Chemogenetic stimulation of grafted neurons and changes in spinal afferent input in paraplegic rats

by

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Abstract

Over 2,000 yearly new cases of spinal cord injury (SCI) in Canada leave many people with reduced motor function that leads to secondary health complications. Increasing one's ability to walk can significantly curtail these complications but there is currently no treatment for recovering locomotor function after SCI in humans. Preclinical research with rodent models was used for my studies described in this dissertation to seek a better understanding of mechanisms underlying the recovery of walking.

The purpose of the first study was to examine changes in networks transmitting sensory input to motoneurons between the first and the fifth week after a complete SCI. The hypothesis that afferent input from the tibial nerve (TIB) in the lumbar segments is distributed differently after SCI was examined. A complete spinal transection injury in rats and *in vivo* electrophysiological measurements of evoked potentials following TIB stimulation were used. TIB afferents evoked intraspinal field potentials in the dorsal horn that showed a significant reduction in amplitude at one-week post-SCI. By five weeks post-SCI, however, TIB afferent transmission efficacy in these pathways was similar in rats without SCI. These findings suggest that TIB afferent input combined with dorsal horn neural excitability of spinal interneurons drives the reorganization needed for recovery of some walking capacity.

My second study utilized a genetically modified rat model in which neurons producing serotonin (5-HT) could be activated to escalate locomotor recovery. By using grafting of embryonic 5-HT neurons below the level of SCI, we tested the hypothesis that activation of grafted 5-HT neurons by chemogenetic means in paraplegic rats improves locomotor activity (compared to rats in which only grafting was done). Behavioural analysis by kinematic and hindlimb EMG assessments during treadmill walking were performed before and after chemogenetic stimulation of grafted 5-HT cells and immunohistochemistry on spinal tissue was used to verify the targets of this stimulation. We found that locomotor cycle regularity and left-right coordination was altered. The findings suggest that selective targeting of neural subpopulations may be a better strategy for improving recovery of walking after complete SCI.

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"He who does not thank the people has not thanked their Lord."

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Dedication

This dissertation is dedicated to the memory of Dr. Larry Jordan (1944-2021). It is under his guidance and supervision I started my PhD journey in the cold plains of Winnipeg. I will forever be indebted for him. I only wish any work I do will be of the rigor that he strived for.

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List of Symbols, Abbreviations and Nomenclature

1w	One week
5w	Five weeks
5-HT	5-hydroxy-tryptamine or serotonin
5-HTR	5-HT receptor
8OH-DPAT	8 hydroxy -di-propylaminotetralin (5-HT 1A agonist)
ACh	Acetyl choline
APV	2-R-amino-5-phosphovaleric acid (Selective NMDA receptor antagonist)
C21	compound 21 (designer drug for DREADD)
CNO	Clozapine N- Oxide (designer drug for DREADD)
CPG	Central Pattern Generator
CZP	Clozapine
DOPA / L-DOPA	Dopamine/ Levo-Dopamine,
DREADD	Designer Receptors Exclusively Activated by Designer Drugs
E14	Embryonic day 14
EMG	Electromyograph
EPSP	excitatory postsynaptic potential
GABA	gamma-aminobutyric acid
GAD	glutamate decarboxylase. (a key enzyme in GABA synthesis)
Gi	gigantocellular reticular nucleus
GLUT	Glutamate
GLY	Glycine
gr I	group I afferent
gr Ib	group Ib afferent
gr II	group II afferent
G-protein	guanine nucleotide-binding protein
IN	interneuron
INT	Spinal cord intact rat cohort
i.p.	intraperitoneal
LPGi	lateral paragigantocellular nucleus
MLR	Mesencephalic Locomotor Region

MN	motor neuron
NA	Noradrenaline
NMDA	N-methyl-D-aspartate
PICs	persistent inward currents
PPN	pedunculopontine nucleus
rAAV	Adeno-Associated Virus
SCI	Spinal Cord Injury
SD	standard deviation
Sol	Soleus (ankle extensor)
TA	Tibialis Anterior (ankle flexor)
T	threshold for activation of most excitable afferents
Th	thoracic
TIB	Tibial nerve
TPH2	Tryptophan hydroxylase
vGAT	vesicular GABA transporter
vGlut	vesicular glutamate transporter
xT	times threshold

Epigraph

"و ما أوتيتم من العلم إلا قليلا"

“And mankind has only been given little knowledge.”

(17:85) The holy Quran.

Chapter I

1.1 General Introduction

One rarely thinks of the precise movements involved in each step they make. Rushing to the bus stop at 8:30 a.m. on a freezing February morning, imagine the precision and urgency required of our nervous system to reach the bus safely and on time. The interaction of the locomotor commands coming from the brain to move forward with the corrective reflexes via peripheral input when slipping on ice and the quick jump on reaching the edge of the sidewalk. All of which is done with little thought. The activation and modulation of different neural circuitry within the spinal cord with complex interactions between the descending supraspinal command and sensory afferents makes such a complicated process appear so simple.

The importance of locomotion is acutely sensed when it is lost, whether to injury or disease. Recovering the ability to move in a stereotypical fashion require understanding the following: 1) The neural circuits involved in locomotion and how they are engaged. 2) The changes to these circuits that occur after injury and how they can be used to facilitate recovery. 3) The neurotransmitters and the neural circuits that are sufficient to restore locomotion after SCI.

1.2 Prevalence of spinal cord injury (SCI)

The profound importance of locomotion in our everyday life is acutely sensed when it is lost. The loss of locomotor ability can be due to traumatic or non-traumatic injury to the spinal cord. Interestingly, up until the late 19th century, the attitude towards spinal cord injury was practically a death sentence where even palliative treatment was considered a waste of resources (Donovan, 2007). There are over 85,000 people living with spinal cord injury in Canada alone, while there are about 2,000 new cases per year (Noonan et al., 2012), and about 17,000 in the United States (Jain et al., 2015). The cause of SCI in 30-40% of the cases is trauma from vehicle accidents, and up to 66% of the cases (especially in older adults) trauma results from falls (ACS, 2022). The life expectancy of people with SCI is close to the same number of years as of people with no SCI. The societal costs are estimated to be in the millions of dollars for each paralyzed person over a lifetime for healthcare expenditures because of problems stemming from lost motor functions and leading to a relative sedentary lifestyle and further health problems (Merritt et al., 2019).

Standard care after SCI involves acute management as well as long-term care. The reduction of pressure from the nervous tissue (by broken bone, for example) is the most critical first step to reduce neural loss. Just like in the standard care of stroke, time is a key factor. The faster the decompression is done, the less is the neural tissue loss. Magnetic resonance imaging (if available) is used to evaluate the internal structure of the nervous tissues within the spine. The surgical decompression intervention is followed by optimizing blood pressure and systemic circulation to allow balanced perfusion of all body parts. Another important management issue is to prevent autonomic dysreflexia, which presents as a life-threatening issue when the injury is above the mid thoracic (T6) level. Internationally accepted spinal trauma classification systems are used to map the patient's muscle strength, sensory neurological status, autonomic function and mobility (Acs, 2022). For long-term care, suggested management range from the neuroprotective to neuro-regenerative methods.

Neuroprotective modalities aim to prevent further nerve damage and protect the remaining structures within the spinal cord from further injury due to the inflammatory cascades that occur after the initial injury. Both pharmacological and non-pharmacological methods have been explored (Flack et al., 2022; Shah et al., 2020). The neuro-regenerative route also employs pharmacologic and non-pharmacologic techniques to either regenerate the lost tissue to regrow axons or to create a scaffold for (re)growth by using biomaterials. Often the combination of these methods is used to modulate the surviving tissue and to better stimulate the growth and function of different remaining neurons around the injury site. Additionally, for functional improvements by spinal or brain stimulation and by different rehabilitative, activity-dependent techniques combined with stimulation is the choice of treatment; albeit these are experimental in nature and not part of standard care protocols yet. Despite the many advancements in the various treatment modalities, the clinical results are promising but modest. None of these methods of treatment has led to complete functional recovery (Flack et al., 2022) The need for an interdisciplinary approach for treatment and rehabilitation is needed and further research should be conducted to investigate the benefits of a combined approach.

1.3 Spinal control of locomotion

An injury to the spinal cord hinders how information from the brain reaches neurons connecting to muscles that will execute motor commands and the flow of peripheral input to the brain. Yet the spinal cord is not merely an axonal highway transmitting information between the brain and the periphery. It also serves as an integration hub with segmentally divided and specialized mini circuits. These specialized circuits are responsible for expressing the stereotypical rhythmic actions of locomotion, whether it be the undulating movement of the swimming lamprey or slithering of a snake or the flying goose or the walking mammal.

The fact that the control of locomotion is within the spinal cord was first described by Graham Brown (1911) in cats that were both spinalized and deafferented (had no supraspinal or sensory input) yet were still able to express coordinated stepping movements. This seminal finding differed from the prevailing thoughts of the time which were also held by Sherrington, whom Brown had worked with, that locomotor activity is the result of a series of spinal reflexes (Stuart & Hultborn, 2008). Sherrington's held the preponderant view that sensory input is the origin of locomotor control and pattern generation and deemphasized the essential spinal origin of locomotor pattern generation which Brown's deafferented model has clearly shown (Stuart, 2005).

Despite his groundbreaking findings, Brown's work was not adopted till the 1960s. At that time, Lundberg and Jankowska conducted a series of studies in which they were able to characterize some properties of these spinal circuits, later referred to as Central Pattern Generators (CPGs), through evoking repeated flexion and extension movements by applying dopamine to spinal cats (Jankowska et al., 1967). They showed that a reciprocal organization between interneurons produced cyclic on and off activity states in interneurons and motoneurons representing the CPG. Since then, the organization of the spinal locomotor CPG has been extensively studied and characterized in different species from invertebrates to humans (Grillner, 2011; Marder & Bucher, 2001).

There are multiple views on which spinal neurons should be considered as part of the CPG. For example, motoneurons (MN) which are nerve cells that innervate, and control muscle tissue directly. The MNs are thus considered as the final output layer of the CPG. This is illustrated in Figure 1.1. On the other hand, spinal interneurons (INs) are neurons that connect with nearby cells creating local circuits are also considered part of the CPG based on various functional role the

neurons may have in enabling locomotor activity (Rybak et al., 2006). There are also other interneurons which enable connections between long distances in the spinal cord (i.e., long propriospinal neurons). The common uniting factor for a subset of interneurons to be considered as part of the CPG is the activity profile during locomotion. Neurons of the CPG even in the absence of input from supraspinal motor centers generate action potentials in relation to the ongoing locomotor pattern that is monitored by MN output (either in nerve cells as electroneurography, ENG or in muscle tissue by electromyography, EMG). The "black box" of the CPGs is often represented by connected interneurons as shown in Figure 1.1, and the identification and nomenclature of the CPG neurons has been done by two major methods. One method entails identification of interneurons based on function, such as input, output, and action potential generation profiling during motor activity. The other method is based on genetic transcription factor identification, that is a newer system which developed in parallel with molecular biology techniques enabling protein detection and genetic manipulations (Kandel, 2013).

Further evidence that the basic pattern of quadrupedal locomotion does not require descending commands from brain or brainstem can be gleaned from the work of Engberg and Lundberg (1969). They were the first to show EMG recordings of hindlimb activity during locomotion where extensor (muscles active during standing or stance phase in locomotion) bursts preceded paw contact consistent with central (spinal) activation. Around the same time, it was observed that decerebrate cats with a cervical level transection (high spinal cats) performed coordinated stepping when their limbs were placed on a treadmill (Miller et al., 1975). Similar observations in humans were reported when electrical spinal cord stimulation evoked rhythmic limb movements in people with SCI (Dimitrijevic et al., 1998).

One key mechanism underlying not only locomotor CPG circuit function but also CPGs involved in other patterned movements, such as breathing or chewing, is the presence of intrinsic bursting cells that can produce sustained and regular neuronal activity. However, in addition to such a kernel module, CPG circuits contain neurons that distribute excitation or inhibition to motoneurons which need to be recruited on a step-by-step basis. The action potentials generated by motoneurons also need to be adjusted frequently, for example, during a stumble, when a disturbance from peripheral input changes the required step. Stumbling is when the dorsum of the foot encounters an obstacle, and the reaction is to step over this obstacle by increasing the elevation

of the foot. Realistic models of locomotor CPGs have come to differentiate between spinal CPG neurons serving as "rhythm generators" or as "pattern formation" cells (Rybak et al., 2006) to allow for such step-by-step adjustments as those observed during stumbling while walking.

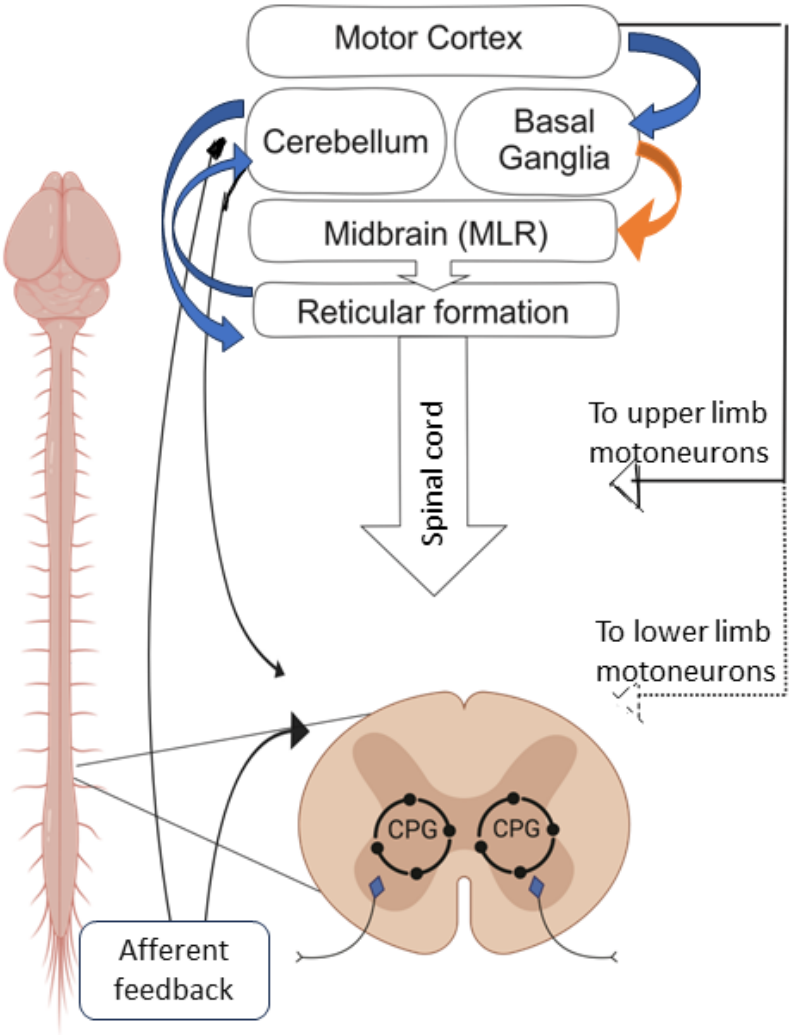

Figure 1.1 Overview of major descending motor centers and the central pattern generator. Illustration of a rat brain and spinal cord (left) indicating the approximate location of the lumbar spinal central pattern generator (CPG) circuitry (right) with the motoneurons (blue) innervating hindlimbs. The box diagram shows major motor command centers of the nervous system influencing motoneurons as well as CPG output. These centers are all involved in voluntary movement generation. Orange arrows imply inhibition while blue is excitation.

1.4 Descending pathways involved in motor control

Voluntary activation of the spinal CPGs for goal-oriented locomotion requires supraspinal input. The major supraspinal motor control centers include the following major regions within the brain: the cortex, basal ganglia, and the cerebellum as summarized in Figure 1.1.

The motor cortex projects directly to the spinal cord, yet its importance is mainly in movements requiring high precision such as walking in difficult terrain to avoid obstacles or precisely placing feet on the rungs of a ladder. The role of the cortex in locomotion is in some sense quite insignificant as mammals can exhibit basic motor functions with it removed entirely (Bjursten et al., 1976; Kawai et al., 2015). Nevertheless, for conscious task-specific movements, the initial descending command center is the primary motor cortex (or M1 region adjacent to the rostral border of the central sulcus) that is organized in a somatotopic manner, i.e., with each muscle being represented over a specific area from which the commands start. The pre-motor cortex, rostral to the M1 region is also thought to play a role in voluntary control, specifically planning of motor actions.

The Basal ganglia which include the striatum, globus pallidus interna and externa, the substantia nigra and subthalamic nucleus have a substantial role in locomotion as is observed from diseases affecting them such as Huntington's or Parkinson's. The role of the basal ganglia is mainly in initiation and motor program selection as well as motor inhibition (Seger, 2008).

The cerebellum is mostly involved in fine-tuning of motor actions, and it is thought to act as a comparator. This comparator function entails that neurons in the deep cerebellar nuclei receive sensory input during the ongoing movements but also input from the planning centers of the basal ganglia. The planned movements are compared to the feedback from the moving muscles and joints to adjust and align the trajectories while executing intended motor actions. In addition, the phylogenetically oldest portions of the cerebellum are organizing postural responses in response to vestibular input a play a key role in balance control (Kandel, 2013). For example, in humans without an intact healthy cerebellum, walking would require a wider base due to the inability of incorporating sensory cues of support provided by an intact, healthy cerebellum that allows balancing while taking steps with a normal (narrow) base of support.

Shik et al. (1966) in a paper translated to English in (1969) identified a region within the midbrain that is responsible for initiation of locomotion without higher brain region input, that is

called the Mesencephalic Locomotor Region (MLR). This region was functionally identified through electrical stimulation in cats but has been shown to be conserved across vertebrate species as reviewed in (Jordan et al., 2008; Ryczko & Dubuc, 2013). Cell populations within this region make connections with neurons of the reticular formation. The medullary reticular formation that is located in the hindbrain and composed of multiple neural pools is the main relay system for the MLR mediating locomotion through descending neuronal pathways that can engage spinal CPGs, as shown by the Jordan lab by using electrical and chemical stimulation in the medulla (Noga et al., 2003).

The reticular formation has several populations of neurons which monosynaptically connect to motoneurons and interneurons in the spinal cord. The majority of the reticulospinal tract cells were shown to be fast-conducting glutamatergic neurons (Du Beau et al., 2012; Noga et al., 1988). In addition to the fast-acting excitatory neurons within the reticulospinal tract there is evidence for an inhibitory GABA and/or Glycinergic reticulospinal tract activated in stopping locomotion as reviewed by (Brownstone & Chopek, 2018). The reticulospinal neurons descend via the ventrolateral funiculus and bilaterally innervate the spinal cord. These neurons are active in a phasic manner during different kinds of locomotion in cats as reviewed by e.g. (Brownstone & Chopek, 2018). The median part of the reticular formation contains several serotonergic nuclei that receive input from the MLR, namely the raphe pallidus, magnus, and obscurus and the parapyramidal (PPR) regions as reviewed in (Jordan, 1998).

1.5 Neurotransmitters eliciting locomotion

Neurotransmitters are chemical messengers endogenous to neurons that exert specific actions on their targets after being released at synapses. All spinal neurons, including motoneurons and interneurons, are genetically defined to express one specific (or a combination of) transcription factors during early embryonic development that determine cellular identity and a neuron-specific transmitter phenotype.

The major excitatory neurotransmitters that induce depolarization of neurons when released are glutamate (GLUT) and acetylcholine (ACh). The major inhibitory neurotransmitters that induce hyperpolarization are glycine (GLY) and gamma-aminobutyric acid (GABA). Neuromodulators, on the other hand, are chemical messengers which when present at the synapse, modify, or modulate the properties of postsynaptic neurons, for example, by shifting the membrane potential

and reducing the threshold for action potential initiation in motoneurons. Neuromodulators usually act through metabotropic receptors and have a slower time course of action than neurotransmitters. Some chemicals, however, such as the monoamines, including serotonin or 5-hydroxytryptamine (5-HT) can be viewed as both transmitters and modulators, depending on the receptors present at postsynaptic sites.

Neurotransmitter profiles of descending neural pathways linked to activating the locomotor CPG include glutamatergic, noradrenergic, and serotonergic (Harris-Warrick, 1988; Larry M. Jordan et al., 2008). Noradrenaline was one of the first transmitters to be implicated in locomotion through studies where L-DOPA, a precursor to dopamine and consequently noradrenaline, was administered to cats with SCI eliciting a pattern of reflexes similar to locomotion. Evidence supporting the role of noradrenaline also stems from the work of Forssberg and Grillner (1973) where they used clonidine, an alpha-2 receptor agonist, to initiate locomotion in chronic spinal cats. Noradrenaline exerts its locomotor related actions in the spinal cord mainly via α-adrenoreceptors (Chau et al., 1998). Both α_1 and α_2-adrenergic receptors are G-protein coupled receptors. The α_2-adrenergic receptors are negatively coupled to adenyl cyclase and are usually expressed as auto receptors which regulate further secretion of noradrenaline. α_1-adrenergic receptors on the other hand mediate excitatory effects through the phosphatidylinositol pathway that affects the intracellular calcium levels associated with the Gq protein (Venugopalan et al., 2006). Both receptor subtypes have been shown to be colocalized with locomotor activated neurons in the thoracolumbar cord from T7 to L7 in the cat (Noga et al., 2011a). Agonists of α_1 and α_2-adrenergic receptors have different effects on spinal locomotion in cats where α_2-adrenergic agonists are more reliably able to initiate locomotion in the acute spinal cat and modulate the established locomotor pattern in the chronic spinal cat. On the other hand, activation of α_1-adrenergic receptors increased weight support on the hind limbs and did not increase the foot drag as observed after administration α_2 adrenergic agonists. (Chau et al., 1998). The receptors by which noradrenaline can exert its facilitatory effects on locomotion can differ according to the type of injury whether complete or incomplete, acute or chronic, as well as differences among species (Kwaśniewska et al., 2020; Nicolas P. Lapointe et al., 2008; N. P. Lapointe et al., 2008).

Dopamine has been found to be evolutionarily conserved in locomotor control. Dopamine has effects on networks identified in the lamprey (Ryczko & Dubuc, 2017), which are similar to those in the rat and in the human nervous system (Sharples et al., 2014). Dopaminergic projections from

the pedunculo-pontine nucleus of the midbrain (PPN) to the reticular formation involving D1 receptors play a major role in the initiation of locomotion (Ryczko & Dubuc, 2017). Moreover, D1 receptors on spinal neurons are vital contributors to sustaining rhythmic locomotor activity. In contrast, the D2 receptor family had a slowing effect on the rhythm when tested *in vitro*, in the neonatal mouse (Sharples et al., 2015).

In general, glutamatergic cells with ipsilateral projections within spinal networks are necessary for rhythm generation as these interneurons provide excitation to motoneurons. I will highlight only a few key findings from the vast amount of research on this transmitter in the context of locomotion, as reviewed in (Jordan et al., 2008). Evidence for a glutamatergic pathway involved in the generation of locomotor activity stems from *in vitro* studies on the lamprey during forward swimming by (Grillner, 1981). Similar experiments to those in the lamprey have been repeated in the neonatal rat preparation originally by (Kudo & Yamada, 1987) and subsequently by several other labs. Fictive stepping or fictive locomotion is the presence of motoneuron activity resembling the same pattern of activity as in overground walking yet evoked in the absence of movement in the neonatal rat preparation with only dorsal and ventral roots intact from which ENG recordings signal activity. A selective NMDA receptor antagonist (APV), was found to interfere with locomotion in the cat and similarly in the neonatal rat by the laboratory of Schmidt (Zaporozhets et al., 2006) and other laboratories. More recent evidence from studies when stimulating the glutamatergic neurons of the reticular formation produced seemingly contradictory evidence for glutamatergic involvement in the initiation of locomotion, as stimulation within the gigantocellularis nuclei (Gi) of the reticular formation in adult mice did not trigger walking (Josset et al., 2018). However, glutamatergic cells of the lateral paragigantocellularis nuclei (LPGi) in adult mice were found to initiate stepping by the work of the Arber lab (Capelli et al., 2017).

Both GABA and GLY are involved in locomotor control. GLY has been recognized to be most important for the maintenance of flexor and extensor alternation during locomotion in what is known as reciprocal inhibition i.e., a state in which a muscle is active, and its antagonist is silent. When glycinergic inhibition is taken away, flexor and extensor motoneurons are mostly activated in synchrony (Cohen & Harris-Warrick, 1984; Cowley & Schmidt, 1995; Kremer & Lev-Tov, 1998). A similar process to reciprocal inhibition is the coordination of muscle activity between the left-right sides. However, that requires involvement of spinal neurons projecting across the midline, the so-called commissural neurons. Ventrally located commissural interneurons identified

in the neonatal rat (Kiehn & Kjaerulff, 1996) and in lamina VIII and the dorsal horn of the lumbar segments in cats have been shown to include glycinergic neurons influencing motor activity (Elzbieta Jankowska & Steve A. Edgley, 2010). The role of GABA is most critical for presynaptic control of sensory input; as the attenuation of responses to peripheral input seems to be one mechanism to ensure smooth movements during walking (Rossignol et al., 2006).

Motoneurons and several classes of interneurons in both the spinal cord and the brain produce ACh. It is accepted that ACh from spinal sources can modify locomotor output in awake, conscious mice (Roseberry et al., 2016). The special synapses, the C-boutons, formed between a group of ACh-producing interneurons and motoneurons determine force generation during movements (Zagoraiou et al., 2009). The cholinergic input to motoneurons has been found to also be involved in modulating the threshold for action potential generation in motoneurons and making the cells more responsive to synaptic input in preparation for fictive stepping (Vasquez-Dominguez, 2016).

Serotonin (5-HT) has diverse effects on rhythmic motor output in different species from invertebrates to vertebrates. 5-HT has been shown repeatedly to be sufficient in activating the CPGs *in-vitro*, in the isolated neonatal spinal cord (Cazalets et al., 1992; Cowley & Schmidt, 1994). However, whether 5-HT neurons are sufficient to initiate walking *in vivo* is not clear. The stimulation of serotonergic neurons within the parapyramidal region of the brainstem in neonatal rat, *in vitro* could trigger stepping as shown by Liu and Jordan (2005). The selective stimulation of 5-HT neurons of this region by optogenetic means in vivo has also shown that fictive stepping could be evoked by recent work in the Jordan lab (Armstrong, 2019).

1.5.1. Subdivisions of serotonergic neurons

Serotonin (or 5-HT) is involved in many neural processes and abundantly available in the CNS but is not inherently necessary for any function (Steinbusch, 1981). There are two main groups of serotonergic nuclei present in the brain; a rostral group in the midbrain and medulla that has more rostral projection and a caudal group in the medulla that provides the innervation to the spinal cord. These groups are named based on an old naming system, referring to B1-B9 subgroups with the rostral groups in the midbrain and pons being the B4-B9 and the caudal groups present in the medulla are the B1-B3. As shown in Figure 1.2, the different populations of 5-HT neurons are known to selectively target more rostral (higher) brain regions (ascending) or caudal (lower brainstem and spinal cord) regions with their projections. Descending populations are the Raphe

Pallidus, Obscurus and Magnus, which correspond to the B1, B2, B3 populations, respectively according to Dahlstrom and Fuxe's (1964) classification see also (Ren et al., 2019).

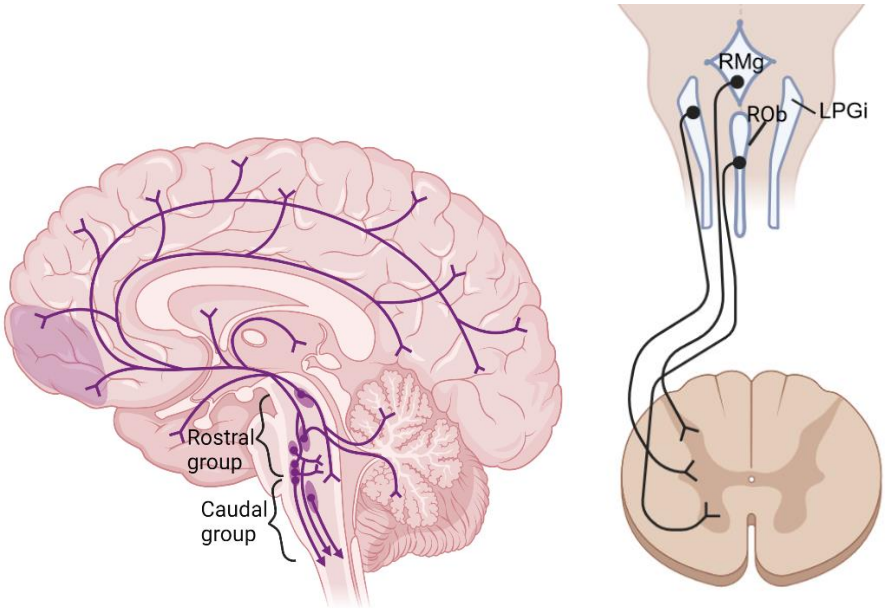

Figure 1.2. Schematic illustration of serotonergic neurons and projection.
Neurons in the human brain (left) producing serotonin (5-HT, purple ovals) and with the lines showing the extent of axonal 5-HT projections to other regions within the central nervous system. Simplified schematic view of brainstem 5-HT nuclei innervating the thoraco-lumbar spinal cord. Neurons in the Raphe Magnus (RMg) innervating the dorsal horn; cells from Raphe Obscurus (ROb) project mainly to ventral horn and neurons of the lateral paragigantocellularis nuclei (LPGi) project mainly to the intermediolateral cell columns. (modified from (Tork, 1990) created with BioRender.com).

A few studies reported that 5-HT maybe produced by neurons within the spinal cord (Newton & Hamill, 1988; Newton et al., 1986) that appear to be associated with the autonomic nervous system. However, it is controversial if these spinal sources produce sufficient amounts of 5-HT. It is also unresolved whether these cells are present only after an insult to the neurons and supporting cells by injury or if there is a spinal population of cells that constantly produce 5-HT like it is, for example, in zebrafish (Huang et al., 2021).

Serotonergic fibers descending to spinal targets create synaptic connections to neurons in the dorsal horn, and motoneurons of the ventral horn, and to interneurons of the intermediolateral cell column (IML) as shown in Figure 1.2. There are also dense 5-HT fibers found close to neurons within the central commissure (ventral to the central canal). Release of 5-HT from descending

axons can affect spinal neurons via post-, and presynaptic actions. The fibers are mostly unmyelinated with small diameter (around 1 μm) and present as varicosities forming "beads on a string"-like structures (Azmitia & Gannon, 1983).

1.5.2. Serotonergic receptors

Serotonin exerts its actions by interacting with multiple types of receptors. There are 7 broad families of receptors, named from 5-HT_1 to 5-HT_7, with up to 14 subtypes identified to date. These types and subtypes of the 5-HTR family were mainly differentiated according to radio-ligand binding studies, with further subtypes proposed based on new ligand and selective binding profiles. The genetic cloning of these different 5-HT receptor subtypes further confirmed them as different molecular entities and expanded on the different known subtypes (Palacios, 2016). All the 5-HT receptors are metabotropic, G-protein coupled receptors, except for the 5-HT_3 receptor, an ion-gated channel. Typical composition of the GPCRs consist of seven transmembrane alpha helices, and these domains divide the receptor into extracellular N-terminus, intracellular C-terminus, three extracellular loops and three intracellular loops (Kandel, 2013). The G-proteins are heterotrimers composed of Gα and Gβ and Gγ subunits.

As reviewed by (Giulietti et al., 2014), the 5-HT_1 receptor family and 5-HT_5 receptors couple with Gi/o protein family to inhibit adenylate cyclase (AC) activity, reducing the intracellular cyclic adenosine monophosphate (cAMP) level which typically leads to a reduction in neural firing. While the 5-HT_4, 5-HT_6 and 5-HT_7 receptors are coupled to the Gs pathway which triggers intracellular AC activation and cAMP production. The 5-$HT_{2\ (2A,\ 2B,\ 2C)}$ receptor family couples with Gq/11 proteins which activate the intracellular activity of phospholipase C (PLC) which consequently increases the intracellular inositol trisphosphate (IP3), diacyl-glycerol (DAG) and Ca^{2+} levels. These events typically lead to increased neural excitability. (Giulietti et al., 2014).

Based on the activity-dependent early gene *c-fos* expression and immunohistochemical staining with colocalization, 5-HT receptors that were found to be localized to neurons active during locomotion were 5-HT_7, 5-HT_{2A} and 5-HT_{1A} receptors (Dai et al., 2005; Noga et al., 2009). These were observed in the lower thoracic (T7) and lumbar cord of the cat.

The 5-HT_1 receptors consist of many subtypes 5-$HT_{1A,\ 1B,\ 1D,\ 1E,\ 1F}$ receptors. They appear to have a rostro caudal gradient in distribution with a scant expression in the cervical segments compared to lumbar and sacral (Marlier et al., 1991) which corresponds to documented expression

of the 5-HT amine itself in the spinal cord (Hadjiconstantinou et al., 1984). They are most abundant in the dorsal horn (Giroux et al., 1999; Marlier et al., 1991), and these have an essential role in modulating afferent signals (see e.g., review on 5-HT_{1A} receptor by (Bajjig et al., 2022). The 5-HT_{1A} receptor is of interest in locomotion as it has been shown to facilitate locomotion in spinalized 5-HT_7 knockout mice. They also colocalized with locomotion activated neurons in the cat (see Table 1.1) 5-HT_1 act through Gi/o negatively couple to the AC/PKA/cAMP pathway that reduces cAMP. The Gi/o also acts directly on the conductance of potassium channels which leads to hyperpolarization and further decreases action potential frequency. Interestingly several 5-HT_1 receptor subtypes show a tendency for constitutive activity, i.e., receptors active in the absence of their ligand, *in vitro* namely 5-$HT_{1A, 1B, 1D}$ yet there is no evidence for such activity *in vivo (*De Deurwaerdère et al., 2020*)*. However, the 5-HT_{1D} receptor has been found to be exclusively associated with gamma MNs (Enjin et al., 2012). This receptor is thought to be important for proprioceptive information processing. When 5-HT_{1D} receptors are removed, smaller monosynaptic reflexes were found in mice suggesting a diminished response to sensory stimulation in leg motoneurons (Enjin et al., 2012).

The 5-HT_2 receptor family includes the 5-$HT_{2A, 2B, 2C}$ subtypes. There is no observed rostro-caudal distribution gradient observed for the 5-HT_2 receptors (Marlier et al., 1991). While they are more highly expressed in the ventral horns and intermediolateral cell column (Marlier et al., 1991). It is notable that 5-HT_{2A} receptors have been found colocalized with *cfos* expression in the ventral horn caused by MLR induced locomotion in the lower thoracic and lumbar cord. 5-HT_{2A} has a role in locomotion and locomotor recovery as shown in (Table 1.1). The 5-HT_{2A} receptors display the least ability to spontaneously activate their internal signaling pathways for the 5-HTRs (De Deurwaerdère et al., 2020). 5-HT_{2B} receptors are expressed on some areas of the brain such as the hypothalamus and frontal cortex and midbrain raphi nuclei yet much higher quantities of 5-HT_{2B} are found in the periphery most notable in the fundus of the stomach and intestines and heart (De Deurwaerdère et al., 2020). The 5-HT_{2C} (previously known as 5-HT_{1C}) receptors are almost exclusively expressed in the CNS of mammals with a very high expression in the choroid plexus epithelial cells and a diffuse low-density expression throughout the brain and spinal cord including the sacral cord (Abramowski et al., 1995; Clemett et al., 2000; Palacios, 2016). Spinalization has been shown to induce changes in expression of the isoforms of this receptor to favor an isoform that shows constitutive activity. The constitutive activity of both 5-$HT_{2B/C}$ has been shown to be a

factor in spasticity after spinalization (Murray et al., 2010) and may play a factor in locomotor recovery after injury(Fouad et al., 2010). This has been shown to be a triggering factor in spasticity after SCI as motoneuron excitability increases via persistent inward currents (or PICs), PICs are depolarizing currents generated by voltage-sensitive channels that tend to stay open as long as the membrane potential remains above their activation threshold (Heckman et al., 2005). A large part of the PICs is mediated via L-type calcium channels (Hounsgaard & Kiehn, 1989). Sodium currents also play an important role in generating PICs in mammalian motoneurons (Lee & Heckman, 2001; Li & Bennett, 2003).

5-HT_3 receptors, as an exception to all other 5-HT receptors, is ionotropic and are broadly expressed in the CNS. They are found in the spinal cord in both the dorsal and ventral horn as well as the dorsal root ganglia (Morales et al., 1998) (Vilaró et al., 2020). They appear to have a role in antinociception (Bardin et al., 2000) while their role in locomotion is not established (Clarke et al., 1996).

5-HT_4 receptors are metabotropic. They has been identified in various peripheral tissue as well as the CNS including the ileum, heart, basal ganglia, and cortex (De Deurwaerdère et al., 2020). 5-HT_5 receptors have been found primarily in the brain with some expression in lamina I and II of the dorsal horn in the spinal cord (Vilaró et al., 2020) and may play a role in antinociception. 5-HT_6 receptors have been shown to be expressed mainly in the brain. 5-HT_6 is known to facilitate cognitive performance and memory therefore is a useful target for treating cognitive impairment such as in Alzheimer's and schizophrenia (De Deurwaerdère et al., 2020). It is also a target for treating neuropathic pain (Sari et al., 2019).

5-HT_7 receptors are found throughout the brain and the spinal cord. 5-HT_7 receptors show a dorso-ventral gradient in the thoracolumbar segments (from T7-L7 with the highest distribution in the L5-L7 segments) with a higher expression dorsal to ventral. The 5-HT_7 receptor appears to also decrease in expression rostrally (Noga et al., 2009). The majority of the *c-fos*-labeled cells observed following MLR evoked fictive locomotion in the adult cat were found to express 5-HT_7 receptors (Noga et al., 2009). This suggested some role in locomotion. Application of 5-HT_7 receptor agonists and antagonists in mice lacking the 5-HT_7 receptor verified their importance in the control of the spinal CPG (Liu et al., 2009). In the 5-HT_7 knockout mice, 5-HT application disrupted the locomotor coordination. Joint work by Jordan and Sławińska also demonstrated that

5-HT_7 receptors are powerful in controlling overground walking as well as the fictive rhythmic motor activity in decerebrate preparations when the CPG works in isolation from the forebrain (Cabaj et al., 2017).

As summarized in Table 1.1 Experiments on intact spinal preparations as well as in spinal injured animals have repeatedly shown that in both *in vitro* and *in vivo*; the activation of several 5-HT receptor subtypes can modulate locomotor-like activity and overall excitability of motoneurons in the lumbar cord. 5-HT receptors are excellent targets for modulating long term plasticity of respiratory movements following an injury as has been shown during experiments in which cervical spinal cord injured rats were exposed to intermittent hypoxia (Vinit et al., 2009). The long-term facilitation of the phrenic nerve resulted from downstream activators of the 5-HT_{2A} (a Gq pathway through PKC) and of the 5-HT_7 through a Gs pathway (Fields & Mitchell, 2017; Fields et al., 2015; Fuller et al., 2005).

The 5-HT receptor density changes with the loss of descending innervations after spinal cord injury. The 5-HT_{2A} receptor gene expression, for example, was shown to increase up to 6 weeks after a Th10 spinal transection (Navarrett et al., 2012) while there was no change in the 5-HT_{2C} receptor gene expression. This was corroborated by the work of (Cao et al., 2018; Kong et al., 2011; Kong et al., 2010) though Cao and colleagues showed an increase in 5-HT_{2C} expression compared to non-injured rats using western blot and immunohistochemical analysis. The expression of 5-HT_{2A} receptors was also found to be increased in the ventral horn after spinalization and it reached a peak level at around 4 weeks post Th10 SCI. This was also seen in phrenic motor neurons after a C2 cervical hemi-section, when 5-HT_{2A} increased (Fuller et al., 2005). Interestingly, though mRNA levels of 5-HT_{2C}R didn't change after spinal cord injury. This difference could be explained by post transcription editing changes that produced an increase in a specific isoform of the receptor active in the absence of its ligands i.e., constitutive activity (Murray et al., 2010). Using quantitative RT-PCR on tissue collected by laser capture (Chopek et al., 2015) mRNA expression of 5-HT_{2A} receptors was found to increase in spinal rats. The 5-HT_{2A} receptor was most abundant on the flexor and extensor motoneurons followed by 5-HT_7 (5-HT_{2A} was ~3x5-HT_7). In contrast Miazga et al. (2018) showed a decrease in both the 5-HT_{2A} and 5-HT_7 receptor gene expression after spinalization in both tibialis anterior and gastrocnemius lateralis motoneurons. However, these seemingly contradictory findings relate to the times after spinal

injury at which the measurements were collected. There are clearly time dependent changes of all receptors tested to date as described in more detail by Miazga et al. (2018).

Serotonin (5-HT) receptor subtype	Animal/preparation	Receptor activation effect on locomotion	Reference
5-HT_{1A} and/or 5-HT_7	5-HT_7 knockout mice with spinal injury, overground walking	Facilitates stepping. 8OH DPAT: 5-HT_{1A}/ 5-HT_7 agonist)	(Landry et al., 2006)
	Fictive locomotor, decerebrate cat, 5-HT_{1A} receptors	Correlation of rhythmic neural activity with walking	(Noga et al., 2009)
	Mice with complete SCI	Facilitates stepping. Buspirone: 5-HT $_{1A}$ 5-HT_7 5-HT_2 agonist	(Jeffrey-Gauthier et al., 2018)
5-HT_{2A}	Rats, neonatal and adult, fictive and treadmill locomotion	Facilitates stepping. Quipazine: 5-HT_{2A} agonist	(Liu & Jordan, 2005) (Sławińska et al., 2012)
	5-HT_7 knockout mice, neonatal	Increase bursting. Ketanserin and spiperone: 5-HT_{2A} antagonists	(Liu et al., 2009)
5-HT_{2C}	Rats after complete sacral SCI And double hemisected rats	Increases tail spasticity (both tonic and phasic bursts) Facilitates stepping. SB206553: 5-HT_{2C} inverse agonist	(Murray et al., 2010) (Fouad et al., 2010)
5-HT_7 selectively	Rats, intact spinal during overground walking and fictive locomotion and spinal rats during treadmill walking	Facilitates stepping and coordination. SB269970 antagonist,	(Cabaj et al., 2017; Sławińska et al., 2012)
	5-HT_7 knockout mice and wild-type, neonatal in-vitro, and adult overground	Facilitates stepping and coordination. SB269970 antagonist	(Liu et al., 2009)

Table 1.1 Effects of different serotonin receptors on rodent locomotion
Overview of some commonly reported effects of various chemicals acting on serotonergic (5-HT) receptors when used to influence locomotor activity in mouse or rats. The 5-HT receptor subtype (column 1), the preparation(s) and species used (column 2), effects on motor activity (column 3) and the reference (column 4) are as indicated.

1.6 Rodent SCI models and locomotor recovery after SCI

Animal models of SCI encompass many approaches, ranging from focal, delimited lesioning of the spinal cord to hemi-section and to full transection levels. These models also differ in the way in which the injury was elicited, by a blunt trauma or by dropping a weight to contuse the spinal vertebrae (Rossignol et al., 2015) or compression using a clip (Cormier et al., 2010) or a partial or complete laceration. Despite the relatively low prevalence of complete spinal cord lesions in humans, animal models incorporated the use of a full transection injury because it is a useful tool for questions addressing recovery from SCI (Rossignol & Frigon, 2011). While incomplete SCI models present relevance to most human SCIs and are helpful models for assessing neuroprotective modes of treatment. Animals with an incomplete SCI preserve more of their physiological functions (such as bladder function) and are more likely to recover some postural control. In addition to the fact that rats can spontaneously recover locomotor ability after an incomplete injury (Fouad et al., 2000; Leszczyńska et al., 2015). The difficulty of applying the incomplete injury type of model is the variability of the tracts that may be spared. As well as the difficulty in assessing if the recovered functionality is due to compensatory behaviors or neural reorganization and plasticity , remyelination of damaged but still intact axons or sprouting of other neurons (Watson et al., 2008). In addition to significant neuropathic pain altering motor function that can develop as a result of an incomplete injury (Gorska et al., 2009; Martinez et al., 2011). Complete spinal injury models enable research to identify contributions of different spinal neural mechanisms, pathways, and networks of locomotion in isolation from supraspinal input. For example, this model allows experiments to focus on interneurons below the level of injury that may contribute to recovery of locomotor ability. In experiments aiming to regenerate the spinal circuits by grafting tissue below the level of injury, complete transection models serve better to reduce the confounding factors that incomplete injury models have. This was the primary motivation to use a complete spinal injury model in my thesis work.

Full transection models have been used commonly in the cat since the 1900s, but it was not until the work of Grillner and colleagues that locomotor movement training has been incorporated as a post-injury intervention to improve functional recovery. The simplest kind of locomotor training after spinal cord injury is to train to stand. Standing is a prerequisite to walking as it requires both loading the limbs and extensors to be recruited in the stance position. Training to stand in the cat model of spinal cord injury showed that it was possible to improve standing (De

Leon et al., 1998) yet these cats did not improve their ability to walk (Hodgson et al., 1994). Spinal cats that were trained for stepping and then trained to stand; lost the improvements gained in stepping. In other words, one kind of training may not be efficient in achieving and maintaining the intended functionality. More recent work on the cat from Frigon's lab would suggest that training for recovery of both standing and locomotion after a complete SCI is not task specific (Harnie et al., 2019).

Complete spinal-transected cats can regain total weight-bearing standing and locomotion after five weeks of rhythmic manual stimulation of triceps surae muscles (non-specific training) and even without any specific training intervention (Harnie et al., 2019). Task specific training, for walking, has been shown to improve locomotor function recovery in all species examined to date, including humans. Directed locomotor training when a cat is placed to walk daily for 30-60 minutes allows these animals to recover locomotor ability within about 21 days of a complete SCI (Barbeau & Rossignol, 1987) while mice can exhibit locomotor-like ability on the treadmill within 14 days by way of spontaneous recovery (Leblond et al., 2003). Rats and mice have also been subjected to exercise training in combination with pharmacological, combinatorial treatments as means to regenerate spinal circuits and locomotor function (Rossignol et al., 2015). Rats, on the other hand, have shown to be able to develop improved hindlimb locomotor ability in the upright posture, when the rat's posture is so that it walks on its hind limbs in an upright position to increase loading (Sławińska et al., 2012; Van Den Brand et al., 2012). The improvement in the upright posture in rats after a complete transection develops without interventions training or otherwise, yet these improvements are not observed in the horizontal position after a complete SCI (Sławińska et al., 2012).

Locomotor testing over a treadmill rather than overground is preferred from the point of view that a treadmill provides continuous afferent feedback. The training effects gained by treadmill walking and those after overground walking have been considered similar, albeit not identical. Stepping movements can be evaluated through the use of markers to follow the changes in joint position over time. Stick diagrams can be constructed by connecting the joint positions of each step as illustrated in Figure 1.3 B Each step during walking consists of two phases: swing and stance. Swing is when the foot is in the air, the flexors are active and there is no weight bearing. Stance is when the foot is in contact with the ground, extensor muscles are active while providing body weight support. Duration of overground stance ranges from 300 to about 800 milliseconds

(ms) in rats. Burst duration reflects the time from the onset to the offset of flexor or extensor motoneuron firing (see Figure 1.3). Step cycle duration is the time between two consecutive foot contacts made with the ground or bursts of activity in a muscle. Step cycle frequency refers to how many full steps cycles occur over 1 s. The number of steps can be quantified by counting the ground contact by the toe or by measuring muscle activity via electromyography and visualizing each burst of muscle activity as illustrated in Figure 1.3 C.

Coordination between flexor and extensor as well as left and right sides is required for locomotion, but it can vary during walking. The phase relationship between the activity of two muscles is expressed by using angles (0 to 360 degrees) based on relative timing. Thus, the shift between the onset of activity in two muscles could account for an entire step cycle (360 degrees) or a quarter of that (90 degrees) or half of the cycle (180 degrees). These numbers are often plotted on a circular plot (or polar plot) as illustrated in Figure 1.3 E. Out-of-phase coupling refers to when for example, one limb is in swing phase while the other limb is in stance phase (left-right coordination: 180°- difference between onset of right *vs* left flexor activity), or extensor muscles are active while the flexor muscles are silent (flexor-extensor coordination: around 90° degree difference of onset of extensor with respect to onset of flexor activity, as the extensors are active during most of the cycle). Rodents can express multiple types of coupling, from the most obvious left-right alternation observed in walking to the completely synchronous type of coordination during gallop (Lemieux et al., 2016).

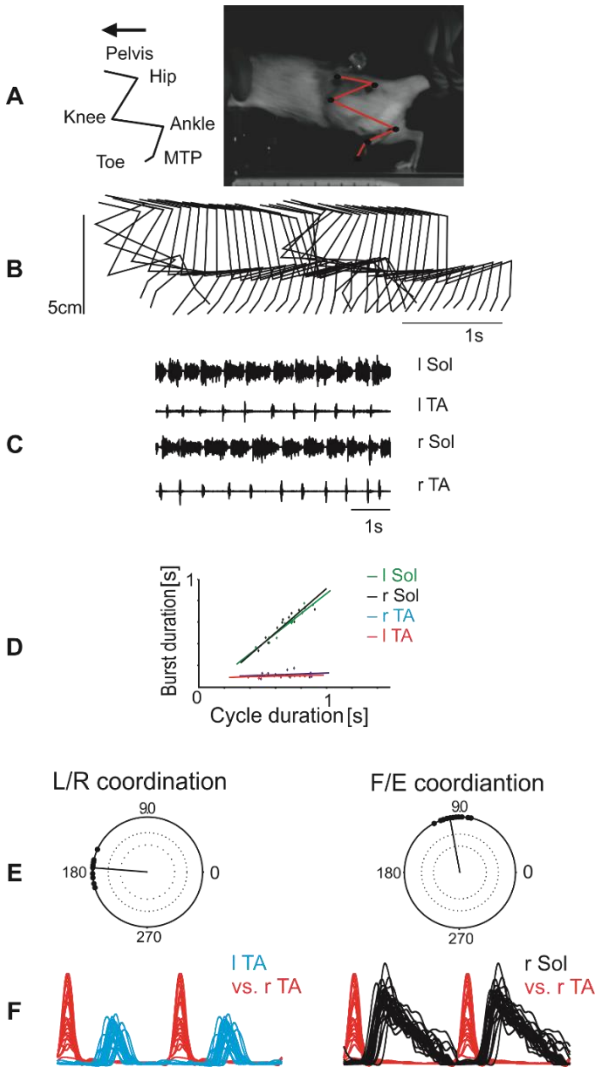

Figure 1.3 Analysis of locomotor activity during stepping.
Testing of a rat after spinalization and grafting in the horizontal plane with front limbs supported on a plate and hindlimbs moving on a treadmill at 10cm/s. **A**. Image of rat during treadmill locomotion with paw plantar stepping (paw contacts surface) during weight support. Marked spots on the hind limb (red) allow for stick figure reconstruction and kinematic analysis. **B** Reconstruction of joint angles for measurement of excursions (horizontal and vertical) and joint range of motion. **C**. Raw EMG recordings from right (r) and left (l) flexors (TA) and extensors (Sol) showing rhythmic alternation over time. **D**. The Sol burst duration is strongly correlated to the step cycle duration while the TA muscle activity is brief and unaltered in duration regardless of the step cycle duration. **E**. Left panel, polar plots depicting phase shifts between right and left muscles, which were expected to be alternating and showing a 180-degree shift (dots show phase shift of each step in the bout analysed). Right panel, polar plots indicating the phase shift between right flexor and extensor activity showing consistent phase shift of about 90 degrees. **F.** Rectified EMG data, and super-imposed step cycles from all steps in the bout analysed showing. Left panel depicts regular left right alternation. Right panel depicts regular flexor and extensor alternation. Note that Sol activity is sustained throughout the stance phase which is an important feature of plantar stepping.

There are different methods to assess recovery after SCI in rodents. They range from assessing the structural recovery to evaluate for the presence or regeneration of lost tissue to the physiological and functional assessment. For structural evaluation one can utilize immuno-histochemistry or anterograde or retrograde axonal tract tracing to visualize and assess specific tracts or population of cells which may contribute to recovery. On the other hand, physiological assessment of the spinal cord can give us important information on severity of the injury or help monitor effectiveness of treatment. Physiological assessment includes but is not limited to electrophysiological assessment such as measuring cord dorsum potentials (CDPs) which shows the conduction of evoked potential along the dorsal surface of the spinal cord. Intraspinal field mapping is another useful method to demonstrate alterations in evoked potentials following afferent or efferent stimulation. Observing differences in size, location, or modality of the responses in the receptive field gives an idea about both functional and anatomical changes within the spinal cord after injury. Mapping the changes in field potentials in the spinal cord demonstrates changes in circuitry and /or neural excitability, this method is utilized in Chapter II. Further functional assessment used with spinal cord injury is to assess locomotor recovery. This can be done using electromyographic recordings to assess coordination of muscle activity. This method was used in Chapter III and is shown in Figure 1.3 C-F.

The use of EMG recordings from hindlimb muscles gives an accurate and objective evaluation of the activity of the antagonistic muscle in each limb. This method allows for observing the activity in muscles, even if it is not strong enough to be expressed as proper locomotion. It also enables us to observe the discrepancies that occur, whether there is an increase or decrease in bursting of some muscles. It also allows us to determine the reason for the inability to express locomotion, whether it is a lack of coordination between the flexors and extensors (intralimb) of one or both limbs or if it is a lack of coordination in the right and left limbs (interlimb), or if there is a minor phase shift in the pattern of activation of the muscles. Displayed in Figure 1.3 C-F are only a few of the analyses available to deduce for locomotor recovery where Figure 1.3 C shows the raw EMG recording, Figure 1.3 D and F show relationship between single muscle burst duration *vs* cycle burst duration. Figure 1.3 E shows the polar plot analysis to show how well the intra and interlimb coordination is. The phase relationship between the activity of two muscles is expressed by using angles (0 to 360 degrees) based on relative timing. Thus, the shift between the onset of activity in two muscles could account for an entire step cycle (360 degrees) or a quarter

of that (90 degrees) or half of the cycle (180 degrees). These numbers are often plotted on a circular plot (or polar plot) as illustrated in Figure 1.3 E. Kinematic evaluation illustrated in Figure 1.3 A-B is a helpful method to evaluate locomotor recovery through quantitative measurements of the horizontal and vertical excursions of the different landmarks on the hindlimbs as well as evaluating changes in the angles of the limb throughout movement.

Other methods for evaluation include the BBB score (Basso et al., 1995) which is a widely used method of assessment used in the open-field overground walking. Limitations of this method is its insensitivity in the lower ranges of assessment as it is limited to the locomotor-like movements while one can have differences in said locomotor-like movements. There are many other methods of assessment such as the Grid walk, inclined plane test, rotary rod test, the catwalk (or ratwalk) for gait analysis. Each method has its indications whether to test for balance, sensory feedback, coordination, muscle strength, or pawprint area and base of support (*Animal Models of Acute Neurological Injuries II*, 2012; Watson et al., 2008).

1.7 Hindlimb muscle afferent input and its role in modifying locomotor activity

Locomotor activity can be modified by sensory (afferent) input. The actions evoked after sensory nerve stimulation depend on the state of activity, such as resting or walking or running; or the phase of walking such as swing or stance during walking. The reflex actions also depend on the type of nerve activated, such as skin or muscle nerve. The types of sensory signals which can alter stepping originate from multiple sensory receptors, such as cutaneous, chemo or muscle receptors (Kandel, 2013). Since the time of Sherrington, when activation of flexor muscles followed by a contralateral extensor burst has been attributed to pain fiber activation; stepping has been viewed as a series of responses to nociceptor input. Cutaneous nociceptors induce flexion to withdraw the limb from the source of the dangerous stimulus and excite extensor muscles contracting on the opposite side to stabilize the rest of the body.

Despite the minimal role of cutaneous input to the locomotor ability of an intact cat (Bouyer & Rossignol, 2003a), cutaneous input has an exaggerated or even more significant role in developing and maintaining locomotion after spinal cord injury, as depicted, in a series of elegant work by Bouyer and Rossignol (2003b). Chronic spinal cats recovered walking ability through locomotor training, but those cats were not able to recover proper plantar stepping in which all cutaneous nerves from the foot were cut. These results showed that cutaneous input is crucial for the

development of proper locomotor output independent of the supraspinal and spinal input. Similarly, in rats, Sławińska et al. (2012) showed that recovery of upright walking was dependent on the presence of afferent feedback from the plantar surface of the foot. The recognition of cutaneous afferents from the paw in rats being just as critical for recovery of walking as it has been found in the cat suggests that the role of these afferents is conserved over many species.

While both dorsal and plantar surfaces of the paw can modify the output of locomotion, the dorsal surface is involved in initiating a correcting reflex during locomotion called the stumbling correction reflex (Forssberg, 1979) while the plantar surface is involved in directing the placement of the foot which allows for consequent weight bearing. The posterior tibial nerve is the main nerve supplying the plantar surface of the foot of the rat (Kambiz et al., 2014) while it also supplies ankle extensor muscles (medial and lateral heads of the gastrocnemius, soleus, plantaris, as well as muscles of the flexor hallucis and digitorum longus, toe flexors and intrinsic muscle of the foot (Badia et al., 2010). The distribution of afferent input from major nerves in this region of the hindleg is summarized in Figure 1.4 .The rostro-caudal gradient of afferent projections with the majority of tibial afferents terminating in the L4-L5 segments has been verified with both anatomical and electrophysiological methods (Swett & Woolf, 1985). The dorso-ventral gradient has also been mapped in terms of the various sensory receptors making up the tibial nerve. Typically, the distribution of cutaneous afferent input is restricted to the dorsal horn (laminae I-III primarily) while the largest muscle afferents (primary spindle endings, or Ia fibers) terminate in the ventral horn and the smaller diameter muscle afferents (secondary spindle endings, group II fibers) terminate in the intermediate zone.

Loading has been considered the most critical component of training –identified to date – that contributes in all species examined to the improved walking (Donelan & Pearson, 2004). It is important to note that even in the incomplete spinally injured rat unloading the hind limbs was detrimental to recovery of walking (Caudle et al., 2011). However, locomotor recovery of spinalized cats and rats, or mice is additionally facilitated and even acutely improved with the addition of exteroceptive stimulation of the tail or perianal region (Leblond et al., 2003; Pearson, 2004). The combination of various afferent stimulation with training primes the cord with information that leads to better responses in terms of the required level of muscle activity.

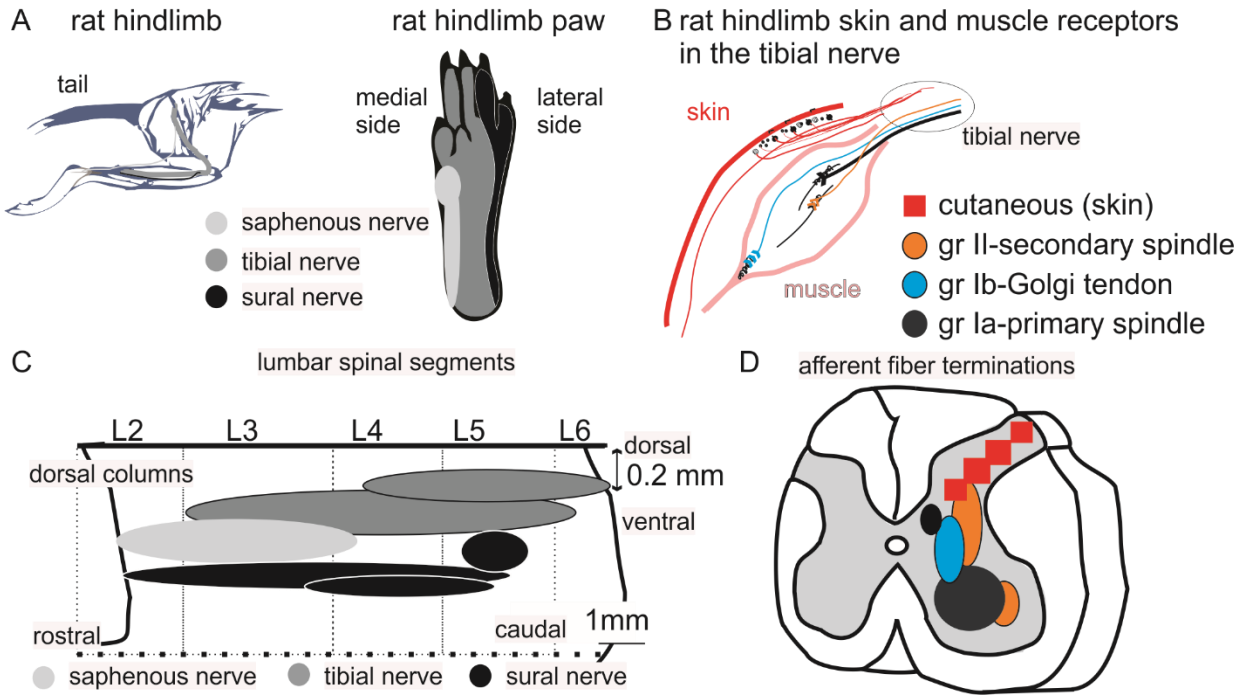

Figure 1.4. Distribution of afferent input from the rat hindlimb.
Schematic illustration of how rat hindlimb sensory input from skin and muscle receptors is distributed in the lumbar spinal cord. **A.** Main nerves innervating the rat hindlimb include the saphenous, the tibial and the sural nerves. The paw receptive fields of each nerve are also indicated here. **B.** The tibial nerve is composed of afferents arising from various receptors located in skin and in muscles tissue (and also joints of the knee and ankle, not illustrated here). The major types of muscle sensory receptor types are described as primary muscle spindle organs (group Ia afferents, gr Ia) Golgi tendon organs (group Ib afferents, gr Ib) and secondary muscle spindle organs (group II afferents, gr II). **C.** Rostro-caudal distribution in the lumbar segments based on fiber terminations (modified from (Swett & Woolf, 1985). D. Typical dorso-ventral distribution of input from various receptors of the tibial nerve.

Tail stimulation in rats leads to immediate modulation of locomotor -like activity in spinalized rats, even without training (Frigon et al., 2022). Tail stimulation is likely to generate both muscle afferent and cutaneous input which improves motor output. Overall, like perineal stimulation, tail pinching is thought to generate ascending input from sacral to lumbar interneural networks and this "extra excitation" to lumbar CPG neurons that is translated into improved motoneuron activity (Frigon et al., 2022). Tail stimulation can be tonic or phasic, and it can be relatively easily adjusted to gain positive functional difference in terms of how stepping is modified. Location of the tail stimulation being at the tip (caudal end) or at the base (rostral end) of the tail can also have differential effects in each animal (unpublished observations from my work in the Slawinska and Jordan laboratories).

Tail electrical nerve stimulation in rats with T10 contusion injury was used at 7 weeks post SCI which temporarily improved walking as measured by BBB scoring (Zhang et al., 2010). This improved walking capacity was not reproducible when the contusive spinal injury was at the L2 - S1 regions which suggested that the interneural networks in these segments are key for the tail-linked pathway. Therefore, tail nerve stimulation has been viewed as a potential intervention for influencing spinal neural network activity within the L2-S1 segments in order to improve motor function.

In a conceptually analogous manner, the spinal cord can also be stimulated using either epidural or transcutaneous electrical stimulation to induce rhythmic stepping as seen in the cat by the work of Mushahwar and Prochazka (Mushahwar et al., 2002; Mushahwar & Prochazka, 2004) and also later in humans as reviewed in (Dimitrijevic et al., 1980) (Mayr et al., 2016). Humans with SCI often undergo a combination of intervention, such as pharmacological and exercise-based training to gain some functional recovery of rhythmic activity and these could be further augmented with electrical stimulation of the spinal cord (Duysens & Van De Crommert, 1998) or by grafting of neural tissue around or below the lesion as reviewed by (Zawadzka et al., 2021).

During training, the barrage of sensory input originating from cutaneous afferents from muscle afferents (load and stretch receptors) provides a complex set of stimuli triggering positive plasticity. This positive plasticity has several aspects, such as increased synaptic efficacy in afferent reflex pathways, in increased sprouting of reminiscent descending, supraspinal axons, sprouting of local neural connections and changes in ion channel expression on the membranes of spinal motoneurons and interneurons. The experiments described in Chapter II of the thesis address the reorganization of afferent input in rats within five weeks after a complete spinal cord injury. The experiments described in Chapter III addresses changes gained in locomotor function via additional excitation of 5-HT neurons introduced by grafted embryonic brainstem tissue below the level of the injury.

1.8 Grafting to improve locomotor function recovery

Cell replacement, or grafting therapy was developed to overcome the limited ability of the spinal cord for regeneration and repair. Cell replacement therapy provides a source for the lost cell populations or connections as well as the scaffolding and substrates needed for growth and regeneration. Cell replacement therapy has been used for many decades, as described in a review by e.g. (Das, 1990). The main questions surrounding cell replacement therapy are regarding 1) the appropriate cell type to be grafted and 2) where and 3) when to graft (Zholudeva & Lane, 2019).

There are many different donor cell types used for grafting after spinal cord injury to induce repair. Each cell type has been chosen with a different rationale and varied level of success. These cell types include Schwann cells, olfactory ensheathing cells, neural stem cell and progenitor cells (adult and or embryonic), and mesenchymal stem cells from the bone marrow as reviewed by e.g. (Tetzlaff et al., 2011). While some grafts improve repair of the spinal cord injury by remyelination or creating a bridge for axonal regrowth, the timing and location of the grafts and the proposed functionality gains all contribute to the decision of the cell type or source of the graft to be used.

When considering the location of the graft, most procedures target the vicinity of the lesion site to overcome the damage and to be as minimally invasive as possible. This approach, however, has its own challenges and caveats, with respect to survival of the graft. There is a hostile environment, fueled by the scar tissue around the lesion, which prevents the integration of the grafted cells (Hosseini et al., 2022) affecting also the development and the phenotype of the cells that were transplanted (Iwai et al., 2014). Placing a graft below the level of the lesion has a better chance of surviving and it also offers more flexibility in the timing of the graft placement (Feraboli-Lohnherr, Orsal, Yakovleff, Gimenez Y Ribotta, et al., 1997) compared to placing a graft within the lesion. A graft that is placed below the level of the injury is thought to influence spinal CPGs and use the remaining intact circuitry for recovering locomotor function rather than regenerating lost connectivity. The timing of the graft placement is essential to consider, as treatments applicable in chronic conditions differ than those after acute injuries.

What type of cells are grafted is also a major factor. Grafting brainstem derived embryonic neurons has been successful since 1977 (Nygren et al., 1977). The embryonic grafted 5-HT neurons re-establish their original targets through mechanisms that are not fully understood but implicate the same factors that guide axons in embryonic neurons (Gimenez Y Ribotta et al., 1996).

In a complete transection model, the grafted 5-HT neurons synapsed below the lesion and serotonergic fibers from the graft remained with a few spinal segments of the grafting levels (Ribotta et al., 2000).

Feraboli-Lohnherr, Orsal, Yakovleff, Gimenez Y Ribotta, et al. (1997) have been successful in grafting 5-HT neurons into spinalized rats to recover locomotor activity. Similarly, Sławińska et al. (2000) showed that grafting of 5-HT neurons is effective at restoring locomotion in chronic spinal rats. This recovery was attributed to 5-HT as applying 5-HT antagonists blocked the improvements Majczyński et al. (2005). The Slawinska lab has repeatedly showed that grafting embryonic cells into the spinal cord one week after spinal cord injury allowed grafted cells to survive and rats displayed improved locomotor function compared to non-grafted rats (Miazga et al., 2018; Sławińska et al., 2000; Sławińska et al., 2013).

Given the role of 5-HT in multiple functions, serotonergic grafts have been applied not only for the recovery of locomotor activity but also for overcoming pain, sensory dysfunction and autonomic dysreflexia. Recent studies from another lab showed the possibility that respiratory function even after 1.5 years of a spinal cord hemi-section in rats could be restored (Warren et al., 2018). After a sever contusive injury (Eaton et al., 2011) showed that differentiated 5-HT cell grafts improved sensory loss and dysfunction such as allodynia and hyperalgesia resulting from SCI in rats. Grafting 5-HT neurons derived from E14 brainstem acutely in the upper thoracic area after complete SCI successfully recovered regulation of autonomic activity and blood pressure in rats (Hou et al., 2020).

Optimizing grafting to generate greater locomotor recovery required knowledge of the serotonergic populations that are successfully activated to produce stepping in isolated, newborn spinal cords, as reported by Liu and Jordan (2005). Specifically, the parapyramidal region (PPR), a serotonergic population located lateral to the pyramidal tract in the medulla, mediates descending serotonergic locomotor commands to the spinal cord (Liu & Jordan, 2005). By grafting all of the B1, B2, and B3 areas—including the lateral portion of the B3 area—which is destined to become the population of serotonergic neurons present in the PPR, attempted to maximize the locomotor activity produced. Previous grafting attempts for locomotor recovery (Feraboli-Lohnherr, Orsal, Yakovleff, Gimenez Y Ribotta, et al., 1997; Majczyński et al., 2005; Sławińska et al., 2000) focused on the midline medullary raphe which are represented by the raphe pallidus (B1) and raphe

obscurus (B2) excluding B3. The improved locomotor activity was observed with the aid of some exteroceptive stimulation by pinching of the tail or perianal area without any shown improvement in overground locomotion. Tail stimulation could be viewed as a possible method to activate the grafted serotonergic neurons as well as components of the CPG present below the level of the injury. In the experiments described in Chapter III, we tested the hypothesis that selective activation of 5-HT neurons in embryonic grafts with chemogenetics leads to better hind limb locomotor performance in spinal grafted rats than in grafted rats without this activation.

1.9 Chemogenetics as a tool for neural activation & inactivation

Chemogenetics refers to a methodology by which receptors are genetically modified so as to render them only responsive to an inert ligand that is not naturally occurring (Sternson & Roth, 2014). One type of the multiple chemogenetic approaches that have become popular for use in understanding neural circuits is the so-called DREADD technology, referring to Designer Receptors Exclusively Activated by Designer Drugs (Armbruster et al., 2007). This method utilized G-protein coupled receptors that are genetically modified so as to render them unresponsive to their native ligand and theoretically only responsive to a specially constructed ligand that only affects these designer receptors.

One ligand used with the DREADDs method was Clozapine-N-Oxide (CNO), a metabolite of the atypical antipsychotic drug called clozapine (CZP) (Armbruster et al., 2007). The basis of the interaction between the synthetic designer drug, CNO and a designer receptor is a point mutation of a naturally occurring human muscarinic acetylcholine receptor, referred to as hM3 (Roth, 2016) which is inserted into neurons of interest when desired to be used for stimulating cellular activity (depolarization). The designer drug is ideally an inert ligand that is a chemical that has high affinity to bind to the mutated muscarinic ion channel but has no other biological effect (Aldrin-Kirk & Bjorklund, 2019).

This technology has been exciting as many potential applications were in sight due to hypothesized zero off-target effects of the designer receptors. DREADDs have been widely used in combination with recombinant Adeno-Associated Viral (rAAV) delivery methods, one of the safest gene delivery platforms for use in humans (with FDA approval since 2017). My work combined DREADDs with rAAVs to deliver hM3s into spinal neurons and to enable excitation of transfected neurons by a designer drug. Another advantage of using DREADDs was that systemic

administration of the designer drug can be done by minimally invasive ways, such as by adding it to the drinking water or by an intraperitoneal injection (Alexander et al., 2009; Roth, 2016).

Even though DREADDs technology seemed sound for my project, some limitations of using hM3-based DREADDs have been recognized since the time I started using this method. One problem was that CNO does not cross the blood-brain barrier and another problem is that it could back-metabolized to clozapine (Gomez et al., 2017) which is more effective in penetrating the BBB and in accumulating in the brain then CNO. It needs to be noted here, that CZP has been considered as a "dirty drug" as it has affinity to several receptors present in the central nervous system. Thereby this drug will induce off-target effects in addition to binding to designer receptors. Because of such off-target effects of the designer drug CNO (Gomez et al., 2017), there has been growing interest in developing new and more efficient designer drugs for hM3 activation. One recently developed ligand is the so-called compound-21 (C21). Thus, the effective use of DREADDs relies on actuator ligands that have minimal off-target effects at minimal concentration levels i.e., as low as possible for naturally limiting any potential off-target effect. Different binding affinity values (pKi) of DREADD ligands constitute a growing list with varying affinity levels (Thompson et al., 2018).

1.10 Rationale, hypothesis, and list of aims

Locomotor activity depends on the integration of descending supraspinal signals and peripheral input from sensory receptors. There is some capacity of the mammalian nervous system to recover locomotor function but the mechanisms underlying this recovery are still ill-understood. My research aimed to investigate plasticity underlying spontaneous locomotor improvements and gain new insights into mechanisms required for improved walking. By utilizing a rat SCI model, with a complete spinal transection, I examined changes in afferent processing within lumbar CPG circuits.

1.10.1. Rationale for assessing changes in afferent input to lumbar spinal neurons after complete thoracic transection

Based on previous preliminary findings in the Slawinska lab, at one week after a complete low-thoracic SCI, rats were not able to plantar step on a treadmill, while at about five weeks after injury, rats exhibited coordinated upright stepping. As the sensory input from the plantar surface of the foot has been reported as critical in rats to facilitate locomotor recovery, we examined changes to sensory input processing from the tibial nerve innervating the plantar surface. The following hypothesis was tested:

Hypothesis 1: The distribution pattern of tibial nerve afferent input within lumbar segments is altered by SCI.

This hypothesis was examined by utilizing a low-thoracic complete spinal transection model, combined with *in vivo,* direct electrophysiological measurements of evoked potentials following tibial nerve (TIB) stimulation. The TIB-evoked responses were collected from the surface of the spinal cord and from intraspinal locations, representing activity of afferents and interneurons excited by TIB. The following aims were sought in these experiments:

Aim 1.1. Map cord dorsum field potentials from TIB muscle afferents at one week and at 5 weeks after a complete spinal cord transection to examine changes in projection patterns.

Aim 1.2. Measure extracellular field potentials in lumbar segments from TIB group II muscle afferent to quantify changes in synaptic efficacy of TIB afferents.

The results of these experiments are described in Chapter II.

1.10.2. Rationale for using embryonic grafts and selective activation of 5-HT neurons below a spinal lesion after SCI

Even partial reconnecting of descending supraspinal input to spinal neurons has been shown to be promising for functional recovery. The transplantation of 5-HT neurons below the level of spinal cord injury has been shown to improve the recovery of locomotor output in rodents to some level. Analogous to how exteroceptive stimulation of the tail can improve locomotor function by activating lumbar neural circuits; extra (additional) activation of the grafted 5-HT neurons "on demand" was one of the goals. By utilizing novel, genetically engineered rats and chemogenetic approaches enabling selective stimulation of grafted 5-HT neurons below the level of spinal injury, my research aimed to improve functional locomotor recovery. As the application of 5-HT in many species has shown superb facilitatory effects on locomotion, the following hypothesis was tested.

> **Hypothesis 2: Stimulation of grafted 5-HT neurons (by chemogenetic means) in paraplegic rats improves locomotor activity compared to rats where only grafting has been performed.**

This hypothesis was examined by utilizing a complete Th9-10 spinal transection model. The lumbar cord below the region was the target site for grafting of embryonic 5-HT neurons equipped with DREADDs. Then these animals were given a designer drug and behavioral trials conducted. The following aims were sought in these experiments:

Aim 2.1. Verify the ability of the dissociated, cultured embryonic neurons to express the designer receptors and to display increased activity levels.

Aim 2.2. Facilitate hindlimb locomotor patterns in spinalized, grafted rats with DREADDs following application of designer drugs (i.e., the excitation of grafted neurons).

Aim 2.3. Verify that grafted neurons in the spinal cord expressed 5-HT and DREADDs.

The results of these experiments are described in Chapter III.

Chapter II

Manuscript Title

Transient reduction of synaptic input from posterior tibial group II afferents after complete spinal cord transection in rat mid-lumbar dorsal horn

Authors

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Author contributions

The studies were conceived and designed by L.M.J. and U.S. and K.S. The experiments were performed by M.N., K.A., U.S., K.S. and L.M.J. The data analysis was done by M.N., E.C-R., K.S. and U.S.; all authors were involved in finalizing the manuscript for submission.

List of abbreviations

1W – 1-week post-injury cohort
5W – 5-weeks post-injury cohort
CDP – cord dorsum potential
gr I – group I afferent
gr Ib – group Ib afferent
gr II – group II afferent
INT – intact rat cohort
SCI – spinal cord injury
T – threshold for activation of most excitable afferents
TIB – posterior tibial nerve
xT – times threshold

2.1 Abstract

Rats with complete spinal cord injury (SCI) improve plantar stepping in the upright posture without any training in about five weeks after SCI. Such recovery might be an effect of spontaneous reorganization of spinal circuits, which depends on sensory input from hindlimb afferents, primarily via the posterior tibial nerve (TIB). This study examined changes in TIB afferent pathways at different times after SCI to better understand mechanisms underlying the recovery of locomotor function.

Cord dorsum potentials (CDPs) evoked by graded electrical TIB stimulation were measured in rats at different rostro-caudal levels of the lumbar spinal cord. The amplitude of components evoked by group Ia, Ib and II fibers were normalized and compared in rats with intact (n=5) and with injured cords at 1 week (n=6) and at 5 weeks (n=6) after SCI. In addition, intraspinal recordings following TIB stimulation were collected (n=5 rats 1 intact and 4 spinalized) to compare latency and amplitude of evoked potentials at multiple dorso-ventral locations at different times after SCI.

The results showed that the TIB group I muscle afferents evoked maximal response at the L4-L5 level in both intact and spinal animals. There was no evidence for a widening rostro-caudal distribution of tibial input to lumbar segments. However, intraspinal field potentials in the dorsal horn evoked by TIB group II afferents were reduced in amplitude at one-week post-SCI. The reduction of synaptic effects seen at one-week post-SCI recovered by about five weeks post-SCI which is also the time point at which rats spontaneously recover some hindlimb walking capacity after a complete spinal transection. The results suggest that plasticity of dorsal group II interneurons from TIB may contribute to the spontaneously improved locomotion after SCI in rats.

2.2 Introduction

Locomotion is the result of the summation of different interactions of descending commands and afferent inputs on spinal neuronal networks that can generate and control the coordinated flexor and extensor motoneuron output. This neuronal network in the spinal cord called the central pattern generator (CPG) can generate locomotor rhythm even in the absence of sensory feedback or descending motor commands. The summative effects of afferent feedback allowing improvements in motoneuron recruitment and firing patterns rely on interneural networks harmonizing sensory input with descending motor commands. The afferent inputs can be muscle or cutaneous acting on motoneurons in a state-dependent fashion to allow for improvements in coordinated motoneuron recruitment and firing patterns (Forssberg et al., 1975, 1977; Mccrea, 2001). Ia muscle afferents or primary muscle spindle organ, along with Ib muscle afferents or tendon organs and group II muscle afferents or secondary spindle organs are responsible for encoding various aspects related to muscle function or location in space including muscle tension developed during contractions, muscle length and dynamic changes in the length of the muscle fibers (Banks et al., 2021; Matthews, 1981). Load-induced enhancement of neural output to support muscle activity relates to the collective feedback from muscle spindle receptors and Golgi tendon organs as well as cutaneous receptors. These receptors transmit multifaceted information about the musculoskeletal system (Nichols, 2018), which is critical for shaping the step-by-step locomotor output.

Proprioceptive information specifically from the plantar surface of the paw provides feedback to shape ongoing CPG as well as flexor and extensor motoneuron activity in cats (Duysens & G., 1980; Mccrea, 2001). The proper plantar paw placement, when the animal brings the plantar surface of the hindlimb into contact with the ground; is one of the key hallmarks of functional recovery of overground stepping in quadrupeds (Basso et al., 1995). In chronic spinalized cats, sensory input from the plantar surface of the foot is mandatory for the recovery of hindlimb walking by locomotor training (Bouyer & Rossignol, 2003b; Rossignol et al., 2006). This was also the case in recovery of bipedal, upright walking in rats after a complete low-thoracic spinalization (Sławińska et al., 2012). Excess excitatory input through tail-pinching allowed for successful plantar stepping of hindlimbs with good interlimb as well as swing-stance phase coordination in the bipedal walking rats in upright posture when the rat forelimbs were held by the experimenter over the treadmill with the hindlimbs touching the moving belt. In the same rats in the air-stepping

configuration (no weight-bearing on hind or forelimbs), poorly coordinated steps, failed plantar placement and abnormal swing and stance durations were evident. Moreover, anesthetizing the plantar surface of the hindlimb foot led to disorganized inter-, and intralimb coordination and no plantar stepping in the bipedal configuration (Sławińska et al., 2012). Thus, the activation of cutaneous afferents innervating the plantar surface of the paw as well as load sensing muscle receptors in the hindlimbs are the key afferents for partial weight bearing and locomotion. Plantar placement and cutaneous input from the plantar surface of the paw are reliant on tibial (TIB) nerve function. The TIB nerve is a mixed nerve as it has branches innervating muscle and skin regions (Badia et al., 2010; Kambiz et al., 2014). This nerve innervates calf muscles that plantarflexes the ankle and allows inversion of the toes.

Group Ia, Ib and II muscle afferent input from the muscles innervated by the TIB nerve is distributed over the L1-S2 spinal segments (Watson et al., 2008). The spatial distribution of the axon collaterals of the triceps surae muscles (medial and lateral heads of gastrocnemius and soleus) in the rat (Vincent et al., 2017) was similar to findings in the cat spinal cord (Brown & Fyffe, 1979). The Ia, Ib and group II afferents converged in the medial half of the deep dorsal horn (Rexed laminae V, V1, and dorsal VII). Monosynaptic actions of group II afferents on target feline motoneurons innervating the gastrocnemius and soleus muscles have been described based on field potentials recorded in the caudal lumbar and sacral segments (Fu et al., 1974). In the mid-lumbar segments, group II field potentials are larger in absolute amplitude and at specific locations, they are not contaminated by the group I afferent-induced field potentials. Therefore it was reasoned that interneurons within mid-lumbar segments are specialized to process information from group II afferents relative to lower-lumbar networks (Edgley & Jankowska, 1987b). Electrical activation of group II afferents in hindlimb nerves is known to evoke negative cord dorsum potentials (CDPs) at the surface of mid-lumbar and sacral segments of the spinal cord in cats (Edgley & Jankowska, 1987b; Jankowska & Riddell, 1993) and also in rats (Riddell & Hadian, 1998). In intact rats, CDPs evoked by group II afferents of the calf muscles *medial and lateral gastrocnemius and soleus*, are largest in caudal regions of the lumbar enlargement with maximal CDPs emerging within the L5 segment in rats (Riddell & Hadian, 1998). Group II afferents of other nerves innervating the ankle and toes (*tibialis posterio*r and *flexor digitorum longus)* evoked responses predominately in the L3 and L4 segments (Riddell & Hadian, 1998). In cats the crossed inhibition of extensor motoneurons elicited by group II afferents was replaced by crossed excitation following spinal transection or

bilateral lesions of the dorsolateral funiculi in anaesthetized cats, indicating strong descending control of the commissural neural circuitry (Aggelopoulos et al., 1996).

There are spontaneous changes that occur in the adult completely transected cat spinal cord which lead to improved weight bearing by about 4-6 weeks after the injury (Harnie et al., 2019). Rats seldom expressed weight bearing or hindlimb stepping one week after a complete thoracic spinalization but after about 5 weeks post-injury, rats increased the number of correct paw placements and improved locomotor coordination was seen when stimulation of the perineal region was applied in addition to locomotor training (Alluin et al., 2015). Improved quadrupedal locomotion was reported by about six weeks post-spinal cord hemi-section in a recent study on rats when repeated excess excitation of large sensory dorsal root ganglion neurons was applied by chemogenetic means (Eisdorfer et al., 2022). The ablation of neurons responsible for relaying proprioceptive sensory input prevented descending detour circuits to innervate neurons below the cord transection level and to bridge circuits for functional recovery in mice (Takeoka et al., 2014). After injury, wildtype mice spontaneously recovered basic locomotor function, whereas mice with deficient muscle spindle feedback failed to regain control over the hindlimbs on the lesioned side. These findings revealed an essential role for muscle spindle feedback in directing basic locomotor recovery and facilitating circuit reorganization after spinal cord injury. Thus, it is important to examine if the activation of spinal sensory neurons involved in proprioceptive pathways underscores motor recovery in rats.

Changes occurring after spinal cord injury include multiple events (Dietz & Fouad, 2014). Greater arborization of myelinated afferents was evident in Rexed laminae I-V in lumbar segments one week after mid-thoracic cord transections (Krenz & Weaver, 1998). Calcitonin gene-related peptide-immuno-reactive fibers in laminae III-V increased in all cord segments at two weeks after transection, but not at one week. As the numbers of neurons immunoreactive for calcitonin gene-related peptide (CGRP) were unchanged, it is thought that increased sprouting and not peptide up-regulation occurs (Krenz & Weaver, 1998). Over-expressing a nerve growth factor in lumbosacral spinal levels facilitated CGRP-positive afferent fiber sprouting two-weeks post-injury in rats with complete mid-thoracic transection (Rabchevsky, 2006). CGRP immune-reactivity has been reported in neurons giving rise to Aβ, Aδ and C fibers (Krenz & Weaver, 1998) and CGRP-positive fibers also colocalize with various other neurotransmitter markers (Lawson et al., 1996; Mccarthy

& Lawson, 1997). That is why the identity of specific afferent fiber populations that sprout after spinal cord injury remains uncertain to date.

Differences in synaptic transmission from TIB afferents after spinal cord injury in rats are unknown even though the functional significance of TIB input for the recovery of locomotion is well established. In this study, we sought to examine the re-organization of the spinal neural circuitry transmitting afferent input from the sole of the hindlimb via the TIB nerve. The hypothesis, that projections of afferents from the TIB nerve innervating the ankle muscles and the plantar surface of the foot evoke different population responses (field potentials) depending on time after SCI was tested.

We used electrophysiological methods similar to those described in earlier studies related to defining the contribution of specific muscle spindle receptor subtypes to spinal neural activation (Banks et al., 2021). Electrical stimulation of nerves allowed to examine the effects of group II afferents, albeit not completely independent of group I afferents. As reviewed by Edgley & Jankowska (Edgley & Jankowska, 1987a) the consensus based on work from multiple laboratories has been that responses evoked by stimuli in the range of two to five times of the motor threshold are predominantly the result of group II afferent fiber activation. Cord dorsum potentials (CDPs) within appropriate latencies reflect the activity of incoming afferent fibers as well as transmission at synapses near the recording electrode. Group II afferent fibers and neurons in the grey matter within laminae IV-V synaptic have contacts, where field potentials with a similar time course to the cord dorsum potentials could be recorded. The amplitude and the latency of cord dorsum (surface) potentials in the 2^{nd} to 6^{th} lumbar segments and of intraspinal field potentials in the 4^{th} lumbar segment were recorded here. Data from rats with intact spinal cord and from those with a complete low-thoracic spinal cord transection at one- or five-weeks post-SCI were compared.

2.3 Methods

All animal care and experimental procedures conformed to the guidelines set by the Canadian Council on Animal Care. All procedures were approved by the University of Manitoba's Central Animal Care Committee. Experiments were performed on a total of 17 female Sprague Dawley (SD) rats (weighing 250 - 320 g). There were three groups of rats: 1) intact spinal rats (n=5), 2) spinal transected with 1-week survival time (n=6) and 3) spinal transected with 5-week survival time (n=6) after injury. Animals in the 1-week-post-spinalization group (1WK) underwent complete thoracic transection 6-8 days before the terminal experiment, while rats in the 5-week-post-spinalization group (5WK) had complete thoracic transection 28-35 days prior to the terminal experiments.

2.3.1. Complete spinal transection surgery and post-op procedures

Complete transection of the spinal cord was performed as described in previous papers (Kwaśniewska et al., 2020; Sławińska et al., 2012). Under sterile conditions, SD rats were anesthetized (isoflurane delivered in 95% oxygen). The spinal cord was completely transected after a laminectomy was performed at the vertebral level of Th9/10 by making two cuts around 1-2 mm apart and removing the severed cord by suctioning. Analgesic and non-steroidal anti-inflammatory (s.c. Tolfedine 4mg /kg b.w. and buprenorphine (0.05 mg/kg) was administered after surgery and antibiotics (gentamycin 5mg/kg b.w. s.c.) were given for 4 days following the transection surgery. Sterile saline was injected s.c. (1-2 mL) in the first 2-3 days post-operatively and the bladder was manually emptied 3 times a day until the voiding reflex was re-established. The animals were kept as a pair-per-cage, housed on a 12-h light-dark cycle and fed ad libitum. The care-fresh bedding was used in combination with normal sawdust bedding and no locomotor training or enriched housing was provided. The completeness of the spinal transection was verified in each animal in two ways: firstly, by monitoring the animals nearly every day for contractions in the hindlimbs (absent for about 7 days), and by dissecting the transected region of the cord after the terminal experiments and verifying the absence of regrown cord tissue at the lesion.

2.3.2. Locomotor assessment

Upright bipedal rat locomotion was assessed on the motorized treadmill while recording marker-based positions of hindlimb joints. An experimenter would hold the rats in upright posture on a running treadmill at speeds of (5 or 10cm/s) with their hindlimbs touching the treadmill. The experimenter manually simulated the tail or perineal area to evoke the best locomotor-like activity

in the hind limbs. Black ink marks were placed on the left hind limb at the iliac crest (pelvis), greater trochanter (hip), knee, ankle (lateral malleolus, 5th metatarsophalangeal joint head (MTP) and the tip of the 4th toe. Video recordings were obtained using an X-Motion™ camera (AOS Technologies) with a capture rate of 120 frames per second (shutter speed 8.3 ms), with 10 seconds of continuous recording. Marker detection in each frame was analyzed using ProAnalyst™ motion detection software (by Xcitex) which plotted each marker position. Positional data were then converted for further analysis using Analysis software developed by the Spinal Cord Research Centre at the University of Manitoba (as used in (Cowley et al., 2015). Changes in the joint angles, as week as horizontal, and vertical excursions of the different markers were analyzed and used to create stick figure diagrams to evaluate hindlimb movement during the recorded bouts at different time points after injury.

2.3.3. Terminal experimental procedure

Under isoflurane anesthesia (delivered in 95% oxygen), eye lubricant (Optixcare) and topical lidocaine (Lidocaine, 2.5% gel) was applied over each region prior to incision. The neck muscles overlying the trachea were dissected. Placement of a tracheal cannula was used to ventilate the rat for the remainder of the experiment. An arterial catheter was placed in the internal carotid artery to measure blood pressure and to supply fluids (0.1 M $NaHCO_3$ and 0.28 M dextrose in sterile water, 0.9-1.2 ml h^{-1}). The core temperature was maintained at 37.5° Celsius, the blood pressure was maintained at above 80 mmHg and the expired mean end-tidal CO_2 levels were monitored and maintained between 2-3.5% by adjusting inspiratory rate (55-75 breath per minute) and volume. The blood oxygenation level was measured (NONIN monitor) regularly. Hindlimbs were dissected to expose the tibial (Tib) and common peroneal (CP) nerves. Dexamethasone (s.c. 0.2 mg/kg) was given and a laminectomy from Th13 to L3 vertebrae was performed. The rat was moved to a specialized frame, fixed, and suspended so that spinal vertebral bodies were clamped, and the head was inserted into a stereotaxic frame. Warm mineral oil pools were created to protect the spinal cord and the isolated nerves in the hind limbs. Immediately prior to the commencement of stimulation protocols, the rats were immobilized using pancuronium bromide (i.p. or arterially, 0.2 – 0.4 mL, Pavulon, 0.4mg kg^{-1}). Four bipolar custom-made silver ball electrodes were placed across the spinal cord at different levels at least 0.5 mm apart (see Figure 2.1). At the end of the experiment, the rats were euthanized (arterial 3M KCl, 1 mL) and the spinal cord was inspected to

confirm the level of each recording electrode and the completeness of the transection in the spinalized rats.

2.3.4. Stimulation protocols

Customized stimulating bipolar silver hook electrodes were placed on the TIB and CP nerves. Stimulus strengths were expressed as multiples of the threshold (T) needed to activate the most excitable fibers (Jack, 1978). After determining the threshold for activating the most excitable fibers in each nerve, the intensities used for stimulation were 1.2xT, 2xT and 5xT. Stimulation with square pulses of 0.2 ms duration at 1 Hz or 2 Hz frequencies were used, and single trials were repeated 27-100 times and recorded for post-hoc averaging.

2.3.5. Data collection and recordings

The CDP recordings were collected from the surface of the spinal cord as shown in Figure 2.1. Electrodes were set to contact the cord between the midline and the dorsal root entry zone of the right side at the four selected locations. In 5 rats, the intraspinal field potentials were recorded using pulled glass (1.2 mm outer diameter, with filament) micropipettes that had a tip inner diameter of 1.6 – 2 µm filled with 3M NaCl (3-5 MΩ). Prior to intraspinal recordings, all but one of the CDP electrodes were removed and the dura was opened where the maximal CDP component evoked by 5xT TIB stimulation was identified. Intraspinal recordings were made by serial electrode penetrations (tracks) in the transverse plane at 0, 5, 10 and 15 degrees with the electrode tip pointing lateral, i.e., pointing to the right side of the right spinal cord with entry points being medial to the dorsal root entry zone. In each of these tracks at the listed angles, 15-30 recordings were collected at 0.2 mm increments, covering up to 2.0 mm distance ventral to the surface of the cord.

All CDP recordings were amplified (10K), analog filtered (0.1–3 kHz bandpass) and digitized (at 20 kHz) by using the custom program running on a Linux PC (Capture and Analysis programs developed by G. Detillieux in the Spinal Cord Research Center, the University of Manitoba, see more open-source documentation at www.scrc.umanitoba.ca). Recordings were used to construct a raster of CDPs and field potentials for each experiment and to measure onset latencies and amplitudes as described below. Measurements from the Analysis program were transferred and processed in Excel (Windows 10).

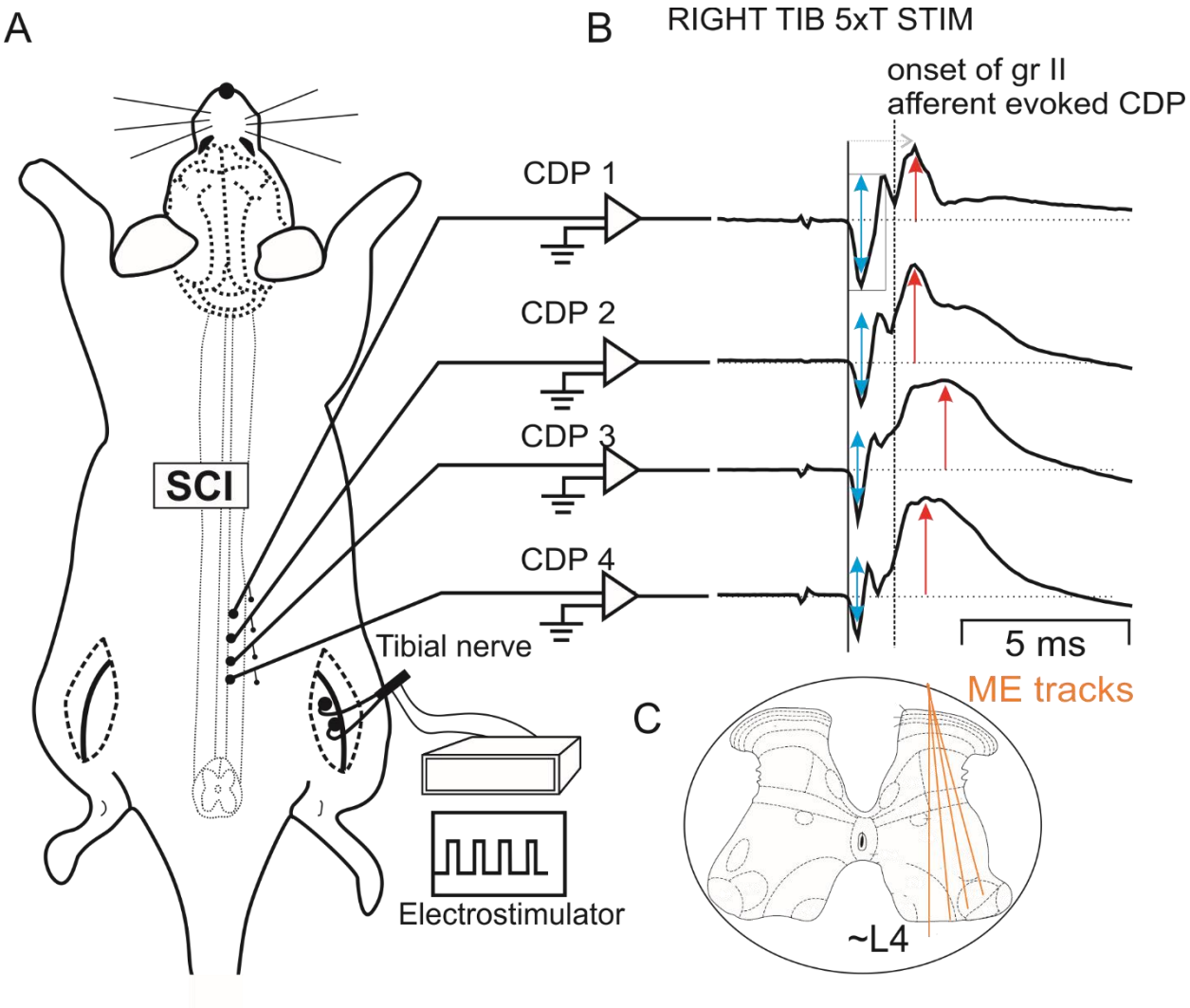

Figure 2.1 . Experimental methods.

Schematics showing the methodology for the cord dorsum potential (CDP) responses to graded electrical tibial (TIB) nerve stimulation when measured at different levels of the lumbar spinal cord. **A**. Positioning of the recording cord dorsum electrodes over the extent of the lumbar segments after laminectomy. **B**. Examples of evoked cord dorsum potentials at the four different locations (electrodes 1-4) following TIB stimulation. The stimulation of the TIB nerve was five times the intensity required to activate the most excitable fibers (5xT). The peak-to-peak amplitude of the group I afferent-evoked (blue double arrows) and the maximum amplitude of the group II (gr II, red arrows, onset marked with dotted line) components were normalized and compared in rats with intact cords and those with an injured cord at 1 week and 5 weeks after a complete spinal transection at the thoracic 9^{th}-10^{th} vertebral level. After the experiment, the segmental location of the four electrodes was verified.

2.3.6. Analysis methods

CDPs recorded at each rostro-caudal levels of the spinal cord during TIB 2xT and 5xT nerve stimulation were averaged from single trials (27-100) to measure onset latency and amplitude. Although, the CDPs represent composites of action potentials in afferent fibers as well as action potentials generated by neurons in the vicinity of the recording electrode, see reviewed in e.g. (Yates et al., 1982), the following generalizations were made for the interpretation of recordings. The triphasic response in the window of 1.1 to 1.8 ms after stimulus onset following stimulation at both 2xT and 5xT was attributed primarily to group Ia afferent fiber activation (Yates et al., 1982). This is referred to as the group I CDP. The response in the window of 1.9 ms to 3.6 ms after 2xT stimulation is attributed primarily to the activation of muscle afferent fibers like the group Ib and low-threshold group II afferents and this is referred to as the group Ib CDP (Jack, 1978; Jankowska & Riddell, 1993; Riddell & Hadian, 1998, 2000). The response in the same window (1.1 to 1.8 ms) after 5xT stimulus intensity, was attributed to group II afferent activation and this is referred to as the group II CDP.

CDP recordings were compared after normalization. The maximal peak-to-peak group I response amplitude was used to normalize the group I CDPs in the same animal at each electrode location (i.e., at each segmental level). The group Ib and II CDPs were also normalized to the maximal respective group Ib and II CDPs measurements in each animal. Based on the post-experiment verification of the segmental location of each electrode in each rat; the normalized averages at the respective segmental levels were used for further statistical comparisons in SigmaStat. Intraspinal field potentials were averaged at each location (at 0.2 mm steps at each angle tested). Then absolute amplitudes of the averaged potentials at each depth in each animal were averaged and these values were transferred to SigmaStat for statistical comparisons. The normal distribution of the data (both CDPs and intraspinal field potentials) was examined by Wilk-Shapiro tests and the Brown-Forsyth test was used for testing equal variance. Statistical comparisons were done by two-way ANOVA and pairwise multiple comparison procedures (Bonferroni t-test) were applied when required.

2.4 Results

Improvement in hind limb elevation and paw placement of spinal rats in upright bipedal posture at 5 weeks after complete transection was typically observed when compared to 1-week post-SCI (Figure 2.2). At 1-week post-SCI, the left hindlimb (LHL) showed minimal movement with no plantar placement of the paw (Figure 2.2A) of one representative rat used in this series. There was dragging of the dorsal surface of the paw and minimal excursions in both horizontal and vertical directions were evident (Figure 2.2A) compared to movements recorded in the same rat at 5 weeks after spinalization (Figure 2.2B). Larger and more regular knee and toe elevation and greater joint angle excursions underlined the improved locomotor ability in the upright posture (Figure 2.2C-D). The vertical excursions of the tip of the 4th toe indicating toe clearance during upright locomotion was irregular and much smaller at 1 week (Figure 2.2C) than at 5 week (Figure 2.2D) post-SCI.

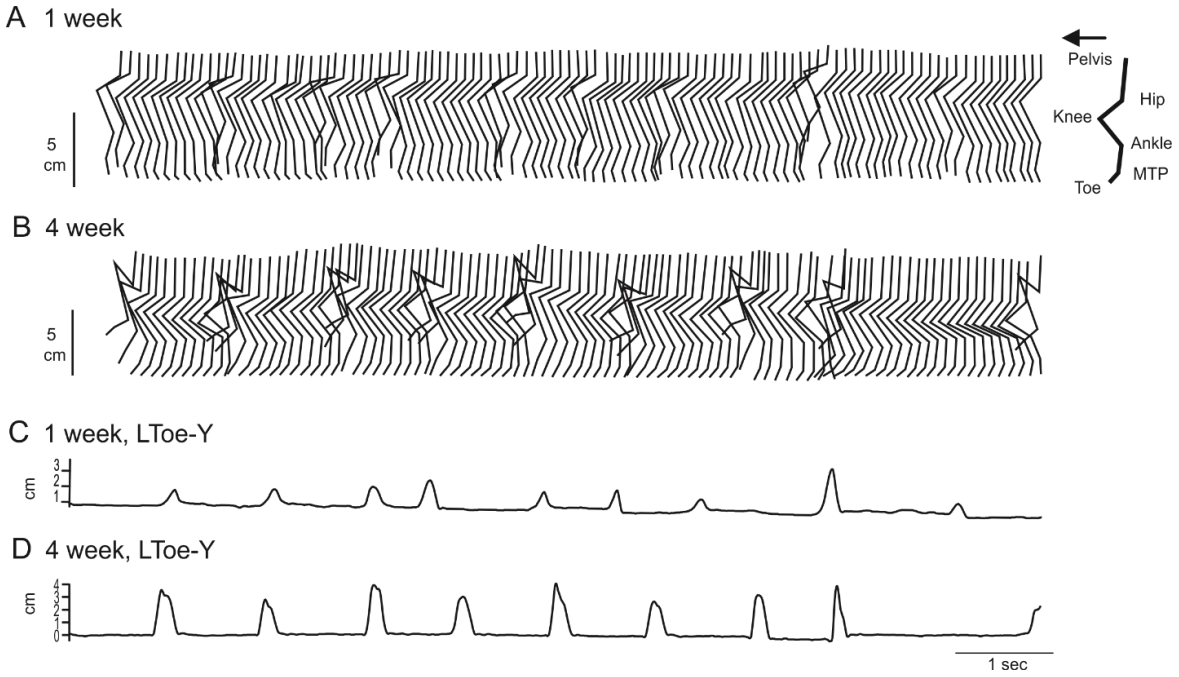

Figure 2.2. Improvements of kinematics of upright locomotion from 1 week to 4 weeks post spinal cord injury.
A. Stick diagram of left hind limb (LHL) at 1 week post spinal cord injury (SCI) at 9th-10th thoracic vertebral level during treadmill upright walking (speed 5cm/s) showing positions of pelvis, hip, knee, metatarsal-phalangeal joint (MTP), and toe over a period of 10 seconds. **B.** Data from the same rat as in A but at 4 weeks after SCI. Note improved range of motion at the hip, knee, and toe. **C**. Vertical displacement of LHL toe from the same bout of activity as in A. **D**. Vertical displacement of LHL toe from the same bout of activity as in B. Note improved vertical displacement.

In order to examine the mechanisms underlying this recovery, systematic cord dorsum potential (CDP) responses from the surface of the lumbar segments following stimulation of TIB were collected in 17 rats. CDPs evoked by graded electrical TIB stimulation were measured from the 3rd to the 6th lumbar (L3-L6) segmental levels of the rat spinal cord (Figure 2.1 A) in intact (INT, n=5) and spinalized rats at 1 week (1WK, n=6) or at 5 weeks (5WK, n=6) after complete thoracic spinal transections. The amplitude of the different components of these potentials (group Ia, Ib, and II) were normalized and compared in the three groups (Figure 2.3 B). Additionally, intra-spinal field potentials evoked by TIB stimulation were recorded in a subset of rats (n=5, 1 INT, 2 at 1WK and 2 at 5WK post-SCI).

2.4.1. Distribution of TIB afferent input based on cord dorsum recordings after SCI

Electrical activation of the TIB nerve produced distinct cord dorsum potentials (CDPs) in the lumbar spinal cord (Edgley & Jankowska, 1987a; Jankowska & Riddell, 1993; Riddell & Hadian, 1998, 2000). These CDPs reflected activity in the incoming afferent nerves as well as nearby neurons excited by group Ia, group Ib or group II afferents. The onset latency of group Ia CDPs in the intact, 1WK and 5WK post-spinalization group was 1.4±0.2 ms, 1.5±0.2 ms and 1.3±0.2 ms, respectively (see Table 2.1).

The distribution of the CDPs was examined after normalizing the amplitude to the maximal responses of each component (Ia, Ib and II) in each rat. Largest CDPs evoked by TIB gr Ia afferents were found in the L4 and L5 segments in intact as well as spinal cord injured rats (Figure 2.3). CDPs from the TIB group Ia afferents were smaller in the L3 and L6 segments, on average. Some of these differences in amplitude between segmental levels were significant (two-way ANOVA, L3 vs. L5 $p=0.01$ and L6 vs. L5 $p=0.03$) There was no significant difference in the amplitude of the group Ia CDPs as a factor of time after spinal cord injury and there was no statistically significant interaction found between the segmental location and the time after injury (two-way ANOVA).

Normalizing the later response after TIB 2xT stimulation i.e. after maximally recruiting Ia and Ib afferents at onset > 2.0 ms showed that the largest responses were also found in the L4 and L5 segments in the intact rats (Table 2.1 and Figure 2.3). The onset latency of group Ib CDPs in the intact, 1WK and 5WK post spinalization group was 2.7±0.3 ms, 2.9±0.4 ms and 2.5±0.3 ms, respectively. The CDPs were significantly greater in L4 than in L3 (two-way ANOVA, $p=0.004$),

and in L6 (p=0.005). The CDPs in L5 were larger than those in L3 (p=0.01) and than those in L6 (p=0.01). Although, the Ib responses showed a tendency for reduction by about 20% in the L4 segment in the 1WK group compared to the spinal intact rats; there was no significant difference in the amplitude between the groups (two-way ANOVA).

gr I	INT	1WK	5WK
L3	59±27% (2)	55±36% (4)	55±42 (5)
L4	84±19% (4)	71±30% (5)	100±0% (3)
L5	92±15% (4)	95±2% (4)	93±15% (4)
L6	63±28% (3)	57±22% (4)	60±7% (5)
gr Ib	INT	1WK	5WK
L3	53±25% (2)	42±29% (5)	48±32% (5)
L4	99±3% (5)	66±33% (5)	100±0% (3)
L5	71±28% (4)	92±17% (4)	82±22% (4)
L6	56±45% (2)	45±24% (4)	44±24% (4)
gr II	INT	1WK	5WK
L3	39±44% (2)	42±26% (4)	55±44% (4)
L4	79±22% (5)	72±28% (5)	97±5% (3)
L5	74±36% (4)	100±0% (4)	99±3% (4)
L6	66±49% (2)	56±28% (4)	41±22% (5)

Table 2.1 Normalized amplitude of cord dorsum potentials evoked by TIB stimulation.
The amplitude of cord dorsum potentials measured at four different lumbar segments (L3-6) from rats with the intact spinal cord (INT) or after spinal cord injury at 6-8 days (1WK) or at 28-35 days (5WK). The measurements were taken from averaged trials following electrical stimulation of the posterior tibial (TIB) nerve at an intensity that was five times the threshold required to activate the most excitable afferents (T). The mean amplitude and standard deviations (±) are reported for group I afferent evoked responses (gr I), for group I afferent-evoked responses (gr Ib), for group II afferent-evoked potentials (gr II) and for group II afferent evoked potentials during light touch (LT) applied to the plantar surface on the stimulated side. The numbers in parenthesis show the number of rats from which recordings were grouped for the respective segments.

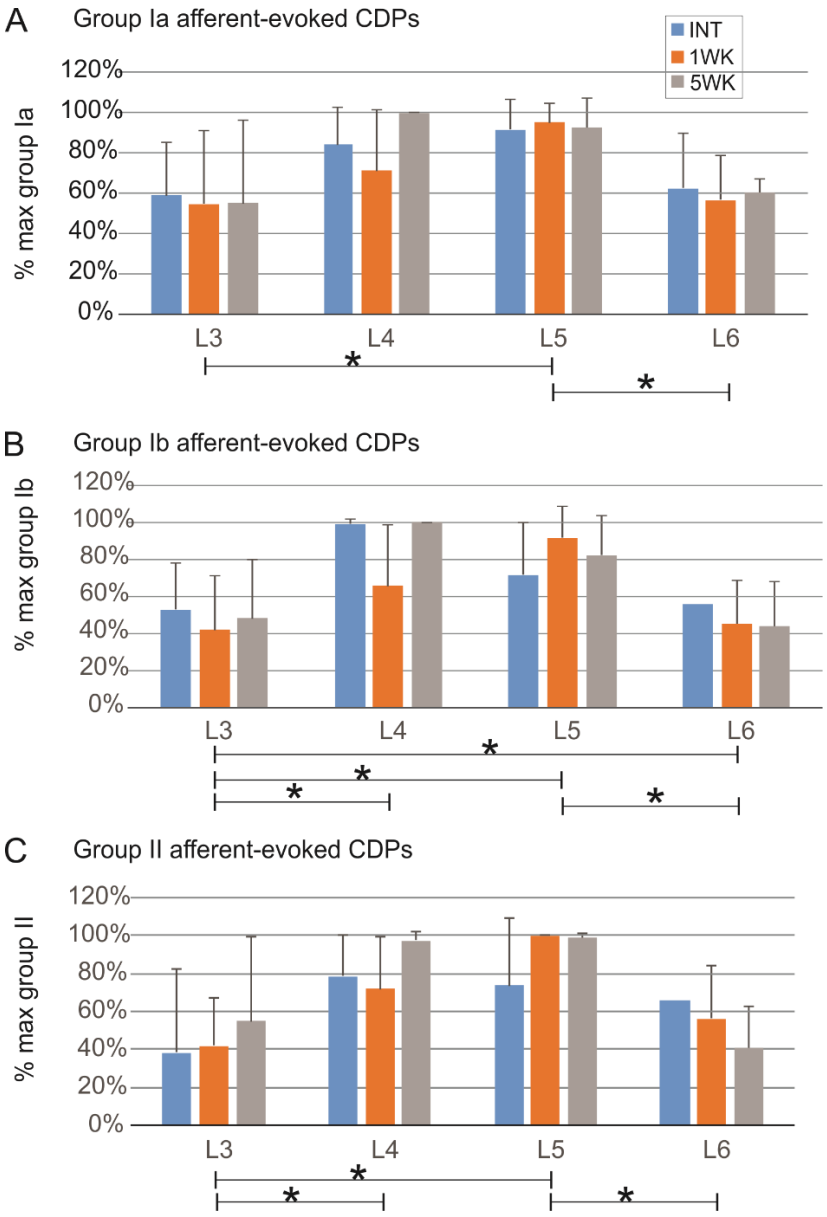

Figure 2.3 Normalized amplitude and distribution of cord dorsum potentials evoked by TIB stimulation

Amplitude changes in the normalized cord dorsum potentials (CDPs) at four segmental locations over the lumbar cord averaged and grouped from all rats measured from averaged trials (n=27-100) after TIB 2xT or 5xT electrical stimulation. **A**. Normalized group I afferent volley (peak-to-peak) amplitude expressed as % maximum in intact (INT, diamonds) and in spinal rats (1WK, squares-1 week post and 5WK, triangles-5 weeks post-injury). **B**. Normalized group Ib afferent-evoked CDP amplitude at each level expressed as % maximum. **C.** Normalized group II afferent-evoked CDP amplitude at each level expressed as % maximum. Maximum values were used to normalize amplitude in each rat before grouping data at respective lumbar (L) segmental levels. Error bars indicate standard deviations and legend in **A** applies to all panels.

The onset latency of the CDPs evoked by TIB 5T stimulation, referred to as the group II CDPs in the intact, 1WK and 5WK post spinalization group was 2.6±0.2 ms, 2.7±0.4 ms and 2.5±0.3 ms, respectively. Confirming the previous finding of Riddell and Hadian (1998), the largest CDPs from the TIB group II afferents were seen in the L4 and L5 segments as shown in Figure 2.3 C. These were, on average, 30% smaller in the L6 segment and 60% smaller in the L3 segment (two-way ANOVA, p=0.02 and 0.03, respectively). There were no differences found in the group II CDP amplitude between the groups of animals as a factor of spinal cord injury. The CDP amplitude at the different segmental levels did not vary as a factor of spinal cord injury (p=0.56).

The time-to-peak values for the group Ib CDPs are listed in Table 2.2. These were 0.6±0.2 ms, 0.7±0.4 ms and 0.9±0.3 ms in the intact, 1WK and 5WK groups, respectively. The time-to-peak values for the group II CDPs were 1.0±0.4 ms, 0.9±0.4 ms and 1.2±0.5 ms in the intact, 1WK and 5WK groups, respectively. There were no statistically significant differences found in the onset latencies or in the time required to reach peak amplitude in intact and spinal transected rats. One notable observation was that the variability of the time-to-peak values of the group Ib CDPs was twice as much in the post-spinalization groups than in the intact group (see Table 2.2). or in other words, the population variance was 0.02, 0.13 and 0.10 for the intact, 1WK and 5WK Ib component.

onset (ms)	**INT**	**1WK**	**5WK**
gr I	1.1±0.17	1.5±0.22	1.3±0.16
gr Ib	2.63±0.25	2.94±0.40	2.47±0.28
gr II	2.59±0.20	2.74±0.45	2.49±0.27
time-to-peak (ms)	**INT**	**1WK**	**5WK**
gr Ib	0.6±0.18	0.72±0.37	0.91±0.33
gr II	1.03±0.44	0.94±0.42	1.15±0.47

Table 2.2 Time-to peak amplitude of CDPs evoked by TIB 2T and 5T stimulation.
The onset latency and the time required to peak amplitude (milliseconds, ms) measured from the segmental cord dorsum potential arrival is shown for each of the cord dorsum potential (CDP) components following tibial nerve stimulation at two times or at five times the intensity required for activating the most excitable fibers.

2.4.2. Reduced group II TIB intraspinal field potential amplitude at one-week post SCI

Based on the observed tendency of smaller cord dorsum field potentials in the L4 segment at one-week post-SCI, the distribution of intraspinal field potentials evoked by TIB stimulation was also examined in a subset of animals. Intraspinal field potentials evoked by TIB were recorded (Figure 2.4A, single trials) and averaged to construct a map of responses at various depths from the cord surface (Figure 2.4B). The field potentials evoked in the dorsal horn between 400 and 600 µm were predominantly the result of group II afferent stimulation because TIB 2xT stimulation did not evoke any (or negligible) responses.

Using similar methods as previously described by Riddell and Hadian (1998) we found that amplitudes were on average, the largest between 400-800 µm below the cord surface (Table 2.3). The amplitude of the group II TIB field potentials recorded at 200, 400, 600 and 800 µm were 467±267, 527±103, 368±136 and 126±269 µV, respectively in intact spinal rats. At one-week post-injury, these amplitudes were found to be 68±43, 127±51, 137±159, 1225±318µV and at five-weeks post injury these were 457±444, 458±491, 328±350, 257±303 µV at 200, 400, 600 and 800 µm, respectively. As shown in Figure 2.4E, statistically significant differences were found between one-week post-SCI measurements compared to those from intact rats (two-way ANOVA, $p=0.016$). This reduction of the evoked potentials at 1WK, however, recovered by 5WK post-SCI; as potentials obtained at 5WK were significantly larger than those at 1WK (two-way ANOVA, $p<0.001$). but not significantly different than those in INT rat ($p=1.0$).

TIB (x5T) potentials	**INT (4 in 1R)**	**1WK (7 in 2R)**	**5WK (7 in 2R)**
Depth from surface	**µV**	**µV**	**µV**
200 µm	467±267	68±43	457±444
400 µm	527±103	127±51	458±491
600 µm	368±136	137±159	328±350
800 µm	126±269	1225±318	257±303

Table 2.3 Amplitudes of field potentials evoked by TIB stimulation (x5T) at different depths from cord surface.
The amplitude of field potentials (expressed as µV) measured at different depth from the surface (200 to 800 µm) in intact spinal (INT) and spinalized rats at one week (1WK) and at five weeks (5WK) after the spinalization. The potentials were evoked by stimulation of the tibial nerve at five times the intensity required for activating the most excitable fibers (x5T) averaged from 27- 100

trials from the number of electrode tracks and the number of rats (R) are indicated in the top row. The ± values represent standard deviations.

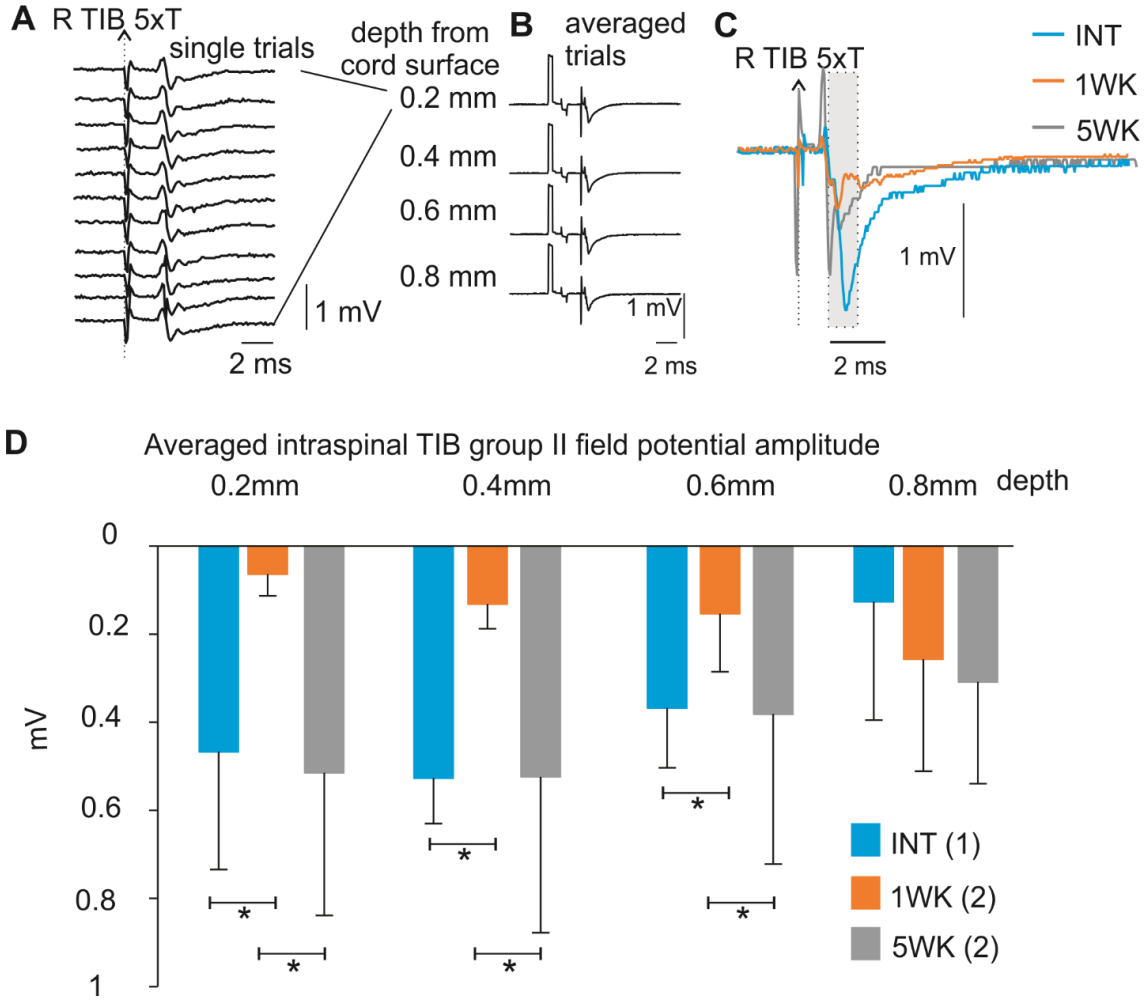

Figure 2.4. Reduced intraspinal field potentials evoked by gr II TIB afferents at one week post spinal cord injury

Field potential responses evoked by TIB stimulation at 5xT at the level of L4 segment at different depths and at different angles of electrode placement (tip lateral). **A.** Rastered traces from a rat displaying individual single test responses to TIB stimulation at 5xT at 0.2mm depth from cord surface. **B.** Averaged test responses (27 trials) from the same rat as in **A**. **C.** Overlay of representative averaged field potentials from each cohort (intact (INT), spinalized at 1 week post SCI (1WK) or at 5 weeks after SCI (5WK). Recordings are aligned by the onset of the tibial nerve stimulation (5 times the threshold, TIB 5T, up arrow). Note reduced amplitude of responses at 1WK. **D.** Grouped, averaged group II field potential amplitude (mV) from all rats (intact, INT n=2, 1-week post-SCI, 1WK n=2, and 5-week post-SCI, 5WK n=2) at the different depths. Field potential onsets ranged 2.9-3.3 ms from the stimulus. Error bars represent SD.

2.5 Discussion

This study has successfully reproduced previous results regarding the distribution of primary (group Ia and Ib) and secondary (group II) muscle afferent input from the tibial nerve (TIB) in rat lumbar spinal laminae (Riddell & Hadian, 1998). Maximal cord dorsum potentials (CDPs) were found in the L4 and the L5 segments and TIB afferents had similar rostro-caudal projection patterns in rats after a complete spinal cord transection to the distribution in rats with intact spinal cords (Figure 2.3). In addition, our investigation demonstrated that dorsal horn intraspinal field potentials in the L4 segments, however, showed a reduction in the amplitude of group II afferent-evoked potentials at one-week post-SCI, which was no longer observed by five-weeks post-SCI. This is the first study to report that TIB group II afferents have a transiently diminished efficacy in exciting dorsal horn mid-lumbar interneurons in rats after spinal cord injury.

2.5.1. Interpretation of cord dorsum potential data

The earliest components of the CDPs (<0.7 ms) reflect mainly group I afferent action potentials. The later components of the CDPs result from afferent fiber action potentials and spinal neural activity in the vicinity of the cord dorsum electrodes. The Ib and the most excitable group II muscle afferents in the TIB nerve were recruited by the 2xT stimulation while after 5xT stimulation, group II afferent-evoked responses were maximal. Our results show that even though the animals exhibited dragging of the hindlimbs in both upright and horizontal posture at 1WK post SCI and developed upright walking abilities at 5WK post SCI the relative rostro-caudal distribution of TIB group I and group II afferent input did not change at any segmental level tested here between one- and five-weeks post-SCI. Testing was done for only the TIB nerve as it has been shown to be the key nerve for the recovery of walking. However, afferent input from other nerves may also change similarly as more loading occurs at all levels of the limb with increased locomotor activity after SCI. Increased afferent input integrated from all joints cumulatively will improve CPG output. The numbers of tendon organs as well as primary and secondary endings of spindles are different in different muscles. Based on previous comparisons in cats and rats. The largest proportion of group II effects were evoked by tibialis posterior and to a lesser degree flexor digitorum longus (Riddell & Hadian, 1998) likely due to the role some muscles play in postural muscle stabilization requiring high level group II afferent based feedback innervation.

2.5.2. Interpretation of intraspinal field potential data

The onset of the TIB group II intraspinal field potentials following 5xT stimulation corresponded well to those reported earlier by Riddell and Hadian (1998). However, there were no comparative data for such potentials evoked in chronic spinal rats. As we did not observe field potentials evoked by 2xT stimulation at depth above 800 µm from the surface of the spinal cord (Figure 2.4E), we posit that TIB group I afferents do not innervate dorsal mid-lumbar segments after spinal cord injury.

Numerous studies suggested that there are no discrete interneural pathways in the intermediate zone processing group Ib and II afferent information independently; as reviewed by (Jankowska & Edgley, 2010). Monosynaptic input from group II afferents not accompanied by input from group I afferents is a common feature of interneurons located within the dorsal horn (laminae IV-V) and our results here suggest that this is the case in rats, even after spinal cord injury. However, a transient reduction of group II-afferent evoked excitation of dorsal horn neurons within a week post-SCI was found. Input to the area of the dorsal horn near the recording sites was mainly from group II TIB afferents with no or negligible contribution from group Ia fibers as illustrated on the schematic in Figure 2.5. In our experimental model, the spinal transection did not damage the TIB afferents or TIB motoneurons or first order interneurons. The complete low-thoracic transection eliminated all descending input emerging from supraspinal sites or from propriospinal neurons above the lesion site. The reason why the effects of TIB group II afferents on lamina IV-VI dorsal horn neurons were weaker at one week after complete spinal cord injury compared to 5-weeks post-injury could be explained by the removal of strong descending control of dorsal horn group II interneurons. We postulate that the dorsal population receive descending excitation which diminished after spinal cord transection and resulted in the smaller field potentials at one-week after injury.

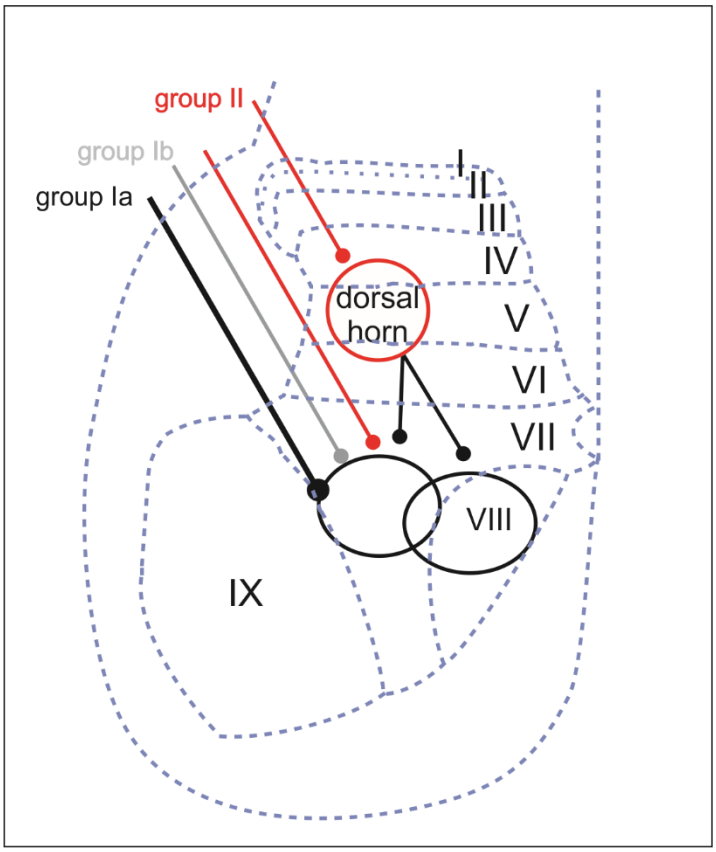

Figure 2.5. Dominant group II afferent contribution to dorsal TIB field potentials recovers by five weeks after a complete spinal transection in rats.
Schematic summary of the input to the area of the dorsal horn in the lumbar (L4) spinal cord showing that dorsal horn neurons receive mainly input from gr II afferents with no or negligible contribution from a gr I fibers even after five weeks after a complete thoracic spinal cord transection. This organization is similar to what has been described previously in the intact rat (Riddell & Hadian, 1998) and also in the cat (Jankowska & Edgley, 2010).

Although, we have explored changes only in the ipsilateral projections of the TIB nerve, it is important to note that previously recognized changes after spinal cord injury described modifications in afferent input processing in crossed (commissural) TIB afferent pathways. The crossed inhibitory pathways transmitting at rest have been examined after acute and chronic spinal transections (Aggelopoulos et al., 1996; Aggelopoulos & Edgley, 1995; Arya et al., 1991a). The crossed inhibition of extensor motoneurons elicited by TIB group II afferents was replaced by crossed excitation following spinal transection in anaesthetized cats (Aggelopoulos et al., 1996). Moreover, crossed TIB-evoked inhibition of other extensors could be restored by serotonergic receptor agonists representing one source of the strong descending control. More recent evidence

from mice, showed that eliminating dI3 interneurons and synaptic transmission mediated by the dI3 population prevented functional motor recovery after spinal cord injury (Bui et al., 2016). The dI3 interneurons are potential candidates for mediating group II transmission, albeit unexplored in mature mice or rats; as 36% of dI3 cells is situated in lamina V (Bui et al., 2013); and that is the same region where the transient changes were observed in our study. Interestingly the dI3 interneuron undergo a transient decrease in $VGLUT1^{+}$ input at 2-3 weeks after a complete transection at the level of T9-T11 in the mouse (Goltash et al., 2023). These changes in the interneuronal circuitry recruited for locomotion after spinal cord injury may contribute to the observed locomotor recovery. After incomplete thoracic spinalization, long propriospinal neurons were also shown to play a role in the locomotor recovery after spinal cord injury (Shepard et al., 2021). The improved synaptic efficacy of group II afferent input in our data, however, does not reflect propriospinal effects as our model was a complete transection.

Bilateral locomotor responses evoked by intraspinal electrical stimulation were more frequent after clonidine injection in spinalized cats (Barthelemy et al., 2006) and noradrenaline facilitated effects from contralateral group II input in neurons that received monosynaptic inputs from the reticular formation (Hammar et al., 2004). Commissural interneurons interposed in crossed reflex pathways are located in laminae VI–VIII in the cat (Edgley et al., 2003; Hammar et al., 2004; Hammar et al., 2007; Jankowska et al., 2009), in regions that are densely packed with locomotor-related α2-noradrenergic receptors (Noga et al., 2011b). Input from both noradrenergic and serotonergic pathways are known as critical for recovering afferent-induced motoneuron recruitment via crossed pathways in cat spinal cord. The effects of clonidine in the rat, however, may be different than in the cat. In the acute spinal cat (Rossignol et al., 2001) activation of noradrenergic receptors, specifically alpha2, will result in weight-bearing and treadmill locomotion. This is not the case in the spinal rat in which serotonergic receptor activation has a predominant role in recovery; likely acting on $5\text{-}HT_{7}$ and $5\text{-}HT_{2A}$ receptors (Sławińska et al., 2013). Noradrenergic agonists and antagonists have opposite effects in rats compared to what was observed in cats (Kwaśniewska et al., 2020) as alpha-2 adrenergic agonist did not facilitate locomotor recovery while yohimbine, an adrenergic antagonist did have facilitatory effects.

Stimulation of the tibial and superficial peroneal nerves in adult decerebrate cats spinalized one month earlier increased the amplitude and duration of crossed extensor responses and triggered locomotor-like activity in approximately one-third of trials following stretch and tibial nerve

stimulation while clonidine, an α2-noradrenergic agonist was administered (Frigon, Johnson, et al., 2012). Differential effects of clonidine on crossed reflexes and on ipsilateral responses to muscle stretch were interpreted in that study as actions of clonidine primarily acting at pre-motoneuronal sites. Clonidine facilitated hindlimb locomotor recovery at four weeks after a complete spinalization in untrained cats by enhancing the excitability of CPG neurons that participate in crossed extensor reflexes (Frigon, Thibaudier, et al., 2012). Even though the hemi-section model has the capacity to spontaneously recover locomotion (Leszczyńska et al., 2015) increased serotonergic innervation directed to motor pools and to Clarke's columns neurons was found. The serotonergic innervation is likely to have played a role in controlling sensory input reaching the motoneurons.

2.5.3. Limitations of this study

The localization and the size of the TIB field potentials were identical to what has been described previously, when intraspinal TIB field potentials were extensively described in intact spinal rats by Riddell and Hadian (1998) During the terminal experiments, the rats were under isoflurane anesthesia while CDP and intraspinal potentials were collected. This anesthesia was different than sodium pentobarbital anesthesia used in the previous studies by Riddell and Hadian (1998). However, as the earliest evoked responses represent primarily excitatory transmission from afferents to interneurons; the TIB CDPs and early field potentials were unlikely to be influenced by either anesthetic.

The popliteal nerve innervating the knee joint has not been removed during the nerve dissection from the main branch of the TIB nerve stimulated in this study. Although joint afferents are considered to be functionally different from muscle or cutaneous afferents, it would have contributed little to the overall TIB group I and group II responses evoked at the levels we tested has been shown in studies in both rats and cats (Riddell & Hadian, 1998, 2000).

As the majority of spinal cord injuries are incomplete (Noonan et al., 2012) the full spinal transection model is not as clinically relevant as other models, such as contusion or compression injury models. Additional studies examining changes in group II afferent transmission after incomplete spinal injuries would be warranted to examine mechanisms which maybe common after different levels of SCI.

2.6 Functional relevance

Despite the disruptive effects of SCI on locomotor output, partial recovery is possible through different techniques as reviewed in e.g. (Dietz & Fouad, 2014; Edgerton et al., 2008; Rossignol, 2006). Methods used to promote recovery may involve increased sensory input from the limbs via retraining of locomotor capacity on a treadmill or pharmacological interventions, or electrical stimulation of spinal neurons or increasingly often a combination of these methods. Loading of the limbs is critical for improved recovery of walking following spinal cord injury in humans (Duysens & G., 1980; Faist et al., 2006; Harkema et al., 1997; Shin et al., 2008; Timoszyk et al., 2005). In rats, after a complete thoracic spinal transection, there is no plantar stepping in the horizontal posture, however, when only the hindlimbs are engaged in upright bipedal-like walking, the plantar stepping improved without any intervention nor locomotor training (Sławińska et al., 2012). The improved plantar stepping in upright (bipedal) walking in rats was thought to be mainly the result of increased hindlimb loading. In rats, recent work examining exercise-based interventions to improve locomotion also found that normal group I and II afferent input was critical to achieve exercise-based reversal of hyperreflexia after spinal cord injury (Ollivier-Lanvin et al., 2010).

In our study, rats received no training or any intervention to facilitate recovery, but the recovery of the improved plantar stepping correlated with the extent to which dorsal horn interneurons are excited by TIB afferents. The ability to modulate speed and to step on a split-belt treadmill in cats was found to be independent of task-specific training (Harnie et al., 2019) but recovery was found to depend on the return of excitability within spinal sensorimotor circuits. In rats, a recent analysis of firing properties and distribution of identified afferents (i.e. Ia, Ib and II) of the triceps surae showed that Ib neurons often fire during passive movement together with group II afferents (Vincent et al., 2017). A weight-bearing-induced reorganization of spinal circuitries has been supported by our findings and more detailed mechanisms leading to this reorganization should be addressed by future animal studies. Changes in the excitability of interneuron pools should reveal more about the factors involved. Interneurons characterized by genetic markers (often by developmentally transient factors) have been available for the mouse as the experimental model while information about the functional subdivisions of spinal interneurons is only available from the feline experimental models. As expressed elegantly by Vincent and colleagues (2017), species differences related to spinal networks need to be examined by more studies on rodents. Future

work in mouse models while surveying functional pools of interneurons could be used to map the modular organization of spinal networks in health and after injury (Abraira & Ginty, 2013).

2.7 Conclusions

This is the first study to describe a transient reduction in synaptic efficacy from TIB afferents, predominantly group II fibers, in rat lumbar spinal segments after SCI. The reduction of the synaptic effects seen at one-week post-SCI recovered by about five weeks after a complete spinal transection injury which is also the time point at which rats spontaneously recover some hindlimb walking capacity. This paper is an important addition to the growing body of work on rodents to determine whether muscle proprioceptors synapse with the same first-order interneurons and contribute to driving the same (or different) spinal networks as presumed from previous data from feline SCI models.

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Chapter III

Manuscript Title Chemogenetic stimulation of embryonic serotonergic grafts to improve locomotor recovery after spinal cord injury in rats

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Author contributions
The studies were conceived and designed by L.M.J. and U.S. and K.S. The experiments were performed by M.N., K.A., U.S., K.S. and L.M.J. Cell culturing was done in the lab of E. E. by N.P. and electrophysiology of cultured neurons was performed in the lab of M. J. by C.P. The data analysis was done by M.N., E.C-R., K.S. and U.S.; all authors were involved in finalizing the manuscript for submission.

List of abbreviations
5-HT - serotonin
DREADDs – designer receptors exclusively activated by designer drugs
E14 - embryonic day 14
EMG - electromyography
Sol - soleus
SCI – spinal cord injury
TA – tibialis anterior
Th - thoracic

3.1 Abstract

Grafting of serotonergic (5-HT) neurons into the spinal cords of paraplegic rats has been used as an experimental method to successfully increase motor function recovery after spinal cord injury (SCI). Additional manipulations introduced with the grafts could offer specific control of activation of cells incorporated into the host spinal cord. Here we utilized designer receptors exclusively activated by designer drugs, DREADDs to stimulate 5-HT neurons in embryonic tissue grafts inserted in rats following a complete SCI. By using cre recombinase-dependent rAAV-mediated incorporation of DREADDs in embryonic tissue grafts from genetically modified rats, we tested the hypothesis that excitation of 5-HT neurons in embryonic grafts leads to better hindlimb locomotor performance in chronic, spinal grafted rats than in grafted rats without the DREADDs. Cultured cells were used to verify the expression of DREADDs by utilizing the fluorescent reporter protein and immunohistochemistry combined with *in vitro* electrophysiological verification of increased excitability in DREADD-positive cells following application of a designer drug (compound-21, C21). Then we grafted embryonic tissue enriched with DREADDs a week after a complete, low-thoracic SCI. Analysis of locomotion consisting of kinematic and hindlimb EMG assessments during treadmill walking were performed before and after C21 injection. Locomotor performance was compared at different time points with respect to C21 application and immunohistochemistry on spinal tissue was used to verify that 5-HT neurons in the grafts were targeted by C21. Increasing 5-HT neuron excitability in grafted rats; reduced hip, pelvis and knee movements in the vertical plane and left-right coordination between both flexors and extensors. A one-time, 5-HT neurotransmitter-based targeting of neural subpopulations within spinal grafts reduced left-right coordination in DREADD-harbouring rats, thus some interference with ongoing motor output can be brought on by excess activation of the grafted cells.

3.2 Introduction

In mammals, locomotion is controlled by the combination of descending supraspinal signals from motor centers and peripheral input from sensory afferents (Jordan et al., 2008); both converge on spinal neural networks. The spinal circuitry driven by descending and sensory input includes the so-called central pattern generator networks, CPGs, which generate coordinated leg muscle activity required for overground locomotion. Spinal cord injury (SCI) disrupts the combined actions of descending and sensory signals. Below the level of injury, intact CPGs incorporate sensory input into a spinally generated motor program enabling stepping. In order to augment and maximize motor function, several approaches have been used in pre-clinical animal models, which allow for the detection of neuronal changes even after a long time after injury, followed by select therapeutic approaches.

Serotonin (5-HT) is at the forefront of neurotransmitters to be utilized in recovering locomotion after spinal cord injury (Courtine et al., 2009; Ghosh & Pearse, 2014; Schmidt & Jordan, 2000; Sławińska et al., 2014b). Descending brainstem neurons are the only source of 5-HT in the spinal cord (Jordan et al., 2008). Following a complete spinal transection, the 5-HT is completely depleted at all spinal levels below the injury. The activation of the serotonergic receptors is thought to be essential for any amount of recovery after SCI (Antri et al., 2005; Antri et al., 2002; Sławińska et al., 2014b) (Fouad et al., 2010). Grafting of 5-HT neurons into spinal rats to recover locomotor activity has been used as an attempt to restore 5-HT levels below the injury (Feraboli-Lohnherr, Orsal, Yakovleff, Giménez Y Ribotta, et al., 1997; Slawinska et al., 2000).

The improvement in the locomotor activity observed after grafting was shown to be due to activity of serotonin from the grafts on the host serotonergic receptors within the spinal cord as shown by (Majczyński et al., 2005) through antagonizing 5-HT_2 receptors with Cyproheptadine or 5-HT_7 receptors by SB269970. To further improve the interlimb coordination in the grafted rats, the grafts were widened to include an additional cluster of neurons (B3) that corresponds to what is presumed to be analogous to the descending serotonergic cluster present in the parapyramidal region (PPR). Stimulating this cluster of 5-HT neurons alone can engage the spinal CPGs through the activation of 5-HT_{2A} and 5-HT_7 receptors in the neonatal rat (Liu & Jordan, 2005). Our lab opted to use grafts that contain the B1, B2, and B3 serotonergic populations, as defined by König et al. (1988) in the embryonic brainstem, which would include all the serotonergic populations

descending to the spinal cord. Such grafts improved coordination in both right and left as well as flexor and extensor activity (Sławińska et al., 2013). Moreover, the improvements in gait were shown to be through serotonergic activity on different neuronal populations within the spinal cord.

One key observation about the locomotor recovery in grafted animals was that in order to maximally optimize the stepping movements in the injured rats, additional exteroceptive stimulation was required most of the time. The best performance, i.e., locomotor activity resembling that observed in spinally intact rats was evoked with the aid of pinching of the tail. These observations suggested that additional excitation of the CPG even in grafted rats is required for maximal recovery of locomotion. In other words, if there was a way to "turn the grafts on and off at will" locomotor recovery could be improved.

Chemogenetic manipulation of cells is widely used for specific activation or inhibition of select neuronal populations (Armbruster et al., 2007; Zhu et al., 2016) and could offer some level of control of grafted embryonic tissue. Specifically, designer receptors activated by designer drugs or DREADDs-based chemogenetic tools have been commonly adopted to manipulate neural circuits, as reviewed in e.g. (Zhu et al., 2016). DREADDs can be inserted into a desired type of neuron within the target graft tissue to lessen the off-target actions. These recently developed genetic tools continue to offer new advantages in deterministically tracing neural circuits and optimizing as well as quantifying the plasticity of spinal neural networks (Eisdorfer et al., 2022; Shepard et al., 2021; Zholudeva et al., 2021).

Here we utilized DREADDs to stimulate 5-HT neurons in embryonic tissue grafted in the spinal cords of paraplegic rats. By using cre recombinase-dependent, rAAV-mediated incorporation of DREADDs into 5-HT neurons in embryonic tissue grafts. We tested the hypothesis that excitation of 5-HT neurons in embryonic grafts leads to better hind limb locomotor performance in chronic, spinal grafted rats than in grafted rats without chemical stimulation. This was achieved by using genetically engineered rats with cre recombinase in 5-HT neurons as donors for cell grafting, which allow the incorporation of DREADDs and the subsequent selective activation of 5-HT neurons. Preliminary reports have been presented earlier (Nazzal, 2017).

3.3 Methods

All animal care and experimental procedures conformed to the guidelines set by the Canadian Council on Animal Care. All procedures were approved by the University of Manitoba's Central Animal Care Committee.

3.3.1. Animals

We used rats from a local colony of TPH2-cre rats (founders obtained from Manheim, Germany, developed by D. Bartsch and colleagues, see e.g., (Weber et al., 2011). These rats have cre gene expression driven under the tryptophan hydroxylase-2 promotor thereby producing cre recombinase in brainstem serotonergic neurons. Experiments from which data is reported here were performed on a total of 12 female TPH2-cre rats and on 9 time-pregnant TPH2-cre rats (weighing 250 - 300 g).

3.3.2. Chemogenetic viral vectors and DREADD constructs

By using recombinant Adeno Associated Viral vectors (rAAVs) carrying the designer receptors exclusively activated by designer drugs (DREADDs) constructs. The rAAV8-hSyn-DIO-hM3D(Gq)-mCherry (Addgene, # 44361-AAV8), original creator Bryan Roth, University of North Carolina, USA). There were two groups of rats from which data are reported here. The first group consisted of 9 spinal transected rats in two cohorts, (n=4) and (n=5), with grafted embryonic neurons and DREADDs conjugated to rAVVs; and the second group consisted of 3 spinal transected control rats with grafted embryonic neurons but no DREADDs using the rAAV8-hSyn-DIO-mCherry construct (Addgene, #50459-AAV8).

3.3.3. Embryonic tissue dissection for culture plating

Primary culture neurons were derived from day 14 embryos (E14; the day following mating is designated as embryonic day zero—E0) of TPH2-cre time pregnant rats. Under isoflurane anesthesia of the pregnant females, embryos were removed by standard, aseptic dissection. The embryos were bathed in cooled 0.05% glucose Hank's buffered solution and further dissected under a microscope to isolate the caudal part of the brainstem containing the populations of serotonergic nuclei of the parapyramidal region (Liu & Jordan, 2005) and the medial raphe serotonergic nuclei corresponding to B1, B2, B3 serotonergic areas (König et al., 1988).

3.3.4. Dissociation and culturing embryonic neurons

The harvested embryonic tissue was digested with 1% papain in Hank's Buffered solution for 15min at 37 C, the tissue was then centrifuged and washed twice with Hank's Buffered Saline solution (HBSS) and then gently titrated in HBSS containing DNAase using a fire-polished Pasteur pipette. After centrifuging and removing the supernatant, the cells were resuspended in the culture media containing neurobasal A, B27 (Life Technologies Inc), L-Glutamine, PSN. Neuronal cells contained in the supernatant were transferred to poly-D-lysine-coated cover slips or 35 mm plates. Each cover slip was placed in a 24-well plate each well containing 1 mL media and around 50 000 cells per well (cover slip). The virus (with a titer of 2.2 x 10^13 GC/mL, Addgene 44361- AAV8) was applied to a concentration of 0.5uL/mL per well with a partial media change at about 24 to 48 hours after the cells were collected and plated. After incubation for 48 hours, a complete media change was done followed by a partial media change (50%) every 3 days until the end of the procedures. The mCherry-expressing neurons were evident by about 10 days after transfection after examining the cultures (Zeiss fluorescent microscope).

3.3.5. Whole-cell patch clamp recordings from cultured neurons

Whole-cell current-clamp recordings were done to detect changes in depolarization and firing in response to different DREADD ligands. Recordings were performed on day 14 after viral transfection, at room temperature (20-22 ˚C) using an Axon MultiClamp 700A amplifier and Digidata 1322A data acquisition system (Molecular Devices, Sunnyvale, CA, USA). Patch electrodes were pulled using a Narishige two-stage puller (PP-83; Narishige, Greenvale, NY, USA) from thin-walled borosilicate glass (TW150-F3; WPI, Sarasota, FL, USA). Electrodes had a final resistance of 3-5 MΩ when filled with intracellular solution (ICS) containing (in mm): 142 potassium gluconate, 10 HEPES, 2 $MgCl_2$, 8 NaCl, pH 7.2 (adjusted with 1M K-OH) and osmolarity between 290 and 295 $mOsmL^{-1}$. Standard extracellular solution (ECS) was composed of (in mm): 140 NaCl, 5.4 KCl, 25 HEPES, 33 glucose, 2 $CaCl_2$, 1 $MgCl_2$, pH of 7.4 (adjusted with 10N NaOH) and osmolarity between 300 and 305 $mOsmL^{-1}$.

A computer-controlled, multi-barreled perfusion system (SF-77B; Warner Institute, Hamden, CT, USA) was used to exchange bath solutions from standard ECS to 5 µM CNO (Clozapine N Oxide) or 5 µM C-21 in ECS. Transfected neurons (identified by presence of the mCherry reporter protein) were clamped at -60 mV and after 1 min of stable baseline, changes in membrane potentials were recorded by the bath application of CNO or C-21. Recordings were filtered (2-2 kHz) and digitized (10 KHz). Data was visualized by using pCLAMP 9.2 software (Molecular Devices) as recordings were collected from three independent experiments.

3.3.6. In vitro electrophysiology

Data collected from cultured neurons was imported into the custom, open-source Analysis software (developed also by Gilles Detillieux in the SCRC). Action potentials evoked either by spontaneous activity or by current injections were quantified over the same time periods (35-50 s) immediately prior to drug administration and after C21 application. The mean frequency of firing was compared by Wilcoxon signed rank test (n=9 trials from 7 different neurons expressing the mCherry reporter protein of the hM3Gq receptor). Similar recordings were collected for cells not positive for the reporter expression.

3.3.7. Immunocytochemistry of cultured embryonic neurons

Cells cultured on a coverslip in 24-well cell culture plates were washed and fixed by incubating with 4% (v/v) paraformaldehyde in PBS for 20 min at room temperature. After washing three times, cells were blocked with 5% skim milk, 1% BSA, 0.3% Triton X-100 in PBS for 1 h at room temperature, and further incubated with the primary antibodies to detect tryptophan hydroxylase (TPH), 5 hydroxytryptamine (5-HT), mCherry, Cre and NeuN at 4 C overnight (for source and characteristic of primary antibodies, see Table 1). Cells were then washed with PBS three times for 10 min and incubated with secondary antibody in 5% skim milk for 2h at room temperature. Cell nuclei were visualized with DAPI-Flouro-mount. Images were captured using a Carl Zeiss microscope.

Antibody	Host	Source and catalogue number	Dilution
TPH	sheep	Millipore AB 1541, lot 7893349	1:175
5-HT	goat	Immunostar 20079	1:1000
Cre	mouse	Millipore MAB3120, lot 2555972, clone 2D8	1:600
mCherry	rabbit	AbCam Ab167453, lot GR312175	1:2000
2^{o} -Cy3, Cy5		Jackson Immuno Research	1:500
2^{o} Alexa 488		Jackson Immuno Research	1:1000
2^{o} Alexa 350	mouse	Invitrogen Lot 939292	1:500
HA	rabbit	Cell-signaling	1:500
mCherry	chicken	ab205402	1:1000

Table 3.1 List of primary and secondary antibodies used
Primary and secondary (2^{o}) antibodies used for immunohistochemical verification of the grafted tissue harvested from the spinal cord of animals used in the behavioral experiments. The name, the host species, the commercial source with catalogue number when applicable and the working concentration used in this study are described.

3.3.8. Experimental timeline and terminal surgery for spinal cord injury

The general set-up and timeline of the experiments are illustrated in Figure 3.1. The general methods and timeline used in the experiments conform to what has been described in (Sławińska et al., 2013). Adult (2-3 months old) TPH2-cre rats underwent a complete spinal cord transection. After a laminectomy at the level of the thoracic 8th vertebra (Th8), the spinal cord was completely transected approximately at the level of the Th9/10 segment by doing two cuts around 1-2 mm apart and suctioning the spinal cord in between to prevent any axonal regrowth. After bleeding was controlled, the muscles and fascia were closed in layers using sterile sutures and the skin was closed with stainless steel surgical clips. Directly after surgery, the rats received analgesic and non-steroidal anti-inflammatory (s.c. Tolfedine 4mg /kg b.w. and buprenorphine (5 mg/kg). The rats received antibiotics for the following week (gentamycin 5 mg/kg b.w. s.c.). The bladder was manually emptied 3 times a day until the voiding reflex was re-established (usually within 7 days). Sterile saline was injected s.c (1-2 mL) to overcome any dehydration in the first 2-3 days postoperatively. The animals were kept as a pair-per-cage, housed on a 12-hour light-dark cycle and fed ad libitum. The care-fresh bedding was used in combination with normal sawdust bedding and no locomotor training or enriched housing was provided.

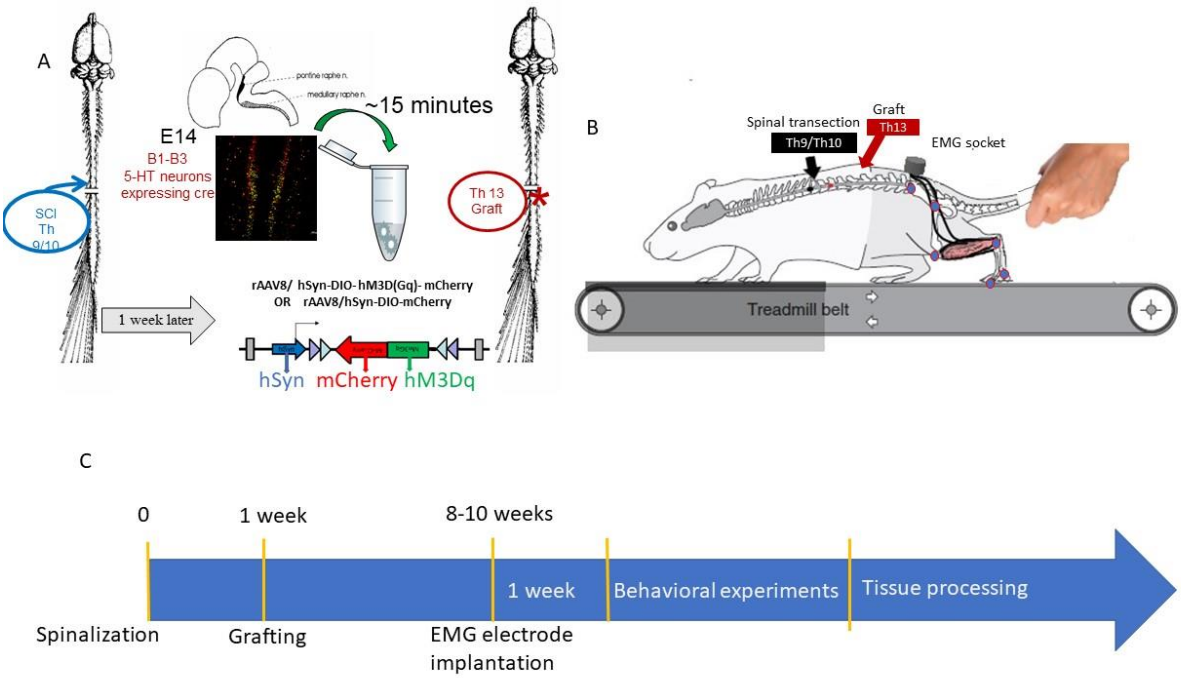

Figure 3.1 Schematics of methods and the timeline of procedures used.
A. Complete thoracic transection at the T9-10 vertebral level was performed one week prior to grafting of embryonic (E14) tissue of brainstem (including B1-B3 serotonergic or 5-HT neurons) harvested from Tph2-cre rats. The embryonic tissue was, incubated with rAAV8-hSyn-DIO-hM3D(Gq)-mCherry) or with rAAV8-hSyn-DIO-mCherry for 15 minutes and then grafted below the transection site at the T12-13 vertebral levels. **B**. Schematics of behavioural testing of locomotor activity. Rats were placed on a treadmill with front limbs supported on a stationary platform but hindlimbs free to move over the belt. Intramuscular EMG recordings (from implanted electrodes in the soleus, and tibialis anterior bilaterally) were recorded. Black ink marks were placed on the left hind limb at the iliac crest (pelvis), greater trochanter (hip), knee, ankle (lateral malleolus, 5th metatarsophalangeal joint head (5MTP) and the tip of the 4th toe and high-speed video recordings were collected at multiple time points after systemic designer drug ligand administration. The hand holding the tail illustrates that exteroceptive stimulation of the tail was used to promote sustained locomotor output in each testing series. **C**. Summary of the timeline indicating spinalization at the start (time 0) then grafting at 1 week later followed by EMG electrode implantation at 8-10 weeks and behavioural testing initiated one week after electrode implantation (lasting for 4-5 weeks) at which point spinal cords were harvested.

3.3.9. Grafting of embryonic neurons after spinal cord injury

Grafted neurons were derived from day 14 embryos (E14) of TPH2cre time pregnant rats. Under the isoflurane of the pregnant females, embryos were removed by standard, aseptic dissection. The embryos were bathed in cooled 0.5% glucose Hank's buffered solution and further dissected under a microscope to isolate the caudal part of the brainstem containing the populations of serotonergic

neurons with descending projections in adult rats to the spinal cord which include the parapyramidal region and the medial raphe serotonergic nuclei corresponding to B1, B2, B3 serotonergic areas. The isolated tissue from the embryonic brainstem was incubated for 15 minutes at room temperature in a vial containing the DREAD containing viral vector. The paraplegic rats received the intraspinal allograft at one-week after the spinal cord transection. A laminectomy was done at the level of Th12 to expose the area of the spinal cord to insert the grafted embryonic and viral mixture. The embryonic tissue then be slowly injected into the spinal cord at the level of Th12/13, below the level of the transection, using a sharpened glass pipette attached to a Hamilton syringe. The needle was pulled out slowly (approximately 10 min) in increments to ensure the graft is not pulled out. Rats were selected randomly to receive either a viral vector that contained the DREADD-conjugate or only a reporter fluorescent protein (mCherry).

3.3.10. EMG electrode implantation

As summarized in Figure 3.1, EMG electrodes were implanted two months after the spinal transection into the *soleus* (SOL, ankle extensor) and *tibialis anterior* (TA, ankle flexor) muscles in both hind limbs. The bipolar electrodes were composed of Teflon-coated stainless steel with hooks implanted into the respective muscles and sutured in place. The ground electrode and the connector were placed on the back of the animal while the wire leads were tunneled from the hind limbs to the mid-back.

3.3.11. Kinematic and electromyographic recordings during treadmill locomotion of spinal rats

Black ink marks were placed on the left hind limb at the iliac crest (pelvis), the greater trochanter (hip), the knee, the ankle (at the lateral malleolus, the 5th metatarsophalangeal joint head (5MTP) and the tip of the 4th toe). Rats were positioned on a moving treadmill with their forelimbs supported on a platform and recording electromyographic activity of hind limb muscles (EMGs) and kinematic marker positions were collected. Video recordings were obtained using an X-Motion™ camera (AOS Technologies) with a capture rate of 120 frames per second (shutter speed 8.3 ms) with 10 seconds of continuous recording. The recordings were digitized (3 kHz sampling frequency) using a Windows laptop. EMGs were amplified, analog band-pass filtered (30 Hz to 3 kHz) and digitized (at 2 KHz sampling rate). The SCRC Capture and Analysis software, an open-source package was used to record signals and perform the first level of analysis (custom programs, developed by G. Dettilieux in the Spinal Cord Research Center, University of Manitoba, www.scrc.umanitoba.ca). Each rat was examined for the occurrence of spontaneous locomotion

before and after receiving intraperitoneal, i.p. injection of 0.3mg/kg compound-21, (C21). Additionally, exteroceptive stimulation was applied to the tail in most animals to induce coordinated steps lasting for 10-30 s or as long as the animal could sustain consecutive steps.

3.3.12. Kinematic and electromyography data analysis

Kinematic motion detection in each video frame was analyzed using ProAnalyst™ (Xcitex) software which recapitulated each marker's position over time. Positional data were then converted for further examinations using the custom Analysis software (developed by the Spinal Cord Research Centre at the University of Manitoba and as described previously in (Cowley et al., 2015). Changes in joint angles, horizontal, and vertical excursions of the different markers were analyzed and used to create stick figure diagrams to evaluate hindlimb movement during the recorded bouts at different time points after injury. Angular joint movements were calculated from the digitized x-y coordinates and displayed as continuous angular displacements for a step cycle and redrawn as "stick diagrams" as described in (Cabaj et al., 2017; Cowley et al., 2015).

Analysis of EMG recordings was performed by the custom SCRC software. EMG amplitude was determined from rectified and filtered EMG records (cutoff 10 Hz). The locomotor pattern was analyzed using polar plots to determine the coordination between flexor and extensor muscles on the two sides, as well as left-right coordination among the muscles of the left and right hind limbs as described previously in (Sławińska et al., 2013). Briefly, the position of each phase differential on these plots is shown around the outmost circle, ranging from 0 to 360 degrees (where 180-degrees position is considered alternation and 0- or 360-degrees position is complete synchrony). The length of the vector (drawn from the center outward) shows the concentration of phase values around the mean (***r*** value, ranging from 0 to 1). This can be interpreted as the strength of coordination between the EMG burst onset of muscles compared (intra limb coordination is established between onsets of EMG activity of different muscles of the same hindlimb e.g. flexor TA vs extensor Sol muscles: interlimb coordination is established between onsets of EMG activity of analog muscles of contralateral hindlimbs e.g. right *vs.* left TA flexor or right vs. left Sol extensor of hindlimb) A built-in Rayleigh's circular statistical test determined if the coordination was phase-coupled if ***r*** was greater than critical Rayleigh's value for a given P value.

Comparison of horizontal and vertical excursions, cycle bursts, slope of linear regression lines, the mean phase shift angles and the mean coupling coefficients for flexor-extensor and left-right

coordination was done by 2-way ANOVA. The presence or absence of designer receptors in the grafts being one factor and the time after the C21 injection (30 and 60 min) being the other factor. Two-way ANOVA was performed in SigmaStat4.0 with first testing the normal distribution of the data by the Shapiro-Wilk test and then testing for equal variance by the Brown-Forsythe test. All data are reported as mean ± SD (standard deviation). For all statistical tests used in this study p-value < 0.05 was considered to be significant.

3.3.13. Immunohistology

The immunohistochemical procedures were the same as those described in (Sławińska et al., 2013). The rats were transcardially perfused at the end of the study with a 4% paraformaldehyde solution and the spinal cord was dissected at the level of transection and several segments above and below this site into 5-6 blocks. The tissue was placed in a cryoprotectant containing 10% sucrose. Serial cross sections of the cord were obtained by a cryostat (15µm thickness and collected on gelatinized glass slides) from all blocks above the transection, through the transection, and below the transection above the graft, through the graft and below the graft. We used immunohistochemical labeling to detect serotonin (5-HT), tryptophan hydroxylase (TPH), cre recombinase (cre), mCherry reporter protein (mCherry) in the transplanted tissue as well as HA-tag protein (HA) in cultured cells. Table 1 depicts detailed information about the primary and secondary antibodies used. The primary antibodies used to label for 5-HT and for TPH were always applied together hence referred to as 5-HT/TPH. They were used together to detect the grafted serotonergic neurons because 5-HT is more highly detectable in the axons while TPH is expressed more in the nuclei. Sections were air dried (for 20 minutes) and then washed with 1.5 TBST solution for 20 min. Primary antibody incubation (~ 200uL per slide, of 1.5 TBST, 10%NDS, AB at respective dilution as shown in Table 1) was done for overnight at 4 ˚C. Secondary AB application was after 3 x 20 min washes in 1.5 TBST for 90 minutes at room temperature. Following a rinse in 1.5 TBST for 20 min, and two rinses with 50 mM Tris buffer, the slides were cover slipped by applying a drop of antifade (DAPI-Flouromount).

Immunofluorescence was examined either on a wide-field Zeiss Imager Z2 microscope or a Zeiss 710 laser scanning confocal microscope using Zeiss Zen Black or Zen Blue image capture and analysis software (Carl Zeiss Canada, Toronto, Ontario, Canada). Data from wide-field and confocal microscopes were collected either as single scan images or z-stack images with multiple scans capturing a thickness of 2 to 4 µm of tissue at z-scanning intervals of typically 0.4 to 0.6 µm

using 10x and 20x objective lenses for wide-field images. Images were collected by using Zeiss ZEN Black and analyzed by using Zeiss ZEN Blue software, and final images were assembled using CorelDraw Graphics (Corel Corp., Ottawa, Canada). Changes made to the background or image color intensity were the same per image set. Images with Cy5 immunolabelling were pseudo-colored either purple or white. Unless otherwise indicated, images depict fields photographed from coronal sections.

The open-source ImageJ program (JaCoP plugin) was used for quantitative analysis. Images of tissue subjected to double labeling were used first to select a field of view containing a distinct fluorescent reporter signal. Assessment of colocalization was determined by using the built-in Costes' automatic threshold setting on 8-bit images (Dunn et al., 2011). Mander's Colocalization Coefficients (Manders CC) and Pearson Correlation Coefficients (PCC) were calculated from one image per animal. The MCC is a measure of colocalization calculated as a fraction (from 0 to 1) of each probe that is colocalized with the other probe and values closer to 1 represent strong colocalization (Dunn et al., 2011).

3.4 Results

3.4.1. Embryonic neurons expressing DREADDs

The first step was to verify that Tph2-cre rat E14 embryos express cre recombinase in 5-HT neurons. The immunohistochemical results as illustrated in Figure 3.2A showed that cre (green) and the 5-HT/TPH proteins (red) were expressed in a large proportion of the same cells. This was verified qualitatively in 2 experiments with the same results as illustrated in Figure 3.2A (no quantification of colocalization was performed in frozen sections of embryonic tissue). Cultured embryonic cells 7-10 days after plating were also examined to verify the colocalization of 5-HT/TPH and mCherry proteins. These tests showed that the reporter mCherry protein was detected in cells that express 5-HT/TPH as illustrated in Figure 3.2B indicating the presence of the DREADD receptors there. Qualitatively similar results were obtained in 2 experiments (without quantification of colocalization).

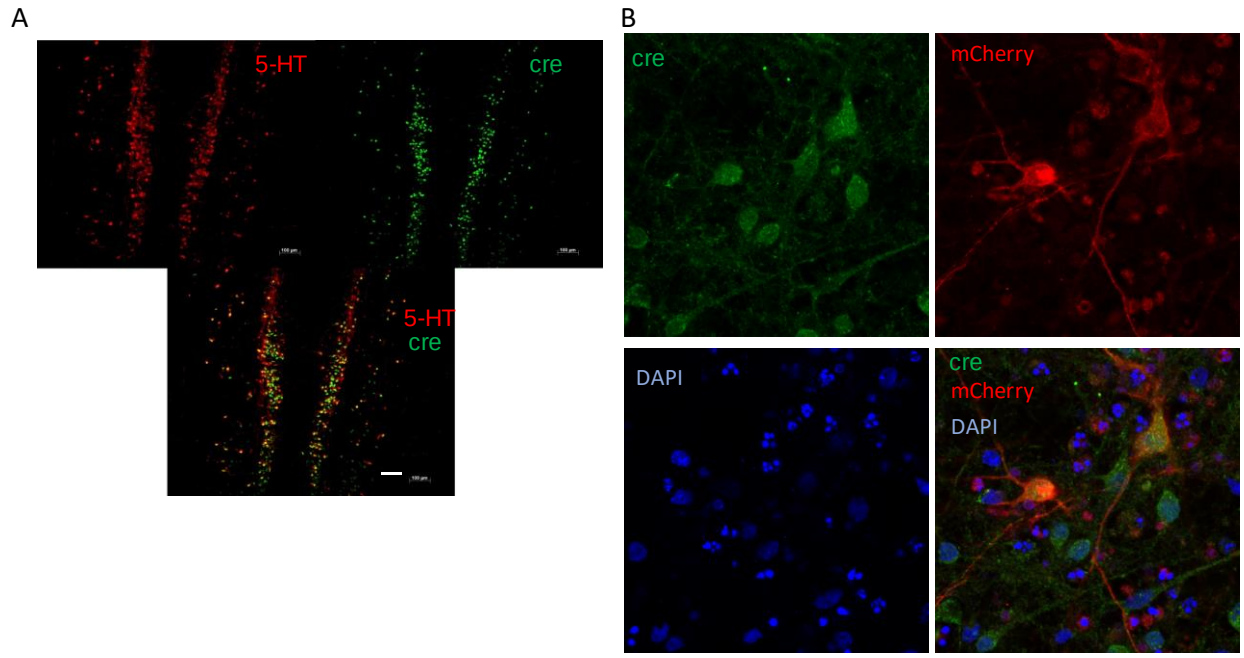

Figure 3.2 Confirmation of cre expression in 5-HT neurons in the brainstem of embryonic day 14 (E14) TPH2-icre rats.
A. Immunolabeling of 14-day old embryonic (E14) rat (Tph2-cre strain) brainstem at 10x magnification showing serotonin (5-HT /TPH, red and cre recombinase (cre, green). The cre expression overlapped with the 5-HT neurons (yellow on merged image, bottom). **B**. Images of immunolabeling in primary cultured neurons derived from E14 Tph2-cre rat brainstem. The mCherry label (red) for DREADD receptors is expressed in cre positive (green) neurons. The cre protein, as shown above, is expressed in 5-HT neurons of Tph2-cre rats. Red: mCherry, Green: cre, Blue: DAPI. Scale bar in 100 μm in A.

The cultured cells were then used for electrophysiological testing of the functional DREADDs. Whole-cell current-clamp recordings were done to detect changes in depolarization and firing in response to DREADD ligand application. The designer drug (compound-21, C21) was applied to the bath solution and whole-cell patch clamp recordings were collected. The number of action potentials in neurons expressing the m-Cherry reporter (Figure 3.3A) was increased after C21 (Figure 3.3B). The firing frequency pre- and post-designer drug application was similarly increased in all tested neurons expressing m-Cherry. The overall increase of firing was significant with a mean firing frequency increase from 1.5 to 2.3 Hz (Figure 3.3C, n=9 trials from 7 cells, Wilcoxon Signed Rank Test, p=0.004).

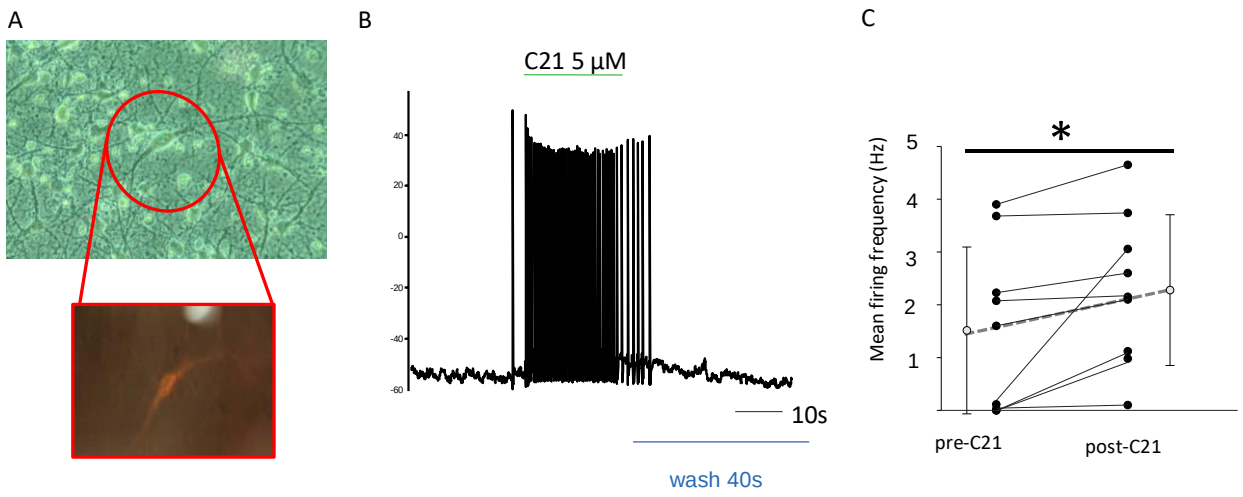

Figure 3.3. Designer drugs increased excitability of cultured cells expressing designer receptors.
A. Photograph of wide-field microscopy image of primary embryonic culture used for recordings. Area in the red circle (expanded in the box) indicates a region where a cell with mCherry expression was identified. **B**. Membrane potential values over 5 minutes from a whole-cell patch recording from the same mCherry positive neuron illustrated in A. **B**. Action potential trains over spontaneous bursts activity with the designer drug compound-21 (C21) 5 µM or without it (wash) is illustrated. **C**. Summary of mean firing frequency (open symbols) in cultured cells from 20-50 s long burst periods with and without C21 in the bath from two independent experimental series (data n=9 cells from 5 different culture dishes). Star indicates significant difference (Wilcoxon Signed Rank Test, p=0.004) and error bars show SD.

The successful excitation of cultured embryonic serotonergic cells after designer drug application led to the use of embryonic cells previously infected with rAAV to express DREADDs *in vivo*. Grafting of embryonic serotonergic cells with rAAV containing DREADD constructs was performed below the level of the complete spinal transection. Behavioural testing regimes commenced 8-10 weeks after spinal injury (7-9 weeks post grafting) with kinematic and EMG recordings during treadmill walking before and after systemic designer drug injection.

3.4.2. Assessment of hindlimb kinematics in spinal, grafted rats with DREADDs after a single exposure to a designer drug

Testing of locomotor activity was performed while rats were supported above a treadmill with hindlimbs free to move when touching the moving belt, as the forelimbs were kept motionless with forelimbs on a stationary platform above the moving belt. During the locomotor testing, the rats hindlimb locomotor movement was triggered by afferent inputs from the moving treadmill belt touching the hindlimbs as well as the experimenter pinching the tail. Testing included recordings of joint positioning of the left hindlimb during walking and these recordings were used to reconstruct stick diagrams as exemplified in Figure 3.4A, D. These examples show 2s of activity (Figure 3.4B and E highlights) from the 10s long bouts of activity from a grafted spinal rat at eleven weeks post-SCI prior to C21 application (Figure 3.4B) and 60 min after C21 (Figure 3.4D). Together with the video recordings of movement, EMGs were also simultaneously collected for bouts of 10s activity, as shown in Figure 3.4B, E. The vertical and horizontal displacement of each marked segment was extracted as shown in Figure 3.4C, F. These features were averaged based on the onset of the toe movements in each step. The averaged values of horizontal and vertical displacements were compared at different time points with respect to C21 injection (Figure 3.4). As illustrated from data collected in one rat in Figure 3.4, the limb trajectory before C21 injection (Figure 3.4A) was similar to the trajectory recorded 60 min later (Figure 3.4D) in terms of the posture and position of each segment. There were some disparities in the height of knee elevation during flexion (red traces in Figure 3.4A, D). The vertical displacement of all joints showed a tendency to be smaller, while the horizontal displacement of the 5th metatarsal and toe tended to be larger after C21 application (Figure 3.4G). Significant differences were identified in this cohort of four rats after the C21 application as indicated by the asterisk in Figure 3.4G. The vertical movement of the pelvis, the hip, the knee, and the ankle were reduced significantly by 30 min post C21. Compared to pre-C21, the pelvis vertical displacement decreased from 0.59 to 0.27 mm ($p=0.036$); while the hip excursion decreased from 0.53 to 0.24 mm ($p=0.024$); the knee excursion decreased the most from 1.18 to 0.68 mm ($p=0.002$) and the ankle movement decreased from 0.77 to 0.53 mm ($p=0.038$, 2-way RM ANOVA) at 30 min post C21. Interestingly, the toe excursion increased from 0.50 to 0.82 mm ($p=0.038$) at 15 min post C21, but it decreased to 0.49 mm by 30 min post C21 ($p=0.030$), and no changes were evident in the vertical or horizontal displacement of the 5th metatarsal.

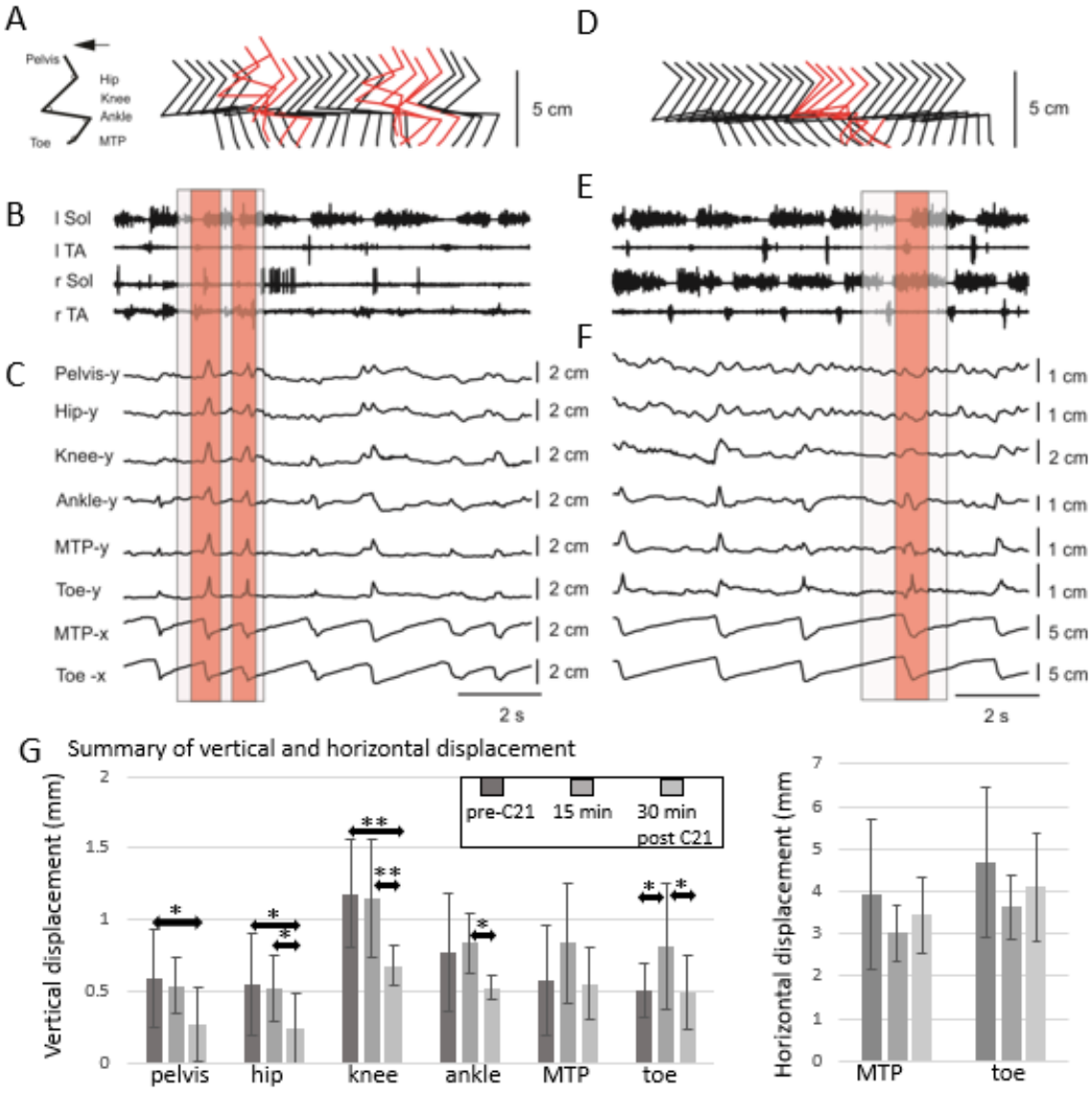

Figure 3.4. Kinematics of stepping after designer drug application.

A. Stick figure reconstructions of left hindlimb movements during treadmill walking at 5 cm/s of a grafted, spinalized rat at 11 weeks after a complete spinal transection at T9-10 vertebral level. Grafted rat with DREADDs before C21 and 1 hour after C21. The stick figures represent 2 seconds (highlighted (red) in C). **B**. Example of implanted raw EMG recordings from soleus (SOL) and tibialis anterior (TA) from the left (L) and right (R) sides over a 10s interval that is synchronized with the kinematic recording prior to C21 injection. **C**. Horizontal and vertical excursions of selected markers (pelvis, hip, knee, ankle, 5MTP and toe) in the same rat as in A and B. **D**. Example of stick figure reconstructions of left hindlimb movements during treadmill walking at 5 cm/s 60 min after C21. **E**. Raw EMG recordings from L and R SOL and TA 60 min after C21. **F**. Horizontal and vertical excursions of selected markers as in C but 60 min after C21 injection. Grouped data of horizontal and vertical excursions from n=4 grafted and spinalized rats showing mean values before (pre) and at 5-15 min, and 30 min (min) after C21 (0.3 mg/kg) injection.

3.4.3. Assessment of EMG activity in grafted spinal rats with DREADDs after a single exposure to a designer drug

We then analyzed hind limb muscle activity. The raw EMG signals were rectified and integrated before quantitative analysis. As illustrated in Figure 3.5A, the grafted spinal rats exhibited relatively good, coordinated locomotion when supported by tail stimulation as described in previous studies (Sławińska et al., 2013) with the SOL burst duration typically corresponding to the step cycle durations and short TA burst durations. The rectified and integrated EMG was used to define the start and end of the bursts in each muscle as illustrated for L SOL in Figure 3.5B. There was no or only minimal overlap between flexors and extensor EMG alternating activity in Sol and TA muscles on one side. The cycle (arrow in Figure 3.5B) and burst (double arrow in Figure 3.5B) durations were measured and quantified for all steps in all four muscles. The bursts from all steps in the left TA and SOL of one rat overlaid in Figure 5C represent typical results when EMG envelops were normalized, taking the onset of TA muscle EMG burst activity as the onset (two cycles repeated for clarity).

The quantified cycle durations from all five rats (n=5) in this cohort, at multiple time points with respect to designer drug application are summarized in Figure 3.5D-F and

Table 3.2. The cycle durations in this cohort ranged from 0.5 to 2.2 s while walking at 5 cms^{-1}. The cycle durations were the same before and after the C21 injection and no significant differences were detected (2-way ANOVA).

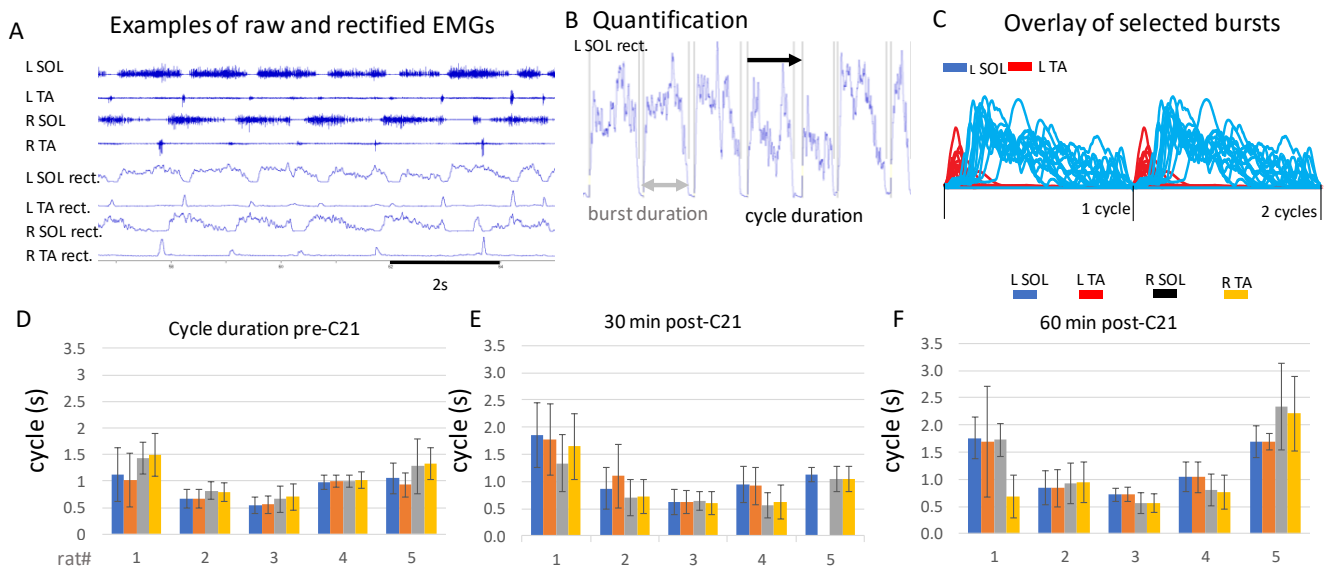

Figure 3.5. Hindlimb locomotor cycle durations unaltered after C21

A. Raw EMG data from hindlimb nerves left (L) and right (R) soleus (SOL) and tibialis anterior (TA) during treadmill walking front-feet supported and tail held. **B**. The cycle durations were visually set by selecting a threshold crossing value for the EMG onset and offset point determination. Time from onset to offset of muscle activity was measured as burst duration (grey double arrow). Time from onset to the next onset in muscle activity was measured as the cycle duration time (black arrow). **C**. Overlay of selected cycles depicting L SOL (blue) and TA (red) EMG activity over 2 normalized cycles (X-axis). **D-F**. The average cycle durations are shown by the circles for L SOL (blue), L TA (red) and R SOL (open) and R TA (orange) muscles quantified from consecutive steps (10-16) taken before (pre, **D**) and 30 min (**E**) and 60 min (**F**) after C21 (0.3 $mgkg^{-1}$ intraperitoneal) injection (grouped in n=5 rats indicated on the X-axis). The error bars represent standard deviation.

Cycle duration (s)	L SOL	L TA	R SOL	R TA
Pre-C21 – controls	0.82±0.2	0.81±0.2	0.79±0.2	0.79±0.2
Pre-C21 – Gq-inj.	0.83±0.3	0.81±0.2	0.98±0.4	1.00±0.4
30 min post-controls	0.54 (2)	0.62 (2)	0.6 (2)	0.62 (2)
30 min post-Gq-inj.	1.08±0.5	1.11±0.5	0.81±0.4	0.77±0.4
60 min post – controls	0.84±0.2	0.90±0.2	0.89±0.2	1.40±0.7
60 min post- Gq-inj.	1.21±0.5	1.19±0.5	1.27±0.7	1.03±0.7
Burst vs. cycle duration linear regression line slope	**L SOL**	**L TA**	**R SOL**	**R TA**
Pre-C21 – controls	0.92±0.06	-0.11±0.08	0.780±0.21	0.07±0.21
Pre-C21 – Gq-inj.	0.90±0.04	0.02±0.07	0.98±0.087	-0.03±0.07
30 min post-controls	0.99±0.07	0.24±0.21	0.96±0.11	-0.04±0.06
30 min post-Gq-inj.	0.96±0.05	0.02±0.06	0.79±0.36	-0.02±0.05
60 min post – controls	0.94±0.14	0.01±0.08	0.67±0.54	0.05±0.06
60 min post- Gq-inj.	0.82±0.19	0.13±0.13	0.68±0.31	0.03±0.08

Table 3.2 Summary of cycle durations and burst *vs.* cycle linear regression line slopes.
The cycle durations of the indicated muscles (soleus, SOL; tibialis anterior, TA) from the left (L) and right (R) sides and the slope of the linear regression lines of the cycle-burst ratio graph. Data from implanted EMGs were obtained in rats with embryonic grafts (controls, n=3) and rats with embryonic grafts combined with DREADDs (Gq inj., n=5) during treadmill walking with supported front limbs and tail before (pre) and after (post) a one-time, systemic administration of compound-21 (C21, intraperitoneal, 0.3 $mgkg^{-1}$). All data represent mean ± standard deviations.

Further analysis was done by plotting the burst *vs*. the cycle duration values. The relationship between burst and cycle durations was expected to be linear for extensor SOL with the slope of the regression lines between 0.6 to 1.0 and close to zero for the flexor TA based on previous results in grafted, spinal rats (Sławińska et al., 2013). Examples of the burst *vs.* cycle duration and obtained regression lines from one rat are illustrated in Figure 3.6A-C before (Figure 3.6A) and after C21 injections at 30 min (Figure 3.6B) and 60 min (Figure 3.6C). The regression lines established for the left and right SOL EMG burst activity showed a clear positive relationship between burst and step cycle durations at all time points after C21 (blue and black lines in Figure 3.6A-C). Unlike the TA burst durations which were not proportional (orange and red lines in Figure 3.6A-C). Grouped data analysis from all animals showed that the slopes of these regression lines were in the range of 0.8 to 1.1 (Figure 3.6D) for SOL and nearly identical in all rats without statistically significant differences and below 0.2 for all conditions of TA EMGs.

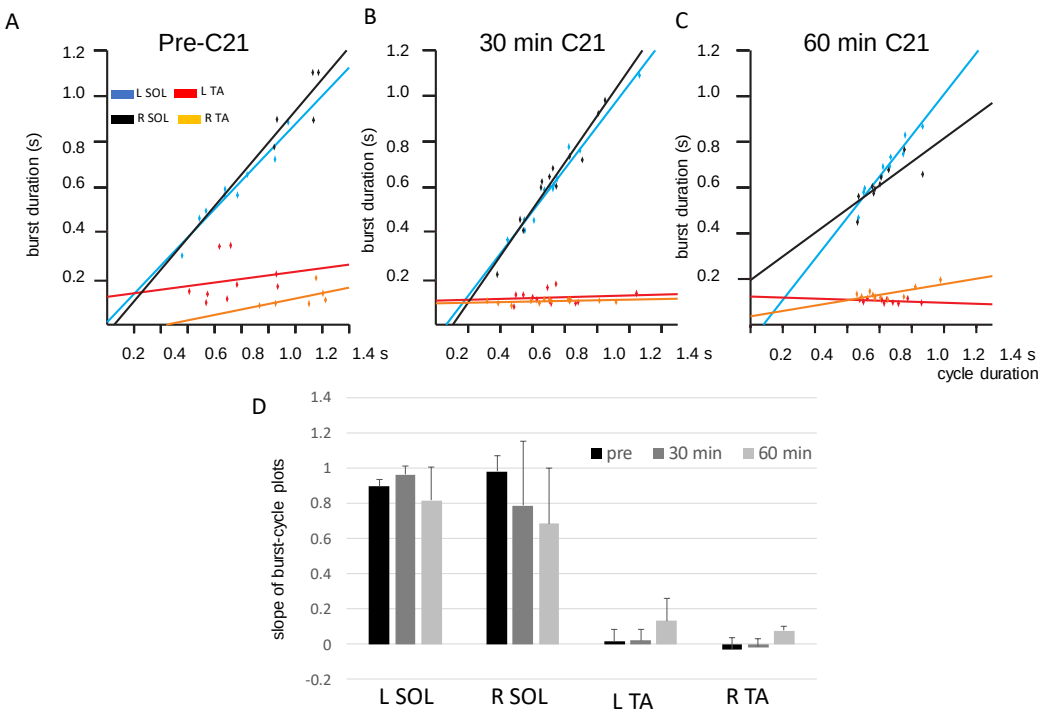

Figure 3.6. Burst vs. cycle duration linear regression line slope unaltered after C21
A-C. Hindlimb EMG burst vs. cycle duration graphs during treadmill locomotion from one rat with DREADDs before (pre C21) and 30 min or 60 min after systemic (intraperitoneal) C21 injection 30 min, 60 min). Individual dots represent single cycles from the bout of locomotion analysed based on cycle settings from SOL (blue, black) and TA (red, orange). **D**. Slope of the regression lines generated after plotting bursts vs. cycle durations and calculating a linear regression equation pre C21 (black) and 30 min (light grey) and 60 min after C21 (dark grey). Error bars represent standard deviations.

To determine whether C21 application affected coordination and the regularity of stepping, phase shifts between flexor and extensor (intralimb) or left-right (interlimb) EMG bursts were examined. The comparison of intralimb coordination was done by comparing SOL and TA EMGs separately on the L and R sides, as illustrated in Figure 3.7A-B and C-D. The strength of coupling was compared by selecting a random series of 6 consecutive steps at different time points after C21 in each rat, as exemplified from one animal in Figure 3.7A and C. The mean phase shift between L SOL and TA muscles was 73 degrees and 52 degrees on the R side. The ***r***-values represented by the length of the vectors in Figure 3.7A and C were 0.78 and 0.94 for the L and R sides, respectively. Grouped data from all animals at each time point showed similar phase angles (phase shifts) as well as relatively high ***r***-values (greater than 0.75) at all time points suggesting the recovery of left-right coordination after SCI that was not significantly altered by C21 (2-way RM ANOVA).

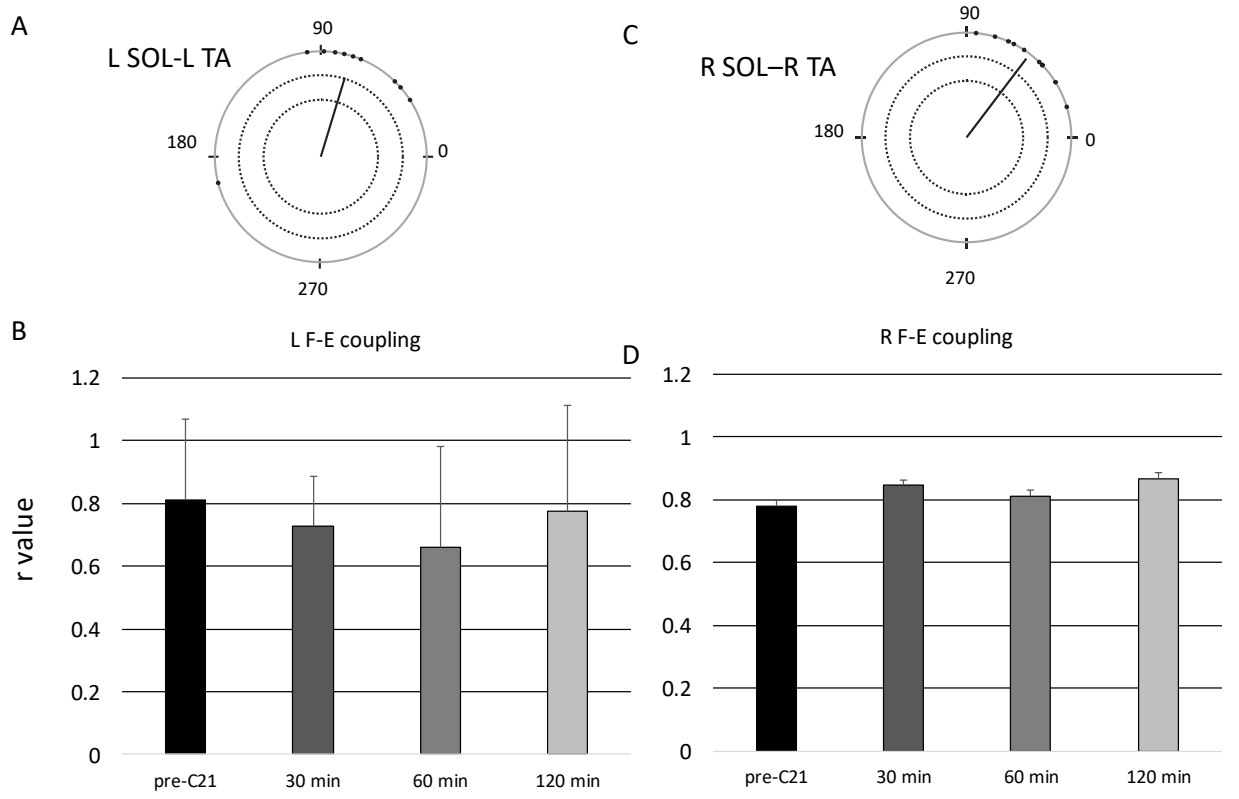

Figure 3.7. Flexor-extensor coordination unaltered by C21
A. Circular plots of phase shift values between consecutive extensor (E) soleus (SOL) and flexor (F) tibialis anterior (TA) EMG bursts on the left (L) from a bout of walking in one rat before compound-21 (C21) administration (0.3 mg/kg, intraperitoneal injection). The circles represent the circular plots of phase shifts, and the direction of the vector represents the mean phase angle of all steps averaged with the length of the vector indicating the strength of coupling (***r*** value). Maximal correlation is one (outer most circle). The dotted circles represent 0.01 (inner dotted circle) and 0.05 significance level (middle, dotted circle). **B**. Bar graphs of mean ***r*** values between F-E muscles on the L side at different time points relative to C21 injection; before or pre-C21: black, 30 min post-C21: dark grey, 60 min post-C21: light grey, 120 min post-C21: lightest grey). **C**. Circular plots of phase shift values between consecutive extensor (E) soleus (SOL) and flexor (F) tibialis anterior (TA) EMG bursts on the right (R) from a bout of walking in one rat, same format as in A. **D.** Bar graphs of mean ***r*** values between L and R TA at different time points relative to C21 administration (same format as in **B**). Error bars represent standard deviations.

The assessment of left-right (interlimb) coordination was done by comparing the onsets of SOL and TA EMGs in both hindlimbs, as illustrated in Figure 3.8A and C (from one rat). The mean phase shift between L and R SOL was 223 degrees and between L and R TA was 221 degrees. The ***r***-values were 0.78 and 0.82 for SOL and TA, respectively in this animal. Grouped data from all rats at all time points tested revealed similar r values as shown in Figure 3.8.8B and D. There were

statistically significant differences, between pre-C21 and at 30 min post-C21 as the SOL ***r***-value decreased from 0.58 to 0.43 and the TA ***r***-value also decreased from 0.63 to 0.47 (2-way rm ANOVA, p=0.03 for the SOL and p=0.03 for the TA, respectively).

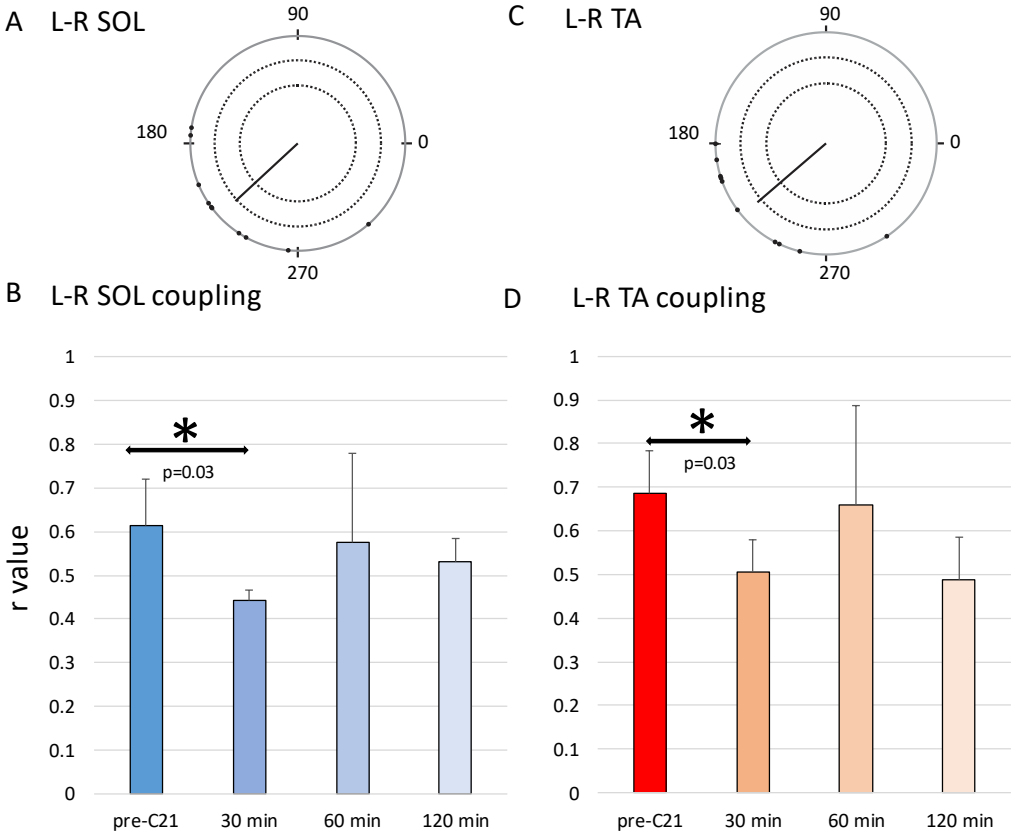

Figure 3.8. Left-right coordination strength reduced after C21

A. Circular plots of phase shift values between consecutive soleus (SOL) EMG bursts on the left (L) and right (R) sides from a bout of walking in one rat before compound-21 (C21) administration (0.3 mg/kg, intraperitoneal injection). The circles represent the circular plots of phase shifts, and the direction of the vector represents the mean phase angle of all steps averaged with the length of the vector indicating the strength of coupling (***r*** value). Maximal correlation is one (outer most circle), dotted circles represent p values 0.01 (inner dotted circle) and 0.05 (middle, dotted circle) of significance. **B**. Grouped data of mean ***r*** values between L-R SOL muscles at different time points relative to C21 (before or pre-C21: black, 30 min post-C21: dark grey, 60 min post-C21: light grey, 120 min post-C21: lightest grey). **C**. Circular plots of phase shift values between consecutive extensor (E) soleus (SOL) and flexor (F) tibialis anterior (TA) EMG bursts on the right (R) from a bout of walking in one rat, same format as in A. **D.** Grouped data of mean ***r*** values between L and R TA at different time points relative to C21 administration (same format as in **B**). Error bars represent standard deviations. Stars indicate significant differences (2-way ANOVA, p=0.03).

3.4.4. Summary of results from control grafted, non-DREADD expressing rats

The control cohort received grafts of embryonic tissue with constructs lacking the DREADD receptors (i.e., only the reporter protein based on the same promoter and rAAV sequence). These animals were examined for changes in locomotor activity following systemic C21 administration. As shown in Figure 3.10A-C, cycle durations were similar after C21 injection to pre-C21 levels and there were no changes in the slope of the burst *vs.* cycle plots (Figure 3.10D). The flexor-extensor coupling was also similar before and after C21 injections (Figure 3.10E-F), however, there was a tendency of the left-right coupling to decrease after C21 (Figure 3.10G-H, red arrows).

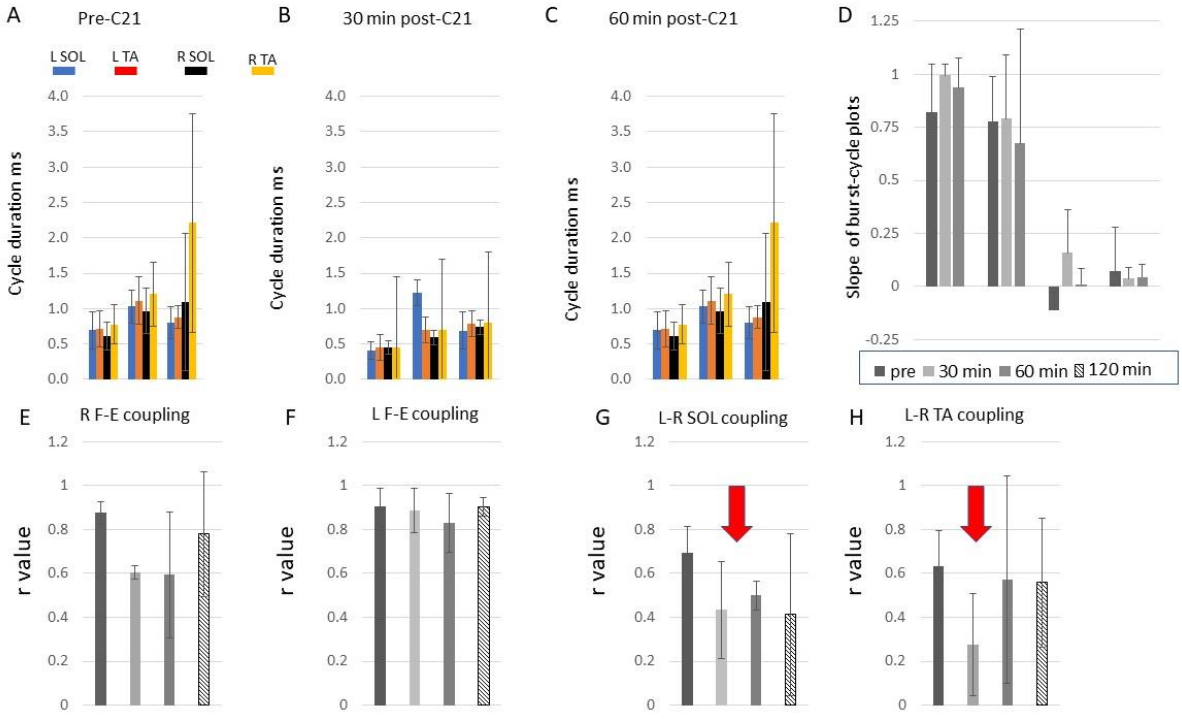

Figure 3.9. Control rat cohort step cycle analysis summary
A-C. The average cycle durations are for L SOL (blue), L TA (red) and R SOL (open) and R TA (orange) muscles quantified from consecutive steps (10-16) taken before (pre, **A**) and 30 min (**B**) and 60 min (**C**) after C21 (0.3 mgkg^{-1} intraperitoneal) injection (grouped in n=3 rats indicated on the X-axis). **D**. Slope of the regression lines generated after plotting bursts vs. cycle durations and calculating a linear regression equation pre C21 (black) and 30 min (light grey) and 60 min after C21 (dark grey). **E-F**. Bar graphs of mean ***r*** values between flexor (F, TA) and extensor (E, SOL) muscles on the left (L) side in **E** and on the right (R) side in **F** at different time points relative to C21 injection; before or pre-C21 (black), 30 min post-C21(dark grey), 60 min post-C21 (light grey), 120 min post-C21(hashed). G-H. Bar graphs of mean ***r*** values between L and R SOL in **G** and TA in **F** at different time points relative to C21 administration (legend in D applies for E-H). Tendency for the L-R coupling to reduce at 30 min after C21 injection (red arrow). Error bars represent standard deviations.

3.4.5. Histological assessment of grafted tissue expressing 5-HT

The behavioral experiments were followed by tissue collection and Figure 3.10 illustrates an example of clear 5-HT/TPH positive neurons of fetal brainstem origin identified in the host spinal cord. This example shows grafted neurons identified in the dorsal horn and around the central canal. The fibers of the grafted neurons labeled for the reporter protein (m-Cherry, red) of the designer receptors extended into the intermediate zone as well as the ventral horn. In each animal, the grafts were confirmed to contain 5-HT neurons using immunohistochemical detection of markers, i.e., 5-HT and TPH. Each graft contained TPH-positive cell bodies giving rise to 5-HT containing processes as illustrated in Figure 3.10B. These processes infiltrated the grafts and extended to some distance from the initial site of the graft. In grafted spinal rats the extent of the 5-HT/TPH labeled processes was estimated and the distances ranged from 128 to 600 µm, similar to previously published results from experiments with E14 grafts by our lab (Sławińska et al., 2013) and also by other labs (Gimenez Y Ribotta et al., 2000).

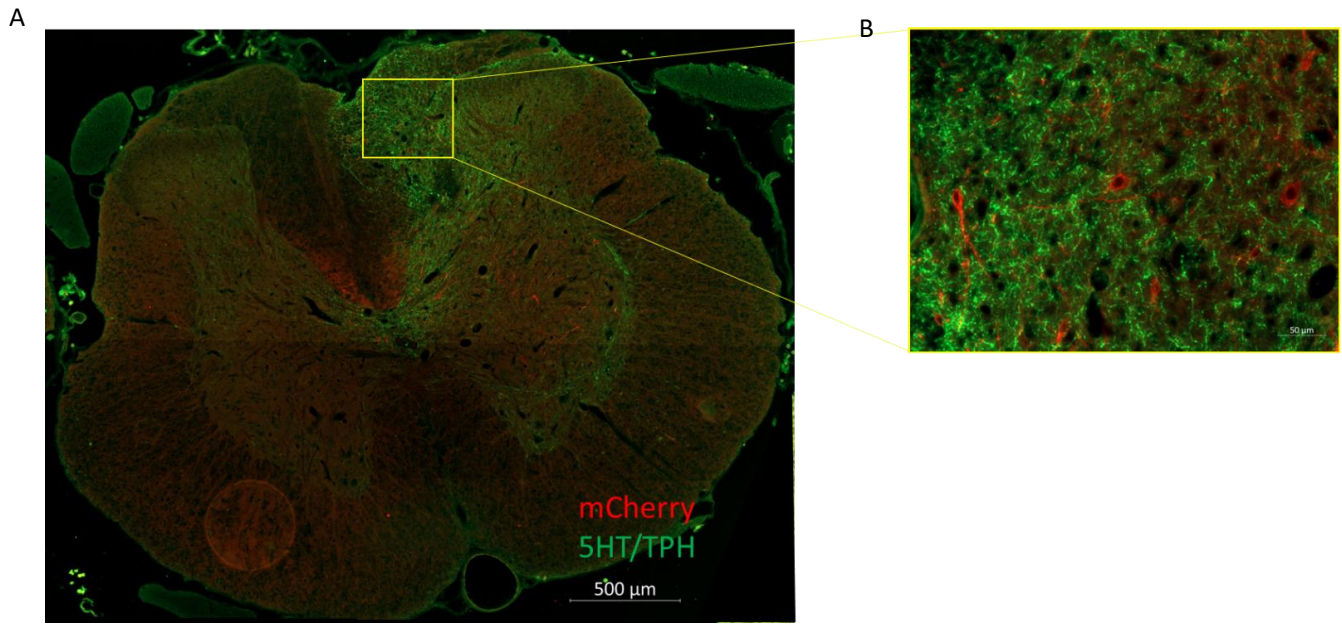

Figure 3.10. Grafted tissue assessment for 5-HT and mCherry colocalization
A. Fluorescent microscopic image of a transverse spinal cord section showing a region with a graft (square) after immunolabeling of 5-hydroxytryptophan, 5-HT and tryptophan-hydroxylase, TPH (green) and the mCherry report protein (red). **B**. Confocal image showing cytoplasmic labeling with mCherry that is visible in the processes of the same cells.

In order to ascertain that designer receptors activate 5-HT neurons, colocalization of 5-HT/TPH markers and the reporter protein, mCherry was established by analyzing fluorescent microscopy images. This was done by randomly selecting regions with visually identifiable grafts as illustrated in Figure 3.11A and obtaining high-resolution images of smaller regions as shown in Figure 3.11B. Triple-labeling of markers for 5-HT/TPH, mCherry and DAPI, as exemplified in Figure 3.11 showed that the reporter protein was localized in cells or processes positive for 5-HT/TPH (arrows Figure 3.11B). The automated, unbiased assessment of colocalization showed 59±45% overlap of 5-HT with mCherry. The overlap of mCherry with 5-HT was 70±22%. Correlation coefficients were similar in terms of means from values obtained from both DREADD cohorts (n=9 in total), thus the combined data were analysed.

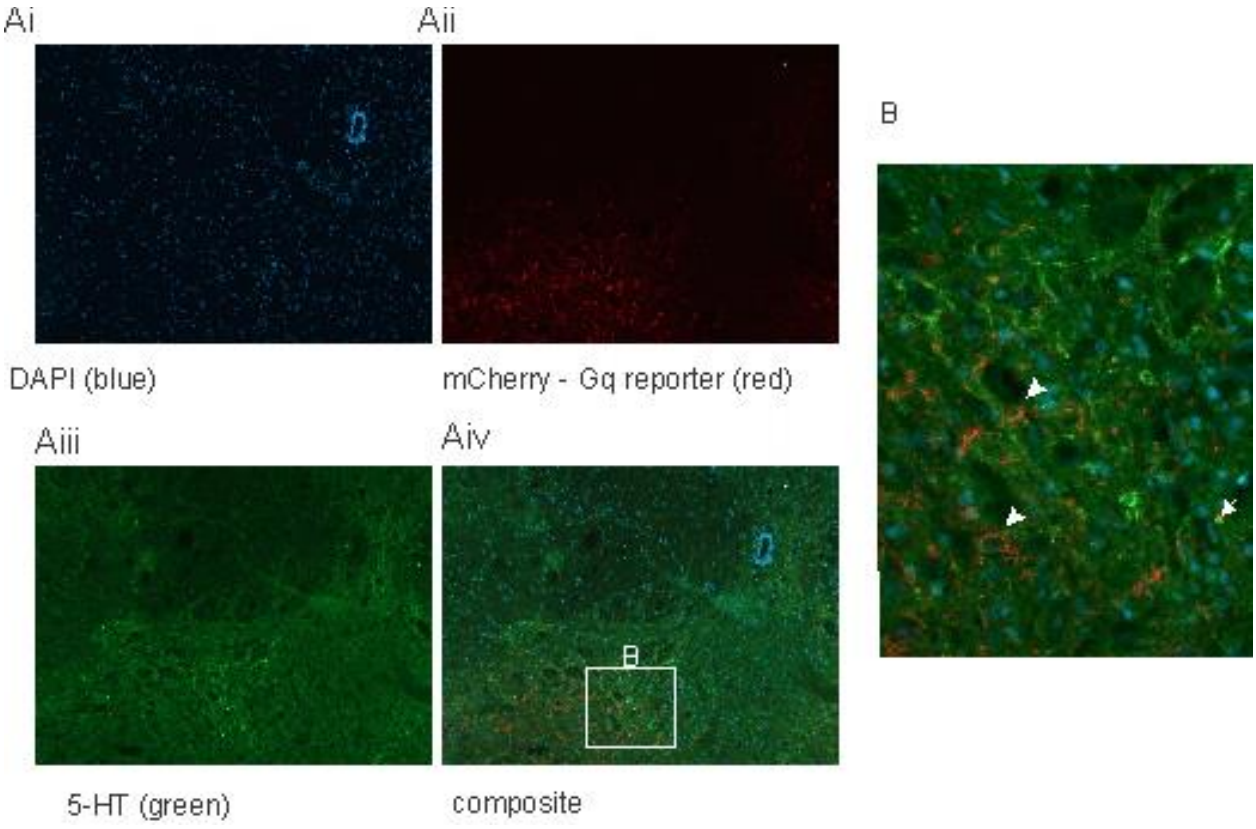

Figure 3.11. Colocalization of 5-HT and mCherry
A. Fluorescent microscopic images of immunolabeling with three different markers. Expression of the 5-HT/TPH (green, Ai), the mCherry reporter protein of the designer receptors (red, Aii) and DAPI, a nuclear marker (Aiii) as well as the composite image with all colors overlaid (Aiv). **B**. Confocal image from the region (in Aiv) by the box showing that the mCherry and the 5-HT /TPH signal colocalized (arrow, yellow).

3.5 Discussion

In this study, we induced excitation of 5-HT neurons in embryonic grafts in chronic, spinal cord injured rats by chemogenetic approaches to examine effects on hindlimb locomotor activity. With recombinant adeno-associated viral transduction approaches, we utilized excitatory DREADDs for the excitation of 5-HT neurons within grafts from embryonic origin. As a result of the DREADD-induced excitation, vertical displacement of the left pelvis, hip, knee, and ankle was smaller at 30 min after designer drug injection than before that (Figure 3.4). Based on muscle activation assessments from an ankle flexor (TA) and extensor (SOL) electromyographic recordings (EMGs), we found significant changes in left-right coordination by 30 min after C21. The flexor-extensor coordination remained the same and no significant alterations of step cycle durations or cycle-burst ratios were found.

3.5.1. Activation of grafted neurons and putative mechanisms contributing to changes in the flexor and extensor muscle activation

This study has confirmed previous findings, regarding the locomotor performance of spinalized rats with embryonic tissue grafted below the lesion. We confirmed that rats with embryonic brainstem grafts recover some degree of coordinated hindlimb stepping as reported previously (Sławińska et al., 2014a). The reduced correlation coefficients between activity of interlimb coordination (on the left and right) sides by 30 min after C21 administration (Figure 3.7) resulted in a similar drop as reported previously by (Sławińska et al., 2013; Sławińska et al., 2014a) after antagonizing 5-HT receptors in spinal grafted rats. The effects of a 5-HT_2 and a 5-HT_7 receptor antagonist both resulted in a similar reduction of left-right coordination as observed here. Our findings further support the idea that facilitatory effects of the grafts on locomotor activity are mediated by 5-HT as shown previously (Sławińska et al., 2014a).

Although, not quantified in this study, but reported previously by the Slawinska laboratory (Sławińska et al., 2013) (Kwaśniewska et al., 2020), the labeling of grafted cells expressing 5-HT as verified by immunohistochemical labeling send their fibres down the spinal cord and innervate the area around the central canal, as well as the dorsal and ventral horn and to some extent the IML region. Visual inspection of histological samples collected from our rats (Figure 3.10) also shows the 5-HT fibres of graft origin with terminals in the same regions. The area of the grafted tissue and the region occupied by it in the cord could be somewhat different from rat to rat; but the

functional assessment of locomotion showed high uniformity at pre-C21 levels for all cohorts based on cycle-burst duration and cycle-burst relations (Figure 3.4 and Figure 3.5) as well as inter- and intralimb coordination (Figure 3.7 and Figure 3.8).

The linear relationship between extensor and flexor burst duration *vs.* step cycle durations established during treadmill stepping remained the same after C21 administration (Figure 3.6). The C21 administration thus had no significant effect on cycle duration measure, suggesting that 5-HT neuron excitation in the grafts did not influence the CPG in terms of recruiting extensor or flexor motoneurons for a longer time and increasing the burst durations than it has before C21 administration. The coupling between flexor and extensor motoneuron activity on the same side was unaltered which corroborates that CPG elements responsible for reciprocal inhibition were not influenced by the activation of 5-HT neurons in the graft. Interestingly, we found that left-right coordination recovered to a lesser extent than that of flexor-extensor correlation (compare maximal values in Figure 3.7 and Figure 3.8) which is a finding not reported previously, to our knowledge. This may be attributed to a larger portion of grafts incorporating into one side of the spinal cord, which needs to be followed-up in future experiments.

3.5.2. Unwanted effects of designer drugs contributing to the deterioration of the locomotor pattern

After the administration of C21, the grafted rats did not exhibit the expected full body weight support throughout the stepping cycle which can be seen in the reduced elevation of the hips. Reduction of pelvis, hip, and knee movements i.e., elevation suggests that there was less force generated by some of the leg muscles. This can be due to multiple factors. One factor being a depolarization block or accommodation which is the phenomenon where the depolarization threshold current intensity for initiation of an action potential is increased. Either due to partial inactivation of sodium channels or an increase in potassium conductance (Schlue et al., 1974). In this case it may have been brought on by the over-excitation of interneurons after C21 effected cells within the grafts via DREADDs. Another factor can be the interaction of C21 on 5-HT_2 and 5-HT_7 whereby it occupies the receptors preventing activation of the receptors by the 5-HT released from the grafts. Consequently, motoneurons and/or interneurons with those receptors would not receive the excitatory input to generate the timely response required for left-right coordination. This possibility is supported by a recent report describing C21 as a competitive antagonist of 5-HT_2 and 5-$HT_{1A/7}$ receptors as well as α-1 adrenergic receptors (Goutaudier et al.,

2019, 2020; Jendryka et al., 2019). The tendency for a decrease in inter-limb (left-right) coordination in the cohort group gives some credibility to the unwanted effects of C21. The back metabolism of CNO to clozapine is undisputed; but since there is no evidence for C21 to be converted to CNO or clozapine, C21 is a more favorable designer drug of choice. Affinity to the same serotonergic receptor subtypes; however, that we targeted by DREADDs would influence the outcomes. The binding of C21 to 5-HT receptors would block binding of 5-HT produced by the grafted cells thereby occluding the desired effects on the grafted 5-HT neurons. Another reason that could lead to undesired effects from DREADD activation can be excessive excitation of the interneurons or motoneurons in response to increased release of serotonin which may decrease the coordination, this could have been tested using a dose-response test of C21 and its locomotor effects. Furthermore, since DREADDs are expressed on all serotonergic neurons irrespective of the location of their final terminations dorsoventrally and the DREADD actuator was administered systematically all cells expressing the designer receptor would respond. The global excitation of all serotonergic neurons within the spinal cord may not depict the physiological activity required or observed during coordinated overground locomotor activity. This method of activation may further contribute to the decrease in coordination of locomotor output.

3.5.3. Spinal neurons contributing to changes after C21 administration

The reduced ***r***-value of the interlimb (left-right) coordination obtained by 30 min after C21 (Figure 3.8) administration suggest that commissural neurons responsible for harmonizing muscle activity between the two sides were affected by the designer receptor-based activation of grafted 5-HT neurons. In the cat, a prominent population of commissural interneurons abundant in lamina VIII has been described as receiving strong reticulospinal input (Bannatyne et al., 2003) and also generating action potentials synchronized with hindlimb locomotor activity (Huang et al., 2000). Moreover, commissural interneurons mediating crossed inhibition or crossed excitation of hindlimb motoneurons from group II muscle spindle afferents in cats have been shown to depend on the modulatory actions of serotonin. For example, short latency inhibition of contralateral extensor motoneurons is present in the intact spinal cord (Aggelopoulos et al., 1995) but after SCI this disappears (Arya et al., 1991b). After administration of 5-HT_{1A} and 5-HT_{7} receptor agonist (+/−)-8-hydroxy-2-(di-n-propylamino)-tetralin-hydrobromide (8-OH-DPAT), however, the crossed inhibition reappears (Aggelopoulos et al., 1996). This suggests that activation of inhibitory "group II commissural" interneurons is modulated by descending 5-HT-dependent control. The

facilitation of responses (i.e. number of action potentials) produced by these interneurons after group II afferent input by localized 5-HT application further corroborates the sensitivity of crossed interneural actions to 5-HT (Hammar et al., 2004). Another study in cats found that 5-HT_2 agonists facilitate responses in all investigated commissural interneurons within lamina VIII while application of 5-HT_{1A}, 5-HT_7 and 5-HT_3 agonists elicit depression of commissural neural activity (Hammar et al., 2007). For a schematic representation of the group II interneurons that express different 5-HT receptors see Figure 3.12. These previously described effects of 5-HT on feline commissural neurons align with our findings in the rat.

Spinal commissural interneurons have been anatomically classified into four different types in rats and mice; based on the direction of their axonal projections: ascending (axons travel rostrally after crossing the midline), descending (axons travel caudally), bifurcating (axons bifurcate after crossing the midline and travel both rostrally and caudally), and short-range intrasegmental commissural neurons (axons remain locally within 1.5 segments of their somas) (Zhong et al., 2006). Information regarding commissural spinal interneurons in the rat is much more limited, however, as data on the effects of 5-HT were only obtained in embryonic and neonatal rats. Commissural interneurons with axons ascending after crossing the midline and those with axons descending after crossing; but not bidirectionally projecting commissural interneurons were excited by 5-HT and rhythmically active during fictive locomotion in neonatal mice when tested *in vitro* (Zhong et al., 2006). As recently reviewed in (Zawadzka et al., 2021) replacement of 5-HT neurons has been shown repeatedly as a viable approach for enhancing functional recovery after spinal cord injury. Our work has focused on the recovery of locomotor function, however, a recent series of studies found similarly promising effects for cardiovascular function benefits (Hou et al., 2020). The 5-HT positive neurons within the graft and host brainstem neurons were found to be reconnected and the regenerated serotonergic circuits improved autonomic function.

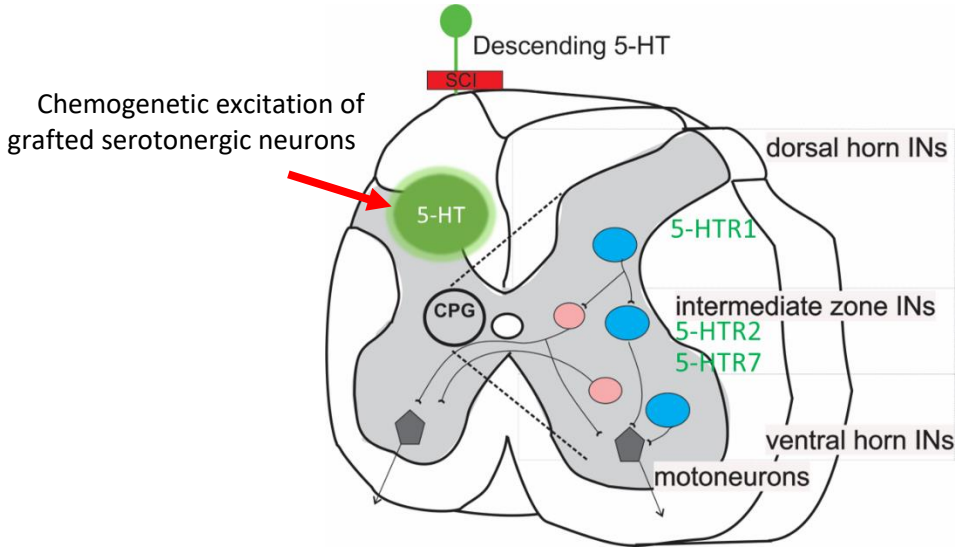

Figure 3.12 Spinal interneuron distribution within the spinal cord with serotonergic receptor expression that are affected by both serotonergic release from the graft as well as the DREADD ligand C21.
Schematic summary of the putative group II populations of interneurons that may contribute to the CPG and express serotonergic receptors that can be affected by chemogenetic activation of the grafted serotonergic neurons or by off target effects of C21. The interneurons can be ipsilaterally projecting (blue) or contralaterally projecting (commissural) (pink) that are only present in the intermediate zone and ventral horn and receive input from interneurons in the dorsal horn. The serotonergic receptors are expressed on the interneurons as well as the motoneurons. The 5-HT_1 and 5-HT_7 receptors have dorso-ventral expression gradient with less expression in the ventral horn while the 5-HT_2 receptors are more highly expressed in the intermediate and ventral horn.

3.5.4. Limitations

The lack of changes in the locomotor relationship between of the burst *vs.* cycle durations might be due to the frequency of designer drug administration. In a recent study, stimulation of large-size dorsal horn neurons with DREADDs was found to be functionally significant only after 6 weeks of repeated (three times weekly) applications of the designer drug to excite the dorsal horn neurons (Eisdorfer et al., 2022). The effects of longer-term, repeated stimulation of the 5-HT graft may likely show greater contribution manifesting in additional changes in muscle activity and kinematic patterns than those observed here. Further work is needed to explore this issue.

Histochemical analysis was performed without labeling the left and right sides prior to cutting. Therefore, it could not be identified in these samples if the grafted tissue was shifted to one side and if that had any relationship with the unilateral functional differences noted in the findings (Figure 3.8). Moreover, examining the full extent of the grafts by random sampling from a region

in which grafts were identified (instead of only ~10-20% of the entire R-C length) the proportion of 5-HT and mCherry overlap would have been likely different. Although, we have examined locomotor activity in control animals without DREADDs in the spinal grafts, the sample size of this cohort was small precluding firm conclusions about the similarity of changes in control and DREADD-harbouring rats brought about by C21.

3.5.5. Conclusions

Our results showed that the local circuits formed by cells in the grafts contribute to functional differences, especially for recovery of flexor and extensor coordination. The significant changes in left-right coordination of both extensor and flexor muscles were attributed to DREADDs-based activation of grafted 5-HT neurons by a one-time exposure to the designer drug C21. The reduced left-right coordination in DREADD-harbouring rats suggest that some interference can be brought on by excess activation of the grafted cells or off target effects of C21. However, because of the trend of a reduction in left-right coordination also observed in the control cohort, more investigations are necessary to understand how spinal neurons are influenced by grafting combined with chemogenetic stimulation when targeting improved locomotor outcomes, and the feasibility of the current chemogenetic ligands in a system dependent on serotonergic receptor activation.

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Chapter IV General Discussion

4.1 Summary of results considering current knowledge about changes in lumbar networks after spinal cord injury in rodents

My research addressed changes after spinal cord injury (SCI) in a rat complete transection model, which leads to an overall loss of hindlimb locomotor function. The rats lost all ability to use hindlimbs for walking, and this model allowed us to examine the innate capacity of the spinal cord in terms of its spontaneous plasticity in the experiments presented in Chapter II. Using the same SCI animal model, augmented with the introduction of serotonergic embryonic brainstem grafts and DREADDs, I examined the effects of altered neural excitability in the experiments described in Chapter III.

As described in Chapter II, I found that input from muscle sensory afferents responding to static stretches (the group II afferents) evoked responses in the lumbar cord that became transiently reduced by one week after SCI. By five weeks post-injury, these same afferents evoked responses with similar amplitude to those that were evident before SCI, suggesting that afferent transmission efficacy in these pathways has recovered. The reduced synaptic efficacy of group II afferents at one week after SCI and its recovery correlated with an absence of locomotor capability. There was no evidence for a wider rostro-caudal distribution of tibial nerve input to lumbar segments and for new projections from group I muscle afferents within the dorsal horn at five weeks compared to one-week post-SCI. These findings fit the current consensus that proprioceptive group I and II afferents are less likely to sprout after SCI than other afferents, such as unmyelinated C-fibers. These findings suggested that recovery of interneural activity levels in some populations of interneurons to its pre-lesional extent is important for regaining stepping.

The activation of spinal neurons below the lesion was the focus of the experiments described in Chapter III. I aimed to boost the activity of neurons caudal to the spinal lesion by utilizing embryonic cells grafting and chemogenetic excitation based on a methodology using designer receptors exclusively activated by designer drugs, or DREADDs. The goal was to reinstate the effects of a key neurotransmitter and modulator, serotonin (5-HT) below the spinal lesion. Given the critical role of 5-HT in multiple physiological functions, serotonergic grafts have been somewhat successful in animal models of SCI for improving the recovery of stepping and other bodily functions. My results align with the current consensus that grafting of embryonic 5-HT

neurons below the lesion ensues improved stepping compared to the walking pattern of rats without grafts. Moreover, my findings add novel information regarding the effects of increased activity (by chemogenetic excitation) of grafted 5-HT neurons. I found that albeit feasible in grafted rats, it is not effective to facilitate stepping stimulating 5-HT neurons as a one-time application. Contrary to my expectations, designer-receptor-induced excitation of 5-HT neurons reduced left-right alternation thereby deteriorated the locomotor pattern of the rats.

The overarching theme that connects both studies is the recovery of spinal neural circuits serving a role in locomotion.

A large body of evidence regarding spontaneous recovery of spinal motoneurons after SCI has been generated by the ground-breaking work of the Bennett lab. The SCI model developed by Bennet focused on the sacral spinal circuits primarily controlling tail muscles (Bennett et al., 2004). This model allowed a systematic examination of various neurotransmitters' effects on motoneurons and some reflex pathways. Bennett and colleagues extensively characterized the effects of different chemicals acting on 5-HT receptors. One of the most unexpected findings from this "sacral cord model" was that 5-$HT_{2B/C}$ receptors became active within weeks after SCI due to constitutive receptor activity i.e., activity in the absence of any ligand (Murray et al., 2010). This activity resulted in the resurgence of large persistent inward currents (or PICs) in motoneurons. These currents increase motoneuron excitability and contribute to the exaggerated late excitatory postsynaptic potentials (EPSPs) that trigger spasms in chronic SCI. Although other 5-HT receptors, such as the 5-HT_1 have also been found to have constitutive activity in single cell cultures (Newman-Tancredi et al., 1997), during testing in neural circuits that are more complex than a cell culture environment, the 5-HT_1 receptor did not show such behaviour (De Deurwaerdère et al., 2020). These findings lead to an important conclusion that 5-HT receptor constitutive activity is different for the various receptor subtypes, isoforms, and it may depend on the circuitry. Even though receptor structure and protein conformation seem to allow constitutive activity in a single cell environment, more complex circuits and the presence or absence of descending neuromodulators (like the lack of 5-HT in a complete transection model) can influence this feature differentially for various 5-HT receptor subtypes (De Deurwaerdère et al., 2020). The potential effects of constitutive activity shown by different 5-HT receptors could offer alternate explanations for my findings, but before paralleling my results to the knowledge gained from the sacral model many unresolved differences need to be addressed. The 5-HT_{2A} levels are reduced in hindlimb

muscles but increased in tail extensor motoneurons after complete SCI, while the 5-HT_7 levels are reduced similarly in hindlimb and tail muscles (Miazga et al., 2018). Another unknown is the effects of 5-HT on the excitability of mature, adult interneurons of the lumbar cord as nearly all studies examined 5-HT effects on motoneurons are in neonates (Garcia-Ramirez et al., 2021). The lack of afferent input in the sacral, in vitro preparation would also be expected to influence the balance of 5-HT receptor activity as synaptic input is amplified largely by the activity of 5-HT_{2C} receptors (Ren et al., 2013).

Reorganization of the central nervous system in terms of somatotopic structuring is common after an injury, such as stroke. Similar reorganization in the spinal cord is less understood and this was examined in Chapter II. My findings show no expansion of the spinal segments receiving tibial afferent input after SCI were contrary to the expectations based on evidence from brain circuits where neighbouring regions extend to process sensory input after an injury. However, alternative explanations regarding sensory input from other muscles than the hindlimbs could be a driver in sensory remapping and should be considered. This is supported by a report on the recovery of body weight support during walking in rats after a complete spinal cord injury (Manohar et al., 2017). In that study, physical therapy induced significant functional recovery by about 12 weeks post-transection. The reorganization of the motor cortex with an increase in the representation of the trunk was identified as an underlying mechanism related to the functional recovery. Activation of the forelimbs when walking also induces activity in muscles controlling the trunk, which in turn will cause excitation in spinal neurons by afferent feedback both above and below the level of the spinal transection. After SCI there is a shift in the weight-bearing, so animals load the forelimbs to a greater extent than the hindlimbs when attempting to move around their cage. This modified body weight distribution has been examined in detail (Cummings et al., 1981; Giszter et al., 2010) and found that recovery of locomotion in neonatal spinal rats was dependent on changes in the weight support caused by a forelimb-trunk-controlled steering of the pelvis.. These findings point to the consideration of reorganization of afferent input from the trunk rather than that of muscle afferent input from the hindlimbs spinal changes in future studies.

In awake, conscious animals, steering of the pelvis and the excessive use of the hindlimb is a highly desired outcome of allowing escape when needed. In the series of experiments of Chapter II, this was likely one of the undescribed factors, which I referred to as the “spontaneous” recovery or “unassisted locomotor recovery”. Rats have different levels of motivation and quantifying the

"unassisted locomotion" could help in understanding the variability observed here in the recovered locomotor coordination indexes.

It is interesting that even when transected neonatal rats received grafts, there was still no recovery of hindlimb motor cortex representation but rather the more caudal axial muscle representation in the motor cortex was rescued (Giszter et al., 1998). Although the type and location of grafts used by Giszter et al differ from the ones I used described in Chapter III (spinal embryonic at the lesion site vs brainstem embryonic below the lesion) the role of the muscle of the trunk can not be overlooked in contributing to locomotor recovery after a complete transection and it would be interesting to know if the motor cortex representation differs with the use of different graft techniques with different levels of integration into the host tissue.

With the knowledge of serotonergic influence on group II afferents (Bras et al., 1990), where the lack of serotonin, due to bilateral lesioned dorsolateral funiculi, abolishes crossed inhibition of extensor motoneurons. with excitation of those same motoneurons taking place to the same stimuli. The activation of 5-HT_{1A} receptors reestablishes this inhibition (Aggelopoulos et al., 1996). Since those experiments were acute it would be interesting to explore if these actions of group II afferents are reestablished over time in correlation with my findings in the changes in the group II afferent responses after a complete SCI, and how that can correlate with spontaneous recovery in the upright position. It would be interesting to expand on this with the use of our knowledge on genetically identified interneurons to correlate the genetic identity with the functional identity which would greatly enrich our knowledge on some of the interneurons that play a role in the CPG network. A good candidate would be the dI3 INs that showed to be phasically active in the extensor phase of drug induced locomotor activity which may indicate the role of the dI3 INs during loading (Bui et al., 2013; Bui et al., 2016). Investigating how this corresponds to group II afferent circuitry and how the presence or lack of serotonin affects their function would be an exciting avenue for future study.

4.2 Limitations of techniques and methods used in this research

One limitation was the use of female rats without testing males. Several reasons were considered for using only females for these studies. Firstly, the handling of male vs. female rats for periods extending from several weeks and even months. The long period of experimentation could have resulted in more stress for male rats as males cooperate less with handlers than female

rats. Male rats have been known to have higher incidents of non-compliant and aggressive behaviour than females. Secondly, after spinal cord injury, major complications resulting from urination and erection/ejaculation dysfunctions in males had to be considered. These incidents would have caused additional health issues such as the high inflammation rates of the penis when recovering bladder function after a complete SCI. Thus, experiments on male rats, from an ethical point of view, would have been difficult to justify for the length required in these studies. Also, most of the previous research on spinal rats has been carried out on females. Nevertheless, there are known differences in how male and female individuals respond to SCI. For example, for a given level and degree of neurologic injury, men tend to do better functionally than women at the time of discharge from rehabilitation (Sipski et al., 2004). In addition to reported differences between male and females in response to DREADDs (Goutaudier et al., 2020).

Another limitation of these studies was the complex, multiple surgical interventions that the rats had to undergo. To maximize the success rates, the Sławińska lab has developed multiple approaches that were also implemented here. First, the experiments were performed on rats that were no more than 6 months old by the end point of the experiments. Thus, the rats were within the young adult stage and handled the physical stress of the multiple procedures better than older rats thus decreasing health complications. The surgical procedures themselves, especially the spinal transection, is an invasive procedure, but when all necessary precautions were considered with preoperative care, the complications could be mitigated. For example, giving antibiotics prior to the surgeries and providing anti-inflammatory medication after the surgeries as well as maintaining a sterile field during the procedures all limited potential complications. The rats were always kept in pairs to maintain their social wellbeing as they are not solitary animals. The only time rats would be caged separately is after EMG implantation so that they would not injure each other by tearing out the implanted cables. After spinalization the rats were provided with modified soft bedding for less trauma to the paralyzed hindlimbs. Subcutaneous saline injections were given postoperatively to ensure hydration and decrease the incidence of urinary infections. Food and water were provided in a more accessible fashion. With water being provided through longer sipping tubes. Nutrigel was provided for the animals with severe impairment especially in the acute post surgical period for increased hydration and nutrient replenishment. Highly palatable jelly-based food in addition to food pellets were placed at floor level for easy access. They were closely monitored after surgical procedures checking for any signs of distress with regular manual bladder

emptying. The rats' weight was monitored with none exceeding 10% loss after spinalization. A week after recovery from spinalization the rats were able to use their forelimbs to climb along the cage to reach their food ad libitum. We had 1/30 mortality rate after spinalization with no complications in any of the other procedures.

The grafting procedures have a 60-70% success rate based on the experience of the Slawinska lab with these methods over the last ten or so years. When examining the locomotor effects of the grafts, in all cases, the rats that are reported on are those that exhibit the best locomotor activity. In our series, we had 2/10 rats in which grafts could not be identified with our histological tests, therefore those were deemed rafting. These were within keeping of the success rates seen previously.

One interesting observation here that should be examined further is that in the grafted embryonic tissue within the host rats extended 1-2 segments caudally which was less than what is usually the extent in the same grafting procedures of this tissue conducted by the same experimenter (Sławińska et al., 2013). This difference maybe related to the fact that the grafts used in my studies were incubated with an AAV vector to allow for expression of the DREADD receptor. AAV is a non-pathogenic neuron that has not shown to cause inflammation or be associated with toxicity (Gray et al., 2010). All surgical procedures were done in a sterile field with antibiotic cover postoperatively with close monitoring for health and activity as well as any infections, or lack of wound healing. One can speculate that the viral vector may have affected the embryonic tissue's ability to become incorporated into the host. Another difference between this experiment and previous grafting experiments is the strain of rat used, here we use a TPH-cre rat that is homozygous therefore the grafts were allografts minimizing chances for rejection.

Another limitation was the off-target effects of designer drugs. First described for CNO, which was found to have dose-dependent undesired effects *in vivo*. The off-target effects could be related to two factors: one is the conversion of the designer drug to another compound through back-metabolism; and the other is the unrecognized affinity of the designer drug to other endogenous receptors (as described in Chapter III). Several studies have examined the implications of CNO back metabolism to clozapine (Mahler & Aston-Jones, 2018) including reports showing this in rats and mice (Manvich et al., 2018) and in non-human primates (Raper et al., 2017). CNO was found to be converted to clozapine; hence DREADD-free control animals also had some changes in

various behaviour tested (Maclaren et al., 2016). Furthermore, there is the observed inability of CNO to cross the BBB unlike clozapine its back-metabolite, which confounds the effects with clozapine being a known 5-HT_7 and 5-HT_{2A} antagonist(Gomez et al., 2017). Yet when Maclaren et al. (2016) examined the effects of CNO on locomotion in rats they didn't observe significant changes. This can be expected in their case where they used intact free moving rats as there are different levels of redundancies in the intact locomotor system allowing for compensations. This is not the case when locomotor activity under different constraints could be altered by CNO. In the grafted, spinalized rats as the locomotor system controlling the hindlimbs is reduced to only spinal neurons and any reinnervation supplied by the grafts. Therefore, even small alterations of receptor activity could result in detectable changes. The balance of active 5-HT receptors in grafted rats, for example, could easily be offset by this side-effect of the designer drug. While that led to the reasoning for the use of C21, yet its effectiveness due to its interactions with the serotonergic receptors becomes limited (Goutaudier et al., 2019, 2020; Jendryka et al., 2019).

In addition to this problem, the DREADD receptors have shown to have some constitutive activity whereby the mere expression of the DREADD receptor independent of the presence of the ligand can exert functional changes on the neurons harboring it (Saloman et al., 2016) (Dulka et al., 2020). The constitutive activity of designer receptors was not tested in our case (due to the lack of available inverse agonists).

Serotonergic neurons can express different transmitters such as substance P, GABA and glutamate among a few others (Okaty et al., 2019). The caudal serotonergic brainstem populations that project to the spinal cord have been found to express GAD, which relates to GABA as a transmitter. This could also have implications on how the cells differentiate and preferentially express one neurotransmitter over another after grafting below the spinal lesion. Another consequence could be that with the application of a designer drug, that causes repeated overexcitation of the grafted serotonergic neurons there could be switching of neurotransmitters released from the grafted cells (Meng et al., 2018). If the grafted cells changed from being predominantly 5-HT-producing cells to another transmitter-producing type, then the excitation triggered by the designer drug could have resulted in the release of GABA, for example. That in turn, would have reduced motoneuron excitability and EMG activity during locomotion. While this is a potential factor in my studies, it is likely not a major influence as we limited the analysis of locomotor pattern testing to the first (and a single) application of designer drugs likely causing

less possibility for switching compared to a more extended period of excitation (i.e. after multiple designer drug applications).

Other factors to consider is the downstream effects of 5-HT receptor activation of motoneuron output. This has been examined and proven to be very complex in the respiratory networks. Both 5-HT_2 and 5-HT_7 receptors contribute to long-term potentiation of the phrenic nerve when activated through intermittent hypoxia after SCI. However, there is crosstalk between the downstream actuators that influences phrenic motor output when repeatedly activating them. This repeated activation may cancel out or tip the balance towards increasing (or decreasing) the activity of a given receptor subtype (Fields & Mitchell, 2017; Fields et al., 2015; Fuller et al., 2005; Hoffman & Mitchell, 2013). It would be important to understand how different downstream pathways of the 5-HT receptors within the spinal cord interact in the presence of grafted tissue. This would be possible by performing western blot analysis of tissue collected from spinal cords and pharmaceutical manipulation of downstream effectors and it can be warranted in future work.

4.3 Functional significance of results in pre-clinical and clinical context

Severe SCI followed by loss of locomotor function has no definite treatment strategy. A combination of interventions is used to prevent neural tissue loss and improve the functional outcomes. Although, the majority of past clinical trials in humans focused on pharmaceutical treatments; this trend has shifted recently. Considering all clinical trials targeting spinal cord injury, nearly 28% of research focused on neuromodulation either by chemical or electrical stimulation or exercise/training as reviewed recently (Dietz et al., 2022). Therefore, my pre-clinical investigations should be considered timely; given the increasing interest of neuromodulation within spinal networks.

Despite the disruptive effects of SCI on locomotor output, partial recovery is possible as reviewed in e.g., (Dietz & Fouad, 2014; Edgerton et al., 2008; Rossignol, 2006). Methods used to promote recovery in clinical trials involve pharmacological interventions, electrical stimulation, or retraining of walking on a treadmill or often, a combination of these methods. When excess excitation of large sensory neurons was induced in rats by chemogenetic means, improved quadrupedal locomotion was reported by about six weeks post SCI (Eisdorfer et al., 2022). The crossed inhibition of extensor motoneurons elicited by group II afferents was replaced by crossed excitation following spinal transection or bilateral lesions of the dorsolateral funiculi in

anaesthetized cats, indicating strong descending control of the commissural neural circuitry (Aggelopoulos et al., 1996). Stimulation of the tibial and superficial peroneal nerves in adult decerebrate cats spinalized one month earlier increased the amplitude and duration of crossed extensor responses and triggered locomotor-like activity in approximately one-third of trials following stretch and tibial nerve stimulation was while clonidine, an α2-noradrenergic agonist was administered (Frigon, Johnson, et al., 2012). Differential effects of clonidine on crossed reflexes and on ipsilateral responses to muscle stretch were interpreted in that study as actions of clonidine primarily acting at pre-motor neural sites and that clonidine facilitates hindlimb locomotor recovery at four weeks post complete spinalization in untrained cats by enhancing the excitability of CPG neurons that participate in crossed extensor reflexes (Frigon, Johnson, et al., 2012).

With respect to grafting and controlling the excitability of neural circuits incorporating the grafted tissue in the host, clinical trials are less prevalent. Firstly, grafting of embryonic tissue raised major controversy regarding the source of the tissue being grafted. Even though, embryonic stem cells are an excellent source to be transplanted as they can differentiate into multiple cell types, including neurons and supporting cells, no active clinical trials can be found. To overcome the ethical issues surrounding the use of embryonically sourced stem cells the use of human inducible pluripotent stem cell technology offers a promising route for grafting treatment (Kleiman & Engle, 2021) Secondly, stem cells trials in general, have a tendency to shift from investigator-driven to profit-driven arrangements; and often end prematurely without conclusions due to the high costs (Yamazaki et al., 2020). Therefore, effort should be placed into exploring the best methods for deriving the cell type of choice from the pluripotent stem cells used. And more pre-clinical work is desired to maximize the details about effective interventions. Expanding on my studies to test 5-HT receptor subtypes contributing to the changes in the locomotor activity; testing different time-points post SCI and after grafting would add relevant details to this pre-clinical study.

4.4 Future research directions

Clinical research has shown repeatedly that in people after SCI, the chances of responding to motor improvement during physical therapy and active rehabilitation strongly depend on measures of volitional trunk and leg strength as reviewed by (Field-Fote et al., 2017). This association shows the important influence of supraspinal centers on spinal networks organizing walking. As the main source of 5-HT is from descending tracts; replacing this by grafting the appropriate cells below the lesion could recapitulate one component of the missing descending drive (Enjin et al., 2012).

The restoration of the lost serotonergic influence via the use of grafts is an attractive method due to the possibility of a more physiologically relevant supply of serotonin with a possibility of modification according to demand. This is in accordance with the effects of 5-HT_2 and 5-$HT_{1A/7}$ receptor agonists do improve locomotor recovery in the horizontal position of the spinal rat while impairing the spontaneously recovered upright position which indicates for a need for a balanced amount of serotonergic neuronal excitation to accompany the afferent feedback. This can be further understood with the ever-complicated world of serotonergic receptors where not only do we have several subtypes, these subtypes interact with each other and create heterodimers (Grinde & Herrick-Davis, 2017; Renner et al., 2012; Sławińska & Jordan, 2019). For example, 5-HT_7 with 5-HT_{1A} can create a heterodimer that decrease the 5-HT_{1A} Gi inhibitory activity. While this interaction can explain the control of seemingly opposing actions of serotonin on locomotion it certainly introduces another level of nuance to understanding how serotonin affects sensorimotor control. An understanding of the changes of the receptor interactions after spinalization would be an interesting avenue to explore for a better understanding on how to tailor treatments after SCI for better recovery.

As 5-HT is a powerful modulator of supporting cell activity in the CNS; it is likely that some of the effects of our grafted cells are exerted on glia. Recovery of function after reorganization in spinal circuits depends on neurons as well as other cell types, such as the supporting cells to neurons. Especially with the knowledge of serotonergic receptors have been found to be expressed on different supporting cell lines (Carson et al., 1996; Merzak et al., 1996). The increasing recognition of neuron-glia interactions following SCI as a factor in shaping network regeneration should be considered in future studies. Supporting cells influence the viability and potentially the function of neurons (Hosseini et al., 2022). Those effects were missed by the outcomes measured.

Implementing additional evaluation of the tissue collected from my experiments, such as immunohistochemical staining for supporting cell types would disclose novel information.

4.5 Summary & conclusions

In summary, my dissertation contributed to previous knowledge regarding the plasticity of neural networks after spinal cord injury. The results of my experiments highlight that sensory input is one of the key regulators of functional recovery, and the novel finding in Chapter II regarding a transient reduction in group II afferent transmission at one week followed by a recovery by about five weeks could explain the partial recovery of stepping. The unique contributions of Chapter III are two-fold. Firstly, I show that embryonically derived brainstem neurons can be successfully transduced to express DREADD receptors and be incorporated into the host tissue. These grafted serotonergic neurons recover a similar degree of coordination to what has been shown in previous work. Secondly, I found that a one-time chemogenetic stimulation of 5-HT neurons with C21 impaired left-right coordination during treadmill walking.

In conclusion, my findings highlight that combining means to artificially change neural excitability after SCI requires more refined means to improve locomotor function rather than to excite lumbar neural circuits based on a single neurotransmitter phenotype.

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