influence that inter-enlargement circuitry has on human locomotion after spinal cord injury is widely contested. A 2011 study from the Behrman lab demonstrated lower limb locomotor training has been shown to increase the presence of arm swing in SCI patients, suggesting that intraspinal pathways maintain some level of functional integrity when trained (Tester

et al 2011). This may appear to contradict our findings, however, this study provides evidence for the previously discussed concept of activity-based training. The use of training may be acting to direct plasticity in a way that improves the involvement of ascending propriospinal pathways. It is also important to remember that behavioral differences seen while silencing intraspinal pathways in a quadrupedal animal will vary drastically from that of a bipedal human. For one, humans do not rely as heavily on their arms during locomotion, which seems like a basic difference in principle. However, given that humans are bipedal, this poses an interesting dilemma: are humans under top-down control during stepping or are they primarily lower limb-driven bottom-up spinal circuitry reliant?

Some would say that humans are more reliant on their “hindlimb” equivalent for locomotion, therefore, locomotion must be driven by circuitry in the lumbar cord and the inputs onto it. Others would postulate that maintaining postural control and integrating visual input is most essential for humans while walking, which would suggest a top-down control largely reliant on supraspinal input. The truth likely falls in the middle of both theories, with supraspinal control and peripheral input contributing equally to the generation of patterned stepping. Maintaining postural control and seeing where you are going is as important as the peripheral input from the walking surface. If we apply this principle to the pathways of interest here, there is potential for several different outcomes. First, LAPNs and LDPNs may have similar influence on lumbar circuitry to account for the relative importance of supraspinal and peripheral input balance, rather than the disparate influences we saw here. It is also possible that these pathways may take on a different role entirely, based upon the reduced reliance on arm-leg interaction. The

opposite could be true, as well, in which the importance of propriospinal neurons skyrockets as a result of injury, thus suggesting that intraspinal circuitry should be targeted much more than it currently is. Whether these issues will be able to be explored in the future remains to be seen, as currently there are no techniques available that would provide unarguable proof of these interactions in the human spinal cord.

Spinal cord stimulation: the be-all and end-all for locomotor recovery?

In the field of spinal cord injury, there has been a disproportionate amount of focus on spinal cord stimulation, likely due to the relative ease with which this therapy can be applied to patients and its continued success in improving patient outcomes after coupling with locomotor stand or step training (Behrman & Harkema 2000; Harkema et al 2011, Harkema et al 2012; Angeli et al 2014). But the question remains: is spinal stimulation the best therapy for injured patients moving forward? Our findings would suggest this is not the case, given the potential for stimulating potentially maladaptive pathways after SCI leading to negative locomotor outcomes. Alternatively, the maladaptive role of propriospinals described here may be mitigated after SCI if a prosthetic stimulator could restore signals that these pathways would typically interact with in the uninjured condition, thereby eliminating any inappropriate communication regarding anticipatory limb and joint angles.

We have often assumed that, in the case of locomotion after SCI, stimulation would override the decreased drive that is present in lumbar circuitry as a result of the injury. Yet, it seems that we only see very limited improvements, leaving speculation that stimulation can really push the system to its highest capacity. This is where the concept of the ceiling effect comes into play. In short, there is only so much potential for recovery

after injury due to the presence of the injury itself, especially in incompletely injured animals that retrain themselves (Kuerzi et al 2010). This concept also applies to injury severity with both positive and negative impacts. For instance, animals with increased SCI severity have the capacity to regain essential motor movements with retraining given that they will not reach their functional “ceiling” by self-retraining alone. However, the overall ceiling is lower, due to the fact that fewer pathways remain uncompromised at the injury epicenter. In the clinical setting, this may play a key role in the limitations of regaining locomotor function post-SCI. As it currently stands, there is not an effective treatment that would result in regaining voluntary motor function in individuals classified as motor complete.

Exploration of the mechanisms that lead to improvements in humans are essential as we look to future experiments. Combinatorial therapy will inevitably be the answer, in which we can combine the best aspects of certain treatments like epidural stimulation while simultaneously eliminating the worst aspects of those same treatments. In an ideal world, we will be able to break the ceiling effect by somehow silencing maladaptive pathways and stimulating adaptive pathways, inducing plasticity in functionally meaningful pathways and preventing it in others, and focusing retraining efforts only on pathways that are of critical importance to recovered function, thus creating an idyllic environment for locomotor improvement post-injury.

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LIST OF ABBREVIATIONS AND SYMBOLS

Δ	Change
θ	Calculated angle
μ	Coefficient of static friction
$^{\circ}\text{C}$	Degrees centigrade
μl	Microliter
μm	Micrometer
3D	Three dimensional
A	Ankle
AAV2	Adeno-associated virus, serotype 2
ANOVA	Analysis of variance
BBB	Basso, Beattie, and Bresnahan locomotor scale
B.P.	Binomial proportions
BL	Baseline
Cm/s	Centimeters per second
CMV	Cytomegalovirus
Con	Control
CPG	Central pattern generator
CPI	Central pattern index
D1D5	Dox1 ^{ON} -Day 5

D1D8	Dox1 ^{ON} -Day 8
D2D5	Dox2 ^{ON} -Day 5
D2D8	Dox2 ^{ON} -Day 8
D2D13	Dox2 ^{ON} -Day 13
D3D8	Dox3 ^{ON} -Day 8
D3D21	Dox3 ^{ON} -Day 21
DAPI	4'6-diamidino-2-phenylindole
Dbx1	Developing brain homeobox 1
Dox	Doxycycline
DSI	Dorsal stepping index
EGFP	Enhanced green fluorescent protein
EnvA	Envelope protein A
eTeNT	Enhanced tetanus neurotoxin, light chain F.E.
F _f	Force of friction
F _g	Force of gravity
Fig.	Figure
FL	Forelimb
GFP	Green fluorescent protein
H	Hip
HAT	Hip-ankle-toe angle
HiRet	Highly-efficient retrograde transport
HL	Hindlimb
HRP	Horseradish peroxidase

Hz	Hertz
I	Iliac crest
i.m.	Intramuscular
i.p.	Intraperitoneal
IgG	Immunoglobulin, G
IHA	Iliac crest-hip-ankle angle
KS	Kolmogorov-Smirnov
LAPN	Long ascending propriospinal neurons
LDPN	Long descending propriospinal neuron
LF	Left forelimb
LH	Left hindlimb
MANOVA	Multivariate analysis of variance
Mg/kg	Milligram per kilogram
Mg/ml	Milligram per milliliter
Mm	Millimeter
N	Normal force
n.s.	Not significant
NeuN	Neuronal Nuclei
PBS	Phosphate-buffered saline
PD1	Pre-DOX1
PD2	Pre-DOX2
PEST	Peptide sequence rich in protein (P), glutamic acid (E), serine (S), and threonine (T)

PostD1	Dox ^{OFF} 1
Post D2	Dox ^{OFF} 2
PSI	Plantar stepping index
RF	Right forelimb
RH	Right hindlimb
RI	Regularity Index
rtTAV16	Reverse tetracycline transactivator, variant 16 RVdG
	Rabies virus, glycol-deleted
s.c.	Subcutaneous
S.D.	Standard deviation
SCI	Spinal cord injury
Syn	Synaptophysin marker
T	Toe
Tet ^{ON}	Tetracycline ^{ON}
Trans.	Transformed
TRE	Tetracycline response element
V0	Ventrally-derived interneurons, subclass “0”
V1	Ventrally-derived interneurons, subclass “1”
V2a	Ventrally-derived interneurons, subclass “2a”
V2b	Ventrally-derived interneurons, subclass “2b”
V3	Ventrally-derived interneurons, subclass “V3”
VAMP2	Vesicle-associated membrane protein 2
Vp/ml	Viral particles per milliliter

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Excellence in Neuroscience Research Graduate Student Award – 1st place
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University of Louisville
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PUBLICATIONS

Pocratsky, A.M., **Shepard, C.T.**, Morehouse, J.R., Riegler, A.S., Hardin, J.T., Beare, J. E., Hainline, C., States, G.J.R., Whittemore, S.R., Magnuson, D.S.K. (2019)
Long ascending propriospinal neurons provide flexible, context-specific control of interlimb coordination. (Submitted: eLife).

Shepard, C.T., Pocratsky, A.M., Brown, B.L., Van Rijswijck, M.A., Burke, D.A., Morehouse, J.R., Zalla, R.M., Riegler, A.S., Whittemore, S.R., Magnuson, D.S.K. (2019)
Silencing long ascending inter-enlargement propriospinal neurons after spinal cord injury restores hindlimb stepping ability in adult rats. (In final preparation).

PRESENTATIONS AND ABSTRACTS (*presenting author)

Brown, B.L.*, **Shepard, C.T.**, Zalla, R.M., Reigler, A.S., Howard, R.M., Van Rijswijck, M.A., Parsch, M.V., Whittemore, S.R., Magnuson, D.S.K. Silencing a spared ascending pathway improves locomotor outcomes and illustrates the importance of understanding plasticity after contusive SCI. The International Symposium on Neural Regeneration. January 2020. Poster Presentation.

Shepard, C.T., Brown, B.L., Van Rijswijck, M.A., Zalla, R.M., Parsch, M.V., Reigler, A.S., Pocratsky, A.M., Whittemore, S.R., Magnuson, D.S.K. Silencing long ascending propriospinal neurons improves hindlimb coordination and stepping precision after incomplete SCI. Society for Neuroscience Annual Meeting. October 2019. Poster Presentation.

Danner, S.M.*, **Shepard, C.T.**, Rybak, I.A., Magnuson, D.S.K. Central control of speed-dependent gait expression in intact rats and following spinal cord injury. Society for Neuroscience Annual Meeting. October 2019. Poster Presentation.

Shepard, C.T., Brown, B.L., Zalla, R.M., Van Rijswijck, M.A., Burke, D.A., Morehouse, J.M., Reigler, A.S., Whittemore, S.R., Magnuson, D.S.K. Silencing long ascending interneurons after spinal cord injury restores hindlimb stepping ability in adult rats. Research!Louisville Annual Meeting. September 2019. Poster Presentation.

Shepard, C.T., Brown, B.L. "Long Ascending Propriospinal Neurons: Where We Are and Where We're Going" KSCIRC Seminar Series. Louisville, KY. May 2019. Oral Presentation.

Brown, B.L.*, **Shepard, C.T.**, Zalla, R.M., Howard, R.M., Parsch, M.V., Magnuson, D.S.K., Whittemore, S.R. Silencing long ascending propriospinal neurons improves hindlimb coordination and stepping precision after incomplete SCI. 25th Annual Kentucky Spinal Cord and Head Injury Research Trust Symposium. Louisville, KY. May 2019. Poster Presentation.

Shepard, C.T., Van Rijswijck, M.A., Pocratsky, A.M., Spicer, N.D., Reigler, A.S., Whittemore, S.R., Magnuson, D.S.K. Making big strides: silencing ascending propriospinal neurons improves locomotor outcomes after spinal cord injury. Society for Neuroscience Annual Meeting. November 2017. Poster Presentation.

Van Rijswijck, M.A.*, **Shepard, C.T.**, Pocratsky, A.M., Whittemore, S.R., Magnuson, D.S.K. Two is better than one: long ascending propriospinal neuron silencing on two sensory surfaces. 2017 Research!Louisville. Louisville, KY. August 2017. Poster Presentation.

Shepard, C.T. Propriospinal neuron silencing improves locomotor outcomes after spinal cord injury. KSCIRC Seminar Series. Louisville, KY. August 2017. Oral Presentation.

Shepard, C.T. Propriospinal neuron silencing improves locomotor outcomes after spinal cord injury. Indiana University School of Medicine Motor Neuron Club. June 2017. Oral Presentation.

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Shepard, C.T., Van Rijswijck, M., Pocratsky, A.M., Spicer, N.D., Riegler, A.S., Whittemore, S.R., Magnuson, D.S.K. A tale of two surfaces: walking surfaces influence locomotor outcomes when propriospinal neurons are silenced after spinal cord injury. 27th Annual Neuroscience Day. Louisville, KY. April 2017. Poster Presentation.

Shepard, C.T. Propriospinal neuron silencing improves locomotor outcomes after spinal cord injury. 27th Annual Neuroscience Day. Louisville, KY. April 2017. Oral Presentation.

Shepard, C.T. Silencing after spinal cord injury: unmasking the role of long ascending propriospinal neurons and sensory inputs before and after contusion. KSCIRC Seminar Series. Louisville, KY. July 2016. Oral Presentation.

Shepard, C.T., Cary, R., Van Rijswijck, M., Pocratsky, A.M., Tran, K., Whittemore, S.R., Magnuson, D.S.K. Walking surface influences on stepping phenotype when long ascending propriospinal neurons are silenced" National Neurotrauma Society Symposium. Lexington, KY. June 2016. Poster Presentation.

Shepard, C.T., Cary, R., Van Rijswijck, M., Pocratsky, A.M., Tran, K., Whittemore, S.R., Magnuson, D.S.K. Getting a grip: walking surface influences on stepping phenotype when long ascending propriospinal neurons are silenced. 26th Annual Neuroscience Day. Louisville, KY. April 2016. Poster Presentation.

PROFESSIONAL ASSOCIATIONS

2018 – present	American Association of Clinical Anatomists (AACA)
2018 – present	American Association of Anatomists (AAA)
2016 – present	Society for Neuroscience (SfN)
2017 – 2019	Women in Neurotrauma Research (WINTR)
2017 – 2019	National Neurotrauma Society (NNT)

TEACHING EXPERIENCE

Senior Teaching Assistant for Dental Gross Anatomy, Upper Limb and Thorax Block, University of Louisville, Louisville, KY	Spring 2019
Lab Group Leader for Medical Neuroanatomy Lab University of Louisville, Louisville, KY	Fall 2018
Senior Teaching Assistant for Team-Based Medical Gross Anatomy University of Louisville, Louisville, KY	Fall 2018
Senior Teaching Assistant for Dental Gross Anatomy University of Louisville, Louisville, KY	Spring 2018
Lecturer: Central Pathways of CN IX-XII, Dental Gross Anatomy University of Louisville, Louisville, KY	Spring 2018
Lecturer: Central Pathways of CN VII, Dental Gross Anatomy University of Louisville, Louisville, KY	Spring 2018
Review Session Leader on Neuroanatomy Lecture Content University of Louisville, Louisville, KY	Fall 2017
Teaching Assistant for Medical Gross Anatomy University of Louisville, Louisville, KY	Fall 2017
Lead Teaching Assistant, Medical Neuroanatomy University of Louisville, Louisville, KY	Fall 2017
Problem-Based Learning (PBL) Mediator and Evaluator, Medical Gross Anatomy University of Louisville, Louisville, KY	Fall 2017
Observer for Student In-Lab Peer Evaluations, Dental Gross Anatomy University of Louisville, Louisville, KY	Fall 2017
Teaching Assistant for Advanced Head and Neck (Dental Resident Course) University of Louisville, Louisville, KY	Spring 2017
Teaching Assistant for Fundamentals of Neuroscience (Graduate Student Course)	

University of Louisville, Louisville, KY	Fall 2016
Teaching Assistant for Medical Gross Anatomy, Head and Neck Block University of Louisville, Louisville, KY	Fall 2016
Teaching Assistant for Medical Neuroanatomy University of Louisville, Louisville, KY	Fall 2016
Tutor for Medical Neuroanatomy University of Louisville, Louisville, KY	Fall 2016
Teaching Assistant for Normal Body Systems 3: Neurological Systems University of Louisville, Louisville, KY	Spring 2016