

**THE ROLE OF BDNF AND DURAL DAMAGE IN SPONTANEOUS  
LOCOMOTOR RECOVERY AFTER SPINAL CORD INJURY**

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by  
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## ABSTRACT

The present study aims to elucidate the mechanisms underlying locomotor recovery following spinal cord injury (SCI) through the investigation of Brain-Derived Neurotrophic Factor (BDNF) delivery, and inflammatory responses associated with different spinal transection methods.

Chapter 2 focuses on characterizing lumbar interneurons' activity during air-stepping following chronic intrathecal BDNF delivery to the lumbar spinal cord. BDNF has demonstrated the potential to elicit full locomotor recovery in untrained spinal animals, suggesting therapeutic benefits for SCI patients. However, the effects of BDNF on large populations of neurons responsible for this recovery are not well understood. The hypothesis is that intrathecal BDNF delivery will result in significantly increased neuronal activity in the L3-L4 segments during air-stepping. A programmable, implantable mini-pump was used to deliver BDNF at 50 ng/day for 35 days post-transection. Kinematic data was collected before and after BDNF delivery, and multiunit extracellular recordings were obtained using 64-channel microelectrode arrays. Results from analysis suggest that while BDNF evidently increases neuronal excitability in treated cats, development of locomotor recovery seems to be achieved through subtle changes in neuronal activity.

Chapter 3 investigates the mechanisms behind instances of spontaneous locomotor recovery observed in the literature, which could involve endogenous BDNF or other beneficial mechanisms. It compares locomotor recovery between open-dura and closed-dura spinal transection methods in cats. Previous studies have reported inconsistent outcomes regarding spontaneous recovery and the need for treadmill training. The

hypothesis is that an open-dura transection will lead to better recovery during treadmill locomotion in untrained spinal cats. Kinematic data and ground reaction forces were measured to assess locomotor parameters and weight-bearing abilities, providing a quantitative analysis of recovery. The results show that an open-dura transection is associated with the development of spontaneous locomotor recovery in untrained spinal cats.

Chapter 3 also examines differences in the inflammatory response at the lower thoracic cord between the two spinal transection methods, given the significant role of inflammation in CNS repair and recovery. The hypothesis is that the open-dura method will result in a higher inflammatory response, characterized by increased macrophages, microglia, and BDNF levels caudal to the transection site. Immunohistochemistry (IHC) and RNA in-situ hybridization assays were used to analyze the cellular and molecular environment near the injury site. Open dura animals show a decrease inflammatory response to injury and show no evidence of endogenous BDNF caudal to the injury. These results suggest the development of spontaneous locomotor recovery associated with a transected dura can be elicited through inflammatory mechanisms alone without the need for neurotrophic intervention.

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# CHAPTER 1

## INTRODUCTION

### **Clinical Outlook on Spinal Cord Injury**

The spinal cord is part of the central nervous system and extends down the vertebral canal as a nervous tissue structure that interfaces and exchanges information between the brain and body. Damage to this structure can critically impair bodily function and limit the extent to which an individual is able to use and feel their body. Unfortunately, the incidence of this injury takes a significant toll on society even with modern medicine. It is estimated that in 2023, there were up to 388,000 people living with spinal cord injury in the United States (National Spinal Cord Injury Statistical Center, 2023). There are approximately 18,000 new cases every year which are largely due to vehicle accidents. The second leading cause of injury are falls followed by acts of violence (National Spinal Cord Injury Statistical Center, 2023). Recovery from these incidents is extremely rare since less than 1% of the patients experience complete neurological recovery by the time of discharge from the hospital. Close to half of all injury cases result in incomplete tetraplegia which involves some degree of weakness or paralysis in all four limbs and up to 30% of patients will return for re-hospitalization due to further lesions or subsequent diseases. In addition, the cost of healthcare and living expenses is estimated to range from \$500k to \$1.4M in the first year of injury alone (Merritt et al., 2019). Similarly, mortality rates are the highest during the first year of injury. Life expectancy has plateaued since the 1980s, with no further progress in how long SCI patients live after the injury.

Respiratory and heart complications are the primary causes of death within the first year of injury (DeVivo et al., 2022). However, while the physical limitations and pain

consequences present challenges when coping with long-term disability, the psychiatric symptoms that result from the physical limitations have been estimated to play a greater role in determining the quality of life after SCI (O'Donnell et al., 2013).

## **Spinal Cord Anatomy and Structure**

The spinal cord is the extension of the central nervous system caudal to the brain. It is responsible for communicating sensory and motor information to and from the brain to guide and execute motor movement. The human spinal cord consists of a total of 33 segments composed of 7 cervical vertebrae, 5 lumbar, 5 sacral and 4 coccygeal (Hashmi et al., 2022). The spinal cord communicates with the body via spinal nerves. There are a total of 31 pairs of spinal nerves that exit the spinal cord and in general the dorsal spinal nerves receive information about the body and the ventral spinal nerves transmit motor commands to the body. At every level, spinal nerves communicate with a different part of the body. The high cervical nerves innervate the neck, lower cervical innervate the shoulders, arms and hands. Thoracic nerves innervate the trunk, lumbar innervate the lower limbs while the sacral nerves innervate the back of the legs, genital, and anal regions. There are two enlargements, one at the cervical (C3-T1) and one at the lumbar levels (L1-S2), that contain sensory and motor nerves conveying information to and from the upper and lower limbs. While general anatomy and tract organization are very similar in the feline, they have a slightly different number and distribution with 7 cervical vertebrae, 13 thoracic, 7 lumbar, and 3 sacral (Thomas & Combs, 1962).

The spinal cord consists of white matter and grey matter. White matter is primarily highly myelinated axons that perform long distance communication while grey matter is composed primarily of cell bodies that are more involved in local circuitry. The white matter

is divided into posterior, anterior and lateral funiculi. The posterior funiculus contains mainly sensory signals being carried to the brain such as proprioception, pressure, and touch. The anterior funiculus contains motor information descending from the brain. The lateral funiculus contains a combination of ascending and descending tracts carrying sensory and motor signals back and forth from the brain. Two primary motor tracts found in this area are the corticospinal (CST) and the rubrospinal tracts while the primary sensory tract is the spinothalamic tract (STT), carrying nociceptive, temperature, and crude touch to supraspinal centers.

The grey matter core is shaped like a H with posterior/dorsal and anterior/ventral horns connected by the intermediate zone. At the center of the grey matter core is the central canal which is connected to the ventricular system and transports cerebrospinal fluid (CSF). The grey matter contains more cell bodies by volume than white matter and can be divided into ten laminae (Kandel et al., 2000; Rexed, 1952). The dorsal horns are composed of laminae I–VI. Laminae I and II contain nociceptive neurons that receive ipsilateral pain information from small diameter primary afferents (Haines, 2012; Kandel et al., 2000). Laminae III and IV receive ipsilateral non-noxious input. Lamina IV and V send fine touch and proprioception information to supraspinal areas via the dorsal columns. Furthermore, the intermediate zone of the grey matter contains laminae VII, VIII, X, and receives projections from primary afferent fibers and projections from muscles and joints and is concerned with sensorimotor integration of the descending commands and sensory feedback. Laminae VII and VIII also respond to contralateral stimuli and contain polysynaptic connections of nociceptive input which is transmitted to the brain through the spinothalamic tract. The ventral horn grey matter is composed of laminae VII-IX. Laminae

VII contains Clarke's nucleus known as the location of second order neuron cell bodies of the dorsal spinocerebellar tract (DSCT), terminating at approximately the 4<sup>th</sup> lumbar segment (Haines, 2012). Lamina IX exist as distinct volumes of motoneuron pools which control the axons that innervate musculature of the body and are paramount for general movement (Haines, 2012; Kandel et al., 2000). Finally, the region that connects the lateral portions of grey matter is Lamina X, which is centered around the central canal.

The spinal cord is responsible for communicating sensory and motor information which can be roughly located to the dorsal and ventral halves. However, the spinal cord also contains local circuitry responsible for lower-level processing, allowing it to elicit responses independently from the brain (Kandel et al., 2000; Sherrington, 1910; Stuart & Hultborn, 2008). Reflex pathways can process and transfer information between the dorsal and ventral halves of the cord resulting in the integration of sensory information to recruit an appropriate motor response. Sir Charles Sherrington postulated the existence of reflex arcs contained within the spinal cord as the fundamental mechanism through which locomotion is executed (Sherrington, 1910). This finding led to a new understanding of the spinal cord as more than just a bundle of axons and encouraged further investigation of behavioral capacity localized in the spinal cord.

### **Spinal locomotion**

Sherrington dissected the stretch reflex arc and determined that movements could be carried out independently from the supraspinal command centers as sequencing of stretch reflex responses from agonist and antagonist muscles, but he did not fully resolve the extent of the circuitry responsible for locomotor movement. Following studies showed that, in a decerebrate and deafferented cat preparation, one could elicit the alternation of

antagonist muscles in the cat's hindlimbs (Graham Brown, 1911). The alternation between flexor and extensor was observed while recording from hindlimb gastrocnemius and tibialis anterior in the absence of afferent feedback or descending commands. This suggested the existence of a more central set of circuits that does not depend on external information to produce a locomotor output. Graham Brown proceeded to develop the 'half-centre' hypothesis in which he proposed that the spinal cord contained a set of interneurons that gives rise to two functional groups or half-centers that are mutually connected by reciprocal inhibitory connections (Brown, 1924; Graham Brown, 1911; Stuart & Hultborn, 2008). These half-centers are then able to recruit extensor and flexor motor neuron pools in alternation, even if inputs from higher centers were to be tonic in nature. Later, the noradrenergic precursor L-DOPA was shown to affect ipsilateral flexor reflex modulation and contralateral extensor activity (Jankowska et al., 1967a) which is normally inhibited in acute spinal animals, suggesting the interneuronal basis for reciprocal inhibition since the effect was not mediated pre- or post-synaptically. Further exploration of this model led to the discovery of rhythmic output bursts during nerve recordings (called fictive locomotion) in the acute spinal cats that were made possible when the cord was bathed with DOPA in the absence of afferent feedback (Grillner & Zangger, 1979). It was also shown that after an injection of noradrenergic antagonist clonidine, a decerebrate acute spinal cat can perform real locomotion on a treadmill at different speeds (Grillner & Zangger, 1973).

The half-center model was the first central pattern generator model. A central pattern generator (CPG) is a circuit of neurons that can produce neural oscillations and rhythmic outputs from tonic/non-rhythmic input independently from the brain. The body of work started by Sherrington and Graham Brown, inevitably led to the development of

this idea and indicated that its location was restricted to the spinal cord. The structure and connectivity of the CPG model has evolved over time to accommodate for the complexity of behavior observed from experimental data. Initially, the half-center model proposed from Graham Brown's experiments was sufficient to account for the observations at the time. The structure of the CPG was suggested to be composed of two spinal half-centers, each composed of a flexor and extensor module that reciprocally inhibit each other (Brown, 1924). Inhibitory interneurons mediate the interaction between spinal half-centers such that only one half center can be active at a time. This model assumed that motoneurons were directly linked to either the flexor or extensor half-center. However, the pattern of motoneuron activation is more complex and includes periods of overlapping activity in ankle flexor and extensor muscles as seen during paw shake (Carter & Smith, 1986; Koshland & Smith, 1989). Grillner suggested the unit burst generator (UBG) as a more flexible model (Grillner, 1981), proposing that UBGs are separate rhythmogenic modules that control separate limb joints while weighing neighboring UBG activity, resulting in local orchestration of the locomotor pattern from a mosaic of UBGs. However, despite the promise at the time, a strictly UBG-dependent CPG model fails to account for non-resetting deletions observed in treadmill locomotion in spinal animals (Duysens, 1977) or fictive locomotion (Grillner & Zangger, 1979). Non-resetting deletions are cases where activity fails simultaneously in multiple synergist motoneuron pools resulting in tonic or rhythmic activity in multiple antagonists, suggesting a more complex locomotor spinal system since they cannot be accounted for by simple inhibition of synergistic motoneuron pools. To address this issue, McCrea and Rybak proposed a CPG architecture composed of two layers, separating the pattern formation and rhythm generation modules (McCrea & Rybak,

2008). In this model, perturbations that affect the pattern formation layer do not reset the rhythm generation layer, allowing for the clock-like behavior implied in behavioral observations during non-resetting deletions.

Even though Jankowska and Graham Brown had shown evidence for the CPG location within the lumbar spinal cord (Graham Brown, 1911; Jankowska et al., 1967b, 1967a), the extent of the network in the cord was still unknown. In the feline model, it was shown that the caudal portion of the lumbar spinal cord, specifically segments L6 to S1, was capable of producing an alternation between extensor and flexor muscles at the ankle joint when isolated from the rest of the spinal cord (Grillner & Zangger, 1979). Moreover, interneurons in the L4 segment play a significant role in locomotor orchestration. The mid-lumbar (L4) interneurons receive group II afferent input while having direct excitatory and inhibitory effects on hindlimb motoneurons (Edgley et al., 1988). Later, the importance of L4 interneurons in the generation of locomotor behavior was confirmed by the Rossignol group. They showed that chronic spinal animals (T13) could no longer carry out stepping behavior after an additional spinal transection at the L4 level (Langlet et al., 2005), while locomotion was not lost when secondary transections were carried out at more rostral lumbar segments. Interneuronal damage at the L4 segment resulted in hindlimb hyperextension and the abolition of locomotor patterns. Neither extensive training nor clonidine delivery could bring back stepping behavior after an L4 lesion, showing a more rostral extension of the CPG network than previously thought. This CPG model has further evolved through the incorporation of distinct classes of interneurons according to studies that explored interneuronal identification from a developmental and genetic standpoint

(Gosgnach, 2011; Jessell, 2000; Kiehn, 2011; Rybak et al., 2015; Talpalar et al., 2013; Y. Zhang et al., 2008).

### **Spontaneous Locomotor Recovery**

It has been extensively shown that the CPG network can be re-engaged after a spinal cord injury. Locomotor ability is conserved in mammals as shown in mice, rats and cats (Barbeau & Rossignol, 1987; Cai et al., 2006; Cha et al., 2007; De Leon et al., 1998b, 1998a; Edgerton et al., 1997, 2001; Fong et al., 2005; Lovely et al., 1986; Timoszyk et al., 2005). While the spinal cord retains stepping capabilities after removal of the supraspinal input, treadmill training is required for the cord to exhibit locomotor behavior in a well ordered and consistent manner. Treadmill training usually consist of reproducing the locomotor pattern over a moving treadmill, either manually or robotically. Evidence points to the idea that appropriate sensory feedback is required to train and modulate locomotor activity in the lumbar cord after SCI (Brownstone et al., 2015; Edgerton & Roy, 2009; Rossignol & Frigon, 2011). Similarly, it has been shown that the spinal cord possesses a certain level of plasticity after an injury since it can learn a different motor task than the ones it was trained on prior to transection (Hodgson 1994). In further support of this learning plasticity, it has been shown that the lumbar cord can be trained for specific tasks such as stepping or standing (De Leon et al., 1998b, 1998a; Edgerton et al., 1997; Hodgson et al., 1994). Mechanisms behind the effectiveness of training is thought to be the activation of spinal networks that are associated with the specific task (Edgerton et al., 2001). Training increases afferent efficiency and allows the circuitry to effectively integrate proprioceptive and cutaneous input resulting in the adaptive capacity to locomote at varying treadmill

speeds (Côté et al., 2003; De Leon et al., 1998b, 1998a; Edgerton et al., 1997; Lovely et al., 1986).

Even though studies have shown overall poor recovery in adult spinal cats, untrained control groups contain unusual variability in some cases and even outliers showing spontaneous recovery after a full thoracic transection (De Leon et al., 1998b; Eidelberg et al., 1980; Hodgson et al., 1994; Lovely et al., 1986). Similarly, the protocols for performing the transection vary across studies with differences in transection metrics such as the preservation of the dura matter, the amount of spinal retraction following the transection, and the preservation of the ventral spinal artery after transection. The specific question of spontaneous locomotor recovery in cats was investigated by DeLeon with a cohort of trained and untrained cats that were kept for up to 12 weeks after transection (De Leon et al., 1998b). In their untrained group of spinal cats (T12-T13), 1/9 animals was able to carry out weight-bearing treadmill locomotion at high walking speed (0.8m/s) at 5-weeks post transection and by the end of the experiment (12 weeks), 2/9 animals still displayed spontaneous locomotor recovery, walking at 0.6-0.8m/s treadmill speeds consistently. In a different study, the average top treadmill speed for the control group (untrained spinal cats), was 0.075 m/s at 4 weeks post transection and 0.2m/s at 7 months post transection (Lovely et al., 1986). The highest speed achieved by an untrained cat was 0.35m/s 7 months after transection. This study showed no evidence of spontaneous locomotor recovery since none of the 6 animals in the control group were able to perform at a speed of 0.4m/s at any time point over the course of 7 months after spinal injury. We have reported similar observations from studies carried out in our lab (Marchionne et al., 2020). In a cohort of 6 untrained spinal cats, only 1/6 animal was able to walk at 0.4m/s at 5 weeks post-transection. In

another study with a group of 3 untrained spinal cats, the results showed no instances of spontaneous recovery since the cats were not even able to carry out plantar weight bearing during treadmill locomotion at 0.2m/s (Boyce et al., 2007). Krupka et al. reported no spontaneous recovery, showing 0/2 untrained spinal cats could perform plantar weight bearing at 0.4m/s 5 weeks post-injury (Krupka et al. 2017).

However, other studies report different outcomes when assessing spontaneous locomotor recovery. In a study that aimed to assess the relevance of task-specific training in locomotor recovery, an entire control group composed of 4 untrained spinal cats showed the ability to spontaneously recover locomotor ability (Harnie et al., 2019). All 4 animals were able to perform weight-bearing locomotion at 0.4m/s and even reach maximal speeds of 0.8-1.0m/s. An earlier study also reported recovery of stepping in 6/6 cats by 6 weeks post transection tested at 0.4m/s and 0.6m/s (Eidelberg et al., 1980). While the bulk of the data presented in this study had the spinal cats being supported by a sling and showing limited standing and weight-bearing ability, animals were able to step with full weight-bearing once perineal stimulation was provided.

In addition to being divided in terms of locomotor outcomes, the studies can also be divided into two categories according to the extent of dural damage during spinal transection surgery. First, DeLeon et al. (1998b) followed spinal transection methods and care described by Roy et al. (1992) where additional damage to the dura is prevented by performing a longitudinal incision to access the spinal cord for transection. Similarly, Lovely et al. (1986) describe a similar transection procedure where the dura is largely preserved. In our lab, we employed a conservative transection method where an initial incision in the dura is sutured close after the spinal cord is transected (Boyce et al., 2007;

Krupka et al., 2017; Marchionne et al., 2020). These studies with limited dural damage show rare incidences spontaneous locomotor recovery. On the other hand, Eidelberg et al. (1980) report a more invasive approach where the dura is cut along with the spinal cord. Harnie et al. (2019) describe a similar approach where the dura is completely cut during spinal surgery and significant retraction of the spinal cord ends into the spinal canal is observed (~1 cm). These studies show a higher incidence of spontaneous locomotor recovery in untrained spinal adult cats.

The reports on untrained rats show a complete absence of any spontaneous locomotor recovery regardless of the surgical approach used to produce the spinal cord injury. Rats with a complete spinal cord transection, where the dura was transected, and studies with rats with severe contusions, where the dura is preserved, have been conducted and offer insights on the influence of the dura condition on the level of spontaneous locomotor recovery in that animal model. With both approaches, the BBB scores or kinematic measures showed a complete absence of locomotor performance (Moshonkina 2004, Lee 2010, DeLeon 2006, Kubasak 2008, Ichiyama 2009, Carvalho 2008, Heng and DeLeon 2009, Zhang 2007, Nothias 2005, Lenkhorst 2001). A review of the existing literature presented below showed that no similar studies exist for the feline model.

In order to compare recovery across multiple studies in the cat, the success criteria for a walking spinal animal was defined to be the following: consecutive stepping (10-20 steps) with plantar weight-bearing at a treadmill speed of 0.4m/s. The speed of 0.4m/s as a success threshold for walking locomotion in cats was derived from literature reports and justified from an energetics standpoint. Rossignol consistently used 0.4m/s to represent normal walking in the feline model when summarizing findings and advancements in the

field of neural control of limb movement during locomotion (Rossignol, 1996). Moreover, a study that quantified metabolic cost with percent of energy recovery showed the highest energy return/lowest metabolic cost for walking between 0.5-0.7m/s, implying that the open field locomotor speed of a cat would naturally fall in that range since cats show a poor adaptation for efficient locomotion at higher speeds since they've had no need to adapt for steady long-distance locomotion (Bishop et al., 2008). For our analysis, we decided to use 0.4m/s as a threshold for success when evaluating spontaneous locomotor recovery in untrained spinal cats in the literature (see Table 1). Statistical analysis (Fisher's Exact test,  $p < 0.001$ ) shows that the proportion of animals with spontaneous locomotor recovery is significantly higher in cats with a transected dura (3/26 preserved dura *versus* 10/10 transected dura). A summary of spontaneous recovery outcomes in adult spinal cats with a thoracic spinal injury is summarized in Table 1. The results of Table 1 show that there is a correlation between the development of spontaneous locomotor recovery in untrained spinal cats and the extent of dural damage during spinal transection surgery.

A different level of inflammatory response might result from varying levels of damage to the blood-spinal cord barrier (BSCB) and these differences would consequently change the cellular and molecular environment at the spinal cord. Inflammation has been shown to have a wide range of effects on functional recovery and it could be playing a role in either hindering or promoting locomotor recovery in the feline model (Alexander & Popovich, 2009; Donnelly & Popovich, 2008; Gensel et al., 2012; Kigerl et al., 2009; Novak & Koh, 2013; Torres-Espín et al., 2018). The present study is aimed at quantifying behavioral and molecular changes associated with a transected dura in untrained spinal cats with a thoracic spinal cord injury.

## **Inflammation after SCI**

There are two components of the pathophysiology underlying SCI. The first component called primary injury is a consequence of initial trauma to neural tissue and surrounding vasculature resulting from external mechanical forces. This results in acute cell dysfunction as well as cell death (Alizadeh et al., 2019; Dumont et al., 2001; Lambert et al., 2016). Secondary injury follows initial trauma and consists of the propagation of cell dysfunction through different cascading mechanisms including neuroinflammation, ischemia, and blood-CNS barrier dysfunction which have been shown to be heavily regulated by the elicited immune response (Chio et al., 2021; Lambert et al., 2016; Yates et al., 2019). The innate response to injury will recruit immune cells through cytokine signaling as a mechanism against injury and/or infection resulting in neuroinflammation (Bethea & Dietrich, 2002; Popovich & Jones, 2003) which in part ensures glial scar formation (Gadani et al., 2015; Popovich et al., 1997). Interleukin-1b (IL-1b), interleukin-6 (IL-6), and tumor necrosis factor alpha (TNF $\alpha$ ) have been identified to play a primary role in the complex inflammatory cascade that influences cellular dynamics (Garcia et al., 2016; Glaser et al., 2006). The upregulation of inflammatory cytokines triggers subsequent macrophage and microglia infiltration (Hellenbrand et al., 2021). Furthermore, with the use of flow cytometry, it has been confirmed that inflammation consists of a time-dependent multiphasic response where the initial phases are largely composed of neutrophils at 1-day after the initial injury, followed by an increase in microglia/macrophage population at 7-days and a peak in T-cells at 9 days post injury (Beck et al., 2010; Carlson et al., 1998; Popovich et al., 1997; Taoka et al., 1997).

Since macrophages and microglia are the primary responders to surround the fibrotic scar after injury and interface with remaining spinal tissue, the evidence points to them playing a key role in mediating the recovery. Macrophages play a critical role in priming the neuronal landscape by removing debris through phagocytosis and releasing a mix of pro-inflammatory and anti-inflammatory cytokines (Lee et al., 2006). Macrophages are known to take part in cases of maladaptive inflammation and axonal retraction where studies have shown that depleting peripheral macrophage populations after an injury improves recovery outcomes by (Horn et al., 2008; Popovich et al., 1999). Macrophage mediated effects on inflammation can harm the nervous system and hinder recovery since it is commonly associated with apoptotic factors and demyelination at the site of injury (N. Zhang et al., 2012). Inflammation in the lumbar spinal cord has also been shown to have adverse effects by inducing locomotor deficits that work against step training treatment (Jeffrey-Gauthier et al., 2021).

However, the therapeutic potential of macrophages has also been widely observed showing motor recovery along with increase in axonal growth (Gensel et al., 2009; Kigerl et al., 2009; Rapalino et al., 1998; Vallières et al., 2006). Macrophage activation through intraspinal zymosan injections, has been shown to result in a transient state where axonal growth was stimulated (Gensel et al., 2009). Similarly, the implantation of stimulated macrophages into a transected spinal cord promoted tissue repair and partial motor recovery reflected as increased plantar weight-bearing ability and hindlimb movement in rats (Rapalino et al., 1998).

So even though the mammalian nervous system quickly loses its ability to regenerate due to the maturation of the immune system (Mladinic & Wintzer, 2002; Tran et al., 2021), contributing to the notion that inflammation is largely maladaptive, inflammatory responses can positively affect the recovery outcome after an injury. The immune system can be leveraged to improve recovery outcomes by promoting the pro-regenerative aspects of neuroinflammation through selective activation and silencing of immune cells (Gensel et al., 2012; Kigerl et al., 2014). While resident immune cells are known to be involved in a variety of processes where the effects can range from harmful to beneficial (Gensel et al., 2012; Novak & Koh, 2013), the time window shortly after transection seems to be associated with enhanced plasticity and remodeling capabilities. Chen et al. showed that neurotrophin 3 (NT-3) overexpression increased plasticity and sprouting in spared corticospinal axons but only if expressed within the first 2 weeks after the injury (Chen et al., 2006). NT-3 did not have the same effect in the chronic stage of the injury. However, the plasticity window seems to be highly dependent on the inflammatory response. By manipulating the immune system and eliciting mild neuroinflammation in chronic stages of spinal injury, the window of plasticity can be reopened, enhancing the efficacy of training further showing the key role of the immune system in recovery (Torres-Espín et al., 2018).

Immune responses in the CNS are also heavily dependent on microglia, who were thought to be primarily or strictly involved in normal CNS tissue maintenance. However, this might be an oversimplified and outdated description (Nimmerjahn et al., 2005; Van Rossum & Hanisch, 2004; Vilhardt, 2005). Microglia can express a variety of receptors enabling them to respond to cytokine signaling from other cells. They have been shown to

have an active role in plasticity and maintenance of the CNS in adults. They do so through the release of cytokines and neurotrophic factors such as BDNF (Parkhurst et al., 2013; Tremblay & Majewska, 2011). Having a wide range of effects, microglia have the potential to radically change the cellular and molecular environment of the lumbar spinal cord. In particular, activated microglia have been known to promote neuronal excitability by causing a shift in the polarity of currents activated by GABA via BDNF signaling in peripheral nerve injury (Coull et al. 2003, 2005). The presence of endogenous BDNF linked to spinal microglia have also been shown to be present in response to spinal injury in rats (Boulenguez et al., 2010). Considering our own work that has shown that BDNF promotes locomotor recovery in the absence of training in spinalized felines (Boyce et al., 2007; Krupka et al., 2017; Marchionne et al., 2020; Ollivier-Lanvin et al., 2015) and the spontaneous locomotor recovery observed in association with dural transection (Eidelberg et al., 1980; Harnie et al., 2019), it is possible that dural damage is associated with increased inflammation and consequently higher levels of BDNF-producing microglia. However, it is also possible that dural damage is affecting macrophage population in a way that promotes the neuroprotective and regenerative potential of the inflammatory response. This work is aimed at addressing the inflammatory outcomes associated with extensive dural damage in the untrained spinal cat and explore the underlying mechanisms that could be responsible for the development of spontaneous locomotor recovery.

### **BDNF's roles in axonal growth & survival, synaptic plasticity and motor recovery**

Neurotrophins are a class of proteins that are paramount for nervous system communication and function. They were initially discovered in the field of neuroembryology during the search for target-specific factors for neuronal survival and

growth. Nerve growth was observed in neighboring neurons after tumor implantation in chick embryos, leading researchers to isolate the responsible protein called nerve growth factor (NGF) (Levi-Montalcini, 1987, 1992). Since then, it has been shown that there is a family of proteins, consisting of BDNF, NT3, NT4/5, and NGF, which are essential for neuronal development (Greene & Kaplan, 1995; Lewin & Barde, 1996). The effects of neurotrophins on the nervous system are mediated by interactions with high and low affinity receptors, Trk and p75, respectively. Through these receptor interactions, this family of proteins plays a critical role in neuronal survival, growth, differentiation, and repair. Of particular interest is BDNF, a primary player in synaptic modulation (Kang & Schuman, 1995; Lessmann et al., 1994; Lohof et al., 1993; Stoop & Poo, 1996). BDNF has been shown to effectively change the firing frequency of spontaneous synaptic currents (Stoop & Poo, 1995, 1996), which inevitably led the field to explore BDNF in the context of spinal cord injury for its therapeutic potential.

Brain-derived neurotrophic factor was the second protein from the neurotrophin family to be isolated. BDNF, in its mature form, is cleaved from pro-BDNF and has a high affinity for the receptor ligand-specific tropomyosin receptor kinase B (TrkB) and a lower affinity for the p75 neurotrophin receptor (p75NTR). Upon binding to TrkB, the receptor activates intracellular cascades that regulate synaptic efficacy, plasticity, cell survival, and neurite growth (Weishaupt et al., 2012). P75NTR can have effects ranging from neuronal differentiation to apoptotic cell death; however, it has a preferential binding affinity for pro-BDNF (Weishaupt et al., 2012). While BDNF works as a primary regulator of LTP and synaptic plasticity, spinal cord injury typically changes the molecular landscape and, thus, its behavior.

In the context of spinal cord injury, BDNF's ability to counteract the up-regulation of pro-apoptotic factors in axotomized neurons has been shown to aid in neuronal survival and the further restructuring of neuronal circuits (Novikova et al., 2000; Weishaupt et al., 2012). Significant neuroprotective effects of BDNF have been observed in experiments involving cervical lesions affecting rubrospinal projections and red nucleus neurons. For instance, axotomized rubrospinal neurons typically experience atrophy, with studies indicating up to 50% neuronal loss within the red nucleus by week five after injury, but BDNF infusion preserved 76% to 98% of the neuronal population depending on the delivery window (Novikova et al., 2000). BDNF treatment, when administered near cell bodies, has demonstrated the ability to rescue a substantial number of these neurons (Kobayashi et al., 1997; Kwon et al., 2007; Ruitenbergh et al., 2004). Various delivery methods, including genetically modified fibroblasts, viral vectors, and subarachnoid infusions, have yielded similar results (Kwon et al., 2007; Liu et al., 2002; Novikova et al., 2000; Tobias et al., 2003).

Adaptive neuronal rearrangements, known as plasticity, play a crucial role in rewiring neuronal networks and restoring function after spinal cord damage (Fouad & Tse, 2008; Onifer et al., 2011). BDNF is a potent promoter of neurite outgrowth and is thus useful for boosting axonal plasticity. Progress has been made in promoting sprouting of the CST through BDNF delivery. For example, BDNF treatment of motor cortex neurons led to sprouting of axotomized CST neurons into healthy tissue rostral to the injury but not into a peripheral nerve graft bridging a thoracic lesion (Hiebert et al., 2002). This result was corroborated by further studies, which also reported increased contacts between CST fibers and propriospinal interneurons above the lesion, correlating with improved horizontal

ladder walking performance (Vavrek et al., 2006). Cell body treatment with BDNF also facilitated substantial sprouting of the spared CST below a lesion (L. Zhou & Shine, 2003). This study combined BDNF-expressing viral vector injections into the motor cortex with NT-3 expression in lumbar motoneurons, leading to significantly enhanced axonal sprouting of the spared CST.

However, BDNF's effects are well known to extend beyond neuronal survival and plasticity. Following the observation of up-regulated TrkB mRNA in white matter and motor neurons after spinal cord injury (Frisén et al., 1992), which showed a correlation between observed axonal regeneration and neurotrophic activity, further studies confirmed BDNF's essential role in promoting neural regrowth in mature spinal tissue of adult rats after exogenous neurotrophic treatment (Bregman et al., 1997; Coumans et al., 2001). While neurotrophin administration has enhanced axonal regrowth within the transplant and the cord caudal to the lesion site, BDNF administration rarely results in axons growing past the injury site (Cao & Shoichet, 2003; Lu et al., 2004; Taylor et al., 2006).

Using different methods of delivery, BDNF has been explored as a therapy to increase locomotor capacity after a complete or partial spinal cord injury. In rats, intrathecal delivery of BDNF after a midthoracic transection resulted in spontaneous air stepping in unloaded hindlimbs as well as improvements in locomotor ability during open field testing (Jakeman et al. 1998). Axonal growth limited to the caudal portion of the cord after BDNF treatment was sufficient to result in locomotor improvements (Jakeman et al., 1998). Animals with incomplete spinal cord injuries show significant recovery of either hindlimb stepping or forelimb function when treated with BDNF following injury (Y. Jin et al., 2002; Liu et al., 1999, 2002; Sasaki et al., 2009). In the case of a complete transection, recovery

outcomes are maximized when neurotrophic treatment is paired with exercise training (Boyce et al., 2007); however, neurotrophic treatment alone is capable of increasing neuronal excitability caudal to the site of the lesion, resulting in comparable locomotor outcomes (Blits et al., 2003; Boyce et al., 2012; Jakeman et al., 1998; Nothias et al., 2005; Ziemińska et al., 2014).

In the SCI feline model, neurotrophin treatment has been explored as an alternative to exercise therapy after a spinal cord injury (Boyce et al., 2007, 2012; Krupka et al., 2017; Marchionne et al., 2020), based on the premise that the elevation of BDNF-gene expression associated with training can be mimicked through exogenous delivery (Côté et al., 2011; Gómez-Pinilla et al., 2001; MacIas et al., 2007). Although locomotor capacity was maximized when neurotrophic treatment was paired with exercise training (Boyce et al. 2007), NT3 or BDNF on their own have the potential to restore locomotor ability up to the full range of speeds tested prior to the transection (Ollivier-Lanvin et al. 2015). Moreover, cats were able to achieve the full range of speeds at an earlier time point when treated with neurotrophic factors alone than when paired with exercise training (Boyce et al., 2007). Locomotor outcomes were nonetheless the same by 5 weeks post-graft implantation (Boyce et al., 2007). The neurotrophic effects were further examined by Krupka et al. (2011), who showed that autologous grafts releasing BDNF into the spinal cord after injury could restore locomotor ability in untrained cats, regardless of when the grafts were implanted (acute, sub-chronic, or chronic stages of injury). Upon implantation, the timeline for recovery was the same, with speeds of up to 0.8 m/s achieved by 5 weeks post-implantation. Furthermore, a more controlled approach was taken by Marchionne et al. (2020), by delivering BDNF intrathecally using a programmable mini-pump. Intrathecal

delivery of BDNF resulted in full recovery of untrained spinal cats by 5 weeks post-injury, showing a promising approach for clinical translation that overcomes immune limitations associated with fibroblast implants and viral vector approaches, as well as the risks associated with those implantation surgeries. While BDNF has shown to be an effective treatment, it is not completely understood how it results in locomotor recovery in spinal animals.

Previous studies (Boyce et al., 2007; Krupka et al., 2017; Marchionne et al., 2020) as well as other explorations of BDNF delivery (Cao & Shoichet, 2003; Lu et al., 2004; Taylor et al., 2006) show no axonal growth through the injury site associated with functional recovery. In addition, studies have characterized synaptic efficacy changes in response to BDNF showing increased sensitization of lamina II neurons (Garraway et al., 2003) and modulation of short and long latency response of Ia afferent pathways (Bilchak et al., 2021; Garraway & Huie, 2016; Mendell et al., 2001). These results indicate that long-distance axonal sprouting is not responsible for untrained locomotor recovery in spinal animals. In a study of the effects of BDNF on locomotor recovery following spinal cord injury (Boyce et al., 2012), reduction of motoneuron rheobase and enhanced neuronal excitability was associated with increased c-fos staining in the intermediate zone. Increased intermediate zone activity, as suggested by c-fos staining results, indicates that there might be larger network dynamics at play in the intermediate zone that could help elucidate the relationship between BDNF and locomotor recovery.

## **Specific Aims**

### **Aim 1: Characterize lumbar interneurons activity during air-stepping in chronic spinal cats treated with intrathecal BDNF delivery to the lumbar spinal cord.**

BDNF has been shown to elicit locomotor recovery in spinal animals in the absence of locomotor training (Boyce et al., 2007, 2012; Jakeman et al., 1998; Krupka et al., 2017; Marchionne et al., 2020; Ziemlińska et al., 2014). However, the mechanism that leads to this behavioral outcome is not fully understood. While there have been studies that have explored some of the mechanisms behind BDNF in the spinal cord, they have been largely limited to intracellular or small-scale recordings (Boyce et al., 2012; Garraway et al., 2003; Mendell et al., 2001). Multiunit extracellular recordings could offer a better understanding of how BDNF affects mid-lumbar interneuron activity and how locomotor behavior correlates to the neural mechanism. The working hypothesis for this aim is that intrathecal BDNF delivery will result in significantly more neuronal activity in the L3-L4 segments during air-stepping. We will collect kinematic data during treadmill locomotion at different speeds (0.2-0.8 m/s) using a high-speed motion capture system (Vicon Motion Systems Inc., USA) to assess locomotor recovery. Following the last locomotor evaluation session, lumbar interneuronal firing will be evaluated during air-stepping using two 64-channel microelectrode arrays (Neuronexus, Ann Arbor, MI) inserted at the dorsal root entry zone to either ~3000 $\mu$ m or ~1500 $\mu$ m depth in two lumbar segments. Neural data will be recorded concurrently with the activity of 14 muscles of the right and left hindlimbs.

**Aim 2: Determine kinematic differences in locomotor recovery between spinal transection methods.**

It has been shown that training can reengage the lumbar spinal circuitry and recover body-weight support and locomotor function in cats, rats, and mice (Barbeau & Rossignol, 1987; Cai et al., 2006; Cha et al., 2007; De Leon et al., 1998b, 1998a; Edgerton et al., 1997, 2001; Fong et al., 2005; Lovely et al., 1986; Timoszyk et al., 2005). However, there has been disagreement on whether cats can recover locomotor stepping spontaneously or if treadmill training is required (Boyce et al., 2007; De Leon et al., 1998b; Eidelberg et al., 1980; Harnie et al., 2019; Lovely et al., 1986; Marchionne et al., 2020). This discrepancy may make cats an unreliable model in the field of SCI. By looking at the differences in locomotor recovery between cats with an open and closed dura, we are able to explore a potential factor responsible for the inconsistency within the model, with the potential to settle the disagreement over spontaneous recovery. The working hypothesis for this aim is that an open-dura transection (transecting the dura along with the cord) will show significantly better recovery during treadmill locomotion in untrained spinal cats. We will collect kinematic data during treadmill locomotion at different speeds (0.2-0.8 m/s) using high-speed motion capture systems (Vicon Motion Systems Inc., USA). This data will yield information such as stance length, hip height and step height which will enable us to quantitatively assess locomotor recovery. Similarly, we will assess weight-bearing ability during locomotion using our own integrated force-plate treadmill system. The trials will be processed using Vicon Nexus and analyzed using MATLAB.

**Aim 3: Determine differences in the inflammatory response produced at the lower thoracic cord between spinal transection methods.**

Microglia and macrophages carry out a dynamic role in the CNS by mediating recovery and plasticity after an injury (Gensel et al., 2009; Nimmerjahn et al., 2005; Torres-Espín et al., 2018; Van Rossum & Hanisch, 2004; Vilhardt, 2005). Consequently, they have the potential to set off a cascade of events that could critically change the environment near the site of injury, especially in response to the invasion of exogenous factors that result from the open-dura method. Not only can a different cellular and molecular environment change how pharmacological interventions interact with the neurons, but also how neurons themselves respond after a lesion. Exploring this aspect of injury gives us the opportunity to learn to leverage cellular and molecular changes in a way that could promote locomotor recovery in human SCI patients. The working hypothesis for this aim is that the open-dura method of transection will result in a higher inflammatory response, showing significant increases in macrophages, microglia, and BDNF caudal to the transection site. We will use immunohistochemistry (IHC) to stain for activated microglia and macrophages using calcium-binding protein Iba-1 near the site of transection in the perfused spinal cord of both study groups. In acute animals, we aim to verify microglia BDNF production using an RNA in-situ hybridization assay from ACD's RNAscope® technology, where we can target BDNF RNA at the lumbar and thoracic cord with high specificity and low background noise.

Table 1. Studies with untrained chronic spinal cats with a thoracic injury are divided according to dural damage described in their respective methodology. Third column shows the number of instances of weight bearing locomotion at 0.4m/s in cats with no treadmill training or any pharmacological intervention. Fourth column shows percentage of spontaneous recovery observed in each untrained spinal cat cohort. Spinal cats with a transected dura show a significantly higher proportion of cats with spontaneous locomotor recovery (3/26 preserved dura versus 10/10 transected dura (Fisher’s Exact test,  $p < 0.001$ )).

<b>Dura</b>	<b>Study</b>	<b># of cats with spontaneous recovery</b>	<b>Total # of spinal cats</b>	<b>Percentage of spontaneous recovery</b>
Preserved	DeLeon et al. 1998	2	9	22%
	Lovely et al. 1998	0	6	0%
	Boyce et al. 2007	0	3	0%
	Krupka et al. 2017	0	2	0%
	Marchionne et al. 2020	1	6	17%
Transected	Eidenberg et al. 1980	6	6	100%
	Harnie et al. 2019	4	4	100%

## CHAPTER 2

# INTRATHECAL DELIVERY OF BDNF TO THE LUMBAR SPINAL CORD MODULATES LUMBAR INTERNEURONS ACTIVITY IN A FELINE MODEL OF SPINAL CORD INJURY

### Introduction

A greater understanding of the neuronal activation patterns within the spinal cord during locomotion and the modifications to these patterns brought on by interventions would advance the development of therapeutic interventions to restore locomotor ability following SCI. In-vivo recordings of spinal extracellular signals have located the neural networks responsible for the control of locomotion in the intermediate zone and ventral horn of the lumbar enlargement (Antri et al., 2011; Edgley et al., 1988; Kjaerulff & Kiehn, 1996; Langlet et al., 2005; Norton & Mushahwar, 2010). Earlier reports (Butt et al., 2002; Deliagina et al., 1983; Kjaerulff & Kiehn, 1996; Langlet et al., 2005) have located the segments of the lumbar enlargement fundamental for rhythm generation of the hindlimbs at the L3-L5 spinal level in the adult cat. Consistent with these reports, previous studies conducted in our laboratory showed that a large proportion of the interneurons from L3-L7 are significantly tuned to the locomotor step cycle and are active during either the flexion or extension phases of air-stepping (AuYong et al., 2011; McMahon et al., 2023). The rhythmic limb movements generating capability of the spinal cord is maintained even in the absence of supraspinal input and this is demonstrated by the possibility of re-engaging the lumbar locomotor circuit following spinal cord injury via intensive body weight supported locomotor training (Bélanger et al., 1996; De Leon et al., 1999) or delivery of neurotrophins such as Brain Derived Neurotrophic Factor (BDNF) or Neurotrophin-3 (NT-

3) to the injury site via cellular grafts (Boyce et al., 2007; Krupka et al., 2017; Ollivier-Lanvin et al., 2015) or intrathecally to the lumbar spinal cord via implantable and programmable mini-pump (Marchionne et al., 2020).

Several studies have characterized synaptic efficacy changes in response to BDNF. It has been shown through slice preparation of lumbar spinal tissue, that neurons in lamina II show increased sensitization when treated with BDNF (Garraway et al., 2003). BDNF has also been shown to modify short and long latency responses of the Ia afferent pathways to motoneurons (Mendell et al., 2001) as well as other synaptic pathways (see Bilchak et al., 2021; Garraway & Huie, 2016; Stoop & Poo, 1996 for reviews). In a study of the effects of BDNF on locomotor recovery following spinal cord injury, Boyce et al. used adeno-associated virus (AAV) constructs to administer BDNF to the lesion site in the thoracic region (T10) after SCI. Using intracellular recordings from triceps surae motoneurons, it was shown that BDNF was associated with a significant reduction of motoneuron rheobase and enhanced neuronal excitability (Boyce et al., 2012). They also showed enhanced activity, as suggested by c-fos staining, in the intermediate zone of the lumbar cord, but the firing of the interneurons in the region was not explored. We explored the firing of those intermediate zone interneurons in our animal model and hypothesized that BDNF could be driving differences in locomotor network dynamics. We theorize that greater knowledge of these changes might help uncover the relationships between neuronal changes and complex behavioral outcomes such as locomotor recovery.

This study characterizes lumbar interneuron population activity in spinalized felines treated with intrathecal delivery of BDNF compared with the activity in animals receiving saline delivery during a terminal experiment involving locomotor activity. Multiunit

activity was obtained and the activity of isolated single units characterized by measures such as firing frequency, signal-to-noise ratio (SNR), and number of active units per trial were compared between the two groups. Preferred phase of firing was calculated for all units and used to classify the units' behavior exhibited during air stepping. In addition to single unit activity, power in multiunit activity was also calculated. Moreover, we used several quantitative measures to characterize changes in the interactions between simultaneously recorded spike trains. Cross-correlation was used to explore changes in correlation and synchrony of firing and peristimulus time histogram (PSTH) analysis to explore projections between units. We also used a point process generalized linear model approach to quantify the association between neural firing and relevant covariates such as self-history of firing and ensemble activity. Using these approaches, we address the differences in lumbar interneuronal firing between spinalized cats treated with intrathecal BDNF or saline during a locomotor behavior.

## **Materials and Methods**

### ***Animals and Experimental Procedures***

All animal care and procedures were performed according to the National Institute of Health guidelines, as well as to the USDA regulations for the use of felines in research and were approved by the Institutional Animal Care and Use Committee of Temple University. Six adult female domestic shorthair cats (Liberty Research Inc. Waverly, NY, weight: 2.2-3.6 Kg) underwent spinal transection at the T11/T12 thoracic level. In three cats, a 50 ng/day dose of BDNF (recombinant human BDNF (Abnova catalog #H00000627-P02), concentration: 1 µg/mL, flow rate: 2 µL/h) was delivered intrathecally to the lumbar spinal cord for 35 days post-transection through a programmable mini-pump

(iPrecio SMP-4200, MISZU, Tokyo) implanted subdermally. The remaining three animals underwent spinal transection and catheter/pump implant as well, but the pump was filled with 0.9% NaCl and those animals served as controls. Kinematic and ground reaction forces were evaluated at two-time points post-transection, 3 and 5 weeks after injury, during locomotion on a treadmill at a range of velocity between 0.3 and 0.8 m/s. Results corroborate the effectiveness of intrathecal BDNF delivery to the lumbar spinal cord to restore stepping in untrained spinalized animals (Marchionne et al., 2020). Following the last locomotor evaluation session, animals underwent a terminal experiment to evaluate lumbar interneuronal firing and EMG activity during air-stepping (Auyong et al., 2011; AuYong et al., 2011; McMahon et al., 2022, 2023). Once air-stepping was controllably inducible through perineal stimulation, lumbar grey extracellular signals were recorded, concurrently with the activity of 14 muscles of the right and left hindlimbs, using two 64 channels microelectrode arrays (model A8x8-5mm-200-200-177, Neuronexus, Ann Arbor, MI) inserted at the dorsal root entry zone to either  $\sim 3000\mu\text{m}$  or  $\sim 1500\mu\text{m}$  depth in two lumbar segments. The level of interneuronal activity for the two groups was assessed in terms of average firing rate, signal-to-noise ratio ( $\text{SNR}_{\text{spk}}$ ) (Joshua et al., 2007), and number of active units per air-stepping locomotor trial. The relative power in multiunit activity was also compared between groups. Relationships between spike trains were examined using cross correlation, peristimulus time histograms (PSTHs), and point process generalized linear models (PP-GLMs).

### ***Surgical Procedures at Terminal Experiment***

Cats were initially injected with atropine (0.05 mg/kg IM) and anesthetized with isoflurane (1.5-3.5% in oxygen) supplied through an endotracheal tube. Heart rate, blood

pressure, end-tidal CO<sub>2</sub>, tidal volume, arterial oxyhemoglobin saturation, respiration rate and temperature were monitored and recorded every 15 minutes. IV fluids enriched with Sodium Bicarbonate (3.4g/L) and Sucrose (25g/L) were administered throughout the experiment. Dexamethasone (2 mg/kg, IV) was given prior to the laminectomy in order to reduce spinal swelling. A spinal laminectomy was performed, and the cord was exposed at the lumbar level between the L3 and L7 spinal segments. Muscles of the hindlimb were exposed via small incisions and bifilar electrodes (AS 633; Cooner Wire, Chatsworth, CA) were implanted into the body of seven hindlimb muscles bilaterally: *soleus* (SOL -Ankle extensor), *gastrocnemius medialis* (MG - Ankle extensor), *tibialis anterior* (TA - Ankle flexor), *vastus lateralis* (VL - Knee extensor), *sartorius anterior* (SA – Hip flexor/ Knee extensor), *biceps femoris anterior* (BFA – Knee extensor), *biceps femoris posterior* (BFP – Knee flexor/Hip extensor). Electrodes were implanted into the body of the muscles and proper electrode placement was verified by stimulation of the electrode and observation of the resulting muscle's twitches.

Following laminectomy and EMGs electrodes implant, animals were transferred to a stereotaxic frame where the spinal vertebrae were securely clamped to the frame. The pia was opened in order to facilitate electrode insertion. A midcollicular decerebration was performed to discontinue isoflurane since it has been shown to disrupt spinal activity (Jinks et al., 2005).

### ***In-vivo Extracellular Neural and Muscle Activity Recordings and Processing***

One hour after decerebration, clonidine (500 µg/kg) was administered IV in order to induce air-stepping. Once air-stepping was controllably inducible through perineal stimulation, in-vivo recordings of spinal extracellular signals were conducted using two 64

channels microelectrode arrays (model A8x8-5mm-200-200-177, Neuronexus, Ann Arbor, MI) inserted at the dorsal root entry zone to either  $\sim 3,000\mu\text{m}$  or  $\sim 1,500\mu\text{m}$  depth in two lumbar segments. The planar 8 shaft arrays were inserted sagittally, i.e. in the rostrocaudal direction, so that the recording sites covered a range of  $1450\mu\text{m}$  rostrocaudally and  $1450\mu\text{m}$  dorsoventrally (from  $\sim 50-1,500\mu\text{m}$  or  $\sim 1,500-3,000\mu\text{m}$  deep) for each array. In the experiment, the rostral electrode array was statically placed at L3 while the caudal array was successively moved from L7 to L4. On some occasions the caudal array could not be installed at L4 while the rostral array was at L3. In those cases, the rostral array was moved to L4 and additional recordings with the caudal electrode moved from L7 to L5 were conducted. The walking trials at each location and depth involved 5 seconds resting period, followed by 50 seconds of air-stepping induced by perineal stimulation and a final 5 seconds period of rest (Figure 1- 1A).

Neural and muscles activity were recorded using the Tucker Davis Technologies RZ2/RS4 system with 128 channels capability for recording of multiunit activity (sampling rate 24 KHz) and 14 analog channels for EMGs activity (sampling rate 12 KHz). Extracellular and muscles activity were processed with customized Matlab scripts (The Mathworks, Natick, MA). EMGs voltages were amplified and band-pass filtered between 10-1000 Hz (Differential Amplifier Model 1700, A-M systems Inc., Carlsborg, Wa). We assessed the quality of stepping in terms of rhythmic left/right hindlimbs alternation and alternation of the flexor/extensor muscles during air-stepping trials. Raw multiunit data from the RS4 was first band-pass filtered between 300 Hz and 4000 Hz (sampling rate 24 KHz). Filtered data were then processed through the UltraMegaSort2000 Matlab toolkit,

which contains Matlab codes for spike sorting of extracellular neurophysiological data (Hill et al., 2012) (see Figure 1- 1B)

#### A. Single unit selection criteria

The units chosen had an average firing frequency greater than 1 Hz throughout the trial, a number of refractory period violations (RPVs) less than 5% for a refractory period of 1.5 msec and  $SNR_{nospk} > 1.5$  (Joshua et al., 2007). For the GLM models we tightened our criteria to only include units with an average firing frequency greater than 5 Hz to avoid sparse matrices of binned spike times used to construct the GLM.

#### I. Characteristics of single unit activity

Gross measures of neuronal activity were evaluated for each air-stepping trial. The number of active units per trial was noted along with the average firing frequency and amplitude ( $SNR_{spk}$ ) of those units for each trial and subject.

#### II. Identification of unit's preferred firing phase during air-stepping

To determine the preferred phase of activity of the units recorded, we first defined the locomotor cycle using EMG data from the right soleus as reference. The cycle was defined as starting on the onset of soleus EMG burst and ending on the next burst onset with cycle going from 0 to 1. Once the cycle was defined, we performed the circular statistics' Rayleigh Test of Uniformity to determine whether each unit was significantly tuned to a specific phase of gait. Units that did not pass the Rayleigh test with  $p < 0.05$  were classified as tonic.

Significantly tuned units were modeled using the Generalized Von Mises distribution (Gatto, 2008; Gatto & Jammalamadaka, 2007), resulting in the parameters ( $\mu_1, \mu_2, k_1, k_2$ ) used for quantitative assessment of each unit's behavior. Following the criteria listed in

(Gatto & Jammalamadaka, 2007), tuned units were subdivided into unimodal (single peak of activity), bimodal (two peaks of activity) or irregular (tuned, but without clear single or double peaks of activity). The proportions of each category (tonic, unimodal, bimodal and irregular) were compared across study groups.

The preferred phase of firing of unimodal units were plotted and the distributions were then compared using the Kolmogorov-Smirnov test of independence. The comparison was limited to unimodal units since there were not enough bimodal units in either group to construct a circular histogram and perform any analysis.

## B. Spike train analyses of neuronal interactions

To identify changes in neural interactions, recorded single unit spike trains were analyzed using cross correlation analysis, peristimulus time histograms (PSTHs), and point process generalized linear models (PP-GLMs).

### I. Cross correlation

Cross correlation analysis was used to probe differences in the short latency interactions between spike trains that may be present during air-stepping. The analysis was implemented using the MLIB Matlab toolbox for analyzing spike data (Stüttgen 2015). The cross correlation between every distinct pair of neurons was calculated within 1 ms of the reference neurons to quantify synchrony of firing present over the course of each trial (~60 seconds). The average of the cross correlation between every pair of neurons in a trial was calculated and used as a measure of synchrony and common drive for that trial. The trial averages were then compared between the control and BDNF-treated groups.

### II. Peristimulus time histograms (PSTHs)

We constructed PSTH between all simultaneously recorded units for each trial using the `mpsth` function of the MLIB Matlab toolbox (Stüttgen 2015). One spike was considered the trigger, and the responses of the paired spike were aligned with that “triggering” spike. For each PSTH we evaluated the presence of significant peaks or troughs within 5 msec of the “triggering” spike. Significance was determined if the firing frequency during that window went above or below the 95% confidence interval of the firing frequency of the unit calculated over the 15 msec preceding the “triggering” spike (Abeles, 1982). We extracted the percentage of the total number of units that each unit projected to with a significant peak (%peak projections) or trough (%trough projections) and averaged that percentage for all the units in a trial. We also extracted the average normalized magnitudes of the peaks (normalized to the 95% CI) ( $\text{mag}_{\text{peak}}$ ) and troughs (normalized to the 5% CI) ( $\text{mag}_{\text{trough}}$ ) for each trial. These values were established for all units recorded at the rostral and caudal locations, and between the units recorded from the caudal or rostral locations to investigate if neurons in closed proximity showed greater relationship between spike trains.

### III. Generalized Linear Models (GLMs)

Connectivity strength between spike trains was also evaluated using point process generalized linear models (PP-GLMs) implemented using the `nSTAT` Matlab toolbox (Cajigas et al., 2012). The software was developed to account for the three covariates that influence neural activity: extrinsic covariates such as sensory stimuli and behavior, the neuron’s past activity, and the activity of other neurons (Cajigas et al., 2012; Truccolo et al., 2005). In our application, we used a bin size of 5 ms to limit the number of bins with no spiking activity and only the neuron’s past activity and other neurons’ firing as

predictors (no sensory stimulus). We obtained good fit for our neuronal spike trains using a neuron self-history of four bins, i.e. going back 20 ms, an ensemble history of 1 bin, i.e. going back 5 ms, and a background activity term,  $\mu$ . In the analysis, we considered the values of the neuron's self-history and ensemble terms; we do not report the background activity term. The GLM model coefficients ( $\beta$ ) describe the extent to which spiking activity in a given neuron is related to concurrent ensemble spiking activity (ensemble terms) or prior firing activity (self-history terms). For each trial, we extracted the percentage of spike trains with significantly good fit (K-S test within 5% CI), the average value of the significant self-history terms, and the average values of the significant ensemble terms.

### C. Analysis of multiunit power spectral density

Multiunit activity during locomotion was analyzed for the trials showing sustained bouts of locomotor activity (see Figure 1- 1A). The multiunit activity during locomotion was normalized to the activity at rest taken from the same channel as the MUA during the period of air-stepping. The rest period was approximately 5 sec in all the trials and preceded the onset of locomotor activity induced by perineal stimulation.

Analysis was performed per subject initially, and then grouped across subjects according to treatment (saline or BDNF). Multi-taper spectral analysis was performed with a time-bandwidth product of 4 and 7 tapers and covered the frequency band from 0.5 to 50 Hz (Andrews et al., 2008; Bokil et al., 2007, 2010; Zeitler et al., 2006). To compare power across subjects, the air-stepping multiunit power spectrum was divided by the corresponding rest multiunit power spectrum per channel to give a normalized, unitless power spectrum (Burns et al., 2010) as shown in the following equation:

$$R(0.5 - 50 \text{ Hz}) = \frac{\text{Stepping MUA power spectrum (0.5 - 50 Hz)}}{\text{Rest MUA power spectrum (0.5 - 50 Hz)}}$$

We examined the mean and peak normalized power during each trial over 3 frequency bands: 0.5-3Hz, 3-6 Hz and 6-10Hz. These bands were chosen based on our prior reports detailing variations in spectral power between those bands during air-stepping in spinalized felines (McMahon et al., 2023).

### ***Statistical Analysis***

Processed data were transferred to SPSS for statistical evaluation. Statistical analyses were performed to evaluate differences in interneurons activity between the BDNF and Control groups. Linear mixed models with repeated measure variables were used to determine the effects of group and/or recording location on measures of neuronal activity.

#### **I. Single unit activity statistical comparisons**

Gross measures of neuronal activity such as number of active units per trial, and average firing frequency and amplitude ( $\text{SNR}_{\text{spk}}$ ) of those units were compared between groups using linear mixed models with the variable of interest as the dependent variables with group as factor and trial as repeated measure. Results for these analyses are reported in Figure 1-3.

We also assessed for potential differences in those measures between the rostral and caudal recording locations using linear mixed models with the number of active units per trial, average firing frequency or amplitude ( $\text{SNR}_{\text{spk}}$ ) of those units as independent variables and group (Control or BDNF treated) and electrode location (MUE1/rostral or MUE2/ caudal) as factors (along with the interaction term) with trial and location as

repeated variables. The differences at the recording locations were also evaluated for the individual cats using linear models with the variable of interest as dependent variable and recording location as factor and trial as a repeated measure. Results for both sets of analyses are reported in Figures. 1-3A-C and 1-3D-F.

The distribution of unit classifications across groups were examined by calculating the number of units in each category (unimodal, bimodal, tonic, irregular) and comparing the distribution of counts using Pearson's chi-squared test.

The distribution of preferred firing phase for unimodal units was compared between groups by construction a preferred phase histogram for each group. The histogram was constructed using the  $\mu$ -value of each neuron in a group. From each histogram, we calculated the cumulative distribution function and carried out the Kolmogorov-Smirnov test to find the likelihood of the distributions being drawn from the same population. Due to the limited number of bimodal units, the same testing could not be performed for those units. Results are reported in Figure 1-4.

## II. Neuronal Interactions statistical comparisons

The cross-correlation averages for each trial were compared using an independent samples t-test ( $\alpha=0.05$ ) to determine whether the samples were statistically different. In addition, the distribution of  $R^2$  trial averages were compared by constructing a histogram and calculating the CDF (cumulative distribution function) and performing the Kolmogorov-Smirnov test with significance level set to  $\alpha=0.05$ . Results for these analyses are reported in Figures 1-5A,B.

The PSTH results were analyzed using linear mixed models with the percentages of significant peaks or troughs/trial as repeated measures and group as the factor for unit pairs

from the same electrode (pairs within MUE1 or MUE2) and all unit pairs (ALL). The average normalized sizes of the peaks or trough per trial were analyzed using similar linear mixed models. Results for these analyses are reported in Figure 1-6.

The GLM results were analyzed using linear mixed models with the per trial average ensemble  $\beta$  and self-history  $\beta$  as dependent variables and group (Control or BDNF treated) as factor for each of the three set of GLM models (using all neurons, only neurons from the rostral electrode, and only neurons from the caudal electrode). Trial was considered a repeated measure in all linear mixed models. Results for these analyses are reported in Figure 1-7.

### III. Multiunit activity power spectra statistical comparisons

The mean and peak normalized power for the rostral (L3-L4) and caudal electrodes (L5-L6-L7) were compared between the two groups (saline vs BDNF) for each frequency bands: low (0.3-3Hz), medium (3-6Hz) and high (6-10Hz), using linear mixed models relating the mean or peak normalized power at each electrode position for each band with groups as factor and trials as repeated measured. Significance level was set at  $\alpha=.05$  for post-hoc comparisons of the estimated marginal means of the mean or peak power for each group at each of those electrode locations/frequency bands combinations. Results for these analyses are reported in Figure 1-8.

## **Results**

### *Summary*

Data from six spinal cats, three that received BDNF intrathecally for 5 weeks and three that received saline, were collected over 106 air-stepping trials (59 trials in BDNF treated cats, 47 trials in Control cats), with two 64 channels in use for each trial. All recordings

took place mediolaterally at the dorsal root entry zone of lumbar segments L3-L7 at a dorsoventral depth of 1500  $\mu\text{m}$  or 3000  $\mu\text{m}$  from the dorsal surface of the spinal cord. We observed spontaneous locomotor activity in the BDNF treated animals during terminal experiments, with two out of three BDNF treated cats spontaneously stepping during the session following clonidine injection while none of the saline treated or prior spinal animals in the laboratory (Auyong et al., 2011; McMahon et al., 2022) have exhibited spontaneous stepping. Both groups performed bouts of locomotion with nice left-right and flexor-extensor alternations of similar duration during perineal stimulation (average air-stepping bout duration/trial for BDNF treated animals:  $48.2 \pm 0.5$  sec (mean  $\pm$  SD), for saline treated animals:  $52.5 \pm 5.3$  sec (mean  $\pm$  SD), no significant differences, t-test,  $p:0.291$ ). The frequency of walking was slightly higher in control animals but not significantly ( $1.7 \pm 0.1\text{Hz}$  versus  $1.4 \pm 0.2\text{Hz}$  in BDNF group, average of the walking frequency over all steps for each cat, no significance difference, t-test  $p:0.103$ ,  $n=3$  in each group).

### ***BDNF leads to spontaneous locomotor activity in spinal cats***

Figure 1-2 shows alternating flexor/extensor and left/right hindlimb activity following clonidine injections but with no perineal stimulation applied in one of the BDNF treated animal. Two out of the three BDNF treated animals spontaneously locomoted following clonidine administration. In these cats that showed spontaneous locomotor air-stepping, we looked for periods of alternation in left/right and flexor/extensor muscle activity during the air-stepping trials. The muscular activity recorded showed alternation in the flexor/extensor activity within each hindlimb, and left/right alternation between the two hindlimbs. Although the flexor and extensor activity alternated, the agonist and antagonist muscles often co-contracted (Figure 1-2). These spontaneous bouts of locomotion often lasted over

50-60 seconds and we were able to record respectively 6 trials and 2 trials of spontaneous activity in the two BDNF treated cats that showed this phenomenon. We never observed spontaneous locomotion in the three Control cats, or in 13 previously studied untreated spinal animals (Auyong et al., 2011; McMahon et al., 2022). The difference between the 2/3 stepping BDNF-treated cats and 13/13 non-stepping controls is statistically significant (Fisher's Exact test,  $p: 0.025$ ).

### ***Changes in single unit activity following BDNF administration***

We observed some modest signs of higher interneuronal activity in the BDNF treated animals. The number of active units per trial was about the same in both groups (Estimated Marginal Mean (EMM) Control:  $45.7.0 \pm 5.2$  (SE) versus EMM BDNF:  $50.8.5 \pm 4.6$  (SE),  $p: 0.469$ ; Fig. 1-3A) but firing frequency was higher by about 27% in BDNF treated animals (EMM Control:  $18.6 \pm 0.9$  Hz (SE) versus EMM BDNF:  $23.6 \pm 0.8$  Hz (SE),  $p: 0.001$ ; Figure 1-3A). The average size of the units was also similar between both groups (EMM Control:  $1.9 \pm 1.1$  SNR<sub>spk</sub> (SE) versus EMM BDNF:  $1.8 \pm 1.1$  SNR<sub>spk</sub> (SE),  $p: 0.444$ ; Figure 1-3A).

Full factorial linear mixed models with group and MUA location as factors (trial and MUA location as repeated measures) showed significant effects for group ( $p: 0.000$ ) and the MUA location\*group interaction term ( $p: 0.003$ ) on the average firing frequency but the 95% confidence intervals (CI) of the EMMs for the MUA locations of the same group overlapped (see Figure 1-3C). MUA locations had a significant effect on size of the units with the SNR being about 15% larger at the rostral location (Figure 1-3D, MUA1 EMM  $1.98 \pm 0.05$  (SE) versus MUA2 EMM  $1.70 \pm 0.05$  (SE),  $p: 0.000$ ). Similar full factorial linear mixed model showed a significant effects for MUA location ( $p: 0.000$ ) and the MUA location\*group interaction term ( $p: 0.05$ ) on the number of units with the EMMs for the

number of units at the caudal electrode being significantly higher than at the rostral location, this effect was more marked for the Control group (no overlapped in the 95% CI of the two EMMs; EMMs' CIs overlapped for the BDNF group, see Figure 1-3B).

Results in individual animals only showed differences between rostral and caudal locations in the number of units per trial for two control animals (Figure 1-3E), with the number of units being lower at the rostral location in both animals (linear mixed model with trial as a repeated measure, and MUA location as the sole factor). Results for average firing frequencies per trial were mixed, decreasing at the caudal location for one BDNF treated animal, but increasing for another and for a Control animal (Figure 1-3F). Results for the average unit size per trial were more consistent, showing a decrease in the average units'  $SNR_{spk}$  at the caudal location for three animals, but an increase for one of the Control animals (Figure 1-3G).

### ***Proportion of units tuned to a specific phase during air-stepping in saline and BDNF-treated animals***

The classification of units according to behavior about the step cycle shows that there are differences in the proportions of some classes of neurons between the two groups. For the BDNF group, 1853 units were recorded and analyzed. The units were classified resulting in the following proportions: 75.45% of the units had a unimodal distribution, 2.59% of the units had a bimodal distribution, and 17.76% had an irregular distribution (Figure 1-4A left panel). The remaining 4.21% exhibited tonic behavior relative to the step cycle. For the control group, 1321 units were recorded with the following proportions: 82.29% had a unimodal distribution, 11.51% Irregular, 3.94% were Tonic, 2.27% Bimodal (Figure 1-4A right panel). The difference in class proportions was statistically significant

(Pearson's chi-squared,  $p < 0.001$ ). The major difference was between the unimodal and irregular categories. The Control group showed more unimodal units when compared to the BDNF group. On the other hand, the BDNF group showed more irregular units than in the Control group. However, bimodal and tonic classes showed no evident change in response to treatment.

We next examined the distribution of the preferred firing phase for the unimodal units relative to the step cycle. The BDNF group contained  $n=1398$  unimodal units and the control group contained  $n=1086$ . The preferred firing phase of each unit was compiled and is shown as a histogram that bins all unimodal units recorded for each group in Figure 1-4B. The extensor and flexor phase are labeled according to the average EMG phase of activity in a representative animal during air-stepping in order to correlate directional tuning to behavioral data. The control and BDNF distributions were compared using the KS test (Kolmogorov-Smirnov test) resulting in no statistical significance ( $p=0.985$ ). While there seems to be subtle differences, both histograms share similar distributions with peaks around the offset of flexion/onset of extension.

Changes in spike train interactions between the Control and BDNF groups  
Cross-Correlation Results: We extracted the average cross-correlation between units for 45 trials from the BDNF group and 37 trials from the Control group. Each trial had a range from 20 to 40 isolated units and was around 60 seconds in duration. The average correlation for each trial was calculated while excluding correlations between the same neuron and the resulting values were used to compare the two groups (Figure 1-5B). The group average for the BDNF-treated group was  $0.032 \pm 0.009$  (mean  $\pm$  SD) while the Control group

average was  $0.028 \pm 0.006$ . These results were compared using an independent samples t-test ( $\alpha=0.05$ ) and showed to be statistically different ( $p:0.01$ ).

In addition, the cross-correlation trial averages were binned into a histogram to visualize their distribution and examine differences between groups. The BDNF-treated group showed a distribution that skewed towards higher values when compared to the Control group's distribution (Figure 1-5A). The Kolmogorov-Smirnov test was used to determine whether the distributions were statistically different. The difference in distributions was shown to be statistically significant ( $p:0.012$ ). We observed that in the BDNF distribution, the bin containing the mean of the distribution contains a lower proportion of the units in that group. This seems to translate to an overall shift towards higher correlation values.

PSTH Results: Figure 1-6 shows the per trial averages of the percentages of significant peak (Figure 1-6A) or trough (Figure 1-6B) projections between pairs of units recorded at both rostral and caudal locations (MUE1 and MUE2). Linear mixed models with the percentages/trial as repeated measures and group as the factor for unit pairs from the same electrode (pairs within MUE1 or MUE2) and all unit pairs (ALL) showed no significant differences between groups (BDNF or Control) in the percentages of significant peaks or troughs between unit pairs. An overall linear mixed model including unit pair's recording locations (MUE1, MUE2 or ALL) and trial as repeated measures, with group, unit pair's recording locations and interaction terms showed significantly higher percentages of peak projections for units recorded across a single electrode (MUE1 and MUE2, Figure 1-6A) than across both electrodes. The normalized size of the peaks or troughs was neither significantly different between groups or between recording locations, see Figures 1-6C

and D (similar linear mixed models but with average normalized peak or trough magnitudes per trials as dependent variables).

Overall, the cross-correlation and PSTH results seem to indicate greater short term (~1 msec) latency synchrony between units, but no differences in excitatory or inhibitory connectivity between units for BDFN or saline treated animals when the latency period extends to about 5 msec. It should be noted that the detection of significant troughs is dependent on the unit's background firing rate and that low background firing rates may prevent the detection of some inhibitory connections between units.

GLM Results: We compared the within trial percentage of neurons whose GLM models were within the 95% confidence intervals of the Kolmogorov–Smirnov (KS) Goodness of Fit test. We found that a significantly larger percentage of the neurons firing could be explained by the neuron's past firing and that of the other neurons in BDNF treated animals, although that difference was relatively small. On average,  $22.87 \pm 10.53\%$  of the models per trial ( $n=46$  trial) passed the KS test in BDNF treated animals *versus*  $19.06 \pm 8.11\%$  ( $n=55$ ) in saline treated animals ( $t$ -test at  $\alpha=0.05$ ).

The values of the betas for the GLM models, which modeled the effects of the neuron's past firing (self-history  $\beta$ ) for latencies up to 20 msec, and effects from other neurons (ensemble  $\beta$ ) for latencies up to 5 msec, showed similar results to the PSTH analyses about differences in interneuronal connectivity strength between the two groups. The average value of the ensemble  $\beta$ s significantly different than zero was higher for units recorded within an electrode than for the ensemble  $\beta$ s of the models constructed with units from both rostral and caudal electrodes (Figure 1-7A, only models that passed the KS Goodness of Fit test were considered). The ensemble  $\beta$ s were actually significantly higher for the

Control group for units recorded within the rostral region (MUE1), but not significantly different for the ensemble  $\beta$ s of the units recorded caudally (MUE2) or across both electrodes (ALL).

The self-history terms showed the greatest shift in values for GLM models constructed using all units with the average self-history  $\beta$ s actually being negative for the BDNF treated animals, indicative of a lower probability of repetitive firing of the lumbar interneurons in those animals. For models looking only at interactions between units within a segment we found no significant difference in the average self-history  $\beta$ s between the BDNF and Control animals (Figure 1-7B). Overall, the results suggest that the past firing history of the interneurons had similar effects on the firing of the neurons for both groups; when all units were considered together interneurons in BDNF treated animals tended to be more inhibited by past firing.

### ***Changes in power spectra density following BDNF administration***

The average and 95% CI of the relative power spectra during air-stepping (normalized to the power at rest) over all the locomotor trials is shown in Figure 1-8 for the BDNF treated and Control animals. As seen previously (McMahon et al., 2023), we saw peaks of activity around 1 Hz, 3Hz and 6Hz, with the peaks at the higher frequencies more prominent in control animals. We looked at both the mean and peak power in the multiunit activity during locomotor bouts between the two groups over three frequency bands: low 0.5-3 Hz; mid 3-6 Hz; and high 6-10 Hz. Statistical analyses confirmed our visual impressions, with the peak power in the mid and high frequency bands higher in the Control group than in BDNF treated animals, but only for the rostral (L3-L4) region of the lumbar

cord (Figure 1-8B). The mean power showed no significant differences between BDNF and Control groups in any of the frequency bands or lumbar regions (data not shown).

## **Discussion**

This study explored some of the changes in neuronal population firing activity in spinalized animals that showed treadmill locomotor recovery following lumbar delivery of BDNF (Marchionne et al., 2020). By carrying out a wide range of analysis, we have been able to identify some differences in the patterns of neuronal activity in those animals compared to the activity in spinalized animals receiving lumbar delivery of saline.

Two out of the three cats treated with BDNF initiated air-stepping with no perineal stimulation following clonidine injection during the terminal recordings. Even though clonidine facilitates the locomotor behavior, it must be initiated through perineal stimulation in untrained spinalized animals. This spontaneous behavior seems to indicate a higher level of excitability (baseline) induced by BDNF where the resulting state of the neural population primes the spinal cord for locomotor activity such that clonidine alone was able to push the population past the initiation threshold and result in the stepping behavior.

While we initially expected radical differences in neuronal firing between groups due to the extent of the behavioral differences, our data instead suggests that BDNF affects certain neuronal firing parameters without much reorganization of the overall pattern of neuronal behavior and connectivity. In terms of single unit activity, we found that BDNF is associated with increased firing frequency. In addition, a higher proportion of the units recorded in the BDNF-treated group displayed irregular behavior. This class of units displayed statistically significant tuning (Rayleigh test,  $p < 0.05$ ) relative to the step cycle

but did not meet the criteria for unimodal or bimodal tuning. In terms of relationships between spike trains, we found higher average cross correlation as well as a skewing towards higher  $R^2$  values when examining the distribution of the BDNF-treated group. In addition, spectral analysis of multiunit activity showed that BDNF is associated with a drop in the higher frequency peaks of multiunit power.

### ***Single unit activity changes***

Firing frequency was the gross neuronal behavior measure most prominently affected, showing a significant increase for animals treated with BDNF. However, the number of active units per trial and the amplitude of the spikes did not change with BDNF delivery. These results suggest that BDNF is not recruiting more neurons or changing the size of the units recruited. Instead, it seems to set the population in a state of high excitability where firing is facilitated. This response in firing frequency would be consistent with increases in synaptic efficacy associated with BDNF (Garraway et al., 2003) and with observations of increased excitability in lumbar spinal networks and decreased KCC2 expression in the ventral horns following an injury (Ziemlińska et al., 2014).

When looking at how neurons behaved relative to the air-stepping cycle, we found that the vast majority of units had a single preferred phase of firing (unimodal) but there were differences in the number of neurons present in certain classes. More specifically, BDNF-treated animals had a higher proportion of irregular units while the Control group had a higher proportion of unimodal units. Irregular is the label placed on units that were significantly tuned to the step cycle (as opposed to tonic units who fired randomly throughout the cycle) but did not meet the criteria to be classified as unimodal or bimodal. While it is hard to pinpoint the activity patterns involved in this class of neurons, we can

infer that the pattern of activity is not totally random but does not strictly correlate to the step cycle as a unimodal or bimodal unit. It has been shown that in the presence of rhythmic motor output, there are populations of cells that fire at irregular intervals often labeled as ‘fluctuation-driven’ which have a supralinear input output curve associated with increased firing sensitivity (Petersen & Berg, 2016). The presence of irregular spiking is thought to be an indicator of concurrent E/I (Petersen et al., 2014). Concurrent or ‘balanced’ E/I is a combination of excitatory and inhibitory inputs whose integrated response is thought to stabilize the network from reverberating excitation (Berg et al., 2019; Petersen et al., 2014). Perhaps the higher occurrence of irregular spiking associated with BDNF-treatment shown in this study is a byproduct of BDNF-induced stability in the network. The proportions of bimodal or tonic neurons were similar between the two groups, but we noted that bimodal or tonic units were scarce (<5%), limiting our ability to perform any analysis with any degree of confidence. The distribution of the preferred firing phase of unimodal units was similar across groups. Unimodal units were tuned to the late flexor and early extensor portion of the gait cycle regardless of treatment, as seen in our prior studies (AuYong et al., 2011; McMahon et al., 2022) with untreated spinal animals.

### ***Spike train connectivity changes***

Since it has been shown that BDNF can promote axonal sprouting (Blits et al., 2003; Schnell et al., 1994), we expected some changes in spike trains connectivity in BDNF-treated animals. As expected, units grouped according to recording sites showed higher connectivity due to proximity. When examining the PSTH results such as % peak projection and % peak trough, there was no evidence to suggest any changes in connectivity across groups. The GLM results revealed that BDNF-treated neurons showed lower

dependence on self-history of firing when compared to neurons from the Control animals. Lower self-history  $\beta$  would indicate that self-history of firing is a poor predictor of firing in the BDNF-treated populations and in turn, that ensemble  $\beta$ s dominate the model's behavior. We interpret this as to indicate that neuronal spiking in BDNF-treated animals is better predicted by the ensemble activity than in Control animals.

We reason that the self-history  $\beta$ s are low in the BDNF-treated group only when electrodes are binned together and not when considered individually due to sample size and local interactions obscuring the larger group dynamics at play. In further support of this interpretation of the GLM results, cross-correlation results suggest that the BDNF-treated neuronal population displayed more synchronous and ensemble-like behavior. The cross-correlation results showed that on average, the BDNF-treated neuronal populations fired together more than the populations from the Control group. In addition to higher synchrony, the histograms of average cross-correlation/trial showed that the BDNF-treated neuronal population skewed towards higher  $R^2$  values between neuron pairs. This increase in synchrony and ensemble-dependent behavior during rhythmic motor output would be consistent with observations of in-phase variations of synaptic excitation and inhibition during scratch-like behavior (Berg et al., 2007; McPherson & Bandres, 2021). This would suggest BDNF treatment resulted in more balance between excitation and inhibition (E/I). Although behavior did not differ once facilitated with clonidine during terminal recording, it could be a set of observations that inform the default state of the network that is associated with facilitated stepping and spontaneous locomotor recovery observed in the BDNF group prior to terminal recordings.

### ***Multiunit activity changes***

When performing power spectral analysis, we observed a significant difference in the 3-6Hz and 6-10Hz frequency bands when comparing MUA activity at the L3-L4 sites between the BDNF and saline treated animals. The resulting analysis suggests that at the higher lumbar segments, BDNF is associated with a relative taming of the peaks in power apparent in the medium to higher frequencies of the neuronal population of the Control group. It is possible that BDNF plays direct or indirect roles in stabilizing the network such that it concentrates power on the lower frequency band more closely associated with locomotion. Bipedal locomotor models have suggested that locomotion occurs via synchronization of distributed pattern generators to the body's dynamics mode (Pitti et al., 2009). The concentration of power in the lower frequency band in BDNF treated animals may be a neural representation of this hypothesized model of movement generation from distributed pattern generators.

### ***Clonidine's confounding effects on neural firing and connectivity during multiunit recordings***

Clonidine is an  $\alpha$ -2 noradrenergic agonist known to engage locomotion after spinal cord injury (Barbeau & Rossignol, 1987; Chau et al., 1998; Pearson & Rossignol, 1991) and modify afferent pathways. In untrained spinalized cats, clonidine injection has been shown to reduce group I disynaptic inhibition (by about 30%) from several nerve pathways to extensor motoneurons, while the monosynaptic group I responses were not affected. Polysynaptic group I excitation to extensor motoneurons went up by as much as 500% with clonidine (Côté et al., 2003), while responses to cutaneous pathways were quite variables, with a mix of increase or decrease in the initial motoneuronal depolarization (R1 response),

and typical decrease in the longer latency hyperpolarization (R2) of extensor motoneurons, with no effects noted for flexor motoneurons (Côté & Gossard, 2004). More recent studies in untrained spinalized cats using stretch of the ipsilateral triceps surae or contralateral stimulation of afferent pathways (most notably the tibial nerve) confirmed a minimal effect of clonidine on the stretch reflex, but enhancement in the cross-extensor reflex's activation of contralateral extensors and a facilitation to initiate rhythmic behavior of both flexors and extensors with stretch or afferent stimulation (Frigon et al., 2012).

BDNF expression following various mode of delivery (Boyce et al., 2012; Krupka et al., 2017; Marchionne et al., 2020), exercise (Gómez-Pinilla et al., 2001), osmotic pumps (Jakeman et al., 1998) increases in motoneurons and muscles, with recent reports indicating a potentially greater effects on extensors muscles due to better survival of TrkB receptors on those motoneurons following spinal injury (Głowacka et al., 2022). Increases in neural activity, as suggested by c-fos staining, is actually higher in the intermediate zone of higher lumbar segments than in the ventral horn or more caudal lumbar segments (Boyce et al., 2012).

Taken together the results suggest that, at the lumbar intermediate zone explored in this study, clonidine and BDNF increase the excitability of neurons related to either the flexor or extensor phases. The behavioral effect observed following clonidine injection suggests that BDNF alone increases the level of excitability of the locomotor circuitry and that the added effect of clonidine is then sufficient to elicit locomotor behavior without perineal stimulation. The absence of locomotor behavior (or at least sustained bouts of locomotion) in spinalized animals without clonidine prevented us to explore the relative contribution of clonidine or BDNF to the spinal excitability following spinal cord injury. The combination

of both agents is likely non-linear and the increase in excitability may plateau. The lack of a spontaneous locomotor behavior in untrained spinalized animals at various concentrations of clonidine suggests that BDNF may be acting by engaging interneuronal group not susceptible to clonidine.

The distribution of  $\alpha$ -2 noradrenergic (clonidine) and TrkB (BDNF) receptors in the spinal cord supports the idea that different interneuronal populations may be at play. In spinalized cats,  $\alpha$ -2 noradrenergic receptors were found predominantly in the dorsal horns (laminae II, III and IV) and around the central canal (lamina X) (Giroux et al., 1999). In the rat, TrkB immunoreactivity is predominant in laminae I-III and in lamina IX (MacIas et al., 2007), and observed on oligodendrocytes (Skup et al., 2002) and astrocytes as well as neurons. Thus, BDNF may have effects on some interneurons others than clonidine and it might be changes in the firing of those interneuronal population that we observed.

### ***Further recordings needed***

The interneuronal firing differences observed were limited and longitudinal chronic recordings in spinalized untrained, trained, and BDNF-treated animals locomoting on a treadmill would provide a clearer picture of the changes in the neural network associated with actual weight-supported stepping. Despite advances in neural recording technology, those recordings are unfortunately still not available. Our evidence suggests that BDNF has effects on neuronal firing and short latency connectivity, and is priming the spinal network for an efficient integration of cutaneous and proprioceptive input, resulting in the exhibited treadmill locomotor behavior after SCI.

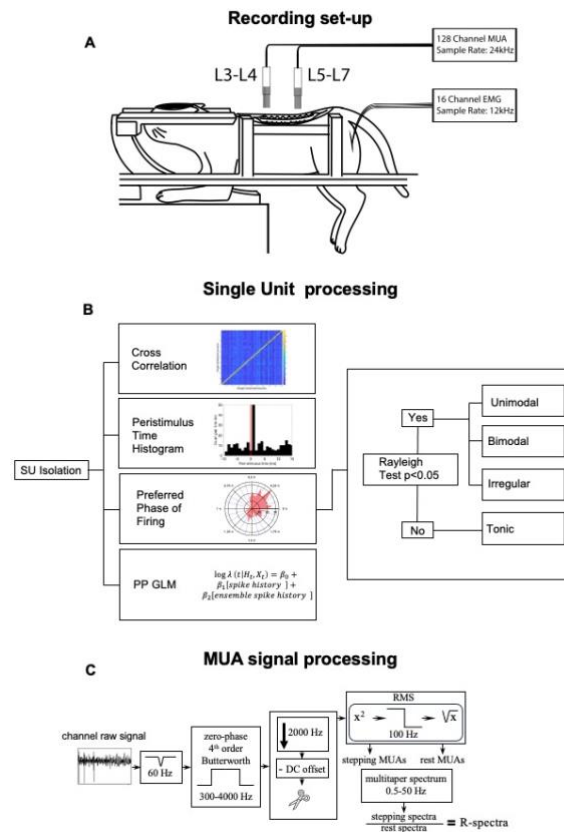


Figure 1-1. Terminal experimental setup. A) The terminal recording set up consisted of two 64 channels microelectrode arrays inserted at the dorsal root entry zone of the L3-L7 lumbar roots of cats sampled at 24kHz and 14 analog channels for EMGs activity sampled at 12 kHz. B) Single units were processed using cross correlation analysis, peristimulus time histogram, preferred phase of firing, and point process generalized linear model (PP-GLM) fitting. Preferred phase of firing was further used to classify units as unimodal, bimodal, irregular, and tonic (see text for details). C) Multiunit activity was filtered and processed, and the stepping spectra was normalized by the spectra at rest to obtain a normalized multiunit activity spectra that was then compared between groups (see text for details).

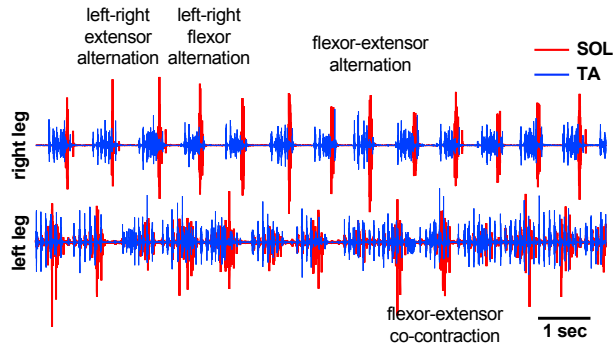


Figure 1-2. Spontaneous locomotor activity in spinalized cats that received BDNF intrathecally for 5 weeks post-transection. The stepping activity was observed in 2/3 BDNF treated animals and showed alternation between the flexor and extensor muscles (*tibialis anterior* (TA) and *soleus* (SOL) shown) activity within a hindlimb and left-right alternation between the two hindlimbs. The locomotion activity also showed periods of co-contraction between flexors and extensors as seen at the end of the locomotor bout pictured.

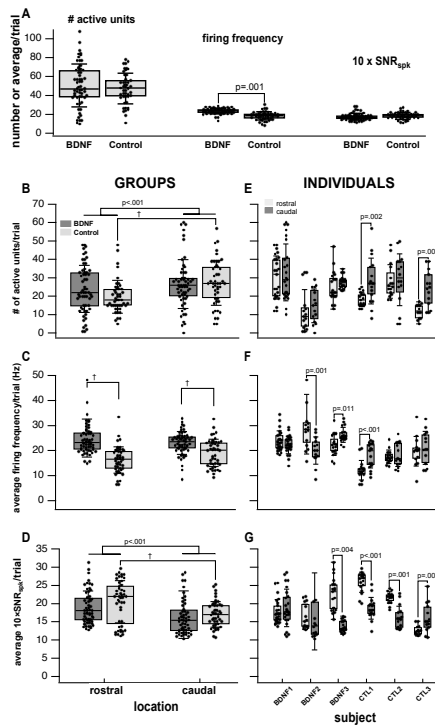


Figure 1-3. Interneuron activity during stepping trials. A) Number of active units per trial (left), average firing frequencies of those units during the locomotor period (middle), and scaled (factor of 10) average SNR<sub>spk</sub> of the active units for each trial (right). Average firing frequency was significantly higher ( $p=.001$ ) in the BDNF treated animals. B) Number of active units per trial for each group at the rostral and caudal electrode locations. C) Same for the average firing frequency of the active units. D) and for the scaled average of the SNR<sub>spk</sub> for the active units. The number of active units was higher at the more caudal locations (L5-L7) for both groups, while average firing frequency of the units active was higher for BDNF treated animals for both electrode locations, and units tended to have larger spikes at the more rostral (L3-L4) locations. E) Number of active units per trial for each individual animals at the rostral (lighter gray) and caudal (darker gray) electrode locations. F) Same as in E but for the average firing frequency of the active units. G) and for the scaled average of the SNR<sub>spk</sub> of the active units. The number of active units per trial showed greater variability for BDNF treated animals (see BDNF1 and BDNF3 *versus*

BDNF2) compared to Controls, while the variability in the other variables between individuals was not as pronounced. Whiskers on the bar graphs indicate the  $\pm 1$  standard deviation. Bars between boxes indicate significant differences between EMMs of the linear mix models, while a cross indicate no overlap in the 98% CI of the EMMs.

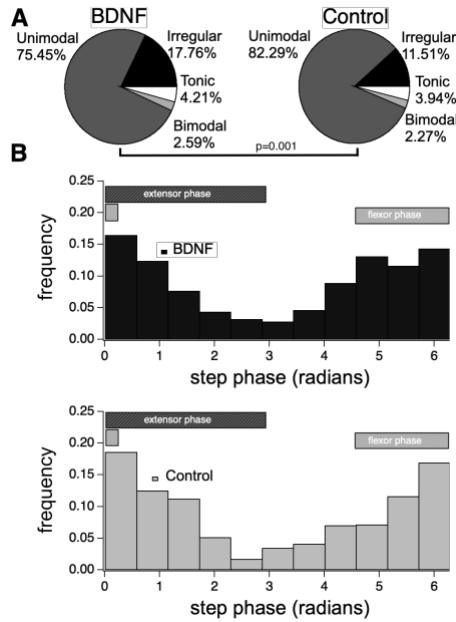


Figure 1-4. The distribution of classes of neurons according to firing behavior during the step cycle. A) On the left panel, the unit class proportions present in the BDNF-treated group. On the right panel, the proportions found in the control group. The units in the BDNF-treated group (n=1853) and control groups (n=1321) showed slight differences in the proportions of classes about the step cycle (Pearson's chi-squared,  $p<0.001$ ). BDNF-treated group showed a higher proportion of irregular units while the control group showed a higher proportion of unimodal units. B) The distribution of preferred firing phase for unimodally tuned units from the onset of right soleus extension to the next. Top panel contains units recorded from the BDNF-treated animals and bottom panel shows units in control animals.

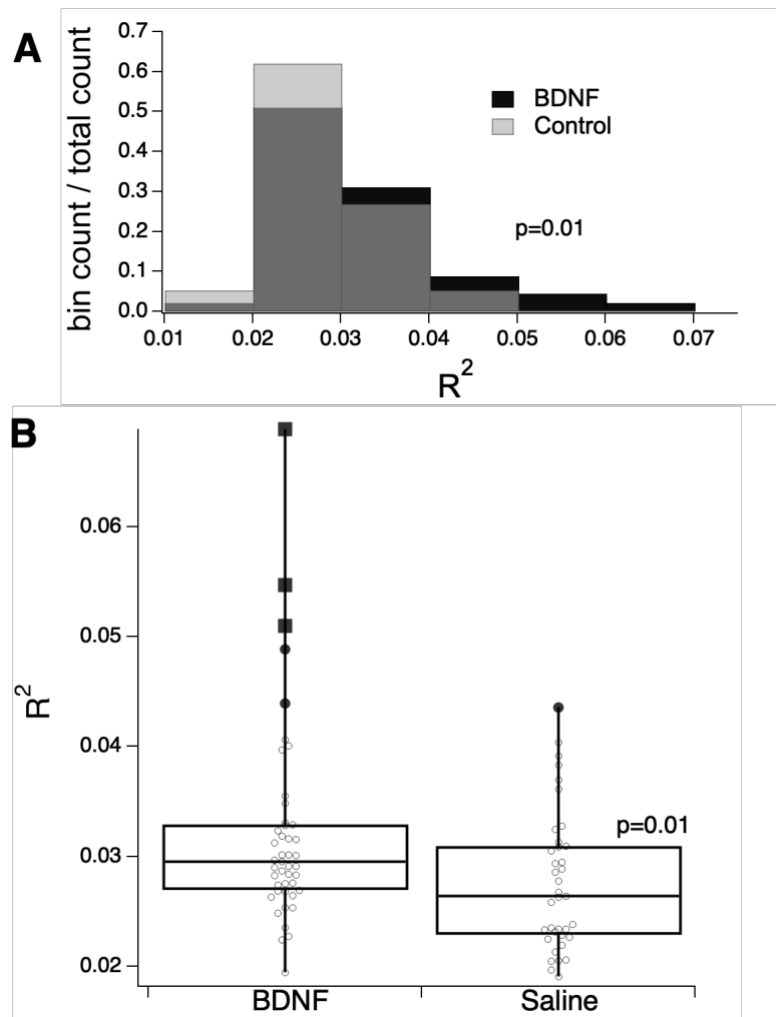


Figure 1-5. Average cross-correlation between units within a trial. A) Histogram overlay showing the differences between the distributions of trial average  $R^2$  between BDNF (black) and Control (grey) groups. Every  $R^2$  value represented in the histogram is the calculated average of  $R^2$  values resulting from very distinct pair of neurons for each trial. B) Box plot showing all the  $R^2$  trial averages along with the group mean and SD for BDNF and Control groups.

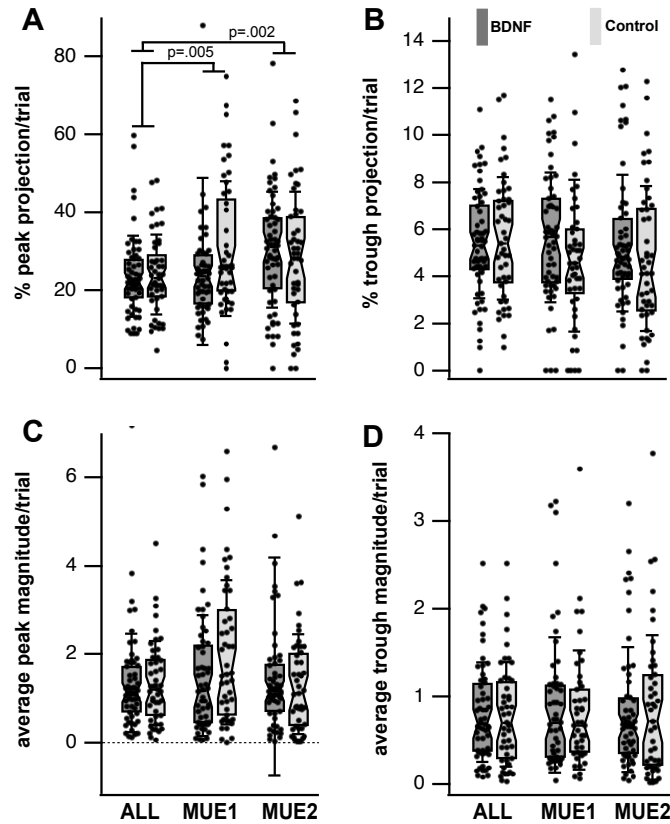


Figure 1-6. Peristimulus time histogram analysis results. A) Averages per trial of the percentages of significant peak projections between all pairs of units recorded at both rostral and caudal locations (ALL), or between pairs of units at the rostral location (MUE1) or caudal location (MUE2). B) Trial averages of significant trough projections. C) Averages per trial of the normalized magnitude of the peaks between pairs of units recorded at both rostral and caudal locations (ALL), or between pairs of units at the rostral location (MUE1) or caudal location (MUE2). D) Same as in C but for the magnitude of the troughs. The number of significant peak projections was higher within an electrode ( $p:0.005$  and  $p:0.002$ , see panel A), but no other differences proved significant and 95% CI of the EMMs overlapped in between groups and locations in all cases.

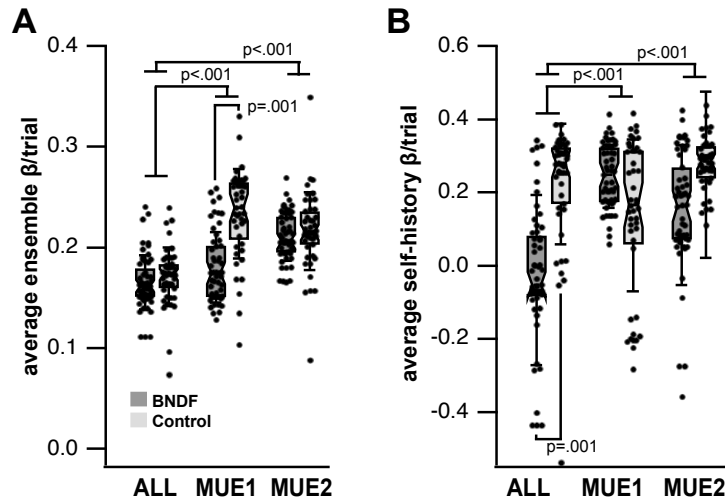


Figure 1-7. Generalized linear model average  $\beta$ s per trial. A) Average value of the ensemble  $\beta$ s per trial for GLM models including all units recorded within a trial (ALL) or models only using the units recorded at the rostral location (MUE1) or caudal location (MUE2). Ensemble  $\beta$ s were significantly greater for units in close proximity, but not different between groups, except for units at the rostral location where average ensemble  $\beta$ s per trial were significantly larger in the Control group. B) Average value of the self-history  $\beta$ s for GLM models including all units recorded within a trial (ALL) or models only using the units recorded at the rostral location (MUE1) or caudal location (MUE2). The self-history  $\beta$ s were again significantly greater for units in close proximity (i.e. within an electrode region), and larger in Control animals for models using all units recorded per trial. Bars between columns indicate significant differences. Ns for average ensemble  $\beta$ s are n=55 trials for all units within a trial, 53 for units within MUE1, and 54 for units within MUE2 for the BDNF group; for the Control group the numbers are 45, 44, and 46 for all units within a trial, units within MUE1, and units within MUE2.

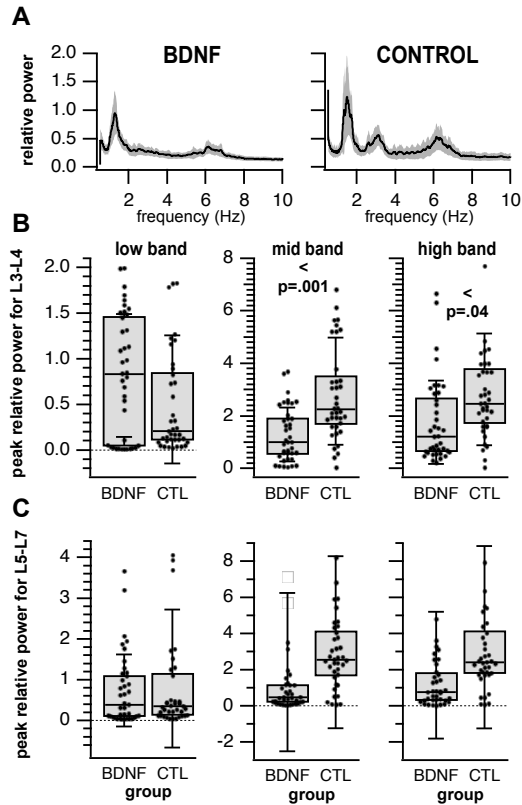


Figure 1-8. Power spectrum of multiunit activity. A) Average (across all trials) and 95% CI of multiunit activity power spectrum for the BDNF treated animals (left) and Control (right). Control animals showed more significant peaks around 3 and 6 Hz compared to BDNF treated animals who only showed a significant peak around 1Hz. The low frequency peak in BDNF treated animals occurred around 1.3Hz (average walking frequency of 1.4Hz), and around 1.5Hz in Control animals (walking frequency of 1.7Hz). B) Peak relative power within the L3-L4 lumbar segments compared between the two groups for 3 frequency bands (low 0.5-3Hz; mid 3-6Hz; and high 6-10Hz). C) Same as in B but for the L5-L7 multiunit activity. Peak relative power was significantly higher in the mid and high frequency bands in Control animals at the more rostral spinal segments. No significant differences were seen at the more caudal segments. n:52 trials for BDNF group, and n:43 trials for Control group.

## CHAPTER 3

# DURA REPAIR CHANGES MACROPHAGE/MICROGLIA POPULATION LEVELS AT THE INJURY SITE AND INFLUENCES LOCOMOTOR RECOVERY AFTER SCI

### Introduction

Spontaneous locomotor recovery has been investigated by multiple studies in early spinal cord literature where it was observed that, while possible, it is rare for cats to spontaneously develop locomotor capability after injury without any training (De Leon et al., 1998b; Eidelberg et al., 1980; Hodgson et al., 1994; Lovely et al., 1986). While the studies included some instances of cats stepping at 0.4-0.6m/s with no prior training, the vast majority of the control animals were unable to perform any locomotor stepping, leading to the consensus that spontaneous locomotor recovery does not occur in this animal model after spinal cord injury (SCI). Another consensus that emerged was that upon a SCI, the spinal cord's capacity for locomotion remains and can be trained even in the total absence of supraspinal commands (Barbeau & Rossignol, 1987; De Leon et al., 1999; Hodgson et al., 1994; Lovely et al., 1986, 1990). It has been extensively shown that hindlimb locomotion can be restored after SCI through intensive treadmill training in mice, rats and cats (Barbeau & Rossignol, 1987; Cai et al., 2006; Cha et al., 2007; De Leon et al., 1998b, 1998a; Edgerton et al., 1997, 2001; Fong et al., 2005; Lovely et al., 1986; Timoszyk et al., 2005). Training usually consists of reproducing locomotor stepping patterns over a moving treadmill, either manually or robotically. The ability to engage in locomotor behavior after SCI is largely due to the central pattern generators (CPGs), a network of neurons capable of producing a rhythmic output independently from the brain

(Grillner, 1981; Grillner & Zangger, 1979; McCrea & Rybak, 2008). Furthermore, evidence shows that appropriate sensory feedback is required to train and modulate locomotor activity in the lumbar cord after SCI (Brownstone et al., 2015; Edgerton & Roy, 2009). In further support of the spinal cord's ability to learn, it has been shown that the lumbar cord can be trained for specific tasks such as stepping or standing (De Leon et al., 1998b, 1998a; Edgerton et al., 1997; Hodgson et al., 1994). Even though several studies have shown overall poor recovery in adult spinal cats after injury, it must be recognized that untrained control groups contain outliers showing spontaneous recovery for unknown reasons after a full thoracic transection (De Leon et al., 1998b; Eidelberg et al., 1980; Hodgson et al., 1994; Lovely et al., 1986).

The traditional view that locomotor recovery in cats is poor in the absence of training has been most recently challenged by an experiment showing not only that locomotor training need not be task specific but that spontaneous locomotor recovery was consistently observed in untrained spinal cats (Harnie et al, 2019). However, differences in the surgical approaches to the spinal transection and how they may affect recovery have been historically overlooked. Studies in the feline literature differ in their description of several factors such as the amount of retraction of the spinal cord ends, the preservation of the ventral spinal artery, and the preservation of the dura mater upon transection. These discrepancies could be engaging physiological systems that affect recovery after SCI, particularly the inflammatory response. Extensive damage to the dura matter will compromise the regulation of the blood-spinal cord barrier and expose the spinal cord tissue to higher levels of exogenous factors which set off a cascade of inflammatory responses (L. Y. Jin et al., 2021; Whetstone et al., 2003). However, inflammation has been

shown to be a complex process that has the potential not only to positively influence recovery outcomes but also to cause further damage to the healthy spinal tissue proximal to the site of lesion (Alexander & Popovich, 2009; Donnelly & Popovich, 2008; Gensel et al., 2012; Kigerl et al., 2009; Novak & Koh, 2013; Torres-Espín et al., 2018)

Activated microglia have been known to increase neuronal excitability by causing a shift in the polarity of currents activated by GABA via BDNF signaling in peripheral nerve injury (Coull et al., 2003, 2005). In addition, spinal microglia have been shown to produce BDNF in response to spinal injury in rats (Boulenguez et al., 2010) and our own work shows that BDNF promotes locomotor recovery in the absence of training in spinalized felines (Boyce et al., 2007; Krupka et al., 2017; Marchionne et al., 2020; Ollivier-Lanvin et al., 2015). We hypothesize that instances of spontaneous locomotor recovery may be due to endogenous BDNF released from microglia associated with an overall increase in neuroinflammation as a response to extensive dural damage.

In this study, we set out to identify differences in locomotor performance associated with different levels of dura damage while quantifying the levels of associated inflammatory response to treatment/dural damage. Although we found no evidence of BDNF being produced by microglia in the thoracic spinal cord, our results show that extensive dural damage is associated with the development of spontaneous locomotor recovery as well as a decreased inflammatory response in the spinal cord caudal to the injury after a complete SCI.

## **Materials and Methods**

### ***Experimental Design***

A total of 10 adult female domestic shorthaired cats (Liberty Research Inc. Waverly, NY) were used for this study. The animals were separated into two groups of 5 cats. In each group, 4/5 cats were kept until the chronic stage of injury (5 weeks post-injury) and 1/5 was kept only during the acute injury (1 week post-injury). This group size was chosen based prior reports showing that statistically significant locomotor performance differences are obtained with that group size. (Boyce et al., 2007; Harnie et al., 2019; Krupka et al., 2017; Marchionne et al., 2020). All animal procedures were performed according to the National Institute of Health guidelines, as well as to the USDA regulations for the use of felines in research and were approved by the Institutional Animal Care and Use Committee of Temple University (protocol 4908). Cats were trained to walk on the treadmill for 20 min/day at speeds ranging from 0.2m/s up to 0.8m/s for 3-6 weeks prior to spinal surgery. Once acclimated and able to perform at the full range of speeds, locomotor kinematics were collected using a high-speed motion capture system. All cats then underwent a complete spinal transection and intrathecal catheter/pump implant where the pump's reservoir was filled with 0.9% NaCl (matching our prior published protocol in Marchionne et al., 2020). One experimental group (open dura, n=5) received a low thoracic spinal cord injury where the dura matter was completely transected along with the spinal cord. The control group (closed dura, n=5) received a spinal cord injury where the dura was opened to reach the spinal cord and closed after transection of the cord. One cat from each group was sacrificed at 1 week post-transection to test for acute changes in the spinal

cord environment. In these animals, no catheter/pump was implanted. The remaining 8 cats were euthanized at 6 weeks post-transection after kinematic data collection at 3 and 5-week post-tx time points. Ground reaction forces (GRFs) were recorded at 5-weeks post-transection (post-tx) to assess weight support ability during locomotion. Once euthanized, histological analysis of the spinal tissue was conducted.

### ***Surgical Procedures: Spinal Transection***

Spinal transection and pump implantation were carried out in the same procedure under aseptic conditions. Anesthesia was induced using ketamine (25 mg/Kg) injection and maintained using isoflurane inhalation (1-5 % in O<sub>2</sub>). A midline incision was made between the T9 and L1 spinal levels. Muscles attached to the dorsal aspect of the spinal column were removed using a surgical spatula and rongeurs were used to perform a narrow laminectomy at the T11-T12 vertebrae junction. For the control group, the dura was carefully punctured and opened along a 5mm longitudinal incision to access the spinal cord. Using microscissors, the spinal cord was transected right above the T11 roots, leaving a 2-3 mm gap between the cut ends. Following the transection, the dura was sutured closed using 7-0 polypropylene.

The experimental group received a spinal cord injury at the same level but the dura was not preserved. As a consequence, after transecting the dura along with the cord above the T11 roots, we observed significantly more retraction of the caudal and rostral portions of the cord (5-7mm). Muscles and skin were sutured closed in both groups following transection. A second incision was made between the L7 and S2 spinal segments where a mini-pump (iPrecio SMP-200, MISZU, Tokyo) was implanted subdermally. The catheter (32G, ReCathCo 22EO) connected to the mini-pump was implanted within the lumbar

cistern through the dura mater at the L7/S1 junction and then run rostrally on the ventral aspect of the cord until approximately reaching the L5-L6 spinal segments in all cats. The pump was programmed to deliver 2 $\mu$ L of solution per hour at a constant flow rate. The pump was filled with 0.9% NaCl saline in all animals and refilled every 15 days through a percutaneous refill port (for more details see Marchionne et al., 2020). Pump and intrathecal catheter implant were conducted to allow comparison between the locomotor performance of the animals in this study with the recovery observed in our historical groups receiving intrathecal delivery of BDNF (or saline as control) following injury (Marchionne et al, 2020).

### ***Locomotor Training and Kinematic Data Collection***

Cats were initially acclimated to the motorized treadmill. Once acclimated, animals were trained to walk at speeds ranging from 0.2-0.8m/s on the treadmill. The treadmill was enclosed in a Plexiglass frame to minimize lateral movements during training and recording. Locomotor performance was recorded using Vicon motion capture system (Vicon Motion System Ltd., Oxford, UK) consisting of three infrared cameras that collected the position of 11 reflective markers placed on each cat's right forelimb and hindlimb. The markers on the hindlimb were placed at the ischium, femoral head, knee joint, lateral malleolus, metatarsophalangeal (MTP) joint and the tip of the third digit. On the forelimb, the markers were placed on humeral head, elbow joint lateral epicondyle, metacarpophalangeal joint and the tip of the third digit. After transection, forelimbs were kept on a platform elevated 1cm above the treadmill during recordings since only hindlimb performance was being evaluated. The cats were held at the base of the tail to provide balance support during locomotion and to provide perineal stimulation to maximize

locomotor output. Perineal stimulation was carefully applied in order to maximize locomotion but avoid excessive stimulation which can interrupt stepping and cause aberrant patterns. Kinematic were recorded for approximately 1 minute per speed starting at 0.2m/s and increasing in 0.1m/s intervals until 0.8m/s. The length of these recording sessions at 3 and 5 weeks post transection have shown no training effects in previous studies carried out in our laboratory (Boyce et al., 2007; Krupka et al., 2017; Marchionne et al., 2020). Criteria for successful locomotion at a certain speed was the ability to execute 10 consecutive plantar weight-bearing steps at that speed and all lower speeds.

### ***Kinematic Data Analysis***

Horizontal and vertical components of each marker were reconstructed and exported using Nexus 9 (Vicon Systems). Post-processing was carried out using custom Matlab (Mathworks, Natick, MA) scripts for processing and separating continuous recording sessions into individual steps for further quantification. Igor Pro (Wavemetrics, Lake Oswego, OR) was used for quantitative analysis of stepping parameters and statistical analysis was carried out using SPSS (SPSS Inc., Chicago, IL) and Rstudio (Boston, MA). Kinematic parameters extracted included stance length, hip height, swing height,  $D_a$  (horizontal foot displacement relative to hip at toe-down),  $D_p$  (horizontal foot displacement relative to hip at lift-off), and hindlimb joint angle excursion for hip, knee and ankle joints (Figure 2-1B). Post-transection values were normalized to pre-transection values for each cat to allow comparisons across subjects. Every parameter was calculated across 10 consecutive weight bearing steps. To assess differences in locomotor recovery, a mixed model analysis was used with normalized kinematic and angular parameters (stance length index, swing height index, hip height index,  $D_a$  index,  $D_p$  index, ankle knee and hip range

index) as dependent variables, and with group and speed as fixed factors. Individual steps were included as repeated measures and cat was included as a subject level factor. Mechanical sensitivity was also tested using paw pressure on each animal on both hind paws at 3 and 5 weeks post-transection to calculate the flexor withdrawal threshold. A control device with 3mm tip hydraulic piston from Topcat Metrology Ltd (UK) was used to deliver and measure pressure applied to the dorsal surface of the paw. Statistical analysis consisted of a linear mixed model using withdrawal threshold as dependent variable, and with group as fixed factor and testing time point (week post-tx) as a repeated measure.

### ***Ground Reaction Forces Data Collection and Analysis***

Ground reaction forces (GRFs) during treadmill locomotion were collected for 7 of the animals at 5 weeks post-transection using a split belt treadmill with each belt motor assembly mounted on a Kistler force plate (Kistler Instrument Corp., Winterhur, SUI) (Dimiskovski et al., 2017). All recordings were carried out at 0.4 m/s which only 5 out of 7 cats could locomote at that time point. The vertical component ( $F_z$ ) of the GRFs was used to evaluate each animal's weight bearing ability. During recording, the cat tail was held at a minimal force to maximize the amount of weight supported by the hindlimbs. Force and kinematic data were collected at 200 Hz and processed in Matlab using custom scripts. The vertical force component was filtered using a Butterworth filter (10Hz cut-off frequency) to remove noise associated with the treadmill motor vibrations. Peak vertical forces of 10 consecutive weight bearing steps were extracted and averaged to compare weight bearing ability across groups. Similarly, mixed model analysis with peak vertical force as dependent variable, and with group and speed as fixed factors.

### ***Tissue processing and histology***

At 6 weeks post-injury, animals were euthanized and perfused intracardially with 0.9% cold saline followed by 4% paraformaldehyde in phosphate buffer. After fixation, the lumbar and thoracic spinal cord was retrieved. Spinal tissue was post-fixed in 4% paraformaldehyde for 2-3 weeks and then cryoprotected in 30% sucrose solution for a minimum of 1 week before segmenting and cutting. The spinal cord was divided into <1cm long segments and embedded using M-1 matrix medium and frozen at -80°C. Tissue was cut into 30µm transverse slices on a cryostat and stored at -20°C. The tissue used for IHC was stored in 10x10 well plate with cryoprotectant in 4% PBS while spinal tissue of the acute animals (n=2) probed using RNAscope in-situ hybridization assay was mounted on charged glass slides. Sections from chronic animals assigned for immunofluorescence were washed in 0.04% Triton X-100 in PBS and blocked with 5% normal goat serum in 0.3% Triton X-100 in PBS. Chronic sections were incubated in primary antibody solution overnight at 4°C. The primary antibody used was goat anti-Iba1 (1:100, Invitrogen, Waltham, MA). Sections were washed the following day and incubated in secondary antibody at room temperature for 2 hours. Sections were then mounted on slides and imaged using a Zeiss Axio Imager M.2. Sections from acute animals were assigned for RNAscope insitu hybridization assay using probes in two different channels (c1:AIF1, c2:BDNF). An AIF1 (allograft inflammatory factor 1) probe was used to tag microglia and macrophages in acute animals since the AIF1 gene is responsible for the production of ionized calcium-binding adapter protein 1 (Iba1). A custom-made probe from ACD BIO (Neward, CA) was used to tag feline BDNF gene in the same assay. Two channels were

used in the RNAscope assay to tag AIF1 gene in the same protocol as BDNF gene to reduce tissue damage observed in hybrid IHC-RNAscope protocols.

Iba1 stain intensity was quantified in terms of mean grey value per  $\mu\text{m}^2$  using ImageJ. Quantification was carried out on 6 slices of thoracic tissue per animal, proximal to the injury site (within half a spinal segment). The average of 6 slices per subject was calculated and used to compare average stain intensity across the closed and open dura groups. RNAscope was carried out within 600  $\mu\text{m}$  of the injury. Positive control for ACD BIO's custom feline BDNF probe was carried out on a brainstem slice of subject CD4, where hippocampus BDNF production served to verify our protocol as well as the probe.

## **Results**

### ***Locomotor speed assessment and walking kinematics***

Both groups' locomotor recovery was assessed using plantar weight-bearing stepping speed as a measure of recovery. Open dura group had a higher locomotor ability with 2/4 cats able to reach speeds of 0.8m/s at either or both testing time points compared with 0/4 cats in the closed dura at either time point. The closed dura group also had a higher incidence of complete inability to step with 2 out of the 4 animals being unable to step at any speed. Using Fisher's exact test ( $p=0.435$ ), we determined the difference in walking speed performance was not statistically significant. We compared kinematic parameters across groups at 5 weeks post-tx for a walking speed of 0.4 m/s. Half of the closed dura group met the walking speed criteria (10 consecutive plantar weight-bearing steps during locomotion at that speed and at the lower speeds) at this point while all open dura animals met the criteria. Locomotor performance was evaluated by comparing the following parameters: stance length, swing height, hip height, Da (horizontal foot displacement

relative to hip at toe-down), and  $D_p$  (horizontal foot displacement relative to hip at lift-off) (Figure 2-1). Parameters were averaged over the course of 10 consecutive weight-bearing steps and normalized according to the animal's pre-transection values. Group averages for stance length, swing height, and hip height are shown on Figure 2-3(A-C). Subject averages for the same parameters are shown in Figure 2-3(D-F). While stance length and hip height during locomotion show variability and overlap between the groups, swing height clearly show that open dura animals have better clearance above the treadmill. Linear mixed model analysis showed group to have a significant ( $p < 0.05$ ) effect on swing height index.  $D_a$  and  $D_p$  indices are shown according to group in Figure 2-4(A-B) and according to subject in Figure 2-4(C-D). The horizontal displacement of the foot relative to the hip at toe-down ( $D_a$ ) in the open dura group shows increased displacement of the foot past the pre-transection values while the closed dura group remained roughly the same as their pre-transection values. In terms of range of motion, angle excursion was evaluated by calculating the range of the ankle, knee, and hip over the same 10 consecutive steps in order to have a better picture of the functional changes between groups during locomotion. Average angle ranges were calculated and examined across groups and across subjects (Figure 2-5(A-F)). Open dura group showed increased ankle and knee angle ranges consistent with the differences observed in swing height and  $D_a$  index. In the subject panel (Fig. 2-5D) for ankle range, we observed OD3 had a unique extension of ankle angle range (Figure 2-3D). Linear mixed model analysis showed group to have a significant ( $p < 0.05$ ) effect on knee angle range index. Overall, open dura animals seem to perform more exaggerated movements that extend the range of motion past pre-transection values while closed dura animals seem to hover around or below pre-transection values.

### ***Weight bearing capability during treadmill locomotion***

Ground reaction forces were examined to evaluate weight bearing abilities in both groups of spinal cats. The vertical component (Fz) of the GRFs was extracted from 10 consecutive weight bearing steps for 5 out of the 6 animals that walked at 0.4 m/s at 5-weeks post transection (recording window was missed in the other animal). The open dura group contains the subject (OD2) with the highest peak vertical force during stepping. However, mixed model analysis shows no group effect on weight bearing indicated by normalized peak vertical GRF component.

### ***Paw Pressure Withdrawal Threshold Comparisons***

Flexor withdrawal threshold was tested using paw pressure on each animal on both hind paws at 3 and 5 weeks post-transection. Withdrawal was tested by delivering pressure stimulus on the dorsal portion of the foot. The results show that withdrawal threshold does not vary over time, indicating no change in sensitivity over the examination period (3-5 weeks post-tx) (Figure 2-7A). In terms of subject measurements, we observed larger variability in the closed dura group where 2 subjects showed elevated thresholds (Figure 2-7B). The variability present in the closed dura group is reflected by the height of the box in Figure 2-7C. The open dura group had a lower withdrawal threshold showing increased mechanical sensitivity to pressure on the hindlimb paws. Linear mixed model analysis show group had a significant effect ( $p < 0.05$ ) on withdrawal threshold. Interestingly, the closed dura animals with the higher withdrawal thresholds were the ones that were able to perform at 0.4m/s (CD1 & CD4). This correlation between withdrawal and walking was only observed in the closed dura group.

### ***Immune Response Variations as Measured by Macrophage/Microglia staining***

Following euthanasia, the spinal tissue was retrieved (T9/T10-S2/S3) to determine the level of injury, completeness of the transection, and to examine the presence of immune cells such as microglia and macrophages at the thoracic level of the spinal cord. Gross analysis of the tissue revealed that the transections were indeed performed just above the T11 roots in all the animals, and that the transections were complete.

The segment right below the site of the injury was used to carry out the histological analysis using Iba-1. Using the intensity of the stain per  $\mu\text{m}^2$  (mean grey value) we quantified the volume of the inflammatory response consisting of macrophages and microglia in the spinal cord (T11). The average stain intensity for every subject was found by calculating the average intensity of 6 transverse slices of the spinal cord caudal to the injury site. We observed a significantly higher average inflammatory response in the closed dura group (Fig. 2-8A, independent samples *t*-test,  $p < 0.05$ ,  $n=8$ ). This overall difference was also observed between individual subjects of each group (Fig. 2-8B).

Overall, our data shows that the level of damage to the dura affects the level of inflammatory response in the spinal cord caudal to the injury at 5 weeks post-transection. A closed dura transection surgery is associated with more widespread inflammation and higher concentrations of microglia and macrophages (Fig. 2-8A,D). Closed dura animals showed higher stain intensity in the lateral and anterior funiculi. The open dura animals showed minimal inflammation in the dorsal horns and lateral funiculus and no noticeable Iba1 labeling in the ventral funiculus.

### ***No BDNF Production from Microglia***

Using ACD BIO's RNAscope insitu hybridization assay, we used a AIF1 probe to label all immune cells that produce the Iba-1 surface antibody found in microglia and macrophages. Using the same assay, an ACD custom-made BDNF-probe was utilized to identify feline BDNF production in the cross sections of the lower thoracic cord. More specifically, this combination of probes targeted BDNF-producing microglia/macrophages. The animals used for this assay had to be euthanized at 7-days post-tx since microglia have been shown to produce BDNF primarily in the acute phase of an injury and are unlikely to be present in that state at the chronic stage of injury (Coull et al. 2005). As predicted from the known time course of microglia activation in other species (Hellenbrand et al., 2021; Perez et al., 2021) RNAscope protocol in tissue from chronic spinal cats which yielded no positive BDNF labeling. The control stain using the feline BDNF-probe was carried out in the hippocampus of one subject. Our control stain showed BDNF-RNA labeling around the DAPI nuclei of which cells/section of the hippocampus (Fig. 2-9) as expected. After the validation of the ACD BIO custom-made probe, the spinal cord of the two acute spinal cats were co-stained using AIF1 and BDNF probes. Our results showed evident AIF1 labeling, indicating the presence of Iba-1 surface antibody in both the open dura and closed dura animals (Fig. 2-10A, 2-10D). However, there was no BDNF being produced even in the acute stages of the spinal cord injury in either group (Fig. 2-10B, 2-10E). There were no signs of positive BDNF labeling around the nuclei stained with Dapi. Fig. 2-10C and 2-10F show both channels used for AIF1 and BDNF probes, clearly establishing only the presence of AIF1 RNA in both groups. This result shows we

found no BDNF production caudal to the injury site at the 7-days post-tx in either the closed dura or the open dura animals.

## **Discussion**

### ***Locomotor recovery in the absence of neurotrophic or exercise treatment***

The ability to recover locomotor capacity in the absence of supraspinal input after injury is a known property of the spinal cord, however, interventions are typically required to reengage the spinal circuitry in a manner such that it results in a significant behavioral outcome. The results from this study show that the method of transection may leverage endogenous effects and elicit significant spontaneous motor recovery. An open dura approach to spinal transection surgery at the T11 spinal level was associated with an increased incidence of spontaneous locomotor recovery during treadmill locomotion. This approach also resulted in the animals being able to perform at the full range of pre-transection speed during treadmill locomotion in 50% of the subjects. Similarly, the method of transection affected most kinematic measures when locomotion was compared at the same walking speed and time point after transection. That differences in the kinematic parameters of walking at the same speed and time point is indicative of a difference in quality of locomotion. The open dura group showed an increased ability to bend the knee joint during the swing phase resulting in a higher swing height and increased knee joint range of motion. Linear mixed model analysis showed the transection method to have a significant effect on both parameters ( $p < 0.05$  in both cases). However, peak ground reaction forces were not different between groups at 5 weeks post-tx when compared at a walking speed of 0.4m/s.

If we consider previous experiments where control subjects also received a closed dura transection and were given saline intrathecally for 5 wks post-transection (Marchionne, 2020), we increase our control group size and obtain a better estimate of that group's performance when assessing spontaneous locomotor ability without training or neurotrophic treatment (Fig 2-11).

When we considered those additional animals in Figure 2-11, we observed a more clearly delineated relationship between treatment and recovery than the one observed in Figure 2-2 containing 4 subjects per group. While high performers in the closed dura group overlap with the low performers of the open dura group, the median top walking speeds of the groups are now separated by a difference of at least 0.5m/s at both time points (Fig. 2-11). The open dura group walked at higher speeds than the closed dura group, and the differences were statistically significant (independent samples t-test,  $p < 0.01$  at 3 weeks and  $p < 0.001$  at 5 weeks,  $n=14$ ). Even with well controlled and consistent surgical procedures in the closed dura animals, we observed instances where those animals achieved speeds of up to 0.5m/s. This is consistent with instances of spontaneous locomotor recovery in other studies where the dura was largely preserved such as in De Leon et al. (1998b) where 2/9 untrained spinal cats recovered to a top speed of at least 0.4m/s or in Marchionne et al. (202) where 1/6 spinal cats walked at 0.8 m/s.

After SCI, mechanical stimulus can be perceived as noxious input resulting in allodynia (Christensen et al., 1996; Siddall et al., 1995). The relationship between SCI and increased nociception is a well-established problem and therapies have been explored in the rat model (Garraway et al., 2014; Nees et al., 2016; Sliwinski et al., 2018, 2024; Tashiro et al., 2015). However, the presence of BDNF after injury is often associated with a further

increase in sensitivity to mechanical stimulus in rats (Coull et al., 2005; L. J. Zhou et al., 2011). These observations led us to expect increase mechanical sensitivity in the open dura group since we expected BDNF to be present after injury. In spite of the present study showing no direct evidence of BDNF from spinal microglia, the open dura showed increased sensitivity during pressure stimulus testing. The open dura group had a lower withdrawal threshold showing increased mechanical sensitivity to pressure on the hindlimb paws. Linear mixed model analysis show group had a significant effect ( $p < 0.05$ ) on withdrawal threshold. Results also show more variability in the closed dura group, suggesting there may be more inconsistent responses to mechanical pressure in the case of increased inflammation.

### ***Inflammation as a potential mechanism for locomotor recovery***

Endogenous release of BDNF from spinal microglia has been observed in the rat model after peripheral nerve injury, and spinal cord transection (Boulenguez et al., 2010; Coull et al., 2005; Keller et al., 2007). Even though microglia function is thought to be largely preserved among vertebrates (Sharma et al., 2021), we noticed no presence of BDNF in the feline spinal cord after injury. Neither an open dura injury or closed dura showed evidence of BDNF-producing microglia in either the acute (1 week) or chronic (5 weeks) period post-injury. Despite the lack of BDNF-producing microglia, our histological results point to a correlation between the inflammatory response and spontaneous locomotor recovery. The animals that received an open dura showed significantly less inflammation in the spinal cord caudal to the lesion site and a greater recovery of treadmill locomotion ability. While we a-priori expected BDNF to play a primary role in the development of spontaneous locomotor recovery, our results show no evidence of BDNF

being produced even in the acute stage of injury. The lack of BDNF in the caudal portion of the cord suggests that the effect of the transection surgical approach on locomotor recovery may be mediated by a mechanism that is not neurotrophin dependent. A more detailed approach to explore the cellular populations over time will be necessary to establish the differences in immune responses time course between an open and closed dura transection approaches and the associated locomotor recovery.

However, the amount of inflammation present in each group suggests that a mild level of inflammation could be promoting recovery in locomotor outcomes. While it is hard to pinpoint a single candidate responsible for mediating such effects, there are multiple candidates within the inflammatory response that could be involved in promoting functional recovery. M2 macrophages are known to take action after M1 macrophages have cleared cellular debris in response to primary injury (Sica et al. 2006). While M1 macrophages can be neurotoxic, M2 macrophages have a primarily anti-inflammatory effect on the spinal environment promoting angiogenesis and matrix remodeling and have been shown to promote axonal growth along with motor recovery in spinal rats (Gensel et al., 2009; Kigerl et al., 2009, Sica et al. 2006, Rapalino et al., 1998). It is possible that the compromised environment resulting from an open dura transection and the associated mild inflammation preferentially activates M2 macrophages, promoting motor recovery. In rats, M2 macrophage induced from bone-marrow derived macrophages have been shown to promote locomotor recovery after spinal injury (Ma et al., 2015) and genetic analysis of immune cells has revealed a positive correlation between locomotor recovery and M2 marker expression (Kisucká et al., 2021). Similarly, the exercise training outcome is associated with decrease in pro-inflammatory markers and enhanced locomotor

performance (Bilchak et al., 2021; Dugan et al., 2020) further suggesting that a combination of mild inflammation and M2 macrophages is critical for improved recovery outcomes observed in open dura animals. Transection method of injury has been shown to result in decreased myelin pathology after injury while contusion methods that imply the preservation of the dura, show increased demyelination due to macrophage recruitment (Siegenthaler et al., 2007). Further investigation is required to probe multiple time points and determine changes in macrophage polarization before the first instances of locomotor recovery are noticed in our animal model (3 weeks post-tx in the open dura group).

The increased levels of inflammation associated with limited recovery in the cats with a closed dura transection suggest the detrimental effects of inflammatory responses. The negative effects of inflammation have been widely observed (Horn et al., 2008; Jeffrey-Gauthier et al., 2021; Kigerl et al., 2009; Popovich et al., 1999; N. Zhang et al., 2012). Inflammation in the lumbar spinal cord has been shown to have adverse effects by inducing locomotor deficits that work against step training treatment (Jeffrey-Gauthier, 2020) and macrophage depletion after a spinal cord injury has been shown to improve recovery outcomes in rats (Popovich et al. 1999, Horn et al. 2008). A closed dura injury where the dura is largely preserved is likely resulting in an upregulation of apoptotic and pro-inflammatory factors that are associated with maladaptive immune response of M1 macrophages (Horn et al., 2008; Kigerl et al., 2014). In addition, damaged microglia have been shown to secrete alarmin interleukin-1 $\alpha$  (Bretheau et al., 2022), a type of interleukin that further exacerbates secondary injury after a lesion by inducing neutrophil infiltration and oligodendrocyte death. Interleukin-1 $\alpha$  gene (IL-1R1) deficiency positively affects locomotor recovery after SCI (Bretheau et al., 2022). The consistently higher inflammatory

response observed in closed dura animals with poor recovery outcomes would suggest large concentrations of M1 macrophages along with a high concentration of injured microglia, promoting secondary injury cascades. However, further investigation is required to determine macrophage polarization.

Due to the differences in behavioral outcomes, our results suggest that an open dura surgical approach is leveraging the neuroprotective aspects of the inflammatory response through a controlled and mild immune response. The compromised barrier could be having a differential effect on monocyte polarization promoting more M2 macrophages which are commonly known to be involved in remodeling and promoting healthy tissue. The present study presents evidence of inflammatory mediated locomotor recovery in untrained spinal cats with a thoracic injury. While it is not commonly considered during study design, inflammation should be a factor taken into account when assessing the locomotor recovery of untrained control groups since differences in surgical methods of transection have the potential to result in spontaneous locomotor recovery.

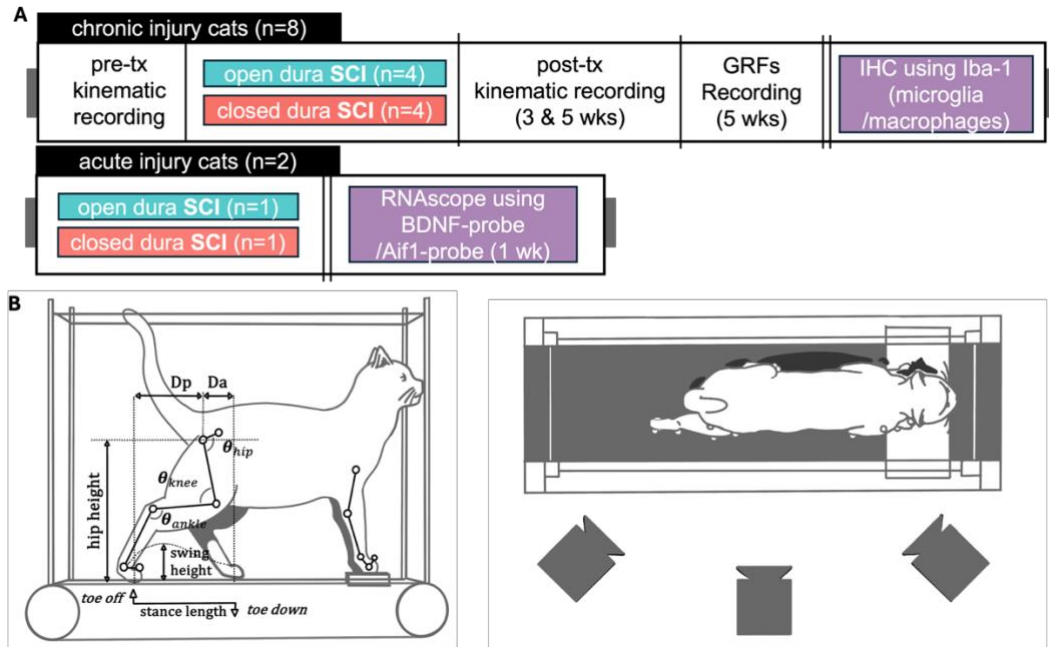


Figure 2-1. Experimental timeline and setup. A) Experimental timeline, groups (teal and red squares) and kinematic recordings + histological testing (purple box). B) Camera acquisition set-up, and kinematic variables analyzed and compared between the two groups. Stance length: horizontal distance traveled by the MTP marker from toe down to toe off, swing height: maximal vertical height of the MTP marker relative to the treadmill surface during swing, Da: horizontal displacement of the foot marker relative to the hip marker at toe down, Dp: horizontal displacement of the foot marker relative to the hip marker at toe off. The hip, knee and ankle angles were measured as shown in panel B.

Table 2-1. Top walking speed for each cat recorded at 3 and 5 weeks post-transection. Animals in the open dura (OD) group were able to reach walking speeds of 0.8m/s which is the maximum speed performed prior to injury. Animals in the closed dura (CD) group had some instances of spontaneous locomotor recovery where 0.5m/s was achieved without any prior training after the injury Acute animals are not included in the table since they were only kept for 1 week post-tx.

<b>Dura treatment</b>	<b>Subject</b>	<b>Top speed at 3-weeks post-Tx</b>	<b>Top speed at 5-weeks post-Tx</b>
<b>open</b>	OD1	0.8 m/s	0.6m/s
	OD2	0.8 m/s	0.8m/s
	OD3	0.5 m/s	0.4 m/s
	OD4	0.2 m/s	0.6 m/s
<b>closed</b>	CD1	0.4 m/s	0.5 m/s
	CD2	-	0.2 m/s
	CD3	-	0.2 m/s
	CD4	0.5 m/s	0.5 m/s

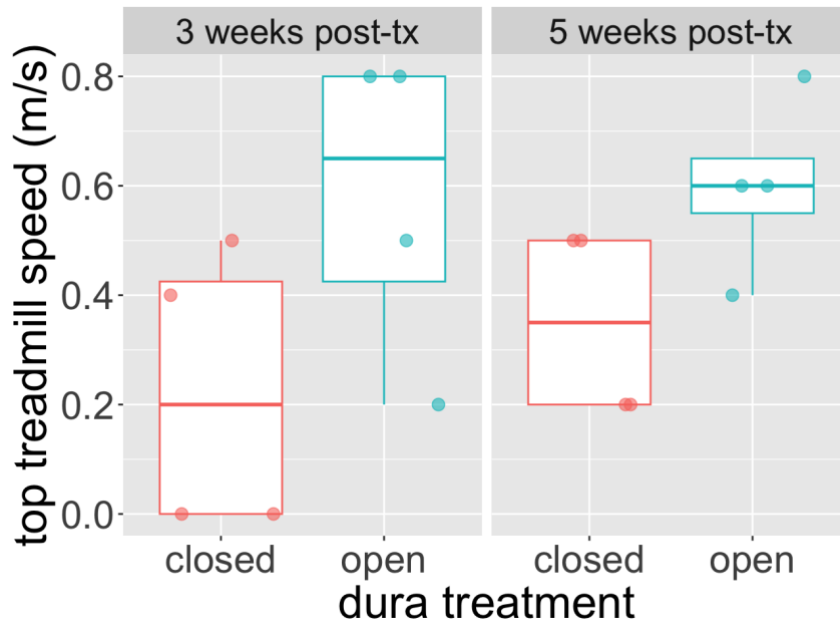


Figure 2-2. Top walking speed at 3- and 5-weeks post injury. The open dura group (n=4) was consistently able to perform at higher walking speeds during testing sessions and showed a higher proportion (2/4) of subject that showed full locomotor recovery when compared to the closed dura group (n=4) with complete absence of full recovery (0/4). Box plots in blue show the open dura group performance at 3-weeks (median = 0.65m/s , mean = 0.58m/s, SD= $\pm$ 0.29m/s) and 5 weeks post tx (median = 0.6m/s , mean = 0.6m/s, SD= $\pm$ 0.16m/s). Closed dura is shown in red at 3 weeks (median = 0.2m/s , mean = 0.23m/s, SD= $\pm$ 0.26m/s) and 5 weeks (median = 0.35m/s , mean = 0.35m/s, SD= $\pm$ 0.17m/s) post-tx. An independent samples t-test was performed comparing the means of each group at both time points showing no statistical significance ( $p > 0.05$  at 3 and 5 weeks post-tx). However, the closed dura group showed little recovery on average and was not able to achieve speeds higher than 0.5m/s. At 3-weeks post-tx, group medians (shown on box plots) are separated by a difference of approximately 0.5m/s. Difference between group medians decreases by week 5 post-tx.

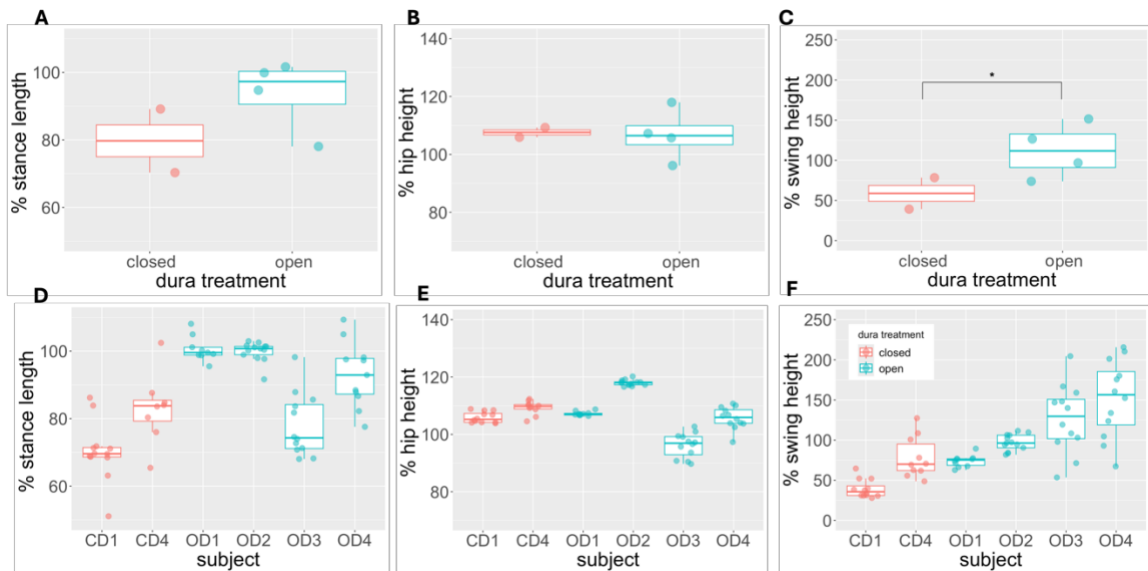


Figure 2-3. Kinematic parameters calculated according to group showing stance length, hip height, and swing height index as a percentage of pre-transection values at 5 weeks post-injury during treadmill locomotion at 0.4m/s. A-C) The average of 10 consecutive steps were extracted from each subject able to meet the walking criteria. Box plots show median, first and third quartile of group data. The open dura group shows higher stance length and swing height indexes that near pre-transection values, while average hip height shows no clear separation between groups. Linear mixed model analysis indicate group has a significant effect on swing height index ( $p < 0.05$ ). D-F) Same kinematic parameters calculated according to subject showing the average stance length, hip height and swing height for every cat. Box plots show median, first, and third quartile of subject data (10 steps).

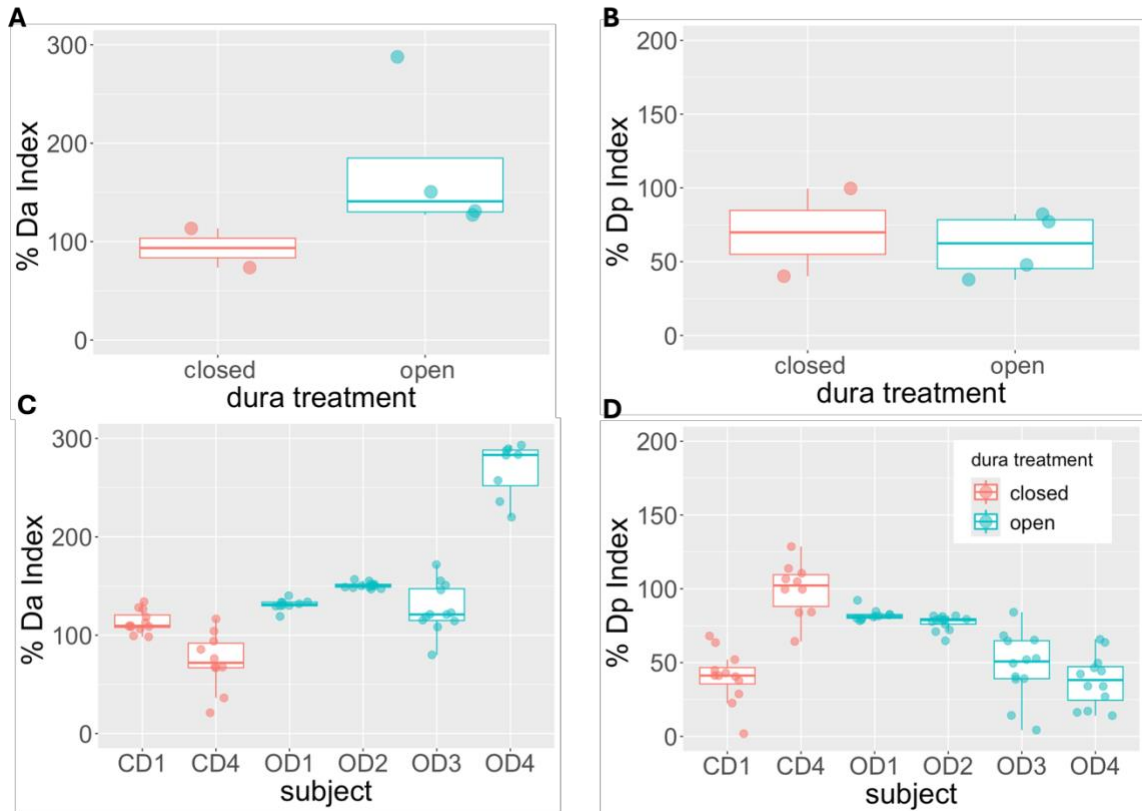


Figure 2-4. Average Da (horizontal foot displacement relative to hip at toe-down) and Dp (horizontal displacement of foot relative to lift-off) indices, calculated as a percentage of pre-transection average, for each group. A-B) Box plots show median, first and third quartile of group data. Da indices in the open dura group (median = 140.89, mean = 174.165,  $SD=\pm 76.40$ ) extend past pre-transection values when compared to closed dura Da index values (median = 93.50, mean = 93.50,  $SD=\pm 28.14$ ). Dp indices show overlap between open dura (median = 62.44, mean = 61.24,  $SD=\pm 21.73$ ) and closed dura (median = 69.88, mean = 69.88,  $SD=\pm 42.06$ ) groups. Open dura however linear mixed model analysis indicated group does not have a significant effect on Da or Dp indices. C-D) Da and Dp indices per subject show consistent stepping across 10 consecutive steps as well as an overlap in subject performance across groups.

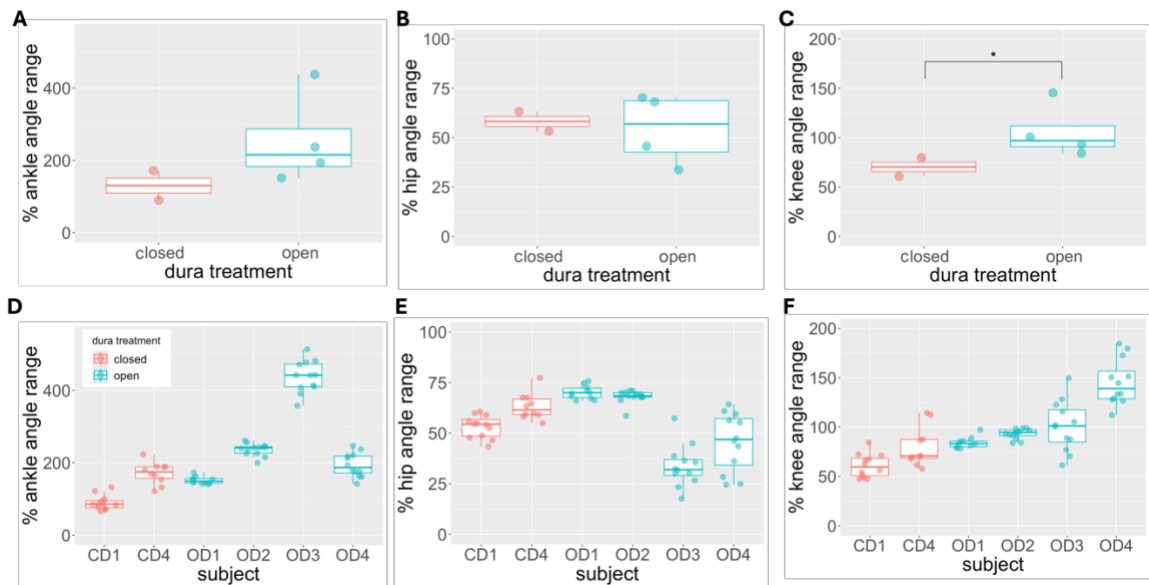


Figure 2-5. Calculated joint angle ranges for the ankle, knee, and hip joints during treadmill locomotion. A-C) Group joint angles illustrated in box plots (showing median, first and third quartile) show increased ankle angle range index and knee angle range index in the open dura group. Linear mixed model analysis showed group to have a significant ( $p < 0.05$ ) effect on knee angle range index. D-F) Subject averages show low variability and consistency of index values across 10 consecutive plantar weight-bearing steps in most subjects.

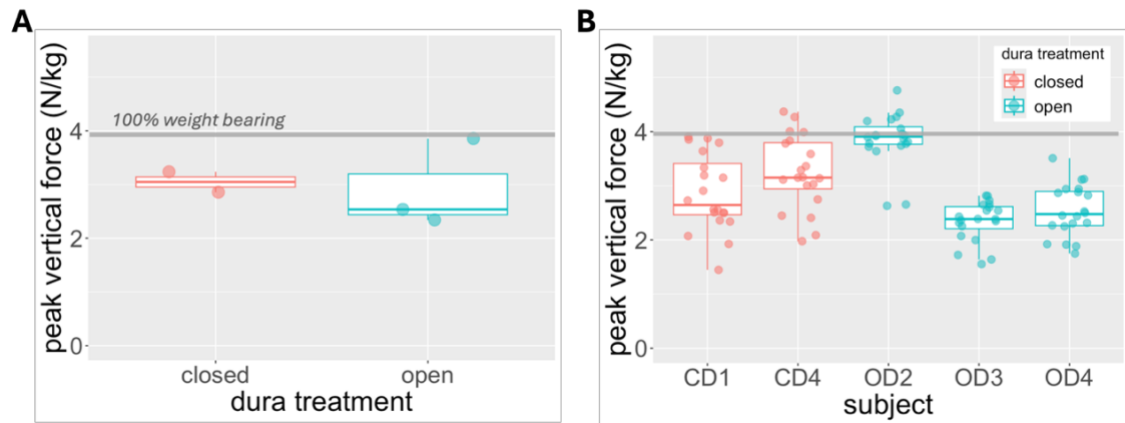


Figure 2-6. Normalized peak vertical ground reaction force. A) Normalized peak vertical component of GRFs during treadmill locomotion at 0.4m/s at 5 weeks post-tx grouped according to treatment. Line drawn at 4 N/kg represents 100% weight bearing vertical GRF for feline hindquarters (Macpherson, 1988; Roberts & Manter, 2005). There was no significant effect of group on weight bearing ( $p>0.05$ , linear mixed model) B) The normalized peak vertical component of GRFs for every step according to subject showing 20 bilateral steps at 0.4m/s and 5 weeks post-tx.

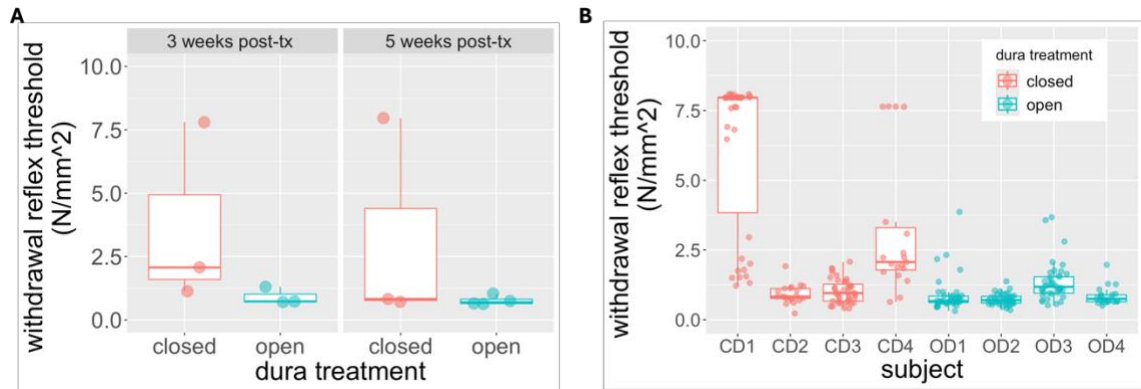


Figure 2-7. Withdrawal reflex threshold measurements. A) Withdrawal reflex threshold shown according to group across time points tested (3 and 5 weeks post-tx). Each data point represents the median withdrawal threshold of each subject and the box plot shown the media for each group as well as the first and third quartiles. Subjects had 20 observations performed at each time point. Differences in withdrawal across groups were significant. The open dura group had a lower withdrawal threshold showing increased mechanical sensitivity to pressure on the hindlimb paws. Linear mixed model analysis show group had a significant effect ( $p < 0.05$ ) on withdrawal threshold. B) Withdrawal reflex according to subject showing bilateral hindlimb testing by two examiners (10 observations each) over both time points amounting to a total of 40 observations per subject with the exception of CD1 and CD4 ( $n=20$ ). CD1 and CD4 show the highest variability and highest withdrawal threshold across all tested animals. There was no effect between points collected by different examiners.

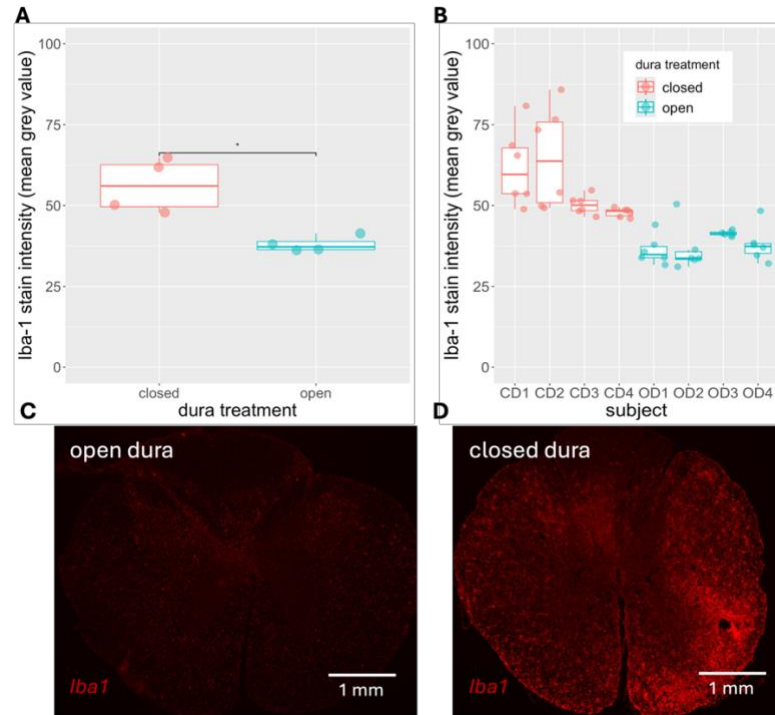


Figure 2-8. Immunofluorescence stain intensity analysis. A) Iba1 immunofluorescence stain intensity per  $\mu\text{m}^2$  according to group and calculated from 6 thoracic spinal (caudal T11) sections per animal. The median, first, and third quartile are shown on box plots. Closed dura group has a statistically higher presence of microglia/macrophages as indicated by average stain intensity (independent samples *t*-test,  $p < 0.05$ ,  $n = 8$ ) (open dura: median = 37.22, mean = 38.0,  $SD = \pm 2.39$ , closed dura: median = 56.03, mean = 56.18,  $SD = \pm 8.41$ ). B) Iba1 stain intensity according to individual subjects showing the average intensity of each of the 6 sections used per subject for the analysis. C) Example of a representative open dura spinal section stained using Iba1 primary antibody shows minimal microglia/macrophage concentration around the dorsal horns and lateral funiculus. D) Example of a representative closed dura spinal section stained using Iba1 primary antibody showing large concentrations of microglia/macrophages around the lateral and ventral funiculus.

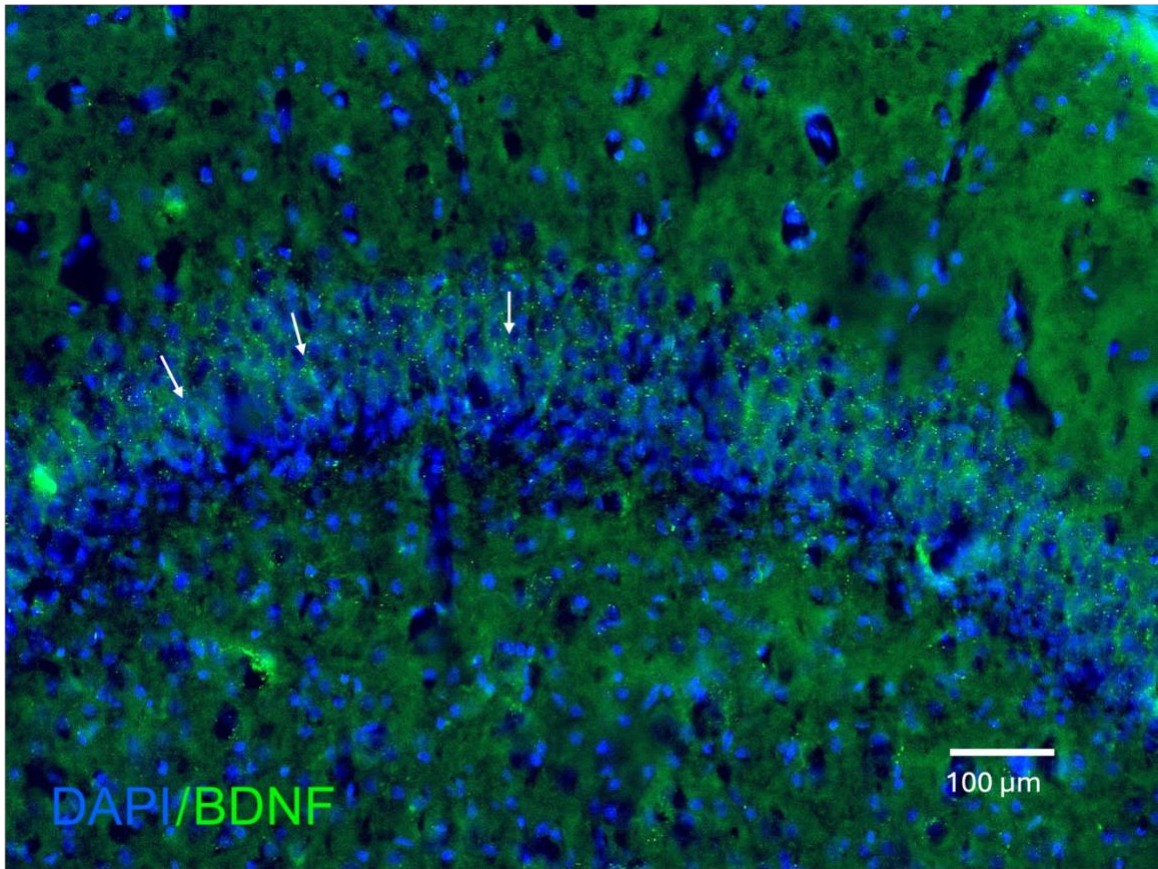


Figure 2-9. Control stain using ACD Bio's RNAscope insitu hybridization assay to tag BDNF production in feline hippocampus (subject CD4). Dapi was used to stain cell nuclei and a green 520 opal dye was used for signal amplification of BDNF-RNA tagging using custom made feline BDNF probe from ACD Bio (Neward, CA).

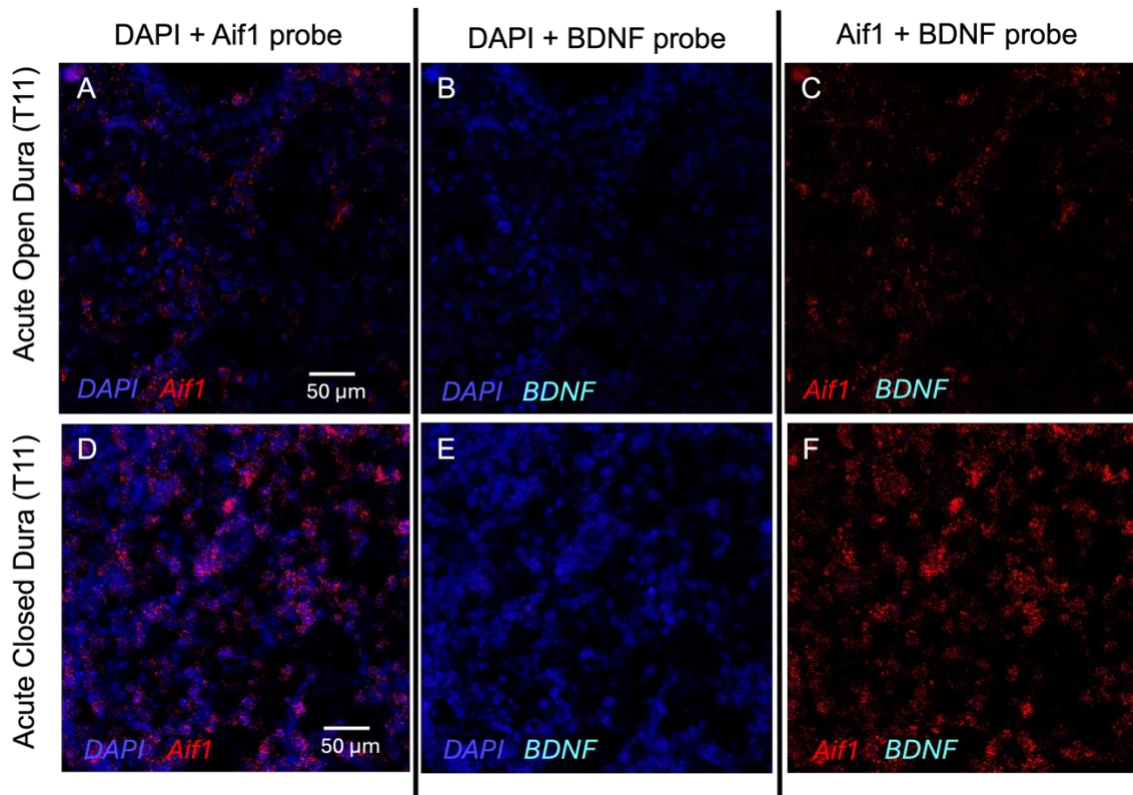


Figure 2-10. RNAscope in situ hybridization results. A) AIF1 gene tagging (red channel) in thoracic spinal section in the open dura animal. B) BDNF tagging (cyan channel) of the same area showing no BDNF production in acute open dura animal. C) Red and cyan channels together showing no BDNF production in any AIF1 positive cells. D) AIF1 gene tagging (red channel) in acute thoracic spinal section in the closed dura animal. B) BDNF tagging (cyan channel) of the same area showing no BDNF production in acute closed dura animal. C) Both channels together showing no BDNF production in any AIF1 positive cells in the acute closed dura animal.



## CHAPTER 4

### SUMMARY AND FUTURE DIRECTIONS

#### Summary

Aim 1: Characterize lumbar interneurons activity during air-stepping in chronic spinal cats treated with intrathecal BDNF delivery to the lumbar spinal cord. The working hypothesis for this aim was that intrathecal BDNF delivery would result in significantly more neuronal activity in the L3-L4 segments during air-stepping. We collected kinematic data during treadmill locomotion at different speeds (0.2-0.8 m/s) using high-speed motion capture systems (Vicon Motion Systems Inc., USA) to assess locomotor recovery. We recorded lumbar interneuronal firing during air-stepping using two 64 channels microelectrode arrays (Neuronexus, Ann Arbor, MI) inserted at the dorsal root entry zone to either ~3000 $\mu$ m or ~1500 $\mu$ m depth in two lumbar segments. Muscle activity from 14 muscles of the right and left hindlimbs were simultaneously recorded. We observed locomotor recovery in BDNF-treated cats as well as spontaneous air stepping. We found BDNF-treated animals had increased firing frequency and a higher proportion of irregular units (not tuned to a phase of gait nor tonic) when evaluating directional tuning. BDNF-treated group also displayed higher cross correlation as well as a drop in high frequency peaks of multiunit power. These results show that BDNF increases some measures of neuronal excitability in locomotor circuitry, and those increases appear sufficient to achieve locomotor recovery during treadmill testing after a thoracic SCI. While we expected more radical differences in the analysis that would account for the behavioral outcomes observed, our results suggest that BDNF achieves this effect largely through subtle changes in neuronal population activity.

Aim 2: Determine kinematic differences in locomotor recovery between spinal transection methods. The working hypothesis for this aim was that an open-dura transection (transecting the dura along with the cord) would show significantly better recovery during treadmill locomotion in untrained spinal cats. We collected kinematic data during treadmill locomotion at different speeds (0.2-0.8 m/s) using high-speed motion capture systems (Vicon Motion Systems Inc., USA). We also assessed weight-bearing ability during locomotion using our own integrated force-plate treadmill system. The results show higher incidences of spontaneous locomotor recovery in the open dura group with 2/4 recovering the ability to step at 0.8m/s while 0/4 in the closed dura achieved full recovery. Peak vertical forces were compared at 5 weeks post-tx and 0.4m/s. Weight bearing ability was not significantly different between animals that could meet the walking criteria in either group. However, the only cat capable of full weight bearing (4N/kg) was a cat who received an open dura method of transection. Our results indicate that an open dura method of transection results in spontaneous locomotor recovery at higher speeds.

Aim3: Determine differences in the inflammatory response at the lower thoracic cord between spinal transection methods. The working hypothesis for this aim was that the open-dura method of transection would result in a higher inflammatory response, showing significant increases in macrophages, microglia, and BDNF caudal to the transection site. We used immunohistochemistry (IHC) to stain for activated microglia and macrophages using Ionized calcium-binding protein-1 (Iba-1) near the site of transection in the perfused spinal cord of both study groups. In acute animals, we aimed to verify that microglia

produce BDNF using an RNA in-situ hybridization assay from ACD's RNAscope® technology, where we targeted feline BDNF RNA at the lumbar and thoracic cord. Contrary to expectations, our results showed that the open dura group had significantly less inflammation in the thoracic cord caudal to the site of injury than the closed dura group. We found no evidence of BDNF production in the thoracic or lumbar cord suggesting that the effects on locomotor recovery observed in the open dura group are largely mediated through a different inflammatory-dependent mechanism.

In summary, the results from this body of work have characterized neurophysiological and inflammatory aspects of spontaneous locomotor recovery in the feline model. Aim 1 explored the key firing properties of interneuronal populations that lead to the spontaneous locomotor recovery after intrathecal BDNF delivery, expanding our understanding of the effects of neurotrophic treatment on the firing dynamics of large neuronal populations. Results from Aim 2 highlight the relevance of spinal transection method employed when studying spinal cord injuries in the feline model. Since we demonstrated that extensive damage to the dura results in the development of spontaneous locomotor recovery, it becomes evident that consistent methods of transection are necessary to successfully explore SCI therapies. The results indicate that a closed dura method of transection should be put forth as the standard injury model in cats as the most clinically relevant considering the lack of spontaneous locomotor recovery in human patients with SCI. This aim shows the necessity of a standardized SCI cat model in order to increase reproducibility across studies, and ultimately, increase clinical translation of potential therapies. Finally, Aim 3 shows a relevant relationship between the different levels of inflammation induced by varying levels of dural damage during spinal transection

surgery and spontaneous locomotor recovery. Results show that an open dura method of transection leads to lower levels of inflammation caudal to the site of injury. Furthermore, lower levels of inflammation are associated with the development of spontaneous locomotor recovery suggesting a new therapeutic target that relies on the immune system without neurotrophic or any pharmaceutical intervention. These results indicate the importance of assessing inflammation and its role when exploring an intervention that aims to improve locomotor recovery outcomes to avoid confounding variables that could bias study results. In conclusion, this exploration of spontaneous locomotor recovery has led to a better understanding of the neuronal dynamics influenced by BDNF, evidence in support of a standardized feline SCI model, and the therapeutic potential of controlled inflammation after a spinal cord injury.

### **Future Directions**

Further exploration of acute and chronic spinal cat tissue could expand upon the implications of this set of studies. First, performing further IHC analysis in the thoracic segment of chronic spinal animals using distinct markers for macrophages and microglia could give us insight into the primary populations present in each group. The proportions of microglia to macrophages present would show which population is more predominant at the chronic stage of each treatment allowing us to infer the likely mechanism behind recovery. Using CD68 as microglia-specific marker and using CD206 as a macrophage-specific marker, each with different fluorescent tags, would address this question. Alternatively, flow cytometry is another reliable method of differentiation between monocytes in the CNS. Second, regardless of population proportions, understanding the macrophage polarization at the acute and chronic stages of injury after different methods

of transection would be beneficial for determining if macrophages are largely behaving in a way that is detrimental or beneficial for motor recovery within the evaluated timespan. By using CD80 for M1 macrophages and CD163 for M2 macrophage identification at multiple time points ranging from acute, to sub-acute, and chronic, would help determine if polarization differs between groups which would further inform the role of dural damage in modifying cellular dynamics in the thoracic spinal cord. Finally, exploring inflammation-related changes in electrophysiological properties using microelectrode arrays in chronic spinal cats would give us further insight into how changes in the cellular and molecular environment affect neuronal firing in the context of spontaneous locomotor recovery. By targeting intermediate zone and dorsal horn regions in the thoracic segments (T11-T13) we can examine the effect of immune cell concentration during spontaneous activity after clonidine administration. Similarly, by targeting interneuronal populations in the lumbar segments associated with locomotor activity (L3-L4), we can compare the effects of open dura induced spontaneous locomotor recovery to recovery induced by intrathecal BDNF administration. These results would determine if different interventions ultimately resulted in the same neuronal firing properties associated with the spontaneous recovery observed with intrathecal BDNF delivery or if these interventions lead to different neuronal changes that result in the same behavioral outcome.

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