Manipulating the Microbiota-Immune Axis to Augment Recovery Following Spinal Cord Injury

by

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Abstract

Spinal cord injury (SCI) not only leads to motor and sensory dysfunction, but just as debilitating are secondary consequences of SCI such as bowel disorders, neuroinflammation, immune suppression, pain and psychiatric disorders. In this thesis, I explore multiple aspects of recovery after SCI in attempt to promote both physical and psychological well-being. In chapter 2, I show that an incomplete cervical SCI alters the composition of the gut microbiome (termed dysbiosis) and increases anxiety-like behaviour in rats. Using a fecal microbiota transplant (FMT) from uninjured donor rats to prevent SCI-induced dysbiosis also prevented the development of anxiety-like behaviour, suggesting a link between these two consequences of injury. I then wanted to determine whether optimal donor selection would influence the efficacy of FMT treatment for SCI. This was explored in chapter 3, where I show that FMT from uninjured rats with increased anxiety-like behaviour was unable to prevent SCI-induced dysbiosis. Furthermore, recipients of this inferior FMT displayed increased anxiety-like behaviour, increased intestinal permeability, and long-term alterations in local and systemic inflammation. Independent of treatment group, this study showed a global downregulation of plasma cytokines and chemokines chronically after injury. I found in chapter 4 that treatment with the antibiotic and anti-inflammatory drug, minocycline, can prevent this SCI-induced suppression of systemic inflammatory markers. In chapters 2, 3 and 4 we explore the complicated relationship between the microbiome, immune system and mental health after SCI. A potential link between these systems is through the bacterial endotoxin, lipopolysaccharide (LPS). LPS can enter circulation through a leaky intestinal barrier (which can be modulated by the microbiome as shown in chapter 3), where it induces an intense inflammatory response. Although increased inflammation is commonly associated with secondary damage following SCI, in certain circumstances inflammation can promote neural plasticity. I explore this dichotomous role of manipulating inflammation after SCI in

chapter 5, where rats were given LPS in the subacute period following SCI. Although LPS significantly improved functional motor recovery of the ipsilesional forelimb, it also induced a chronic anxiety-like state. The results of this thesis show that manipulating the microbiome and inflammation following SCI may be a therapeutic tool to promote both physical and mental well-being, and that considering multiple aspects of recovery in preclinical models is imperative to determine potentially detrimental treatment side effects.

Preface

This thesis is an original work by Emma Doolin (pseudonym Schmidt). The research project, of which this thesis is a part, received research ethics approval from the University of Alberta Research Ethics Board (ACUC), Project Name: "Repairing the injured spinal cord", AUP00000254, December 22, 2020.

Most of the chapters and appendices of this thesis have appeared in published articles. The chapters as they appear have been slightly altered from the published formats to integrate into the thesis. All raw data that has appeared in publications can be found at the Open Data Commons for Spinal Cord Injury.

Chapter 2 and Appendix A: *Schmidt, E. K. A., Torres-Espin, A., Raposo, P. J. F., Madsen, K. L., Kigerl, K. A., Popovich, P. G., Fenrich, K. K., and Fouad, K. (2020b). Fecal transplant prevents gut dysbiosis and anxiety-like behaviour after spinal cord injury in rats. PLOS ONE, 10.1371/journal.pone.0226128*
I was responsible for the majority of the in vivo experiments, data analysis and interpretation, and manuscript preparation. Dr. Torres-Espin was responsible for the analysis of the microbiota data. Ms. Raposo assisted with the in vivo experiments and tissue processing. Dr. Madsen provided her expertise in microbiota research. Dr. Kigerl and Dr. Popovich also provided their expertise in microbiota research following spinal cord injury. Dr. Fenrich performed the spinal cord contusions. Dr. Fouad was the principal investigator and was involved in the concept of the project and surgical procedures. All authors reviewed and edited the manuscript. Data used for figure 2.7 was a separate experiment that did not appear in the published manuscript.

Chapter 3: *Schmidt, E. K. A., Raposo, P. J. F., Madsen, K. L., Fenrich, K. K., Kabarchuk, G., and Fouad, K. (2021). What makes a successful donor? Fecal transplant from anxious-like rats does not prevent spinal cord injury-induced dysbiosis. Medical Biology, 10.3390/biology10040254*

I was responsible for the majority of the in vivo experiments, tissue processing, data analysis and interpretation, and manuscript preparation. Ms. Raposo assisted with the in vivo experiments and tissue processing. Dr. Madsen's team assisted in the intestinal permeability assay and microbiota bioinformatics. Dr. Fenrich performed the spinal cord contusions. Ms. Kabarchuk analyzed the single pellet grasping videos. Dr. Fouad was the principal investigator and was involved in the concept of the project and surgical procedures. All authors reviewed and edited the manuscript.

Chapter 4 and Appendix C: *Schmidt, E. K. A., Raposo, P. J. F., Torres-Espin, A., K. A., Fenrich, K. K., and Fouad, K. (2021). Beyond the lesion site: minocycline augments inflammation and anxiety-like behavior following SCI in rats through action on the gut microbiota, 10.1186/s12974-021-02123-0*

I was responsible for the majority of data analysis and interpretation, manuscript preparation and parts of the in vivo experiments. Ms. Raposo was responsible for the majority of in vivo experiments and tissue processing. Dr. Torres-Espin was responsible for the analysis of the microbiota data. Dr. Fenrich performed the spinal cord contusions. Dr. Fouad was the principal investigator and was involved in the concept of the project and surgical procedures. All authors reviewed and edited the manuscript.

Chapter 5: *Schmidt, E. K. A., Raposo, P., Vavrek, R., and Fouad, K. (2020a). Inducing inflammation following subacute spinal cord injury in female rats: A double-edged sword to promote motor recovery. Brain, behaviour, and Immunity, 10.1016/j.bbi.2020.12.013.*

I was responsible for the majority of the in vivo experiments, tissue processing, data analysis and interpretation, and manuscript preparation. Ms. Raposo assisted with the in vivo experiments and tissue processing. Ms. Vavrek was responsible for a large part of the in vivo experiments. Dr. Fouad was the principal investigator and

was involved in the concept of the project and performed the surgeries. All authors reviewed and edited the manuscript. Data used for Figure 5.9 was obtained during a lab rotation (NEURO501) in Dr. Bennett's laboratory. For this project, Mr. Sanelli performed the spinal cord injuries, Ms. Black assisted in the EMG recordings, and Dr. Bennett was the supervisor involved in project conceptualization.

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Chapter 1

Introduction

1.1 Introduction to spinal cord injury

Spinal cord injury (SCI) has devastating physical, socioeconomic and vocational consequences. There are an estimated 12500 new cases of SCI each year in North America, and the lifetime cost of a SCI patient is an estimated 2.35 million dollars [Hachem et al., 2017]. The majority of these cases are traumatic SCI, which are caused by an external impact such as from motor vehicle accidents, falls, sports-related injuries and violence [Gedde et al., 2019]. Non-traumatic SCI is less common and occurs when an internal process (such as intrasprinal tumours, ischemia or infection) injures the spinal cord [Scivoletto et al., 2011]. SCI cases are classified as either functionally complete, meaning there is a complete loss of motor and sensory function below the level of injury, or functionally incomplete in which there is some residual function [Kirshblum et al., 2011]. The location and severity of the lesion determines the extent of deficits, with the cervical level being the most commonly affected (50-60% of cases) [Hachem et al., 2017]. Traumatic SCI is further broken down into the acute stage (less than 48 hours), the subacute stage (48 hours to 14 days), the intermediate stage (14 days to 6 months) and the chronic stage (over 6 months)

[Ahuja et al., 2017]. The primary injury (the initial mechanical insult to the spinal cord) damages neurons and glial cells, disrupts the vasculature and impairs the blood-brain barrier [Ahuja et al., 2017]. These immediate results of the primary injury initiate a secondary injury cascade of multiple cellular, molecular, and vascular events that can last for months to years after injury [Oyinbo, 2011, Anwar et al., 2016]. Spinal tracts below the levels of injury lose their brain-body connection through impairment of ascending sensory and descending motor pathways [Alizadeh et al., 2019]. In addition to the sensorimotor deficits caused by the primary and secondary injury events, SCI is also associated with various systemic complications that can greatly effect the well being of patients such as spasticity, pain, bladder and bowel dysfunction, sexual dysfunction, autonomic dysreflexia and mental health disorders [Adams and Hicks, 2005, Siddall and Loeser, 2001, Benevento and Sipski, 2002, Krassioukov et al., 2003, Post and van Leeuwen, 2012, Kennedy and Rogers, 2000]. Such system-wide consequences of SCI are not only detrimental to quality of life after injury but can also lead to decreased life expectancy and increased mortality rate [Soden et al., 2000, Savic et al., 2017]. Considering these multiple aspects of recovery following SCI is therefore vital to further understand the response to injury and promote comprehensive treatment options.

1.2 Therapeutic approaches to treat spinal cord injury

Although there is currently no cure for SCI, various therapeutic approaches exist to treat different aspects of SCI pathology. The majority of these interventions have been primarily studied in preclinical animal models, however numerous case reports and clinical trials are ongoing. Therapeutic interventions used in the acute period following SCI focus on promoting neuroprotection and mitigating secondary damage, largely

through attenuation of the acute inflammatory response (which will be discussed in more detail in section 1.3) [Ulndreaj et al., 2017, Lambrechts and Cook, 2020]. While reducing the spread of damage following the initial insult to the spinal cord is important, substantial research is also focused on regeneration and repair of severed axons to repair damaged pathways [Tsai and Tator, 2005]. Since CNS axons do not regenerate as well as peripheral nerve fibres, this area of research offers a particular challenge to scientists and clinicians [Zurn and Bandtlow, 2006, Chen et al., 2007]. While peripheral nerves spontaneously regenerate and re-innervate appropriate targets, central nervous system (CNS) axons retract and their growth cones collapse [Chen et al., 2007, Dontchev and Letourneau, 2003, Abe et al., 1999, Schwab, 1996]. The fact that the local CNS environment contributes to the limited ability of CNS axons to regenerate was first described in 1981 by David and Aguayo, pioneers in the field of CNS regeneration, who showed that CNS axons could regenerate in the injured spinal cord through peripheral nerve "bridges" [David and Aguayo, 1981]. This discovery inspired countless studies on promoting regeneration either by enhancing growth-promoting molecules (such as brain-derived neurotrophic factor (BDNF), mTOR, and neurotrophin (NT)-3) or by neutralizing growth-inhibitors (such as NOGO-A and chondroitin sulphate proteoglycans) [Keefe et al., 2017, Weishaupt et al., 2012, Kanno et al., 2012, Merkler et al., 2001, Bradbury et al., 2002]. In addition to axon regeneration, recovery following SCI can be due to plasticity within the CNS; plasticity is a term that encapsulates every adaptive change such as synaptic changes, sprouting of new connections, loss of existing connections, changes in cellular properties and cortical map reorganization [Onifer et al., 2011, Wieloch and Nikolich, 2006, Ding et al., 2005]. The ability of the nervous system to adapt as such is integral to recovery following SCI and other CNS injuries. In fact, injury to the CNS itself can enhance neural plasticity, which may involve interaction with the immune system

[Wieloch and Nikolich, 2006, Ding et al., 2005, O'Reilly and Tom, 2020]. However, plastic changes are not always beneficial and may have detrimental consequences such as neuropathic pain, spasticity, autonomic dysfunction and mental health disorders [Weaver et al., 2001, Brown and Weaver, 2012, Tan et al., 2012, Deumens et al., 2008, Kays et al., 2012]. Potential negative side effects of promoting plasticity are therefore an important caveat to consider when exploring therapeutic strategies.

1.3 The dual role of inflammation following spinal cord injury

Neutrophils are the first peripheral responder to SCI and enter the spinal cord within hours and are cleared rapidly (within days to weeks) [Kigerl et al., 2006]. The first central responders following SCI are the resident CNS cells [David and Kroner, 2011]. Microglia (the resident macrophages of the CNS), astrocytes and oligodendrocytes release proinflammatory cytokines and chemokines which trigger an inflammatory cascade including the recruitment of blood-borne monocytes to the lesion [David et al., 2012]. Within 3 to 7 days post-injury, blood-borne monocytes enter the spinal cord where they differentiate into macrophages and adopt a phenotype almost indistinguishable from activated microglia (often referred to as microglia/macrophages) [David and Kroner, 2011]. This acute inflammatory response at the lesion coincides with increased blood-brain-barrier permeability within the first week of SCI [Whetstone et al., 2003]. Vascular permeability is further enhanced upon upregulation of pro-inflammatory cytokines such as TNFα and IL-1β [Pan et al., 2011]. In addition to the release of cytokines, activated neutrophils and microglia/macrophages can release free radicals, neurotoxic enzymes, eicosanoids, nitric oxide and proteases that can cause

further cell death [Brady et al., 2006, Chandler et al., 1995, Chao et al., 1992, Liu et al., 2006, Liu et al., 2006, Shamash et al., 2002]. Within weeks, reactive astrocytes in addition to microglia and oligodendrocyte precursors form to create a physical and molecular barrier around the lesion known as the glial scar [Leal-Filho, 2011, Yuan and He, 2013]. For decades, the glial scar was thought to be a primary inhibitor of SCI recovery. Indeed, reactive astrocytes in the glial scar release various axonal growth inhibiting proteins such as chondroitin sulfate proteoglycans, which, when degraded, can promote substantial axonal growth following SCI [Bradbury and Carter, 2011, Fouad, 2005, Yang et al., 2020].

Multiple methods to inhibit the inflammatory response in acute SCI have had some beneficial yet controversial outcomes. Methylprednisolone is a glucocorticosteroid which has anti-inflammatory and immunosuppressive activity [Short et al., 2000]. It has been shown to protect against secondary injury, inhibit the release of interleukins, and improve neurological recovery in preclinical animal models [Akhtar et al., 2009]. Given these promising results, Methylprednisolone treatment for acute SCI was evaluated in multiple clinical trials and became a standard of care in many hospitals throughout the United States [Bracken et al., 1990, Bracken, 1991]. However, given the severe side effects (such as increased infections, gastrointestinal hemorrhages, sepsis and pneumonia), absence of reproducible results, and inappropriate use of statistical tests, steroid treatment for SCI is no longer recommended [Short et al., 2000, Hurlbert, 2000]. An anti-inflammatory drug with much less side effects and a long safety record in humans, minocycline, has more recently been considered for acute SCI therapy. Minocycline, an antibiotic tetracycline derivative, has been shown to reduce lesion size and promote functional recovery in preclinical models of SCI, attributed to the drug's direct anti-inflammatory and microglial inhibiting properties [Wells, 2003a, Lee et al., 2003, Stirling, 2004, Shultz and Zhong, 2017, Festoff et al., 2006]. Re-

sults from phase I/II clinical trials suggest that minocycline produces modest, yet insignificant improvements for patients with acute SCI [Casha et al., 2012]. Another anti-inflammatory therapeutic target that has displayed beneficial effects in preclinical models of SCI is Etanercept, a TNFα inhibitor. Several experimental studies in rodents showed that Etanercept attenuated inflammation, reduced tissue injury, and promoted functional motor recovery after SCI [Genovese et al., 2006, Marchand et al., 2009, Esposito and Cuzzocrea, 2011, Chen et al., 2011]. A myriad of other studies have reported neuroprotective effects of anti-inflammatory treatment for SCI [Bao et al., 2004, Mabon et al., 2000, Zhou et al., 2009, Song et al., 2015, Machova Urdzikova et al., 2015, Arnold and Hagg, 2011], however clinical trials have been less successful [Casha et al., 2012, Bracken, 1991]. Although inflammation is a contributor to secondary damage following SCI, it is too simplistic to conclude that inflammation is inherently detrimental to recovery. In fact, a proper immune response is critical to promote healing [Koh and DiPietro, 2011], and as we will see throughout this thesis, can play a dual role in the recovery following SCI.

A straightforward example of the dichotomous role of inflammation is the polarization model of macrophage function. Although criticized for being an oversimplification (a fact the original authors admit to), this models provides a useful framework for considering different qualities of inflammation [Mills et al., 2000]. This concept was first proposed by Mill et al., and suggested two distinct macrophage groups (designated M1 and M2) that influence whether a Th1 (release of pro-inflammatory cytokines) or Th2 (release of anti-inflammatory cytokines) inflammatory response occurs [Mills et al., 2000]. Both subsets of macrophages are present at the lesion site within the first week following SCI, however only M1 macrophages persist for longer [Kigerl et al., 2009]. M2 macrophages have been associated with neuroprotection and regeneration of spinal tissues after injury, whereas M1 macrophages generally are considered neurotoxic [Kigerl et al., 2009, Kong and Gao, 2017]. As mentioned pre-

viously, this generalization is likely overly simplistic and more recent views suggest a spectrum of macrophage activation [Nahrendorf and Swirski, 2016]. Evidence of the reparative properties of macrophages comes from optic nerve injury. Oncomodulin, a macrophage-derived growth factor, plays a critical role in retinal ganglion cell regeneration following optic nerve injury [Yin et al., 2009, Yin et al., 2006, Yin et al., 2003]. Macrophages have also been associated with recovery after SCI, since transplantation of peripheral nerve stimulated macrophages into the injured spinal cord promoted tissue repair and motor recovery [Schwartz et al., 1999b, Rapalino et al., 1998]. Activated macrophages and microglia may promote tissue repair and protection via release of various neurotrophic factors (including NGF, BDNF and NT-3), modulating glutamate excitotoxicity, and clearing myelin debris in the lesioned environment [Dougherty et al., 2000, Chen et al., 2008, Krenz and Weaver, 2001, Vinet et al., 2012]. Further evidence on the link between inflammation and plasticity has been shown in a chronic model of SCI, where inducing inflammation systemically with lipopolysaccharide (LPS) augmented sprouting of corticospinal tract (CST) axons and enhanced motor recovery [Chen et al., 2008, Torres-Espín et al., 2018a]. These promising preclinical results highlight the therapeutic potential of augmenting the beneficial aspects of neuroinflammation following SCI. However, because of the dichotomous role that inflammation can play on damage and repair [Hohlfeld et al., 2007], further research on the complex interaction between the immune and nervous systems is required to optimize the therapeutic potential while mitigating damage.

1.4 Systemic inflammation and chronic immune suppression

The CNS and immune systems are integrated to regulate homeostasis [Marques et al., 2016]. These two complex systems functionally communicate via the autonomic nervous system and the hypothalamic-pituitary-adrenal (HPA) axis [Dantzer, 2018]. The autonomic nervous system consists of the parasympathetic nervous system (PNS), the sympathetic nervous system (SNS), and the enteric nervous system [Waxenbaum et al., 2020]. The enteric nervous system that controls gastrointestinal processes has extensive two-way communication with the CNS, but is capable of autonomous functions and thus not directly affected following SCI [Waxenbaum et al., 2020]. SCI can directly and indirectly disrupt the function of the autonomic nervous system, which can have profound effects on the immune system. Not only does SCI increase local inflammation within the spinal cord [Popovich et al., 1997], there is also an increase in systemic inflammation as indicated by elevated concentrations of various pro-inflammatory cytokines in circulation [Bloom et al., 2020, Hayes et al., 2002]. However, in certain cases SCI can lead to a paradoxical chronic immune suppression [Riegger et al., 2007]. Following upper thoracic and cervical SCIs, sympathetic preganglionic neurons of the SNS are disconnected from supraspinal control. Thus, when something happens below the level of injury (e.g., bladder extension, constipation, or simply a sun burn), an automatic reaction of the sympathetic nervous system ensues, resulting in increased blood pressure, release of norepinephrine from lymphoid organs and glucocorticoids from the HPA axis [Weaver et al., 2006, Eldahan and Rabchevsky, 2018]. Furthermore, physical or psychological stress in general can activate the HPA axis, leading to additional glucocorticoid release [Tsigos and Chrousos, 2002]. In the case where preganglionic neurons have been disconnected from supraspinal

control, this response is unable to be properly modulated by the CNS and is termed autonomic dysreflexia [Weaver et al., 2006]. The result of excessive or uncontrolled circulating catecholamines can lead to immune suppression and subsequent risk for infection [Zhang et al., 2013]. Non-neurogenic mechanisms of immune suppression following CNS damage have also been proposed, including the compensatory anti-inflammatory response syndrome following a systemic inflammatory response [Adib-Conquy and Cavaillon, 2009, Meisel et al., 2005]. Regardless of the cause, chronic immune suppression significantly impacts the well being of SCI patients since acute infection is the leading cause of death following SCI [DeVivo et al., 1989, Thietje et al., 2011].

1.5 Gut microbiota and the gut-brain axis

A fundamental and interconnected relationship exists between the immune system and the gut microbiota [Belkaid and Hand, 2014]. Microbiota is the term used to describe the complex community of microorganisms that inhabits the surface of the body, and in this thesis will be focusing on the bacterial community within the lower intestine. The microbiota degrades dietary substances to enhance host metabolic efficiency while supplying nutrients to the microbes [Belkaid and Harrison, 2017]. Over millions of years, this symbiotic relationship has evolved past simple digestive efficiency to a complex and poorly understood microbiota-host interaction [Bercik et al., 2012]. The host immune system has co-evolved with the microbiota to protect the host from pathogens (which can have health implications from inflammation to sepsis) and to foster a diverse microbial community for various metabolic benefits [Lee and Mazmanian, 2010, Fung et al., 2017]. One of the earliest advancements in the study of the gut came in the 1800's when a gunshot wound to Canadian fur-trader, Alexis St. Martin, left him with a fistula, or open window,

into his intestines [Beaumont, 2009]. At the cost of discomfort to his patient, his doctor William Beaumont took this opportunity to study digestion in real time by placing food in St. Martin's stomach and examining it later. Beaumont also made the fascinating observation that the emotional state of his patient greatly affected digestion [Beaumont, 2009]. Ivan Pavlov, famous for his studies on classical conditioning, was directly inspired by Beaumont's work and scientific method. Together with Carl Ludwig they developed the Pavlov pouch, an externalized intestine to study dog digestion (believed to be one of the first chronic animal experiments). In these studies, Pavlov described the cephalic phase of digestion in which sensory perception of food triggers gastric secretions [Wood, 2004]. An early pioneer in the concept that host microbial partners from the gut can influence human health was Élie Metchnikoff. Although perhaps best known for describing phagocytosis in 1883, Metchnikoff believed that toxic bacteria were the cause of aging and therefore drank sour milk every day to prolong his life. Although Metchnikoff died at the age of 71, his probiotic consumption corresponded with improvements in his mood [Underhill et al., 2016]. Observations from scientists such as Beaumont, Pavlov and Metchnikoff alluded to the existence of a "gut-brain-axis". Although the earlier work on the gut-brain-axis focused on digestion and satiety, more recently higher-order processes have been considered, such as neurological and psychiatric conditions, which will be discussed further in section 1.6.

The mechanisms of how the gut microbiota influences the CNS and host behaviour remain largely unknown, however there are multiple potential pathways involved. The bidirectional communication of the gut-brain axis involves the brain, spinal cord, ANS, immune system, and the HPA axis [Cryan et al., 2019] (Fig. 1.1). Gut microbiota interact with the gut-brain axis through modulation of the intestinal barrier, producing local neurotransmitters (for example GABA, serotonin, melatonin and histamine), producing short-chain-fatty-acids, and influencing

mucosal immune activation [Bercik et al., 2012]. The vagus nerve, which makes up the majority of the PNS branch of the ANS, is the fastest and most direct route of communication between the gut and the brain [Bonaz et al., 2018]. A critical non-neuronal route of communication between the gut and the CNS is the HPA axis [Carabotti et al., 2015]. In response to physical or psychological stress, the paraventricular nucleus of the hypothalamus secretes corticotropin-releasing hormone, which initiates the release of adrenocorticotropic hormone from the anterior pituitary into circulation. Once in the bloodstream, this hormone travels to the adrenal cortex of the adrenal glands, leading to the release of glucocorticoids [Herman and Seroogy, 2006, Tsigos and Chrousos, 2002]. Glucocorticoids can have a variety of effects on the body, most notably the "fight or flight" response (in addition to suppression of the immune system), but also initiate a negative feedback loop to inhibit further glucocorticoid release [Gjerstad et al., 2018]. A link between the microbiota and the HPA axis was first shown in germ free mice, who are reared in sterile environments and thus lack a microbiota [Yi and Li, 2012]. Germ-free mice have impaired innate lymphoid cell and organ function, reduced production of interleukins, immature microglia phenotype, and are generally more susceptible to infection, all of which highlight the importance of the gut microbiota in proper immune function [Round and Mazmanian, 2009, Sprinz et al., 1961, Östman et al., 2006, Kennedy et al., 2018]. In response to stress there is an increase in gut permeability (or "leaky gut"), which can cause the translocation of bacteria and bacterial matter (such as LPS) across the epithelial barrier and trigger an immune response, which ultimately activates the HPA axis [Söderholm et al., 2002, Ilchmann-Diounou and Menard, 2020, Ghosh et al., 2020, de Punder and Pruimboom, 2015]. In germ free animals, this HPA response to stress is exaggerated, indicated by elevated plasma corticosterone [Sudo et al., 2004, Neufeld et al., 2011]. Although there is still much to learn about

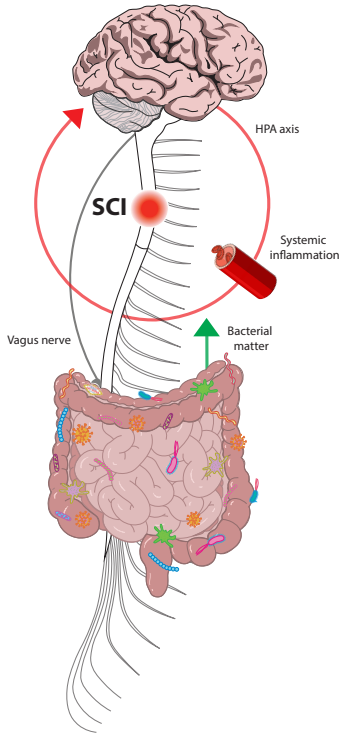

Figure 1.1: Microbiota-immune axis following spinal cord injury

the gut-brain axis, there is clearly a strong link between the function of the microbiota and the host nervous and immune systems, and alterations of this homeostasis can cause or exacerbate various diseases and disorders.

1.6 Gut microbiota involvement in CNS diseases and disorders

A disturbance to the composition of the microbiota, termed dysbiosis, causes intestinal inflammation and has been linked to irritable bowel syndrome and inflammatory bowel disease [Kaur et al., 2011, Chassard et al., 2012]. More recently, the role of the microbiota in the etiology of diseases involving organs distal to the intestines has been explored. As a striking example, germ-free mice do not develop symptoms

of spontaneous experimental autoimmune encephalomyelitis (EAE, a widely used animal model of multiple sclerosis) [Lee et al., 2011]. Furthermore, administration of the probiotic *Lactobacilli* has been shown to alleviate symptoms of EAE through regulation of cytokine responses [He et al., 2019]. The role of the microbiota has also been suggested in autism spectrum disorder based on observations that children with autism have imbalanced proportions of various intestinal bacteria [Pulikkan et al., 2019, Pulikkan et al., 2018]. Psychiatric disorders in general have been widely studied for their strong connection to both the microbiota and inflammation [Carlessi et al., 2021]. Multiple studies in both humans and animals have correlated depression and anxiety symptoms with gut dysbiosis and increased systemic inflammation [Stevens et al., 2018, Sun et al., 2019, Cheung et al., 2019, Luo et al., 2018]. Probiotic treatment, particularly those containing species in the *Lactobacillus* genus, has shown efficacy for treatment of depressive-like symptoms in animals and humans [Desbonnet et al., 2010, Pinto-Sanchez et al., 2017, Pirbaglou et al., 2016, Rudzki et al., 2019, Hadizadeh et al., 2019, Chong et al., 2019]. The link between microbiota and mental health was clearly shown in a study by Li et al. in which mice received a fecal microbiota transplant (FMT) from control mice or mice with high levels of anxiety-like and depressive-like behaviour. The FMT not only colonized the recipient mice with a microbiota composition similar to the donors, but the recipient mice also adopted the behaviours of the donors [Li et al., 2019]. That is, mice that received the FMT from anxious- and depressive-like donors displayed increased levels of anxiety-like and depressive-like behaviours themselves [Li et al., 2019]. Furthermore, these mice also displayed increased inflammatory cytokines and upregulation of indoleamine 2,3-dioxygenase 1 (IDO1) in the hippocampus [Li et al., 2019]. A similar and just as fascinating study by Kelly et al. showed that FMT from humans diagnosed with major depressive disorder (MDD) to rats induced depressive-like and anxiety-like behaviours in the recipient animals

[Kelly et al., 2016]. Results from studies such as these suggest that the microbiota is involved in the development of depression and anxiety, which likely involves interaction with the host immune system. Although gut dysbiosis has been suggested as a contributing factor to the development of mental health disorders, this is obviously not the case for traumatic CNS injuries (for which gut dysbiosis is a symptom and not the cause). However, recent evidence suggests that dysbiosis may be a disease modifying factor in stroke since treating dysbiosis with probiotics or FMT improved stroke outcome in animal experiments [Chen et al., 2019, Akhoundzadeh et al., 2018]. Similarly in experimental SCI research, Kigerl et al. recently showed that treatment with probiotics improved locomotor recovery and reduced the lesion size following a thoracic SCI in mice [Kigerl et al., 2016a]. Although the interaction between the microbiota and CNS diseases and disorders is a novel and constantly evolving field, the microbiota offer extraordinary therapeutic potential that deserves further exploration.

1.7 Mental health after spinal cord injury

Early research on psychological disorders following SCI considered depression to be a necessary part of the grieving process in order to cope with the injury [Holmes, 1975, Boekamp et al., 1996]. More recently, research has supported that not all individuals with a SCI develop depression or anxiety disorders, with an estimated prevalence around 30% [Kennedy and Rogers, 2000]. Although studies report that most individuals with a SCI do not develop diagnosable psychological disorders, the rates of depression and anxiety are still significantly higher than the general population [the SCIRE Research Team et al., 2009]. There has been extensive research investigating mood disorders following SCI in the human population. Although multiple meta-analyses and longitudinal studies show a

significant and clear increased risk of mental health disorders following SCI, the etiology and mechanisms underlying comorbid psychological disorders has not been elucidated [the SCIRE Research Team et al., 2009, Kennedy and Rogers, 2000, Bonanno et al., 2012, Craig et al., 2009]. Some studies report that depression and anxiety scores decrease with time after injury and particularly after discharge, whereas others report that there is little resolve of these disorders over time [Craig et al., 1994a, Hancock et al., 1993, Kennedy and Rogers, 2000, Cairns et al., 1996]. Not surprisingly, although perhaps under appreciated, is the consensus that individuals with comorbid mood disorder following SCI have less adaptive coping strategies, poorer subjective health, more difficulty in daily functioning, increased risk of developing pressure ulcers, urinary tract infections and cognitive impairment [Bombardier et al., 2004, Bonanno et al., 2012, Malec and Neimeyer, 1983, Craig et al., 2017, Krueger et al., 2013, Herrick et al., 1994, Dorsett and Geraghty, 2004]. Given the detrimental impact of mood disorders on both psychological and physical recovery following SCI, it is concerning that mood disorders are both under appreciated and under treated in adults with SCI [Fann et al., 2011].

Experimental animal studies have confirmed the clinical data indicating an increased prevalence of anxiety-like and depressive-like behaviours following SCI [Brakel and Hook, 2019, Luedtke et al., 2014]. Furthermore, given the complicated nature of mental health, animal studies exclude socioeconomic factors and provide the opportunity to study neurophysiological factors contributing to mental health disorders. In humans, the diagnostic and statistical manual of mental disorders is used by healthcare providers to classify and diagnose psychiatric disorders including MDD and generalized anxiety disorder (GAD) [American Psychiatric Association, 2013]. The criteria for MDD is to display at least 5 of the following symptoms: depressed mood, loss of interest/pleasure (known as anhedonia), weight loss or gain, changes in sleep, fatigue, psychomotor agitation/retardation, feeling worthless, decreased concentra-

tion, and thoughts of suicide or death [American Psychiatric Association, 2013]. GAD is highly comorbid with MDD with the primary symptom being excessive worry in addition to restlessness, fatigue, impaired concentration, irritability, difficulty sleeping and muscle tension [American Psychiatric Association, 2013]. Of course, it is impossible to ask a rodent how they are feeling or diagnose them with a human mental health disorder. Instead, scientists use sensitive behavioural tests to measure different aspects of depression and anxiety, and subsequently determine whether the animals exhibit various "depressive"-like or "anxiety"-like behaviours. Common tests for depressive-like behaviour in rodents include the forced-swim test, tail suspension test, sucrose preference test (SPT), and social interaction test [Yankelevitch-Yahav et al., 2015, Castagné et al., 2011, Willner et al., 1987a, D'Aquila et al., 1994]. Common tests for anxiety-like behaviours in rodents include the open-field test, elevated plus maze (EPM) and light/dark box (LDB) [Hogg, 1996, Seibenhener and Wooten, 2015, Kulesskaya and Voikar, 2014]. In this thesis, the forced-swim test was not used as it can cause excessive stress, and the tail suspension test was not used as it is only appropriate in mice due to their small size [Can et al., 2011].

1.8 Incomplete cervical spinal cord injury

The model of spinal cord injury used in this thesis is a dorsal unilateral incomplete cervical contusion or transection injury. Not only are incomplete cervical injuries the most common SCI and therefore clinically relevant to study [Hachem et al., 2017], but this injury model does not significantly affect gross locomotion and long-term motor deficits are only detected with sensitive skilled reaching tasks [Siegenthaler et al., 2007, García-Alías et al., 2015]. This is important for the interpretation of many of the sensitive behavioural tests used throughout this

thesis which rely on the rat's locomotor abilities. The incomplete cervical injury model damages the corticospinal tract (CST) as well as parts of the rubrospinal tract, reticulospinal tract and grey matter, ultimately leading to impairments in skilled reaching of the ipsilesional paw [Lawrence and Kuypers, 1968]. Cell bodies of the CST predominantly originate in the motor cortex and descend to the pyramids in the medulla. There, 90% of the axons decussate and travel through the contralateral ventral part of the dorsal column (in rodents) and 10% remain ipsilateral and form the anterior corticospinal tract [Canty and Murphy, 2008]. The majority of CST axons innervate the grey matter of the cervical spinal cord where they synapse onto motor neurons to orchestrate skilled movements of the forelimbs [Asanuma and Rosen, 1972, Kleim et al., 1998, Rasmussen and Penfield, 1947]. Although the CST is critical for fine motor control of the hand in primates, rats display a great amount of recovery when the CST is lesioned [Whishaw et al., 1998, Kanagal and Muir, 2008, Kanagal and Muir, 2009]. This recovery of fine motor skills may be due to plasticity of CST axons or compensation of spared descending motor pathways [Fouad et al., 2001, Oudega and Perez, 2012, García-Alías et al., 2015]. In the rat, the rubrospinal tract likely plays a more important role than it does in humans and, if spared, may compensate for loss of function following damage to the CST [Nathan and Smith, 1982, Whishaw et al., 1998, Raineteau et al., 2001]. The reticulospinal tract is an anatomically diverse and somewhat poorly defined bundle of axons which originates in the gigantocellular zone of the reticular formation and descends in the ventral and lateral white matter [Wang, 2009]. Given its location, the reticulospinal tract is at least partially preserved following an incomplete dorsal SCI and may serve as a relay for injured CST neurons [García-Alías et al., 2015, Ballermann and Fouad, 2006]. Promoting regeneration or plasticity of CST axons and other descending motor systems using various cell transplantation techniques, increasing growth-promoting molecules, inhibit-

ing growth inhibitors, or simply through rehabilitative training has been widely studied in experimental SCI [Girgis et al., 2007, Fouad, 2005, Merkler et al., 2001, Li and Lepski, 2013, Weishaupt et al., 2012]. Often, therapeutic interventions are most (or only) effective when combined with rehabilitation [Weishaupt et al., 2013, Torres-Espín et al., 2018a, García-Alías et al., 2009, Kubasak et al., 2008], which is why rehabilitative training is included in this thesis.

1.9 Chapter aims

The overall aim of this thesis is to investigate multiple systemwide consequences of SCI. Although we do aim to enhance motor recovery of the ipsilesional forepaw, this is not the only goal and I believe that it is important to consider the whole body and mind when examining potential therapeutics for CNS injuries or diseases.

In chapter 2 our aims were two fold. First, we wanted to determine whether using a (relatively) mild cervical SCI would elicit changes in anxiety-like behaviour as well as changes in the gut microbiota. Second, given the link between dysbiosis and mental health disorders in uninjured populations, we aimed to determine whether there is a link between gut dysbiosis and the development of anxiety-like behaviour following SCI using a fecal transplant from uninjured donors. Next, in chapter 3 we wanted to determine whether optimal donor selection would influence the efficacy of FMT for SCI treatment. In chapter 4 we again manipulate the microbiota following SCI, however this time using minocycline. Although minocycline has been widely studied for its anti-inflammatory properties, its impact on the gut microbiota and systemic inflammation following SCI is unknown. Furthermore, in chapters 3 and 4 we monitor systemic inflammatory markers over time following SCI to evaluate both acute and chronic changes and how treatments targeting the gut microbiota alter systemic inflammation. Finally in chapter 5, we investigate the dual role that inflammation

can play by systemically injecting the bacterial product LPS in the sub-acute time point following SCI to determine what effect this has on rehabilitative training. In each experiment, we assess not only lesion size and functional recovery, but (perhaps just as important), we monitor anxiety-like and depressive-like behaviours as well.

Chapter 2

Fecal transplant prevents dysbiosis and anxiety-like behaviour following spinal cord injury [1]

2.1 Introduction

In addition to physical and sensory impairments, SCI is associated with an increased prevalence of anxiety and depression, and a reduced quality of life [Lim et al., 2017, Kennedy and Rogers, 2000]. As a result, suicide is a leading cause of death following SCI [Thietje et al., 2011, DeVivo et al., 1989]. It is therefore crucial to determine safe and effective treatments, or preferably prophylactic strategies, to improve mental well-being following SCI. To do this, the link between SCI and affective disorders must be further elucidated. Given the drastic lifestyle changes and complications such as pain and autonomic dysfunction associated with SCI, it is likely that psychosocial factors are involved in the etiology of depression and anxiety after injury [Post and van Leeuwen, 2012]. However, evidence suggests that biological changes

[1] This chapter has appeared in "Fecal transplant prevents gut dysbiosis and anxiety-like behaviour after spinal cord injury in rats" [Schmidt et al., 2020b]

caused by central nervous system injury can also contribute to the development of mood disorders [Fenn et al., 2014, Maldonado-Bouchard et al., 2016a].

Research in animal models confirmed the association between SCI and the prevalence of mood disorders observed in humans. After a thoracic spinal contusion, Luedtke et al. showed that rats displayed various depressive-like behaviours, which were reversed by treatment with the antidepressant Fluoxetine [Luedtke et al., 2014]. These depressive-like behaviours following SCI have been associated with increased inflammation [Luedtke et al., 2014, do Espírito Santo et al., 2019]. Outside of SCI research, depression and anxiety have also been associated with pathological alterations of the gut microbiota (dysbiosis) [Dantzer et al., 2008, Foster and McVey Neufeld, 2013]. Microbiota changes have recently been shown after SCI in both human and rodent studies [Gungor et al., 2016, Kigerl et al., 2016a, O'Connor et al., 2018]. In mice, dysbiosis caused by a severe thoracic SCI was associated with increased intraspinal inflammation and reduced functional recovery, both of which could be reversed with chronic oral probiotic treatment [Kigerl et al., 2016a]. Given the profound effect that intestinal dysbiosis can have on behaviour, the microbiota may be a key player in reduced mental well-being following SCI. However, it is currently unknown whether SCI-induced gut dysbiosis is involved in the etiology of anxiety and depression following SCI.

In the present study, we show that an incomplete unilateral cervical SCI induces dysbiosis and long-term changes in anxiety-like behaviours. This model of SCI has negligible effect on the rat's locomotor ability, thus minimizing the effect of reduced locomotion on behavioural outcomes. A relationship between anxiety-like behaviour and dysbiosis was demonstrated by administering a fecal microbiota transplant (FMT) at the time of injury and for two consecutive days after SCI. The FMT attenuated gut dysbiosis and alleviated SCI-induced anxiety-like behaviour. The

present study shows for the first time that the development of anxiety-like behaviour after SCI in a rodent model is linked to gut dysbiosis.

2.2 Methods

2.2.1 Animals

All animal use was approved by the animal care and use committee for Health Sciences at the University of Alberta and complies with the Canadian Council for Animal Care and ARRIVE guidelines. Experiments were performed using adult female Lewis rats (Charles River Laboratories, Montreal, QC Canada) weighing 180 to 220 g. Upon arrival, rats were handled daily (5 min per rat) for one week prior to behavioural testing. Rats were group housed (5-6 rats per cage) and kept in a 12 h light/dark cycle (lights on at 08:00 h) with ab libitum access to standard chow and water. Experimental groups were housed separately to avoid cross-colonization by coprophagia. Behavioural testing and analyses were performed by an experimenter blind to the group assignment. The first experiment consisted of two groups: (1) a sham operated group (n = 6) and (2) a group that received a cervical contusion SCI (n = 6). For the second experiment, two cohorts of rats were used and randomly divided into four experimental groups (n = 45): (1) healthy (n = 10); (2) sham (n = 11); (3) SCI with control gavage (n = 10); and (4) SCI with FMT (n = 14). Both cohorts underwent identical experimental conditions but only the second cohort of rats were used for the 16s rRNA gene analysis (healthy n = 10, SCI-FMT n = 10, sham n = 5, SCI n = 5). The healthy group served as the donor animals for the FMT.

2.2.2 Surgical procedures

Surgeries were conducted under isoflurane anesthesia (5% for induction; 2.5% for maintenance) supplied with a 50:50 air/oxygen mixture. The dorsal neck was shaved

and disinfected with 10% chlorhexidine digluconate. Eye drops were applied to prevent corneal dehydration and body temperature was maintained with a heating blanket. The cervical vertebra was exposed and a laminectomy of C5 was performed. The animal was placed in the Infinite Horizons impactor (Precision Systems & Instrumentation, Lexington, KY) at an angle of 15 degrees using a customized frame to induce a unilateral injury on the right side (1.25mm off of midline). The impactor tip was lowered until just in contact with the spinal cord, raised 2.5 mm, and the force was set at 125 kdyns (with the mean measured force of 137.65 kdyn, the mean displacement of 1015.7 μm and a velocity of 124.04 mm/s). Muscles were sutured with 5-0 vicryl and the skin closed 9 mm stainless steel clips. Buprenorphine was injected s.c. immediately post-op (0.03 mg/kg) and again 8-12 hours later (0.02 mg/kg) for analgesia. Animals were hydrated with 4 ml saline s.c. immediately postoperatively and a 2 ml dose the day after surgery. Bladders were manually expressed when necessary (evidence of wet abdomen and full bladder) until voiding was re-established.

2.2.3 Behavioural testing

Behavioural testing was performed during the light cycle (08:00 - 20:00 h). With the exception of the cylinder test (as this was a measure of physical function and not anxiety-like behaviour), behavioural testing did not take place within 24 hours of fecal collection to avoid any potential interaction between these tests and performance outcome. Behavioural apparatuses were cleaned between sessions with odourless detergent and dried with paper towel. The light-dark box, elevated plus maze and open field tests were never performed on the same day to avoid interference between these outcome measures.

Light-dark box

The light-dark box was made of white and black opaque Plexiglas (21×21×21 cm light chamber, 21×21×21 cm dark chamber). The light (100 lux) and dark (0 lux) chambers were connected by a 7x7 cm door. Animals were placed in the middle of the dark chamber facing away from the door and allowed to freely explore the chambers for 10 minutes while video recorded from above. Offline video analysis was performed to analyze the time spent and latency to enter the light chamber. Increased number of entries and time spent in the light chamber as well as decreased latency to enter the light chamber is associated with decreased anxiety-like behaviour [Bourin and Hascoët, 2003]. This test was performed once before SCI, then again 1 and 4 weeks after SCI.

Cylinder test

The cylinder test is used to measure forelimb asymmetry following unilateral injuries in rodents [Schallert et al., 2000, A Geissler, 2013]. Rats were placed in a transparent Plexiglas cylinder (21x25 cm) and recorded as they explore the vertical environment with their forepaws for three minutes or a minimum of ten rears. The number of left and right paw placements on the cylinder wall were recorded at baseline, 1 and 3 weeks after injury. Any contralateral bias in forepaw placements (i.e., increased reliance on the uninjured paw) is associated with physical deficits and expressed as a percentage of right paw placements.

Sucrose preference test

Rats had access to two bottles in their home cage (same treatment group per cage), one with water and the other with a 2% sucrose solution. The amount of sucrose solution or water consumed over 48 hours was determined by weighing the bottles. The location of the bottles was switched at 24 hours to avoid any side preference.

Decreased consumption of the sucrose solution is associated with anhedonia-like behaviour (a symptom of depression in humans) [Willner et al., 1987b]. This test was performed twice before SCI and weekly for 3 weeks thereafter. The first week of the sucrose preference test was used to allow the rats to acclimatize to the sugar water and was thus excluded from analysis. Sucrose consumption was analyzed as a percent of total fluid consumed over 48 hours and expressed as a percent change from baseline values.

Elevated plus maze

3 weeks after SCI, rats were placed in the junction of two open arms and two closed arms, facing towards an open arm and allowed to explore the arena for ten min (100x100 cm and elevated 65 cm above ground). Percent time spent and entries into the open and closed arms as well as the total distance travelled were recorded from above as measures of anxiety-like behaviour [Pellow et al., 1985]. This test was used only once to avoid habituation to the maze, known as "one-trial tolerance" [Albrechet-Souza et al., 2009, Bertoglio and Carobrez, 2002, File et al., 1990, Rodgers and Shepherd, 1993, Zhou et al., 2015]. Offline video analysis was performed using customized software.

Open field

Rats were placed in the center of a rectangular Plexiglass enclosure 100×80x30 cm) and videotaped from above for five minutes as they freely explored the arena [Prut and Belzung, 2003]. Customized motion-tracking software was used to measure the distance traveled and the percentage of time spent in the inner 60% of the arena versus the periphery (along the wall and in corners). This test was performed once before, 1 and 3 weeks following surgery.

2.2.4 Fecal collection

Fecal pellets were collected during the dark cycle to ensure the fastest defecation time. No fecal collection occurred within 48 hours of the sucrose preference test. Fecal pellets were immediately collected in individual sterile eppendorf tubes and stored at -80°C until further processing. For bacterial cultures, fecal pellets were collected weekly before and after injury. For 16s rRNA sequencing, fecal collection was performed at three time points: one week prior to injury, 3 days post-injury and 4 weeks post-injury. For the microbiota transplant, fecal pellets were immediately collected from healthy donor rats and processed to make a slurry solution.

2.2.5 Bacterial culture

Stool samples were homogenized in phosphate-buffered saline (PBS), filtered to 40 μm and diluted to 104 in PBS. 100 μl of solution was plated on CHROMagar orientation plates and incubated at 37 °C for 48 hours.

2.2.6 Fecal microbiota transplant

Fresh fecal matter from healthy uninjured rats was diluted to a concentration of 1:10 in PBS (10%), L-cysteine HCL (0.05%), glycerol (20%) and sterile water (60%) and filtered to remove fiber content (with a filter size of 100 μm). Vehicle treated rats (Sham and SCI groups) received a filtered solution without fecal content. All rats (with the exception of the Healthy group) were fed via an oral gavage once a day on the day of injury and for 2 days after with 500 μl of either the fecal slurry or control solution. Although less common than the lower gastrointestinal tract route, upper gastrointestinal tract administration of an FMT has also been proven effective in humans [Chapman et al., 2016, Kassam et al., 2013].

2.2.7 Fecal transplant from SCI rats into uninjured rats

A total of 10 rats were used for this experiment (Uninjured n = 5, Uninjured + FMT n = 5). Fecal matter was collected from rats 3 days post SCI and processed to make the transplant solution as described above. 3 weeks following the FMT rat behaviour was assessed in the light-dark box, EPM and open field.

2.2.8 16s rRNA analysis

Frozen fecal samples were shipped on dry ice to Microbiome Insights Inc. (Vancouver, Canada) for sequencing and bioinformatics. Bacterial 16S rRNA gene V4 amplicons from fecal samples were generated on an Illumina MiSeq and quality-filtered and clustered into 97% similarity operational taxonomic units (OTUs) using the mothur software package [Schloss et al., 2009]. 1.558896 x 106 high quality reads were obtained and the resulting dataset had 39427 OTUs with a read range of 3423 and 3.4401 x 104. The potential for contamination was addressed by co-sequencing DNA amplified from fecal samples and from four each of template-free controls and extraction kit reagents processed the same way as the samples. Two positive controls, consisting of cloned SUP05 DNA, were also included (number of copies = 2*106). OTUs were considered putative contaminants and removed if their mean abundance in controls reached or exceeded 25% of their mean abundance in specimens.

2.2.9 Perfusion and lesion analysis

All rats were euthanized 5 weeks post-SCI with a lethal dose of Sodium Pentobarbital (100mg/kg) and transcardially perfused with saline containing 0.02 g heparin/l followed by 4% paraformaldehyde (PFA) in 0.1M phosphate-buffered with 5% sucrose as fixative. Spinal cords were removed, post-fixed in 4% PFA overnight at 4 °C and cryoprotected in 30% sucrose for 5 days. 1 cm cervical spinal cord blocks with the

lesion in the center were embedded in O.C.T., mounted onto filter paper and frozen in 2-methylbutane (-40 °C). Serial cross sections of the spinal blocks were cut at a thickness of 25 μm on a NX70 cryostat (Fisher Scientific), staggered across eight sets of slides and stored in a -20 °C freezer until further processing. Two sets of slides were stained with 0.5% cresyl violet and imaged under a light microscope to analyze lesion size. Total lesion volume was calculated as the percentage of damaged tissue using ImageJ software (National Institute of Health, USA).

2.2.10 Data Analysis

Statistical analysis

GraphPad prism 7 (GraphPad Software Inc., La Jolla, CA) was used for all statistical analysis, excluding microbiota data. The Shapiro-Wilk and D'Agostino & Pearson tests were used to assess normality. When the assumption of normality was complied, one and two-way ANOVAs were used for single and longitudinal tests (with repeated measures), respectively followed by Tukey's multiple comparisons post hoc test. When the assumption of normality was rejected, the non-parametric Kruskal-Wallis test (for multiple groups) or Mann-Whitney t-test was applied appropriately. An α of 5% was used as statistical cutoff. Values in results are expressed as mean ± standard deviation.

2.2.11 OTU analysis

Microbiota data were analyzed using R through Rstudio by an analyst blind to the experiment [Team, 2013, RStudio, 2018]. OTU tables extracted (see 16s rRNA analysis section) were read, managed and analyzed using the phyloseq R package [McMurdie and Holmes, 2013], an R extension for analyzing microbiome census data. Shannon index of alpha diversity, a measure of the number of different OTUs (gener-

ally Genus/Species) present in each sample, was obtained using the phyloseq package before OTU filtering. Linear mixed model (LMM) was fitted for statistical inference considering group, time and their interaction as fixed effect terms and the animal as a random effect. For the determination of the number of significantly different genus-species frequency representation within (Fig. 5B) and between (Fig. 5C) groups, limma method implemented in the limma package was used [Ritchie et al., 2015]. We applied limma as a multivariable linear model with empirical bayes correction [Phipson et al., 2016]. Before fitting the model, the OTU table was aggregated by the estimated genus and species that each OTU represents, their relative frequencies were calculated and a logarithmical (log (x + 1)) transformation applied. Then a factorial model was fitted using limma with the genus-species relative frequency as the response and the group, time and their interaction as explanatory terms. After empirical bayes regulation of the coefficients, the different contrasts were tested, and the p value computed and adjusted for controlling false discovery rate using the Benjamini & Hochberg method. To reduce the chances of false positive with respect to the standard type 1 error of 5%, we set a cut-off of significance at 1% (an adjusted p-value$<$0). The same algorithm was used for the OTU, family, class, order and phylum aggregated taxonomical level determination. Unsupervised ordination was conducted blinded to the experimental condition by Non-metric Multidimensional Scaling (NMDS) using the phyloseq package. The transformed data (see above) were used and a NMDS computed over the centered Bray-Curtis dissimilarity restricted to 5 dimensions. A stress of 0.086 was reached after 20 iterations. A permutation analysis of variance (PERMANOVA) was computed using the vegan package over the Bray-Curtis dissimilarity matrix to test the hypothesis of whether the centroids of the multivariate space were different by the terms of group, time and their interaction [Oksanen et al., 2013]. Beta dispersion was used to test the multivariate homogeneity of variance.

2.2.12 PICRUST analysis

The functional inferences of each library were performed using the PICRUST algorithm (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States), based on 16S rRNA gene data present in the Greengenes database and the KEGG database [Langille et al., 2013]. A total of 328 inferred functional pathways were categorized. Limma was used as described above to detect the functional pathways statistically over- or down-represented within and between groups considering adjusted p value <0.05 as cutoff for significance. After standardization (data centered and divided by the standard deviation) across pathways for each animal, a hierarchical cluster analysis was performed with all the pathways that showed a main group effect in limma at any time point. That same dataset was then analyzed by principal component analysis (PCA) to establish the 'functional microbiota composition' as the principal components (PC) and the loadings and scores were computed. Scree plot was used as diagnostic for number of PC selection and an absolute loading (interpretable as a correlation) higher than 0.6 was considered important. The scores for PC1 and PC2 were used as response variable in hypothesis testing by Kruskal-Wallis test followed by a Conover tests with Bonferroni adjust of p value. PC1 and PC2 were also used to compute a second PCA together with the behavioural data to determine the relationship of the 'functional microbiota composition' components and the performance of the animals at the multivariate 'behavioral testing' space. In both PCAs, stability of the loadings to outliers was analyzed by iteratively running the PCAs with the data of one animal out each time (aka leave-one-out cross-validation). Then the computed leave-one-out PCs were compared with the original (all animals) PCs by Pearson correlation, and the averages and 95% confidence interval for r calculated.

2.3 Results

2.3.1 An incomplete unilateral cervical SCI induces anxiety-like behaviour and alterations in gut microbiota

Our first goal was to determine whether rats develop anxiety-like behaviours in parallel to gut microbiota changes after a cervical spinal contusion injury. To assess anxiety-like behaviour, rats were tested in the elevated plus maze (EPM) task 3 weeks following SCI or sham operation (Fig. 2.1 A). Increased time spent and entries into the open arms of the maze indicates reduced anxiety-like behaviour. To determine whether the SCI had an effect on locomotion in the EPM, the total distance the rat moved within the open and closed arms of the maze was calculated. There was no significant difference in the total distance moved in the maze between sham or SCI rats (Fig. 2.1 B). However, rats with a SCI spent significantly less time in the open arms compared to sham animals, suggesting a SCI-induced increase in anxiety-like behaviour (Fig. 2.1 C.). To assess whether SCI rats also develop changes in microbial composition in parallel with anxiety-like behaviours, fecal samples were plated on CHROMagar orientation plates prior to injury, 3 days following injury, and weekly after SCI. Qualitative analysis showed a clear change in the composition of bacteria, with maximal changes observed at 3 days after SCI (Fig. 2.1 D). These data confirm that after an incomplete unilateral cervical SCI, rats display a rapid onset and persistent alteration in the gut microbiota and a later increase in anxiety-like behaviour.

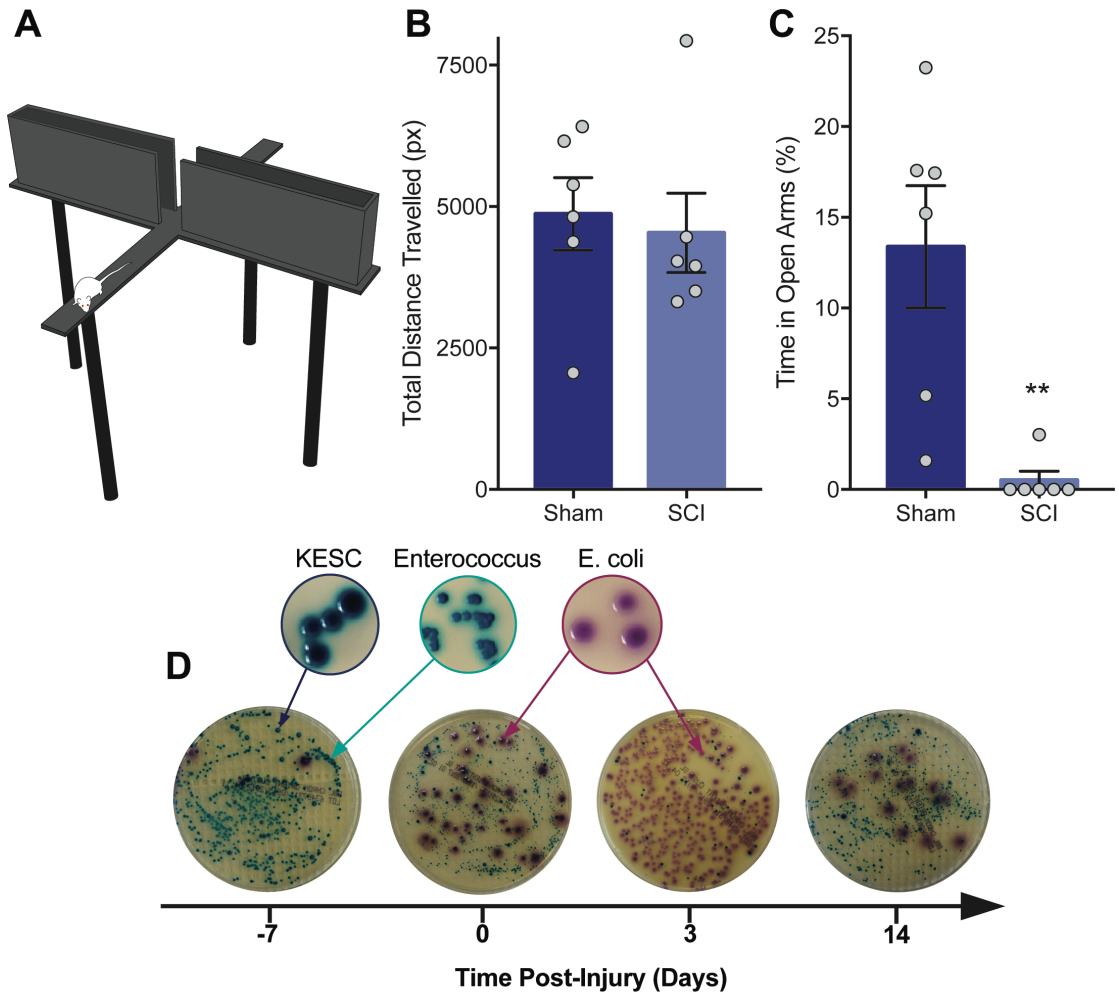

Figure 2.1: The elevated plus maze, shown in (A), was tested 3 weeks following spinal cord injury (SCI). (B) There was no difference in the total distance travelled between SCI and sham operated groups. Sham = 4869 px ± 1579, SCI = 4536 px ± 1713. (C) After SCI, rats spent significantly less time in the open arms compared to sham animals (Mann-Whitney test, p = 0.0043; Sham = 13.37% ± 8.26, SCI = 0.50% ± 1.23) (D) Fecal matter from SCI rats were collected at various time points, filtered and diluted with PBS using sterile techniques, and plated onto CHROMagar orientation plates to assess bacterial growth over 48 hours. Immediately following injury (time 0) there was clear alterations in microbial growth, with maximal changes seen 3 days after injury. **p<0.01.

2.3.2 Fecal microbiota transplant prevents SCI-induced dysbiosis and anxiety-like behaviour

We next tested whether there was a link between the observed alterations in microbial composition and increased anxiety-like behaviour after SCI. Rats were given an FMT from healthy (i.e., uninjured), non-anxious-like donor rats at the time of injury and for two consecutive days after SCI. Experimental groups consisted of rats that had surgery but no SCI (Sham), a group that received a cervical SCI but no fecal transplant (SCI), a group that received an SCI and a fecal microbiota transplant (SCI-FMT), and a group that did not undergo any operation and served as the donors for the FMT (Healthy). Upon arrival, rats were allowed one week to acclimatize to the new environment before any testing took place. Fecal collection for microbiota analysis took place prior to, 3 days and 4 weeks following surgery. All experimental groups underwent a battery of behavioural tests before and for 4 weeks after surgery, followed by perfusions and lesion analysis (2.2).

2.3.3 Fecal microbiota transplant reduces anxiety-like behaviour in the elevated plus maze and light-dark box

Rats were tested in the EPM 3 weeks following surgery (Fig. 2.3 A). Differences between groups emerged in the total distance travelled in the EPM. Healthy animals travelled significantly further than SCI and Sham animals (Fig. 2.3 B). There was no difference in the total distance travelled between Sham, SCI and SCI-FMT groups. There was also no significant difference in the distance travelled between SCI-FMT and Healthy animals. The most robust behavioural results were seen in the percent time spent in the open arms. Compared to SCI rats that spent most of their time in the closed arms, SCI-FMT and Healthy groups spent significantly more time in the open arms, indicating reduced SCI-induced anxiety-like behaviour in the EPM.

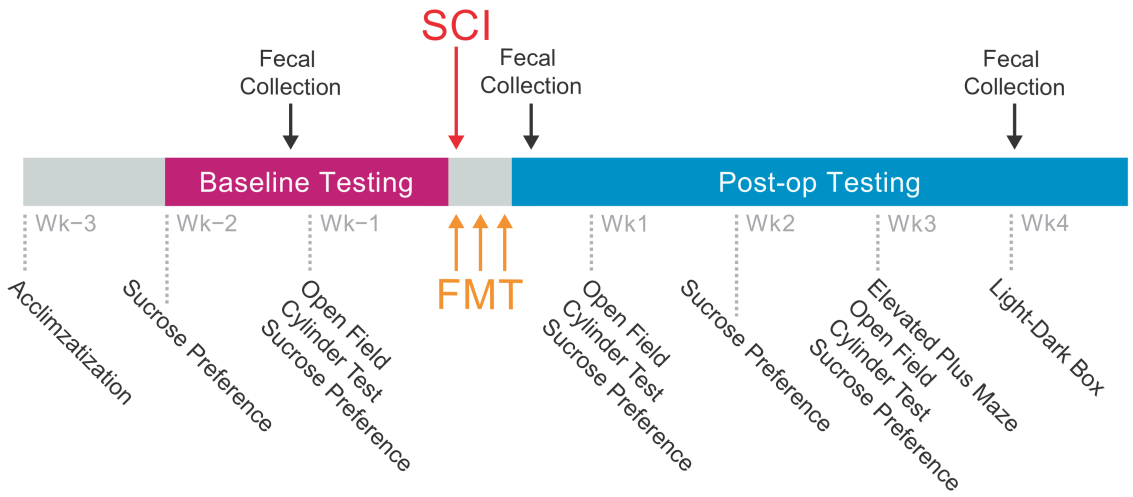

Figure 2.2: Upon arrival, rats were allowed one week to acclimate to their environment before testing. Prior to injury, baseline measures were obtained in the cylinder test, open field, light-dark box and sucrose preference tests. With the exception of the healthy group, all rats were gavaged with a fecal slurry (FMT: fecal microbiota transplant) or control solution at the time of injury and for 2 days following injury or sham surgery. Stool samples were collected for 16s rRNA sequencing prior to, 3 days and 4 weeks following surgeries. After surgeries, rats were tested weekly on a battery of behavioural tests followed by perfusions (5 weeks following surgeries) and tissue analysis.

There was no difference in the percent time spent in the open arms between SCI-FMT and Healthy groups (Fig. 2.3 C.). A similar trend between groups was found in the percentage of open arm entries (Fig. 2.3 D.). To further assess anxiety-like behaviour, rats were tested in the light-dark box (LDB) at baseline (pre-injury) then again at 1 and 4 weeks after surgery. There were no significant differences between the groups in the time spent in the light compartment at baseline or 1 week after surgery (Appendix A.1.). 4 weeks following SCI, FMT treated rats displayed decreased anxiety-like behaviour, spending significantly more time in the light compartment compared to both SCI and Healthy groups (Fig. 2.3 E.), a trend that was also seen in the latency to enter the light chamber (Fig. 2.3 F.). To assess depressive-like behaviour, the sucrose preference test was used at baseline and once a week for 3 weeks following injury (Fig. 2.3 G). There was no difference between the groups in the percent of sucrose water

consumed after surgery, indicating that our model of a unilateral cervical SCI did not induce anhedonic behaviour in the sucrose preference test.

2.3.4 Fecal microbiota transplant did not affect functional recovery or lesion severity

To supplement data from the EPM task, rat movement was tested in an open field (Fig. 2.4 A). There, the total distance travelled and the distance travelled in the inner 60% of the arena were measured to quantify overall activity and anxiety-like behaviour, respectively. 1 week following surgeries, both SCI groups travelled significantly less distance than Sham and Healthy animals (Fig. 2.4 B.). By three weeks, all groups had declined in their overall locomotion and there were no differences between the groups in the total distance travelled. We next measured the proportion of distance travelled in the inner arena as an indicator of anxiety-like behavior. At both time points tested (1 and 3 weeks) following surgeries, there were no significant differences between groups (Fig. 2.4 C). To determine whether the FMT had an effect on lesion pathology, total rostral-caudal lesion extent and the percent area of damaged tissue per spinal cord cross section were analyzed. Consistent with the finding that the FMT did not improve locomotor recovery, there were no significant differences in lesion extension or total lesion area between SCI and SCI-FMT groups (Fig. 2.4 E). Forepaw function was assessed using the cylinder test and expressed as a percentage of ipsilesional paw placements. Sham and healthy animals did not display forepaw asymmetry at any time point, and there were no significant differences in forepaw asymmetry between any groups pre-injury (Fig. 2.4 F). 1 and 3 weeks following SCI, both SCI and SCI-FMT groups displayed forepaw asymmetry, making significantly more contralesional forepaw placements compared to uninjured animals. 1 one week after injury, SCI-FMT rats made significantly fewer ipsilesional paw placements compared to the untreated SCI group, however this difference was gone 2 weeks later.

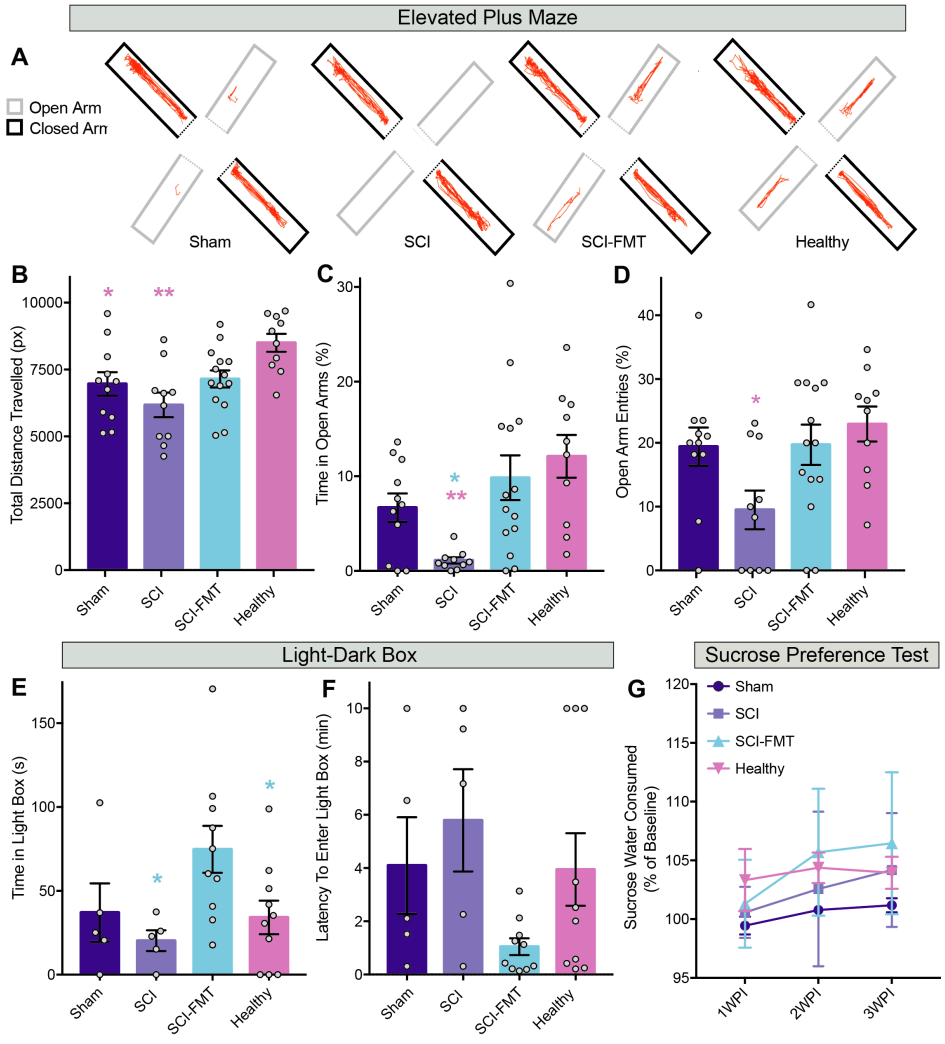

Figure 2.3: (A) Representative images from the motion tracking software used to analyze the elevated plus maze. (B) Healthy animals travelled significantly further than the SCI group (one-way ANOVA; p = 0.0014) and sham group (p = 0.0469). (C) SCI rats spent significantly less time in the open arms compared to FMT treated and Healthy groups (one-way ANOVA; SCI vs. SCI-FMT p = 0.0119; SCI vs. Healthy p = 0.0027. (D) Similarly, SCI rats displayed a reduced percentage of open arm entries compared to healthy rats (one-way ANOVA; p = 0.0267; SCI vs. Healthy p = 0.027). Although not significant, SCI rats also displayed a reduced percentage of open arm entries compared to sham (p = 0.1352) and SCI-FMT rats (p = 0.0909). (E) 4 weeks after injury, rats were tested in the light-dark box with increased time and reduced latency to enter the light component indicating decreased anxiety-like behaviour. FMT treated animals spent significantly more time in the light component compared to the SCI group as well as the Healthy group (Two-way repeated measures ANOVA; SCI vs. SCI-FMT p = 0.01, SCI-FMT vs. Healthy p = 0.03). (F) FMT rats displayed a reduced latency to enter the light compartment compared to the SCI group, although this failed to reach significance (two-way repeated measure ANOVA; p = 0.0843). (G) There were no differences between groups in the percentage of sucrose water consumed at any time point tested. Error bars indicate standard error mean. *p<0.05, **p<0.01, ***p<0.001

Together these results indicate that acute FMT treatment did not affect lesion size and did not improve functional recovery.

2.3.5 Fecal microbiota transplant prevents SCI-induced dysbiosis

In the first experiment (Fig. 2.1) gross changes in fecal microbiota content were observed 3 days after SCI. For a more comprehensive analysis of bacteria present in the gut, 16s rRNA sequencing was performed from fecal samples obtained before SCI, then again 3 days and 4 weeks after SCI. Shannon index of alpha diversity (Jost, 2007) was used to evaluate bacterial diversity and showed that bacterial diversity was similar between groups at baseline. A time effect was observed with an increase in bacterial

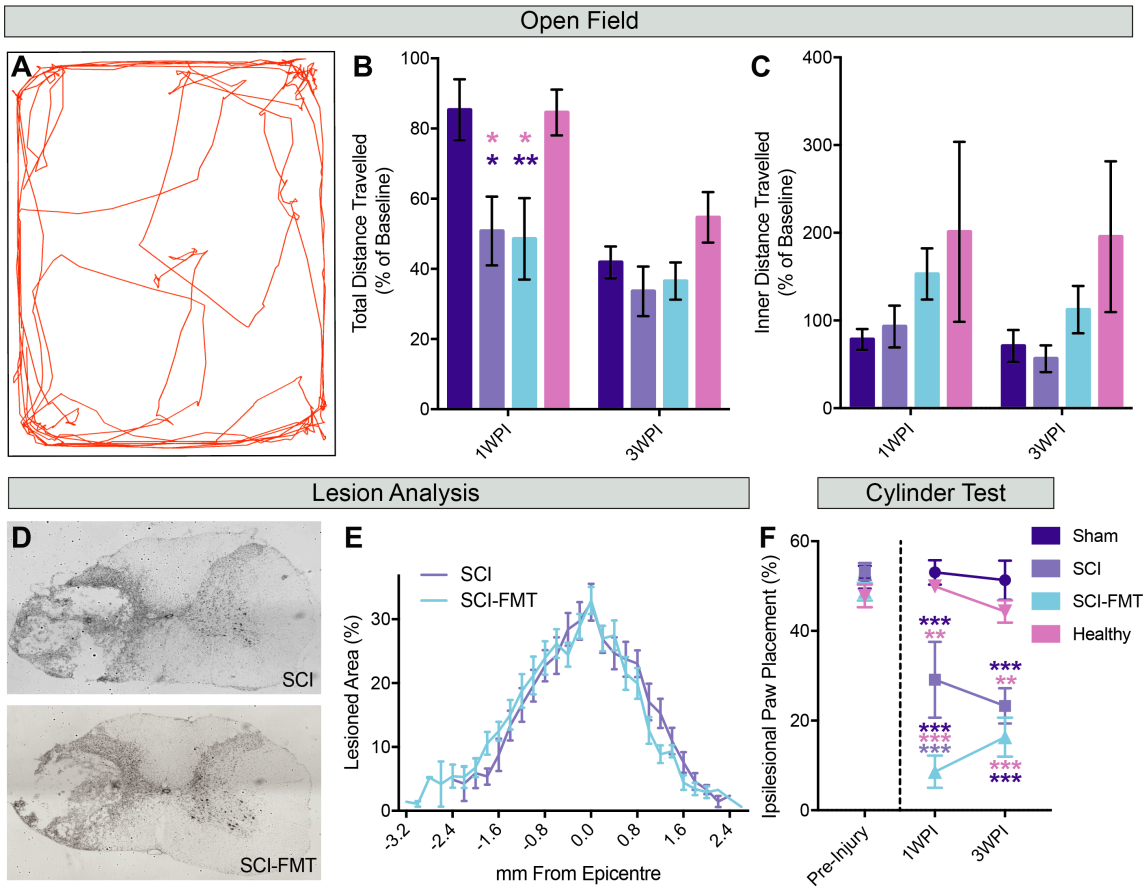

Figure 2.4: Caption on next page

Figure 2.4: (Previous page) Rats were tested in the open field before surgeries, then again 1 week and 3 weeks following later. (A) Representative image from the motion tracking software used to analyze the movement of rats in the open field. (B) 1 week following injury, both SCI and SCI-FMT groups travelled less distance compared to baseline than sham and healthy animals Repeated measure two-way ANOVA: Sham vs SCI $p = 0.026$, Sham vs. SCI-FMT $p = 0.007$, SCI vs. Healthy $p = 0.036$, SCI-FMT vs. Healthy $p = 0.011$). By 3 weeks following injury there was no difference in the distance travelled in the open field relative to baseline between groups. (C) We next assessed the percent distance travelled in the inner 60% of the arena relative to baseline. At both 1 and 3 weeks following injury there was no difference between groups in the percent inner distance travelled. (D) Representative images of the maximum lesion site from SCI-FMT and SCI groups stained with cresyl violet. (E) There was no significant difference between SCI and SCI-FMT groups in the lesion progression (Mann-Whitney test; $p = 0.3688$). The average maximum lesion severity for SCI group was 32.67% and 32.95% for SCI-FMT groups. The average lesion extension was 4.06 mm for the SCI group and 3.886 mm for the SCI-FMT group. (F) Sham and Healthy rats did not show any forelimb asymmetry at any time point. Prior to SCI, rats did not display any contralateral bias in their paw placements in the cylinder test. 1 week post-injury, SCI and SCI-FMT rats made significantly fewer ipsilesional (right) paw placements than Healthy and Sham animals. At one week post injury, the SCI-FMT group had a more significant contralateral bias in their paw placements than the SCI group (SCI vs. SCI-FMT $p = 0.0008$). 3 weeks after injury, the differences between SCI and SCI-FMT groups were abolished, with all SCI animals making fewer ipsilesional paw placements than uninjured groups (Sham vs. SCI $p = 0.0001$ (1WPI & 3WPI), sham vs. SCI-FMT $p < 0.0001$ (1WPI & 3WPI), SCI vs. Healthy $p = 0.0015$ (1WPI) $p = 0.0013$ (3WPI), SCI-FMT vs. Healthy $p < 0.0001$ (1WPI & 3WPI). Error bars indicate standard error mean.*$p<0.05$, **$p<0.01$, **$p<0.001$

diversity 3 days after surgery in all four groups (Fig. 2.5 A. LMM: group effect $p = 0.37$, group x time interaction $p = 0.052$, time effect $p < 0.001$). Taken together, these data indicate that the mean bacterial diversity between groups was comparable at each time point tested. Next, we analyzed the effect of injury on the microbiota composition by contrasting the relative abundance of each genus-species aggregated operational taxonomic unit (OTU) at 3 days and 4 weeks post-injury with respect to baseline (pre-injury) (Fig. 2.5 B). Animals in the Healthy group did not show changes in the genus-species frequency over time. Looking at the overall microbiota composition, 3 days after injury SCI rats presented 112 statistically different OTUs

(adj. p value <0.01). FMT reduced the total number of altered genus-species OTUs (12 at 3 days post injury vs. baseline), which suggests a prevention of SCI-induced dysbiosis. By 4 weeks after injury, the number of significantly different genus-species OTUs was reduced compared to baseline in all groups with respect to those observed at 3 days, indicating a normalization of the microbiota composition. To further explore the time-dependent changes in the microbiota composition following SCI, pairwise comparisons were performed between groups at each time point (Fig. 2.5 C). At 3 days after injury, major changes were observed in Healthy vs. SCI groups (155) and SCI-FMT vs. SCI groups (153), but not between Healthy and SCI-FMT groups (9). When analyzing the overlap of OTUs between the Healthy vs. SCI group and the SCI-FMT vs. SCI group, 138 OTUs were the same. This supposes a 90.2% overlap with respect to the 153 OTUs changing by SCI-FMT treatment, suggesting that FMT treatment overrides changes in the microbiota composition induced by SCI to a "healthy" composition. By 4 weeks after injury, most differences between groups were reduced. To explore the microbiota composition at the multivariate space, an unsupervised ordination was performed by non-metric multidimensional scaling (NMDS) at the genus-species level (Fig. 2.5 D and E, see Fig. A.2 for all other taxonomic levels). This analysis further indicates the proximity of Healthy and SCI-FMT animals across time. We also observed a deviation of the microbiota composition in SCI animals from the Healthy multivariate space at 3 days after SCI. There were significant main effects of group and time (group effect R2 = 0.079 p <0.001; time effect R2 = 0.091 p <0.001) as well as a time-group interaction (R2 = 0.112 p <0.001). These differences can be observed when the NMDS scores are plotted by timepoints (Fig. 2.5 E). These differences are also confirmed at the OTU and family levels (Appendix A.2.).

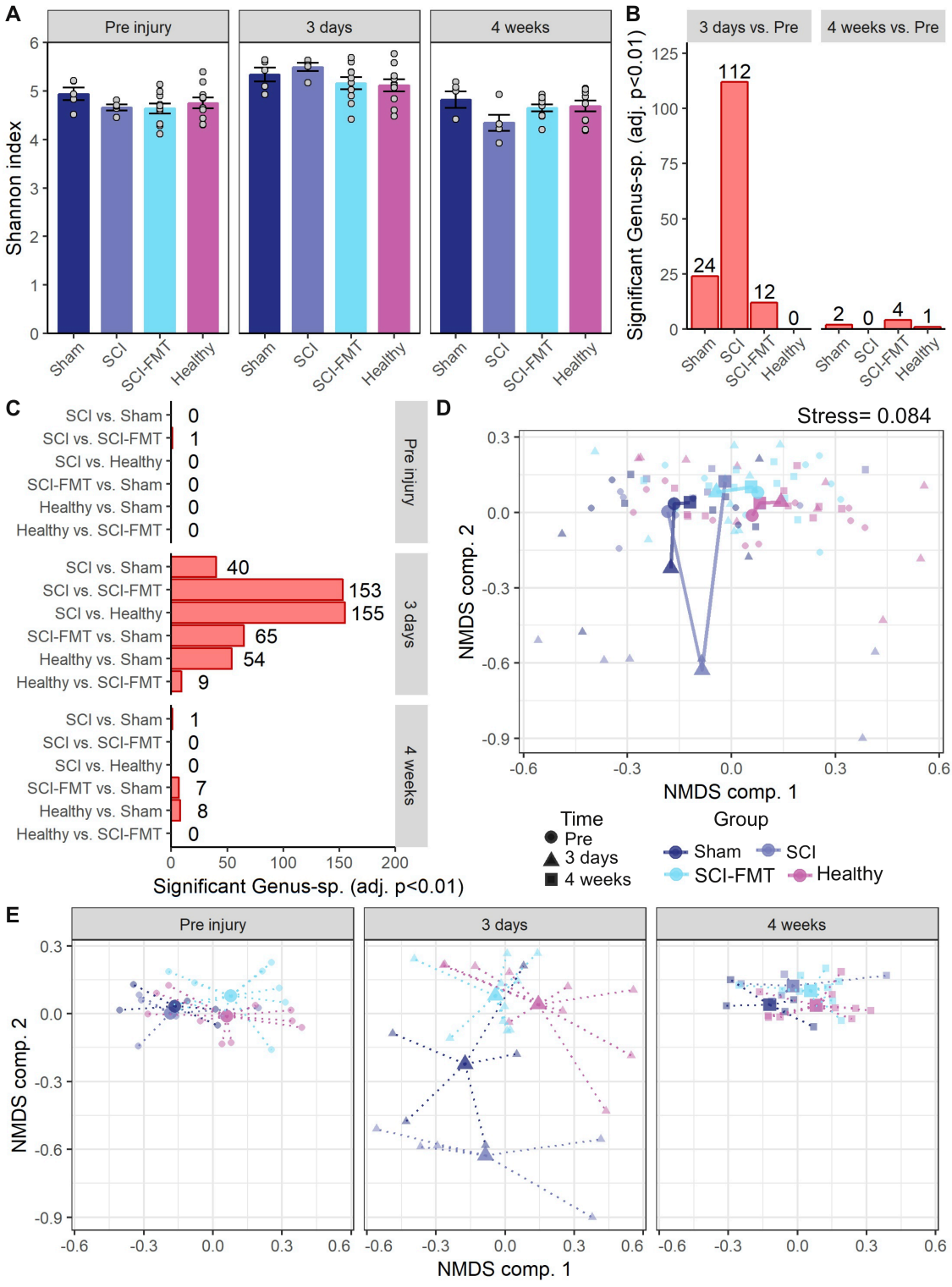

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Figure 2.5: (Previous page) (A) Shannon index of alpha diversity was calculated from the operational taxonomic unit (OTU) table (see methods) and a LMM was fitted revealing no statistical differences between groups. A significant time effect was found with an increase in alpha diversity at 3 days and normalization by 4 weeks. (B) The number of differentially represented OTUs at the genus-species level (measured by the limma method) between 3 days and pre-injury was higher in SCI group, followed by Sham, and SCI-FMT groups. Healthy animals did not show significant differences at the specified cutoff (adj. $p<0.01$), indicating the stability of the microbiota in healthy rats over the course of the experiment. By 4 weeks the number of differences were highly reduced demonstrating the normalization of the microbiota composition by the end of the follow up. (C) Pairwise comparisons were performed between groups at each time point by contrasting the coefficients of the limma model. When comparing between groups, major differences were observed at 3 days post-injury, especially between SCI and Healthy, and SCI and SCI-FMT groups. Notice that the number of differentially represented genus-species comparing Healthy vs. SCI-FMT was the smallest of all, confirming the proximity of these two groups. (D) Unsupervised ordination was performed over all the samples by NMDS and Bray-Curtis dissimilarity of the genus-species OTUs level (stress of 0.084 after 20 iterations). Considering group and time, the 2D-plot of the NMDS two first components shows a cluster cloud of the 'microbiota composition' and the deviation of the SCI animals from that cluster at 3 days post-injury. The big points represent the 2D centroids of each group and timepoint, and the lines join the time trajectory for each group. A PERMANOVA was used to perform hypothesis testing in the Bray-Curtis dissimilarity matrix between groups, timepoints and their interaction (group effect $R2=0.079$ $p<0.001$; time effect $R2=0.091$ $p<0.001$; interaction $R2=0.112$ $p<0.001$). Notice that only around 30% of the variance is explained by group, time and their interaction, indicative that other factors might contribute to the big individual differences. Nonetheless, the dispersion between groups, especially SCI compared to Healthy and SCI-FMT can be appreciated when that same analysis is plotted by timepoints (E). Dotted lines in (E) represents the 2D distance of each animal with the respective centroid at each timepoint in the NMDS space.

2.3.6 Spinal cord injury-induced changes in the microbiota metagenomic functional pathways are abolished with a fecal microbiota transplant

PICRUSt [Langille et al., 2013] was used to infer the functional potential of gut microbial communities after SCI and as a result of FMT. Confirming the results of the microbiota composition analysis, major changes in the metagenomic functional path-

ways between groups were observed at 3 days (Fig. 2.6), but not at baseline or 4 weeks after injury. Hierarchical clustering analysis clearly show the Healthy and SCI-FMT groups cluster together and have an inverted relationship with SCI and Sham groups (Fig. 2.6 A). To determine the pathways that contribute to major variance and their interrelation, a principal component analysis (PCA) was performed. The score plots for the first and second PCA components show clustering of Healthy and SCI-FMT animals on one side, and SCI and Sham rats on the other side (Fig. 2.6 B). The first component of the PCA statistically distinguished between these two binomials (Healthy and SCI-FMT vs. SCI $p<0.001$) (Fig. 2.6 D). The loadings (relative contribution of each variable into a given PC) for the first component are presented in Fig. 2.6 C (see Appendix A.3 and A.4 for the complete list). The functional pathways in the microbiota at 3 days post-injury that are more likely over- (positive loadings) or under- (negative loadings) represented in SCI and Sham rats are inversely represented in Healthy and SCI-FMT animals. Finally, a second PCA was used to study whether the overall functional microbiota components were associated with behavioural outcomes (Fig. 2.6 E). Component one, which distinguishes between Healthy/SCI-FMT and SCI/Sham animals (Fig. 2.6 D), was inversely associated with increased time spent in the open arms of the EPM and light component of the LDB, as well as with decreased latency to enter the LDB at one week after injury. The second component of the functional microbiota was inversely associated with decreased anxiety-like behaviour in the EPM and LDB. This indicates that anxiety-like behaviour contributes to variance for both functional components of the PCA, while distinguishing between groups (Fig. 2.6 B and D). Taken together, the analysis of the microbiota composition after SCI and FMT treatment, both at the composition and metagenomic functional levels, confirmed that a cervical SCI induces transient dysbiosis that can be prevented by a FMT. Moreover, functional microbiota changes correlate with the behavioural differences observed between groups.

Since we have shown that FMT from healthy rats improves anxiety-like behaviour following SCI, we aimed to determine whether FMT from injured rats would induce

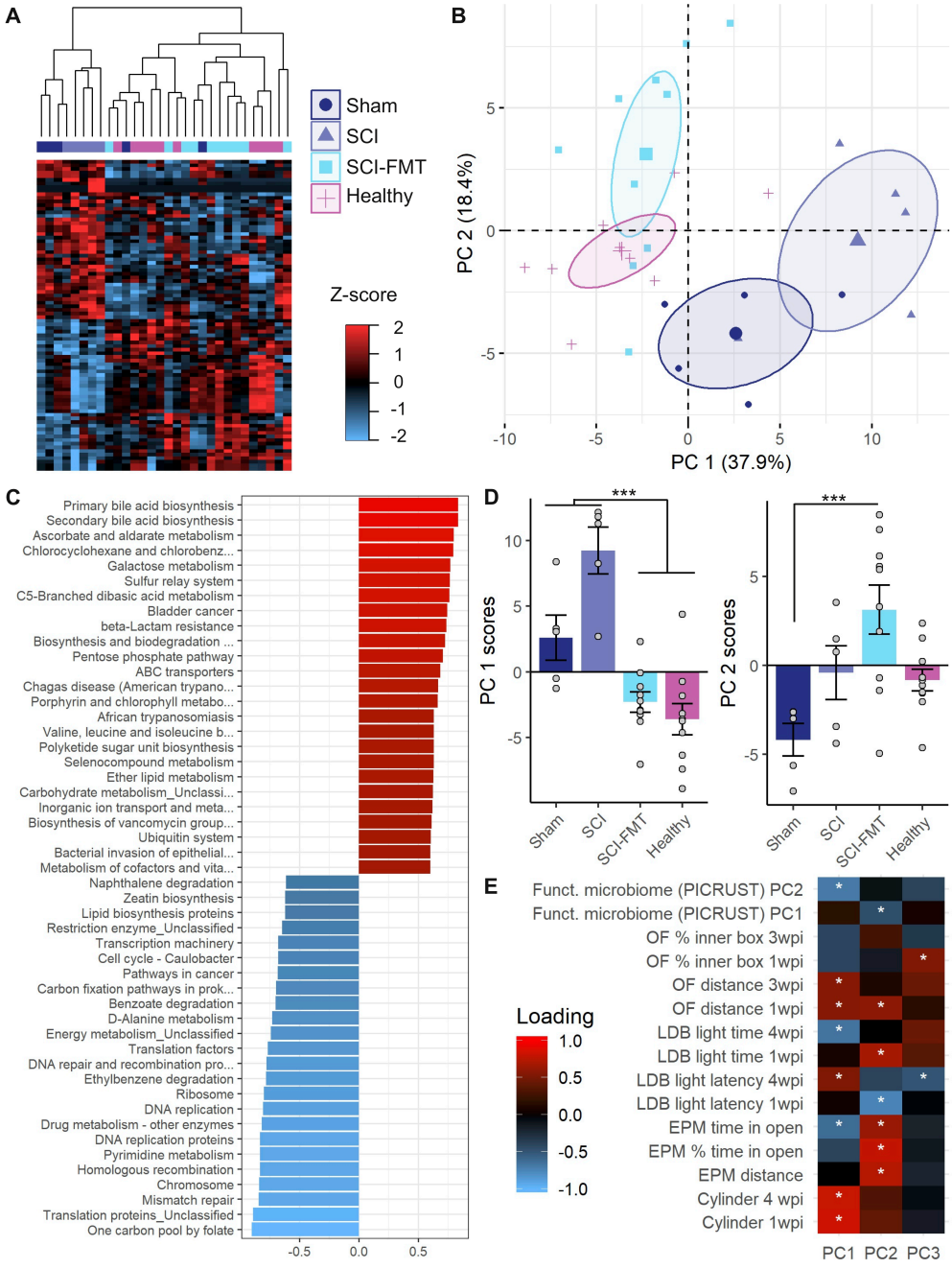

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Figure 2.6: (Previous page) Of the 328 functional pathways found by the metagenomic analysis of the 16s RNA by using PICRUST, 86 were found differentially represented between groups (determined by limma method and cutoff of adj.p<0.05). (A) Hierarchical clustering with these 86 functional pathways was performed, demonstrating the proximity between SCI and Sham, and Healthy and SCI-FMT. This analysis revealed two clusters of functional pathways that followed different direction between the SCI/Sham binomial and the Healthy/SCI-FMT pair, with over-representation and under-representation of these pathways respectively and vice versa. (B) A PCA was performed to determine the major components of functional pathways that explains the observed variance between animals in a unsupervised manner. The 2D plot of the animals' scores for the two first components (explaining 56% of the variance) shows a clear differentiation between the aforementioned group pairs, PC1 being he component that distinguish between SCI/Sham and Healthy/SCI-FMT. Leave-one-out cross-validation demonstrated high stability of the PCA results (Pearson's PC1 r = 0.99 ± 0.0003; PC2 r = 0.99 ± 0.0029). (C) List of the most important (—loading— <0.6) functional pathways that contribute to PC1. (D) Hypothesis testing of the PC1 and PC2 scores by Kruskal-Wallis showed statistical differences between groups in the scores (PC1 p<0.0001, PC2 p<0.01). Conover test for multiple comparisons revealed that PC1 scores were statistically different between SCI and Healthy and SCI-FMT (p<0.001) but not between SCI and Sham (p=0.56). PC2 scores were statistically different comparing Sham animals and SCI-FMT rats (p<0.01). (E) A second PCA between the first and second components of the 'functional microbiota PCA and the behavioural analysis was conducted to study the interrelation of the microbiota and the animal performance. This second PCA shows how the 'functional microbiota PC1 correlated with the performance on the cylinder test and the LDB 4 weeks post injury, while 'functional microbiota PC2 was associated with EPM and LDB at 1 week. The direction of the loadings scores 'functional microbiota PC1 was invers to EPM distance, EPM % time in the open arms and EPM time in the open arms absolute value. That can be interpreted as higher score in 'functional microbiota PC1 (pointing to SCI/Sham in B) being associated with less distance and open arm time in the EPM. Similar interpretation can be done for the 'functional microbiota PC2 and the LDB at four weeks. Overall this second PCA shows an association between the functional pathway composition of the metagenomic microbiota and the behavioural outcomes. Leave-one-out cross-validation also showed stability of the second PCA (Pearson's PC1 r = 0.96 ± 0.025; PC2 r = 0.95 ± 0.029). In D *** p<0.001. In E * —loading—<0.4

anxiety-like behaviour in healthy rats. To test this, uninjured rats received either FMT from SCI rats (fecal matter collected 3 days post injury) or a vehicle solution and their behaviour was assessed 3 weeks later. FMT from SCI to uninjured rats had no significant effect on behaviour in the light-dark box, EPM or open field (Fig. 2.7

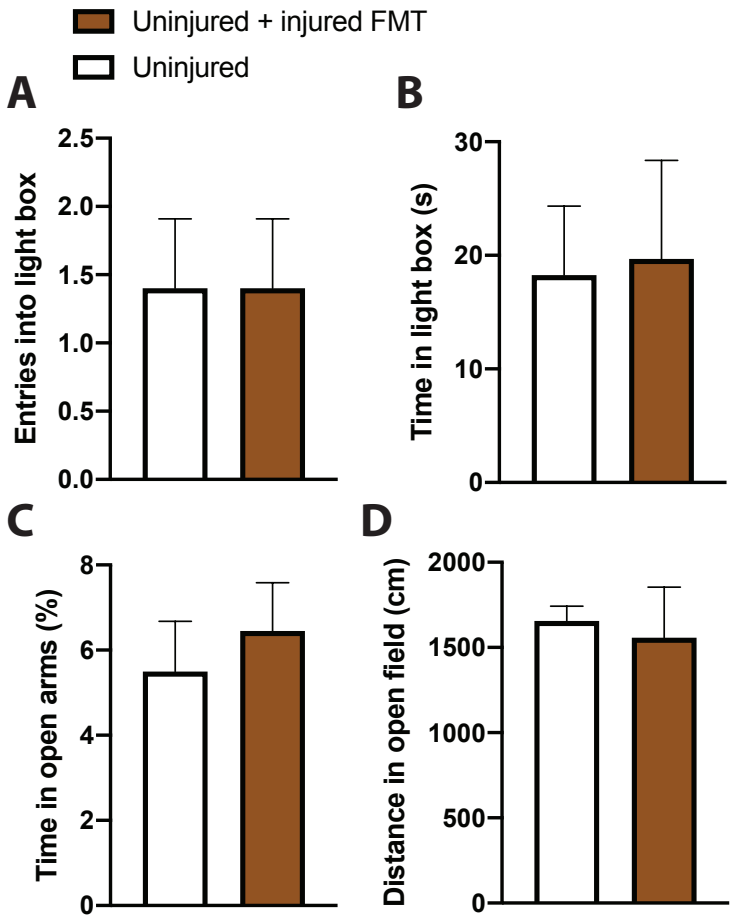

Figure 2.7: The number of entries (A) and time spent (B) in the light component of the light-dark box. (C) Percentage of time spent in the open arms of the elevated plus maze. (D) Total distance travelled in the open field. Error bars represent standard error mean.

A - D). Since healthy uninjured rats have a stable microbiota composition (as shown in this study), they are likely less susceptible to perturbations in their microbiota and subsequent behavioural changes.

2.4 Discussion

The present results show that a unilateral cervical spinal contusion in rats induces a transient change in the microbiota composition (measured 3 days following injury) that returns to baseline within 4 weeks. We show that this SCI-induced gut dysbiosis is involved in the development of anxiety-like behaviour following SCI, since both gut dysbiosis and anxiety-like behaviours were significantly reduced following treatment with an FMT. Functional analysis of the microbiota composition confirmed this treatment effect, and showed an inverse relationship between SCI-FMT/Healthy and SCI/Sham groups. Together these data demonstrate that acute onset SCI-induced intestinal dysbiosis can have profound long-term behavioural consequences, which are preventable by FMT treatment in the acute post-injury period. Targeting the gut microbiota may therefore provide a novel therapeutic target to treat multiple consequences of SCI.

It is unclear how the relatively mild SCI used in the present study induced gut dysbiosis. Alterations of the intestinal microbiota may occur for a variety of reasons, including psychological or physical stress [Galley et al., 2014, Myers and Hawrelak, 2004]. In addition, disruption of the autonomic nervous system following SCI can alter gut and immune function and thus indirectly impact the gut microbial communities through alterations in gut motility [Krassioukov, 2009, Carabotti et al., 2015, Lucin et al., 2007, Zhang et al., 2013, Lynch et al., 2000, Pop et al., 2014, Bik and Relman, 2014]. Another possible contributor to gut dysbiosis following SCI are surgeries per se. Supporting this idea, animals that received

a sham SCI displayed an altered microbiota composition 3 days after surgery. Although not as significant as the changes seen in SCI animals, sham operated rats also displayed minor behavioural abnormalities compared to healthy animals. Specifically, sham rats travelled significantly less distance in the EPM than healthy animals, which cannot be explained by locomotor deficits. These findings indicate the acute effects of surgery had an effect on both microbiota composition with lasting changes in behaviour. Results from both experimental stroke and SCI corroborate our findings. For example, it has been shown that a sham stroke in mice induced intestinal dysbiosis, although to a lesser extent than a severe stroke [Singh et al., 2016]. Following a sham SCI, Espírito Santo et al. found that rats displayed increased depressive-like behaviour in the sucrose preference test, again to a lesser extent than the SCI group [do Espírito Santo et al., 2019]. Although our results and others suggest that surgery alone can induce both dysbiosis and behavioural changes, these effects were more severe following SCI. It is therefore likely that multiple factors are involved in the development of gut dysbiosis following SCI.

Our results indicate that changes in the microbiota are linked to the development of anxiety-like behaviour after SCI. Although it is unknown what triggers these behavioural changes following SCI, the relationship between the microbiota and mental health in the uninjured population is becoming increasingly clear. Initial experiments on the link between the microbiota and stress-related behaviours found that germ-free mice have an exaggerated stress response and a reduced anxiety-like phenotype [Sudo et al., 2004, Neufeld et al., 2011]. Since then, many animal studies have strengthened the connection between microbiota changes and behaviour, and have shown that treating dysbiosis can improve anxiety or depressive-like behaviours [Foster and McVey Neufeld, 2013, Huang et al., 2016, Desbonnet et al., 2010]. Similar results have been shown in humans, finding significant correlations between depression and the composition of the intestinal microbiota [Naseribafrouei et al., 2014,

Valles-Colomer et al., 2019]. The gut microbiota can influence brain and behaviour via the gut-brain axis, which involves the nervous, autonomic, endocrine and immune systems [Alam et al., 2017]. Pathological alteration of the gut microbiota together with compromised intestinal barrier function can influence immunity and inflammation and thus have a profound effect on the health and behaviour of the host [Foster and McVey Neufeld, 2013, Fung et al., 2017, Smith, 2015]. Both human and animal studies have found that increased blood levels of proinflammatory cytokines are linked to anxiety and depression [Dantzer et al., 2008, Dowlati et al., 2010, Zorrilla et al., 2001, Miller et al., 2009, O'Donovan et al., 2010], and various anti-inflammatory treatments have antidepressant and anxiolytic effects [Köhler et al., 2014]. Given the acute and chronic inflammatory state associated with SCI [Popovich and McTigue, 2009, Schwab et al., 2014], it is likely that inflammation plays a role in the etiology of mental health disorders following SCI [Maldonado-Bouchard et al., 2016a]. Currently, however, it is unclear whether SCI-induced systemic inflammation is the cause or result of dysbiosis and breakdown of the intestinal barrier.

Kigerl et al. showed that SCI increases intestinal barrier permeability [Kigerl et al., 2016a], which would allow bacteria or microbial components (e.g. endotoxins) to enter the circulation, leading to increased systemic inflammation [Berg, 1992]. Indeed, one study found that after a thoracic SCI in rats, there is a significant increase of the bacterial endotoxin LPS in circulation [Liu et al., 2004a]. This increase in circulating endotoxin can further compromise the integrity of the intestinal barrier [O'Dwyer, 1988, Guo et al., 2013]. In addition to increasing intestinal permeability and causing an acute systemic inflammatory response, systemic injection of LPS into rodents is an established model of depression [Yirmiya, 1996a, Frenois et al., 2007], further supporting the evidence that inflammation is critically involved in mental health disorders [Miller et al., 2009]. Recent studies highlight the role that in-

flammation plays in the development of anxiety- and depressive-like behaviour after SCI. Maldonado-Bouchard et al. found that SCI-induced depression- and anxiety-like behaviour was associated with increased peripheral (serum) and central (spinal cord and hippocampus) levels of pro-inflammatory cytokines [Maldonado-Bouchard et al., 2016a]. Similarly, do Espírito Santo et al. also showed that depression-like behaviour following SCI is associated with increased plasma concentrations of pro-inflammatory cytokines [do Espírito Santo et al., 2019]. Similar results have been shown in animal models of the chronic inflammatory disease, multiple sclerosis, where increased hippocampal inflammation was associated with increased anxiety-like behaviour [Peruga et al., 2011]. These findings indicate that multiple injuries and diseases of the central nervous system, which involve active neuroinflammation and systemic inflammation, also affect well-being. Therefore, we hypothesize that treating intestinal dysbiosis after SCI may improve the integrity of the intestinal barrier and reduce systemic inflammation, preventing the subsequent development of mental health disorders. However, future research is required to determine the mechanisms of the relationship between SCI-induced dysbiosis and mental health disorders.

Although we showed that SCI-FMT rats adopted a similar reduced anxiety-like behaviour as their uninjured donors, the reverse was not true. That is, when FMT was transferred from acute SCI rats to uninjured rats, we did not observe any behavioural differences in the recipients. Since stool samples were not analyzed in this preliminary study, we cannot confirm whether the fecal transplant from SCI donors perturbed the microbiome of uninjured rats. Indeed, healthy adults typically have a stable and resilient microbiome composition [Lozupone et al., 2012]. If the FMT was successful in perturbing the healthy microbiome state, this suggests that some other consequence of injury, for example increased inflammation, is critical for the interaction between the microbiome and anxiety-like behaviour. In support of this,

there is a temporal discrepancy between the gut dysbiosis observed at 3 days and the behavioural changes observed at 3 weeks after SCI. As hypothesized above, the FMT from uninjured rats may indirectly affect behaviour by reducing both systemic and central (i.e., brain) inflammation, which may take weeks to progress. If central inflammation is critical for the development of anxiety-like behaviour (as has been shown by [Maldonado-Bouchard et al., 2016a] and [do Espírito Santo et al., 2019]), it is possible that the FMT from SCI did not produce lasting central inflammation and thus did not induce anxiety- or depressive-like symptoms.

In the present study, acute FMT treatment did not improve functional recovery in the open field or the cylinder test, and there was no difference in lesion size between SCI and SCI-FMT groups. On the other hand, a study in experimental stroke found that treating intestinal dysbiosis had neuroprotective effects, likely through an anti-inflammatory mechanism [Singh et al., 2016]. Furthermore, following a thoracic SCI in mice, Kigerl et al. showed that treating dysbiosis with probiotics increased functional recovery and reduced secondary damage [Kigerl et al., 2016a]. Possible reasons why we did not find neuroprotective effects of treating dysbiosis include the mild cervical SCI used does not induce significant long-term deficits in locomotion or lasting gut dysbiosis. Second, to ensure permanent colonization of the gut by beneficial microorganisms in the FMT, repeated administrations may be needed to realize a lasting benefit of FMT. Indeed, Kigerl et al. gave daily doses of probiotics for 35 days following SCI, whereas our rats received an FMT for only 3 days [Kigerl et al., 2016a]. Thus, using a more severe injury model or extending the period of treatment may have a greater influence on recovery. Nonetheless, the finding that FMT treated and untreated rats did not differ in the distance travelled in the open field or EPM makes it easier to interpret the behavioural differences between groups as being independent of locomotor deficits due to SCI. Indeed, Luedtke et al. showed that depressive-like

signs did not correlate with motor recovery following a thoracic contusion in rats [Luedtke et al., 2014].

We found clear results in both the time spent and entries into the open arms of the EPM that both SCI-FMT and Healthy animals displayed significantly reduced anxiety-like behaviour compared to SCI animals. However, in the LDB only the SCI-FMT group displayed a reduced anxiety-like phenotype, with no differences between Sham, SCI or Healthy groups. A potential reason for this is that the LDB was tested at multiple time points, which may reduce sensitivity to the apparatus as shown with the EPM [File, 1990]. We also did not find any differences between groups in their behaviour in the inner area of the open field. This may be due to the reduced sensitivity of the open field to assess anxiety-like behaviour [Prut and Belzung, 2003, Carola et al., 2002]. Finally, we did not find any differences between groups in the sucrose preference test, suggesting that our model of SCI does not induce anhedonia, which is indicative of depressive-like behaviour. This is in contrast to both Luedtke et al. [Luedtke et al., 2014] and Espírito Santo et al. [do Espírito Santo et al., 2019], who found a reduction in sucrose water intake following a thoracic SCI. These confounding results may be due to differences in lesion severity, lesion level or subtle differences in the methods of testing sucrose consumption. Therefore, since we did not find differences between groups in the sucrose preference test, we cannot conclude whether rats experience depressive-like behaviour following the present model of SCI. Additional tests such as the forced swim test social interaction test would be needed to confirm whether or not the present model of SCI induces a depressive-like phenotype.

These data show for the first time that a fecal transplant following SCI in rats prevents the development of anxiety-like behaviour. Future work is required to determine the mechanisms of SCI-induced dysbiosis, such as measuring intestinal barrier function, levels of systemic proinflammatory markers and microbial metabolites, as

well as to determine whether lesion level and size have an effect on the severity of dysbiosis and anxiety-like behaviour. Taking central, peripheral and psychological consequences into consideration will provide a more comprehensive treatment approach not only for SCI, but other central nervous system diseases as well.

Chapter 3

Optimal donor selection is critical for successful fecal transplant following spinal cord injury [1]

3.1 Introduction

In chapter 2 we prevented SCI-induced dysbiosis by transferring fecal matter from uninjured donor rats into recipient rats immediately after SCI. This fecal microbiota transplant (FMT) from uninjured, non-anxious-like rats not only successfully re-established a healthy microbiota composition after injury, but also improved symptoms of anxiety-like behaviour [Schmidt et al., 2020b]. Clinically, FMT is defined as the administration of fecal matter solution from a healthy donor into the intestinal tract of a recipient [Bakken et al., 2011, Smits et al., 2013]. Unfortunately, the definition of a healthy donor is less straightforward. Currently, donors are selected primarily to exclude known pathogens and mitigate the risk of transferring infectious diseases [Duvallet et al., 2019, Paramsothy et al., 2017, Bafeta et al., 2017,

[1] This chapter has appeared in "What makes a successful donor? Fecal transplant from "anxious" rats does not prevent spinal cord injury-induced dysbiosis"

van Nood et al., 2013]. While ensuring recipient safety is a priority above all, research on optimal donor selection beyond the exclusion of transmissible pathogens is still at an early stage [Duvallet et al., 2019, Barnes and Park, 2017]. Although the choice of donor does not influence the efficacy of FMT to treat *Clostridium difficile* infections (currently the only FDA approved use of FMT [Administration, 2013]), it is unknown how critical donor selection is to treat diseases and disorders with more complex host-microbiota interaction, such as SCI [Kassam et al., 2013, Osman et al., 2016].

In this chapter, we aimed to determine whether the mental state of FMT donor rats would influence the therapeutic benefits of FMT after SCI. Rats who displayed naturally reduced baseline activity levels and increased anxiety-like behaviour (referred to as *anxious* donors) were selected as FMT donors. Notably these rats were uninjured and had a diverse microbial community, which has been shown to be an indicator of FMT success for treatment of ulcerative colitis and Clostridium Difficile infections [Barnes and Park, 2017, Kump et al., 2018]. We therefore hypothesized that FMT from *anxious* rats would yield similar therapeutic benefits as FMT from non-*anxious* rats as in the previous chapter [Schmidt et al., 2020b]. Here, rats in the experimental groups received either vehicle or FMT treatment for 3 days following a cervical contusion SCI and underwent 7 weeks of rehabilitative training in a reaching task targeting their impaired forearm. Fecal matter and plasma were collected throughout the experiment, and anxiety- and depressive-like behaviours were assessed at the end of the rehabilitation period. The inherently increased anxiety-like behaviour of the FMT donors was associated with a decreased abundance of *Lactobacillus* in their stool and thus in the FMT solution. Contrary to our hypothesis, FMT from *anxious* donors did not prevent SCI-induced gut dysbiosis and even resulted in some negative side effects. Rats which received the FMT displayed chronically increased anxiety-like behaviour, minor but long-term alterations in local and systemic inflammation, and increased intestinal permeability. These results indicate

that donor selection is critical for successful FMT following SCI and possibly other CNS injuries and diseases as well.

3.2 Methods

3.2.1 Animals

40 female adult Lewis rats (Charles River) were group housed (n = 5 per cage, experimental groups housed separately) on a 12 hour light-dark cycle and received ad libitum access to standard rat chow and water. During training periods, rats were food restricted to 10g per rat per day. Behavioural testing and all analyses were performed by an experimenter blinded to the experimental groups. Three groups of rats were used: SCI + vehicle (n=15), SCI + FMT (n=15), and FMT donors (n=10). The two cages which displayed the highest baseline anxiety-like behaviour in the open field were chosen as uninjured age and sex matched fecal donors and were not trained in the single pellet grasping (SPG) training. SCI + vehicle and SCI + FMT groups were chosen to average each group's pre-injury success rate in the SPG task.

3.2.2 Experimental timeline

Prior to SCI, rats in the two experimental groups were pre-trained on the SPG task and underwent baseline testing on the open field and von frey tests. Immediately following SCI and for two consecutive days thereafter, rats were gavaged with FMT solution or a vehicle control solution. Following 7 weeks of rehabilitative training on the SPG task, rats underwent behavioural testing. Fecal matter was collected for 16s rRNA analysis at baseline, on the day of injury, 3, 7, 14 and 56 days post-SCI. Blood was collected to measure inflammatory plasma analytes at baseline, 3, 21 and 77 days post-SCI (Fig. 3.1).

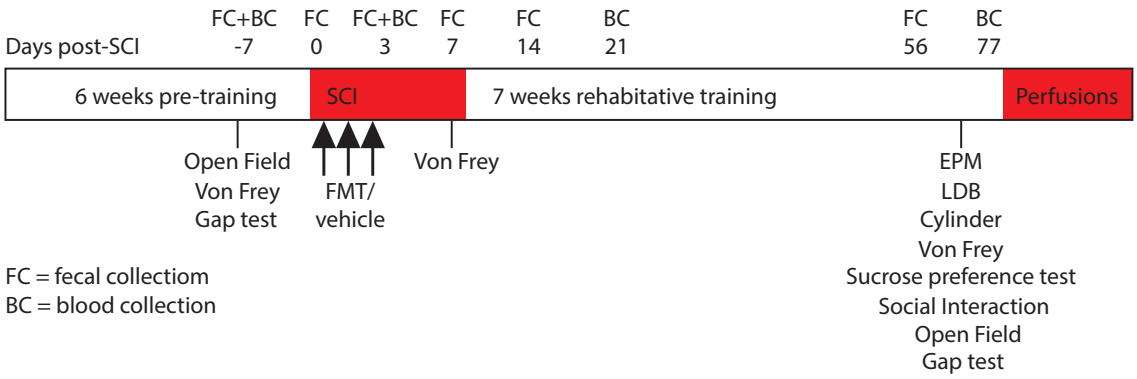

Figure 3.1: Experimental Timeline

3.2.3 Single pellet grasping training

The SPG protocols and equipment were used as previously described [Torres-Espín et al., 2018b]. Rats were first acclimatized to the SPG double-window enclosure and each rat's preferred paw was established by manually counting the number of left and right reaching attempts for a sucrose pellet. Once the preferred paw was established, the pellet dispenser was positioned so the rat could only reach the pellet with its preferred paw. Rats were trained to reach for a pellet on one side of the enclosure and then travel to the opposite end where another pellet was dispensed, and so on. Training consisted of 10 minutes per rat per day, 5 days a week for 6 weeks prior to SCI. Rehabilitative training began 10 days following SCI and continued for 7 weeks. Training sessions were video recorded and analyzed offline. The total number of attempts made (rat reached towards the pellet) and number of successes (rat successfully reached, grasped and consumed the pellet) were quantified. Success rate was defined as the total number of successful attempts divided by the total number of attempts multiplied by 100.

3.2.4 Spinal cord injury

SCI cervical contusions were performed as previously described [Schmidt et al., 2020b]. Rats were anesthetized with isoflurane (5% induction; 2.5% maintenance; 50:50 air/oxygen mixture) and the dorsal neck was shaved and disinfected with 10% chlorhexidine digluconate (Sigma-Aldrich). The Infinite Horizons impactor (Precision Systems & Instrumentation) was used to deliver a 125 kdyn unilateral contusion 1.25mm lateral to the midline (on the side of the preferred paw) at an angle of 15 degrees (towards midline) at cervical level 5. Synthetic braided sutures were used to suture the muscles and the skin was closed using 9mm stainless steel clips. Buprenorphine was injected immediately after SCI and again 8 hours after (0.03 mg/kg; subcutaneous; WDDC). Saline was injected (4 ml, subcutaneous) post operatively and bladders were manually expressed until voiding was re-established (within 2 days post SCI).

3.2.5 Behavioural testing

Light dark box

Rats were placed in the light component of a customized light-dark box apparatus (dark compartment 0 lux; light compartment 100 lux; each chamber 30 cm long x 30 cm wide x 30 cm high) and allowed to freely explore for 10 minutes while video recorded from above. The time spent in the light component was analyzed as measures of anxiety-like behaviour.

Elevated plus maze

Rats were placed in the center of the elevated plus maze apparatus (2 closed arms: each 50 cm long x 10 cm wide x 50 cm high, and 2 open arms: each 50 cm long x 10 cm wide x 1 cm high) and video recorded from above for 10 minutes. Customized

tracking software (https://github.com/cdoolin/rat-apps) was used to quantify the percent time spent in the open arms and the total distance travelled. This test was used only once to avoid one-trial tolerance [18].

Sucrose preference test

Rats were exposed to two water bottles in their home cage: one with a 2% sucrose solution and one with regular drinking water. The percentage of sucrose water consumed over 48 hours was calculated as a measure of anhedonia. The location of the bottles was switched at 24 hours to avoid side preference.

Open field

Rats were placed in the center of an open field arena (100 cm long x 80 cm wide x 30 cm high) and video recorded from above for 5 minutes. Offline video analysis was performed using customized tracking software (https://github.com/cdoolin/rat-apps) to quantify the total distance travelled.

Cylinder

Rats were placed in an acrylic cylinder (21 cm diameter x 23 cm high) with mirrors located behind so that the rat could be observed from all sides using one camera. Each rat was video recorded for 3 minutes and offline analysis was used to quantify the number of left and right paw placements made on the side of the cylinder. Forepaw asymmetry was expressed as the percentage of ipsilesional paw placements.

Von Frey Test

Rats were acclimatized to the testing chamber (IITC Life Science, CA, USA) prior to testing. Tactile sensitivity was assessed on both forepaws (when the animal was weight-bearing on its forepaws). A rigid probe connected to the automated Von

Frey apparatus was applied in increasing pressure until the rat displayed a defined nociceptive response (paw retraction, licking) and the maximum pressure that elicited a withdrawal was recorded. This was repeated 3 times per paw, with a minimum of 3 minutes between measures. The average of the 3 measures per paw was used for analysis.

Social Interaction

The test rat was placed in the open field apparatus with an unfamiliar, uninjured rat for 10 minutes while video recorded from above. The time spent in active interaction (sniffing, nipping, grooming, following, mounting, kicking, boxing, wrestling, jumping on, and crawling) was recorded as a measure of anxiety-like behaviour [File and Hyde, 1978].

3.2.6 Fecal collection and transplantation

Fecal samples were collected as previously described [Schmidt et al., 2020b]. During the dark cycle, rats were placed into individual sterile cages. Fecal pellets were immediately collected, placed into sterile eppendorf tubes and stored in a -80 °C freezer until further processing. For the fecal transplant solution, pellets were collected from uninjured FMT donors (pooled from all 10 rats) and immediately processed to make the transplant solution. The fresh fecal matter was diluted 1:10 in sterile PBS (10%), L-cysteine HCL (0.05%), glycerol (20%) and sterile water (60%) and passed through a 100 μm filter. The solution was frozen at -20 °C and thawed at room temperature for 12 hours prior to use (the use of frozen fecal matter for FMT has proven to be effective [File, 1990]). The SCI + vehicle group received the filtered solution that did not contain fecal matter. 2 hours after SCI and for 2 consecutive days after, rats were gavaged with 500 μl of either FMT or vehicle solution.

3.2.7 16s rRNA sequencing

DNA was extracted as previously described [Laffin et al., 2019]. Fecal microbial DNA was extracted with AquaStool solution (Multitarget Pharmaceuticals LLC, Colorado Springs, USA) as per the manufacturer instructions. Briefly, 100mg of mouse fecal pellet was homogenized in the AquaStool solution with 0.1mm beads at 0.6m/s for 40s. AquaRemove was added to remove potential PCR inhibitors per manufacturer's instruction followed by ethanol/NaCl precipitation for further purification. DNA Samples were sent to Genome Quebec (McGill University, Montreal, Canada) for Illumina Miseq sequencing. V3-V4 region of universal 16S rRNA primers with 341 forward primer: 5'-TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG CCT ACG GGN GGC WGC AG-3' and 805 reverse primer: 5'- GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA GGA CTA CHV GGG TAT CTA ATC C-3' were used.

Demultiplexed paired-end sequences were merged and performed quality control implementation (mean sequence quality score 000 30) and features table construction (amplicon sequences variants, ASVs) via DADA2 [Callahan et al., 2016] plugin in QIIME2 (version 2019.10) [Bolyen et al., 2019]. An even sequence depth of 9,452 reads per sample was used to conduct microbiome diversity and composition analyses. Taxonomy assignments from the phylum to genus levels were conducted by a pre-trained Naive Bayes classifier [Bolyen et al., 2019] (Silva 132 99% OTUs database) and the q2-feature-classifier function in QIIME2. Alpha-diversity of Shannon index and community balance of Pielou's evenness index, and beta-diversity analysis (unweighted unifrac emperor distance) were conducted using the QIIME2.

3.2.8 Blood collection

The area over the tarsal joint was shaved and the saphenous vein was punctured using a sterile needle. Blood was collected into a microvette CB300 capillary tube

(Sarstedt Inc, Nümbrecht, Germany) and immediately centrifuged for 5 minutes at 3000 rpm. Plasma was then pipetted into sterile microcentrifuge tubes and stored at -80 °C freezer until further processing.

3.2.9 Cytokine analysis

Frozen plasma samples were sent to Eve Technologies (Calgary, Canada) and diluted 2-fold for the Rat Cytokine 27-Plex discovery assay. Cytokines and chemokines measured were: Eotaxin, EGF, Fractalkine, IFN-gamma, IL-1a, IL-1b, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12(p70), IL-13, IL-17A, IL-18, IP-10, GRO/KC, TNF-alpha, G-CSF, GM-CSF, MCP-1, Leptin, LIX, MIP-1alpha, MIP-2, RANTES, and VEGF. GRO/KC values are not reported as they were out of range in our samples. For heatmap visualization, plasma analytes were expressed as a change from baseline (x2 - x1 / x1).

3.2.10 Intestinal permeability assay

Once the uninjured FMT donor rats had completed all of their baseline testing and fecal collections, they were used to assess intestinal permeability. These rats were randomly divided into an SCI + vehicle group (n = 5) and an SCI + FMT group (n = 5) and received identical treatment as the original treatment groups (2 hours after SCI and for 2 consecutive days after, rats were gavaged with 500 µl of either FMT or vehicle solution). The day before injury and again 7 days following SCI, rats were fasted for 4 hours and then gavaged with 0.6g/kg FITC dextran (4 kD, Sigma-Aldrich) diluted in sterile PBS. Blood was collected 4 hours later via the saphenous vein and plasma was collected as described above. Plasma samples were diluted 1:10 with sterile PBS and transferred to an opaque-bottom 96-well plate. Samples were run in duplicates and a PBS blank and standard curve measurements were measured on the same plate. Fluorescence was determined at 530 nm with an excitation at 485

nm on a plate reader (SpectraMax, Molecular Devices). Intestinal permeability was quantified as a fold change from baseline levels.

3.2.11 Perfusion and tissue cutting

At the end of rehabilitative training and all final behavioural assessments, rats were euthanized with sodium pentobarbital (240 mg/kg). Rats were transcardially perfused with saline containing 0.02 g heparin/L followed by 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS) and 5% sucrose. Spinal cords were extracted and post-fixed in 4% paraformaldehyde 4 °C for 4 hours and transferred to a 30% sucrose solution for 5 days. A 1 cm block around the lesion site was embedded in O.C.T. (Sakura Finetek, USA), mounted onto filter paper and frozen at -40 °C in 2-methylbutane. A NX70 cryostat (Fisher Scientific) was used to section the cord at a thickness of 25 µm. Every second section was kept and staggered across eight slides and stored at -20 °C.

3.2.12 Lesion analysis

Frozen slides were thawed for 1 hour at 37 °C and washed in TBS (2 x 10 min). Slides were placed into 0.5% cresyl violet for 3 minutes, rinsed with filtered water and serially dehydrated in EtOH (2 minutes in 50%, 75%, and 99%). Slides were then placed in xylene (2 x 2 minutes) and coverslipped with Permount™. Images of the entire lesion extension were taken with an epifluorescence microscope (Leica DM6000B, camera Leica DFC350 FX) at 5x magnification and analyzed using ImageJ (National Institute of Health, USA). Lesion size was calculated as the percent of damaged tissue divided by the total area of the spinal cord cross section. IBA1

3.2.13 Microglia analysis

Sections were thawed at 37 °C for 1 hour and rehydrated in PBS (2 x 10) minutes followed by PBS with 0.3% Triton™ X-100 (PBS-T) (1 x 10 min). Blocking buffer consisting of 5% normal donkey serum in PBS-T was applied 1 hour at room temperature. Sections were incubated overnight at room temperature in rabbit-anti-IBA1 (1:500, Wako) antibody with blocking buffer. The next day, sections were washed with PBS (3 x 10 minutes) and incubated with donkey-anti-rabbit AF488-conjugated (1:500, Life Technologies) antibody in the blocking buffer solution for 2 hours. Sections were then rinsed in PBS (2 x 10 min) and cover slipped with Fluoromount™. Images were captured with an epifluorescence microscope (Leica DM6000B, camera Leica DFC350 FX) and analyzed using ImageJ (National Institute of Health, USA). 5x magnification images were taken to visualize the entire spinal cord cross section 0.25cm rostral to the lesion, at the lesion epicenter, and 0.25cm caudal to the lesion. The IBA1 optical density per spinal cord cross section was quantified and expressed as a percentage area of positive staining.

3.2.14 Statistical analysis

Statistical analyses were performed using GraphPad Prism 8 (San Diego, CA) and an alpha value of 5% or less was considered significant. Normality was analyzed using the D'Agostino-Pearson omnibus test. Data at a single time point was analyzed using an unpaired parametric t-test for two groups and an ordinary one-way ANOVA for three groups (non-parametric tests were used for data that did not pass normality). Data with multiple time points was analyzed using an ordinary repeated measures two-way ANOVA followed by Sidak's multiple comparison test.

3.3 Results

3.3.1 Fecal microbiota transplant from *anxious* donors

Although the rats used in the present experiment are genetically identical, there is a natural variability in their baseline levels of anxiety-like behaviour, which can be further influenced by environmental stressors. To determine how important optimal donor selection is, the two cages of rats who naturally displayed decreased baseline activity in the open field (as an indicator of anxiety-like behaviour [Russell, 1973, Gould et al., 2009]) were chosen as the FMT donors ($p = 0.0052$) (Fig. 3.2A). This altered behavioural phenotype was associated with significantly reduced levels of *Lactobacillus* in the FMT donor's stool compared to the experimental groups (SCI + Vehicle and SCI + FMT) at baseline ($p = 0.0006$) (Fig. 3.2B). Reflecting the lack of *Lactobacillus* in the donor stool, the FMT solution also contained a lack of Lactobacillus (Fig. 3.2B). FMT donors displayed a similar alpha diversity (the bacterial variance within the samples) as the experimental groups, which was also reflected in the FMT solution (Fig. 3.2C). Compared to previously successful FMT donors (which, when transferred to rats after SCI, prevented both SCI-induced dysbiosis and anxiety-like behaviour [Schmidt et al., 2020b]), *anxious* FMT donors spent significantly less time in the open arms of the elevated plus maze, confirming their increased anxiety-like phenotype ($p = 0.0002$) (Fig. 3.2D). *anxious* FMT donors also displayed significantly lower proportions of *Lactobacillus* compared to the non-*anxious* FMT donors described in chapter 2 ($p < 0.0001$; [Schmidt et al., 2020b]) (Fig. 3.2E). These data suggest that, although the FMT donors were uninjured and had a diverse microbiota composition, they had an increased anxiety-like phenotype and reduced proportion of the genus *Lactobacillus*, a commonly prescribed probiotic [Sanders and Klaenhammer, 2001, Maragkoudakis et al., 2006, Lebeer et al., 2008].

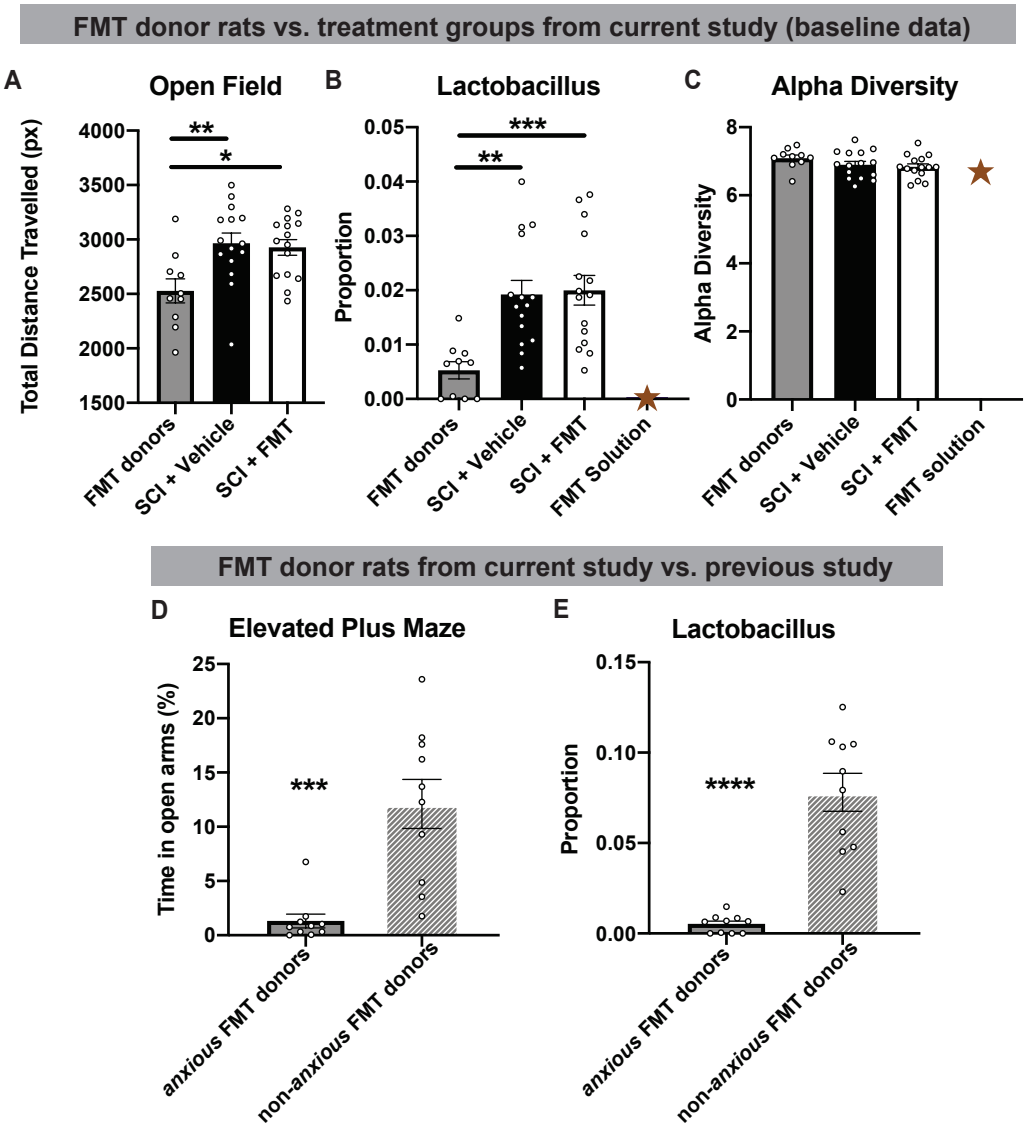

Figure 3.2: (A) Fecal microbiota transplant (FMT) donors travelled significantly less distance in the open field compared to the SCI + vehicle and SCI + FMT treatment groups in the present experiment (measured at baseline prior to SCI). (B) Fecal matter from FMT donors had significantly decreased baseline proportions of Lactobacillus, which is also reflected in the decreased amount of Lactobacillus found in the FMT solution. (C) All groups had similar baseline levels of baseline alpha diversity, including the FMT solution. (D) FMT donors in the current study displayed significantly increased anxiety-like behaviour in the elevated plus maze (indicated by the percent of time spent in the open arms) and (E) had significantly less fecal proportion of Lactobacillus relative to successful FMT donor rats from previous experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. Gold star represents the FMT solution (A single value and therefore not included in statistical analysis). Error bars represent standard error mean.

3.3.2 FMT from *anxious* rats did not prevent dysbiosis after SCI

Fecal samples were collected prior to injury, on the day of injury, then 3, 7, 14 and 56 days after SCI for 16s rRNA sequencing. The differences in microbial abundance between the fecal samples was visualized using beta diversity plots. On the day of injury, 3- and 14-days post-SCI there was a deviation in the samples away from baseline values, confirming our previous results that a cervical SCI induces acute dysbiosis. At 7- and 56-days post-SCI, the samples clustered closely with baseline values (Fig. 3.3A). When looking at the beta diversity of the two treatment groups across all time points, there was no difference between FMT or vehicle treated groups (Fig. 3.3B). Although SCI resulted in acute dysbiosis visualized in the beta diversity plots, there was no significant effect of injury or FMT on the alpha diversity (Fig. 3.3C). Next, we looked at the four most abundant bacteria at the Phylum level: Bacteroidetes, Firmicutes, Cyanobacteria and Proteobacteria. There was no effect of SCI or FMT in the proportion of Bacteroidetes or Firmicutes (Fig. 3.3D & E). The proportion of Proteobacteria was increased on the day of injury and 3 days post injury (Fig. 3.3F) and the proportion of Cyanobacteria was increased 3 days post-SCI ($p<0.0001$ for both) (Fig. 3.3G), however there were no significant effects of FMT treatment. The proportion of the genus *Lactobacillus*, a common bacteria present in probiotics [26], was reduced chronically after SCI in both FMT treated and untreated groups ($p<0.0001$) (Fig. 3.3H). There was no significant difference between groups in any bacteria at the genus level (Figs. B.1 and B.2). These results indicate that the FMT from *anxious* donor rats was not successful in preventing SCI-induced dysbiosis.

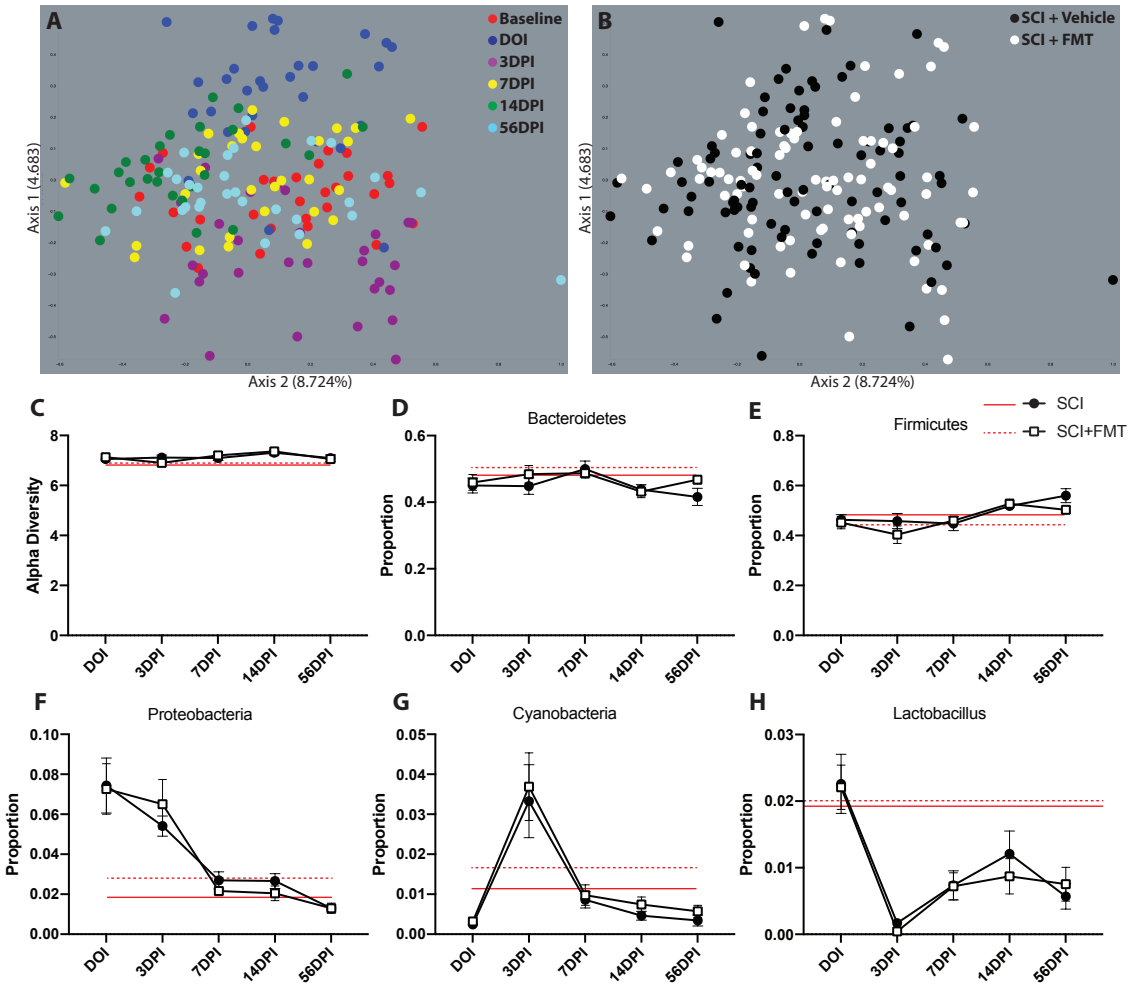

Figure 3.3: (A) PCoA plot of beta diversity shows the diversity between fecal samples over time on the day of injury (DOI), 3-, 7-, 14- and 56-days post-injury (DPI). (B) The same PCoA plot is shown with the colors representing the groups instead of timepoints. Axis 1 and 2 explain 4.683% and 8.724% of the variance between samples, respectively. (C) There was no effect of injury or treatment on the alpha diversity. The four most abundant operational taxonomic units at the phylum level also show no differences between experimental groups in the proportion of (D) Bacteroidetes, (E) Firmicutes, (F) Proteobacteria and (G) Cyanobacteria. (H) The proportion of the genus Lactobacillus was reduced after SCI but not affected by FMT. Red lines represent baseline values. Error bars represent standard error mean.

3.3.3 FMT from *anxious* rats did not affect functional recovery from SCI

10 days following SCI, rats began 7 weeks of rehabilitative therapy in the SPG task which targeted their impaired forepaw (Fig. 3.4A). There was no difference between FMT or vehicle treated rats in the number of attempts made to reach for the pellet, indicating that the FMT did not influence participation in rehabilitation (Fig. 3.4C). There was a significant decrease in success rate following SCI, which gradually improved for both vehicle and FMT groups throughout the rehabilitation period (Fig. 3.4D). To prevent compensatory pellet-scooping strategies, rats were tested in a modified task where a gap was introduced between the pellet and the training chamber (Fig. 3.4B). Rats which received an FMT performed better in the gap test at the end of the rehabilitation period, however this did not reach statistical significance ($p = 0.089$) (Fig. 3.4E). FMT treatment did not alter mechanical sensitivity, however both groups experienced reduced sensitivity of the ipsilesional forepaw at 1- and 9-weeks post injury (Fig. 3.4F). At the end of the rehabilitative training period, rats were tested in the cylinder task to measure forepaw asymmetry and in the open field to assess locomotor activity; there were no differences between groups in either of these tests (Fig. 3.4G & H). Although there was no significant treatment effect in the efficacy of rehabilitative training or motor recovery following SCI, treatment with FMT from *anxious* donors resulted in a chronic (11 weeks post injury) decrease in the density of microglia caudal to, but not rostral to or at, the lesion site compared to vehicle controls (Fig. 3.5A-F). This decreased microglial density was not due to differences in injury size, as the lesion extension and area were similar between groups (Fig. 3.5G-I).

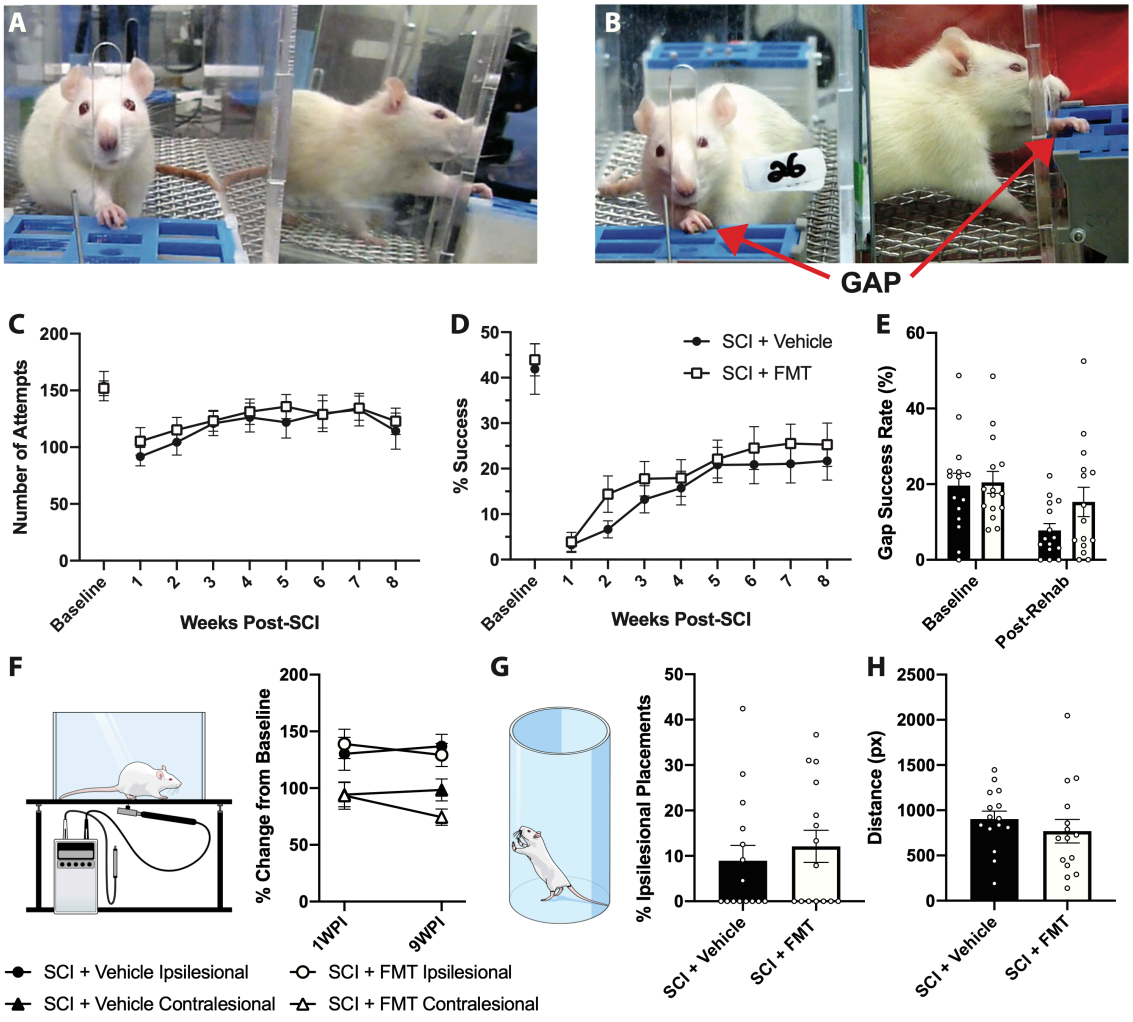

Figure 3.4: (A) Image of a rat in the regular single pellet grasping apparatus, reaching through a narrow opening for a food pellet. (B) Image of a rat reaching in the single pellet grasping apparatus that has been modified to include a gap between the pellet and the opening of the chamber (to eliminate compensatory pellet scooping behaviour). (C) There was no difference between FMT and vehicle groups in the number of attempts or (D) the success rate in rehabilitative training. (E) The success rate in the modified gap task was measured once at baseline and again at the end of the rehabilitation period. There were no significant differences between FMT and vehicle treated groups in the von frey test (quantified as the force required to elicit a withdrawal response, expressed as a percentage of baseline values) (F), the cylinder test (G) or the distance travelled in the open field (H). Error bars represent standard error mean.

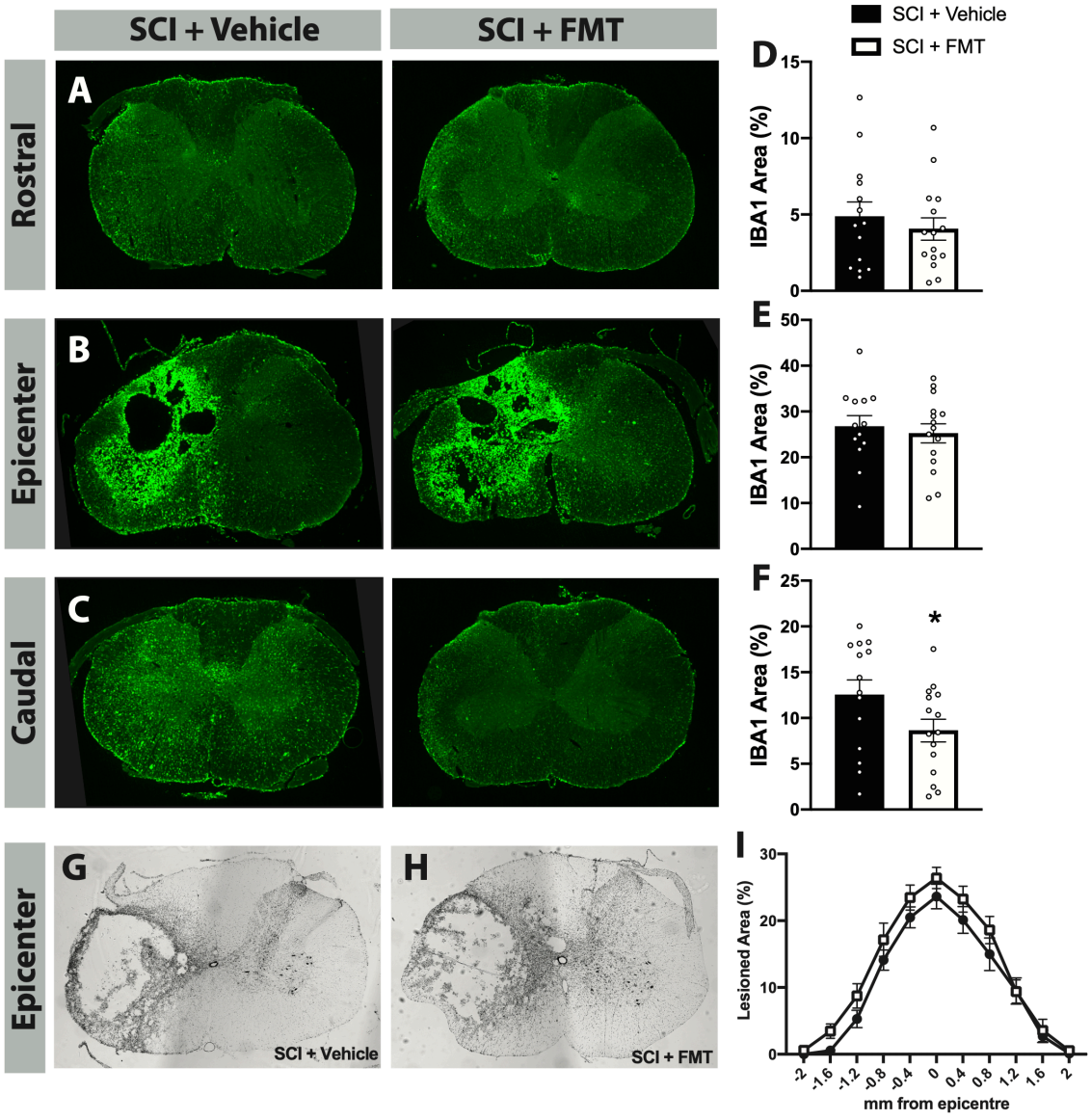

Figure 3.5: Representative images of IBA1 positive cells in the cervical spinal cord immediately rostral to the injury (A), at the injury epicenter (B) and immediately caudal to the injury (C). The percent area of IBA1 positive staining rostral to, at and caudal to the lesion is quantified in (D-F), respectively. Immediately caudal to the injury, SCI + FMT rats displayed a significantly reduced density of IBA1 positive cells compared to vehicle controls. Representative cross sections of the maximum injury site for SCI + Vehicle and SCI + FMT groups are shown in (G) and (H), respectively. (I) Quantification of the rostral (negative measurements) to caudal (positive measurements) extension of the lesion area was expressed as a percentage of lesioned tissue. * $p < 0.05$. Error bars represent standard error mean.

3.3.4 FMT from *anxious* donors increased anxiety-like behaviour

Nine weeks after SCI, at the end of rehabilitative training, rats were tested for depressive- and anxiety- like behaviours. Rats that received an FMT from *anxious* donors spent significantly less time in the open arms of the elevated plus maze ($p = 0.0341$), although both groups travelled a similar total distance (Fig. 3.6A-C). SCI + FMT groups also spent less time in the light component of the light-dark box (Fig. 3.6D) and drank significantly less sucrose solution ($p<0.0001$) (Fig. 3.6E) compared to vehicle controls. Both FMT and vehicle groups spent a similar amount of time interacting in the social interaction test (Fig. 3.6F).

3.3.5 Temporal profile of plasma analytes following spinal cord injury

To determine the effect of both SCI and the FMT on acute and chronic systemic inflammation, plasma analytes were measured before SCI, then 3, 21 and 77 days after injury. There was an overall trend of increased levels of all plasma analytes at 3- and 21-days post SCI, and a drastic downregulation by 77 days in both experimental groups (Fig. 3.7). Looking at the concentrations of each plasma analyte over time, rats which received the FMT displayed significantly increased concentration of LIX at 77 days ($p = 0.009$), reduced levels of RANTES at 21 days ($p = 0.012$) and higher levels of RANTES by 77 days post injury ($p = 0.023$) (Fig. 3.8B). There was no significant treatment effect in any of the other chemokines, cytokines or other analytes measured (growth factors, glycoproteins and the hormone leptin) (Fig. 3.8A & C).

3.7 FMT from *anxious* donors increased intestinal permeability

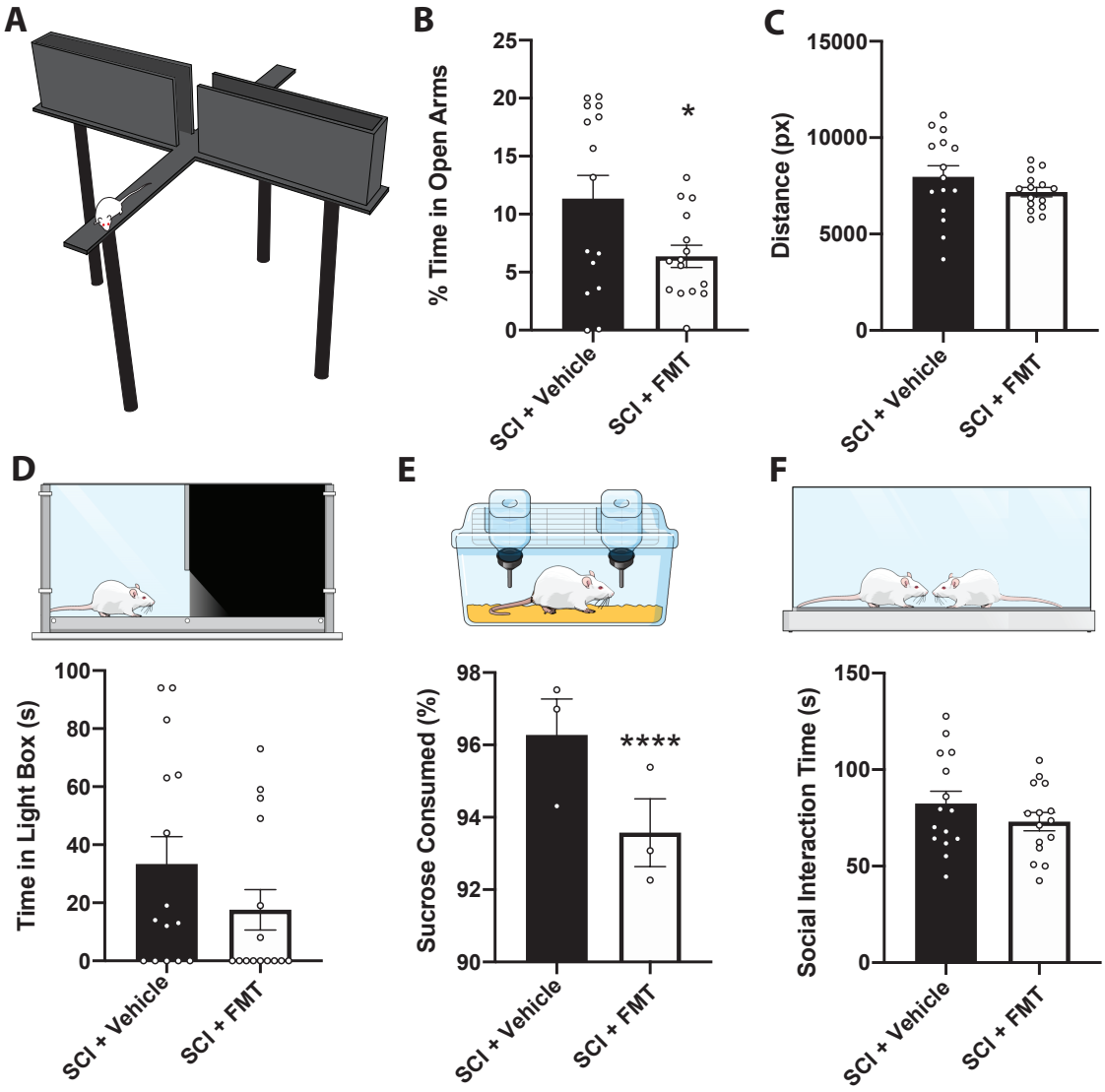

Figure 3.6: At the end of rehabilitative training, rats were tested for anxiety-like and depressive-like behaviours. (A) Schematic of a rat in the open arm of the elevated plus maze. (B) SCI + FMT rats spent significantly less time in the open arms compared to untreated rats. (C) Both groups of rats travelled a similar amount of distance in the elevated plus maze. (D) SCI + FMT rats spent less time in the light-component of the light-dark box and (E) drank less sucrose water than untreated rats (each data point represents a cage containing 5 rats, each of which were considered for statistical analyses). (F) Both fecal transplant treated and untreated rats spent a similar amount of time interacting in the social interaction test. * $p < 0.05$, * $p < 0.0001$. Error bars represent standard error mean.

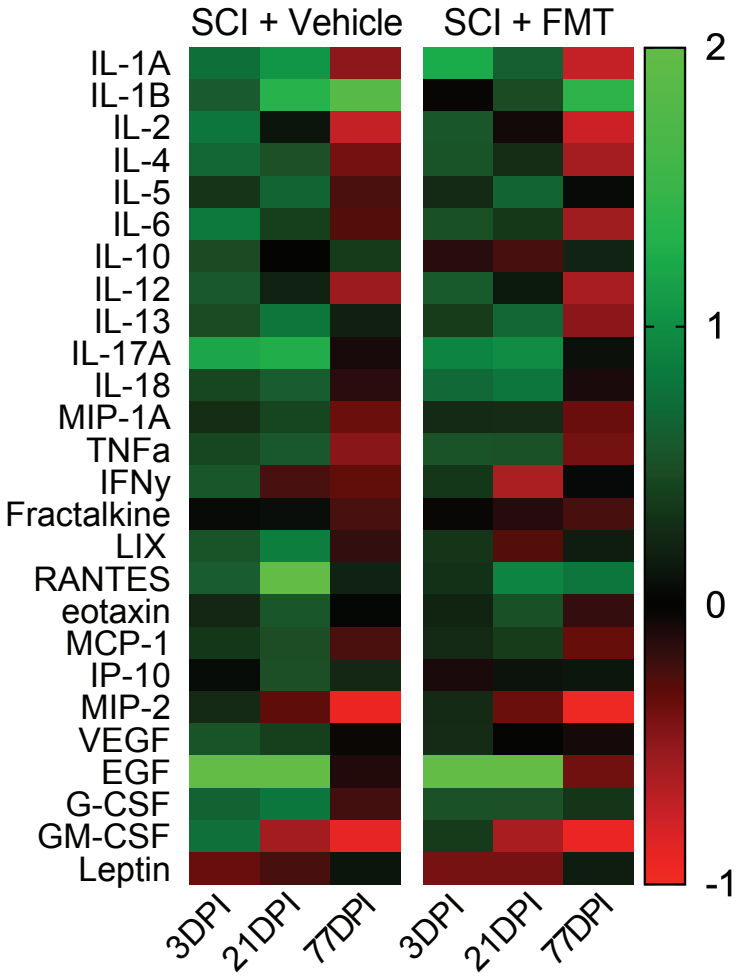

Figure 3.7: Plasma markers (cytokines, chemokines, growth factors, glycoproteins and hormones) were expressed as a change from baseline values and plotted over time (positive numbers represent an increase from baseline values and negative numbers represent a decrease from baseline values). Values above 2 were set at 2 for visualization purposes (RANTES and EGF were affected).

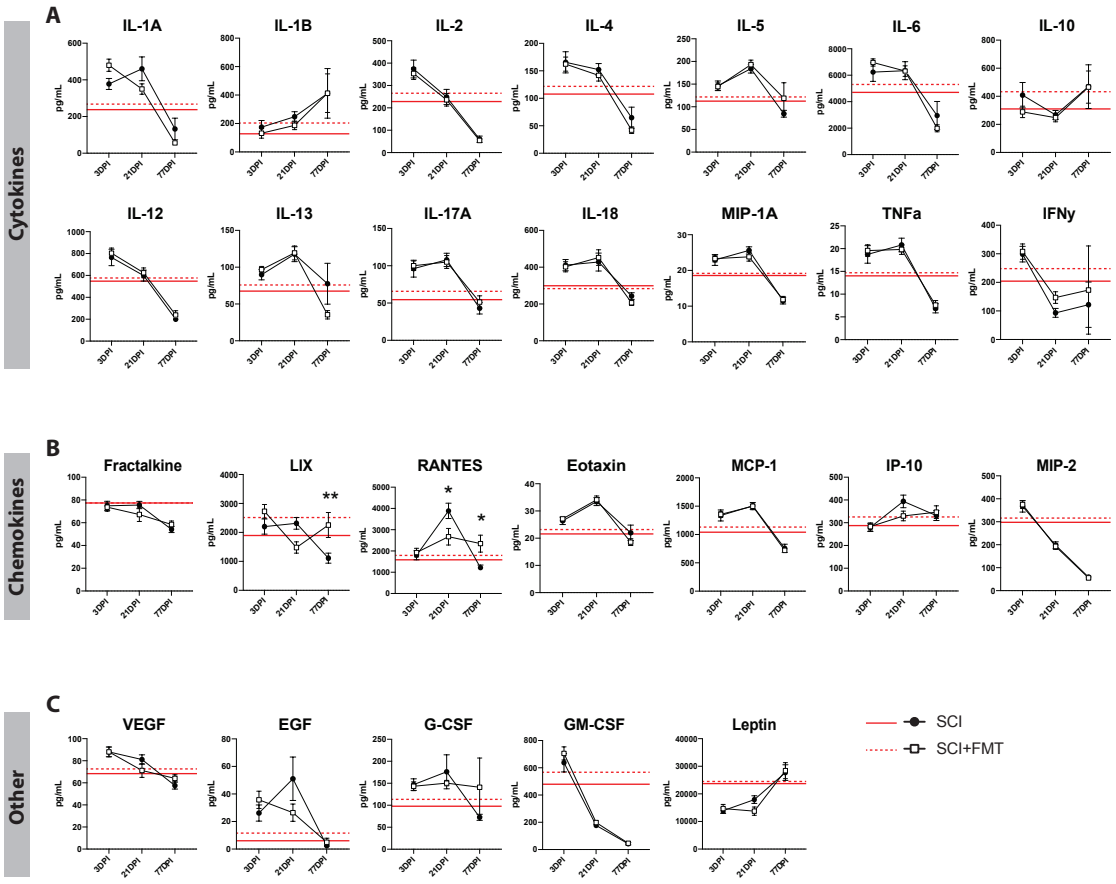

Figure 3.8: (A) Temporal profile of plasma cytokines 3 days post injury (3DPI), 3 weeks post injury (3WPI) and 11 weeks post injury (11WPI) for SCI + Vehicle and SCI + FMT groups. (B) Temporal profile of plasma chemokines show SCI + FMT rats have significantly increased levels of LIX and RANTES at 11 weeks compared to vehicle controls. (C) Profile of other plasma markers (growth factors, glycoproteins and hormones) over time after injury. Red lines represent baseline values. * $p < 0.05$. ** $p < 0.01$. Error bars represent standard error mean.

Increased intestinal barrier permeability has previously been shown in mice 7 days following a thoracic SCI, which can allow bacterial and other matter to translocate across the impaired epithelial tight junctions [Kigerl et al., 2016a, Ghosh et al., 2020]. To test whether a cervical contusion SCI in rats also triggers an increase in intestinal permeability, rats were gavaged with FITC-dextran and the concentration of FITC was measured in blood 4 hours later (Fig. 3.9A). This test was performed before SCI and again 7 days later and expressed as a fold change from baseline to account for individual differences. SCI alone did not alter intestinal permeability, however FMT from *anxious* donors increased intestinal permeability by nearly 20% compared to baseline (Fig. 3.9, SCI + Vehicle vs. SCI + FMT $p = 0.043$). This increased intestinal permeability was not due to differences in lesion size (Fig. 3.9C). To determine whether differences in intestinal permeability between groups was associated with changes in systemic inflammation at the same time, plasma cytokines/chemokines were analyzed in these rats 7 days post injury. There was no difference between FMT or vehicle controls in plasma concentrations of cytokines, chemokines, or other growth factors, glycoproteins and hormones (Fig. 3.9D – F).

3.4 Discussion

The use of healthy human stool to treat diseases has been documented in Chinese medicine for over 1700 years [Zhang et al., 2012]. However, the first report of FMT treatment in modern Western medicine was not until 1958 [Eiseman et al., 1958], and it was not until 2013 that FMT was included in the treatment guidelines for recurrent *Clostridium difficile* infections [Surawicz et al., 2013]. The popularity of FMT as a treatment is increasing rapidly for various other diseases, such as: irritable bowel disease, irritable bowel syndrome, obesity, autism, Parkinson's disease, multiple sclerosis, metabolic syndrome, stroke and SCI [Schmidt et al., 2020b, Sun et al., 2018,

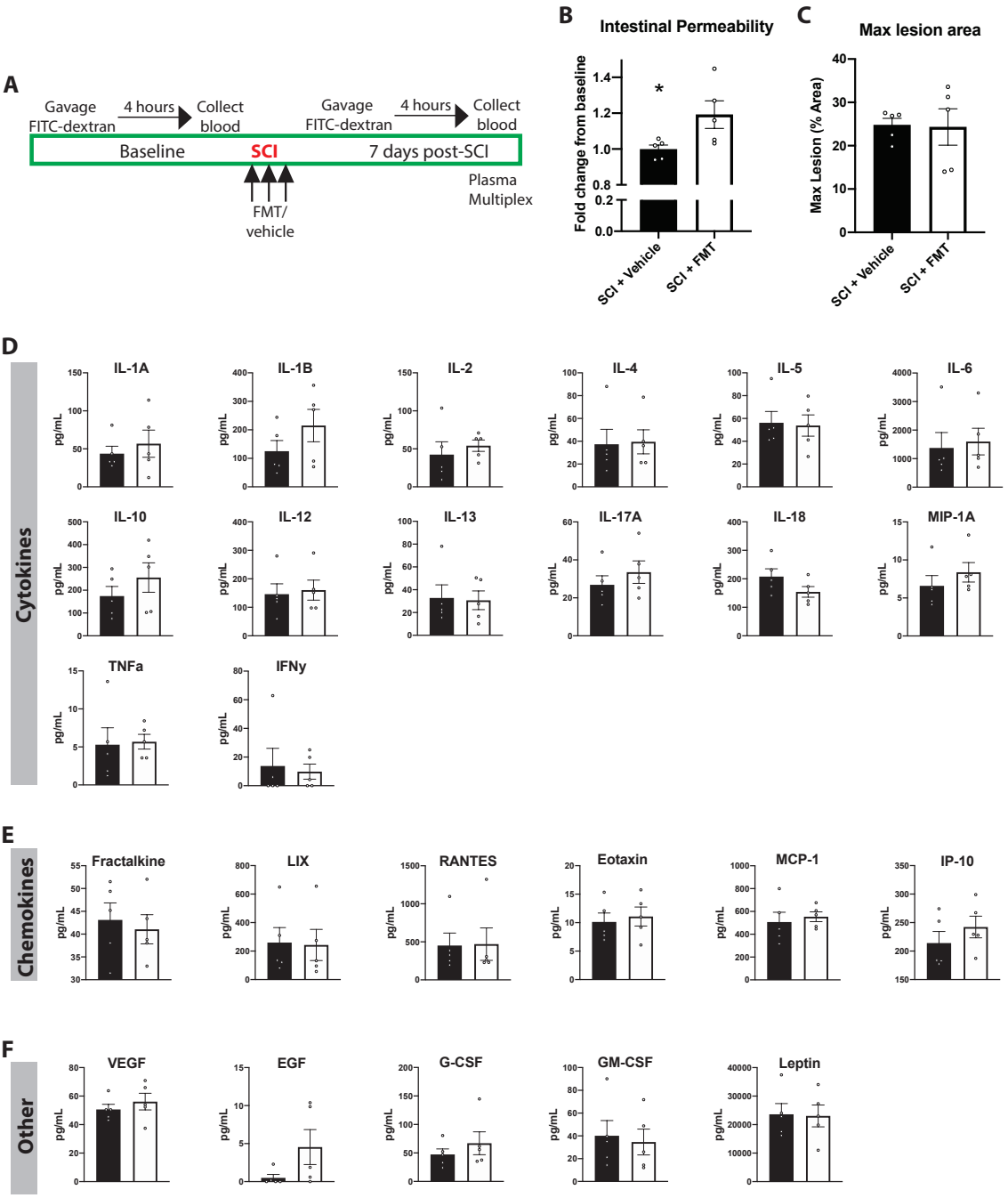

Figure 3.9: (A) The FITC-dextran test for intestinal permeability was performed at baseline prior to spinal cord injury and again 7 days after injury. (B) SCI + FMT rats displayed significantly increased intestinal permeability relative to vehicle controls. (C) There were no differences between groups in the maximum lesion size. 7 days following injury, plasma was extracted and analyzed for levels of various cytokines (D), chemokines (E) and other growth factors, glycoproteins and hormones. * $p < 0.05$. Error bars represent standard error mean.

Xue et al., 2020, Xu et al., 2019]. Aside from excluding donors with known fecal matter pathogens, the selection of FMT donor does not appear to influence the success of treatment for *Clostridium difficile* infection [Kassam et al., 2013, Osman et al., 2016]. However, the same is not necessarily true for other disorders, especially those with more complicated microbiota-disease interactions such as SCI. Donor selection criteria beyond the exclusion of known pathogens is therefore a crucial area of research that is still in its infancy [Duvallet et al., 2019, Barnes and Park, 2017].

In chapter 2 we showed that FMT from uninjured, non-*anxious* rats prevented both acute dysbiosis and the development of anxiety-like behaviour following SCI [Schmidt et al., 2020b]. Contrary to our hypothesis, here we show that optimal donor selection is essential for successful (i.e. prevents SCI-induced dysbiosis) FMT treatment following SCI. Critically, the FMT donor rats in the present study were uninjured, free of pathogens and are genetically compatible to the recipients and would likely have passed screening criteria used clinically for FMT donors. In FMT trials, potential donors undergo a preliminary interview to rule out potential risk factors such as drug use and medical history [Duvallet et al., 2019, Barnes and Park, 2017, Bibbò et al., 2020, Woodworth et al., 2017, Wilson et al., 2019]. Individuals who pass the preliminary interview then undergo blood and stool testing to exclude the risk for transferring infectious diseases [Duvallet et al., 2019, Barnes and Park, 2017, Bibbò et al., 2020, Woodworth et al., 2017]. Although a history of psychiatric conditions is a risk factor for potential FMT donors [Cammarota et al., 2017], it is often not considered for donor screening [Duvallet et al., 2019, Barnes and Park, 2017, Bibbò et al., 2020, Woodworth et al., 2017, Wilson et al., 2019]. This is particularly relevant for studies on the efficacy of FMT for depression and anxiety. While there are relatively few human studies on FMT for treating psychiatric disorders, the existing results show short-term success but inconsistent long-term improvement [Mizuno et al., 2017, Kurokawa et al., 2018, Mazzawi et al., 2018,

Paramsothy et al., 2015, Huang et al., 2019]. The results of the present study in rats suggest that even minor behavioural abnormalities can impact the success of FMT and may help explain the inconsistent long-term results of FMT treatment for psychiatric disorders. Indeed, multiple animal studies show that the behaviour of the FMT donor can be transferred to the recipient [Li et al., 2019, Lv et al., 2019, Siopi et al., 2020, Kelly et al., 2016, Zhao et al., 2020].

In the present study, the FMT donors had increased baseline levels of anxiety-like behaviour which was associated with a significant reduction in the proportion of *Lactobacillus* in their stool. Supporting this association is the finding that humans diagnosed with major depressive disorder have reduced levels of *Lactobacillus* compared to controls [Aizawa et al., 2016]. Furthermore, *Lactobacillus* is one of the most frequently used probiotic bacteria and has been shown to improve anxiety and depression in multiple preclinical studies [Liu et al., 2016, Liang et al., 2015, Bravo et al., 2011] and clinical trials [Slykerman et al., 2017, Lew et al., 2019, Wallace and Milev, 2017]. In a recent double-blind, randomized, placebo controlled study, treatment with the probiotic *Lactobacillus* was shown to significantly reduce kynurenine concentrations in patients with major depressive disorder [Rudzki et al., 2019]. The kynurenine pathway can be activated by inflammation and is thought to play a significant role in the pathogenesis of depression [Ogyu et al., 2018, Savitz, 2016]. Reducing kynurenine concentrations by blocking indoleamine 2,3-dioxygenase (the rate-limiting enzyme in the kynurenine pathway of tryptophan metabolism [Savitz, 2020]) has also been shown to block LPS induced depressive-like behaviour in rodents [O'Connor et al., 2009]. The kynurenine pathway may therefore be an important player in the microbiota-immune-brain axis involved in the pathogenesis of depression and anxiety following SCI. The lack of *Lactobacillus* present in the FMT donor stool may indicate alterations in the kynurenine pathway and be, at least, partly responsible for the unsuccessful FMT. However, there were no significant differences

between FMT and vehicle groups in the proportion of *Lactobacillus* following SCI at the time points measured. This may be because both groups displayed no detectable amounts of *Lactobacillus* at 3 days post SCI, presenting a floor effect. Furthermore, more detailed sequencing may be required to detect differences at the species level, as there are over 260 metabolically unique *Lactobacillus* strains and only some species are used in probiotics [Maragkoudakis et al., 2006, Zheng et al., 2020]. Nonetheless, sequencing at the Phylum level indicated a global acute shift in the microbiota composition on the day of injury and 3 days post-SCI, similar to previously reported [Schmidt et al., 2020b]. However, in the present study, using FMT from *anxious* donors with low levels of *Lactobacillus* was unsuccessful in preventing SCI-induced dysbiosis.

Although the FMT from *anxious* donors used in the present study did not improve SCI-induced dysbiosis, there were some long-term effects on inflammation and anxiety-like behaviour. There is a strong link between increased inflammation and the development of mental health disorders. In rodent models of SCI, increased local (brain and spinal cord tissue) and systemic inflammation have been associated with the development of anxiety and depressive-like behaviours [do Espírito Santo et al., 2019, Maldonado-Bouchard et al., 2016a, Wu et al., 2014]. Here, rats that received the FMT from *anxious* donors displayed increased anxiety-like behaviour, which may suggest an increased inflammatory phenotype. In support of this, FMT from *anxious* donors resulted in increased intestinal permeability of the FMT recipient rats, which can allowed bacterial matter such as LPS to translocate across the impaired epithelial tight junctions [Kigerl et al., 2016a, Drewe et al., 2001]. Once in circulation, LPS triggers a strong immune response that can reach the central nervous system and last for months after exposure [Lu et al., 2008, Qin et al., 2007]. An important caveat to the intestinal permeability assay was that the study was done in rats with increased baseline levels of anxiety-like behaviour, which may

have also affected their baseline intestinal permeability levels. This may also explain why we did not observe a SCI-induced change in intestinal permeability, as has been shown by others [Kigerl et al., 2016a]. Although we did not measure systemic LPS, the chemokines LIX and RANTES (both of which are upregulated by LPS [Li et al., 2016, Nonaka et al., 1999, Arima et al., 2000]) were significantly increased in FMT treated rats 77 days after injury, suggesting a chronic increased inflammatory state compared to vehicle controls. However, in both groups, we observed a significant increase in both pro-inflammatory and anti-inflammatory cytokines and chemokines at 3 and 21 days after SCI. This is likely due to the acute systemic inflammatory response initiated following trauma to the spinal cord [Bloom et al., 2020, Gris et al., 2008]. By 77 days, both FMT and vehicle groups displayed a drastic downregulation in the majority of inflammatory cytokines, which may reflect a symptom of SCI-induced immune depression [82]. This immune depression is hypothesized to be triggered by sympathetic dysregulation associated with upper thoracic and cervical SCIs and generally takes time to develop following injury [Riegger et al., 2007, Zhang et al., 2013].

In conclusion, these results highlight the importance of optimal donor selection for successful FMT treatment following SCI. Although the FMT donors were otherwise healthy and pathogen free, they displayed naturally increased anxiety-like behaviour and reduced proportions of *Lactobacillus*. FMT from these *anxious* donors did not prevent SCI-induced dysbiosis and had some negative side effects including increased intestinal permeability, increased anxiety-like behaviour, and chronic alterations in both local and systemic inflammation. While recipient safety must prevail above all, vigilant donor selection beyond the exclusion of known pathogens is essential to improve the success of FMT as shown here in the context of SCI.

Chapter 4

Minocycline treatment alters inflammatory and microbiota profiles following spinal cord injury

[1]

4.1 Introduction

Minocycline is a synthetic tetracycline derivative with anti-inflammatory and neuroprotective properties [Wells, 2003a, Smith and Leyden, 2005, Garrido-Mesa et al., 2013]. Numerous animal studies have shown that minocycline has anti-inflammatory, anti-oxidative and direct neuroprotective effects after SCI [Lee et al., 2003, Wells, 2003a, Stirling, 2004, Teng et al., 2004, Festoff et al., 2006, Yune et al., 2007, Shultz and Zhong, 2017]. These positive preclinical results, coupled with minocycline's long safety record in humans [Goulden et al., 1996], resulted in a phase II placebo-controlled randomized clinical trial to test the therapeutic effects of minocycline treatment for acute

[1] This chapter has appeared in "Beyond the lesion site: minocycline augments inflammation and anxiety-like behavior following SCI in rats through action on the gut microbiota"

SCI [Casha et al., 2012]. Results from the trial showed modest, though not statistically significant, motor recovery in SCI patients that received minocycline treatment [Casha et al., 2012]. Beneficial effects of minocycline have also been shown in amyotrophic lateral sclerosis, stroke, multiple sclerosis, and Parkinson's disease [Brundula et al., 2002, Thomas and Le, 2004, Gordon et al., 2007, Matsukawa et al., 2009]. The pharmacological effects of minocycline have primarily been attributed to modulation of neuroinflammation [Elewa et al., 2006, Soczynska et al., 2012]. However, some studies have failed to reproduce the neuroprotective effects of minocycline treatment following SCI, Parkinson's disease and Huntington's disease [Diguet et al., 2004, Scott et al., 2018, Lee et al., 2010].

Minocycline is also a broad-spectrum antibiotic, modulating the composition of the intestinal microbiota [Hasebe et al., 2019, Schmidtner et al., 2019]. Recently, an imbalanced intestinal microbiota composition (dysbiosis) has been linked to impaired functional recovery and increased anxiety-like behaviour following SCI [Kigerl et al., 2016a, Schmidt et al., 2020b]. Bidirectional communication between the microbes that colonize the gastrointestinal tract and the central nervous system can have a profound impact on disease progression and likely involves interactions with the host immune system [Fung et al., 2017, Fung, 2020]. Although the local tissue response to minocycline treatment for SCI has been well characterized, minocycline's (and antibiotics in general) impact on the microbiota-immune axis following SCI is unknown. This is particularly relevant since 93.2% of SCI patients receive antibiotic treatment in the first week after injury [Geisler et al., 1991, Geisler et al., 2001b, Geisler et al., 2001a]. Given the broad influence of modulating the intestinal microbiota and systemic immune response, these systemwide effects of antibiotics could help explain the contradicting evidence of minocycline's efficacy as a treatment for SCI.

The aim of the present study is to elucidate multiple systemwide changes induced by minocycline treatment in a rodent model of cervical SCI. Four groups of rats were used: uninjured, uninjured + minocycline, SCI, and SCI + minocycline. We show, for the first time, that minocycline treatment for SCI has a profound acute effect on the fecal microbiota diversity and composition, and subsequently prevents SCI-induced suppression of cytokines/chemokines and attenuates anxiety-like behaviours.

4.2 Methods

4.2.1 Animals

All animal use was approved by the Animal Care and use Committee for Health Sciences at the University of Alberta. Adult female Lewis rats (Charles River, n = 40) were group housed with five rats per cage (experimental groups housed separately). Rats were kept on a 12 h light/dark cycle (lights on at 08:00) and they received ad libitum access to standard rat chow and water. Behavioural testing and analyses were performed by an experimenter blind to the experimental groups. Rats were divided into four groups; four rats were excluded (one died after surgery, one had no lesion, one had a lesion size greater than 50%, and one was a multidimensional outlier for the plasma and microbiota analysis) for a total of 36 rats: uninjured n = 10, uninjured + minocycline n = 10, SCI n = 8, SCI + minocycline n = 8.

4.2.2 Drug administration

50 mg/kg minocycline (Sigma Aldrich) was dissolved daily in sterile water and administered via oral gavage daily for 7 days beginning 2 hours after SCI. Rats that did not receive minocycline were gavaged with 0.5 ml sterile water daily for 7 days beginning 2 hours after SCI.

4.2.3 Spinal Cord Injury

Surgeries were performed similarly to previously described [Schmidt et al., 2020b]. Under isoflurane anesthesia (5% induction; 2.5% maintenance, supplied with a 50:50 air/oxygen mixture) the dorsal neck was shaved and disinfected with 10% chlorhexidine digluconate (Sigma-Aldrich). A 125 kdyn unilateral contusion was performed 1.25 mm right of midlien at an angle of 15 degrees (pointed towards midline) at C5 using an Infinite Horizons impactor (Precision Systems & Instrumentation). Muscles were sutured with synthetic braided sutures and the skin was closed with 9 mm stainless steel clips. Buprenorphine (0.03 mg/kg, WDDC) was injected subcutaneously immediately post-op and again 8–12 hours later. Animals received 4 ml saline (subcutaneous) for hydration immediately after surgery. Bladders were manually expressed when necessary (evidence of wet abdomen and full bladder) until the animal re-established bladder control.

4.2.4 Behavioural Testing

Open field

Rats were placed in the centre of an open field arena (100 x 80 x 30 cm) for 5 minutes while video recorded from above [Walsh and Cummins, 1976]. Offline video analysis of the distance travelled was performed using customized tracking software (https://github.com/cdoolin/rat-apps).

Elevated plus maze

Rats were placed in the junction of two open arms and two closed arms (each arm is 50 cm long and 10 cm wide) elevated 65 cm above the ground while being video recorded from above for 10 minutes [Walf and Frye, 2007]. Offline video analysis was performed using customized motion tracking software (https://github.com/cdoolin/rat-apps) to

analyze the time spent in the open arms and total distance travelled. Entries into the open and closed arms of the elevated plus maze (EPM) were counted when all 4 paws were located in the arm.

Cylinder

Rats were video recorded while they explored the walls of a clear plexiglass cylinder (21 cm diameter x 25 cm height) for 5 minutes [Schaar et al., 2010]. Number of left and right paw placements were recorded and expressed as a percentage of ipsilesional paw placements.

Light dark box

Rats were placed in the dark compartment of the light dark box (LDB) (dark compartment 0 lux, light compartment 100 lux) and video recorded from above for 10 minutes [Bourin and Hascoët, 2003]. Total distance travelled and the integer number of entries (considered when all 4 paws enter the light box) into the light compartment were recorded using custom software.

Sucrose preference test

Rats received access to 2 water bottles in their home cage; one with a 1% sucrose solution and the other with regular drinking water [Liu et al., 2018]. The percentage of sucrose water consumed over 2 hours was recorded during the dark cycle when the rats were more active. The location of the water bottles was switched after 1 hour to control for side preference.

4.2.5 Fecal collection

To collect fresh fecal matter for 16s rRNA analysis, rats were placed in individual sterile cages at the beginning of the dark cycle as previously described

[Schmidt et al., 2020b]. Fecal pellets were collected in sterile eppendorf tubes and immediately placed in a -80 °C freezer until further processing.

4.2.6 Blood collection

Animals were gently restrained and the area over the tarsal joint was shaved. The saphenous vein was punctured using a sterile needle and blood was collected into a microvette CB300 capillary tube (Sarstedt Inc, Nümbrecht, Germany) and kept on ice. Blood samples were then centrifuged for 5 minutes at 3000 rpm at 4 °C, plasma was pipetted into sterile microcentrifuge tubes and transferred to a -80 °C freezer until further processing.

4.2.7 Cytokine analysis

Frozen plasma samples were shipped on dry ice to Eve Technologies (Calgary, Canada) for analysis. Samples were diluted 2 fold and run on the Rat Cytokine 27-Plex and Rat Stress Hormone 2-Plex discovery assays that measured: Eotaxin, EGF, Fractalkine, IFN-gamma,IL-1a, IL-1b, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12(p70), IL-13, IL-17A, IL-18, IP-10, GRO/KC, TNF-alpha, G-CSF, GM-CSF, MCP-1, Leptin, LIX, MIP-1alpha, MIP-2, RANTES, VEGF, corticosterone and melatonin. All plasma analytes were normalized to baseline (before injury) values for analysis.

4.2.8 Multivariate analysis of plasma analytes

The fold increase respect to baseline was used to normalize the expression of each analyte to its respective pre-injury levels. A multivariate analysis of variance (MANOVA) considering repeated measures was used to test the hypothesis that the mean vector of the groups, and their interaction over time, were different in the plasma analytes variable space. Wilks test was used to determine significance. To study temporal

patterns, Multiple Factor Analysis [Thurstone, 1931] was computed using the FactoMiner R package [Lê et al., 2008] to extract the multivariate scores of each animal in the plasma analyte landscape, and the loadings of each analyte at different time points. Each time point constituted a group of variables of plasma analytes using the fold increase with respect to baseline. We considered the first 2 dimensions for further analysis, explaining 24.3% and 12.4% of the total variance by dimension 1 and dimension 2, respectively. Linear mixed model (LMM) was fitted for dimension 1 for statistical inference considering group, time and their interaction as fixed effect terms and the animal as a random effect using the lme4 and lmerTest R packages [Bates et al., 2015, Kuznetsova et al., 2017].

4.2.9 16s rRNA analysis

Frozen fecal samples were shipped on dry ice to Microbiome Insights Inc. (Vancouver, Canada) for sequencing and bioinformatics. 16Sv4 amplicons were generated from the fecal samples and MiSeq-generated Fastq files were quality filtered and clustered into 97% similarity operational taxonomic units (OTUs) using the mothur software package (v. 1.39.5) [Schloss et al., 2009]. The resulting dataset consisted of 184614 OTUs with an average of 33185 reads per sample. OTUs were removed if their mean abundance in controls reached or exceeded 25% of their mean abundance in specimens. Alpha diversity was estimated with the Shannon index on raw OTU abundance tables after contaminants were filtered out. Putative contaminants were described as OTUs whose mean abundance in controls was equal to or greater than 25% of their mean abundance in the sample specimens.

4.2.10 Multivariate analysis of microbiota composition

OTU data was analyzed using R through Rstudio [Team, 2015]. Abundance tables for each time point were normalized with respect to each animal's value at baseline. Unsu-

pervised ordination of the normalized abundance table was conducted (for each of the aggregated taxonomic levels: species, genus, family, class, order and phylum) blinded to the experimental condition by Non-metric Multidimensional Scaling of the Bray-Curtis distance between animals using the vegan R package [Oksanen et al., 2019] with a maximal of 20 iterations and keeping the first 5 dimensions. A permutation multivariate analysis of variance (PERMANOVA) was computed using the vegan package over 999 permutations of the Bray-Curtis dissimilarity matrix to test the hypothesis of whether the centroids of the multivariate space were different by the terms of group, time and their interaction. Pairwise comparisons were performed using the pairwiseAdonis R package [Martinez Arbizu, 2020], adjusting p-values using Bonferroni's correction.

4.2.11 Perfusion and tissue cutting

Animals were euthanized 5 weeks post-SCI with a lethal dose of Sodium Pentobarbital (240 mg/kg). Rats were perfused transcardially with saline containing 0.02 g heparin/l followed by 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS) with 5% sucrose. Spinal cords were extracted and post-fixed overnight in 4% PFA at 4 °C followed by 30% sucrose for 5 days. The lesioned spinal cord (1cm block) was embedded in O.C.T. (Sakura Finetek, USA) mounted onto filter paper and frozen in 2-methylbutane (-40 °C). Serial cross sections of the spinal blocks were cut at a thickness of 25 µm on a NX70 cryostat (Fisher Scientific), staggered across eight sets of slides and stored at -20 °C until further processing.

4.2.12 Lesion analysis

Tissue was stained with cresyl violet acetate solution and imaged using a light microscope. Frozen sections were thawed for 1 hour at 37 °C and rehydrated in TBS (2 x 10 minutes). Slides were placed in 0.5% cresyl violet for 3 minutes and serially

dehydrated in EtOH (50%, 75%, 99%) for 2 minutes each, followed by xylene (2 x 2 minutes) and coverslipped with Permount™. The total rostral-caudal extension of the lesion was imaged and quantified using ImageJ software (National Institute of Health, USA). Lesion size was expressed as a percentage of the total cross section area.

4.2.13 Immunohistochemistry

Frozen sections were thawed at 37 °C for 1 hour and rehydrated in PBS for 2 x 10 minutes followed by PBS with 0.3% Triton™ X-100 (PBS-T) for 10 minutes. 5% normal donkey serum in PBS-T was applied as a blocking buffer for 1 hour at room temperature. Sections were then incubated overnight at 4 °C in rabbit-anti-IBA1 (1:500, Wako) antibody with blocking buffer. The next morning, sections were washed with PBS (3 x 10 minutes) and incubated with donkey-anti-rabbit AF488-conjugated (1:500, Life Technologies) antibody in blocking buffer for 2 hours at room temperature. Sections were then rinsed 3 x 10 minutes in PBS and cover slipped with Fluoromount™.

4.2.14 Image analysis

Images were captured using an epifluorescence microscope (Leica DM6000B, camera Leica DFC350 FX) and analyzed using ImageJ. Images were acquired at 5x magnification to visualize the entire spinal cord cross section 0.25cm rostral to the lesion, at the lesion epicenter, and 0.25cm caudal to the lesion. IBA1 optical density was quantified and expressed as a percentage area of positive staining using thresholding. To assess microglial morphology, 40x magnification images were taken of the ventral horn of the grey matter on both the ipsilesional and contralesional side rostral (0.25cm), at, and caudal (0.25cm) to the lesion. 3 representative cells per image were chosen and the process length and number of endpoints per cell were measured using the ImageJ plugin NeurphologyJ (Ho et al., 2011).

4.2.15 PICRUSt2 analysis

PICRUSt2 (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States) software was used following the developer's instructions to predict the functional abundances based on the 16s rRNA gene sequences [Langille et al., 2013, Douglas et al., 2019]. The relative abundance was calculated by dividing the abundance of each pathway by the total abundance of all pathways per sample. The top 10% most abundant pathways were used for analysis and presented as a fold change from baseline values.

4.2.16 Statistical analysis

Behavioural, tissue and plasma statistical analysis was performed using GraphPad Prism 8 (San Diego, CA). A 5% or less alpha value was considered significant. Time-course data was analyzed using a repeated-measure two-way ANOVA with the Geisser-Greenhouse correction followed by Tukey's multiple comparison post hoc test, with individual variances computed for each comparison. For data with only one time point, a one-way ANOVA was used followed by Fisher's LSD test. All summary values in the text represent mean ± standard deviation if not otherwise stated.

4.3 Results

4.3.1 Minocycline treatment did not affect lesion size

To determine whether minocycline treatment reduced lesion size following SCI, the rostral to caudal extension of the lesioned area in the coronal plane was analyzed 5 weeks after injury. There was no difference between minocycline treated or untreated rats in the size (SCI: 27.74% ± 11.48%; SCI + minocycline: 31.38% ± 10.07%) or

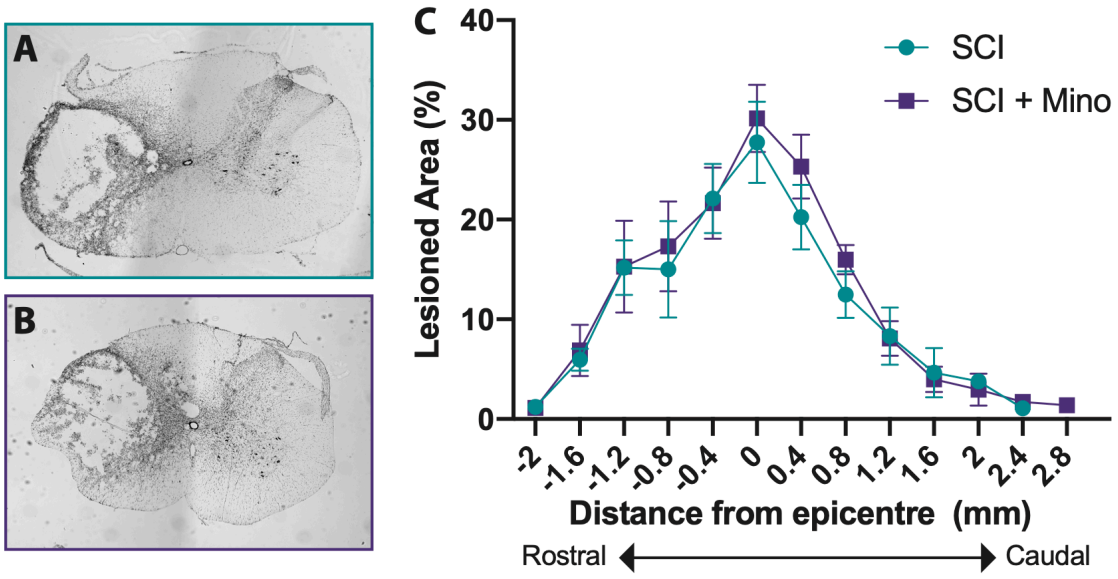

Figure 4.1: Representative images of the maximum lesioned area for the SCI group (A) and SCI + minocycline group (B). (C) The rostral (negative numbers) to caudal (positive numbers) extension of the lesion was quantified and expressed as the percentage of lesioned area for each coronal section. Error bars represent the standard error of the mean.

extension (SCI: 3.25 mm ± 0.89 mm; SCI + minocycline: 3.75 mm ± 0.56 mm) of the lesion (Fig. 4.1A, B and C).

4.3.2 Minocycline altered microglial density and morphology

The density and distribution of the microglia marker, IBA1, was analyzed around the lesion site at C4, C5 (at the maximum injury location) and at C6 (Fig. 4.2A-D). It is well characterized that SCI results in the activation of microglia [David and Kroner, 2011], which was confirmed by the increased area of IBA1 staining in all SCI rats relative to uninjured groups. Minocycline treatment following SCI resulted in an increased area of IBA1 immunoreactivity rostral to the injury at C4 (ipsilesional: $p = 0.003$) and caudal to the injury at C6 (contralesional: $p = 0.048$, ipsilesional: $p = 0.0001$) compared to untreated SCI rats (Fig. 4.2E-G). There was

no effect of minocycline treatment between uninjured groups in the area of IBA1 staining at any location measured.

Microglial morphology was further assessed in the ventral grey matter by quantifying the length of the microglial processes and the number of endpoints per cell (Fig. 4.2H-K). Increased process length and endpoints suggests a ramified microglial morphology, whereas a reduction in the length and number of endpoints indicates a more activated state [Davis et al., 1994]. SCI resulted in a significant increase in the activation of microglia characterized by increased process length (Fig. 4.2L-N) and increased number of endpoints (Fig. 4.2O-Q) per cell. Focusing on the effect of treatment within injury groups, rats that received minocycline displayed a general increase in the process length, which was significant between uninjured groups at C4 (Fig. 4.2L, ipsilesional: $p = 0.032$). Similarly, minocycline treatment resulted in an overall increase in the number of endpoints per cell in both uninjured and injured groups. This was significant between uninjured groups at C5 (ipsilesional: $p = 0.027$) and C6 (ipsilesional: $p = 0.008$, contralesional: $p = 0.025$) and between SCI groups at C6 (ipsilesional: $p = 0.035$). In summary, 7 days of minocycline treatment induced a more ramified spinal microglial morphology in both uninjured and SCI rats relative to untreated control groups measured 28 days after the offset of treatment.

4.3.3 Minocycline promoted affective but not motor recovery following SCI

Rat behaviour was assessed at baseline (prior to SCI) and for 4 weeks following injury. Both SCI and SCI + minocycline groups had a drop in body weight following injuries which returned to uninjured values by 4 weeks (time x group effect $p < 0.0001$, time effect $p < 0.0001$, group effect $p = 0.006$). Uninjured rats that received minocycline consistently had the highest body weight (Fig. C.1). At 7 days post-injury, both SCI groups travelled significantly less distance in the open field compared to uninjured

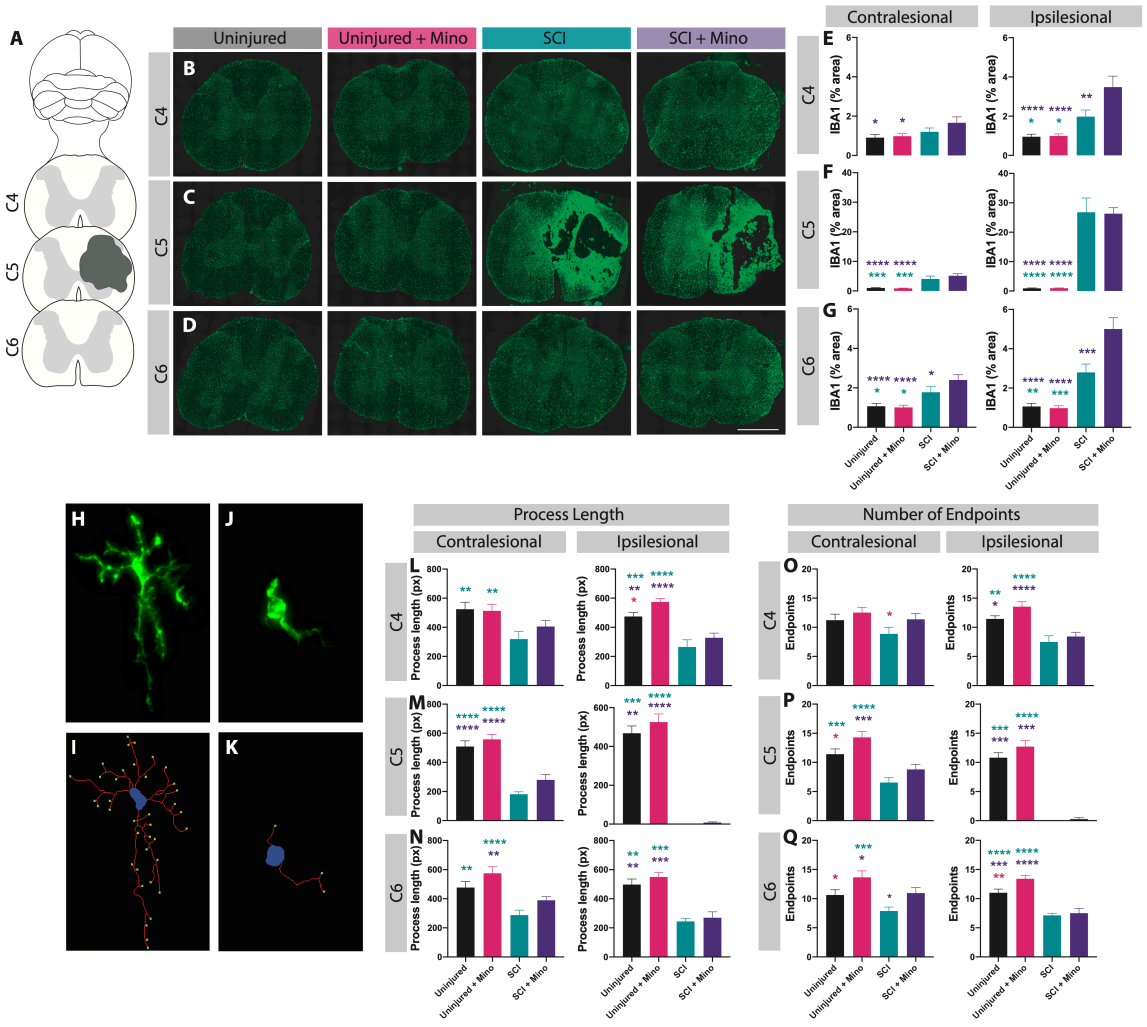

Figure 4.2: (A) IBA1 immunohistochemical marker was used to visualize microglia in the cervical spinal cord at C4, at the maximum lesion site (C5), and caudal to the lesion site at C6. Representative spinal cord images are shown from each group at C4 (B), C5 (C), and C6 (D). Quantification of the area of IBA+ staining is shown at C4 (E), C5 (F) and C6 (G) on the contralesional (left graphs) and ipsilesional (right graphs) spinal cord. Microglial morphology in the ventral grey matter was assessed by quantifying the length and number of endpoints per cell. (H) Image of a ramified microglia and (I) the automated analysis shows the soma in blue, processes in red and the endpoints in green. (J) Image of an activated microglia and (K) the corresponding output of the analysis. Quantification of the average process length per cell is shown at C4 (L), C5 (M) and C6 (N) on the contralesional (left graphs) and ipsilesional (right graphs) spinal cord. Quantification of the average number of process endpoints per cell is shown at C4 (O), C5 (P) and C6 (Q) on the contralesional (left graphs) and ipsilesional (right graphs) spinal cord. Error bars represent the standard error of the mean. Scale bar represents 1mm. *p<0.05, **p<0.01. ***p<0.001, ****p<0.0001.

rats (Fig. 4.3A-B; time x group effect $p < 0.0001$, time effect $p < 0.0001$, group effect $p = 0.001$). By 14 days post-injury, untreated SCI rats travelled significantly less distance than uninjured + minocycline animals. By 28 days post-SCI, all groups regardless of injury had declined in the overall distance travelled in the open field (Fig. 4.3A and B). SCI resulted in significantly reduced use of the ipsilesional forepaw in the cylinder test, with no effect of minocycline treatment (time x group effect $p < 0.0001$, time effect $p < 0.0001$, group effect $p < 0.0001$) (Fig. 4.3C and D).

A single testing session 3 weeks post-SCI was used for the EPM and LDB to avoid one-trial tolerance [Bertoglio and Carobrez, 2002, File, 1990]. There was no difference between all four experimental groups in the total distance travelled in the EPM (Fig. 4.4A and B). Similar to previous studies reporting SCI-induced anxiety-like behaviour in the EPM [Schmidt et al., 2020b], untreated SCI rats spent less percent time in the open arms and made significantly fewer open arm entries than both uninjured groups (uninjured vs. SCI $p = 0.047$, uninjured + minocycline vs. SCI $p = 0.028$) (Fig. 4.4C and D). Paralleling the SCI-induced anxiety-like behaviour observed in the EPM, untreated SCI rats spent the least amount of time in and entries into the light compartment of the LDB (Fig. 4.4E, F and G). SCI + minocycline rats made significantly more entries into the light compartment compared to untreated SCI rats, indicating a reduced anxiety-like behavioural state ($p = 0.022$). Anhedonic behaviour was assessed in the sucrose preference test 7 days post-injury (at the offset of minocycline treatment). SCI + minocycline rats consumed the least amount of sucrose water; however, this did not reach significance (Fig. 4.4H). Taken together, results from these behavioural tests suggest that minocycline treatment had a long-term (i.e. at least 2 weeks after the offset of treatment) attenuation of anxiety-like behaviour in the LDB but did not promote motor recovery following SCI.

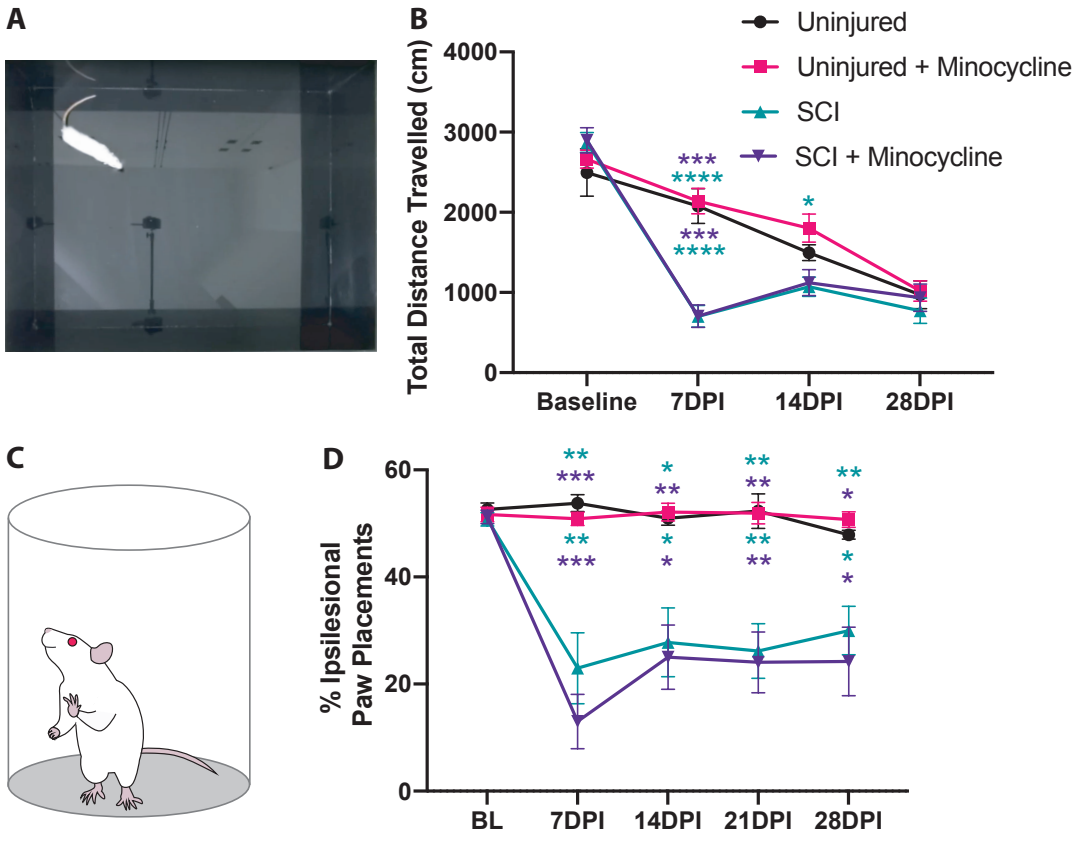

Figure 4.3: (A) Image shows a rat in the center of the open field apparatus. (B) Both SCI and SCI + minocycline groups travelled significantly less distance than uninjured rats in the open field at 7 days post-SCI. (C) The cylinder test was used to assess forepaw use asymmetry. (D) SCI resulted in decreased use of the ipsilesional paw compared to uninjured rats. Error bars represent the standard error of the mean. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. Top asterisks represent uninjured + minocycline groups vs. SCI (green) and SCI + minocycline (purple). Bottom asterisk represent uninjured vs. SCI (green) and SCI + minocycline (purple).

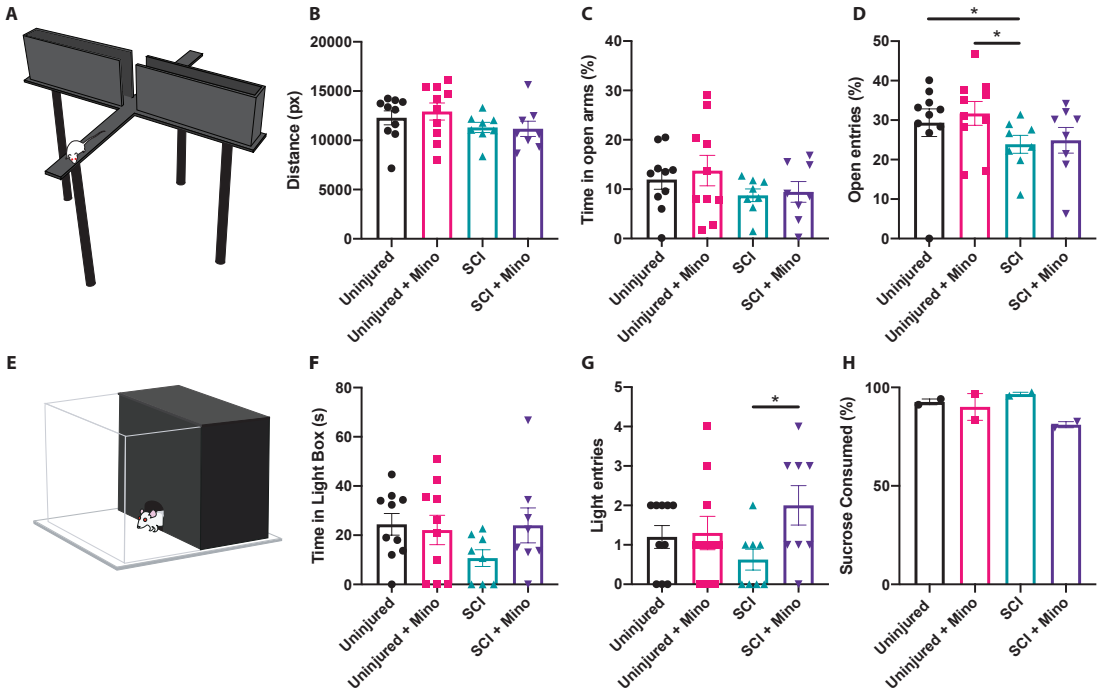

Figure 4.4: (A) Rat in the open arm of the elevated plus maze. (B) The total distance travelled in the maze. (C) Percent time in the open arms was calculated as a percentage of the time spent in the open arms divided by the total time spent in the maze. (D) Percent open arms entries was calculated as a percentage of the number of open arm entries divided by the total open and closed arm entries. (E) Schematic shows a rat entering the light component of the light-dark box. (F) The amount of time spent in the light component and (G) the number of entries made into the light component of the light-dark box. (H) The percent of sucrose water consumed where each data point represents a cage. Error bars represent the standard error of the mean. *$p<0.05$

4.3.4 Minocycline prevented SCI-induced suppression of inflammatory cytokines/chemokines

A total of 29 plasma analytes (cytokines, chemokines and hormones) were measured at 5-, 14- and 28-days post-SCI and expressed as a fold change from baseline values. Multivariate analysis of variance resulted in a significant group by time interaction (MANOVA group x time p=0.005), indicative of differences across groups and time at the overall profile of the 29 plasma analytes. Multiple factor analysis was used to study the multidimensional relationship between plasma analytes, group and time. Global scores show differences between groups across time, particularly in dimension 1 (Fig. 4.5A). When looking at the dimension 1 scores at each time point measured, there is a deviation of untreated SCI rats from uninjured groups by 28 days post-injury (Fig. 4.5B). Minocycline treatment prevented this SCI-induced long-term change in the plasma analyte composition. Looking at the relationship of the individual plasma analytes with multidimensional dimension 1, all plasma markers (with the exception of G-CSF and corticosterone) moved towards the same positive direction (Fig. 4.5C). Given that SCI + minocycline and uninjured groups are mostly positive in dimension 1, this indicates that there is a correlation between these groups and the changes in plasma analytes, which is opposite in direction to the untreated SCI group. Looking at individual plasma analytes, there was a substantial downregulation in the majority of analytes at 5 days post injury in all groups (Fig. 64.6). By 14 days post-injury, there were minimal differences between treatment groups in levels of plasma analytes (Fig. 64.6). By 28 days post-injury, SCI induced a significant suppression of the majority of plasma cytokines and chemokines, which was normalized with minocycline treatment (Fig. 64.6). Figure 4.7 shows the univariate adjusted p-values for individual plasma analytes that were significantly different between groups. At 5 days post-injury, the only significant difference between groups was an increase in Leptin in uninjured rats relative to uninjured + minocycline and SCI + minocycline groups, which remained

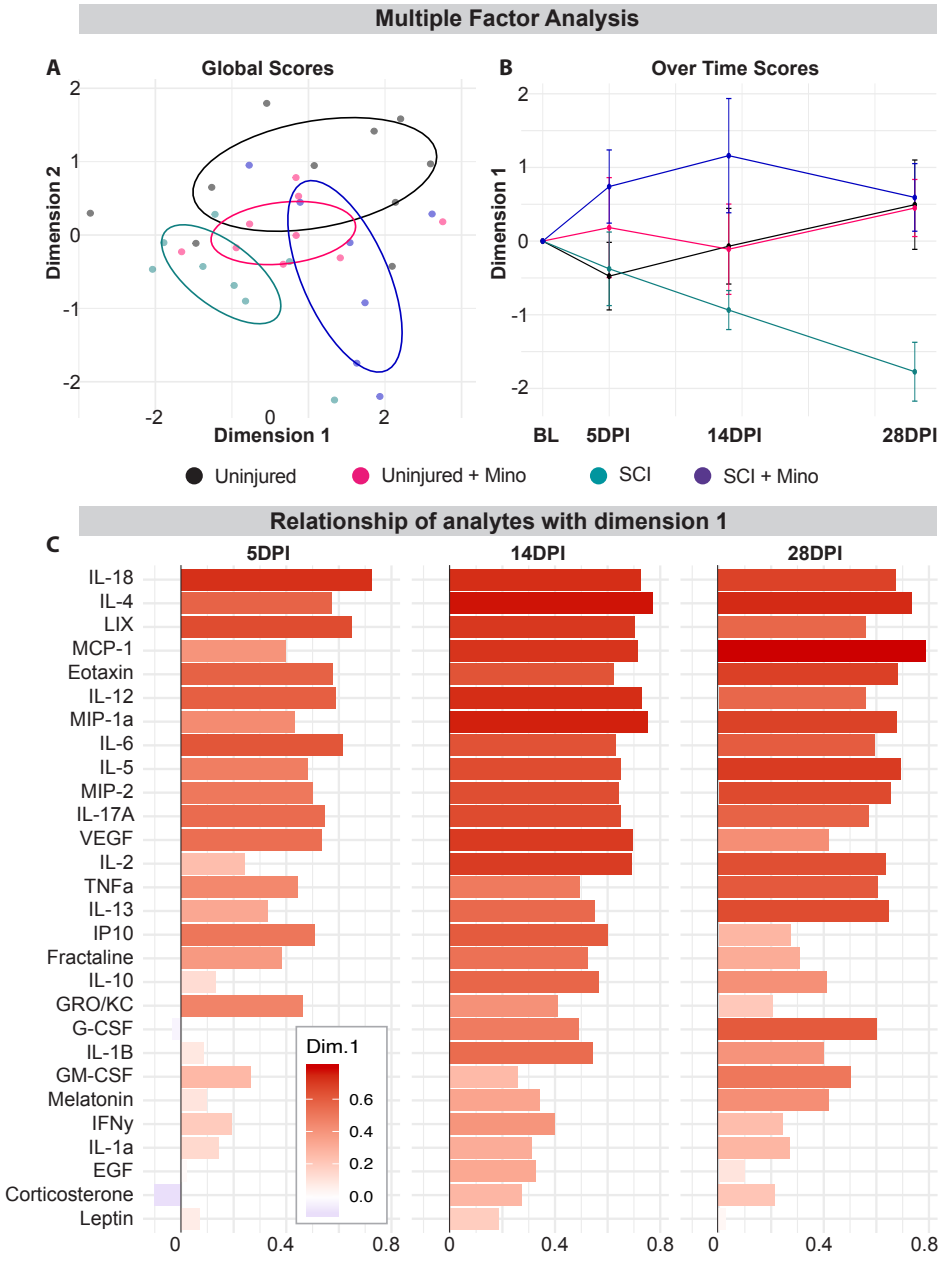

Figure 4.5: (A) Multiple factor analysis of plasma analytes (measured with respect to baseline values) shows the relationship of each subject with the multidimensional factors 1 and 2 across all time points. (B) Scores in multidimensional factor 1 are shown for each group over time. (C) The importance of each plasma analyte to multidimensional factor 1 is shown over time using their loadings. Ellipses in A represent the 50% bivariate distribution. Error bars represent the standard error of the mean.

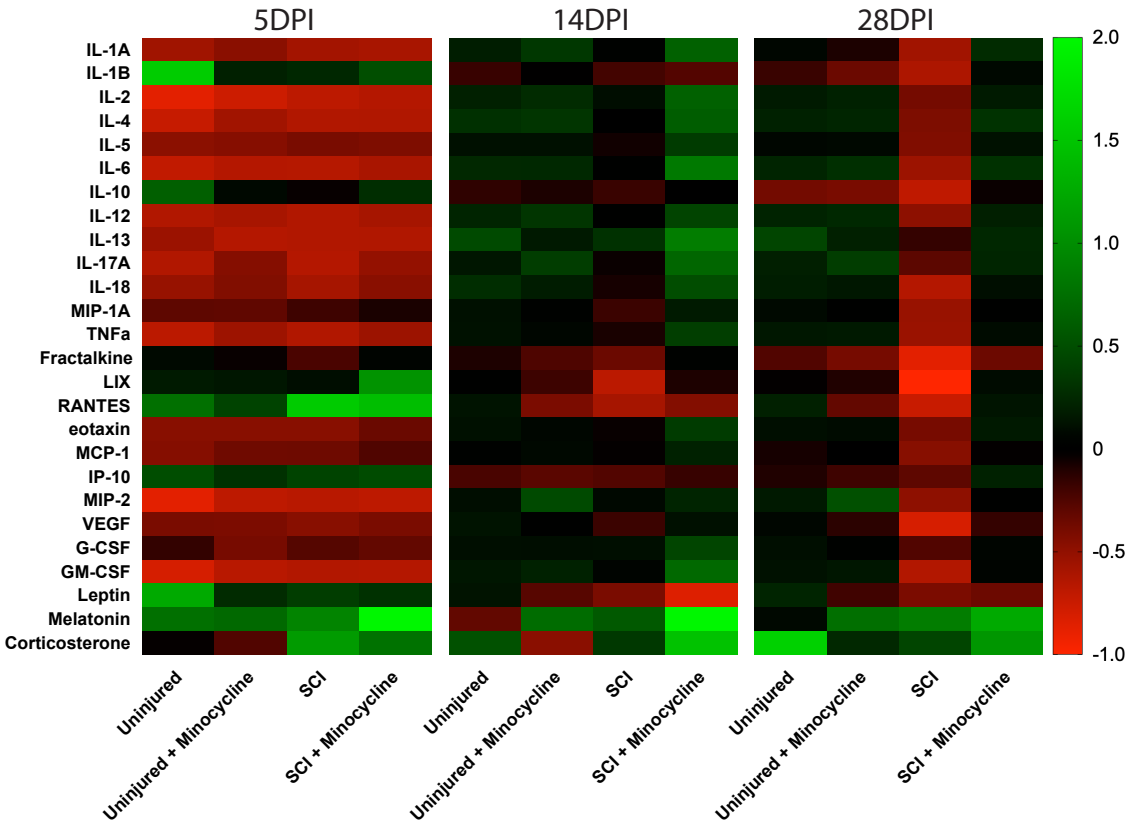

Figure 4.6: Heatmap shows the relative change of plasma analytes at 5-, 14- and 28-days post injury. Positive numbers reflect an increase and negative numbers reflect a decrease from baseline values.

significant at 14 days post-SCI for the SCI + minocycline group. By 14 days post-injury, melatonin was significantly reduced in uninjured rats compared to all other groups. The majority of statistically significant differences were observed between SCI rats and all other groups at 28 days post-injury. Compared to the uninjured group, SCI rats had reduced plasma levels of IL-12, Eotaxin, MIP-1a, IL-6, IL-5, MCP-1, VEGF and Fractalkine. Compared to uninjured + minocycline rats, SCI animals had significantly reduced plasma levels of LIX, IL-17a, IL-12, GM-CSF, Eotaxin, TNFa, MIP-1a, IL-4, IL-6, IL-5, IL-18, MCP-1 and Fractalkine. Finally, compared to SCI + minocycline rats, SCI animals had decreased levels of MCP-1, Eotaxin, GM-CSF, MIP-1a, IL-6, TNFa and Fractalkine. 28 days post-injury, there were no differences between uninjured vs. uninjured + minocycline as well as uninjured + minocycline

vs. SCI + minocycline groups. Uninjured vs. SCI + minocycline rats only differed in plasma levels of melatonin. Together these results show that SCI induced a relative reduction of blood levels of various cytokines and chemokines over time compared to uninjured rats, and that acute minocycline treatment prevented this SCI-induced suppression of inflammatory cytokines/chemokines.

To determine the spectrum of microbiota changes induced by both SCI and minocycline, fecal samples were collected and 16s ribosomal RNA (rRNA) gene sequencing was performed at baseline, on the day of injury (DOI), 5, 14- and 28-days post-injury (DPI) and expressed relative to baseline values. Minocycline treatment for 7 days resulted in a significant decrease in the Shannon index of alpha diversity (Fig. 4.8A). This reduction in bacterial diversity lasted longer in uninjured + minocycline rats (reduced alpha diversity on the first day of treatment, 5 and up to 14 days), and was shorter but more severe in SCI + minocycline rats (significantly decreased relative to all other groups at 5 days post-injury) (time x group effect $p < 0.0001$, time effect $p < 0.0001$, group effect $p < 0.0001$). The ratio of firmicutes to bacteroidetes (the two major bacterial phyla) was differentially affected by minocycline treatment and SCI (Fig. 4.8B). Minocycline treatment resulted in a transient but significant decrease in the firmicutes/bacteroidetes ratio that lasted up to 14 days post-SCI in both uninjured + minocycline and SCI + minocycline rats compared to untreated uninjured rats. SCI alone (without minocycline treatment) also decreased the firmicutes/bacteroidetes ratio relative to untreated uninjured rats, which reached statistical significance at 28 days post injury (time x group effect $p < 0.0001$, time effect $p = 0.004$, group effect $p = 0.002$).

Non-metric multidimensional scaling (NMDS) was used to extract the landscape space of the microbiota composition at each taxonomic level. Focusing on the phylum level, minocycline resulted in a significant alteration of the microbiota composition beginning on the first day of treatment (on the day of injury) regardless of whether

	5DPI	14DPI	28DPI
Uninjured vs. Uninjured+Mino	Leptin (p = 0.023)	Melatonin (p = 0.049)	
Uninjured vs. SCI		Melatonin (p = 0.041)	IL-12 (p = 0.021)
			Eotaxin (p = 0.041)
			MIP-1a (p = 0.005)
			IL-6 (p = 0.041)
			IL-5 (p = 0.018)
			MCP1 (p = 0.043)
			VEGF (p = 0.044)
			Fractalkine (p = 0.020)
Uninjured vs. SCI+Mino	Leptin (p = 0.026)	Leptin (p = 0.037)	Melatonin (p = 0.026)
		Melatonin (p = 0.012)	
Uninjured+Mino vs. SCI			LIX (p = 0.003)
			IL-17a (p = 0.002)
			IL-12 (p = 0.003)
			GM-CSF (p = 0.003)
			Eotaxin (p = 0.010)
			TNFa (p = 0.005)
			MIP-1a (p = 0.001)
			IL-4 (p = 0.021)
			IL-6 (p = 0.039)
			IL-5 (p = 0.010)
			IL-18 (p = 0.029)
			MCP1 (p = 0.025)
			Fractalkine (p = 0.017)
Uninjured+Mino vs. SCI+Mino			
SCI vs. SCI+Mino			MCP-1 (p = 0.018)
			Eotaxin (p = 0.005)
			GM-CSF (p = 0.015)
			MIP-1a (p = 0.034)
			Fractalkine (p = 0.018)
			IL-6 (p = 0.014)
			TNFa (p = 0.035)
Colour represents which group is significantly increased			
Uninjured	Uninjured + Minocycline	SCI	SCI + Minocycline

Figure 4.7: Minocycline treatment attenuated spinal cord injury-induced suppression of cytokines/chemokines. Table shows plasma analytes that are significantly different between groups at each time point measured following SCI. P value was calculated using Tukey's multiple comparison test following a repeated measures two-way ANOVA.

the animals received a SCI or were uninjured (Fig. 4.8C-E) (NMDS1 p = 0.0016). The difference between minocycline treated and untreated groups is seen primarily in NMDS component 1, which was maximal at 5 (Fig. 4.8 F–H) and 14 days (Fig.

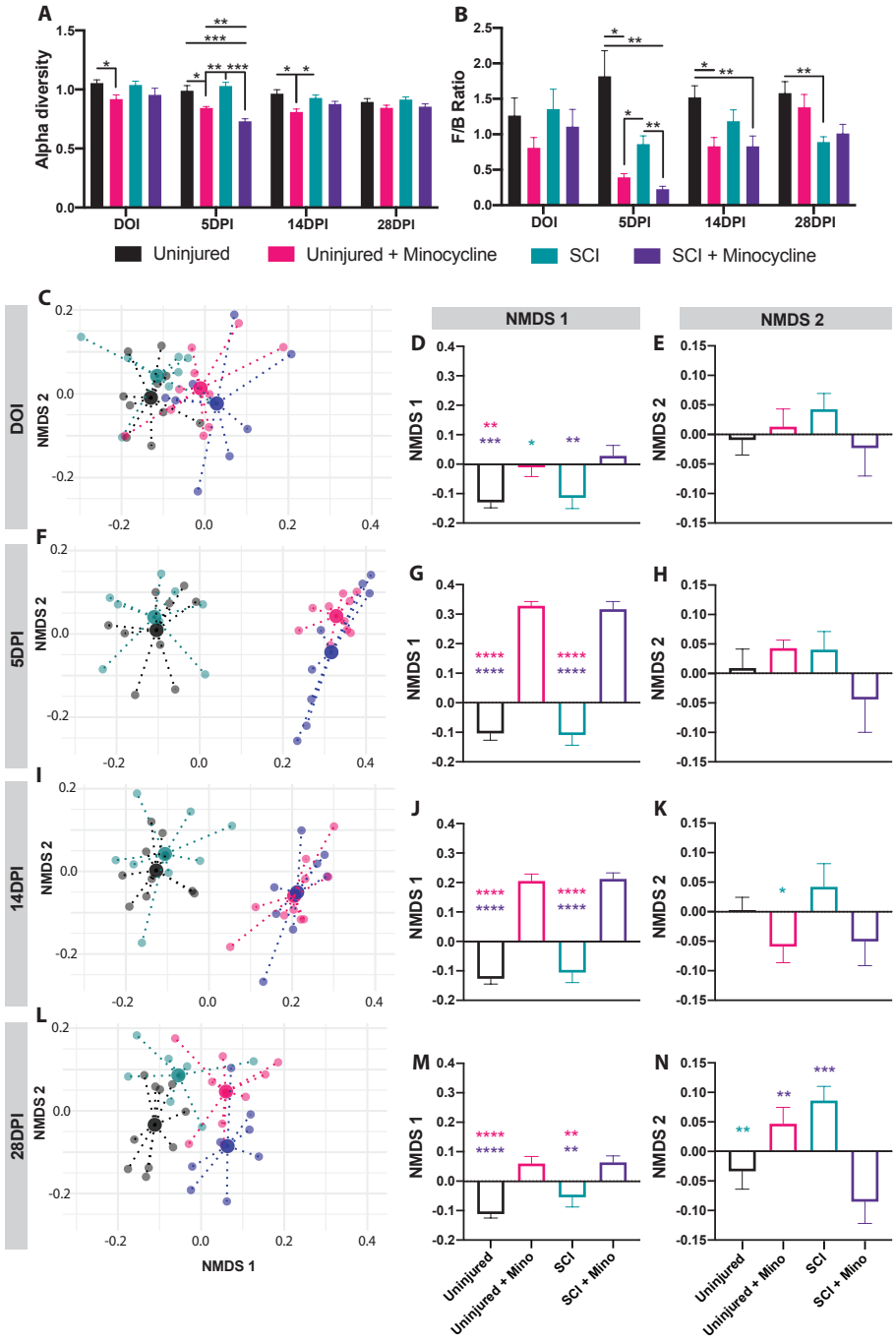

Figure 4.8: Caption on next page

Figure 4.8: (Previous page) (A) The Shannon index of alpha diversity and (B) the Firmicutes/Bacteroidetes ratio are shown over time for each treatment group. (C-N) Non-metric multidimensional scaling (NMDS) was used to visualize the overall microbiota composition at the Phylum level. (C) 2D plot of the NMDS first 2 components shows the centroid of each group (large points) and each individual rat (small points) on the day of injury. Individual NMDS 1 scores (D) and NMDS 2 scores (E) are shown for each group on the day of injury. Similar plots of shown for 5 days post injury (F-H), 14 days post injury (I-K) and 28 days post injury ((L-N). All data is normalized to baseline values. Error bars represent the standard error of the mean. *$p<0.05$, **$p<0.01$, ***$p<0.001$.

4.8I-K) post injury, and was reduced but still significant by 28 days post injury (21 days after the offset of minocycline treatment) (Fig. 4.8L-N) (5DPI $p < 0.0001$; 14DPI $p < 0.0001$; 28DPI $p < 0.0001$). Significant differences between groups in NMDS component 2 emerged beginning at 14 days and were maximal by 28 days post injury, when uninjured and SCI + minocycline groups moved in the opposite direction to uninjured + minocycline and SCI groups (Fig. 4.8N) (NMDS2 $p = 0.002$). NMDS plots for all other taxonomic levels can be found in Appendix C. Looking at the family, class and order taxonomic levels, untreated SCI rats deviated from all other groups at 28 days in NMDS component 2. Although there was a significant effect of SCI at 28 days post injury in NMDS component 2 at multiple taxonomic levels, minocycline treatment accounted for the majority of changes in the microbiota composition regardless of injury. A similar trend is seen in the functional gene profile of the microbiota composition (Fig. 4.9). Looking at the top 10% most relatively abundant genetic pathways, the majority of differences were observed at 5 days post-injury between minocycline treated and untreated groups regardless of injury, suggesting that minocycline treatment also had a significant acute effect on the microbiota functional profile (Fig. 4.10).

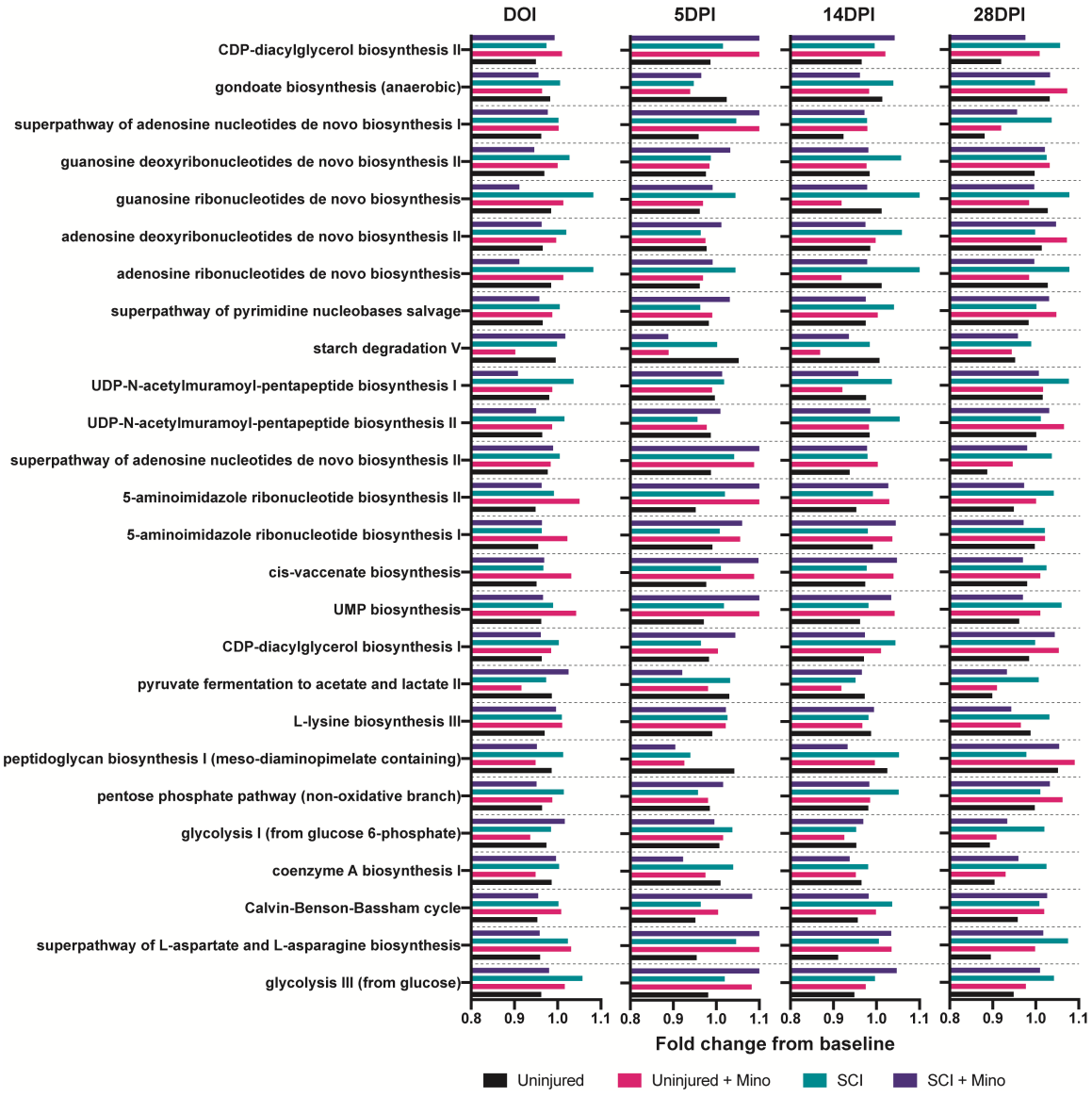

Figure 4.9: Top 10% most relative abundant PICRUSt pathways with respect to baseline values.

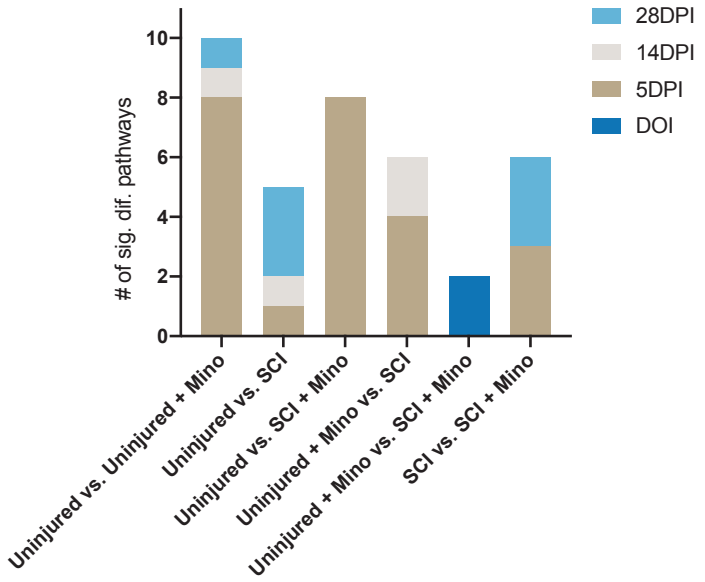

Figure 4.10: The number of pathways significantly different between groups (out of the top 10% most abundant PiCRUST pathways). On the day of injury, differences were only observed within minocycline group. At 5 days, minocycline treatment accounted for the majority of differences between groups. By 28 days, the majority of differences were between SCI vs. uninjured and SCI vs. SCI + minocycline groups.

4.4 Discussion

Minocycline has been widely studied for its direct anti-inflammatory and neuroprotective properties for central nervous system diseases and injuries including amyotrophic lateral sclerosis, stroke, multiple sclerosis, Parkinson's disease and SCI [Wells, 2003a, Shultz and Zhong, 2017, Thomas and Le, 2004, Gordon et al., 2007, Matsukawa et al., 2009, Brundula et al., 2002]. However, minocycline's impact on the intestinal microbiota and systemic immune response following SCI has not yet been investigated. Using a comprehensive analysis of plasma inflammatory analytes and fecal microbiota, we show for the first time that minocycline treatment has a profound acute effect on the microbiota composition followed by the prevention of SCI-induced suppression of inflammatory cytokines/chemokines. Although minocy-

cline did not reduce lesion size or improve motor recovery, it did have a potential anxiolytic effect after SCI.

In addition to being a broad spectrum antibiotic, minocycline is highly lipid soluble and can pass through the blood-brain barrier to produce a variety of anti-inflammatory, anti-oxidative and neuroprotective effects [Shultz and Zhong, 2017, Festoff et al., 2006, Elewa et al., 2006, Yong et al., 2004]. Minocycline has been shown to inhibit caspase-1, caspase-3 and microglial activation, protect neurons from oxidative stress and free radicals, prevent glutamate-induced apoptosis, and protect blood-brain barrier integrity [Garrido-Mesa et al., 2013, Wells, 2003a, Shultz and Zhong, 2017]. These promising preclinical results prompted a phase II placebo-controlled randomized clinical trial of minocycline to treat acute SCI [Casha et al., 2012]. Although recognized for its lack of adverse side effects, there was no statistically significant effect of minocycline efficacy for motor recovery after SCI [Casha et al., 2012]. Furthermore, a pivotal animal study reporting neuroprotective benefits of minocycline for cervical SCI was unable to be replicated in a follow up study [Pinzon et al., 2008]. Another group found no behavioural or histological benefits of minocycline treatment for cervical contusion in rats [Lee et al., 2010]. Contradicting results of minocycline treatment have also been implicated in stroke, Parkinson's and Huntington's diseases [Diguet et al., 2004, Scott et al., 2018]. In line with these studies, we found no beneficial effect of minocycline treatment on lesion size or motor recovery after SCI. However, the utilized motor tests in the present study may not have been sensitive enough to detect changes in fine motor skills. We did confirm the inhibitory properties of minocycline treatment on microglial activation in the spinal cord of both injured and uninjured rats [Yune et al., 2007, Festoff et al., 2006, Marchand et al., 2009]. Curiously, SCI rats treated with minocycline also displayed an increased density of IBA1 immunoreactivity above and below the injury site, which has not been reported previously.

Although IBA1 is upregulated upon activation of microglia [Sasaki et al., 2001], we showed that the observed increased area of IBA1+ cells in SCI + minocycline rats above and below the injury site was not due to increased microglial activation. This was determined by the increased complexity (i.e., increased process length and number of endpoints), indicating a ramified (less activated) microglial phenotype in rats that received minocycline. The increased density of IBA1 immunoreactivity in SCI + minocycline rats may otherwise be due to the observed increase in length and number of microglial endpoints or a general increase in the number of microglial cells.

Many of the anti-inflammatory properties of minocycline have also been studied for their beneficial effects on depression and anxiety [Soczynska et al., 2012, Rosenblat and McIntyre, 2018, Reis et al., 2019]. For example, minocycline can block LPS-stimulated inflammatory cytokine secretion, sickness behaviour and anhedonia [Henry et al., 2008]. On the other hand, minocycline is also an antibiotic, and antibiotic treatments have been shown to increase the risk for depression and anxiety [Guida et al., 2018, Lurie et al., 2015]. Multiple other treatments that target the gut microbiota have also been shown to modulate affective behaviours, which was also highlighted in Chapter 2 [Pirbaglou et al., 2016, Zheng et al., 2016, Schmidt et al., 2020b]. We show similar results in this experiment such that rats with SCI displayed increased anxiety-like behaviour, which could be partly alleviated with minocycline treatment. Anxiolytic and antidepressive effects of minocycline have been previously shown, mainly attributed to the drug's anti-inflammatory properties [Schmidtner et al., 2019, Reis et al., 2019, Zhang et al., 2019a, Camargos et al., 2020].

Minocycline's anti-inflammatory and neuroprotective effects have been well characterized for a variety of diseases/injury conditions; however, little is known about minocycline's antibiotic effects on the gut microbiota following SCI and how this

would influence other outcome measures. Additionally, looking at the microbiota composition provides a measure of the efficacy of minocycline's dose and route of administration. Here we show that 7 days of minocycline treatment had a significant effect on the microbiota diversity and composition regardless of injury, proving that the drug was effective. In addition to transiently reducing the alpha diversity, minocycline treatment also significantly decreased the ratio of Firmicutes to Bacteroidetes. Interestingly, a relative decrease in the abundance of Firmicutes and decreased abundance of Bacteroidetes has been shown in humans with major depressive disorder [Jiang et al., 2015]. However, we did not find minocycline to induce depressive- or anxiety- like symptoms, which may be because the decreased Firmicutes to Bacteroidetes ratio was not permanent. By 28 days post-injury (21 days after the offset of minocycline treatment), the differences between minocycline treated and untreated groups were reduced, and untreated SCI rats began to display an altered microbiota composition (particularly in the firmicutes/Bacteroidetes ratio, estimated functional gene profile, and at the order, family and class taxonomic levels). Research in mice also found significantly altered microbiota composition 28 days after a thoracic SCI [Kigerl et al., 2016a]. Although we did not observe significant differences between SCI and uninjured rats at 5- or 14-days post-injury, it is possible that there was an acute SCI-induced dysbiosis between the day of injury and 5 days post-injury, as previously reported in Chapters 2 and 3 [Schmidt et al., 2020b].

Although minocycline treatment had a profound effect on the fecal microbiota composition and diversity, a different trend and time course was observed in the systemic inflammatory markers. At 5- and 14-days post-injury, there were minimal differences between groups in the relative change in systemic cytokine/chemokine levels. There was a distinct trend in the plasma analytes such that there was a decrease in the majority of cytokines/chemokines measured relative to baseline in all groups at 5 days post-SCI, including uninjured rats. This may be a result of

the stress of surgeries and/or gavaging having a greater acute impact on systemic inflammation than the SCI itself, since stress is known to affect the inflammatory response [Rohleder, 2014, López-López et al., 2016]. Nevertheless, the majority of differences in plasma analytes were observed at 28 days, when untreated SCI rats displayed a reduction in plasma cytokines/chemokines relative to baseline compared to both uninjured groups and SCI + minocycline rats. Although SCI results in a local inflammatory cascade at the injury site [Stammers et al., 2012], it has been shown that the temporal systemic (i.e. plasma) and local spinal cytokine profiles can be entirely different [Mukhamedshina et al., 2017]. One study also found a general SCI-induced downregulation of blood levels of cytokines, which was greater in cervical SCI compared to thoracic SCI [Hong et al., 2018]. This long-term suppression of cytokines and chemokines may indicate a symptom of SCI-induced immune suppression syndrome. SCI-induced immune suppression is hypothesized to be caused by autonomic dysreflexia triggered by upper thoracic and cervical SCIs [Riegger et al., 2007, Zhang et al., 2013]. Accordingly, minocycline treatment has been shown to reduce the severity of autonomic dysreflexia after SCI, which may explain how minocycline prevented SCI-induced suppression of inflammatory cytokines/chemokines in the present study [Squair et al., 2018]. Squair et al. report that, although minocycline treated rats had no observable differences in motor recovery, they had increased preservation of sympathoexcitatory axons and improved cardiovascular control measured 8 weeks following SCI [Squair et al., 2018]. This chronic setting is when autonomic dysreflexia typically manifests (around 3-6 months after SCI in humans). Similarly, in the present study we did not observe SCI-induced suppression of plasma cytokines until the latest time point measured at 28 days post-SCI. Results from chapter 3 corroborate this result and showed even more drastic downregulation of blood levels of cytokines/chemokines at 11 weeks after a cervical contusion SCI. Complications due to infection are a leading cause of death follow-

ing SCI [Soden et al., 2000], therefore preventing immune suppression may mitigate the risk of infection and thus reduce mortality rate. However, future work would be needed to determine whether the observed SCI-induced reduction of systemic cytokines/chemokines is indeed a symptom of immune suppression, and whether minocycline can indeed prevent SCI-induced immune suppression.

The present results point towards a temporal relationship between minocycline's effects on the fecal microbiota followed by the reduction in systemic cytokines/chemokines and attenuation of anxiety-like behaviour. The gut microbiota plays a critical role in the host immune system, and modulation of the microbiota can have a profound influence on the body's response to infection and disease [Fung, 2020, Fung et al., 2017, Thaiss et al., 2016, Ma et al., 2019]. Research in germ free mice (i.e., without any microorganisms) has revealed the vital interplay between the gut microbiota and immune homeostasis [Wu and Wu, 2012]. For example, germ free mice have altered macrophage and microglia phenotypes, are neutropenic, and have an impaired innate immune response [Ohkubo et al., 1990, Mikkelsen et al., 2003, Erny et al., 2015]. Germ free mice and mice given antibiotics have also been shown to have a significantly attenuated severity of autoimmune encephalomyelitis via modulation of the peripheral immune response [Ochoa-Repáraz et al., 2009, Lee et al., 2011]. Furthermore, ongoing research on the gut microbiota strongly suggests a causal link between intestinal dysbiosis and the development of anxiety and depressive-like behaviours [Zheng et al., 2016, Wong et al., 2016, Valles-Colomer et al., 2019, Pearson-Leary et al., 2020].

Although our present results are descriptive in nature, they highlight two important concepts. First, although minocycline has direct local anti-inflammatory properties, its impact on the microbiota may also affect the systemic immune and affective consequences of SCI. Second, changes in plasma cytokine/chemokine levels were preceded by minocycline-induced changes in the fecal microbiota composition,

suggesting that the microbiota may be involved in the suppression of inflammatory cytokines/chemokines following SCI. In conclusion, our work underscores the importance of the microbiota-immune axis for recovery following SCI, which should be considered when investigating potential therapeutics that may modulate this axis, such as minocycline. The results of the present study are critical for a comprehensive understanding of the full spectrum of minocycline activity beyond the lesion site. This is particularly relevant for the potential clinical application of minocycline to treat acute SCI in humans.

Chapter 5

Inducing inflammation following subacute spinal cord injury: a double-edged sword to promote motor recovery [1]

5.1 Introduction

In chapters 2, 3 and 4, we augmented the immune system either indirectly (through the microbiota) or directly (through minocycline's direct anti-inflammatory mechanisms) in the acute and subacute stages of SCI. In chapter 3, we showed that FMT from *anxious* donors increased intestinal permeability and exacerbated anxiety-like behaviour following SCI. These negative effects of the FMT may have been due to a translation of LPS across the impaired intestinal barrier and subsequent increase in inflammation, as evidenced by increased concentrations of chemokines associated with LPS in rats that received the FMT from *anxious* donors. However, as discussed

[1] This chapter has appeared in "Inducing inflammation following subacute spinal cord injury in female rats: a double-edged sword to promote motor recovery" [Schmidt et al., 2020a]

in section 1.3, inflammation can also play a beneficial role in regeneration and plasticity of the CNS. Further exploring the dichotomous role that inflammation can play following SCI is the purpose of this chapter.

Rehabilitation is currently one of the most effective treatment options to promote motor recovery following incomplete spinal cord injury (SCI), with early intervention generally providing more favourable outcomes [Nam et al., 2017, Norrie et al., 2005, Scivoletto et al., 2005, Sumida et al., 2001]. This observation corroborates research indicating that the acute injury environment increases the capacity for plasticity [Biernaskie, 2004, Ding et al., 2005, Scivoletto et al., 2005, Sumida et al., 2001]. Neuroinflammation likely plays a role in this process, as early components of the inflammatory process have been shown to be beneficial for the natural but limited repair process following SCI [Anderson et al., 2016, Arnett et al., 2001]. Indeed, Chen et al. showed that over-expression of neurotrophin-3 promoted sprouting of CST axons in the acutely lesioned, but not chronically lesioned or unlesioned spinal cord [Chen et al., 2006]. This enhanced growth of the CST is important for motor recovery as the CST is critical for fine motor control [Martin, 2005, Piecharka et al., 2005]. Neuronal sprouting of the CST could be re-established in the chronically lesioned spinal cord by triggering an immune response with LPS, implicating immune activation as a key component of neurotrophin-3 induced axonal growth [Chen et al., 2008]. More evidence for the link between injury-induced inflammation and plasticity comes from research in the optic nerve. Using a model of optic nerve crush, Benowitz et al. showed that oncomodulin, a protein released from macrophages, is a formidable growth promoting signal from the immune system [Yin et al., 2009]. Further support of the association between inflammation and neuronal plasticity is the finding that the window of opportunity for effective rehabilitative training can be reopened by injecting LPS in rats with chronic SCI [Torres-Espín et al., 2018a]. LPS treatment resulted in increased CST

sprouting and a robust increase in rehabilitative training-induced motor recovery [Torres-Espín et al., 2018a]. This points towards a dichotomous role of inflammation as it can both exacerbate tissue damage and yet is an essential promotor of plasticity following SCI [Gensel and Zhang, 2015, Jones et al., 2005, Rust and Kaiser, 2017]. Although the above evidence suggests that the acute neuroinflammatory response may promote plasticity, there is also substantial research implicating inflammation as a key factor in secondary damage following SCI [Gris, 2004, Zhou et al., 2014]. We therefore hypothesized that enhancing inflammation in the subacute stage of SCI using LPS, at a time point when the lesion environment is still in a proinflammatory state [Popovich et al., 1997], would not have the same beneficial effect as chronic administration. To test this, rats received a systemic injection of LPS 10 days following a mild cervical SCI to trigger an immune response followed by 6 weeks of rehabilitative training.

5.2 Methods

5.2.1 Animals

Adult female Lewis rats (n=60 in 2 cohorts, Charles River Laboratories) were group housed (n=5 per cage, treatment groups housed separately) with ad libitum access to water and 12h on-off light cycle. Rats were food restricted during training periods (10g per rat per day) and otherwise had ad libitum access to standard rat chow. The study was approved by a local animal care and use committee (Health Sciences) at the University of Alberta and complies with the guidelines of the Canadian Council for Animal Care.

5.2.2 Single pellet reaching and grasping training

The single pellet grasping (SPG) enclosure, motorized pellet dispenser and training protocols were used as previously described [Torres-Espín et al., 2018b]. First, the rat's preferred paw was established by manually presenting a pellet and recording the number of left and right paw attempts. Once the preferred paw had been established, the pellet dispenser was positioned in a way to enable the rat to only use this paw. A high-intensity dual-window enclosure system was used to train the rats; once the rat had completed an attempt on one side of the enclosure, a pellet was presented on the other side, and so forth. Training consisted of 10 min sessions per rat per day, 5 days a week for 6 weeks before SCI. Ten days following SCI, rats received intraperitoneal injections of LPS/saline (see below for details) and rehabilitative training started 4 days after (14 days post-SCI). After 6 weeks of training the final assessment was conducted. Performance on the SPG task was analyzed once a week from video recordings. At the offset of rehabilitative training, rats were tested in a modified SPG task with a 7 mm wide gap between the pellet and the opening of the enclosure. This set up prevented 'scooping' of the pellet into the mouth, which is a common compensatory strategy. The parameters used to analyze the SPG task (both regular and gap) were the number of attempts the rat made to reach for a pellet and the success rate. Success rate was defined as the number of successful attempts divided by the total number of attempts, expressed as a percentage. An attempt was defined as each time the rat reached for a pellet, and a success as an attempt that resulted in the pellet being eaten.

5.2.3 Single pellet reaching and grasping high speed analysis

At the offset of pre-training (baseline) and at the offset of rehabilitative training after SCI, the pattern of movements to successfully grasp and retrieve a pellet was analyzed as previously described [Metz and Whishaw, 2000]. Rats were placed in the training

enclosure and 3 successful reaching attempts were recorded at high speed (120 fps, Panasonic DMC-FZ200; resolution of 1280 × 720 pixels). These 3 successful attempts were scored and averaged for each animal. This skilled reaching analysis consisted of 11 components each rated from 0 (movement is absent), 0.5 (movement is present but abnormal) to 1 (movement is normal).

5.2.4 Spinal cord injury

Rats were anaesthetized using isoflurane (3% in 50:50 air:oxygen mix) and their dorsal neck shaved and cleaned with 10% chlorhexidine digluconate (Sigma-Aldrich). An incision was made in the skin above vertebrae C2-C5, the muscles above C3-C4 were split and a laminectomy was performed at C4. A dorsolateral quadrant SCI was performed at C4 on the side of the preferred paw using custom made blades. The muscle layers were sutured with 5-0 Vicryl and the skin was stapled with 9mm surgical clips. Animals received 4 ml saline for hydration and 0.2ml of buprenorphine (0.03mg/ml) as analgesic immediately postoperatively and a second dose of 0.1ml buprenorphine (0.03mg/ml) was given 8 h after injury.

5.2.5 LPS administration

LPS was derived from Escherichia coli endotoxin (serotype 055:B5, Sigma-Aldrich) and dissolved in sterile saline for injection. Rats received a single intraperitoneal dose of 0.5mg/kg LPS or saline 10 days following SCI. Skin temperature, weight and general sickness behaviour (piloerection, social isolation and reduced activity) was monitored pre-injection, 4, 8, 24, 36, 48 and 72 h after injection.

5.2.6 Behavioural Testing

Open Field Activity

Animals were placed in the centre of a black acrylic open field arena (100 x 80 x 30 cm) and video recorded from above for 5 minutes. The total distance moved was analyzed using custom motion-tracking software. This test was performed at baseline (before SCI), after SCI (before LPS/saline injections), 1- and 3-days post LPS/saline injections, and again at the offset of rehabilitative training.

Horizontal Ladder Task

Rats were filmed as they traversed a horizontal ladder (100 cm long, 12 cm wide, 12 cm high, 3 mm diameter cross bars spaced between 2 and 3 cm with a 45° mirror underneath). If a rat paused or turned around, the trial was considered invalid. 6 continuous ladder crosses per rat (3 per side) were used for analysis and the average between the three videos was used for each animal. For each paw, the total number of correct paw placements, paw slips (the paw contacted the ladder rung but slipped) and paw misses (the paw did not make contact with the ladder rung) were calculated. The final outcome measure was the percentage of correct paw placements for each paw. This test was performed at baseline, after SCI (before LPS/saline injections) and at the offset of rehabilitative training.

Cylinder Test

Rats were placed in an acrylic cylinder (21 cm wide x 23 cm tall) and video recorded for 3 minutes or until a minimum of 10 rears were made. The number of left and right forepaw placements were counted, and forepaw asymmetry was expressed as a percentage of ipsilesional paw placements.

Von Frey Test

Rats were acclimatized to the testing chamber prior to testing (IITC Life Science, CA, USA). Tactile sensitivity was assessed on both forepaws; if the rat was placing weight on the forepaw the score was not recorded. The Von Frey rigid tip probe was applied gradually in increasing pressure until the rat displayed a defined nociceptive response (paw retraction, licking) and the maximum pressure that elicited a withdrawal was noted. This test was repeated 3 times per paw, with a minimum of 3 minutes between measures. For each rat the average of the 3 measures for each paw was used for analysis.

Elevated Plus Maze

Anxiety-like behaviour was assessed in the elevated plus maze (EPM) four weeks after LPS or saline injections in the second cohort of rats (n=14 per group included regardless of participation in rehabilitative training). Rats were placed in the junction of two open arms and two closed arms, facing towards an open arm and allowed to explore the arena (100 x 100 cm and elevated 65 cm above ground) for 10 min. Time spent and entries into the open and closed arms as well as the total distance travelled were recorded from above as measures of anxiety-like behaviour. This test was used only once to avoid habituation to the maze. Offline video analysis was performed using customized software.

5.2.7 Corticospinal tract tracing

At the offset of rehabilitative training and after all behavioural testing, the anterograde tracer biotinylated-dextran amine (10% BDA; 10 000 MW, Life Technologies, New York, USA) was injected into the contralesional forelimb motor cortex to trace the ipsilesional corticospinal tract (CST). Using a dental drill, a 1.5 mm square window over the motor forelimb cortex was made (1-2.5 mm rostral and 1-2.5 mm lateral

to bregma). Three injections of 1 µl BDA were made at a depth of1.5 mm into the cortex using a Hamilton syringe. Following tracing, the skin was sutured with 5-0 Prolene and the animals received 0.1ml buprenorphine (0.03mg/ml). Rats were euthanized and perfused 12 days following tracing surgeries.

5.2.8 Perfusions and tissue processing

Twelve days following CST tracing, rats were euthanized with Sodium Pentobarbital (240mg/kg) and transcardially perfused with saline containing 0.02 g heparin/l followed by 4% paraformaldehyde (PFA) in 0.1M phosphate-buffered with 5% sucrose as fixative. Spinal cord and brain tissue were extracted, post-fixed in 4% PFA overnight at 4 °C and cryoprotected in 30% sucrose for 5 days. Spinal cord tissue was cut into a 0.5cm block above the injury (cervical levels 2-3, C2-C3) and a 0.5cm block around the lesion site (cervical level 4, C4). Spinal cord blocks were embedded in O.C.T. and frozen in 2-methylbutane at -50°C. Spinal cord cross sections were cut at 25µm on a CryoStar™ NX70 cryostat (Thermo Scientific) and stored at -20°C until further processing.

5.2.9 Immunohistochemistry

To visualize BDA traced CST axons, frozen sections were acclimatized at 37°C for 1 hour and rehydrated in TBS (2 x 10 min) and TBS-TX (TBS with 0.5% Triton X-100) (2 x 10 min). The sections were incubated for 2 hours at room temperature with 1:200 Streptavidin, Alexa Fluor™ 488 conjugate (Invitrogen) diluted in TBS-TX. The sections were then washed in TBS (4 x 10 min) and coverslipped with Fluoromount (Southern Biotech). For lesion analysis, slides were thawed at 37°C for 1 hour and rehydrated in TBS (2 x 10 min). Slides were then placed in 0.5% Cresyl Violet solution for 3 min followed by a serial dehydration in 50%, 75%, and 99% EtOH for 2 min each. Slides were then cleared in Xylene for 2 x 2 min and coverslipped using Permount

mounting media. For IBA1 and GFAP analysis, slides were thawed for 1 hour at 37°C and rehydrated in TBS for 10 minutes followed by TBS with 0.3% Triton™ X-100 (TBS-T) for 10 minutes. A blocking buffer of 5% normal goat serum in TBS-T was applied for 1 hour at room temperature. Sections were then incubated overnight at room temperature in rabbit-anti-IBA1 (1:500, Wako) and mouse-anti-GFAP (1:500, Sigma) antibodies with blocking buffer. 20 hours later, sections were washed with TBS (3 x 10 minutes) and incubated with goat-anti-rabbit AF488-conjugated (1:500, Life Technologies) and goat-anti-mouse AF555-conjugated (1:500, Life Technologies) antibodies in blocking buffer for 2 hours at room temperature. Sections were then rinsed in TBS-T (2 x 10 minutes) followed by TBS (2 x 10 minutes) and cover slipped with Fluoromount™.

5.2.10 Image analysis

For lesion analysis, Cresyl Violet stained sections were imaged with an epifluorescence (Leica DM6000B, camera Leica DFC350 FX) microscope. The maximum lesioned area was calculated as the percentage of damaged tissue using ImageJ software (National Institute of Health, USA). For BDA analysis, a confocal (Leica DMi8 and TCS SP8) microscope was used and 5 spinal cord sections from the C2-C3 block and 5 sections from the C4 block were imaged to quantify the total number of traced CST axons and CST collaterals using imageJ software. The number of descending BDA+ CST axons were manually counted and the BDA+ CST collaterals in the grey matter were manually outlined in each image. All of the images were aligned with one another and the xy coordinates of the BDA+ pixels were extracted using customized ImageJ macros. Then, using a customized R script, the xy coordinates were summed for each group and heat maps were generated using the kde2d function in the R MASS package [Team, 2013]. The number of BDA+ pixels divided by the number of labelled descending CST axons (for each respective spinal segment) was

calculated as a measure of normalized CST sprouting into the grey matter. IBA1 and GFAP stained sections were imaged with an epifluorescence microscope immediately rostral to the injury, at the maximum injury site, and immediately caudal to the injury. 10x magnification was used to image the entire spinal cord cross section and imageJ was used to quantify the IBA1 and GFAP optical density, calculated as the percentage area of positive staining in the selected ROI area. 40x magnification was used to image microglia cells in the ventral grey matter for morphological analysis. 6 representative microglia cells per cross section (3 ipsilesional and 3 contralesional were chosen and the number of endpoints and process length were measured using the imageJ plugin NeurphologyJ [Ho et al., 2011].

5.2.11 Statistical Analysis

Graphpad prism (version 8.0.0 for Mac, GraphPad Software, California USA) was used for statistical analysis. Normality was assessed using the D'Agostino-Pearson omnibus test. For time-course data, a repeated measures two-way ANOVA was used followed by Sidak's multiple comparison test, with a single pooled variance. For the high-speed analysis of reaching and grasping movements, an ordinary two-way ANOVA was used. A parametric unpaired t-test was used for data analyzed at a single time point. 20 animals were excluded based on lack of participation in rehabilitative training. 9 animals were excluded based on deviation of pre-defined lesion size (spared corticospinal tracts and rubrospinal tract). 3 animals died following SCI. Final analysis included 13 animals in the LPS group and 15 animals in the saline group. One rat in the LPS group did not participate in the gap test (made no attempts) and therefore was excluded from that particular analysis.

5.2.12 Neuronal excitability experiment

In a separate experiment, neuronal excitability of the tail was assessed in chronically spinalized Sprague-Dawley rats (n = 5) before and after LPS treatment.

Spinal cord injury

Under sterile conditions, rats received general anesthetic (sodium pentobarbital, 58.5 mg/kg) and a laminectomy was performed on the L2 vertebrae to expose the S2 spinal cord. A transverse split in the dura was made and 0.1 to 0.3 ml of Xylocaine (1%) was topically applied. SCI was performed under a surgical microscope; the pia was held with fine forceps and a fine suction tip was used to suck under the pia (suction tip made by heating and pulling a 1 ml syringe to a 0.1 to 0.2 ml tip). Following injury, the dura was closed with two 8-0 silk sutures and the muscle layers and skin were securely sutured.

LPS Administration

LPS was derived from Escherichia coli endotoxin (serotype 055:B5, Sigma-Aldrich Canada, Ltd., Canada) and administered i.p at a concentration of 0.4 mg/kg.

Measuring Spasms in Awake Rats

Tail muscle spasms were measured with Cooner wires (AS631) wires on 23g needles that were inserted into the tail muscles while the rat was in a Plexiglass tube and the tail was restrained from moving. Stimulation wires were placed at the base of the tail and EMG wires were placed in the mid-tail with a ground wire in between. All wires were separated by at least 1 inch and placed on opposite sides of the tail (one dorsal, one ventral). Threshold was determined as the minimal stimulus intensity to elicit a motor response. Stimulation with 0.2 ms current pulse at 1-2x threshold was recorded for 3 seconds every 40 seconds at a latency of 200 ms. EMG recording lasted

a minimum of 60 minutes for each rat and the first 30 minutes (45 sweeps) were used for analysis.

EMG and Statistical Analysis

Offline EMG analysis was performed using Clampex version 9.0. Tail EMG activity was segmented into 2 reflexes with respect to the stimulation pulse at 200 ms (time 0): An M-wave was seen immediately following the stimulation followed by an h-reflex (monosynaptic) at a 10 - 20 ms latency. The polysynaptic reflex was analyzed between 20 and 40 ms. All data was transformed to absolute value (rectified) and the mean amplitude area was calculated for each reflex. An ordinary one-way ANOVA was used to analyze the raw EMG data and follow up multiple comparisons were used to compare each time point after LPS administration to baseline values.

5.3 Results

5.3.1 Systemic LPS administration in the subacute stage of spinal cord injury induces a transient sickness response

Rats received a cervical SCI that selectively impaired the reaching and grasping ability of their preferred forepaw. Ten days after injury rats received either saline or LPS injection and were monitored for sickness behaviour, weight and temperature immediately prior to injection and 4, 8, 24, 48, and 72 hours later. LPS treatment resulted in visible sickness behaviour beginning at 4 hours and lasting up to 36 hours post-injection (time x treatment effect ($F\ (6, 156) = 125.0$, $p<0.0001$), time effect ($F\ (6, 156) = 125.0$, $p<0.0001$) and treatment effect ($F\ (1, 26) = 517.4$, $p<0.0001$)) (Fig. 5.1 A). This sickness behaviour was accompanied by a significant reduction in weight relative to saline controls between 24 and 72 hours post injections (time x treatment effect ($F\ (7, 182) = 43.30$, $p<0.0001$), time effect ($F\ (4.003, 104.1) =$

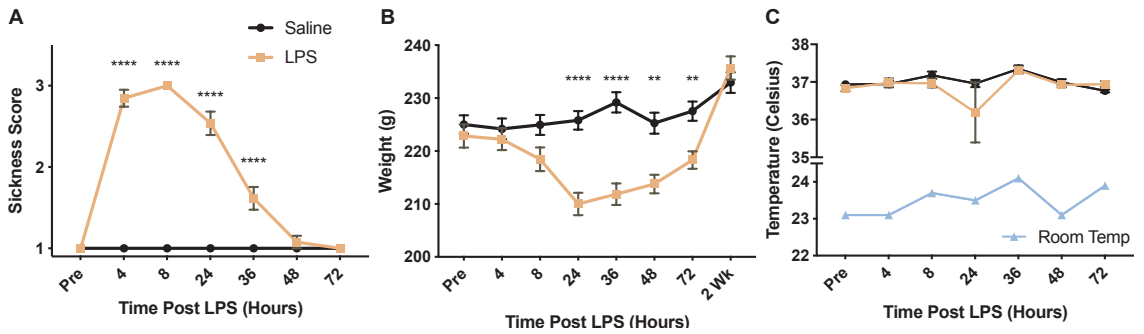

Figure 5.1: (A) Sickness behaviour was scored on a scale from 1 (no sickness behaviour) to 3 (full sickness response visually characterized by piloerection, isolation and immobility) following LPS or saline injection. LPS rats showed sickness symptoms for up to 36 hours post LPS injection relative to Saline rats. (B) LPS rats lost weight relative to saline treated controls that lasted up to 72 hours post injections. (C) There were no differences in body temperature between Saline or LPS rats. Error bars indicate SEM. *p<0.05, **p<0.01, ****p<0.0001.

86.51, p<0.0001) and treatment effect (F (1, 26) = 8.947, p=0.001) (Fig. 5.1 B). There were no significant differences measured in body temperature between LPS or saline groups (Fig. 5.1 C).

5.3.2 Inducing inflammation in subacute SCI promotes recovery in a reaching and grasping rehabilitative training task

One week following SCI, all rats experienced a drastic (approximately 40%) reduction in success rate in the SPG task when compared to baseline/preinjury (Fig. 5.2 A). After receiving LPS or saline injections and beginning rehabilitative training, both groups followed a similar trend and exhibited an improved success rate over the first 3 weeks of training. However, at this same time point the saline group plateaued in their recovery, whereas in weeks 4 through 6 the LPS treated group showed a modest increase in success rate compared to the saline group (Fig. 5.2 A). There was a significant effect of treatment over time (F (6, 156) = 2.716, p=0.016), as well as a significant time effect (F (4.161, 108.2) = 28.76, p<0.0001) (Fig. 5.2 A).

There were no differences between LPS or saline groups in the number of attempts made to reach for the pellet throughout the rehabilitative period (Fig. 5.2 B). To discriminate between compensatory strategies and true functional recovery, a gap was introduced in the pellet dispenser at the offset of rehabilitative training to prevent the rats from scooping the pellets into their mouths (Fig. 5.2 C). Once this compensatory behaviour was eliminated, it revealed that the LPS treated animals performed significantly better than the saline group (p=0.046) (Fig. 5.2 D). To further analyze the skilled reaching and grasping pattern, these movements were decomposed and analyzed in 11 components (Fig. 5.2 E). Prior to SCI, both LPS treated and untreated groups displayed a similar reaching and grasping pattern (Fig. 5.2 F). At the offset of rehabilitative training, rats that received LPS had generally better performance in the grasping, supination and release movements compared to rats that received saline only (significant treatment effect $F(1, 275) = 5.397$, p=0.021) (Fig. 5.2 G). In summary, these results reveal that untreated rats develop compensatory strategies to improve their success rate in the SPG task, whereas rats that received a single dose of LPS 10 days after SCI recovered true grasping ability of their uninjured paw.

5.3.3 Subacute LPS treatment enhances recovery in an untrained task

Non-trained behavioural tasks were used to determine whether subacute LPS treatment had an effect outside of the trained SPG task. There was no effect of LPS treatment on performance in the horizontal ladder (Fig. 5.3 A), however rats that received LPS injections showed significantly improved recovery in the cylinder test relative to saline controls ($p = 0.015$) (Fig. 5.3 B and Fig. 5.3 C). To assess general locomotor activity, the distance travelled in an open field arena was analyzed at baseline, 1 week post SCI (before LPS or saline treatment), 1 day, 3 days and 6 weeks after treatment. As a result of the LPS-induced sickness response, rats in the LPS group

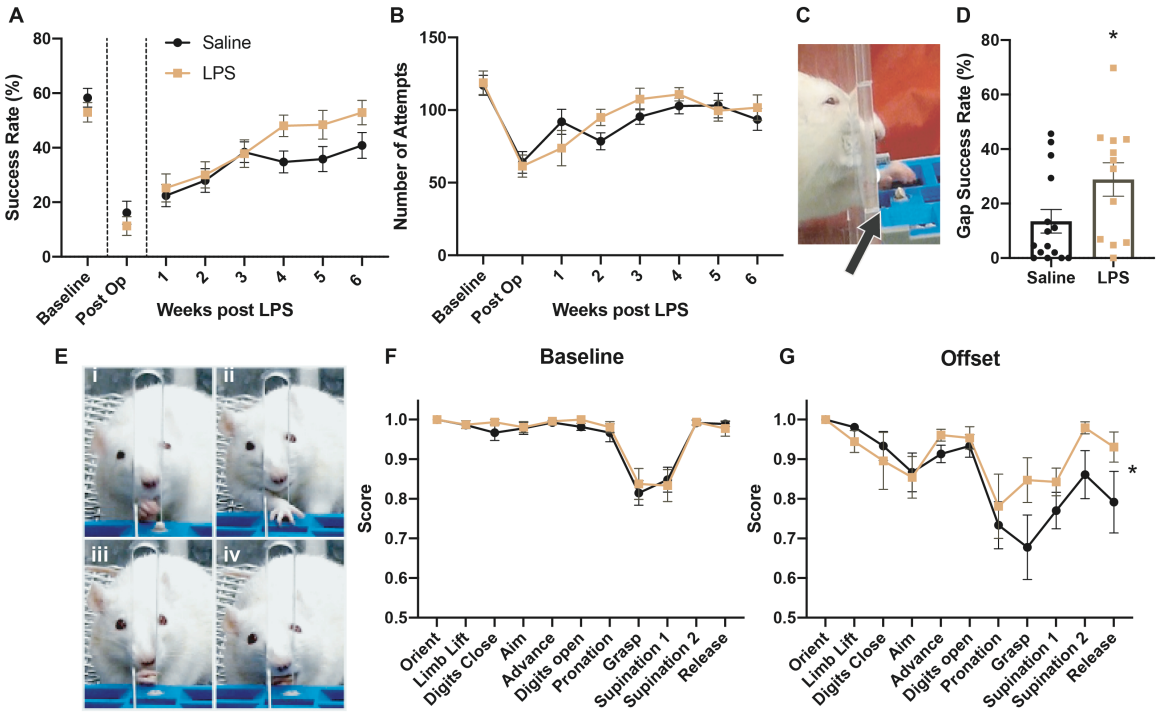

Figure 5.2: Rats underwent 6 weeks of rehabilitative training on the SPG task. (A) Rats that received a single LPS injection 10 days post-SCI showed modest but insignificant improvement in success rate relative to saline controls. (B) There was no difference between groups in the number of attempts made to retrieve the pellet. (C) To discriminate between compensatory strategies (scooping) and true functional recovery, rats were tested at the offset of rehabilitative training in a modified SPG task with a gap (indicated by the arrow). (D) LPS treated rats had a significantly higher success rate compared to Saline controls in the gap SPG task. (E) Reaching and grasping movements were broken down and analyzed into 11 sequences. Representative frames from high speed video recording show the rat's paw advancing and the digits opening (i), initiating a grasp (ii), supination (iii) and release (iv). (F) There were no differences between treatment groups at baseline before SCI. (G) At the offset of rehabilitative training there was a significant main treatment. Error bars indicate SEM. *p<0.05. *p<0.05

travelled significantly less distance in the open field compared to the saline group on day 1 post treatment; this difference became insignificant by 3 days and there was no difference in activity in the open field by 6 weeks ($p<0.0001$ at 1 day, time x treatment effect ($F(4, 104) = 6.779$, $p<0.0001$)) and time effect ($F(3.251, 84.54) = 38.06$, $p<0.0001$) (Fig. 5.3 D). There was an effect of injury on mechanical sensitivity such that the contralesional forepaw had increased sensitivity and the ipsilesional forepaw had reduced sensitivity (Post op: saline $p=0.041$, LPS $p=0.043$; Post LPS: saline $p=0.0002$, LPS $p=0.0009$; Offset (6 weeks post LPS): saline $p<0.0001$, LPS $p=0.0003$), however there was no effect of LPS (Fig. 5.3 E).

5.3.4 Subacute LPS treatment did not affect lesion size or CST fibre sprouting into the cervical grey matter

To determine whether subacute LPS treatment induced plasticity of the injured CST, BDA was injected into the contralesional forelimb motor cortex and the sprouting of CST fibres into the grey matter was normalized to the number of BDA labelled descending CST axons (Fig. 5.4 A) [Bareyre et al., 2004, Lindau et al., 2014, Mitchell et al., 2016, Torres-Espín et al., 2018a]. The density of BDA+ CST axon collaterals projecting into the cervical grey matter was quantified rostral to the lesion site (C2-C3, Fig. 5.4 B) and immediately above the maximum lesioned area (C4, Fig. 5.4 C). Both saline and LPS groups displayed a reduction of CST collaterals extending into the grey matter at the injury site compared to above the lesion at C2 – C3 (Fig. 5.4 D, F, E, G). The overall density of CST collaterals projecting into the cervical grey matter was not significantly different between groups above (Fig. 5.4 L) or at the injury site (Fig. 5.4 M). There was no correlation between the grey matter CST density and success rate in training (Fig. 5.5). To determine whether there were statistical differences between groups in the distribution of CST projections, the grey matter was sectioned into 75 μm^2 bins. Differences were observed between

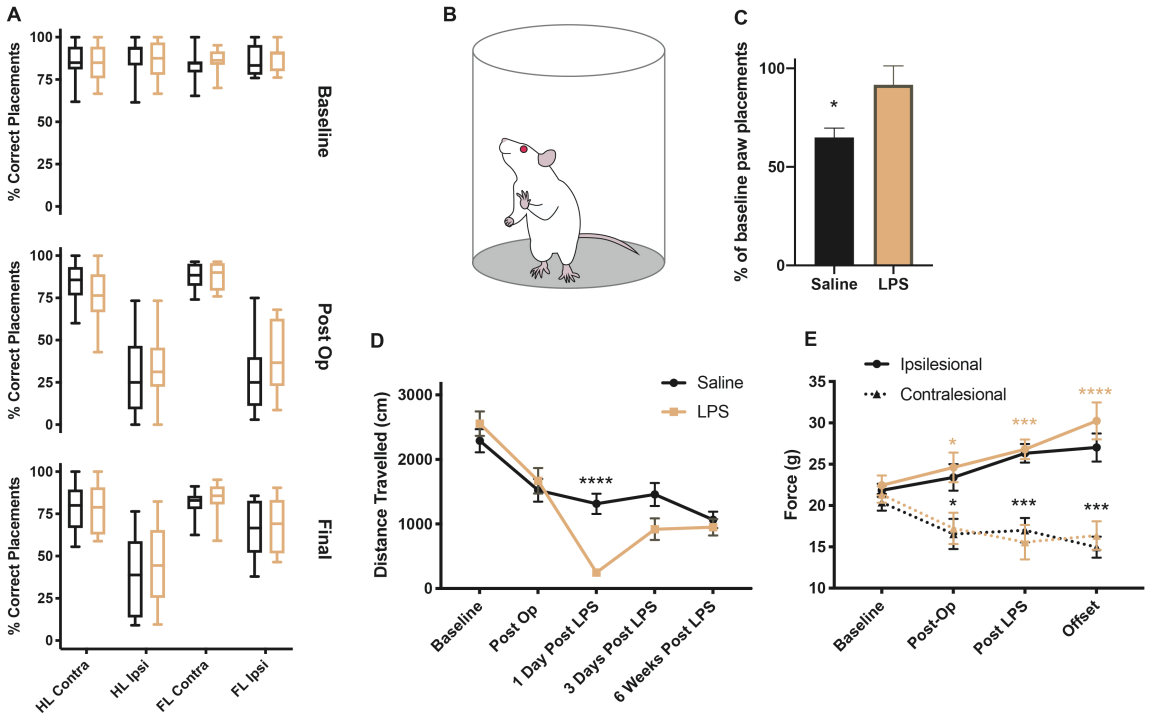

Figure 5.3: (A) Rats were tested on the horizontal ladder at baseline (top), post op (middle) prior to LPS injections, and a final assessment (bottom) at the offset of rehabilitative training. There were no differences between LPS or Saline groups at any time tested. (B) Depiction of a rat in a clear cylinder making a paw placement. (C) LPS treated rats showed significantly greater recovery in the cylinder task compared to Saline controls. (D) General locomotor activity was analyzed in the open field. (E) Mechanical sensitivity was assessed using an electronic Von Frey apparatus. Following SCI, the ipsilesional forepaw required greater force to elicit a withdrawal response, while the contralesional forepaw required less force to elicit a response regardless of treatment group. Error bars indicate SEM. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

groups both above (Fig. 5.4 H) and at the injury site (Fig. 5.4 I). However, there were a similar number of bins where either the saline or LPS group was significantly increased, suggesting no overall increase of one group over another. To further analyze the distribution of CST projections in a different manner, the grey matter was sectioned into 7 evenly spaced rings originating at the center of the spinal cord and propagating outwards. In both groups, the density of CST collaterals was highest in

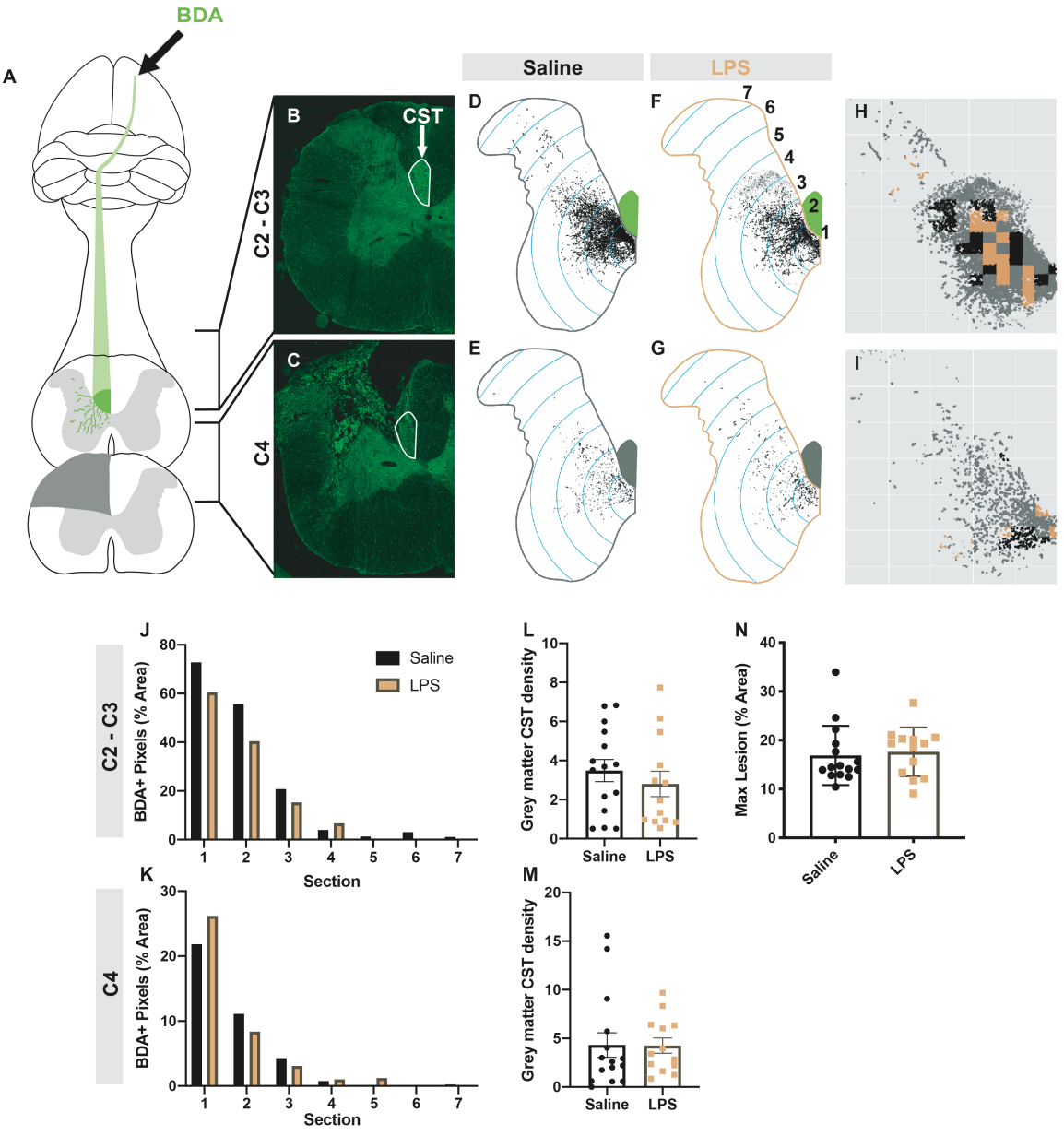

Figure 5.4: Caption on next page

Figure 5.4: (Previous page) (A) At the offset of rehabilitative training, the CST was labelled by injecting BDA into the contralesional forelimb motor cortex. Two weeks later the labelled CST axons were analyzed in two separate spinal cord blocks: above the lesion site (C2 - C3) and at the lesion site (C4). Grey matter CST fiber density was normalized to the total number of traced descending CST axons. Representative images of the labelled CST axons are shown (B) above the lesion site and (C) at the lesion site. An overlay of all of the outlined collaterals above and at the injury site are shown for saline (D, E) and LPS (F, G) groups. Statistically different areas of CST collateral density above and at the lesion site are shown in (H) and (I), respectively. Colours denote which group is significantly increased. To further examine CST axonal projections, the grey matter was split into 7 sections of rings propagating from the center of the spinal cord. Quantification of BDA+ pixel area above the lesion site (J) and at the lesion site (K) show that the majority of CST collaterals project to the intermediate grey matter. Quantification of the total normalized CST density above (L) and at the lesion site (M) revealed no significant differences between groups. (N) The lesioned area was calculated as a percentage of the total cross-sectional spinal cord area. LPS treatment had no effect on the lesion size. Error bars represent SEM.

the first few ring sections closest to the central canal. Compared to the saline group, rats that received LPS displayed decreased density of CST collaterals near the central canal above the injury site, but a slightly increased density of axons at the lesion site in the first ring section (Fig. 5.4 J and 5.4 K). Overall, these data indicate that there was no significant effect of LPS treatment on CST projections into the cervical grey matter. Furthermore, there was no difference in lesion severity between saline or LPS treated animals (Fig. 5.4 N).

5.3.5 LPS induces a long-term increase in anxiety-like behaviour

Because inflammation has frequently been linked to anxiety and depression [Dantzer et al., 2008, De La Garza, 2005, Raison et al., 2006, Vogelzangs et al., 2013, Yirmiya, 1996b], we wanted to determine whether enhancing inflammation in the subacute stage after SCI had any long-term consequences on anxiety-like behaviour. Therefore, rats were tested in the EPM 4 weeks following LPS or saline injections

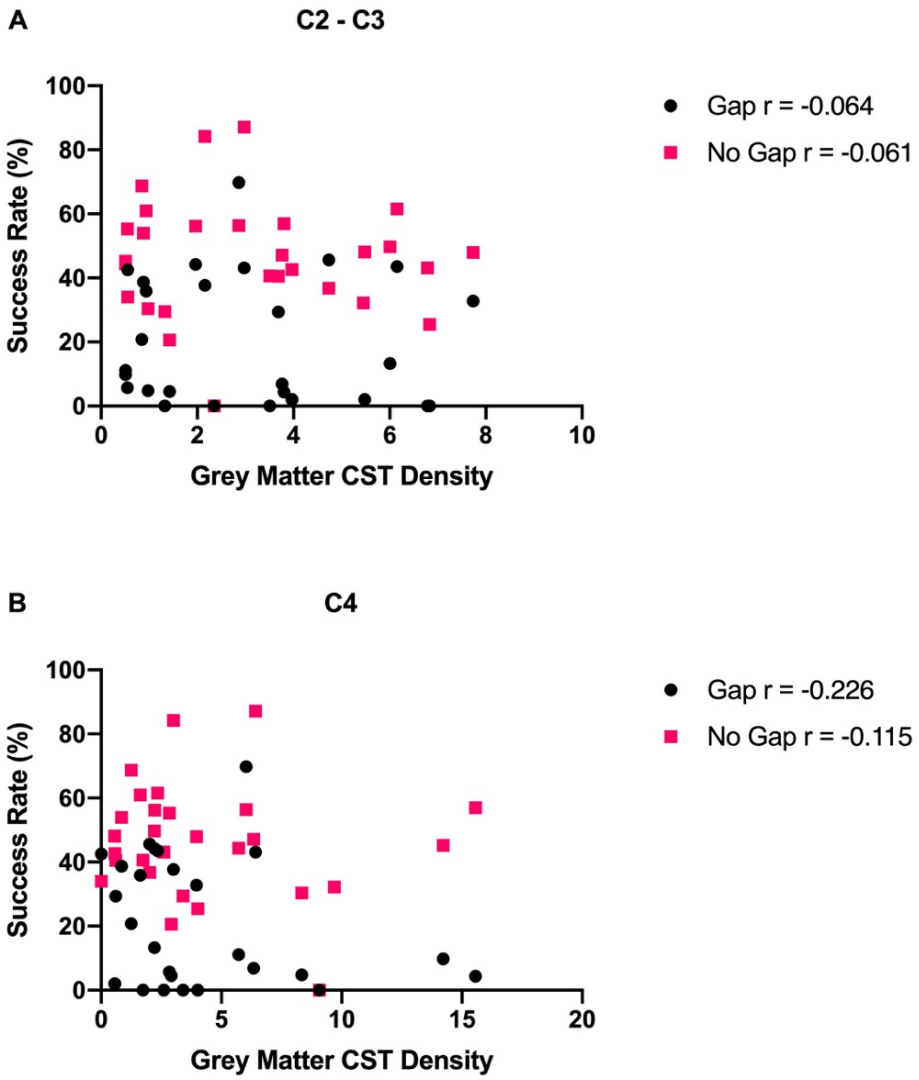

Figure 5.5: (A) There was no correlation between the CST grey matter density rostral to the injury (cervical level C2 – C3) and the success rate in rehabilitative training with or without a gap. (B) There was no correlation between the CST grey matter density at the maximum injury site (C4) and the success rate in rehabilitative training with or without a gap.

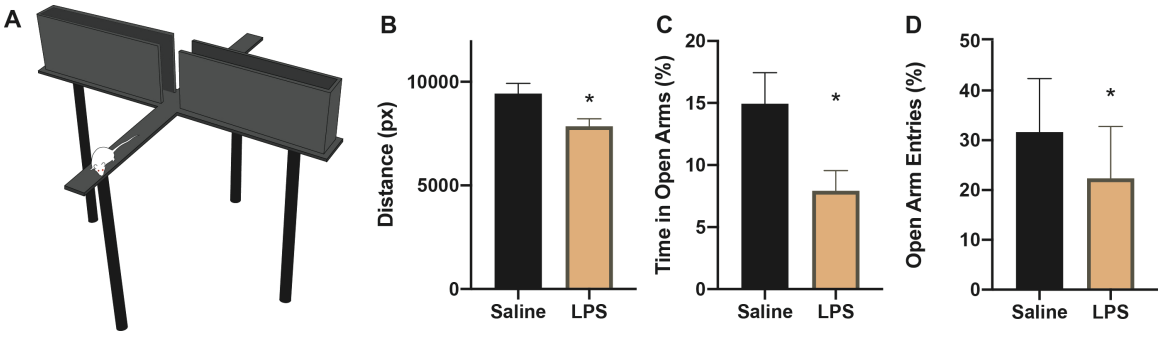

Figure 5.6: Rats were tested in the EPM 4 weeks following LPS or Saline injection. (A) Depiction of the elevated plus maze apparatus with a rat in an open arm. (B) LPS rats travelled significantly less distance in the EPM than Saline treated animals. (C) LPS rats spent significantly less percentage of time in the open arms of the maze, suggesting increased anxiety-like behaviour compared to Saline controls. (D) LPS rats made significantly fewer percentage of open arm entries compared to Saline rats. Error bars represent SEM. *p<0.05.

(Fig. 5.6 A). LPS treated rats travelled significantly less distance in the maze compared to saline controls (p=0.015) (Fig. 5.6 B). Rats that received LPS spent significantly less time in (p=0.027) and made less entries into (p=0.028) the open arms of the maze (Fig. 5.6 C and Fig. 5.6 D). Thus, a single dose of LPS in the subacute period following SCI induces a long-lasting increase in anxiety-like behaviour.

5.3.6 Subacute LPS treatment attenuates the expression of IBA1 and GFAP around the lesion site

10 weeks following SCI, IBA1 and GFAP expression was assessed immediately rostral, at the lesion epicenter and immediately caudal to the injury (Fig. 5.7 A–I). Rats that received LPS displayed significantly reduced density of IBA positive cells rostral to the lesion (p = 0.016) and at the maximum injury location (p = 0.009) (Fig. 6J, L). Caudal to the injury, LPS treated rats also displayed reduced IBA1 expression, however this did not reach significance (Fig. 5.7 N). There was no effect of LPS on

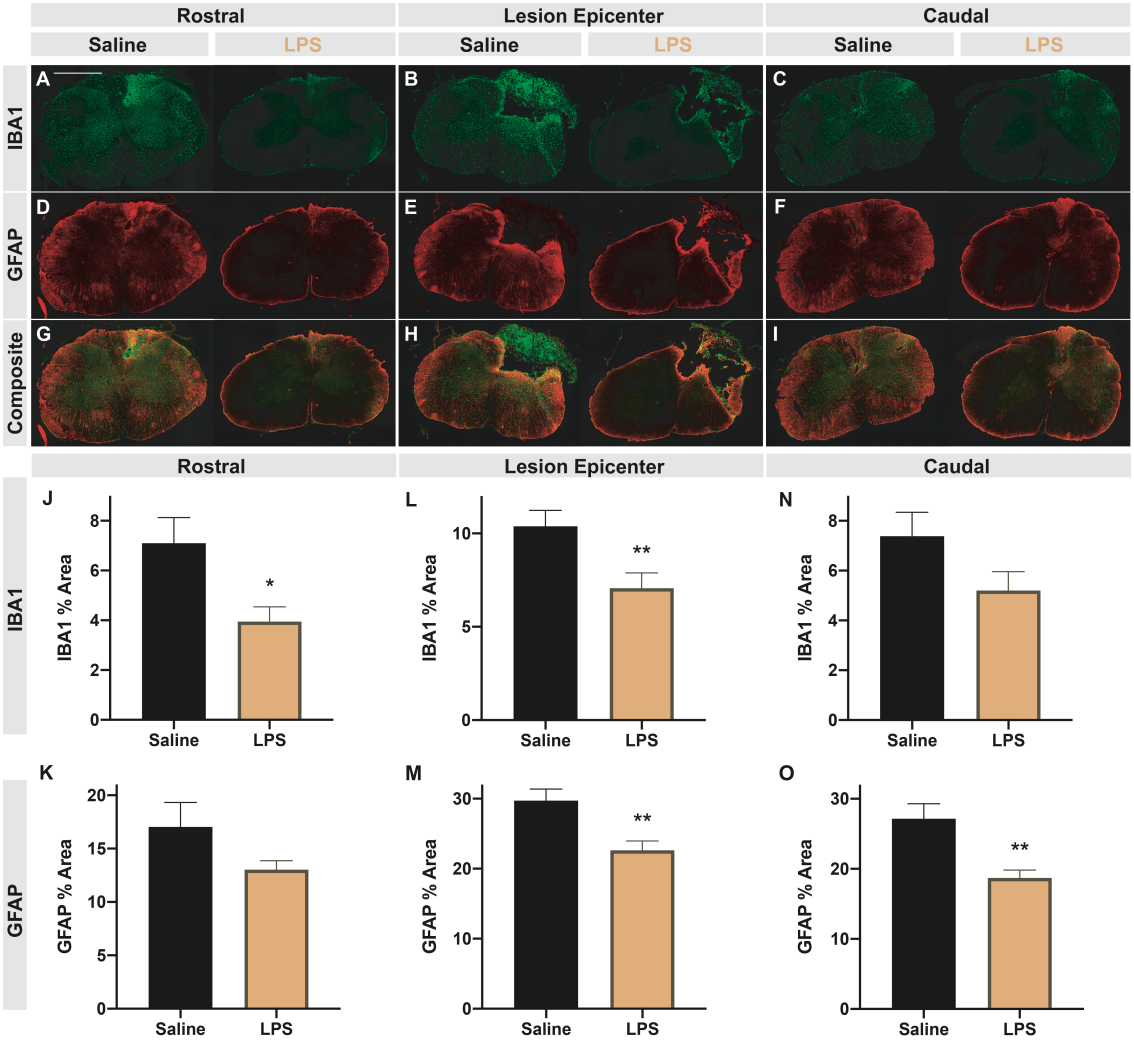

Figure 5.7: IBA1 optical density was assessed immediately rostral to the injury (A), at the lesion epicenter (B) and immediately caudal to the injury (C). GFAP optical density was assessed immediately rostral to the injury (D), at the lesion epicenter (E) and immediately caudal to the injury (F). (G-I) Composite images of IBA1 and GFAP staining. Quantification of the percent area of IBA1 positive cells rostral (J), at (L), and caudal (N) to the injury show reduced IBA1 density in LPS treated rats. Quantification of the percent area of GFAP positive cells rostral (K), at (M) and caudal (O) to the injury show reduced GFAP density in LPS treated rats. Scale bar represents 1mm. Error bars represent SEM. *$p<0.05$, **$p<0.01$.

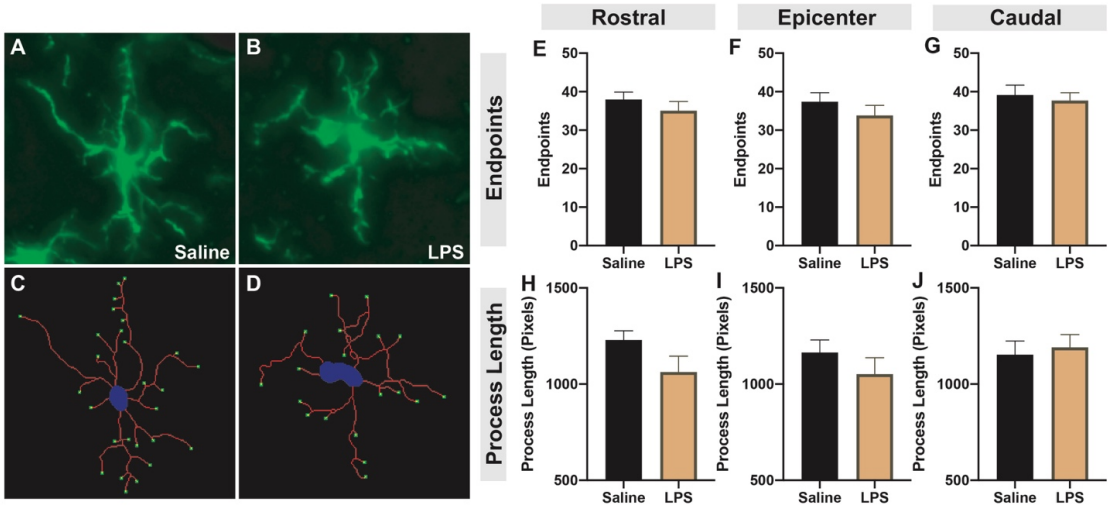

Figure 5.8: (A) and (B) show representative images of an IBA1 positive microglia cell in saline and LPS groups respectively. The images below show the output of the automated analysis used to quantify the number (green) and length (red) of microglia process (C & D). The number of microglia endpoints were quantified 8 weeks after a single intraperitoneal LPS injection immediately rostral (E), at (F) and immediately caudal (G) to the injury at cervical level 4. The average length of microglial processes per cell was quantified -immediately rostral (H), at the injury epicenter (I) and immediately caudal (J) to the injury. Error bars represent SEM. *$p<0.05$.

the morphology of the microglia, assessed by the length of microglial processes and number of process endpoints per cell (5.8). LPS treatment also resulted in a long-term decrease of GFAP expression rostral, at ($p=0.003$) and caudal to the lesion ($p=0.002$) (Fig. 5.7 K–O). These data suggest that a single dose of LPS 10 days after SCI can attenuate immune cell expression long-term (i.e., 8 weeks) after injection.

5.3.7 LPS reduces neuronal excitability

In a separate experiment, we aimed to determine whether inducing inflammation with LPS had any long term effects on neuronal excitability. To test this hypothesis, we used chronically injured rats in which the S2 sacral spinal cord was completely transected. This injury eliminates all ascending and descending inputs from supraspinal

levels, leaving the circuitry within the sacral spinal cord intact. This allows us to measure tail muscle spasms in the awake rat using electromyography (EMG). The base of

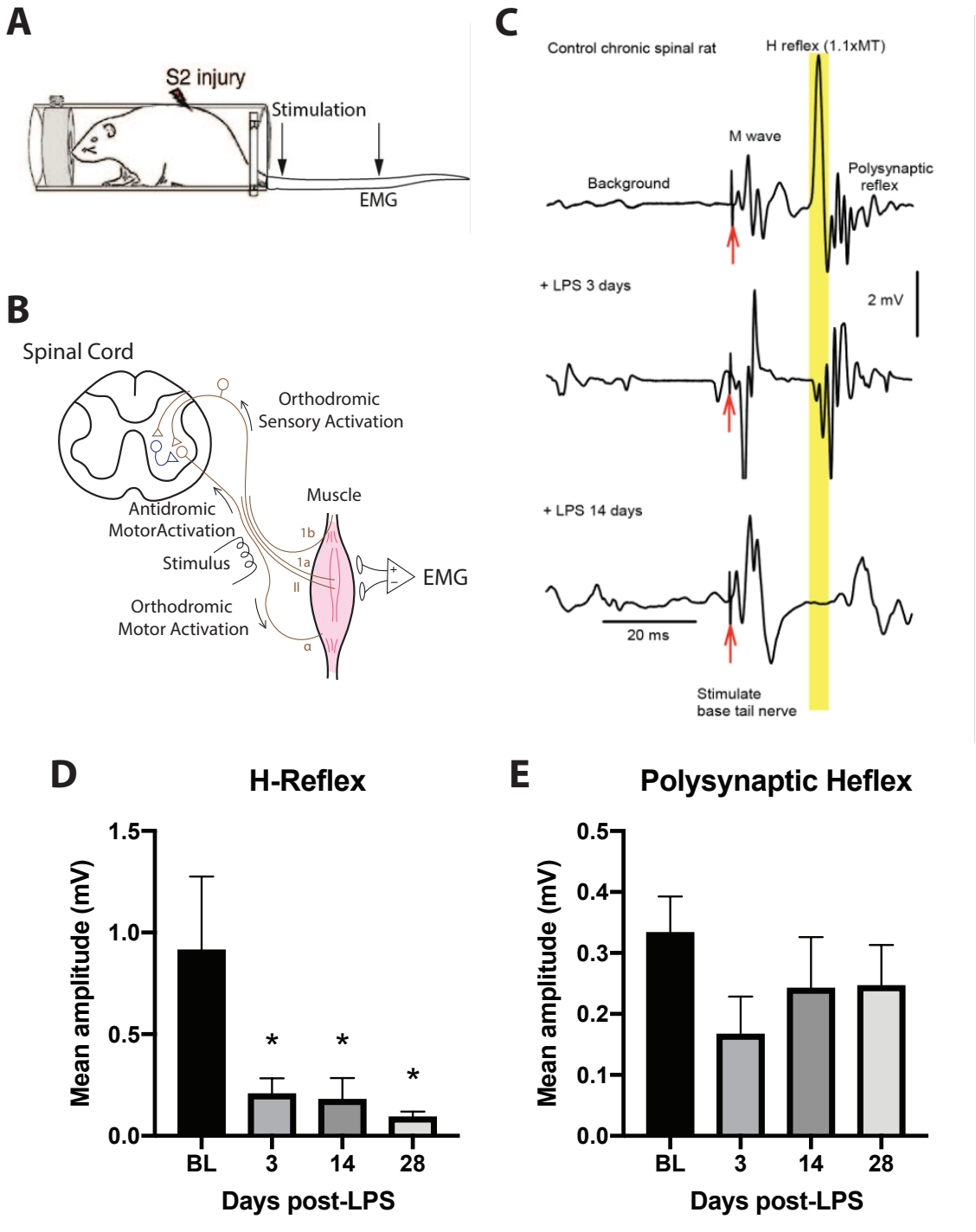

Figure 5.9: Caption on next page

Figure 5.9: (Previous page) (A) Schematic of tail spasm in awake chronic sacral SCI rat. Wires are placed in a homonymous configuration: stimulation wires are placed at the base of the tail and recording wires are placed in the middle of the tail. (B) Schematic of monosynaptic reflex. Electrical stimulation of 1a afferents causes a reaction of muscles in their innervating nerves. (C) Representative images of decreased monosynaptic (H reflex) reflex 3 and 14 days following administration of LPS in the chronic spinalized rat. Yellow highlight indicates the H reflex 10-20 ms following electrical stimulation, followed by a polysynaptic response. (D) Quantification showing LPS significantly decreased the monosynaptic (h-reflex) and is maintained for up to 28 days. (E) There was no statistically significant change in the polysynaptic reflex. Error bars represent SEM.

the tail was stimulated briefly (0.2 ms) at 2x motor threshold and the monosynaptic (h-reflex) and polysynaptic EMG activity was recorded (Fig. 5.9 A & B). A single injection of LPS resulted in a significantly reduced amplitude of the h-reflex 3, 14, and up to 28 days after injection ($p = 0.05$, $p = 0.041$, $p = 0.022$, respectively) (Fig. 5.9 C & D). There was no effect on LPS treatment on the polysynaptic reflex (Fig. 5.9 E).

5.4 Discussion

Previously we have shown that inducing inflammation with LPS 8 weeks after SCI amplifies the efficacy of rehabilitative training, which is often less effective in these chronic stages of injury [Norrie et al., 2005, Scivoletto et al., 2005, Sumida et al., 2001, Torres-Espín et al., 2018a]. In the present study we sought to determine whether LPS treatment earlier after SCI would also be able to increase training efficacy. We initially hypothesized that enhancing inflammation at this subacute time point would not have the same effect as chronic application since levels of inflammation and the capacity for motor recovery are already relatively higher [Popovich et al., 1997, Scivoletto et al., 2005, Sumida et al., 2001]. Contrary to our hypothesis, we found that a single dose of LPS given 10 days following SCI had a beneficial effect on improving functional recovery of the injured forepaw that

translated beyond the trained grasping task. Paradoxically, inducing inflammation with LPS resulted in a chronic decreased expression of microglia and astrocytes around the injury site. The beneficial effects of LPS came at a cost however, since LPS treatment induced a long-term increase in anxiety-like behaviour.

Although we found similar effects of inducing inflammation in both the current study and our earlier work (chronic SCI, [Torres-Espín et al., 2018a]), there were some important differences in the methods and results. First, a single injection of LPS was used for the subacute time point (10 days post injury), however two injections were given in the chronic setting (8 and 11 weeks post-injury) [Torres-Espín et al., 2018a]. Our results suggest that a single injection is sufficient to produce a treatment effect. However, in comparison to chronic administration, LPS given in the subacute time point had a more modest effect on training efficacy. This may be due to the lack of a second injection, the subacute time point being less effective as inflammation is still present, and/or the saline group displaying a more robust recovery due to the earlier training onset. Regardless, inducing inflammation in either the subacute or chronic stages of SCI promoted the restoration of grasping function of the ipsilesional forepaw. In the present study, the beneficial effects of LPS were not associated with significant changes in CST sprouting rostral to injury. When applied chronically after SCI, LPS treated rats displayed increased CST collateral density and further projection of CST collaterals into the cervical grey matter both at the lesion site and one to two spinal segments rostral to the lesion site [Torres-Espín et al., 2018a]. Since we did not observe such structural plasticity of the CST fibres, the beneficial effects of subacute LPS treatment on rehabilitative training could otherwise be due to functional plasticity (ex. synaptic plasticity) or modification of other descending motor tracts such as the rubrospinal and reticulospinal tracts [Morris and Whishaw, 2016].

One of the most robust effects of LPS treatment was its ability to produce meaningful, functional recovery of the rat's forepaw. This finding was demonstrated at the

end of rehabilitative training when a gap was introduced into the pellet dispenser to discourage compensatory scooping of the pellet. Rats that did not receive LPS performed poorly in this task, displaying a similar success rate as they did immediately following SCI. This suggests that the saline group's improved success rate over the 6 weeks of rehabilitative training was largely due to learning a compensatory strategy. In comparison, rats that received LPS treatment in either the subacute or chronic setting after SCI displayed restorative grasping and supination movements to retrieve the pellet, without relying on compensatory movements [Torres-Espín et al., 2018a]. Notably, when applied in the subacute setting, LPS treatment promoted recovery in the cylinder test. Therefore, LPS-induced motor recovery was not task-specific and effectively translated to an untrained task. This finding is significant as it indicates that LPS-induced motor recovery increased injury-induced recovery likely via enhanced neuroplasticity.

It is well recognized that upon binding to the CD14/TLR4/MD2 receptor complex on immune cells in the periphery, LPS triggers an immune response therefore promoting the secretion of nitric oxide, reactive oxygen species and pro-inflammatory cytokines [Lu et al., 2008, Qin et al., 2007]. Once LPS-induced inflammation reaches the brain, it initiates a self-propagating process that can last for months after peripheral injection [Qin et al., 2007]. This LPS-induced neuroinflammation is characterized by the activation of macrophages and microglia, and the upregulation of a variety of proinflammatory factors such as tumor-necrosis factor alpha, IL-1β, nuclear factor kappa B, nitric oxide and cyclooxygenase-2 [Zhao et al., 2019]. There is extensive literature on the destructive nature of neuroinflammation, and many immunosuppressive therapies have been shown to be effective for central nervous system disorders and damage including multiple sclerosis, Parkinson's disease, depression, stroke, and SCI [Kohler et al., 2016, Liebigt et al., 2012, Rocha et al., 2015, Thompson et al., 2018, Wells, 2003b]. However, neuroinflammation and adverse CNS outcomes do not go

hand-in-hand, and multiple studies report significant benefits of neuroinflammation [Schwartz et al., 1999a, Schwartz et al., 1999b, Yong et al., 2019]. For example, serial prophylactic injections of LPS can promote a reactive and neuroprotective microglia phenotype that promotes recovery and protects against neuronal loss following SCI [Freria et al., 2020]. Furthermore, transplantation of peripheral nerve-activated macrophages into the injured spinal cord has been shown to promote tissue repair and functional recovery [Rapalino et al., 1998, Schwartz et al., 1999b]. The reparative effects of macrophages may be dependent upon oncomodulin, as this macrophage-derived protein has been shown to promote regeneration of retinal ganglion cells [Yin et al., 2009]. Immune cells other than macrophages have also been implicated in the promotion of CNS repair. Leukocytes and microglia are well known to promote the secretion of a variety of neurotrophic factors that are important for neurogenesis and remyelination [Sousa-Victor et al., 2018, Yong and Rivest, 2009]. The role of the LPS receptor, TLR4, which is found on a variety of cell types including dendritic cells, neutrophils, mast cells, macrophages, microglia and neurons, has also been implicated in CNS repair. Antagonizing TLR4 inhibited neurological recovery following intracerebral hemorrhage in a rat model, while another group found that agonizing TLR4 improved Alzheimer's pathology in mice [Lei et al., 2016, Michaud et al., 2013]. Histamine may also play a role in the neuromodulator effects of LPS. LPS stimulates an increase in mast cells which can pass the blood brain barrier and release histamine [Silverman et al., 2000, Wang et al., 2020], which has been shown to modulate neuronal and central inflammatory circuits [Coslovich et al., 2018, Dong et al., 2014, Wei et al., 2016, Zhu et al., 2014].

There is strong evidence that peripheral inflammation leads to increased neuronal excitability [Riazi et al., 2008]. In culture, activation of the TLR4 receptor evokes sensory neuron excitability and hyperalgesia, and this effect can be reversed by a TLR4 antagonist [Due et al., 2012]. We therefore did not expect to see a decrease in

excitability of the h-reflex following peripheral inflammation induced by LPS. However, it is important to note that the animals used for this study had spasticity of the tail and thus have hyperexcitability of spinal reflexes and potentially an altered environment than intact animals [D'Amico et al., 2014]. Increased spasticity results in unwanted movements which may impede rehabilitative training. Therefore, the improvement of training efficacy following LPS may not be to increased excitability, rather increased inhibition which may modulate neural activity in an adaptive manner when combined with rehabilitative training. There is little evidence of inflammation reducing neuronal excitability, however Hellstrom et al., [Hellstrom et al., 2005] found that, in CA1 pyramidal neurons, exposure to LPS resulted in an increased action potential threshold and an increase in synaptic GABAergic input. Therefore, LPS may decrease neuronal excitability through GABAergic mechanisms in the presynaptic neuron which may result in the observed reduction in EMG amplitude of monosynaptic reflexes.

We have previously shown in chronic SCI that LPS enhances the expression of microglia at the lesion site within hours after administration [Torres-Espín et al., 2018a]. Although LPS-induced acute microglia and astrocyte activation is well characterized [Ryu et al., 2019], we describe a novel finding in which LPS resulted in a chronic (i.e. measured 8 weeks after injection) attenuation of microglia and astrocyte expression. This result may or may not be particular to SCI, in which immune cells can persist at the lesion site chronically [Beck et al., 2010, Fleming et al., 2006, Sroga et al., 2003]. It is possible that exposure to LPS following SCI resulted in a compensatory anti-inflammatory response syndrome, whereby excessive inflammatory stimuli produces an adaptive immune suppression [Adib-Conquy and Cavaillon, 2009, Vergadi et al., 2018]. The beneficial effects of LPS for treatment following subacute SCI may therefore be due to the promotion of immune resolution at the lesion site, which has previously been associated with improved recovery following SCI

[Francos-Quijorna et al., 2017]. The improvements in motor recovery with LPS treatment took time to develop (approximately 4 weeks following injection) in both subacute and chronic applications [Torres-Espín et al., 2018a]. This time point may coincide with the resolution of inflammation observed in LPS treated rats, however future work would be required to support this hypothesis.

There is a strong link between inflammation and mental health disorders [Miller and Raison, 2016, Raison et al., 2006]. Increasing evidence shows that this holds true after SCI, which causes a drastic post-traumatic immune response, prolonged neuroinflammation, and an increased prevalence of depression and anxiety [Hausmann, 2003, Williams and Murray, 2015]. This has been shown to be independent of lesion severity or location, and does not necessarily improve over time after injury [Craig et al., 1994b, Dryden et al., 2005]. Reducing levels of blood proinflammatory cytokines with a 12 week anti-inflammatory diet is effective in decreasing symptoms of depression after SCI, implicating inflammation as a key factor in the development of mental health disorders in the context of SCI [Allison and Ditor, 2015]. Further evidence from preclinical SCI research in rodents has shown an association between anxiety- and depressive-like behaviours and increased levels of inflammation in the brain, spinal cord and blood [do Espírito Santo et al., 2019, Maldonado-Bouchard et al., 2016b, Wu et al., 2014]. Furthermore, alterations in the intestinal microbiota composition and a leaky gut (which can allow bacterial matter such as LPS to enter the circulation [Fukui, 2016, Liu et al., 2004b, Valentini et al., 2014]) have been linked to motor outcome and the development of anxiety-like behaviour following SCI in rodents [Kigerl et al., 2016b, Schmidt et al., 2020b]. Outside of SCI research, inducing inflammation with LPS is commonly used as a model for anxiety- and depressive-like behaviour in rodents [De La Garza, 2005, Yirmiya, 1996b]. Following intraperitoneal LPS administration, rats display sickness behaviour characterized by decreased mo-

tor activity, decreased appetite, and social isolation. This LPS-induced behavioural response is considered acute and transient and is therefore studied within 24 hours after injection [Nava and Carta, 2001, Salazar et al., 2012]. Indeed, in the present study, rats experienced decreased locomotion in the open field, weight loss and sickness behaviour lasting up to 3 days after LPS injection. More importantly, in our study a single injection of LPS elicited long-term (i.e. 4 weeks after LPS) effects on anxiety-like behaviour in the EPM. It is unclear whether uninjured rats would have a similar long-term increase in anxiety-like behaviour following LPS administration. It is possible that the combination of SCI and LPS acted similar to the two-hit hypothesis suggested for other mental health disorders, where previous immune activation can prime the immune system to be more susceptible to a second adverse event [Feigenson et al., 2014]. Nonetheless, LPS-induced anxiety-like behaviour did not interfere with the rat's ability or motivation to participate in rehabilitation training as evidenced in the similar attempt rates between groups. Furthermore, in line with research indicating that LPS causes mechanical allodynia only in male but not female rats [Sorge et al., 2011], our female rats did not experience LPS-induced pain behaviours. However, the von frey test may not have been an appropriate method of testing allodynia of the forepaws , and additional pain assays may be necessary. Nonetheless, given the sex differences in response to the LPS-induced inflammatory response [Kuo, 2016], the present research should be replicated in males.

We show that triggering an immune response in the subacute period following SCI in combination with rehabilitative training can enhance functional recovery. This recovery may in part be due to the paradoxical chronic resolution of neuroinflammation at the lesion site following subacute LPS treatment. However, inflammation can be a double-edged sword and therefore its manipulation should be considered cautiously. Although eliciting inflammation with LPS promoted functional recovery

following SCI, it also caused a long-term increase in anxiety-like behaviour. Inducing neuroinflammation with LPS may generally enhance the plasticity of a variety of neural substrates, as evidenced by LPS-induced changes in the limbic system, pain sensitivity, and motor recovery [Calil et al., 2014, Guo and Schluesener, 2006, Torres-Espín et al., 2018a, Yirmiya, 1996a]. Given the widespread immune response triggered by LPS, future research should explore the temporal systemic and local immune response to LPS treatment for SCI. Furthermore, the timing of treatment intervention may still be an important factor. Although we have shown that inducing inflammation 10 days or 8 weeks following SCI both have a beneficial effect on functional recovery without exacerbating lesion size, it is very likely that inducing a systemic immune response in the acute (i.e., within days) lesion environment would have a detrimental effect on SCI pathology. Future work should be considered to optimize inflammation-induced plasticity by separating its beneficial aspects from detrimental behavioural consequences.

Chapter 6

Conclusion

6.1 Summary of results

In this thesis, we investigated various therapeutics targeting the microbiota-immune axis to augment multiple aspects of recovery following SCI in rats. In chapter 2, we first showed that an incomplete cervical SCI induced acute intestinal dysbiosis and a long-term increase in anxiety-like behaviour. We next showed that there was a link between these two consequences of SCI, since preventing SCI-induced dysbiosis with a FMT also prevented the development of anxiety-like behaviour. We showed in chapter 3 that optimal FMT donor selection is critical for successful transplant following SCI. FMT from rats with increased baseline levels of anxiety-like behaviour and reduced stool proportions of *Lactobacillus* (which we will term *inferior* FMT) was not only unsuccessful in preventing SCI-induced dysbiosis, but the recipient rats also displayed increased intestinal permeability, adopted an increased anxiety-like behavioural state and displayed altered local and systemic inflammation. Furthermore, we showed that an incomplete cervical SCI resulted in a chronic suppression of systemic inflammation (reflected by the decreased concentration of plasma cytokines and chemokines). In chapter 4, we showed that treatment with the antibiotic and anti-

inflammatory drug minocycline can prevent this SCI-induced suppression of plasma cytokines and chemokines. This suppression of circulating inflammatory markers was preceded by minocycline's drastic impact on the microbiota composition, suggesting a temporal relationship between these events. Finally in chapter 5 we used the bacterial endotoxin, LPS, to show the dichotomous role that inflammation can play in recovery after injury. Although LPS treatment enhanced motor recovery, it also had a long-term negative effect on anxiety-like behaviour following SCI.

6.2 Potential mechanisms and future directions

6.2.1 lipopolysaccharide translocation

In chapter 2, we show striking results from successful FMT treatment (i.e., FMT from non-anxious-like donors successfully prevented SCI-induced dysbiosis), however the mechanisms of how this treatment worked remain elusive. We hypothesize (as one possibility) that preventing SCI-induced dysbiosis also prevents a leaky gut, which would allow the translocation of bacterial matter such as LPS across the impaired epithelial tight junctions [Ghosh et al., 2020]. Once in circulation, LPS initiates a potent inflammatory response which has been strongly linked to the development of mental health disorders [Yirmiya, 1996a, De La Garza, 2005, Raison et al., 2006]. Therefore, optimal FMT treatment may protect against a leaky gut via modulation of the microbiota and prevent the subsequent increase in inflammation from endotoxin translocation, thus preventing the development of anxiety-like behaviour following SCI. Although we did not test this directly, we showed that FMT is able to modulate the intestinal barrier, since *inferior* FMT treatment enhanced intestinal permeability. Although in this experiment, untreated SCI rats did not display a change in intestinal permeability following SCI, it is possible that a leaky gut may have been observed at different timepoints as tight junctions are highly dynamic and

can open or close rapidly [Chelakkot et al., 2018]. Furthermore, the intestinal permeability assay was run in rats with increased baseline levels of anxiety-like behaviour. Since stress itself can induce a leaky gut [Zheng et al., 2017], this may explain why we did not observe a change in intestinal permeability following SCI in control rats. Further evidence that the negative effects of *inferior* FMT treatment were a result of endotoxin translocation are the parallels between LPS and *inferior* FMT treatment. Both LPS and *inferior* FMT treated rats displayed improved motor recovery in the modified gap test (although this did not reach significance for *inferior* FMT rats). Furthermore, both *inferior* FMT and LPS treated rats displayed a seemingly paradoxical chronic reduction in microglial density around the lesion site, which may suggest a compensatory anti-inflammatory response following systemic inflammation [Adib-Conquy and Cavaillon, 2009]. Interestingly, we found similar (yet opposite) results from minocycline, such that direct anti-inflammatory treatment with minocycline resulted in an increase in microglial immunoreactivity around the lesion site. These confounding results suggest that manipulating systemic inflammation after SCI can disrupt the balance of the immune system that can have long-term repercussions within the CNS.

Going back to potential mechanisms of FMT treatment, both intestinal permeability and systemic LPS concentrations should be measured at various timepoints following SCI and with optimal FMT treatment. Since we show that both administration of LPS or increasing intestinal permeability (with the *inferior* FMT) results in a long-term increase in anxiety-like behaviour, it is possible that similar mechanisms are responsible for the development of anxiety-like behaviour observed in these studies. If optimal FMT treatment does reduce the translocation of LPS across the intestinal barrier, it would be important to consider that this may have a detrimental effect on rehabilitative training and reduce the opportunity for plasticity. In support of this, successful FMT treated animals performed significantly worse in the cylinder

task compared to untreated SCI rats. Clearly there is a complicated relationship between the microbiota and systemic inflammation, and it is important to consider these interactions when testing potential therapeutics. It is unknown whether LPS will be able or proven safe enough to be used clinically, so determining downstream mechanisms of LPS induced plasticity will be important to minimize the negative side effects such as long-term increases in anxiety-like behaviour. On the other hand, it will also be important to consider potential negative side effects of anti-inflammatory treatment such as reducing the opportunity for beneficial plasticity following injury.

6.2.2 The kynurenine pathway as a potential mechanism underlying treatment results

The kynurenine pathway of tryptophan metabolism may play a role in the treatment effects of FMT, minocycline and LPS observed throughout this thesis, whether beneficial or detrimental. Tryptophan is a dietary essential amino acid whose metabolites are integral to a variety of physiological and immune functions [Le Floc'h et al., 2011]. Less than 5% of tryptophan is metabolized into serotonin [Michael et al., 1964]; the majority of tryptophan is metabolized into kynurenine via the rate-limiting enzymes tryptophan-2,3-dioxygenase and indoleamine-2,3-dioxygenase (IDO) [Bender, 1983]. Tryptophan-2,3-dioxygenase is localized in the liver and is activated by increased glucocorticoid concentrations induced by stress [Danesch et al., 1983]. IDO is widely distributed throughout the periphery and CNS and is induced by inflammatory stimuli such as LPS and pro-inflammatory cytokines [Moreau et al., 2005, Dinel et al., 2014]. Not only does activation of the kynurenine pathway reduce the bioavailability of tryptophan and subsequent synthesize into serotonin, kynurenine metabolites can have drastic effects on inflammation, neuronal activity and behaviour [Terness et al., 2002, Lapin, 1978, Lapin, 1983]. The two most widely studied kynurenine metabolites are kynurenic acid and quinolinic acid. Kynurenic acid acts as an NMDA receptor antag-

onist and has been proposed to be neuroprotective and suppress multiple inflammatory pathways [Savitz, 2016]. On the other hand, quinolinic acid acts as an NMDA receptor agonist and exerts various neurotoxic effects such as inhibiting astrocytic glutamate reuptake, disrupting the BBB, destabilizing the cytoskeleton of cells and inducing apoptosis [Lugo-Huitrón et al., 2013]. These opposing roles of the kynurenine pathway may help explain the dichotomous role that inflammation can play. Kynurenine metabolites have been shown to be essential for LPS-induced depressive-like behaviour; for example, blockade of IDO activation with minocycline prevents LPS-induced depression without interfering with sickness behaviour or cytokine activation [O'Connor et al., 2009, Dantzer, 2017]. Combining minocycline with LPS treatment may therefore prevent some of the negative side effects of inducing inflammation on mental health while still activating some immune pathways which may promote plasticity. Furthermore, minocycline's prevention of SCI-induced suppression of systemic inflammation observed in chapter 4 may be mediated through inhibition of IDO, since kynurenine and its metabolites can be immunosuppressive and downregulate the inflammatory response to LPS [Kimura et al., 2009, Bessede et al., 2014]. On the other hand, kynurenine can stimulate cell proliferation and growth [Chalisova et al., 2019], which may in part explain LPS-induced motor recovery.

In addition to the many routes of communication between the gut and the brain, the gut microbiota can modulate circulating tryptophan and kynurenine, which are able to pass through the BBB through large amino acid transporters where their metabolites can cause a variety of neuroactive effects in the CNS [Fukui et al., 1991, Wiedlocha et al., 2021]. Administration of *Lactobacillus* in rats, non-human primates and humans can attenuate IDO activity and the kynurenine pathway, which, as previously mentioned, have been associated with depression and anxiety-like behaviours [Rudzki et al., 2019, Valladares et al., 2013, Vujkovic-Cvijin et al., 2015, Rudzki et al., 2019]. This may explain why the *in-*

ferior FMT was not effective in preventing SCI-induced anxiety-like behaviour, as it did not contain *Lactobacillus*. In the case where inflammation is produced centrally, such as in SCI, the majority of kynurenine is produced locally by microglia and astrocytes [Kita et al., 2002, Guillemin et al., 2005]. Although the kynurenine pathway is relatively understudied in the context of SCI, existing studies suggest that targeting this pathway may provide multiple therapeutic benefits such as neuroprotection and improved mood. For example, blocking the metabolism of quinolinic acid after SCI has been shown to reduce functional deficits associated with the injury [Blight et al., 1995, Yates et al., 2006]. On the other hand, the kynurenine pathway may also be a target of neuroprotection, since administration of kynurenic acid into the spinal cord improves recovery of motor function after SCI [Jacobs and Lovejoy, 2018, Wrathall et al., 1992]. Another study found that increasing kynurenic acid synthesis in individuals with SCI (with gene transfer of human KAT-II) improved bladder function, perhaps by blocking NMDA receptors in the spinal cord [Wang and Liao, 2017]. Alterations in the kynurenine pathway may also explain the increased rate of depression and anxiety in SCI patients; Allison and Ditor found that the kynurenine/tryptophan ratio was significantly correlated with depression scores in patients with SCI [Allison and Ditor, 2015]. Targeting kynurenine 3-monooxygenase, which is the essential regulator of the metabolic fate of kynurenine to either the neuroprotective kynurenic acid or the neurotoxic quinolinic acid, may be a therapeutic target to mitigate the detrimental aspects of neuroinflammation [Parrott and O'Connor, 2015]. For example, a recently discovered compound termed KMO inhibitor 1 has shown unique potential for its ability to cross the BBB (via a prodrug variant) and lower neurotoxic kynurenine pathway metabolites locally [Zhang et al., 2019b]. Furthermore, since kynurenine metabolites act as endogenous ligands to NMDA receptors, they may also be involved in inflammation-mediated plasticity, however this hypothesis has yet to be explored. Nonetheless, the kynure-

nine pathway likely plays a part in multiple aspects of SCI consequences, including systemic inflammation, immune suppression, psychiatric disorders and neuronal plasticity.

6.2.3 The dichotomous role of inflammation-induced plasticity

Throughout this thesis, we have eluded to the hypothesis that neuroinflammation is a driving force behind plasticity that occurs following injury to the CNS, regardless of whether that plasticity manifests as a beneficial or detrimental process. One of the most infamous examples of neuronal plasticity was proposed in 1949 by Donald Hebb, who put forth the idea that "neurons that fire together wire together" [Donald Olding Hebb, 2005]. This theory of activity-dependent plasticity was later proven by Bliss and Lomo, who demonstrated that high-frequency stimulation of hippocampal afferents resulted in persistent increase in synaptic strength, termed long-term potentiation [Bliss and Lømo, 1973]. Over 20 years later it was shown that the pro-inflammatory cytokine IL-1 was critically involved in maintaining long-term potentiation [Schneider et al., 1998]. Since then, countless studies have verified the involvement of immune mediators in neural plasticity, however the nature of this involvement and how it transitions from a beneficial to detrimental role are poorly understood [Yirmiya and Goshen, 2011]. The fact that triggering an inflammatory response in a rodent paw increases spinal dorsal horn neuron receptive field size and excitability associated with hyperalgesia is a critical example of detrimental inflammation-induced neuronal plasticity [Dubner and Ruda, 1992, Kitagawa et al., 2005]. After SCI, there is a critical window where spontaneous plastic changes are observed at multiple levels including cortical map changes, neuronal excitability changes, axon regeneration and sprouting [Peruzzotti-Jametti et al., 2014, Carmichael, 2006, Endo et al., 2007, Thomas et al., 2017, Houle and Côté, 2013]. However, this plasticity is a double

edged sword; the same processes that foster recovery can also cause unwanted plastic processes such as neuropathic pain, spasticity, depression, and potentially gut dysbiosis [Hulsebosch et al., 2009, Adams and Hicks, 2005, Allison and Ditor, 2015, Kigerl et al., 2016a, Miller and Raison, 2016]. Further manipulating the immune response following SCI can exacerbate these processes; it is therefore prudent to be cognizant of the dichotomous role inflammation-induced plasticity can have and monitor multiple aspects of recovery when considering potential plasticity-promoting therapeutics for CNS injuries.

6.2.4 Limitations and future direction

In many ways, the results presented in this thesis prompt more questions than they answer, leading to numerous potential future studies. Primarily, how can we determine the mechanisms that underly the results? For example, the kynurenine/tryptophan ratio could be measured both systemically (i.e., in plasma) and locally (i.e., in spinal cord and brain tissue) to delineate how this pathway is involved in the pathophysiology of SCI. Inhibiting IDO1, the rate limiting enzyme of the kynurenine pathway, in conjunction with LPS treatment may elucidate whether the kynurenine pathway is involved in the deleterious effects of LPS on the development of anxiety-like behaviour following SCI. The downstream role of TLRs following systemic LPS injection could also be considered to determine whether the effects of LPS are mediated by TLR2 or TLR4 [Takeuchi et al., 1999]. Given that anxiety/depressive-like behaviours are a main focus of this thesis and we do not look in the brain, there is a missing link between the systemic and behavioural effects observed. Therefore, brain structures associated with affective behaviours, such as the amygdala, hippocampus, anterior cingulate-prefrontal cortex and striatum [Zhang et al., 2018], should be examined both on a structural and functional level. In addition to looking at brain structures, the role of the HPA axis in recovery following SCI could be examined as the HPA axis

is involved in many of the topics covered in this thesis such as psychiatric disorders, inflammation and immune suppression [Tapp et al., 2019, Iob et al., 2020, Sudo, 2012]. SCI-induced immune suppression should be measured directly (for example by measuring lymphoid organ atrophy and circulating leukocytes) and the role of the microbiota in the development of immune suppression should be considered. This is especially important since immune suppression enhances the risk of infection, which is a leading cause of death acutely following SCI [DeVivo et al., 1989]. In addition to directly monitoring symptoms of immune suppression, assessing gut motility or neurogenic bowel syndrome in our model of SCI would also be prudent to determine whether gut dysbiosis is a symptom or a cause of these clinically important outcome measures. Another future direction would be to investigate probiotics as a treatment for SCI-induced gut dysbiosis and anxiety-like behaviour. Indeed, probiotics may sound like an "easier pill to swallow" for patients. However, FMT would likely be more effective as it is able to establish a durable alteration in the recipient's microbiome [Grehan et al., 2010], whereas probiotics only temporarily colonize the gut lumen [Tannock et al., 2000]. Furthermore, the efficacy of this transient probiotic engraftment is highly variable between individuals based on their existing microbiome composition [Zmora et al., 2018]. Nonetheless, investigating the efficacy of transferring specific strains of bacteria is a worthwhile venture to broaden our understanding of how bacteria contribute to various disease states. Finally, these studies should be replicated in males to determine what effect sex has on outcome measures, especially since males have an altered inflammatory response to LPS [Marriott et al., 2006, Sens et al., 2017]. Overall, the results presented in this thesis are largely descriptive in nature and could use further research to determine the underlying mechanisms. This will be essential for clinical translation since both the *inferior* FMT and LPS treatment had some negative side effects. In order for these

treatments to be clinically viable, a deeper understanding of the factors mediating both beneficial and harmful processes is essential.

6.3 Epilogue

The first documented case of SCI was approximately 2500 BC, where it was described as "an ailment not to be treated" [Hughes, 1988]. This therapeutic nihilistic philosophy continued for millennia until the 20th century, when Dr. Donal Munro refused this defeatist attitude and opened the first spinal cord unit in 1936 [Silver, 2005]. Dr. Munro had the unique perspective for his time that with attentive care and a holistic treatment approach (including neurological, urological, orthopedic, psychological and social care), his SCI patients could live longer, better lives [Bodner, 2009, Silver, 2005, Trieschmann, 1988]. Since then countless clinicians and researchers have dedicated their careers to finding a treatment for the SCI. This thesis contributes to this enormous undertaking and emphasizes the importance of considering the whole body and mind when investigating treatment options. As SCI impacts almost every system in the body (from the acute damage of sensory and motor tracts, to the commensal micro-organisms inhabiting the gut, to mental health), it is likely that multiple therapeutic approaches targeting multiple aspects of recovery will have to be utilized. Then, perhaps, SCI will be considered an ailment to be cured.

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Appendix A

Appendix

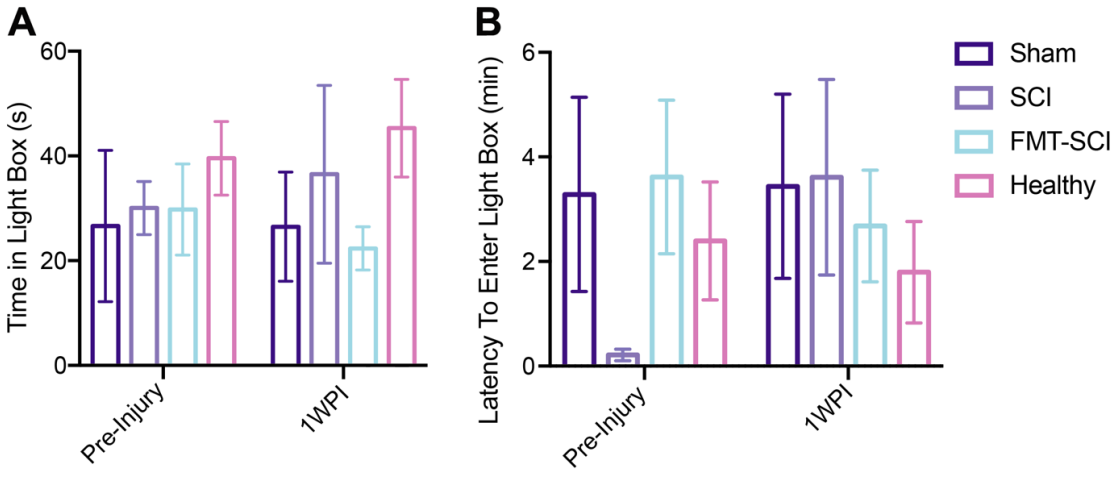

Figure A.1: There was no significant difference in the time spent in light chamber (A) or latency to enter the light chamber (B) before or one week post-injury (1WPI) (repeated measure two-way ANOVA). Error bars indicate standard error mean.

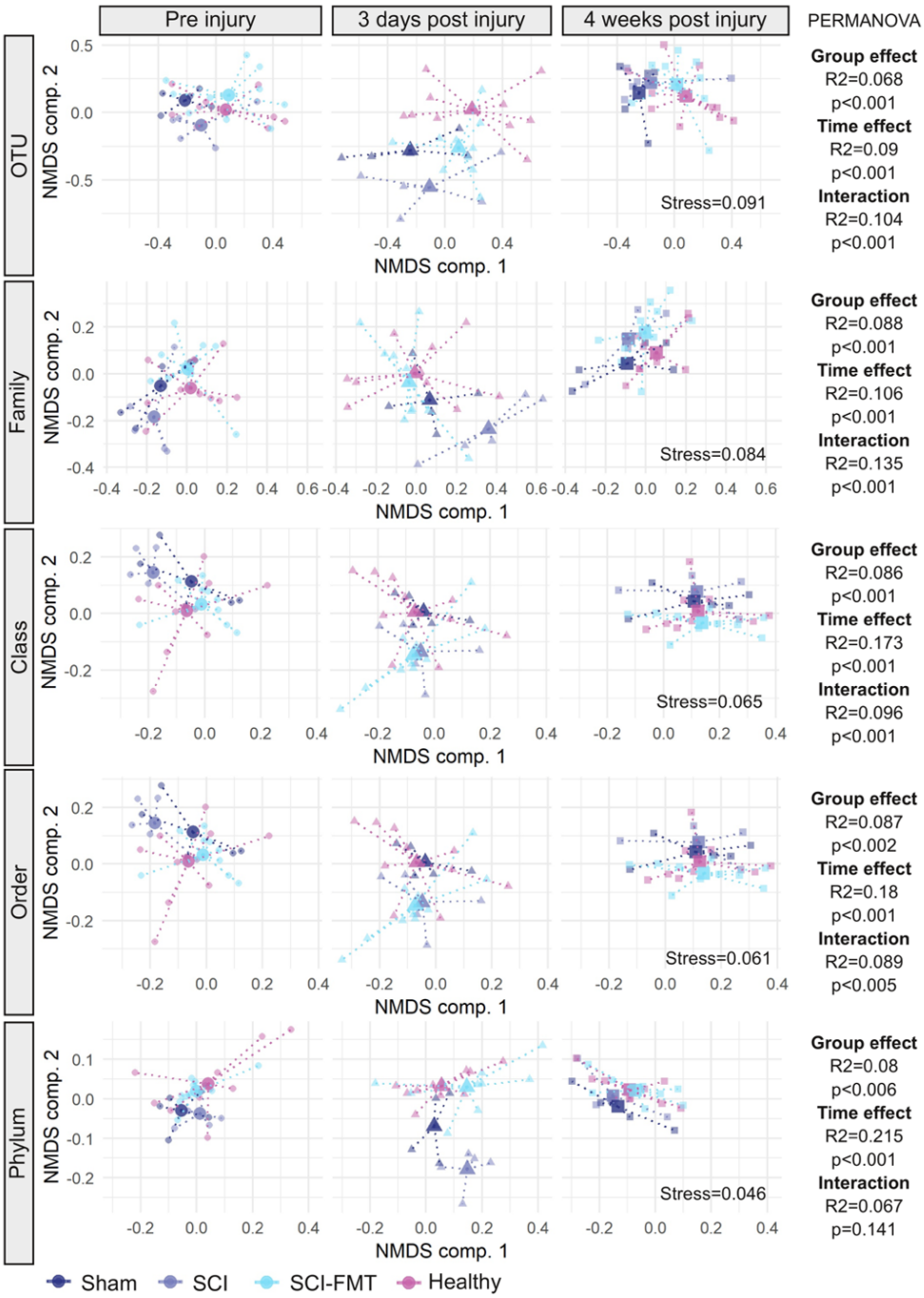

Figure A.2: Unsupervised ordination was performed by non-metric multidimensional scaling (NMDS) and Bray-Curtis dissimilarity at the Phylum, Order, Class, Family and OTU levels. Dotted lines represents the 2D distance of each animal with the respective centroid at each timepoint in the NMDS space.This analysis indicates a deviation in the microbiome composition three days post-injury, with fewer differences between groups pre-injury and four weeks post-injury. The proximity between healthy and SCI-FMT groups can be seen at the OTU, family and phylum levels.

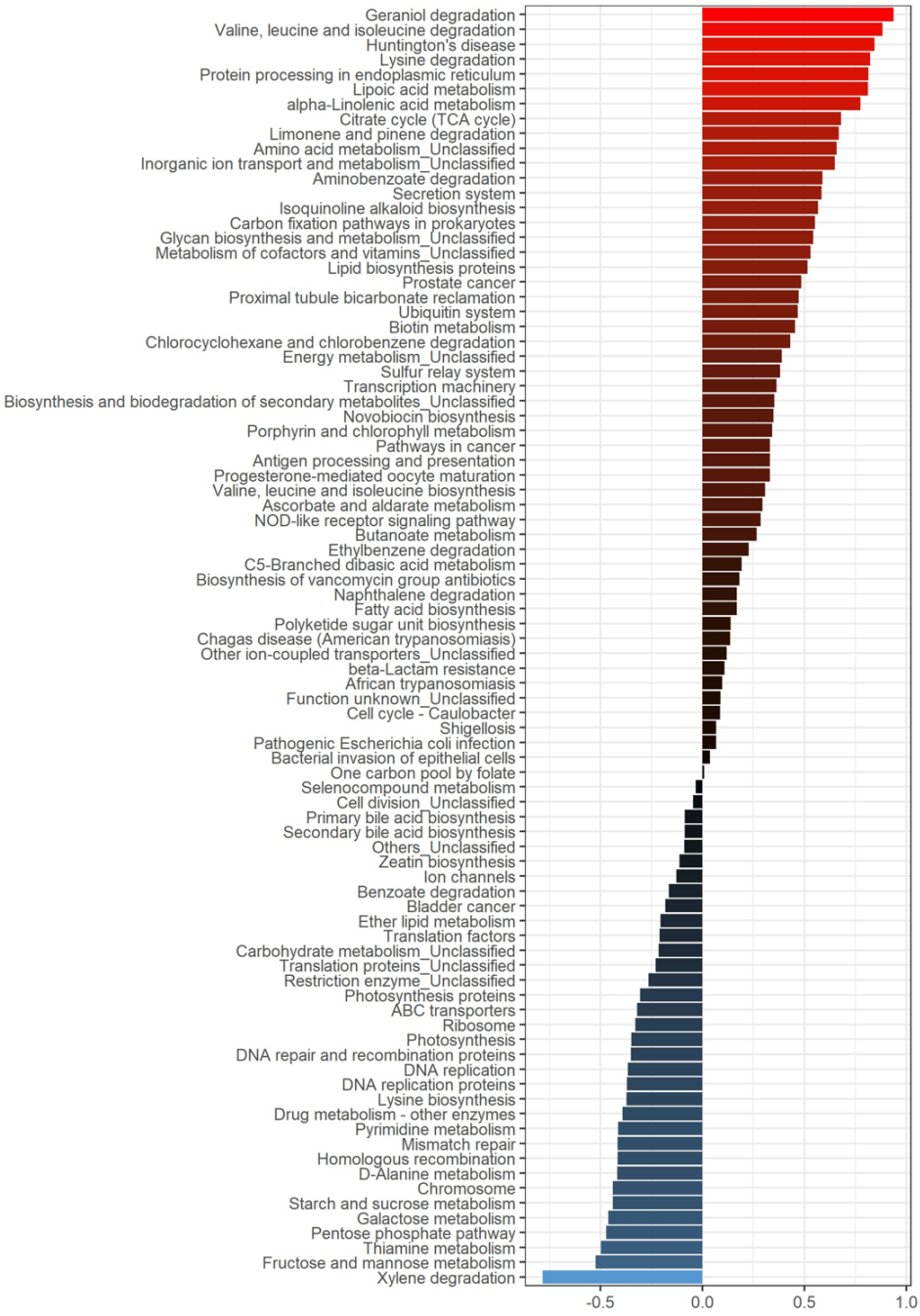

Figure A.3: Complete list of the functional pathways that contribute to the second principal component (explaining 18.4% of the variance) of the PICRUST analysis three days after spinal cord injury or sham operation (Fig. 6B). Pathways that are more likely positively correlated to the second principal component are shown in red, and pathways that are more likely negatively correlated are shown in blue.

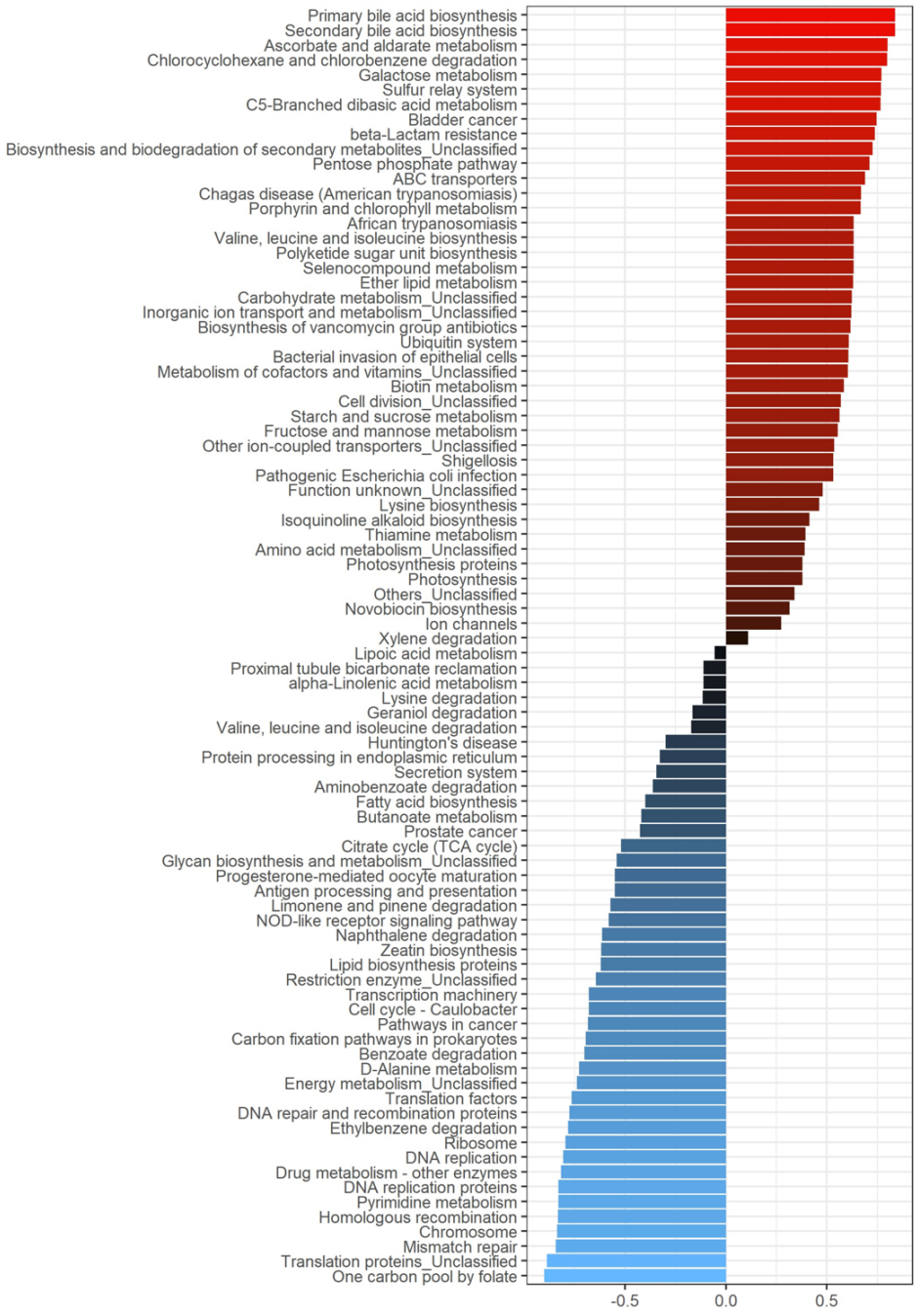

Figure A.4: Complete list of the functional pathways that contribute to the first principal component (explaining 37.9% of the variance) of the PICRUST analysis three days after spinal cord injury or sham operation (Fig. 6B). Pathways that are more likely positively correlated to the first principal component are shown in red, and pathways that are more likely negatively correlated are shown in blue.

Appendix B

Appendix

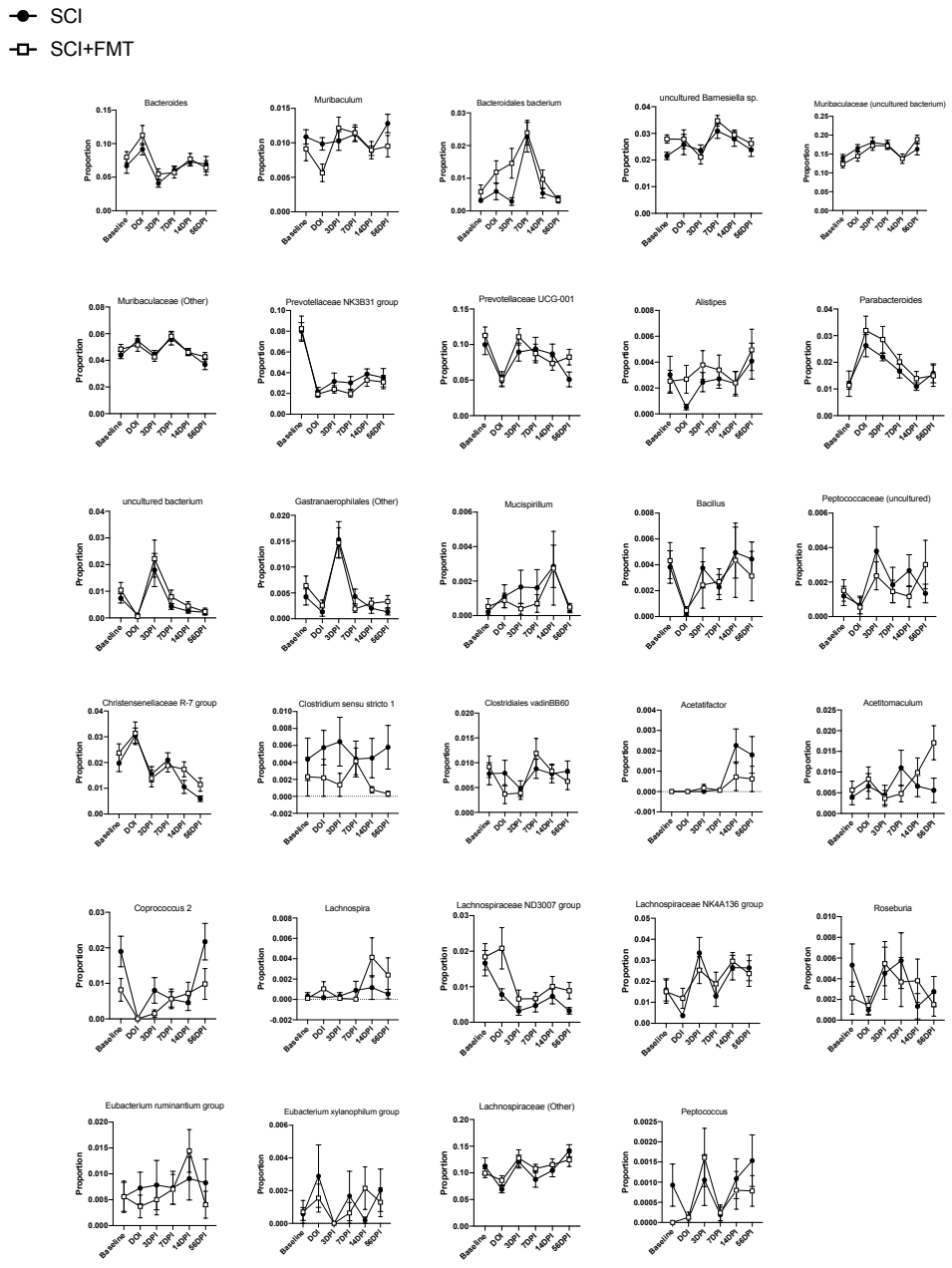

Figure B.1: Proportion of bacteria at the genus level over time following spinal cord injury. Error bars represent standard error mean.

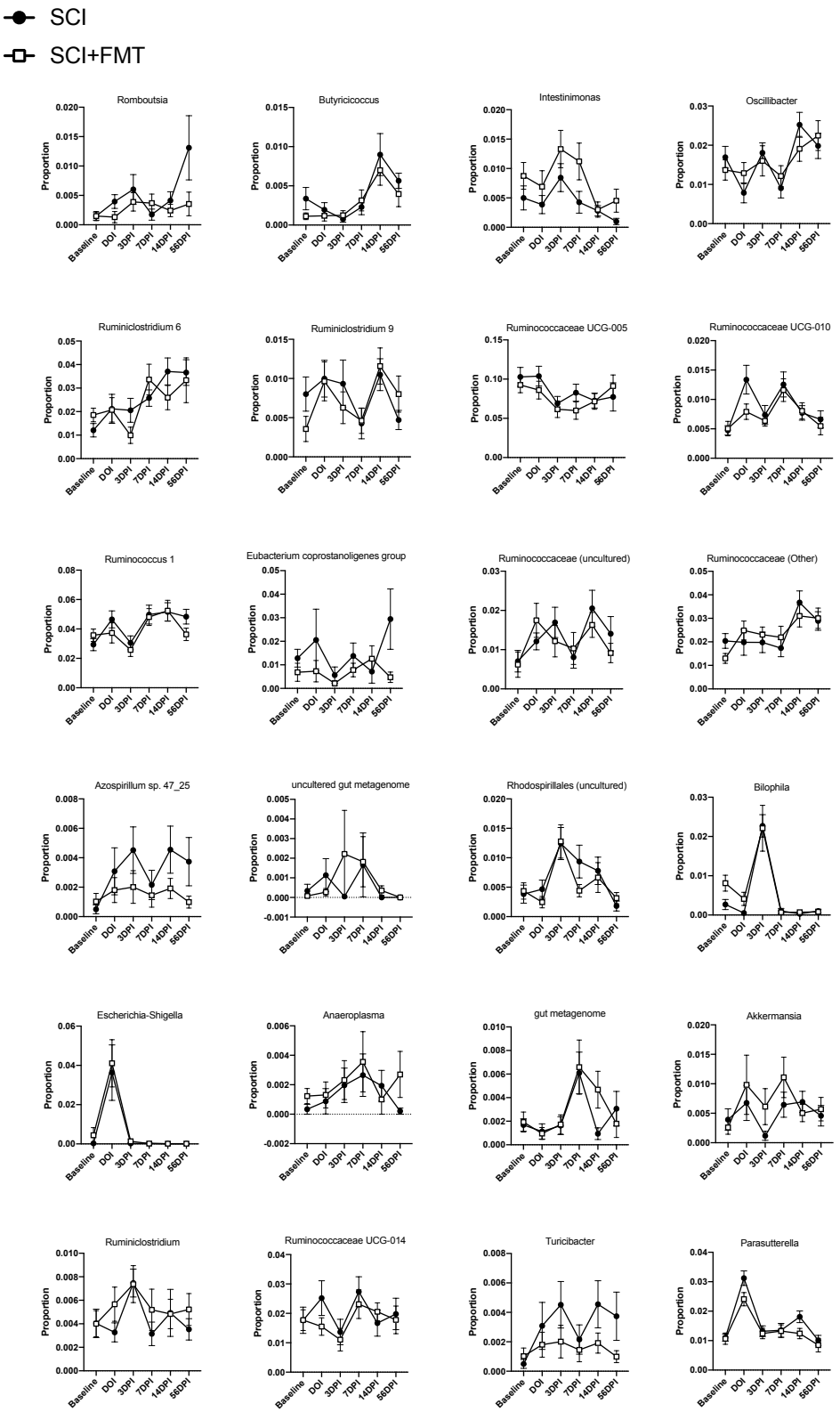

Figure B.2: Proportion of bacteria at the genus level over time following spinal cord injury. Error bars represent standard error mean.

Appendix C

Appendix

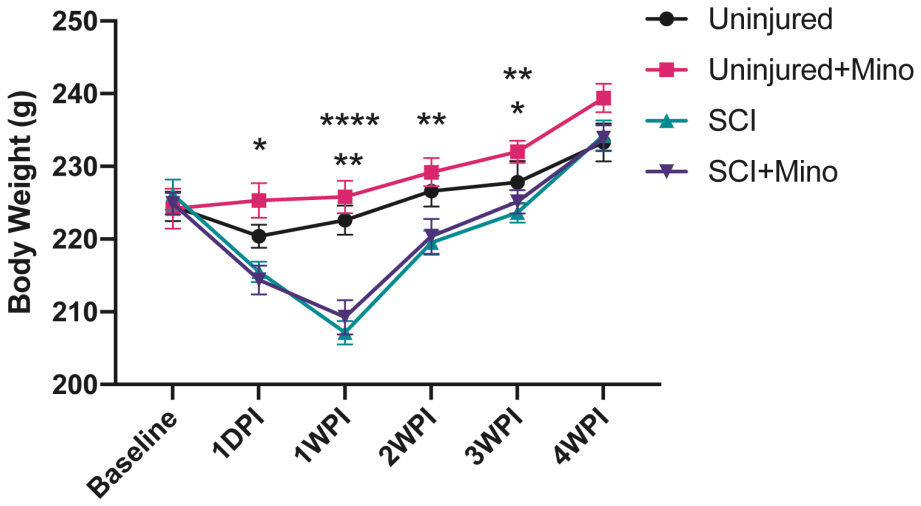

Figure C.1: Body weight was monitored at baseline and weekly following SCI. SCI rats lost weight relative to uninjured animals that remained significant until 4 weeks post-injury, particularly in comparison to uninjured + minocycline rats that consistently weighed slightly more than untreated rats. Error bars represent the standard error of the mean. *p¡0.05, **p¡0.01, ***p¡0.001, ****p¡0.0001

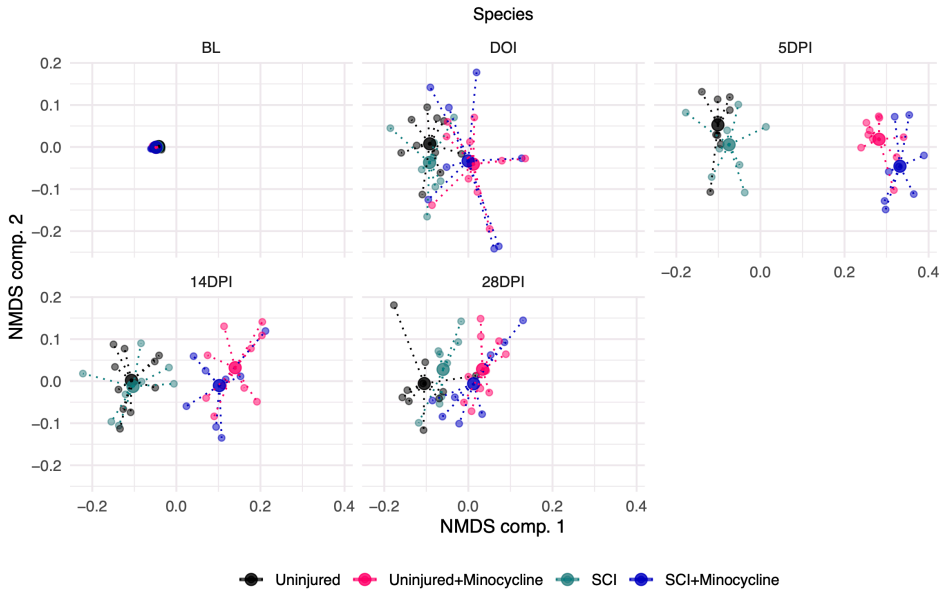

Figure C.2: Non-metric multidimensional scaling at the species level shows an effect of minocycline treatment on the overall microbiota composition at 5 and 14 days.

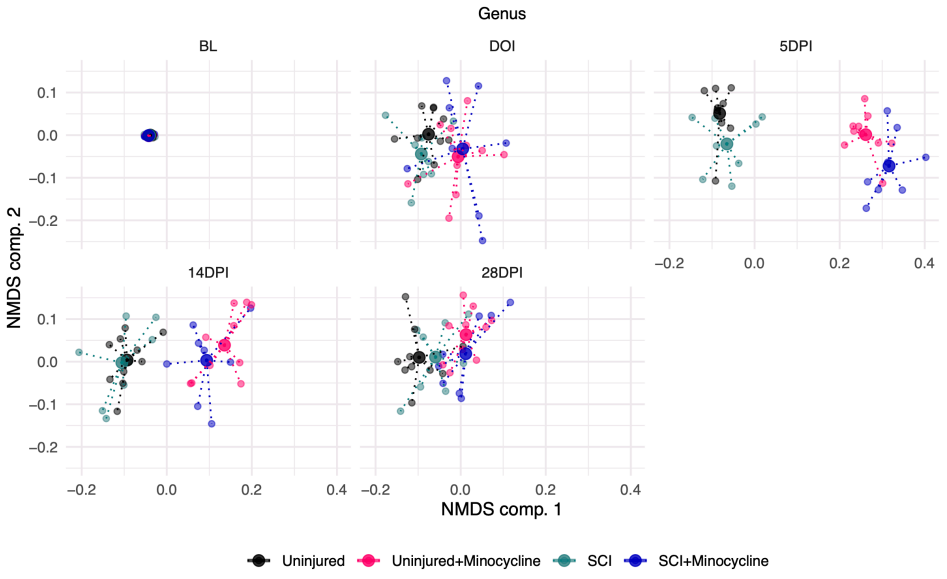

Figure C.3: Non-metric multidimensional scaling at the genus level shows an effect of minocycline treatment on the overall microbiota composition at 5 and 14 days.

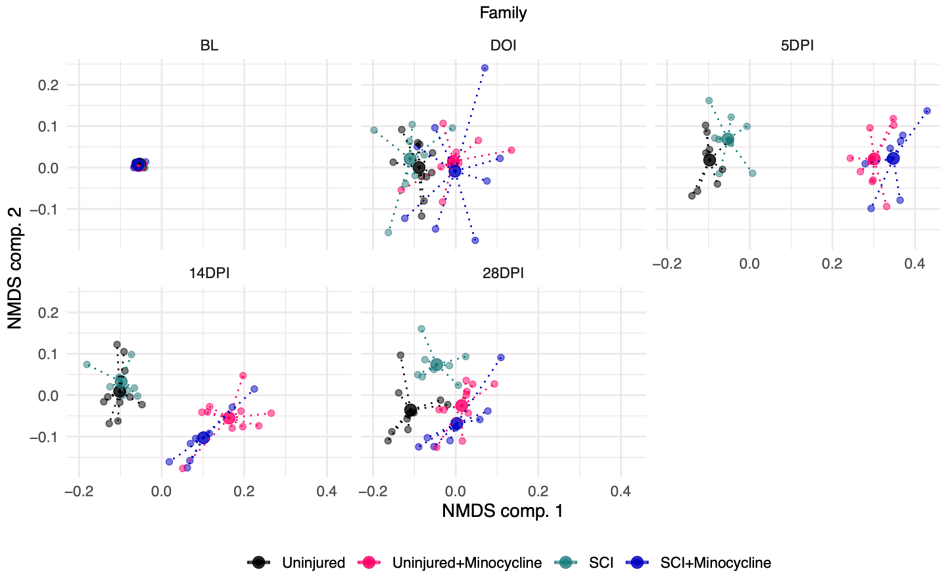

Figure C.4: Non-metric multidimensional scaling at the family level shows an effect of minocycline treatment on the overall microbiota composition at 5 and 14 days.

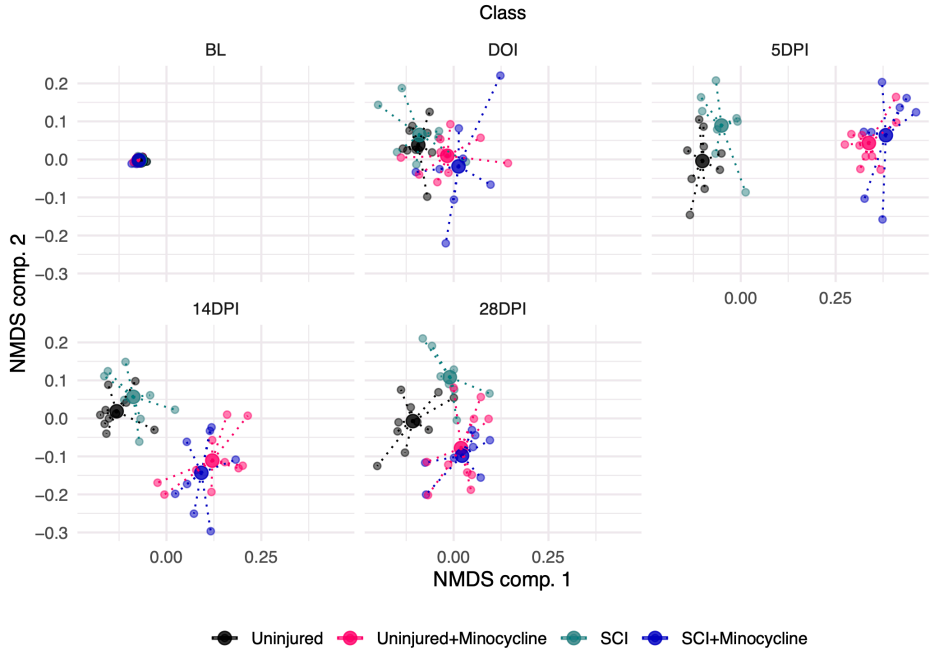

Figure C.5: Non-metric multidimensional scaling at the class level shows an effect of minocycline treatment on the overall microbiota composition at 5 and 14 days.

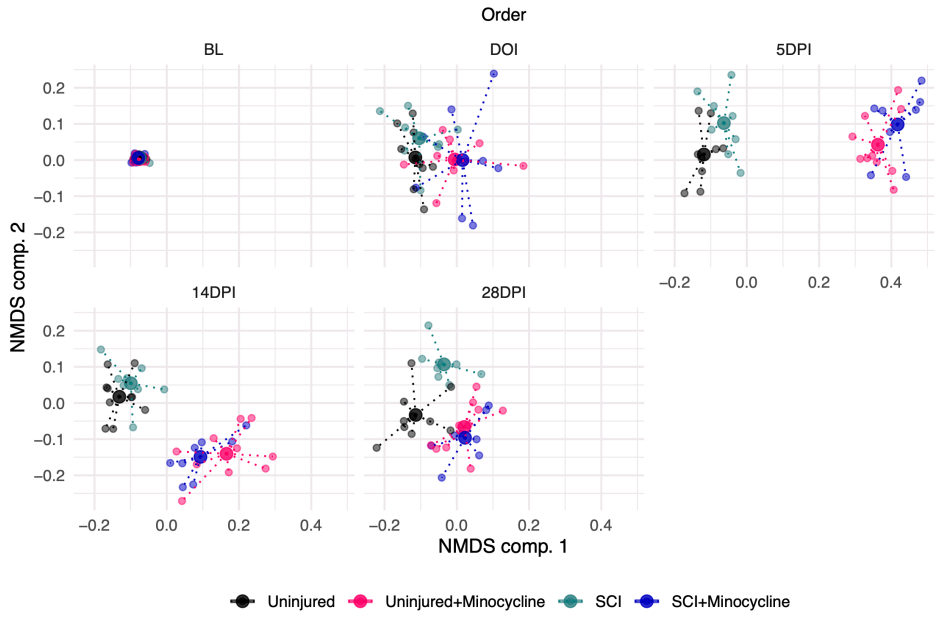

Figure C.6: Non-metric multidimensional scaling at the order level shows an effect of minocycline treatment on the overall microbiota composition at 5 and 14 days.