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Estrogen Nanoparticles in Spinal Cord Injury

April Ann Cox
Medical University of South Carolina

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by

April Ann Cox

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Approved by: _____

Chairman, Advisory Committee Narendra Banik

_____

Advisory Committee Deanna Adkins

_____

Chris Gregory

_____

James Krause

_____

Abhay Varma

Table of Contents

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“Let gratitude be the pillow on which you kneel to say your nightly prayer”
Maya Angelou

I’d like begin by acknowledging MUSC for giving me the opportunity to earn this degree. My path to acceptance into a Ph.D program was unconventional, and deemed by many to be an unlikely candidate. I am grateful for this institution placing confidence in me as an applicant worthy of the challenges that lay ahead. During the course of the program there have been many individuals that have helped me be successful during difficult times. In particular, I would like to acknowledge Dr. Krug and Dr. Adkins for their valuable time and insight. I would like to acknowledge Dr. Mulholland for his generosity in providing his lab and resources to train me in slice culture techniques. To my dissertation committee members, I thank you all for your thoughtful consideration and suggestions for improving my research. Within Dr. Banik’s lab I would like to thank Denise Matzelle for training on the surgical model as well as providing help in proofreading this dissertation. I would like to acknowledge Dr. Samantaray for being an endless source of advice and wisdom regarding all laboratory matters, large and small. Without her support, these data would not be possible. I would also like to acknowledge Dr. Knaryan for her many kind words and encouragement. I want to especially thank my mentor, Dr. Banik, who welcomed me into his lab and has provided me with the tools and guidance to be successful in attaining this degree. His infinite kindness and optimistic outlook cease to inspire me. Finally, I would like to thank all of my friends and family for patience and support along the way. My parents in particular, have provided me with endless encouragement during difficult times and celebration in successful times. To them I am eternally grateful.

LIST OF FIGURES

LIST OF ABBREVIATIONS

1. ESTROGEN (17-β ESTRADIOL)……E2
2. CEREBROSPINAL FLUID……CSF
3. CENTRAL NERVOUS SYSTEM……CNS
4. POLY-ETHYLENE GLYCOL……PEG
5. INTRAVENOUS……IV
6. GLIAL DERIVED NEUROTROPHIC FACTOR……GDNF
7. GLIAL FIBRILLARY ACIDIC PROTEIN……..GFAP
8. CYCLOOXYGENASE……..COX
9. NITRIC OXIDE……NO
10. INDUCIBLE NITRIC OXIDE SYNTHASE……..INOS
11. IONIZED CALCIUM BINDING ADAPTOR MOLECULE 1……IBA1
12. INTRAPERITONEAL……IP
13. WESTERN BLOT……WB
14. IMMUNOFLUORESCENCE……..IF
15. CHONDROITIN SULFATE PROTEOGLYCAN……CSPG
16. BASSO, BEATTIE, AND BRESHNAHAN SCALE……BBB
17. LUXOL FAST BLUE……LFB
18. BOVINE SERUM ALBUMIN……..BSA
19. PHOSPHATE BUFFERED SALINE……..…PBS
20. MAXIMUM CONCENTRATION……..CMAX
21. TIME AT WHICH CMAX IS OBSERVED……TMAX

ABSTRACT

Spinal cord injury affects more than 12,000 individuals in the US annually. Currently, no FDA approved drug treatment is available for the acute care of these individuals. Estrogen (E2), as a naturally occurring steroid hormone, is a potential answer. As a highly pleiotropic agent, E2 is known to have anti-inflammatory, anti-apoptotic, angiogenic, and neurotrophic properties, making it ideal for use in the treatment of a highly complex, multi-faceted condition such as SCI. Safety concerns around the use of high doses of E2 have limited its application clinically. To address these concerns, a nanoparticle delivery approach was used. Low doses of E2 (25 - 2.5 µg) were formulated in nanoparticles and embedded into gel patches that were placed directly on contused spinal cord tissue, allowing for focal delivery. Plasma E2 levels revealed exposure profiles distinct from traditional dose routes, potentially conferring increased efficacy. To further understand novel early, intermediate, and chronic neuroprotective mechanisms that E2 exerts, a 6hr, 48hr, and 28 day rat model of SCI was utilized. In the peracute (6hr) model, cytokine and chemokine profiling was conducted in plasma, tissue, and CSF which allowed for identification of biomarkers as well as novel targets of E2 regulation. Data revealed a panel of factors regulated by E2, including IL-6, MCP-1, and GRO-KC, etc. The intermediate model was used to test the hypothesis that E2 may drive neuroprotection through modulation of glial cell response. Data suggest E2 decreases various markers of glial cell reactivity and inflammation. Finally, a chronic model was used to assess the potential of a single low dose nanoparticle delivered E2 to drive improvements in locomotor function. The lowest single dose at which functional improvements may be seen, was found to be 5.0 µg of E2. These findings illustrate the ability of focally delivered nanoparticle formulated E2 to reduce plasma exposures to physiologically relevant levels while maintaining efficacy in a functional model. Taken together, these findings may help advance E2 into clinical evaluation at safer doses, thus providing a treatment options for SCI individuals.

CHAPTER ONE

SIGNIFICANCE AND REVIEW OF LITERATURE

SIGNIFICANCE

An estimated 150,000-200,000 worldwide cases of spinal cord injury (SCI) are diagnosed annually, with some 2.5 million people living chronically with paralysis secondary to SCI. The societal cost of medical and rehabilitative care for these individuals is immense with loss of mobility, independence, and productivity. The cost to the individual is immeasurable. This is a population in dire need of effective and safe therapeutics.

Currently, no FDA-approved drug exists for the treatment of acute SCI. Methylprednisolone, has been widely used, albeit off-label, in the care of acute SCI with limited efficacy and remains a controversial therapeutic that has yet to garner FDA approval (Hall 2003, Bracken 2012, Breslin and Agrawal 2012, Lammertse 2013, Schroeder, Kwon et al. 2014).

In the last thirty years, the field of neurotrauma research has seen an abundance of novel and promising pharmaco-therapeutic interventions. The use of single-agent targeted therapeutics in an enormously complex condition that includes numerous pathophysiologies, such as inflammation, oxidative stress, ischemia, glial scarring, etc., has not proven efficacious. Thus, evaluation of broadly acting drug candidates, including the steroid hormone estrogen, is justified.

Estrogen (E2) is a highly pleiotropic agent that has been reported to activate transcription of some 137 E2 regulated genes (Lin, Strom et al. 2004), and it is known to have anti-inflammatory , anti-oxidant, anti-apoptotic, and

neurotrophic properties (Sribnick, Ray et al. 2004, Cuzzocrea, Genovese et al. 2008, Kumar, Lata et al. 2010, Samantaray, Sribnick et al. 2010, Kipp, Berger et al. 2012, Evsen, Ozler et al. 2013, Elkabes and Nicot 2014). E2 has exhibited a neuroprotective effect in SCI models (Yune, Kim et al. 2004, Cuzzocrea, Genovese et al. 2008, Ritz and Hausmann 2008, Kachadroka, Hall et al. 2010, Sribnick, Samantaray et al. 2010, Samantaray, Smith et al. 2011, Lee, Choi et al. 2012, Siriphorn, Dunham et al. 2012, Mosquera, Colon et al. 2014). Additionally, E2 has shown a neuroprotective effect in traumatic brain injury models (Soustiel, Palzur et al. 2005, Gatson, Liu et al. 2012, Zlotnik, Leibowitz et al. 2012, Asl, Khaksari et al. 2013, Day, Floyd et al. 2013, Zhang, Hu et al. 2013) and stroke models (Merchenthaler, Dellovade et al. 2003, Lebesgue, Chevaleyre et al. 2009, Zhang, Raz et al. 2009, Ardelt, Carpenter et al. 2012, Perez-Alvarez, Maza Mdel et al. 2012), suggesting E2 warrants clinical evaluation in the setting of neurotrauma. However, the translation of E2 from preclinical models into clinical practice with the FDA-approved drug Premarin (a cocktail of equine conjugated estrogens also containing lesser amounts of androgens and progestogens) has significant safety concerns (LaCroix, Chlebowski et al. 2011).

Short term E2 treatment used for contraception has been reported to be significantly associated with increased risk of venous thromboembolism, with the greatest risk occurring in less than a year of treatment and at the highest dose of E2 (Lidegaard, Edstrom et al. 2002). These safety concerns with traditional systemic dosing (either tablet or intravenous injection) of E2 pose a hurdle for

movement into SCI individuals. A more sophisticated form of drug delivery, such as nanoparticles that allow for site directed delivery of a drug, may provide for a significant advancement in the drug delivery field, thereby reducing safety concerns of E2.

Nanoparticle drug delivery systems have been shown to improve the therapeutic index of drugs (Zhang, Gu et al. 2008). By focally delivering E2 to the injured spinal cord directly, maximal efficacy may be achieved while keeping systemic levels of E2 in a physiological range. This technique may effectively increase the therapeutic window of E2.

Thus, in conclusion, by pairing the steroid hormone E2 with a nanoparticle drug delivery technique the potential exists for the development of an effective and safe therapeutic for use in SCI individuals.

Hypothesis: *Focal delivery of estrogen via nanoparticles will maximize therapeutic potential through enhanced bioavailability with increased functional recovery..*

Specific Aim 1: Characterize E2 release from nanoparticles and assess anti-inflammatory potential of E2 in a peracute (6hr) rat SCI model

Specific Aim 2: Evaluate E2 nanoparticle neuroprotective effects in acute (48hr) rat SCI model

Specific Aim 3: Assess functional recovery in chronic model (4 week) of rat SCI.

The exploration of these specific aims will add to the knowledge of E2 as a neuroprotectant in SCI and the capacity of nanoparticles to focally deliver E2 to the injured spinal cord by:

RESULT 1: E2 was shown to be released in a time dependent manner from nanoparticles in both an *in vitro* and *in vivo* model. E2 treatment acted as a rapid anti-inflammatory in a 6 hr spinal cord injury model by significantly decreasing various cytokines and chemokines in plasma, spinal cord tissue, and cerebrospinal fluid (CSF).

RESULT 2: Nanoparticle E2 exerted neuroprotective effects in a 48hr model of SCI by decreasing apoptosis, glial reactivity, and inflammation, and by increasing growth factor expression.

RESULT 3: Nanoparticle E2 resulted in significant improvement in various parameters of gait in a 28 day chronic model. These findings were seen in tandem with improved bladder function and enhanced spinal cord tissue preservation.

LITERATURE REVIEW

Introduction

The first known documented case of SCI has been dated to 2500 years BCE. During that era, SCI was considered “an ailment not to be treated” (Donovan 2007). Tremendous advances have been made in the field since this ancient beginning; however, a panacea for spinal cord injury remains elusive. Extensive bench research has lead to a better understanding of the pathophysiology of SCI, thereby uncovering potential therapeutic targets. Novel therapeutics showing promise in preclinical models of SCI have been translated into clinical trials. This review will provide a brief historical overview, followed by a summary of the mechanisms and pharmacological therapeutics studied over the past decade. Finally, a focused review of the E2 spinal cord injury literature will be provided as a background for the work presented herein.

Historical Overview:

The concept that SCI was an “ailment not to be treated” reflects the long-lasting belief that the catastrophic nature of the injury and lack of regenerative capacity of the spinal cord made the injury medically futile to treat. Over the course of the last 4,000+ years, treatment for SCI was centered on surgical interventions to stabilize and decompress the spine (Donovan 2007). Only in the second half of the 20th century did scientists begin using pharmacologic interventions.

The first randomized clinical trial investigating a pharmaceutical agent in SCI was initiated in 1979 when the National Acute Spinal Cord Injury Study I (NASCIS) investigated the efficacy of the synthetic glucocorticoid steroid methylprednisolone (a derivative of prednisone, FDA approved 1955) in SCI (Bracken 1992). Glucocorticoid steroids are potent anti-inflammatory and immunosuppressant drugs, and in the first known publication investigating steroids in spinal cord injury, researchers found that treatment with a high dose of glucocorticoid steroid (dexamethasone) significantly improved the functional recovery in a dog model of SCI (Ducker and Hamit 1969). These preclinical findings, along with findings from many additional studies, were translated into this seminal clinical trial in 1979. NASCIS I was followed by two subsequent studies, NASCIS II and NASCIS III, both investigating doses and timing of methylprednisolone treatment after SCI (Bracken, Collins et al. 1984, Bracken, Shepard et al. 1990, Bracken, Shepard et al. 1997). The reported findings of these trials have led to wide off-label use of methylprednisolone in acute SCI. However, these studies have fallen under intense scrutiny and have not resulted in FDA approval of methylprednisolone treatment in acute SCI.

The largest randomized clinical trial ever conducted in SCI investigated the efficacy of monosialotetrahexosylganglioside sodium (GM-1), proprietary name Sygen. GM-1 is a ganglioside (complex glycolipid predominant in plasma membrane) that through unknown mechanisms can elicit neuroprotective effects in SCI by promoting neural outgrowth, repair and regeneration (Geisler, Dorsey

et al. 1991). As with the NASCIS trials, the potential effectiveness of this therapeutic is controversial. The GM-1 study failed to report statistically significant efficacy in a clinical setting (Geisler, Coleman et al. 2001). Due to lack of efficacy, the current guidelines published by the American Association of Neurological Surgeons (2013), do not recommend treatment with either corticosteroids or GM-1 ganglioside.

While progress certainly has been made through clinical trials over the last 30 years, the need for effective pharmacological intervention in acute SCI remains. The drug that has been most extensively clinically evaluated, methylprednisolone, functions primarily as an immunosuppressant and anti-inflammatory in the setting of acute ACI. Since these early days of SCI research and clinical testing, inflammation is now accepted as only one of many pathophysiological mechanisms in SCI. Animal models have demonstrated that SCI is marked by a primary injury, that results from the mechanical trauma, followed by a more insidious phase referred to as secondary injury (Tator and Fehlings 1991). The secondary injury cascade begins within seconds of the primary injury and results in further tissue damage, disruption of blood vessels, ischemia, cell death, inflammation, Wallerian degeneration and glial scarring. Considerable progress has been made to unravel the complex molecular signals that drive secondary injury. These mechanisms, along with their cognate therapeutic approaches, will be discussed in the following three broad categories: acute, intermediate, and chronic mechanisms of secondary injury.

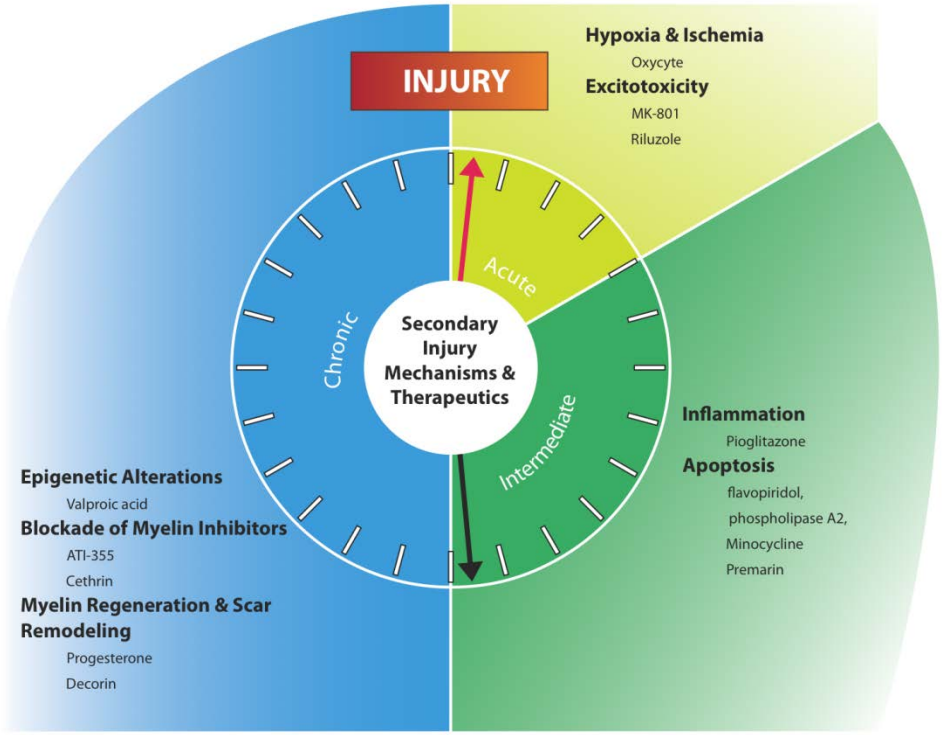


Figure 1. *Metabolic Brain Disease,* Cox et al. 2014

I. Acute Mechanisms

Hypoxia & Ischemia: One of the first pathophysiological changes to occur immediately following a traumatic SCI is disruption of blood flow with resultant hypoxia to the injured tissue. The mechanical trauma results in disruption of cell membrane, vasospasm, hemorrhage, and loss of microvasculature necessary to supply spinal cord tissue with oxygen and other vital nutrients. The loss of both oxygen and nutrients to the spinal cord immediately following injury triggers the subsequent secondary injury with influx of Ca^{2+}, calpain and caspase activation, glutamate excitotoxicity, and inflammation. Hypoxia, therefore, contributes to the expansion of the primary lesion (Tator and Fehlings 1991, Tator and Koyanagi

1997). Pharmacological agents that have the capacity to restore oxygen and nutrients to the damaged region of the spinal cord have been an area of research interest. One such compound that has been studied is Oxycyte (a new generation perfluorocarbon), an oxygen carrier that can be intravenously injected to increase oxygen availability in damaged tissue. Oxycyte treatment in a rat model of moderate-severe contusion spinal cord injury significantly increased oxygen saturation and reduced apoptotic cell death with better tissue and myelin preservation, respectively (Schroeder, Highsmith et al. 2008, Yacoub, Hajec et al. 2013). Additionally, Oxycyte treatment was shown to reduce spinal cord lesion size in a swine model of decompression sickness (Mahon, Auker et al. 2013). Oxycyte may be suitable as an adjunctive therapy in the treatment of SCI; Oxycyte treatment ideally should begin at the earliest possible time point following an acute injury to lessen the detrimental cascade triggered by hypoxia.

The mechanical trauma from the initial injury will cause massive disruption in both macro and micro vasculature that will disrupt blood flow to the spinal cord and result in ischemia. Numerous studies have reported that ischemia contributes to the subsequent neuronal degeneration and loss of motor function in SCI (Anthes, Theriault et al. 1995, Tator and Koyanagi 1997, Muradov and Hagg 2013). Ischemia, unlike SCI, can be studied as a single entity to provide some enlightenment about the contribution it plays in the complex network of mechanisms driving secondary injury. Researchers have attempted to determine what role ischemia plays in SCI using a model of focal ischemia in

the spinal cord to investigate effects on axonal degeneration. Focal ischemia alone has been reported to cause both loss of sensory axons and death of oligodendrocytes (Muradov, Ewan et al. 2013); these findings suggest that restoration of blood flow should be of utmost importance in the treatment of SCI.

In addition to triggering cell death, ischemic injury also activates microglia, the resident macrophages of the CNS. Inhibition of microglial activation has been shown to elicit neuroprotective effects (Cho, Cheong et al. 2011). Activation of the toll-like receptor 4 on microglia may be a mechanism for microglial activation in the setting of ischemic injury (rodent aortic occlusion model) (Bell, Puskas et al. 2013). As research continues to further elucidate the exact signaling mechanisms of ischemia that trigger the activation of microglia, additional pharmacological targets may be identified. Activated microglia are primary drivers of both innate and adaptive immune response through the release of proinflammatory cytokines and chemokines (Schomberg and Olson 2012) and will be discussed in more detail in the inflammation section of this review.

Excitotoxicity: Excitotoxicity is a pathological state in which high levels of the excitatory neurotransmitter glutamate results in toxicity or death to neurons (Doble 1999). Immediately following spinal cord injury, the levels of glutamate can rise to excitotoxic threshold levels (Liu, Thangnipon et al. 1991). Glutamate binds one of three receptors, N-Methyl D-Aspartate (NMDA), Alpha-amino-3-hydroxy-5-methylisoxazoleproprionate (AMPA), or Kainate; the binding of

glutamate will modulate Ca^{2+} influx into the cell thereby regulating Ca^{2+} homeostasis and downstream signaling cascades (Mehta, Prabhakar et al. 2013). The potential critical role the NMDA receptor plays in mediating calcium influx, has made it an attractive pharmacological target for many years now. The NMDA receptor antagonist, MK-801, was reported to attenuate numerous inflammatory markers in a mouse model of SCI (Esposito, Paterniti et al. 2011). Although MK-801 cannot be used in SCI individuals due to toxicity, an opportunity exists for the development of a safe NMDA receptor antagonist. Riluzole (a sodium channel blocker / glutamate receptor modulator), approved for the treatment of amyotrophic lateral sclerosis, has been shown in a preclinical rodent study to act as a neuroprotectant through modulation of excitotoxicity (Springer, Azbill et al. 1997, Schwartz and Fehlings 2001, Wu, Satkunendrarajah et al. 2013) . A phase I safety trial of Riluzole in acute cervical spinal cord injury individuals reported a rate of complication with drug use similar to that of matched individuals, as well as an enhanced improvement in motor score with drug- treated individuals compared to matched individuals (Grossman, Fehlings et al. 2013). Follow-up placebo controlled trials evaluating Riluzole in SCI individuals are anticipated.

Downstream effectors of excitotoxicity, such as the activation of intracellular proteases, provide additional targets for therapeutic intervention. Calpain, a Ca^{2+} activated cysteine protease, has emerged as a potential target in SCI. The role of calpain in spinal cord tissue degeneration has been discussed

in the scientific literature for over 30 years (Banik, Powers et al. 1980, Banik, Hogan et al. 1982). Mechanistically, the role of calpain in spinal cord tissue degeneration has been further elucidated over the past 10 years. Studies have shown that apoptosis following SCI requires *de novo* protein synthesis and can be blocked with a pharmacological inhibitor of calpain (Ray, Matzelle et al. 2001, Ray, Hogan et al. 2003). Rodent studies have shown an improvement in both tissue and motor function recovery after treatment with various synthetic calpain inhibitors (Arataki, Tomizawa et al. 2005, Sribnick, Matzelle et al. 2007, Akdemir, Ucankale et al. 2008, Yu, Joshi et al. 2008). Calpain inhibition by the endogenous inhibitor, calpastatin, has also been shown to be involved in Wallerian degeneration in an optic nerve transection model (Ma, Ferguson et al. 2013). These findings suggest that both early and prolonged inhibiton of calpain may provide protection against both apoptosis and Wallerian degeneration. Over the years, a number of calpain inhibitors have been investigated (leupeptin, calpeptin, E64D) (Ray, Wilford et al. 1999, Momeni and Kanje 2006, Tsubokawa, Solaroglu et al. 2006); however, difficulties with drug safety and solubility have precluded advancement of these compounds into the clinic. Currently, researchers have an intense interest in the development of a targeted safe therapeutic to inhibit pathological calpain activation.

Melatonin, a naturally occurring hormone, has also been reported to show beneficial effects in SCI, potentially through mechanisms modulating calpain activation (Samantaray, Sribnick et al. 2008). Numerous rodent models of SCI

have shown increased neuroprotection with melatonin treatment (Fujimoto, Nakamura et al. 2000, Esposito, Genovese et al. 2009, Park, Lee et al. 2010, Park, Lee et al. 2012, Schiaveto-de-Souza, da-Silva et al. 2013). Melatonin is a pleiotropic agent, and thus may exert neuroprotective effects through its anti-oxidant, anti-nitrosative, and immunomodulatory mechanisms (Samantaray, Das et al. 2009). The abundance of preclinical studies reporting neuroprotection with melatonin treatment as well as melatonin's high safety profile make melatonin a potential candidate for clinical trial investigation as either a stand-alone agent or as an adjunctive therapeutic in acute SCI treatment.

II. Intermediate Mechanisms

Inflammation: The acute mechanisms of hypoxia, ischemia, and excitotoxicity give rise to an inflammatory response that contributes to the expansion of the secondary injury. In rodent models, activation of resident astrocytes and microglia can be seen as early as 2 hours following injury and persist up to 6 months (Gwak, Kang et al. 2012). Human studies have shown that the first peripheral immune cell to enter the spinal cord lesion site is the neutrophil, which arrives as early as 4 hours post injury; activated microglia were found at 1 day post injury, and macrophages were seen by day 5 (Fleming, Norenberg et al. 2006). In animal models, blockade of neutrophils has been found to decrease markers of inflammation following SCI (Chatzipanteli, Yanagawa et al. 2000, Gris, Marsh et al. 2004). These findings suggest that, mechanistically, neutrophils may contribute to the inflammation seen post SCI.

Significant advances in the understanding of the complex role of macrophages in SCI have revealed macrophages play dual roles as both pro- and anti-inflammatory mediators. Results of rodent studies indicate that altering the ratio of M1/M2 macrophages in favor of the anti-inflammatory M2 may promote regenerative growth (Kigerl, Gensel et al. 2009, Busch, Hamilton et al. 2011) The complexity of macrophage signaling and therapeutic potential are beyond the scope of the current review; however, signaling is detailed in two recent review articles (David and Kroner 2011, Ren and Young 2013). While modulating the types of cells present in the setting of acute neurotrauma may represent an avenue for therapeutic intervention, another important approach is regulation of cell signaling.

Inflammation in the central nervous system is thought to be regulated by the nuclear transcription factor, nuclear factor kappa β (NF-Kβ). Blockade of NF-Kβ, thereby, may be a therapeutic approach for decreasing inflammation. A transgenic mouse model of SCI, where NF-Kβ is selectively inhibited in astrocytes, has been reported to show decreased inflammation as well as increased axonal sprouting (Brambilla, Bracchi-Ricard et al. 2005, Brambilla, Hurtado et al. 2009). Regulation of inflammation via modulation of gene transcription has also been tested with Thiazolidinediones (TZDs), synthetic agonists of the ligand-activated transcription factor peroxisome proliferator-activated receptor-gamma (PPARγ). One such TZD, pioglitazone, has been tested in a rat model of SCI as a potential neuroprotectant. The authors reported

a significant decrease in inflammatory gene expression with enhanced motor function recovery in a rat SCI model that were only seen when drug treatment began within 2 hours of injury induction (Park, Yi et al. 2007). These findings highlight the critical role early inflammation may play in SCI.

Apoptosis: Loss of cells in the spinal cord following injury may be attributable to both apoptosis and necrosis. Necrosis, caused by mechanical tissue damage, is considered irreversible. In contrast, apoptosis is regulated through cell signaling and may be triggered by a variety of external or internal stimuli, thereby becoming an attractive candidate for pharmacological modulation. Cellular stressors triggering release of pro-apoptotic signaling molecules from the mitochondria and activation of death receptors are the two broad independent pathways through which apoptosis is triggered (Green 1998). The last 10 years of research into mechanisms of apoptosis specific to SCI have yielded many promising therapeutic targets. One potential modulator of apoptosis in SCI is cell cycle activation. The cell cycle inhibitor, flavopiridol, was shown to reduce both neuronal and oligodendrocyte apoptosis in a rat model of severe SCI (Byrnes, Stoica et al. 2007). Another potential modulator is Phospholipase A2 (PLA2); a lipolytic enzyme thought to contribute to neurodegeneration in secondary injury, which has recently been implicated in the pathogenesis of SCI through its ability to induce neuronal death when injected into normal spinal cord tissue (Liu, Zhang et al. 2006). An animal study has shown that PLA2 is upregulated following

injury *in vivo* and blockade of PLA2 *in vitro* protects against oligodendrocyte cell death (Titsworth, Cheng et al. 2009).

An additional promising anti-apoptotic therapeutic is the antibiotic Minocycline, which has been shown in numerous animal models to decrease apoptosis (Stirling, Khodarahmi et al. 2004, Takeda, Kawaguchi et al. 2011, Watanabe, Kawaguchi et al. 2012, Sonmez, Kabatas et al. 2013). Treatment with Minocycline has also shown functional improvement in a number of preclinical models (Wells, Hurlbert et al. 2003, Teng, Choi et al. 2004). Based on these preclinical studies, Minocycline was evaluated for safety in a placebo controlled phase II clinical trial in acute SCI individuals. The authors report that the drug regimen was safe and well tolerated and suggested improved motor function in individuals with cervical injuries (Casha, Zygun et al. 2012). The positive results of the minocycline phase II clinical trial warrant further investigation of drug efficacy in a phase III multi-center placebo controlled trial.

Hypothermia (both epidural and systemic) has been found to decrease apoptosis in a rat model of SCI (Ok, Kim et al. 2012). Two clinical trials investigating the safety and potential benefit of modest hypothermia in acute cervical spinal cord injury reported promising results for both safety and potential neuroprotection (Levi, Casella et al. 2010, Dididze, Green et al. 2013). As hypothermia treatment is posited to potentially provide an early adjunctive therapeutic in the treatment of SCI, additional clinical studies investigating hypothermia are warranted.

III. Chronic Mechanisms

***Epigenetic Alterations*:** Over the last 10 years, the field of epigenetics has expanded to include research into the mechanisms that may limit the central nervous system's ability to regenerate. More specifically, researchers have speculated that the mature chromatin status of the cells comprising the spinal cord may be blocking these cells from reactivating the developmental programs necessary to successfully rebuild the damaged tissue (York, Petit et al. 2013). DNA methylation, chromatin structure, and histone acetylation status are the broad categories of epigenetic modifications that drive changes in gene expression. Histones, the spool-like proteins that DNA winds around to achieve the highly condensed state in chromatin, can be modified through acetylation. The acetylation status of a histone will then drive gene silencing or transcription. Valproic acid (VPA), a histone deacetylase (HDAC) inhibitor, has been found to reduce gliosis and increase production of both brain and glial-derived neurotrophic factors in a rodent model of SCI (Abdanipour, Schluesener et al. 2012). Another study in rodent SCI has reported that treatment with VPA can decrease gliosis and improve functional outcomes in open-field behavioral assays (Lu, Wang et al. 2013). The field of epigenetic regulation in both spinal cord injury and, more broadly, in neuroregeneration is arguably still in its infancy. A tremendous promise exists in the approach of selectively regulating gene expression to simultaneously decrease degenerative processes and increase regenerative processes that will ultimately drive restoration of damaged nervous

tissue. Hopefully, as this field matures an emergence of new therapeutics will provide the tools necessary to achieve these goals.

Blockade of Myelin Inhibitors: Regeneration of axons following injury is inhibited by a number of molecules, such as NoGo, myelin associated glycoprotein (MAG), and oligodendrocyte myelin glycoprotein (OMgP) (Hunt, Coffin et al. 2002). The existence of these inhibitors and their ability to block regeneration have been known since the late 1980s (Schwab, Kapfhammer et al. 1993). Early publications in the field of axon regeneration have shown that blockade of NoGo with the IN-1 antibody promoted regrowth after injury in animal models (Schnell and Schwab 1990, Brosamle, Huber et al. 2000). The exact contribution of these molecules to successful axon regeneration *in vivo,* however, is not yet clearly defined, as a recent publication investigating the role of these three inhibitors demonstrates. The authors of this publication exhibit this by using mutant transgenic mouse models that block of all three myelin inhibitors (NoGo, MAG and OMgP) and when compared to blockade of any single inhibitor it failed to show additive effects (Lee, Geoffroy et al. 2010). These authors state that while “MAG, NoGo, and OMgp may modulate axon sprouting, they do not play a central role in CNS axon regeneration failure” (Lee, Geoffroy et al. 2010). Regardless of the exact role each of these inhibitors may play in spinal cord regeneration, the wealth of positive preclinical findings with pharmacological blockade has resulted in two agents moving into clinical evaluation. The two agents, ATI-355 (humanized anti- Nogo antibody, Novartis) and Cethrin

(recombinant protein RHO GTPase antagonist, BioAxone BioSciences) are being clinically evaluated for their potential to modulate axon regeneration in SCI. Results from the ATI-355 trials have not yet been released, although the trial was registered as complete in November, 2013. Results from the phase I/IIa clinical trial reported Cethrin to be safe and tolerable in acute SCI individuals, and suggested that Cethrin enhanced motor function recovery (Fehlings, Theodore et al. 2011). Cethrin works by inhibiting the RHO pathway, the final common signaling pathway of the myelin inhibitors. To date, Cethrin is the only drug to attain orphan drug status from the FDA (2005) in the treatment of acute cervical and thoracic spinal injuries. The next step in the development path of Cethrin will be a placebo-controlled efficacy trial.

Myelin Regeneration & Scar Remodeling*:* Progesterone, a naturally occurring steroid hormone, has emerged as a potential therapeutic in SCI through findings that suggest it may serve as both a neuroprotectant and pro-myelinating agent. Results of a study conducted in a rat SCI model indicated that treatment with progesterone resulted in sparing of white matter tissue with concomitant improvement in motor function (Thomas, Nockels et al. 1999). A mechanistic study examining the effects of progesterone in a rat model of SCI reported that progesterone treatment restored myelin levels and increased the density of oligodendrocyte progenitor cells, which are potentially responsible for remyelination (De Nicola, Gonzalez et al. 2006). An additional study reported that progesterone may work by suppressing gliosis at the early stage of SCI

while promoting oligodendrocyte differentiation and remyelination at the later stages (Labombarda, Gonzalez et al. 2011).

Glial scarring and wound cavitation are thought to be major inhibitors of spinal cord regeneration in the chronic phase. One of the major proteins involved in the glial scar is chondroitin sulfate proteoglycan, which is produced by activated glia, and serves to block regrowth of axons across the injury (Cregg, DePaul et al. 2014). The protein tyrosine phosphatase σ has been identified as a key receptor in the process of growth cone dystrophy, and antagonism of this receptor is reported to lead to increased regeneration in injured rats (Lang, Cregg et al. 2015). Another recent approach to enhance regeneration has been to use microtubule stabilizing drugs. Epothilone B, a FDA approved microtubule stabilizing drug that is BBB penetrant, was found to activate microtubule polymerization and growth through the inhibitory environment of the glial scar, resulting in enhanced motor function recovery (Ruschel, Hellal et al. 2015). Recently, a pan Tgfβ 1/2 antagonist, Decorin, was shown to decrease wound cavitation and scar tissue mass through suppression of inflammatory fibrosis (Ahmed, Bansal et al. 2013). The authors of this study also reported that Decorin treatment has potential for dissolution of mature scars through induction of matrix metalloproteinases with subsequent axonal regeneration. The concept that existing scar tissue may be remodeled to drive regeneration is an exciting one, as it would potentially offer a treatment option to individuals chronically living with paralysis due to SCI.

ESTROGEN

Background

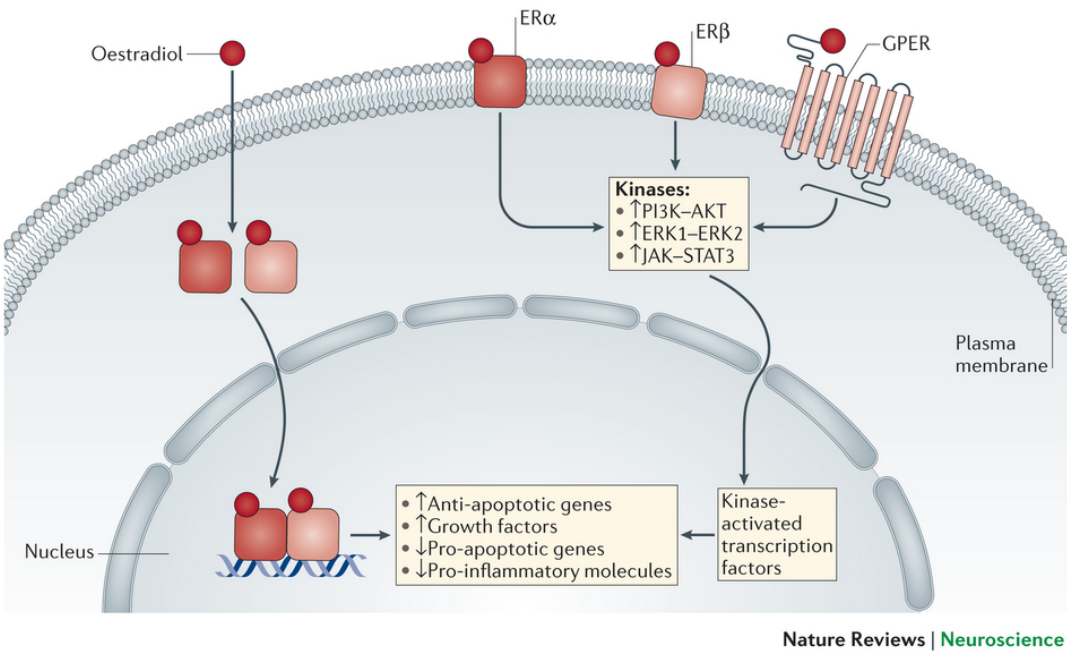
Estrogen (E2) is a widely studied hormone that has over 225,000 PubMed citations. The bulk of these citations are focused on E2 in reproduction, development, and cancer, however. It wasn't until 1988, that a paper was published suggesting E2 may play a neurotrophic role in the injured CNS (Jones 1988). E2 was first reported to attenuate neuronal injury experimentally in an *in vitro* model of hemorrhage, trauma, and ischemia, wherein E2 decreased neuronal loss (Regan and Guo 1997). E2 was then used in a peripheral nerve injury model and found to enhance sciatic nerve regeneration following crush injury (Islamov, Hendricks et al. 2002). The first *in vivo* report of E2 in spinal cord injury was made in 2003, where the authors report that a single 4mg/kg intravenous dose of E2 reduced infiltration of macrophages/microglia into the injured spinal cord (Sribnick, Wingrave et al. 2003). Since then more than 80 papers have been published in the field investigating the effects of E2 administration in various models of spinal cord injury. A recent review article has summarized all the *in vivo* E2 experiments conducted up to 2014 and their primary findings (Elkabes and Nicot 2014).

Mechanisms of Neuroprotection

E2 binds to its cognate steroid hormone receptors ERα and ERβ, translocates to the nucleus, binds to estrogen response elements alongside

additional co-factors, and is responsible for either directly or indirectly activating transcription of some 137 estrogen regulated genes (Lin, Strom et al. 2004). This has been the classical understanding of the steroid hormone receptor signaling pathway; however research has shown that E2 also exerts its effects through an additional receptor as well as non-receptor mediated mechanisms.

E2 binds the G-protein coupled receptor 30 (GPR30), and it has been shown that this signaling pathway can mediate apoptosis in neurons of the injured spinal cord (Liu, Han et al. 2011, Chen, Hu et al. 2015). Activation of ERα, ERβ, and GPR30 result in activation of the extracellular signal-regulated kinase 1 (ERK1) and the phosphoinositide 3-kinase (PI3K) signaling pathways, as recently reviewed and illustrated in the figure below (Arevalo, Azcoitia et al. 2015).



Oestradiol enhances the expression of anti-apoptotic genes and neuroprotective growth factors and represses the expression of pro-apoptotic genes and pro-inflammatory molecules in the brain. This action of the hormone is mediated by two redundant mechanisms: the binding to the intracellular oestrogen receptors (ERs) ERα and ERβ and subsequent regulation of ER-mediated transcription, and the binding to membrane receptors (ERα, ERβ and G protein-coupled ER (GPER)) and subsequent regulation of kinase-activated transcription factors by the activation of phosphoinositide 3-kinase (PI3K)–AKT, extracellular signal-regulated kinase 1 (ERK1)–ERK2, and Janus kinase (JAK)–signal transducer and activator of transcription 3 (STAT3) signalling.

Figure 2. E2 signaling cascades (courtesy of Arevalo et al. The Neuroprotective actions of oestradiol and oestrogen receptors. *Nature Reviews Neuroscience, 2015)*

The signaling cascades diagrammed (Figure 2) are activated in numerous cell types within the CNS. All three major cell types: neurons, astrocytes, and oligodendrocytes have been shown to express the enzymes necessary for production of E2 (Garcia-Ovejero, Azcoitia et al. 2005). E2 receptors ERα, ERβ, and GPR30, are also widely expressed (Spence, Wisdom et al. 2013). Finally, E2 itself may function as an antioxidant directly through its properties as a hydrophobic phenolic type molecule (Moosmann and Behl 1999). Given the potential for E2 to elicit various cell signaling pathways in a variety of cell types, there is enormous potential for E2 to drive neuroprotection through varied mechanisms in an injury setting.

E2, more specifically, may serve as a neuroprotectant in part due to its action as an anti-apoptotic along with its actions as an anti-inflammatory, antioxidant, and as a promoter of angiogenesis. Numerous studies conducted in a rat SCI model have shown a reduction in apoptosis and/or improved locomotor function recovery with E2 (or Premarin) treatment (Chen, Yeh et al. 2010, Sribnick, Samantaray et al. 2010, Samantaray, Smith et al. 2011, Siriphorn, Dunham et al. 2012). A recent study reported that E2 treatment protected against oligodendrocyte cell death mediated via the RhoA-JNK3 pathway in a rat model of SCI (Lee, Choi et al. 2012). Preservation of oligodendrocytes is key to preventing the Wallerian degeneration seen in the secondary injury phase of SCI. E2 may also be exerting neuroprotective effects by modulating excitotoxicity. More specifically, E2 was reported to upregulate expression of the glutamate

transporter 1 (glial specific glutamate transporter) along with the Kir4.1 channel (inwardly rectifying potassium channel) expression in a rat SCI model (Olsen, Campbell et al. 2010). Since E2 binds to its cognate receptor and, thus, can regulate expression of 137 genes, E2 may be simultaneously driving neuroprotection through numerous mechanisms (Lin, Strom et al. 2004). Given the highly pleiotropic nature of E2 and the robust preclinical findings, E2 is a promising candidate for continued development as a therapeutic in SCI.

Clinical Translation & Nanoparticle Delivery

Long term E2 treatment (5 years) in post-menopausal women is known to carry adverse risks of cardiovascular disease, cancer, stroke, and pulmonary embolism; as studies conducted by the Women's Health Initiative (WHI) have reported (Rossouw, Anderson et al. 2002). Shorter term E2 treatment used for contraception is reported to be significantly associated with increased risk of venous thromboembolism- with the greatest risk occurring in less than a year of treatment and at the highest dose of E2 (Lidegaard, Edstrom et al. 2002). Spinal cord injury individuals are at a heightened risk for development of thromboembolic complications (Waring and Karunas 1991). Given this increased risk, special consideration must be made to not compound the risk with pharmacological treatments. Thus, while preclinical data are very promising to suggest E2 as an effective therapeutic, these safety concerns with traditional dosing of E2 pose a hurdle for movement into SCI individuals. More

sophisticated forms of drug delivery, such as nanoparticles, may provide a better option.

Nanoparticles, synthetic polymers capable of carrying and targeting delivery of drugs, have shown great promise for rational, focused drug administration. The first nanoparticle formulated drug to garner FDA approval for the treatment of cancer was Doxil, in 1995 (Gabizon and Martin 1997). Doxil is a pegylated (poly-ethylene glycol coated) liposomal nanoparticle carrying the chemotherapeutic doxorubicin. The liposomal formulation of the drug allowed for preferential accumulation into the skin making it uniquely suited for the treatment of Kaposi's sarcoma. This example highlights the advantage that nanoparticle formulated drugs can offer - targeted drug delivery. The vast majority of research into nanoparticle drug delivery has been conducted in oncology, and has yielded at least 11 FDA approved nanotherapeutics with at least 9 more being evaluated in phase III clinical trials (Marchal, Hor et al. 2015). While the field of nanotherapeutics first gained success in oncology these advances can now be applied to neurotrauma research.

The use of nanoparticles as a drug delivery system for CNS disorders is a burgeoning field that will allow for focal drug delivery. By site-directed administration of E2 in a nanoparticle formulation, sustained high localized tissue concentrations may be achieved while keeping the overall systemic exposure relatively low, thereby reducing risk of high dose E2 exposure related toxicities to

the patient. Indeed, nanoparticle drug delivery systems have been shown to improve the therapeutic index of drugs (Zhang, Gu et al. 2008).

Nanoparticles have the potential to increase the therapeutic index of drugs, however, the question of how to focally deliver E2 loaded nanoparticles to the spinal cord remains. A recent exploratory delivery technique whereby embedded agarose gel plugs with MP loaded nanoparticles that were placed directly onto lesioned tissue in a rat SCI model illustrated the feasibility of our approach (Chvatal, Kim et al. 2008). Given acute spinal cord trauma individuals are likely to undergo spine stabilization surgery, delivery of the nanoparticles directly via a surgically implanted gel patch also remains a translational approach. MP has been shown to be released from the agarose gel preparation and to penetrate the spinal cord exerting its anti-inflammatory effects. High dose methylprednisolone given via traditional oral or intravenous routes have been used for more than 30 years now for the treatment of acute SCI, albeit off label, and remains quite controversial as it acts as an immunosuppressant and lacks robust efficacy. Therefore, further development of nanoparticle methylprednisolone may not be warranted. However, given the robust and promising preclinical data with E2 development of a nanoparticle delivery approach is suggested. By focally delivering E2 to the injured spinal cord directly, maximal efficacy may be achieved while keeping systemic levels of E2 in a physiological range. This technique may effectively increase the therapeutic window of E2.

CHAPTER TWO

ESTROGEN RELEASE FROM NANOPARTICLES AND E2 AS AN ANTI-INFLAMMATORY IN A PER-ACUTE RAT MODEL OF SCI

Introduction

Nanotherapeutics in SCI is a relatively new area of research. Work in this area began with numerous studies investigating the capacity of polymers such as PEG and other nanotechnologies to be used as wound sealants in the damaged spinal cord. The focus herein will be on nanoparticle drug delivery; other reviews may be referenced for these earlier studies (Cho and Borgens 2012). Nananoparticle drug delivery can be divided into two distinct approaches, systemic delivery and site directed. Systemic delivery is administration of a nanotherapeutic via intravenous (IV), oral gavage, or intraperitoneal (IP) dosing techniques such that the nanotherapeutic will be distributed via the bloodstream throughout the body. Site directed delivery approaches will allow for therapeutics to be placed directly onto injured/diseased tissue. This approach may allow for increased tissue concentrations of drug, thus enhancing efficacy while sparing toxicities associated with high systemic dosing regimens.

Recently, a group has published two papers describing methylprednisolone encapsulated into PLGA nanoparticles, embedded into agarose gels, and then placed directly onto lesioned spinal cords (Chvatal, Kim et al. 2008, Kim, Caldwell et al. 2009). Further, this group has evaluated the difference between directly injecting methylprednisolone vs. nanoparticle gel delivery for efficacy and reported that the nanoparticle delivered methylprednisolone had better functional improvement. Another group has reported a similar finding using glial derived neurotrophic factor (GDNF)

encapsulated into PLGA nanoparticles. Here the authors used either an intraspinal injection or a direct injection into lesion tissue and reported that only the site-directed injection of GDNF nanoparticles resulted in functional improvement (Wang, Wu et al. 2008). Prostaglandin-E-1, Neurotrophin-3, and flavopirodol loaded nanoparticles have also been reported to be efficacious in spinal cord injury (Takenaga, Ishihara et al. 2010, Ren, Han et al. 2014, Elliott Donaghue, Tator et al. 2015).

Given the success of these preliminary studies relied on site-directed delivery of drug loaded nanoparticles we aim to apply these advances into an investigation of E2 loaded nanoparticles in spinal cord injury. To begin this work E2 release from the nanoparticle must be characterized in an *in vitro* system and subsequently in a peracute (6hr) *in vivo* rat model of SCI. With these preliminary data, illustrating that the nanoparticles can deliver E2 to the spinal cord, more long term studies may be warranted.

Additionally, while much is known about the neuroprotective effects of E2 in sub-acute and chronic conditions (days to weeks), less is known about its potential to mediate inflammation rapidly in a per-acute setting of SCI (6hrs). To further elucidate the potential for E2 to rapidly modulate inflammation, the cytokines IL-6 and GRO-KC (human IL-8), and the chemokine MCP-1 (candidate biomarkers identified in a human a study (Kwon, Stammers et al. 2010)) were investigated in plasma, cerebrospinal fluid (CSF), and spinal cord tissue of an acute rat model of SCI. The calcium binding protein S100β, the most frequently

cited biomarker studied in SCI (Yokobori, Zhang et al. 2013) was also investigated in spinal cord tissue. As the potential exists for E2 to modulate numerous targets, outside of those previously identified as biomarkers, a complete cytokine/chemokine array will be used to fully characterize how E2 may function as a rapid anti-inflammatory in SCI. Sampling the plasma, CSF, and tissue simultaneously will further understanding of the dynamics of using plasma/CSF biomarkers to reflect the status of the injured nervous tissue. In utilizing nanoparticles, we hypothesize that E2 concentration will be greater in tissue than plasma and aim to keep systemic exposure at physiological levels. Additionally, by using a multiplex cytokine/chemokine array approach in plasma, tissue, and CSF, we hypothesize identification of novel cytokines/chemokines that are targets of E2 modulation. Collectively, the data presented herein may provide additional support for the clinical translation of this novel drug therapeutic into the acute neurotrauma setting.

Materials and Methods

Nanoparticles and Gel Plug Delivery System

Nanoparticles: Nanoparticles were formulated at Clemson University (Bioengineering Department) using the nanoprecipitation method previously described (Maximov, Reukov et al. 2010, Satishkumar and Vertegel 2011). Briefly, 50:50 PLGA (poly-lactic-co-glycolic acid) was mixed with PEG-PLA copolymer, and 17-β estradiol (E2) (Sigma E2758) in a 10:2.5:1 (wt.) ratio. The vehicle control batch was loaded with 0.9% saline. This was dissolved in acetone to obtain a 5 mg/mL polymer solution. This solution was added drop-wise to 20 mL of de-ionized water in an ultrasonic water bath and sonicated for 30 minutes. The formed particles were then separated by centrifugation (7500 rcf) for 2 hours, washed three times with de-ionized water, and lyophilized overnight. Each batch was evaluated for particle size (dynamic light scattering), load efficiency, and zeta potential to ensure consistency among batches. Nanoparticles were stored in sucrose at -20°C.

Gel Plug Delivery System: Gel plugs (0.6% SeaPlaque agarose in PBS) were made by dissolving lyophilized E2 loaded PLGA nanoparticles at either 25 µg or 2.5 µg dose (or saline loaded vehicle control) in 50 µL sterile filtered PBS. The gel (final volume 50uL) was set in PCR amplification tubes overnight to harden. Prior to insertion into animals, gels were sterilized under tissue culture UV lamp for 15 minutes.

Rat Model

All animal experiments were conducted in accordance with guidelines of the Institutional Animal Care and Use Committee, and performed under the protocol ARC #2079. Adult male Sprague-Dawley (SD) rats weighing 200-250 gm were used. Five groups, each with n=6 animals per group were included in this study as follows: (1). Naïve, (2). Sham, (3). Injury + nanoparticle vehicle control (Inj + Veh), (4). Injury + 25 µg E2, and (5). Injury + 2.5 µg E2. The naïve group was used to establish a baseline for cytokine/chemokine expression. Inclusion of this group was intended to aid in identifying analytes that are changed due to laminectomy procedure. Comparison of sham vs. injury + Veh group will identify markers changed due to SCI. To generate the SCI model, animals were anesthetized with ketamine (100 mg/kg) and xylazine (5 mg/kg), and a laminectomy was performed at thoracic vertebrae 9 & 10 (T9 & 10), the dura mater was left intact. The spine was immobilized with a spinal stereotactic device, and a moderate to severe SCI was induced by the weight-drop model (Perot, Lee et al. 1987). A weight of 5 gm was dropped from a height of 8 cm onto an impounder (0.3 cm in diameter) placed on the dural surface of the spinal cord. Sham animals undergo only a T9 &10 laminectomy. Immediately following the injury induction, the sterile gel plug was placed directly on the dural surface of the contused spinal cord, filling the space voided by the laminectomy (Figure 3).

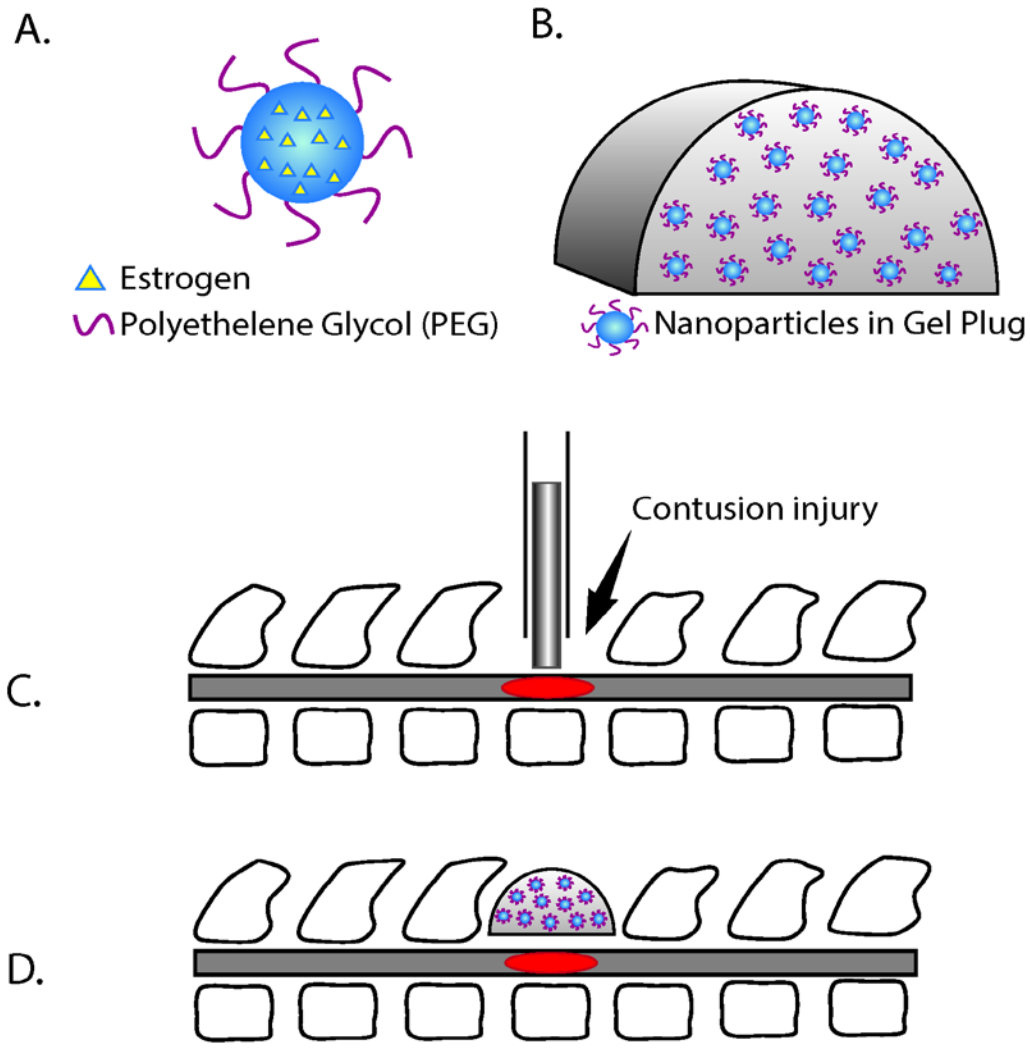


Figure 3. E2 Nanoparticle Gel Plug Approach A. PEG decorated nanoparticle loaded with Estrogen B. Estrogen loaded nanoparticles embedded in gel plug C. Spinal cord contusion injury D. Placement of gel plug onto lesion

The incision was sutured, and animals were monitored for 6 hours until euthanasia. At the 6hr time point, animals were deeply anesthesized with isoflurane, and whole blood was drawn via cardiac puncture into EDTA-coated tubes for E2 concentration analysis and cytokine profiling. CSF was collected via cisterna magna puncture and flash frozen in liquid nitrogen for cytokine profiling via ELISA. For CSF analysis, only clear samples completely devoid of blood contamination were analyzed. Animals were decapitated, and a necropsy was performed to collect a 1 cm section of spinal cord taken centered on the lesion site (0.5 cm caudal to lesion epicenter taken with 0.5 cm rostral to epicenter). At necropsy all gel plugs were observed to have remained in place, and the gel plug was removed and discarded prior to tissue collection. Tissue was flash frozen in liquid nitrogen and stored at -80°C until processing. Naïve animals were euthanized in identical fashion, and samples were taken at same time of day as in the experimental animals to control for potential circadian effects on cytokine/chemokine levels.

Estrogen Concentration *In Vitro* & *In Vivo*

In Vitro: To evaluate the potential for E2 to be released from the nanoparticle embedded agarose gel patch an *in vitro* release experiment was conducted. Gel patches placed in complete cell culture media (RPMI 1640 + 10% FBS) maintained in cell culture incubator for 32 days. E2 concentration was measured via commercially available ELISA kit (Calbiotech ES180S).

In Vivo: Plasma

Whole blood drawn via cardiac puncture was collected into EDTA containing tubes (final concentration EDTA 1.5 mg/mL) and placed immediately on ice. Whole blood samples were spun at 10,000 RPM in a Sorvall Legend Micro21 bench top centrifuge, and plasma was collected and stored at -80°C. 17-β estradiol (E2) concentration was determined using a commercially available ELISA kit (Calbiotech Estradiol ELISA ES180S). Undiluted samples were processed following directions provided by the manufacturer.

In Vivo: Spinal Cord

Spinal cord tissue homogenate (aliquot of identical sample as used for cytokine profiling) was diluted either 1:100 for the 25 µg E2 dose or 1:10 for the 2.5 µg E2 in 6% BSA block, and samples were run on same ELISA kit as plasma analysis. Using protein concentration data gathered from Bradford assay, tissue sample E2 concentration was then converted from pg/mL to pg/µg total protein to account for differences in protein concentrations between samples.

Cytokine Profiling

Tissue: Spinal cord tissue samples were homogenized in 400µL of ice cold PBS with a protease inhibitor cocktail (Sigma P1860, final dilution 1:200). Samples were homogenized with a Polytron hand held tissue homogenizer set at maximum speed for 30 seconds on ice. Samples were then sonicated briefly and spun at 10,000 g for 20 min at 4°C. Supernatants were analyzed via Bradford assay for protein concentration, and samples were normalized to a final protein

concentration of 8 µg/mL with homogenizing buffer. Normalized samples were stored at -80°C then shipped on dry ice to Eve Technologies (www.evetechnologies.com) and run undiluted on the cytokine/chemokine 27-plex discovery assay that included: Eotaxin, EGF, Fractalkine, IL-1α, IL-1 β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12(p70), IL-13, IL-17A, IL-18, IP-10, GRO/KC, IFN-γ, TNF-α, G-CSF, GM-CSF, MCP-1, Leptin, LIX, MIP-1α, MIP-2, RANTES, VEGF.

Plasma:

Plasma samples collected at necropsy were stored at -80°C then shipped on dry ice to Eve Technologies. Samples were run undiluted on the rat cytokine/chemokine 27-plex discovery assay.

Cerebrospinal Fluid: CSF was taken at necropsy, flash frozen in liquid nitrogen, stored at -80°C, then shipped on dry ice to Eve Technologies. Samples were run undiluted on the rat cytokine/chemokine 27-plex discovery assay. Three groups (sham, Injury + 25 µg, Injury + 2.5 µg) contained only n=5 samples due to omission of a sample with blood contamination.

Western Blot S100β

An aliquot of the spinal cord tissue homogenate that was used for cytokine profiling was used for Western blotting, n=6 samples representative of the 6 animals per group (n=24 total samples included in analysis). Using the data from the Bradford assay for protein estimation samples will be diluted to 1.0 µg/µL concentration with sample buffer [62.5 mM Tris–HCl, pH 6.8, 2% sodium dodecyl sulfate, 5 mM β-

mercaptoethanol, 10% glycerol] and boiled for denaturation. 12.5 µL (12.5 µg total protein) of each sample will be loaded onto 4-20% precast gels (Bio-Rad Laboratories, CA, USA) and resolved at 100 V for 1 and 1/2 hrs. Immunoblotting was performed as described in a previous lab publication (Samantaray, Patel et al. 2013). The resolved gel was transferred to Immobilon™-polyvinylidene fluoride microporous membranes (Millipore, MA, USA). The membranes were blocked for 1 hr with non-fat dry milk powder in wash buffer (0.1 % Tween-20 in 20 mM Tris–HCl, pH 7.6) and immunoblotting was performed with primary antibody Anti-S100β (Abcam rabbit monoclonal #ab52642) at 1:1000 overnight at 4°C and Anti-β-Actin (Sigma mouse monoclonal #A2228) at 1:10,000 room temperature for 1hr. The membranes were then incubated with horseradish peroxidase tagged secondary antibodies (dilution 1:2000) tagged with (primary goat anti-mouse or primary goat anti-rabbit antibodies) at room temperature. Chemiluminescent reagents (ECL Prime, GE Healthcare) were used to detect the immunoreactive bands on the membranes, and the images of protein bands were captured via Alpha Innotech FluorChem FC2 Imager. The immunoreactive bands were quantified using NIH's Image J software. Given multiple gels were used to accommodate all of the samples the data was analyzed as a percent of control. Sham animals were defined to be 100% and the relative band intensity of the remaining animals was normalized to the average of the sham group per gel.

Statistical Analysis: To test our hypothesis that E2 may significantly decrease IL-6, MCP-1, and GRO-KC a one-way ANOVA test was performed using

GraphPad Prism 5.0 with a Tukey's multiple comparison post-test to determine any differences between sham, Inj + Veh, Inj + 25 µg E2, and Inj + 2.5 µg E2 treated groups. Each test was conducted for each marker in the plasma, tissue, and CSF compartments such that 9 one-way ANOVA's were run. Significance was defined as $p<0.05$. Additionally, as a screening tool to assess the remaining markers the non-parametric Wilcoxon rank sum test was used to detect differences among naïve, sham, Inj + Veh, and Inj + E2 treated groups. SAS 9.3 software was used to perform the analysis. Each matrix was treated as a separate group, and all of the analytes reported were included in the analysis, such that for plasma, 75 comparisons were made; in CSF, 64 comparisons were made; and in tissue, 75 comparisons were made. Significant differences were defined as $p<0.05$. A false discovery rate (fdr) correction was made to account for the multiple comparisons made within each statistical test. Both the uncorrected exact p value and the fdr corrected exact p values are shown for significant p values Inj + Veh vs. Inj + 25 µg / Inj + 2.5 µg in tabular form (tables 1-3).

Results

In Vitro E2 Release from Nanoparticle Gel Plug:

E2 levels continued to rise for 6 days (144 hours), and then showed a plateau in concentration until day 32 (Figure 4). This data illustrates that E2 is released from nanoparticles embedded in gel patches into cell culture media and provides

the rationale for *in vivo* evaluation. Additionally, there were no overt signs of bacterial or fungal growth in the media (devoid of antibiotics/antimycotics after 32 days of incubation with the E2 nanoparticle gel plug. Given this observation the UV light sterilization technique was deemed acceptable for animal usage.

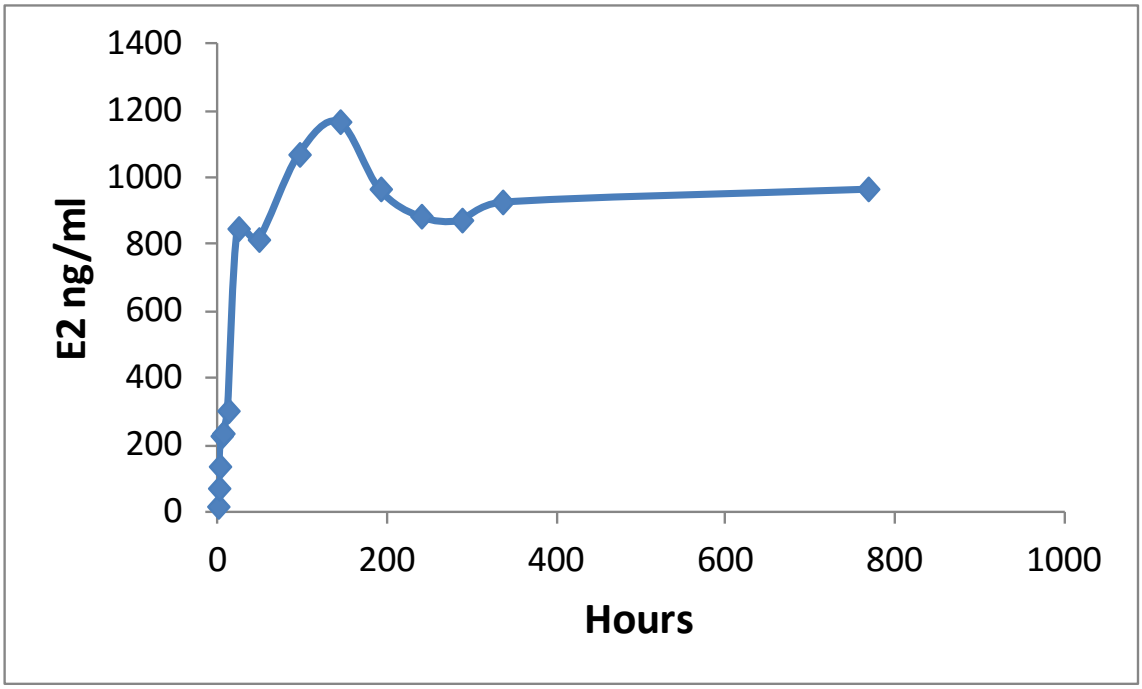


Figure 4. Estrogen *In Vitro* Release Profile

Concentration of E2 released into culture medium from agarose gel patches embedded with 25 µg E2 nanoparticles. E2 concentration assayed on ELISA format. Media serially sampled from 0, 2, 4, 6, 12, 24, 48hrs, and then every other day to day 14 and a final sample taken on day 32.

E2 Plasma Concentration: The E2 plasma concentration (mean ±SEM) of animals in the sham group (14.6 pg/mL ±0.7) was comparable to the Injury + Vehicle group (15.8 pg/mL ±0.9) (Figure.5). These values were within the range of reported serum concentrations in female rats (during estrous/trough E2 levels) using a radioimmunoassay technique (Strom, Theodorsson et al. 2008). E2 concentration in plasma at 6hrs post agarose gel plug placement was nearly ten-fold higher in the 25 µg dose group (725.2 pg/mL ±175.2) vs. the 2.5 µg dose group (81.6 pg/mL ±18.6) (Figure 5). Notably, the E2 concentration in the 2.5 µg dose group was lower than reported E2 concentrations in female rats during proestrous, or peak E2, at 300 pg/mL (Saddick 2014). This suggests that the concerns of supraphysiological E2 dosing, as seen in humans taking high dose contraception, may be avoidable using a focal nanoparticle delivery approach that may allow for physiologically relevant plasma E2 levels .

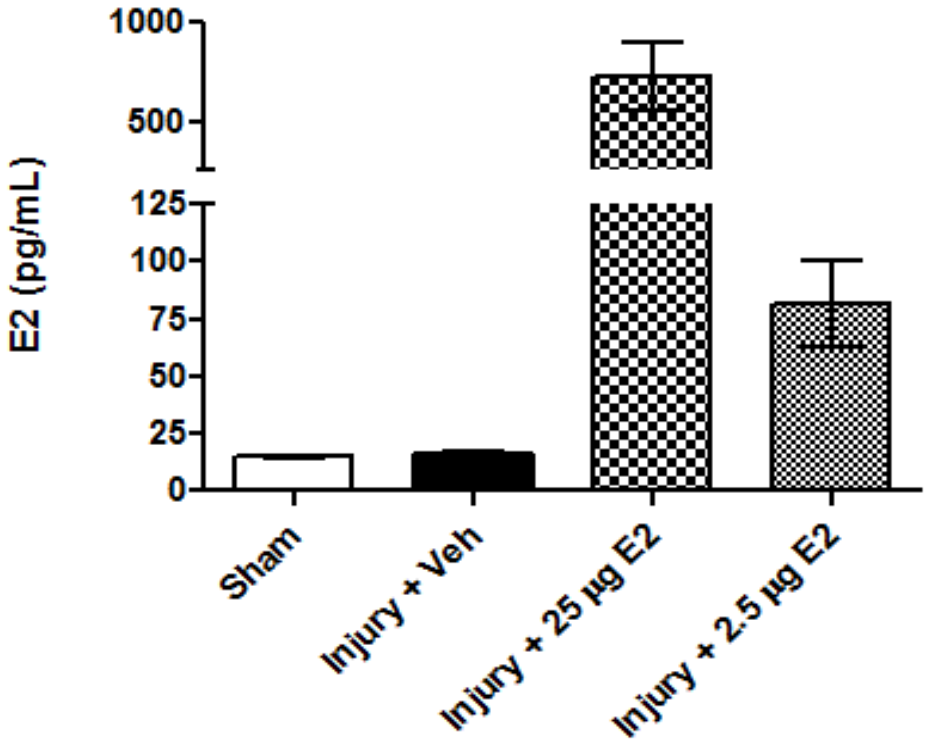


Figure 5. Plasma E2 Concentration 6 hrs Post Injury E2 plasma concentration was nearly ten-fold higher in the 25 µg dose group (725.2 pg/mL ±175.2) vs. the 2.5 µg dose group (81.6 pg/mL ±18.6), n=6 per group.

E2 Tissue Concentration: The E2 tissue concentration (mean ±SEM) of animals in the sham group (1.6 pg/µg ± 0.08) was comparable to the Injury + Vehicle group (0.9 pg/µg ± 0.07) (Figure 6). E2 concentration in spinal cord tissue was also nearly ten-fold higher in the 25 µg dose group (1564 pg/µg ± 366) vs. the 2.5 µg dose group (163 pg/µg ± 47) (Figure 6). This suggests that given the doses chosen in this study, at this single time point, the nanoparticle E2 release is dose linear. Additionally, the concentration of E2 (at both doses) is double in the tissue as compared with plasma (Figure 7). This data provides evidence to support our hypothesis that focal nanoparticle delivery of E2 may allow for increased tissue concentrations over plasma.

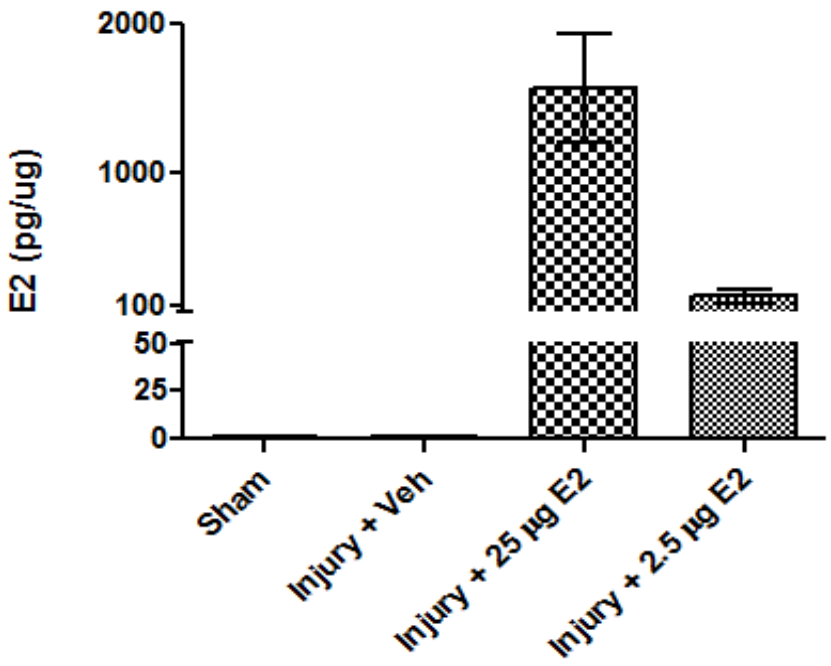


Figure 6. Spinal Cord Tissue E2 Concentration 6 hrs Post Injury E2 concentration in spinal cord tissue was nearly ten-fold higher in the 25 µg dose group (1564 pg/µg ± 366) vs. the 2.5 µg dose group (163 pg/µg ± 47), n=6 per group.

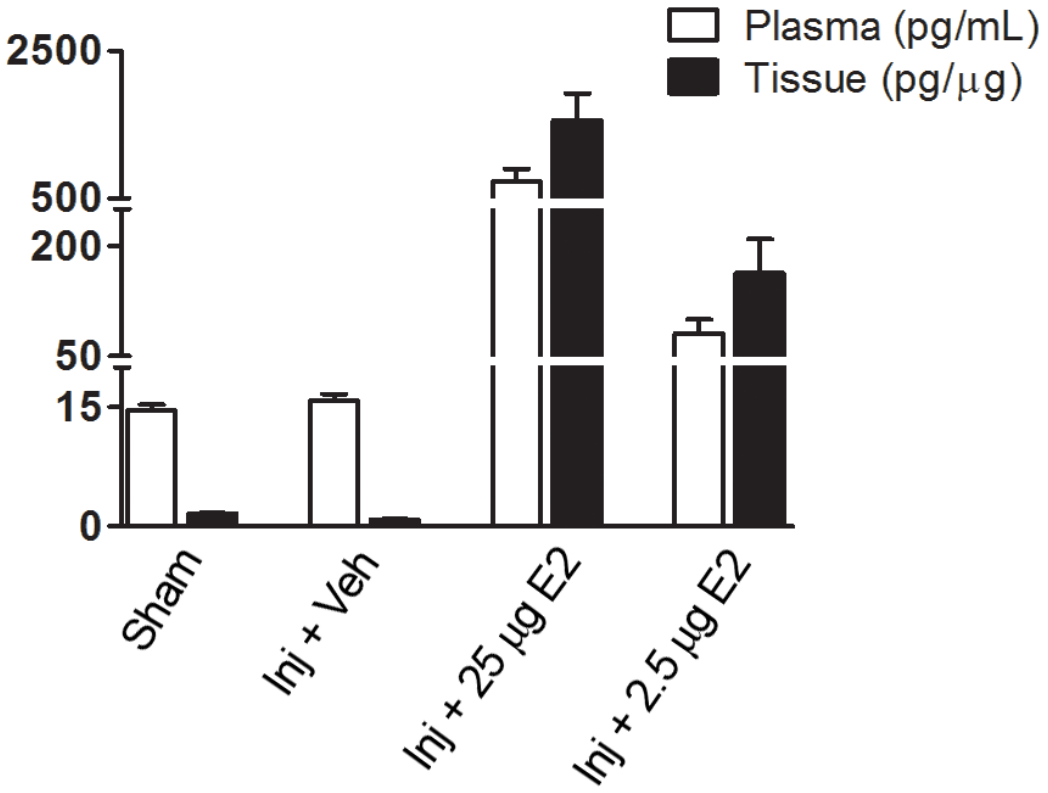


Figure 7. Plasma and Tissue E2 concentrations 6 hrs Post Injury Tissue concentrations were double that of plasma for both 25 μg (1564 pg/μg tissue vs. 725 pg/mL plasma) and 2.5 μg (163 pg/μg tissue vs. 82 pg/mL) E2 doses. N=6 per group

CSF Cytokine/Chemokine Profiling: 18 of the 27 cytokines/chemokines profiled were assayed at measurable levels. G-CSF, Eotaxin, GM-CSF, IL-5, LIX, TNF-alpha, MIP-2, RANTES, and MIP-1A were found to be below the levels of quantification (BLQ), and thus not reported. Both IL-6 and GRO-KC were increased with SCI, and decreased with E2 treatment, however, did not reach significance (Figure 8). MCP-1, however was found to be significantly increased with SCI and significantly decreased at both doses of E2 (Figure 8). Using a screening approach to evaluate the remaining markers IL-10, IL-2, IFN-γ, IL-17a, and TNF-α were found to be increased with injury and decreased with E2 treatment (Table 1). Showing no change with either injury or E2 treatment were IL-4, IL-13, and IL-18 (Table 1). IL-1β, IL-12p70 and leptin showed increased levels with E2 treatment (Table 1). The growth factors EGF and VEGF had slightly decreased levels in the 2.5 μg E2 dose group, and the remaining chemokines (IP-10, Fractalkine, LIX, and RANTES) exhibited no change with either injury or E2 treatment (Table 1). The factors that showed the greatest change between sham and injury, recapitulating findings in a human CSF biomarker study (Kwon, Stammers et al. 2010), were IL-6, MCP-1, and GRO-KC. In summary, data suggest that E2 treatment at either the 25 or 2.5 μg dose may modulate the following cytokines/chemokines in the CSF: IL-1α, IL-6, IL-10, IL-2, IL-10, IFN-γ, IL-17a, TNF-α, IL-1β, IL-12p70, Leptin, EGF, VEGF, MCP-1, and GRO-KC.

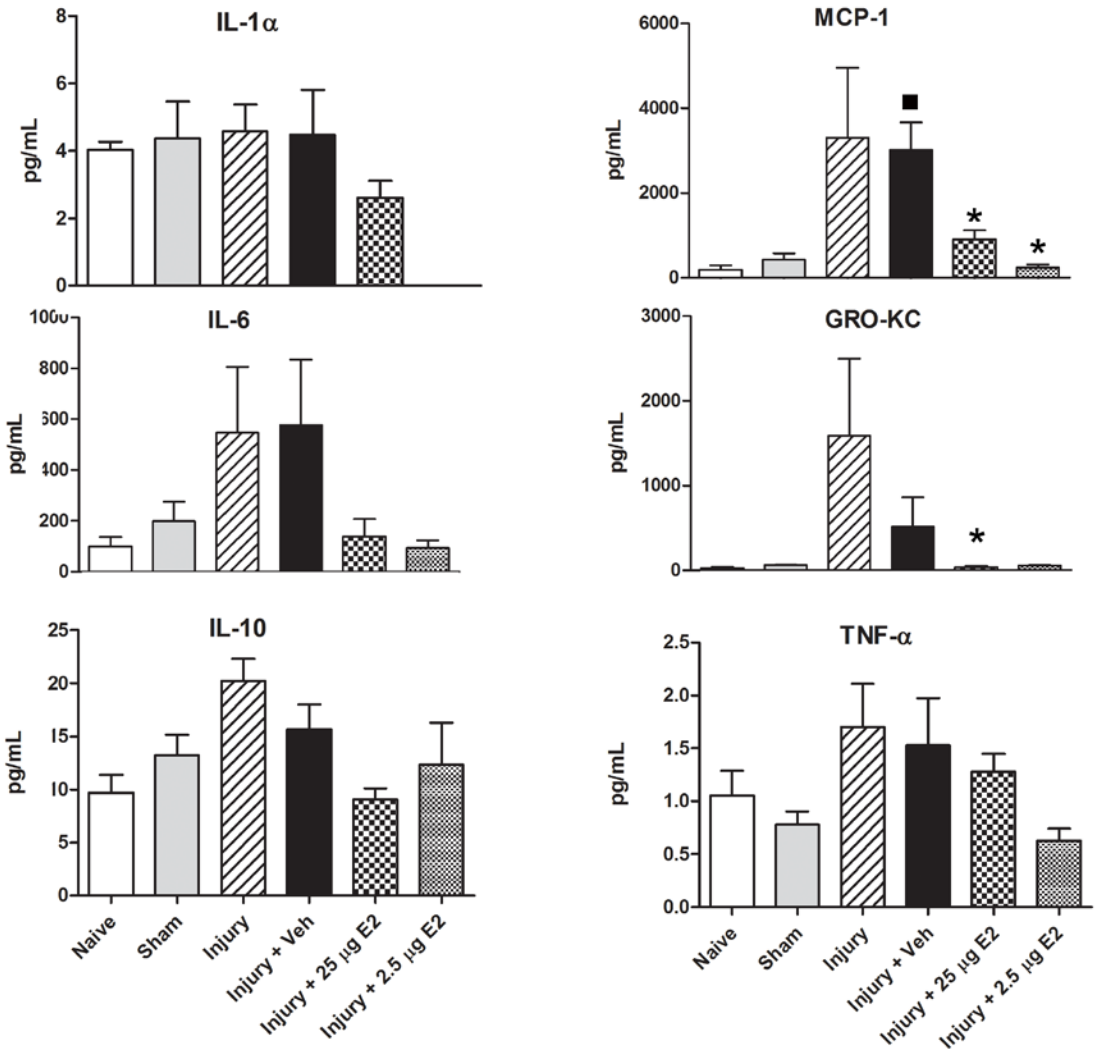


Figure 8. Cytokine and Chemokine Expression in CSF IL-1α was unchanged from sham or naïve animals compared to Injury or Injury + Veh groups. IL-1α was decreased with 25 μg E2 treatment, and levels were BLQ for the 2.5 μg E2 treatment (data not shown). IL-6 was increased following injury, and E2 treatment reduced levels to that of sham and naïve animals. IL-10 was increased with injury; E2 treatment reduced level to sham and naïve animals.

MCP-1 was significantly increased with injury and reduced significantly with E2 treatment. GRO-KC was increased with injury and significantly decreased with 25 μg E2 treatment. TNF-α was increased with injury and decreased with 2.5 μg E2 treatment. ■ Significant differences between Sham and Inj + Veh groups, $p<0.05$. * Significant difference between Injury + Veh group and Injury + 25/2.5 μg E2 treated group, $p<0.05$. n=5 all groups

Analyte	Naïve	Sham	Injury + Vehicle	Injury + 25 µg E2	Injury + 2.5 µg E2	Uncorrected P Value *Inj + 25 µg E2 **Inj + 2.5 µg E2	False Discovery Rate Corrected P Values
IL-1a	omitted	4.4 (1.1)	4.5 (1.3)	2.6 (0.5)	omitted		
Leptin	110 (34)	omitted	116 (34)	194 (86)	202 (39)		
IL-4	7.9 (1.3)	6.2 (1.4)	6.5 (1.1)	4.6 (1.0)	7.2 (0.5)		
IL-1B	143 (51)	59 (29)	58 (24)	141 (58)	156 (52)		
IL-2	22 (7)	omitted	78 (13)	39 (8)	50 (21)	*0.035	
IL-6	100 (38)	198 (78)	577 (258)	omitted	93 (31)	** 0.052	
EGF	0.32 (0.13)	0.32 (0.08)	0.30 (0.05)	0.34 (0.06)	0.17 (0.06)		
IL-13	6.5 (1.6)	10.2 (3.2)	omitted	omitted	9.4 (3.0)		
IL-10	9.7 (1.7)	13.2 (1.9)	15.7 (2.4)	9.1 (1.1)	12.3 (4.0)	*0.069	
IL-12p70	5.9 (1.6)	7.0 (1.6)	6.2 (0.5)	9.8 (4.3)	11.4 (2.5)		
IFNy	24 (8)	56 (7)	27 (7)	BLQ	17 (3)		
IL-17A	5.1 (1.2)	6.4 (2.8)	7.7 (2.0)	4.6 (0.6)	5.1 (1.9)		
IL-18	24 (10)	30 (5)	29 (3)	28 (6)	omitted		
MCP-1	195 (93)	omitted	3009 (655)	907 (208)	235 (72)	* 0.030 **0.011	
IP-10	167 (23)	152 (20)	220 (67)	183 (21)	147 (36)		
GRO/KC	omitted	omitted	514 (348)	40 (12)	55 (6)	*0.038 **0.056	
VEGF	19 (6.9)	18 (0.8)	14.9 (1.7)	14.4 (2.6)	10.2 (1.7)	**0.017	
Fractalkine	43 (3)	49 (22)	40 (4)	46 (5)	46 (3)		
LIX	55 (10)	52 (9)	66 (7)	54 (8)	60 (9)		
TNFa	1.1 (0.2)	1.0 (0.2)	1.5 (0.4)	1.3 (0.2)	0.6 (0.1)	**0.056	
RANTES	30 (9)	26 (3)	23 (5)	32 (2)	23 (3)		

Table 1. Cytokine, Chemokine, and Growth Factor Expression in CSF G-CSF, eotaxin, GM-CSF, MIP-1A, IL-5, and MIP2 were below the limits of quantification (BLQ) in this assay, thus omitted. Values are expressed as: Mean (SEM). A minimum of 3 samples were required for mean and SEM analysis (numerous samples were below limit of quantification), thus analytes with less than 3 were omitted and indicated as such. * P value for Injury + Veh group vs. Injury + 25 µg E2 treated group. ** P value for Injury + Veh group vs. Injury + 2.5 µg E2 treated group.

Spinal Cord Tissue Cytokine/Chemokine Profiling: All 27 of the cytokines/chemokines profiled were assayed at measurable levels. However, G-CSF was BLQ for the naïve and 25 µg E2 dose groups. Both IL-6 and GRO-KC were significantly increased with SCI and significantly decreased at the 2.5 µg E2 dose (Figure 9). MCP-1 was significantly increased with SCI, however E2 treatment only slightly trended for a decrease (Figure 9). Using the screening approach to evaluate the remaining markers the majority of cytokines showed no change in level with either injury or E2 treatment. The cytokines that were unchanged were IL-4, IL-2, IL-13, IL-12p70, IFN-γ, IL-5, IL-17A, and IL-18 (Table 2). Analytes displaying increased levels with injury and decreased levels with E2 treatment were IL-1α, IL-1β, IL-6, IL-10, TNF-α, and leptin (Figure 9, Table 2). The chemokines eotaxin, fractalkine, and LIX were unchanged in level with injury or E2 treatment. G-CSF, MIP-1α, MCP-1, GRO-KC, LIX, MIP-2, and RANTES were increased with injury and decreased with E2 treatment while IP-10 levels were increased with injury but not affected by E2 treatment (Table 2). The growth factors EGF and VEGF were increased with injury and decreased in E2 treated groups (Table 2). The factors that exhibited the greatest change in expression between sham and injury, suggesting their specificity as biomarkers of spinal cord injury, were IL-1α, IP-10, GRO-KC and MIP-2. These factors showed 4-7 fold increases between sham and injury groups. The marker with the largest increase in expression was MIP-1α, with a 20-fold increase in expression between sham and injury groups. In summary, data suggest that E2

treatment at either the 25 or 2.5 μg dose modulate the following cytokines/chemokines in the spinal cord tissue: IL-1α, IL-1β, IL-6, IL-10, TNF-α, Leptin, G-CSF, MIP-1α, MCP-1, GRO-KC, LIX, MIP-2, RANTES, EGF, and VEGF.

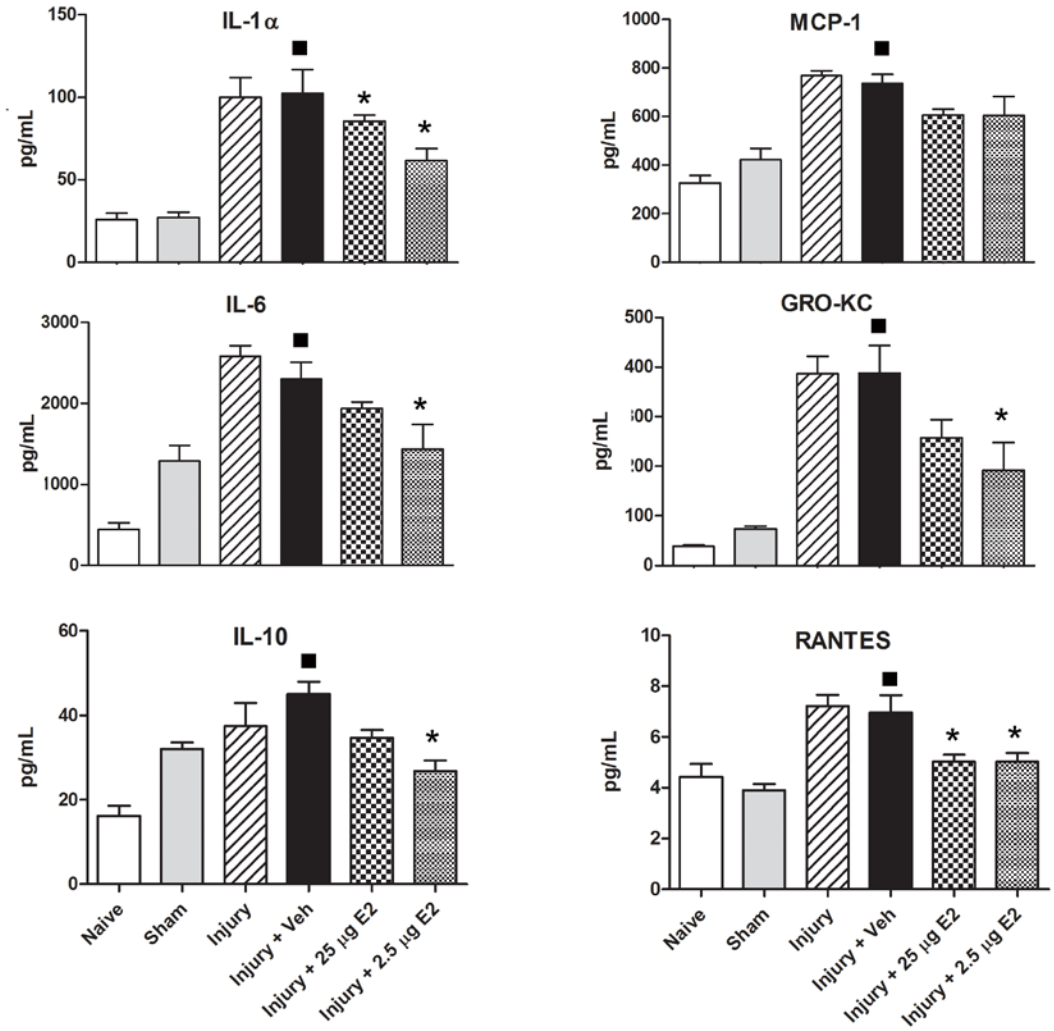


Figure 9. Cytokine and Chemokine Expression in Spinal Cord Tissue IL-1α and RANTES were significantly increased in Injury + Veh group compared to Sham and significantly decreased with either dose of E2. IL-6, IL-10, and GRO-KC were significantly increased in Injury + Veh group compared to Sham, and significantly decreased with 2.5 μg E2 treatment. MCP-1 is significantly increased in the Injury + Veh group, and treatment with E2 showed a trend toward a decrease. ■ Significant difference between Sham and Inj + Veh groups, $p<0.05$. * Significant difference between Injury + Veh group and Injury + 25/2.5 μg E2 treated group, $p<0.05$. n=6 per group

Analyte	Naïve	Sham	Injury + Vehicle	Injury + 25 µg E2	Injury + 2.5 µg E2	Uncorrected P Value * Inj + 25 µg E2 **Inj + 2.5 µg E2	False Discovery Rate Corrected P Values
G-CSF	omitted	7.6 (3.2), n=5	10.5 (1.0)	omitted	omitted		
Eotaxin	8.7 (0.9)	11.3 (0.5)	11.4 (1.0)	9.9 (0.5)	9.0 (1.1)		
GM-CSF	3.9 (0.7)	3.8 (1.0)	6.5 (0.6)	5.8 (1.4)	4.2 (1.2)		
IL-1a	26 (4)	27 (3)	102 (14)	85 (4)	62 (7)	**0.0108	
Leptin	369 (23)	687 (32)	709 (8)	560 (20)	464 (35)	*0.002 **0.002	*0.015 **0.015
MIP-1a	4.3 (0.2)	8.5 (1.2)	173.5 (23)	127.9 (5.9)	94 (21)	**0.0411	
IL-4	15.5 (1.2)	17.9 (0.8)	19.7 (1.0)	18.2 (1.2)	16.7 (0.9)		
IL-1B	997 (91)	1515 (191)	1439 (67)	1019 (48)	1032 (79)	*0.002 **0.004	*0.015 **0.027
IL-2	84 (13)	114 (8)	115 (7)	99 (5)	71 (11)	** 0.009	**0.041
IL-6	464 (97)	1284 (198)	2298 (208)	1933 (87)	1434 (310)	** 0.065	
EGF	0.51 (0.08)	0.80 (0.08)	1.05 (0.11)	0.68 (0.07)	0.63 (0.10)	**0.028	*0.045
IL-13	26.1 (1.5)	24.7 (1.9)	31.8 (3.9)	22.4 (2.5)	23.5 (5.2)		
IL-10	18.2 (1.6)	32.1 (1.6)	45.1 (2.9)	34.7 (1.9)	26.9 (2.4)	*0.0108 **0.002	*0.045 **0.015
IL-12p70	69 (4)	72 (7)	69 (7)	74 (3)	69 (5)		
IFNy	140 (5)	172 (6)	156 (8)	136 (6)	145 (11)		
IL-5	17.4 (2.7)	32.6 (3.0)	21.3 (3.1)	21.8 (1.2)	20.5 (3.6)		
IL-17A	21.6 (3.3)	25.3 (4.4)	17.4 (2.2)	15.7 (1.4)	13.6 (2.0)		
IL-18	1308 (87)	1544 (125)	969 (49)	989 (43)	987 (43)		
MCP-1	351 (24)	422 (47)	737 (38)	606 (25)	605 (77)		
IP-10	57 (5)	95 (12)	359 (12)	347 (13)	333 (50)		
GRO/KC	40 (2)	74 (6)	389 (55)	257 (36)	192 (56)	**0.041	
VEGF	15.4 (2.1)	24.6 (3.8)	18.4 (2.6)	12.5 (1.4)	8.9 (1.4)	**0.026	
Fractalkine	52 (4)	66 (6)	77 (4)	80 (4)	76 (9)		
LIX	176 (6)	172 (3)	186 (11)	174 (3)	154 (12)		
MIP-2	36 (3)	65 (6)	458 (53)	377 (20)	258 (83)		
TNFa	3.5 (0.3)	3.8 (0.6)	5.8 (0.6)	5.0 (0.3)	4.1 (0.4)		
RANTES	4.8 (0.5)	3.9 (0.3)	7.0 (0.7)	5.0 (0.3)	5.0 (0.3)	*0.009 **0.015	*0.041

Table 2: Cytokine, Chemokine, and Growth Factor Expression in Spinal Cord Tissue All 27 analytes were analyzed. Values are expressed as: Mean (SEM). Samples in which the "n" was below 6 were due to values that were BLQ and were omitted. * P value for Injury + Veh group vs. Injury + 25 µg E2 treated group. ** P value for Injury + Veh group vs. Injury + 2.5 µg E2 treated group. n=6 all groups

Plasma Cytokine/Chemokine Profiling: All 27 of the cytokines/chemokines profiled were assayed at measurable levels. IL-6, GRO-KC, IL-12, IL-17, IFN- γ and MCP-1 were all found to be significantly decreased with E2 treatment (Figure 10). Surprisingly, the levels of IL-6, GRO-KC, and MCP-1 were comparable between sham and Injury + Veh animals. Comparison of these markers in naïve animals reveals that all three markers are increased due to the laminectomy procedure, suggesting that in plasma these are general markers of inflammation due to the surgical procedure rather than markers specific to injured spinal cord tissue (Table 3). Using the screening approach to evaluate the remaining markers, the majority of cytokines in the plasma showed dynamic responses to sham, injury, and E2 treatment. IL-1α, IL-4, IL-1β, IL-2, IL-13, IL-10, IL-12p70, IFN-γ, IL-5, IL-17A, IL-18, and TNF-α were increased with either sham or injury and decreased with E2 treatment (Table 3). Both growth factors EGF and VEGF were increased with injury and decreased with E2 treatment, and the chemokines IP-10, fractalkine, MIP-2, and RANTES were unchanged between injury and E2 treated groups (Table 3). Eotaxin, GM-CSF, and MIP-1α were decreased with E2 treatment, but unchanged by injury (Table 3). G-CSF and LIX were increased with injury and decreased with E2 treatment (Table 3). A number of factors showed large increases in expression between the naïve and sham groups: IL-1α, IL-4, IL-1β, IL-6, IL-13, IL-10, IL-12p70, IFN-γ, IL-18 and GRO-KC (55 fold increase). However, given that the sham treatment (laminectomy without

contusion injury) does not involve damage to the spinal cord, these markers could represent systemic response to trauma. Leptin, however, had a 3-fold increase between sham and injury, making it a potential biomarker specific to SCI. In summary, data suggest that E2 treatment at either the 25 or 2.5 μg dose may modulate the following cytokines/chemokines in the plasma after SCI: IL-1α, IL-4, IL-1β, IL-2, IL-6, IL-13, IL-10, IL-12p70, IFN-γ, IL-5, IL-17A, IL-18, TNF-α, EGF, VEGF, Eotaxin, GM-CSF, MIP-1A, G-CSF, MCP-1, GRO-KC and LIX.

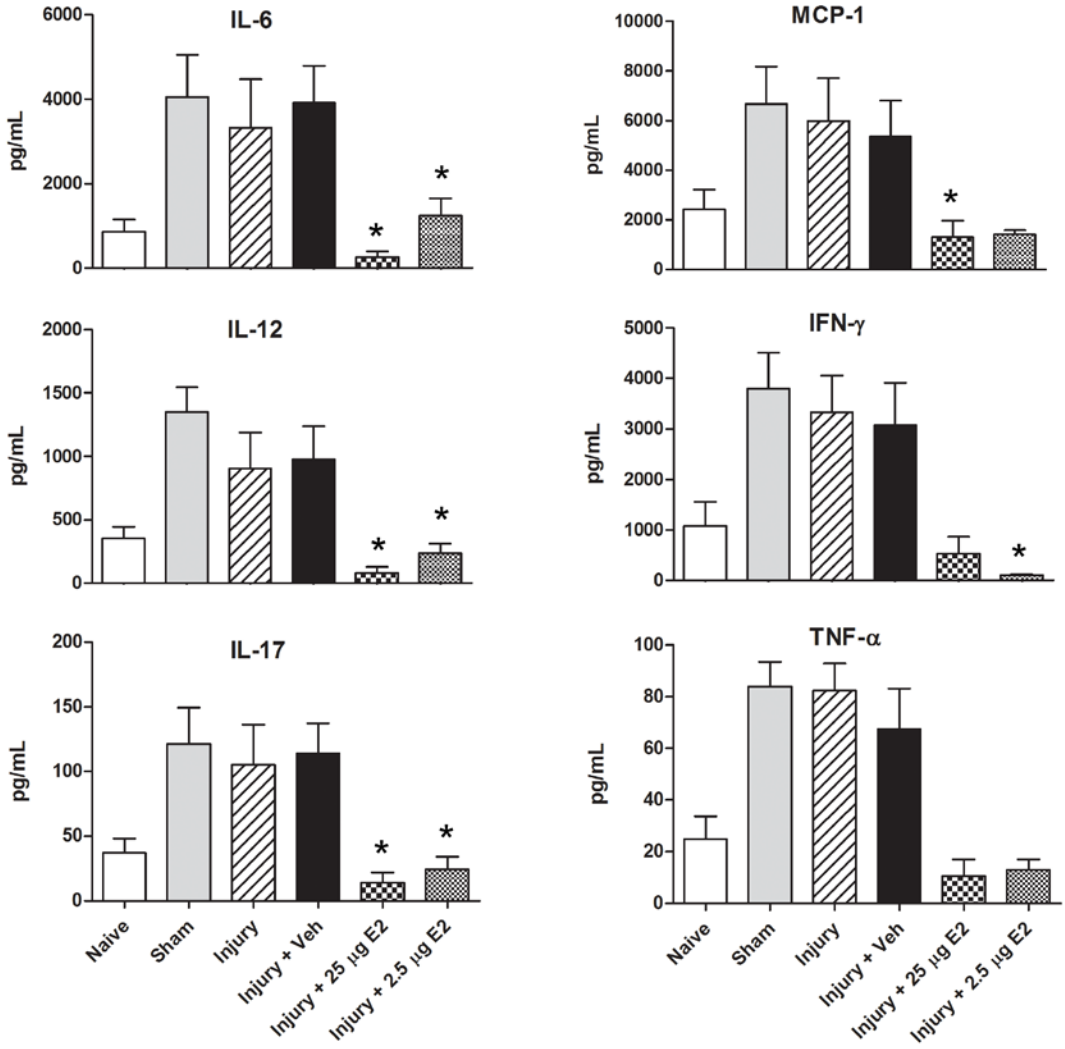


Figure 10. Cytokine and Chemokine Expression in Plasma Levels for IL-6, IL-12, IL-17, MCP-1, IFN-γ, and TNF-α were comparable between Sham and Injury + Veh groups. IL-6, IL-12, IL-17, MCP-1, and IFN-γ were significantly decreased with either or both E2 doses. E2 treatment decreased TNF- α to below that of the naïve group. ***** Significant difference between Injury + Veh group and Injury + 25/2.5 μg E2 treated group, $p<0.05$. n=6 per group

Analyte	Naïve	Sham	Injury + Vehicle	Injury + 25 µg E2	Injury + 2.5 µg E2	Uncorrected P Value * Inj + 25 µg E2 **Inj + 2.5 µg E2	False Discovery Rate Corrected P Values
G-CSF	47 (9)	201 (53)	176 (47)	31 (12)	20 (5)		
Eotaxin	25 (9)	90 (9)	75 (16)	18 (10)	14 (2)	*0.03 ** 0.017	
GM-CSF	99 (40)	245 (57)	228 (48)	56 (26)	145 (55)		
IL-1a	31 (6)	234 (54)	151 (47)	omitted	28 (5)		
Leptin	1816 (408)	1738 (188)	4047 (1085)	11987 (1364)	3407 (1595)	*0.008	*0.0464
MIP-1a	7 (2)	23 (3)	19 (5)	omitted	7 (2)	*0.008	
IL-4	36 (6)	152 (41)	140 (39)	13 (7)	38 (10)	*0.006 **0.025	*0.046 ** 0.093
IL-1B	omitted	106 (42)	85 (48)	omitted	omitted		
IL-2	85 (36)	306 (66)	306 (76)	11 (4)	74 (33)	*0.016 ** 0.017	*0.072 ** 0.072
IL-6	869 (279)	4058 (993)	3913 (887)	257 (139)	1249 (403)	*0.004 **0.03	*0.046 ** 0.093
EGF	1.7 (0.6)	5.4 (1)	6.3 (2.4)	4.9 (3.7)	1.0 (0.3)		
IL-13	82 (18)	349 (41)	239 (70)	55 (34)	50 (11)		
IL-10	39 (11)	173 (28)	150 (34)	45 (17)	66 (27)	*0.009	*0.046
IL-12p70	353 (93)	1248 (194)	975 (265)	80 (48)	236 (78)	*0.004 ** 0.015	*0.046 **0.072
IFNy	1077 (474)	4351 (540)	3073 (835)	537 (329)	106 (22)	*0.009 **0.009	*0.046 **0.046
IL-5	96 (19)	215 (21)	159 (31)	57 (24)	59 (11)	**0 .004	** 0.114
IL-17A	37 (11)	121 (28)	114 (23)	14 (8)	24 (10)	*0.004 * 0.009	*0.046 **0.046
IL-18	74 (27)	331 (79)	307 (82)	omitted	112 (42)	*0.036	*0.1061
MCP-1	2422 (796)	6666 (1504)	5348 (1442)	1305 (661)	1401 (186)	*0.036	*0.1061
IP-10	63 (7)	107 (10)	98 (14)	101 (8)	79 (26)		
GRO/KC	201 (71)	11001 (1257)	8359 (2037)	2635 (958)	469 (34)	** 0.009	** 0.046
VEGF	23 (9)	84 (14)	72 (13)	25 (5)	26 (11)	*0.004 ** 0.031	*0.046 ** 0.01
Fractalkine	21 (4)	48 (8)	45 (6)	32 (6)	49 (8)		
LIX	542 (90)	1137 (248)	1186 (303)	220 (74)	369 (89)	*0.004 ** 0.03	*0.046 **0.01
MIP-2	omitted	101 (20)	109 (30)	83 (11)	133 (18)		
TNFa	25 (9)	84 (10)	67 (16)	11 (6)	12 (5)	*0.008 ** 0.032	*0.046 **0.01
RANTES	186 (14)	131 (18)	151 (35)	141 (16)	176 (50)		

Table 3. Cytokine, Chemokine and Growth Factor Expression in Plasma All 27 analytes were analyzed. Values are expressed as: Mean (SEM). Samples in which the values that were BLQ were omitted. * P value for Injury + Veh group vs. Injury + 25 µg E2 treated group. ** P value for Injury + Veh group vs. Injury + 2.5 µg E2 treated group. n=6 per group

Western Blot S100β

There was a significant increase in S100β protein levels in spinal cord tissue of Inj + Veh group (195% of control) vs. Sham (100% of control) (Figure 11). There was a non-significant reduction of S100β in the Inj + 25 µg E2 vs. Inj + Veh (153% of control vs. 195% of control) , however, a significant reduction was seen in S100β protein expression in the Inj + 2.5 µg E2 group vs. Inj + Veh (89% of control vs. 195% of control) (Figure 11). The Inj + 2.5 µg E2 group had S100β levels comparable to those seen in sham.

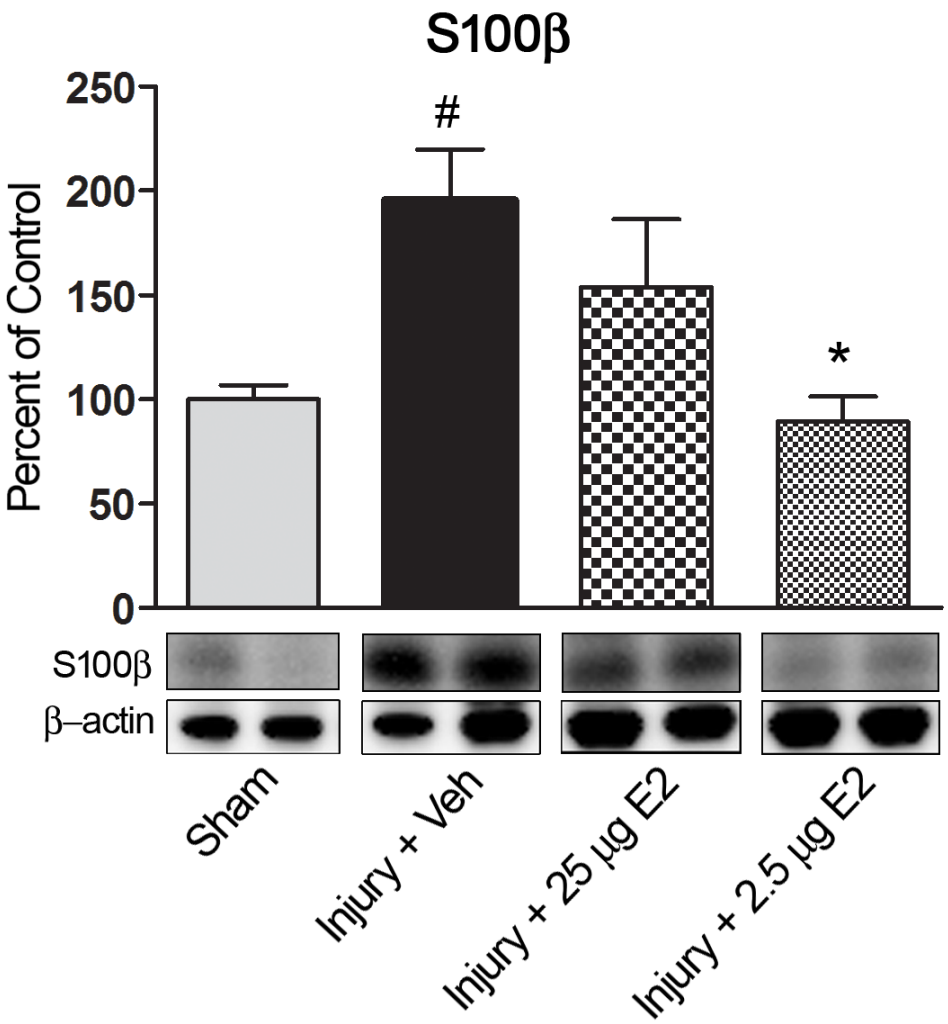


Figure 11. S100β Expression in Spinal Cord Tissue A significant increase in S100β was found in the Injury + Veh group as compared with Sham, and a significant decrease was seen with the 2.5 µg E2 dose. Two representative bands per group displayed (n=6 per group total). # Significant differences between Sham and Inj + Veh groups, $p<0.05$, *****Significant difference between Injury + Veh group and Injury + 25/2.5 µg E2 treated group, $p<0.05$

Discussion

Given safety concerns associated with high doses of E2 (thrombus, feminization, stroke, etc.), a nanoparticle drug delivery system was utilized. We report that by using an E2 nanoparticle embedded gel approach the concentration of E2 in the spinal cord tissue is double that of plasma (Figure 1). Additionally, the plasma concentration at the 2.5 μg E2 dose resulted in plasma levels of 81 pg/mL (Figure 1). These levels are well within physiological range (peak non pregnant plasma E2 levels in females are 300 pg/mL (Saddick 2014)). Therefore, E2 delivered focally to damaged tissue may be efficacious at physiological plasma levels. This approach retains significant translational relevancy, as the majority of acute SCI individuals will undergo spine stabilization surgery, thus providing opportunity for surgical placement of a therapeutic gel patch.

The approach of sampling 3 biological compartments with naïve controls has allowed for a further understanding of how inflammatory signals correspond between plasma/CSF/tissue in both SCI and in surgical intervention (laminectomy). Notably, the plasma levels of IL-6, GRO-KC, and MCP-1 are comparable between sham and injury + vehicle groups (Figure 2), while naïve controls are significantly lower (Table 3). These results suggest that the laminectomy procedure alone can result in the same response of IL-6, GRO-KC, and MCP-1 in plasma, and suggest that IL-6, GRO-KC and MCP-1 may be

primarily derived from a peripheral source rather than injured nervous tissue. The pattern of IL-6, GRO-KC and MCP-1 in tissue and CSF are very different from plasma, however. In tissue and CSF sham levels are lower than injury + vehicle animals, revealing specificity for injury within these compartments (Figure 2). Given the specificity of the CSF for increased IL-6, GRO-KC, and MCP-1 only in the presence of spinal cord injury, the data suggest the pool of IL-6, GRO-KC, and MCP-1 measured in CSF is derived, in part at least, from the damaged spinal cord. As human studies must extrapolate how the CSF or plasma relates to the state of the injured spinal cord, the data presented here provide a comprehensive profile of acute inflammatory signaling in 3 compartments to allow for a greater understanding of how plasma and CSF reflect tissue response to injury across 27 cytokines/chemokines (Tables 1-3). As IL-6, GRO-KC, and MCP-1 have been previously identified in human studies as candidate biomarkers in CSF (Kwon, Stammers et al. 2010) these data, in a “reverse translational” approach, help provide evidence that the markers are reflective of the condition of the injured spinal cord.

E2 treatment resulted in rapid anti-inflammatory effects, and these effects were seen at both doses chosen for the present study (25 µg or 2.5 µg.) In particular, the study focused on IL-6, GRO-KC, MCP-1, and S100β as these markers have been previously identified in human studies as either being elevated in injury or as potential biomarkers of injury severity (Kwon, Stammers et al. 2010, Bank, Stein et al. 2014, Pouw, Kwon et al. 2014). E2 treatment

significantly decreased IL-6 expression in plasma and tissue, and trended towards a decrease in CSF (Fig. 2). IL-6, a prototypical cytokine, is rapidly secreted at the site of injury by a number of cell types including neurons, glia, T-cells, and macrophages, and plays a central role in early neuroinflammatory response (Erta, Quintana et al. 2012). E2 pretreatment has previously been reported to decrease tissue levels of IL-6 at a 24hr time point in a mouse model of SCI (Cuzzocrea, Genovese et al. 2008). However, Ritz and Hausmann reported that a 0.1 mg/kg or 4 mg/kg E2 dose in an acute rat SCI model significantly increased IL-1α, IL-1β, and IL-6 in tissue at 6hrs post treatment (Ritz and Hausmann 2008). Differences in dosages, dose routes, and time points may explain the incongruous results with E2 regulation of IL-6 in acute SCI.

The neutrophil attractant chemokine, GRO-KC (rodent IL-8 homolog), has been reported to be increased in acute SCI (McTigue, Tani et al. 1998, Stammers, Liu et al. 2012). Neutrophils, one of the first peripheral immune cells to migrate to the lesioned tissue, are thought to be key contributors to the secondary injury seen in SCI (Neirinckx, Coste et al. 2014), and further, a reduction of infiltrating neutrophils has shown decreased tissue damage in a rat model (Bao, Fleming et al. 2011). In a traumatic brain injury model GRO-KC has also been associated with blood brain barrier dysfunction and nerve growth factor production, making it a potentially important chemokine in neurotrauma and repair (Kossmann, Stahel et al. 1997). Thus, GRO-KC (IL-8) may be a critical chemokine to regulate in acute SCI. Here, we report that E2 treatment

significantly reduced GRO-KC in the plasma and tissue compartments with a trend for reduction in the CSF (Figure 2), again highlighting the therapeutic potential of E2 in acute SCI.

MCP-1 (CCL2) is a small chemokine induced by a variety of stimuli (injury, inflammation, oxidative stress, etc.) that is produced by a variety of cell types and functions to recruit monocytes/macrophages to the site of injury or inflammation (Deshmane, Kremlev et al. 2009). Studies have found a correlation between macrophage infiltration and MCP-1 levels (Lee, Shih et al. 2000), and MCP-1 has been found to be upregulated in animal models following SCI (Lee, Shih et al. 2000). In a naturally occurring canine SCI, MCP-1 levels in CSF were negatively associated with post-injury outcome (Taylor, Welsh et al. 2014). Here we report that E2 treatment resulted in a significant decrease in MCP-1 levels in plasma and CSF, while tissue levels had a slight trend only for decrease (Figure 2). A link has been shown between estrogen receptor α (ERα) and MCP-1 expression. In an experimental autoimmune encephalitis model treatment with ERα specific ligand resulted in decreased MCP-1 (Spence, Wisdom et al. 2013). Given these prior findings, these data further support the hypothesis that E2 may decrease inflammation through regulation of MCP-1 signaling.

The calcium binding protein S100β, most abundantly found in glial cells, is involved in a number of cellular processes including: inhibition of protein phosphorylation, regulation of cell-cycle progression, astrocyte proliferation, and can act in a concentration dependent manner as either a trophic or toxic factor to

neurons (Donato 1999). A direct link between E2 and S100β expression is not yet known. E2 has been reported to decrease calcium influx through interaction with L-Type voltage gated calcium channels in an *in vitro* setting of glutamate excitotoxicity (Sribnick, Del Re et al. 2009). Given S100β is a calcium binding protein a direct or indirect relationship may exist where E2's modulation on calcium influx would influence S100β expression and/or release. However, it should be noted that S100β is a highly complex signaling protein with the potential to serve widely varying functions in intracellular or extracellular roles that are also dependent on disease state (Donato, Sorci et al. 2009). Given the complexity of both E2 and S100β it may be overly simplistic to hypothesize calcium as the link between the two as numerous additional pathways may be involved. Regardless of this complexity as a signaling molecule, the role as a biomarker has been shown in numerous animal models of SCI where S100β is elevated in serum and/or CSF of injured animals compared to sham (Ma, Novikov et al. 2001, Loy, Sroufe et al. 2005, Cao, Yang et al. 2008). In addition, S100β has been identified in two human SCI as a candidate biomarker, capable of predicting injury severity into either a specific ASIA rating (Kwon, Stammers et al. 2010) or complete vs. incomplete injuries (Pouw, Kwon et al. 2014). Given the growing evidence that S100β may be a valid biomarker for injury severity in human SCI, our findings that E2 nanoparticle treatment significantly decreased S100β expression in spinal cord tissue (Figure 3) suggest that early intervention with E2 may decrease injury severity. Due to the small

volume of CSF that can be collected from a rat analyzing both cytokine/chemokine screens and S100β levels was not possible; however, this warrants further investigation.

While IL-6, GRO-KC, MCP-1, and S100β were the focus of the study the multiplex assay format allowed for an additional screen to be performed for the potential identification of new E2 targets. Using a Wilcoxon rank sum test to screen all 27 markers evaluated it was found that E2 treatment exerted widespread effects on cytokines/chemokines/growth factors such as: leptin, MIP-1α, IL-4, IL-2, IL-10, IFNγ, TNFα, etc . More specifically, 15 analytes were significantly decreased in plasma (Table 3), 6 analytes were significantly decreased in spinal cord tissue (Table 2), and a number trended to be decreased in CSF (Table 1). The lack of significance in the CSF compartment may be attributed to increased variance in these samples as compared with tissue or plasma. These findings further highlight the pleiotropic nature of E2 in the setting of neurotrauma and suggest that the rapid anti-inflammatory signaling exerted by E2 may contribute to the improvement in motor function as previously reported (Cuzzocrea, Genovese et al. 2008, Ritz and Hausmann 2008, Sribnick, Samantaray et al. 2010, Samantaray, Smith et al. 2011, Lee, Choi et al. 2012, Mosquera, Colon et al. 2014). It remains to be seen whether low dose nanoparticle delivered E2 may result in functional improvement. However, as we continue to evaluate this method of E2 delivery in functional models of SCI, we will seek a better understanding of how early anti-inflammatory treatment may

modulate late-stage motor recovery. Thus, by using this targeted nanoparticle drug delivery approach, we hope to advance the highly promising therapeutic E2 into clinical studies, ultimately allowing for development of a safe and effective treatment for acute SCI individuals.

CHAPTER THREE

E2 AS A NEUROPROTECTANT AT 48HRS FOLLOWING SCI AND TIME COURSE STUDY OF E2 RELEASE FROM NANOPARTICLE

INTRODUCTION

Nanoparticle delivery of E2 to damaged spinal cord tissue may allow for sustained drug release such that drug effects may be seen not only in peracute models (hours of injury) of inflammation but also for days following as the nanoparticle continues to deliver drug. This method of drug delivery may be optimal due to several factors: single administration, focal administration, and prolonged release. Traditional dose routes such as IV or IP do not allow for any of these advantages. Additionally, these dose routes will result in very high plasma concentrations at early time points, furthering the risk of toxicities. Nanoparticle E2 delivery may reduce those maximal exposures seen conventionally while prolonging plasma exposure, making it appropriate for treatment of acute SCI. Given this approach requires the surgical application of the nanoparticle gel plug during spine stabilization/decompression surgery (typically within 6-8 hrs of injury), the drug chosen should be optimized for lessening secondary injury mechanisms.

The highly pleiotropic nature of E2 makes it an ideal candidate for treatment of complex pathologies such as those seen in secondary injury. Nanoparticle delivery of E2 may further enhance therapeutic potential for increases in neuronal protection and recovery through numerous mechanisms, including increased neurotrophin expression, decreased inflammation and gliosis, and increased neuronal survival. While these are certainly not the only potentially mechanisms, angiogenesis for example is another mechanism by which E2 may exert protective effects (Chen, Yeh et al. 2010), these will be the focus of the work presented herein.

Growing evidence supports a link between E2 and neurotrophin regulation, for example an E2 responsive element was identified on the brain derived neurotrophic factor (BDNF) gene suggesting a direct regulatory link (Sohrabji, Miranda et al. 1995). As neurotrophins are largely produced by glial cells to signal for neuronal survival and health, a study of how neurotrophins may be regulated by E2 in glia cells is warranted. In cultured astrocytes, E2 receptor alpha has been shown to regulate expression of GDNF, BDNF, and nerve growth factor (NGF) (Xu, Bi et al. 2013). Incubation of astrocyte-neuron co-cultures with 100nM E2 has been reported to result in upregulation of GDNF, specific to astrocytes (Campos, Cristovao et al. 2012). Treatment of an *in vivo* model of traumatic brain injury with Premarin has been reported to upregulate GDNF and was shown by double immunostaining to co-localize with bromodeoxyuridine (Brdu) expressing neurons in hippocampus, suggesting a role in neurogenesis (Chen, Yeh et al. 2010). Given these preliminary findings, we hypothesize that E2 may stimulate an increase in neurotrophins, such as GDNF, in the injured spinal cord.

Inflammation is a natural response to tissue damage and serves a physiological purpose to contain and repair injured tissue. However, excessive inflammation, as seen in neurotrauma, leads to increased injury severity as described in the secondary injury theory (Tator and Fehlings 1991). One of the potential mechanisms contributing to the spread of secondary injury, also a central mechanism to inflammation, is reactive gliosis. Gliosis is a generic term that describes activation of any type of glial cell, including astrocytes, microglia, and

oligodendrocytes. Reactive astrogliosis was thought for many years to be a maladaptive response as the primary driver of glial scarring, and thus the blockade of axonal regeneration in SCI. For years this dogma existed, and prevention of this response became a research focus. This polarizing view has now been disproven as many transgenic models reveal detrimental effects by complete blockade of astrocyte response, as reviewed elsewhere (Sofroniew 2009).

Astrocytes contribute to gliosis in both beneficial and detrimental ways through complex signaling mechanisms, as described in a recent review (Karimi-Abdolrezaee and Billakanti 2012). The release of proinflammatory cytokines TNF-α, IL-1β, and IL-6 during the acute phase of tissue injury may be the key signaling trigger in the activation of astrocytes, as recently reported (Lin, Basu et al. 2006, Karimi-Abdolrezaee and Billakanti 2012). Given our findings that E2 can acutely decrease an array of proinflammatory cytokines and chemokines including TNF-α, IL-1β, and IL-6 in both systemic and local compartments we hypothesize that E2 treatment will result in decreased astrogliosis.

Astrogliosis is pathologically characterized by hypertrophied (as characterized by extended and swollen processes) and proliferated astrocytes in and around lesioned tissue. The hallmark molecular marker of activated astrocytes is glial fibrillary acidic protein (GFAP), an intermediate filament protein required for cytoskeletal modifications such as hypertrophy that mark astrogliosis (Sofroniew and Vinters 2010). Other markers such as glutamine synthase and S100β have

also been proposed as markers specific to activated astrocytes, respectively (Norenberg 1979, Barres 2008, Goncalves, Leite et al. 2008).

Glutamine synthase is a critical enzyme in the conversion of glutamate and ammonia to glutamine within the astrocyte; this recycling allows for protection against glutamate excitotoxicity. The enzyme has been found to be upregulated in astrocytes following spinal cord injury, further illustrating its potential as a marker of astrogliosis (Benton, Ross et al. 2000). However, production of glutamine synthase has been reported in microglia, macrophages, and oligodendrocytes in rat spinal cord; however, only astrocyte and macrophage/microglia produced glutamine synthase contributed to increased levels following injury (Liu, Wu et al. 2013). Given these findings, glutamine synthase may be considered a marker of gliosis, rather than specific to astrogliosis.

Excitotoxicity, the pathological overstimulation of glutamate receptors, is a major contributor to neuronal death following acute injury (Mazzone and Nistri 2014). Thus, it is critical for a potential neuroprotectant to decrease excitotoxicity, thereby sparing neuronal loss. Immediately following SCI there is a rapid rise in the excitatory neurotransmitter glutamate to concentrations high enough to drive neuronal death (Liu, Thangnipon et al. 1991). Glutamate receptors may be broadly classified into two groups, either metabotropic or ionotropic. Ionotropic receptors, more specifically the NMDA receptor, function by forming an ion channel after glutamate binding, and allow for the rapid movement of ions into the cell. The NMDA receptor, once activated, may result in a lethal calcium influx into the cell,

resulting in cell death (Choi 1992). Riluzole (a sodium channel blocker / glutamate receptor modulator discussed in Chapter 1) has recently been evaluated in a Phase I safety and preliminary efficacy trial and reported to have significantly enhanced motor function, particularly among cervical injury patients (Grossman, Fehlings et al. 2014). These results are very promising and suggest the importance of modulating excitotoxicity in the acute stage of SCI. As discussed previously, E2 treatment has been shown to decrease glutamate induced cell death by inhibiting calcium influx through the L-type voltage gated calcium channel (Sribnick, Ray et al. 2006). We hypothesize that E2 treatment may decrease glutamine synthase expression through indirect mechanisms involving the decrease of glutamate signaling, thereby decreasing excitotoxicity.

S100β has been identified as an early biomarker of excitotoxicity released by astrocytes (Mazzone and Nistri 2014). As discussed earlier, S100β, is a calcium binding protein and a complex signaling molecule active in homeostasis as well as disease (Donato 1999). In a pathological setting S100β protein may play a detrimental role. Treatment of cultured astrocytes and neurons with a high concentration of S100β revealed inducible NOS activation, NO production, and subsequent neuronal death (Hu, Ferreira et al. 1997). Currently, no literature exists directly linking S100β and E2 regulation, hence we aim to further explore the potential for E2 to change S100β signaling in SCI.

Microglia, as the resident immune cell of the CNS, also play an active role in gliosis following injury. Activated microglia express ionized calcium binding

adaptor molecule 1 (Iba1), which has been reported to be exclusively produced in microglia, making it an ideal marker for measurement of microglia activity (Ito, Imai et al. 1998). Based on its molecular structure Iba1 is hypothesized to serve as an adaptor molecule mediating calcium signals (Ito, Imai et al. 1998). Given E2 may decrease calcium influx, we hypothesize that E2 treatment will reduce IBa1 expression in microglia. By decreasing microglial activation, the potential to decrease the downstream effects of microglia signaling follow.

Microglia secrete factors such as nitric oxide (NO) and cyclooxygenase (COX-2) that serve to trigger inflammation (Chao, Hu et al. 1992, Giulian, Corpuz et al. 1993). Nitric oxide (NO), a gas and free radical, is an important signaling molecule across the body that serves as a potent vasodilator, neurotransmitter, and also as a free radical that can cause oxidative damage to surrounding tissue. NO is produced by a variety of nitric oxide synthases. In inflammation the isoform inducible nitric oxide synthase (iNOS) is the primary enzyme responsible for its production. It is reported that iNOS production is increased in lesioned spinal cord tissue and that decreasing iNOS activity results in improvements in locomotor activity in a rat model (Jiang, Gong et al. 2014). In addition E2 administration in a mouse model (300μg/kg subcutaneous pre and post-injury induction) was shown to significantly decrease iNOS expression, resulting in improved locomotor recovery (Cuzzocrea, Genovese et al. 2008). COX-2, an enzyme responsible for the conversion of arachidonic acid to prostaglandins, is another biochemical marker of inflammation. COX-2 is upregulated in a rat model of SCI, and blockade

results in improved locomotor function recovery (Resnick, Graham et al. 1998, Cuzzocrea, Genovese et al. 2008).

To further understand the multi-faceted role that E2 may play as a neuroprotectant in SCI, various proteins will be investigated at the 48hr time point. E2 driven effects on proteins related to the neurotrophin GDNF, inflammation (COX-2 and iNOS), glial response (GFAP, Iba1, glutamine synthase, S100β), and apoptosis (BAX and Bcl2) will be studied via Western blot (WB) and immunofluorescence (IF) techniques. The apoptotic markers are chosen given the 48hr time point is the stage in which early apoptosis and changes in injury related proteins can be seen, and therefore any potential therapeutic effects of E2 treatment may be uncovered. In summary, we hypothesize that by delivering E2 in a nanoparticle gel embedded formulation, we will show a prolonged release into the plasma compartment as compared with conventional drug dosing techniques (IV or IP) resulting in improved neuroprotection.

MATERIALS AND METHODS

Nanoparticles and Gel Plug Delivery System: Techniques will be the same as described in Chapter 2.

Rat SCI Model:48Hr Rat SCI Model: Induction of injury and treatment with E2 nanoparticle will be conducted exactly as described for the 6hr model, with the exception being time point for sacrifice will be extended to 48hrs. For western blots n=5 per injury group, n=3 for sham group; and for immunofluorescence experiments n=3 all groups.

Rat Plasma Concentration Time Course Model: Naïve animals with no laminectomy were used for the IV and IP dose groups. For the nanoparticle embedded gel plug delivery a SCI was performed to allow for placement of gel plug onto lesioned tissue. All groups will have n=3, rats were serially bled via tail vein under isoflurane anesthesia.

E2 Delivery: Intravenous and Intraperitoneal Injections

For IP injections animals were injected with 25 μg 17-β estradiol dissolved in corn oil (100 ug/mL, 25 uL dose) as a single bolus dose. For IV dosing animals were injected via tail vein with 25 μg water soluble 17-β estradiol (sigma E4389, 100 μg/mL, 250 uL dose) as a single bolus dose. IV injections were done under isofluorane anesthesia.

In Vivo: Plasma Collection 48 hr profile studies

Whole blood drawn via tail vein puncture was collected into EDTA containing tubes (final concentration EDTA 1.5 mg/mL). Samples were collected at 1, 2, 4, 8, 12, 24, and 48 hrs following drug administration. Animals were serially bled, (n=3 per group) such that each animal underwent 7 tail vein bleeds, and one final cardiac stick. Whole blood samples were spun at 10,000 RPM in a Sorvall Legend Micro21 bench top centrifuge, and plasma was collected and stored at -80°C. 17-β estradiol (E2) concentration was determined using a commercially available ELISA kit (Calbiotech Estradiol ELISA ES180S).

Tissue Collection and Analysis: As described above, spinal cord samples will be harvested into three segments and either snap frozen for protein analysis on WB (n=5) or embedded into OCT and frozen for cryostat sectioning and IF (n=3).

Western Blotting: For this analysis, an additional group, laminectomy + injury (injury), will be included. This will allow for comparison to Injury + Veh group and determination if the agarose gel or saline loaded nanoparticles exert any biological effects. Additionally, only the 25 μg E2 dose group will be analyzed by western blotting. Samples will be homogenized with hand held Polytron in homogenizing buffer with protease inhibitors. Bradford assay will be performed for protein estimation, and normalized samples will be diluted to 2.0 μg/μL concentration with sample buffer [62.5 mM Tris–HCl, pH 6.8, 2% sodium dodecyl sulfate, 5 mM β-mercaptoethanol, 10% glycerol] and boiled for denaturation. Samples will be loaded onto 10-20% polyacrimide gels for SDS-PAGE using 4-20% precast gels (Bio-Rad Laboratories, CA, USA) and resolved at 100 V for 1 and 1/2 hrs. Immunoblotting was performed as described in a previous lab publication (Samantaray, Patel et al. 2013). The resolved gel was transferred to Immobilon™-polyvinylidene fluoride microporous membranes (Millipore, MA, USA). The membranes were blocked for 1 hr with non-fat dry milk powder in wash buffer (0.1 % Tween-20 in 20 mM Tris–HCl, pH 7.6). Immunoblotting was then performed with primary antibodies: COX-2 (Cell signaling #12282), BAX (sc-7480), Bcl2 (sc-130308) GDNF (ab18956), and glutamine synthase (MAB302). Results from WB

will be analyzed via Friedman's method two-way ANOVAs on GraphPad Prizm software.

Immunofluorescence: Spinal cords (caudal penumbra) were sectioned on a cryostat at 8µM and mounted onto slides. For all stains, slides were treated using this basic protocol:

(1). Fixation for 15 minutes in either 95 % EtOH or -20º C acetone (2). Washed 3 x 5 minutes in PBS (3). Blocked in 2% serum protein of the host secondary for 2 hrs at RT. (4). Incubated overnight in primary antibody, 20º C. (5). Washed 3 x 5 minutes in PBS (6). Incubated secondary antibody 2 hrs at RT (7). Washed 3 x 5 minutes in PBS (8). Final wash in deionized water 5 minutes (9). Allow to air dry, mount with ProLong Gold anti-fade mounting media with DAPI (Life Technologies #P36931), and coverslip. The primary antibodies used are summarized in the table below.

Antibody	Target Cell	Source
GFAP	astrocyte	MAB3402
S100 β	astrocyte injury	ab52642
GDNF	glial	ab18956
ED-1	macrophage	ab31630
Iba-1	microglia	ab5076

Table 4. Antibodies and Targets in Immunofluorescent Staining Antibodies, targets, and source information for all of the immunofluorescent staining

Immunofluorescence Signal Intensity Analysis: GFAP, S100β, Ed-1, and Iba-1 antibody stains were analyzed for signal intensity. All images were captured at 20X magnification on an Olympus IX73 inverted fluorescent microscope. Images were taken in the dorsal funinculus, and all images for a given stain were captured on a single day under identical settings. A single image was taken of each animal per group such that n=3 images per group for analysis. Fluorescent intensity was measured using Image J software to determine fluorescent pixel intensity per field, and reported as arbitrary units. Statistical differences were determined by one-way ANOVAs with a Tukey's post-test using GraphPad Prizm software.

RESULTS

48 E2 Plasma Profiling of 25 μg and 2.5 μg E2 Nanoparticle Doses:

The maximum concentration (Cmax) for the 25 μg dose was 500 ± 154 pg/mL and time at which maximal plasma concentration (Tmax) occured was 4hrs following administration. The Cmax for the 2.5 μg dose was 172 ± 99 pg/mL and the Tmax was 2hrs. By 24 hrs both dose groups had returned to plasma E2 levels seen in non-treated rats.

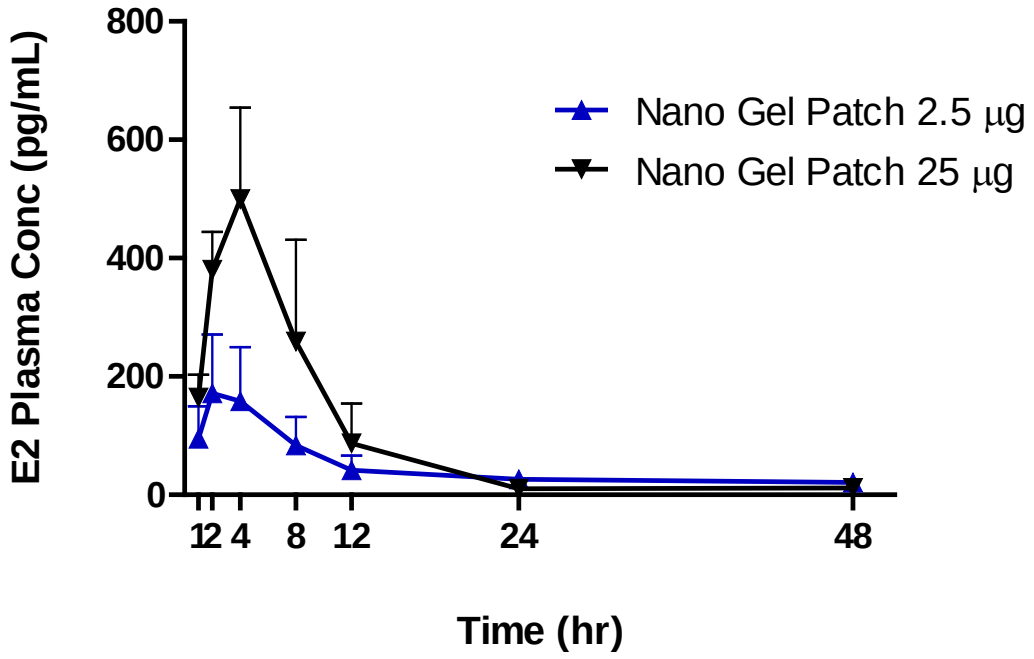


Figure 12. E2 Time Course Plasma Concentration 25 and 2.5 μg E2 SCI rats treated with 25 μg or 2.5 μg dose of E2 nanoparticle embedded gel plug (n=3 per group).

48hr E2 Plasma Profiling of IV, IP, and Nanoparticle Drug Delivery Approaches:

Comparison of different dosing techniques highlights the differences in plasma exposures. The Cmax / Tmax for the IV group was 1064 ± 250 pg/mL / 1hr, the IP group was 324 ± 114 pg/mL / 1hr, and 500 ±154 pg/mL / 4hrs for the nanoparticle group. Both the IV and IP dose groups had a Tmax at 1 hr and then the plasma exposure rapidly decreases, as expected for this dosing technique. The nanoparticle group, however, had a delayed Tmax of 4 hrs, and increased plasma levels out to 12 hrs. This provides evidence of prolonged E2 exposure in the plasma with decreased maximum concentrations using the nanoparticle delivery technique.

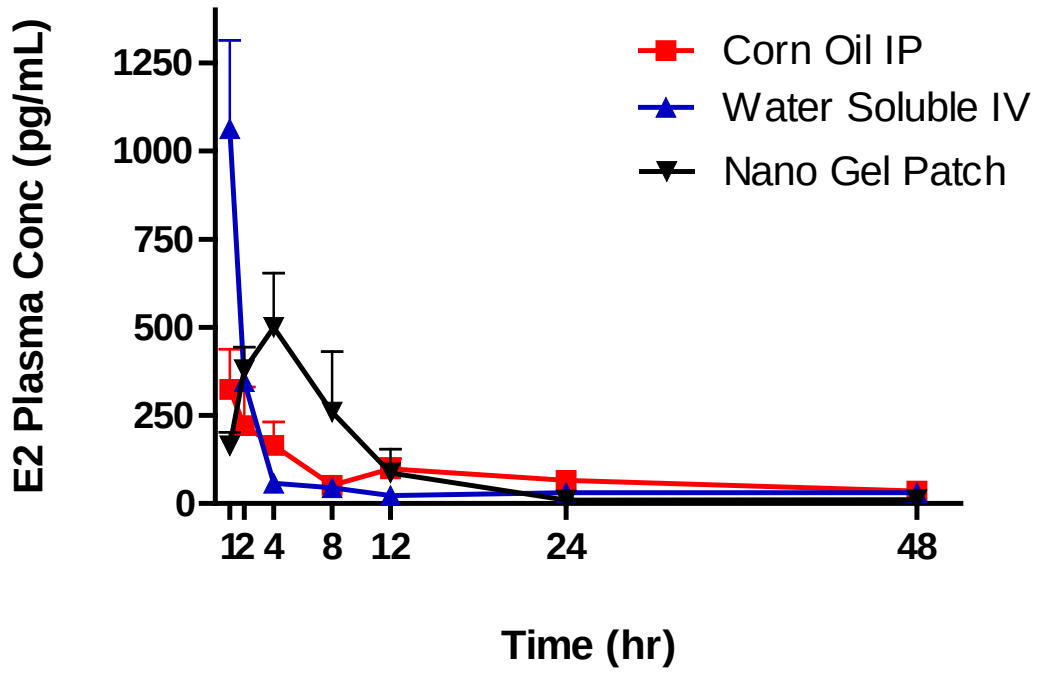


Figure 13. Varying Dose Routes and E2 Time Course Plasma Concentration

All groups dosed with 25 µg E2 delivered by single IP, IV, or nanoparticle embedded gel plug (n=3 per group).

Western Blotting and Immunofluorescence: GDNF

GDNF expression was found to be significantly increased with E2 treatment in the caudal penumbra section of the injured spinal cord. No difference was found between the Injury and Injury + Vehicle treated groups, indicating that the void nanoparticle embedded agarose gel plug did not exert any effects on this marker. There was a slight, although not significant, increase in GDNF in the injury group vs. Sham. Immunofluorescent imaging revealed correlative findings where an increased in immunoreactivity was found in E2 treated sections as compared with vehicle treated.

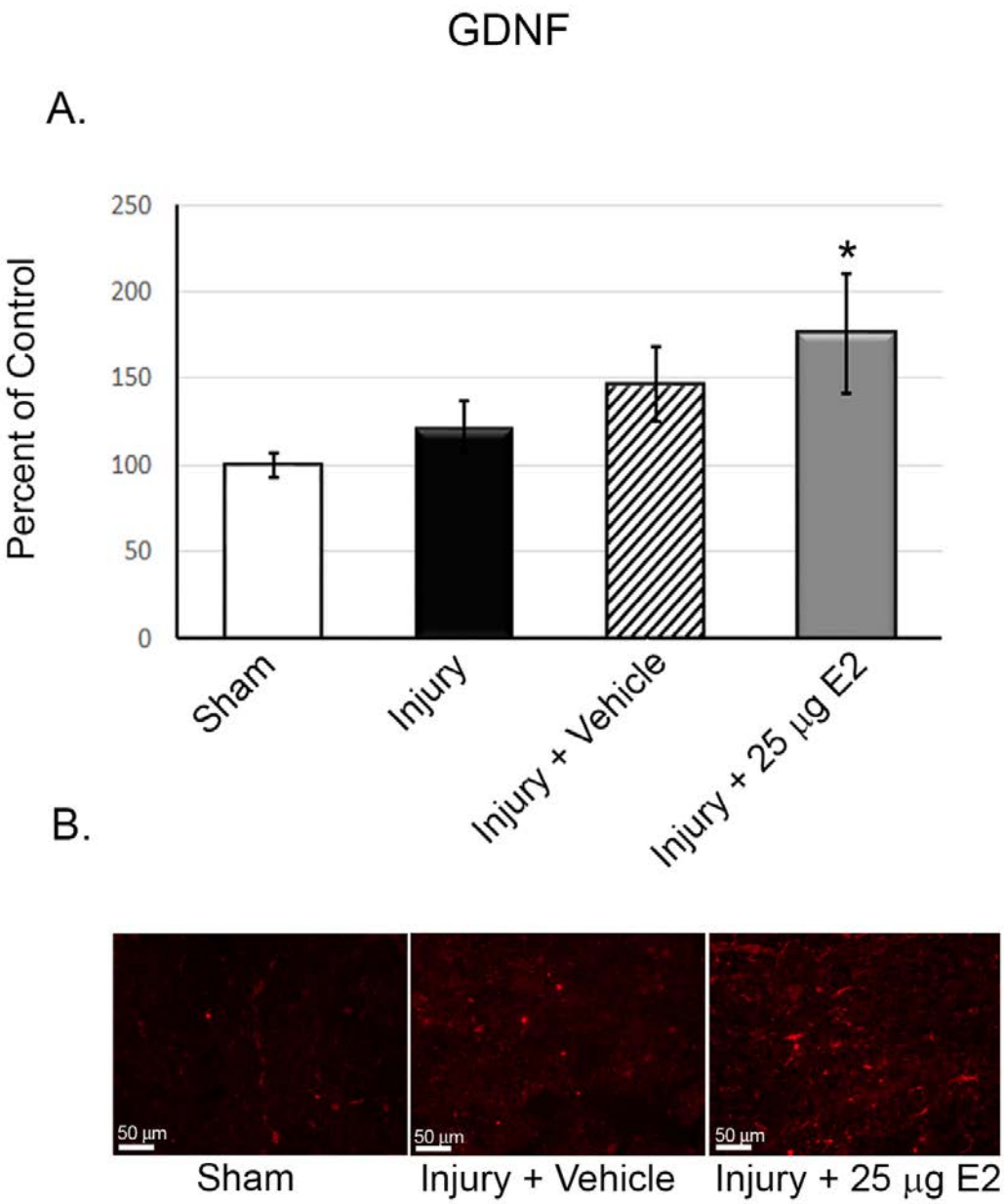


Figure 14. GDNF 48hrs (A). WB of GDNF shows a significant increase in GDNF in E2 treated group. *p<0.05 Injury + E2 nano vs. Injury (B). Immunofluorescence of GDNF imaged in dorsal funinculus (white matter) of caudal penumbra. Images taken at 200x, representative of group average. n=3 per group

Western Blotting and Immunofluorescence: Glutamine Synthase

Glutamine synthase activity was increased with injury and significantly decreased with E2 treatment. No difference was found between the Injury and Injury + Vehicle treated groups, indicating that the void nanoparticle embedded agarose gel plug did not exert any effect on glutamine synthase. Immunofluorescent imaging revealed correlative findings, where an increased in immunoreactivity was found in the Injury and Injury + Vehicle groups as compared with the E2 treated sections.

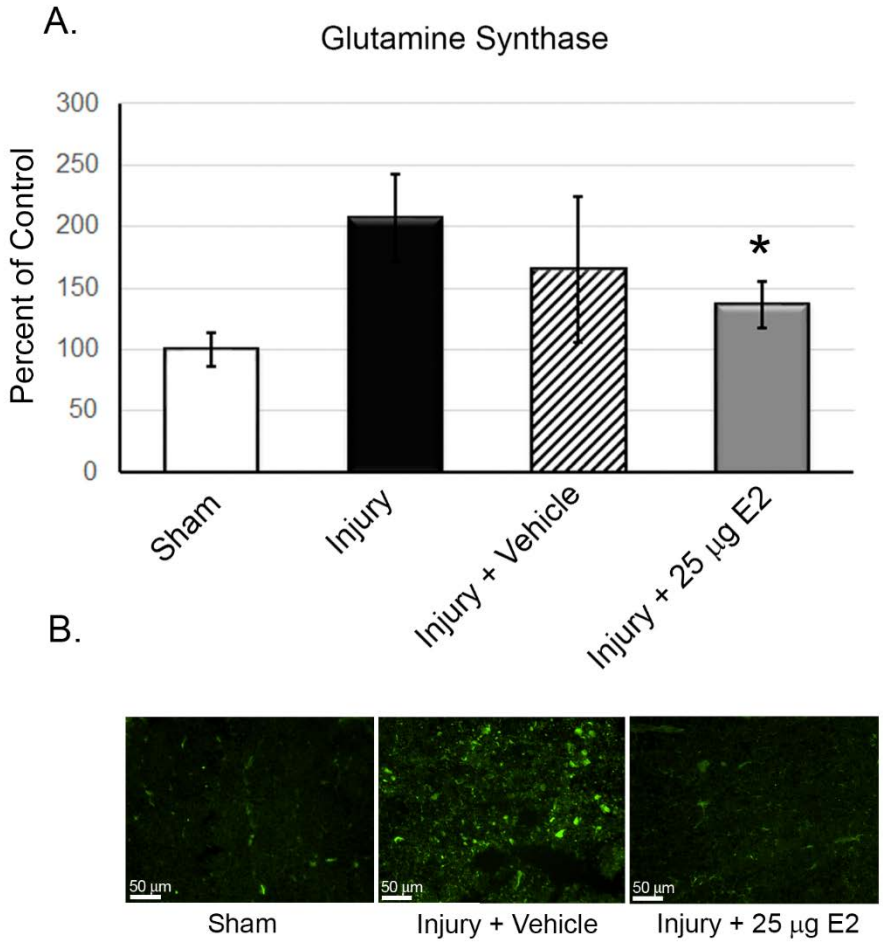


Figure 15. Glutamine Synthase 48hrs (A). WB of glutamine synthase shows a significant decrease in the E2 treated group. *p<0.05 Injury + E2 nano vs. Injury (B). Immunofluorescence images of glutamine synthase in dorsal funinculus (white matter) of caudal penumbra illustrate the increase seen in the Injury Vehicle group vs. E2 and sham groups. Images taken at 200x, representative of group average. n=3 per group

Western Blotting:Cox-2 & Bax/Bcl-2: Cox-2 expression was upregulated nearly 2.5 fold in the Injury group vs. Sham. Cox-2 was significantly decreased with E2 treatment. Bax expression was increased with Injury, and Bcl-2 protein was decreased as compared with sham. The resulting ratio in the Injury and Injury + Vehicle groups is high, illustrating high BAX and low Bcl-2. E2 treatment reduced the amount of BAX and increased Bcl-2, resulting in a ratio below that of sham. Data suggest decreased apoptosis with E2 treatment.

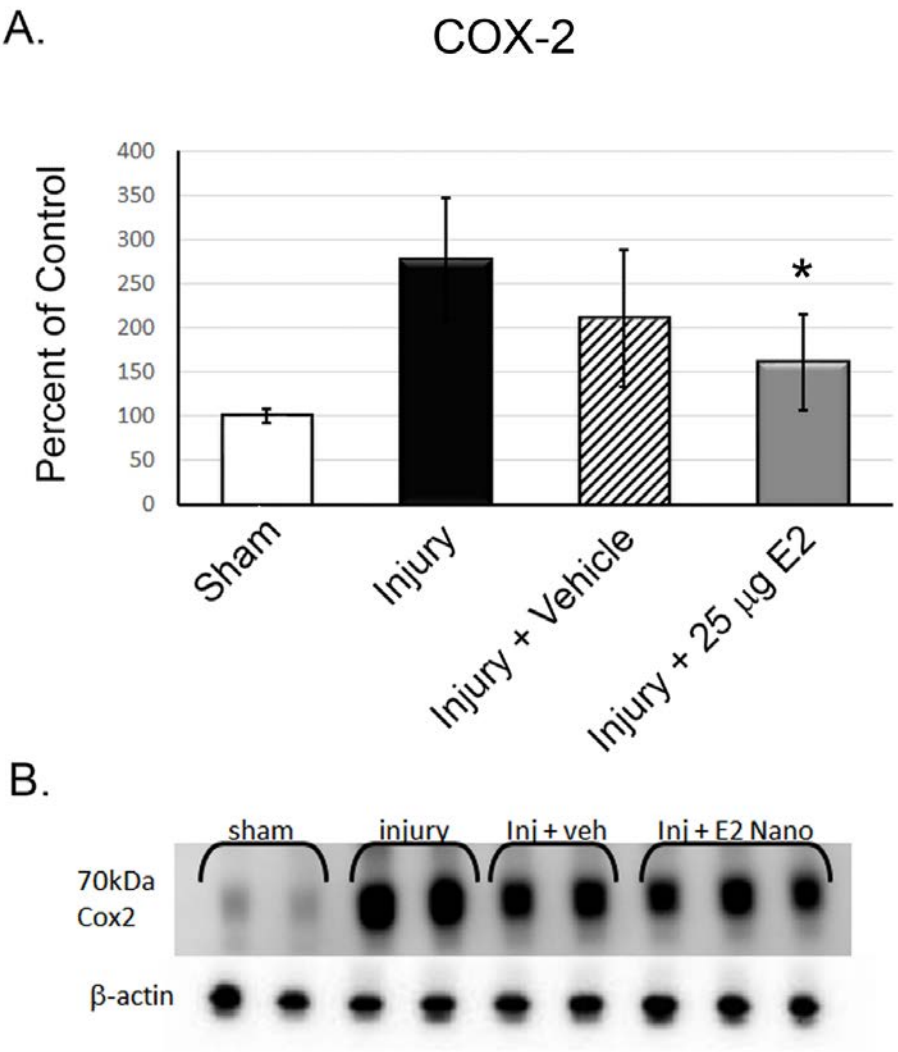


Figure 16. COX-2 48hrs (A). WB of COX-2 shows 2.5 fold increase in the Injury groups vs. Sham. E2 treated group shows significantly decreased COX-2 expression. *p<0.05 Injury + E2 nano vs. Injury (B). Blot image taken of 2-3 representative bands of each group. n=3 in sham group, and n=5 in injured groups

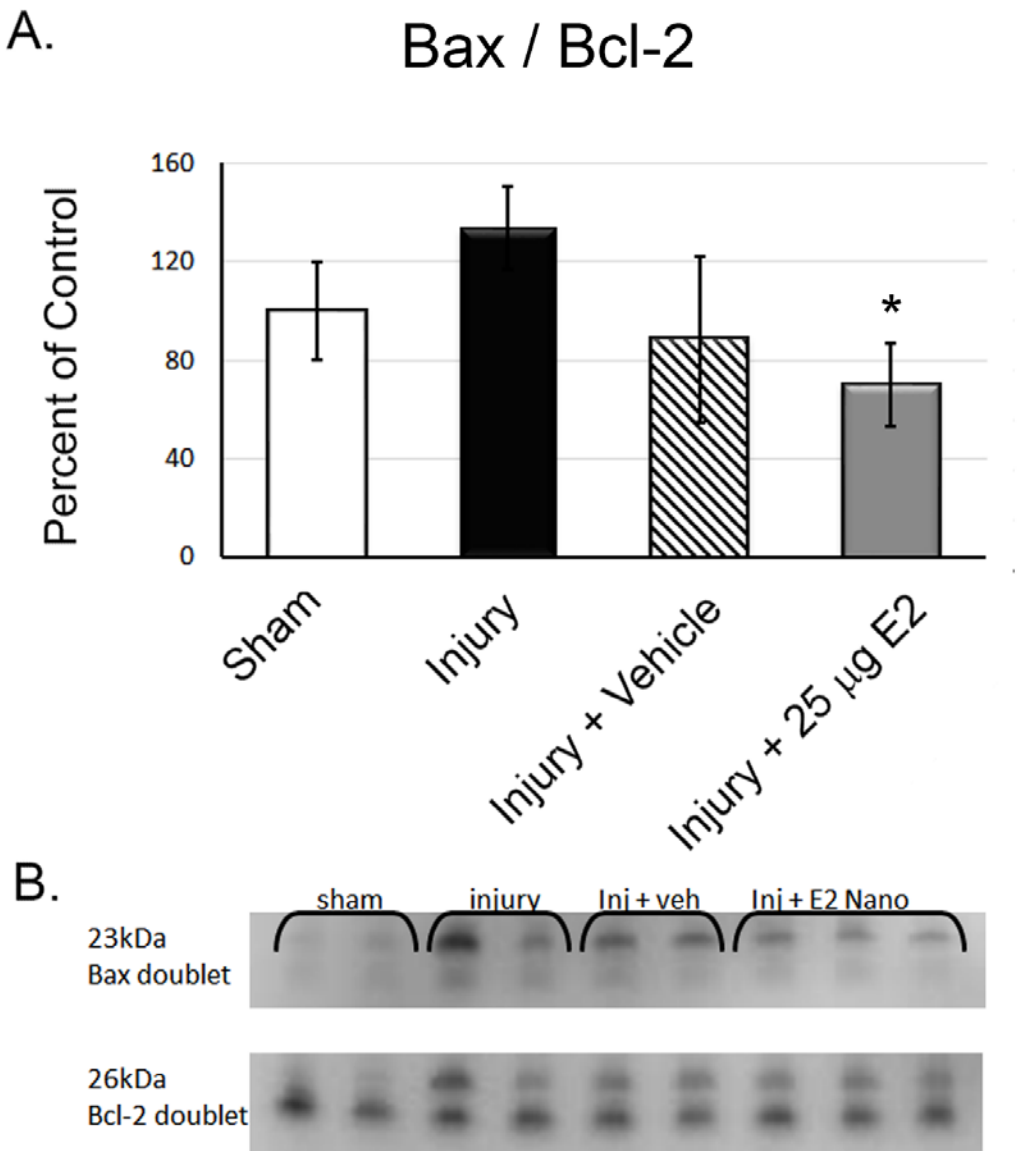


Figure 17. Bax / Bcl-2 48hrs (A). WB of Bax / Bcl-2 ratio shows 30% increase with injury and a 30% reduction below sham treatment with E2 treatment. *p<0.05 Injury + E2 nano vs. Injury (B). Blot image taken of 2-3 representative bands of each group. n=3 in sham group, and n=5 in injured groups

Immunofluorescence Analysis: GFAP

GFAP staining was increased in all Injury groups and found to be significantly decreased at both doses of E2. Additionally, distinct differences were observed in the morphology of the reactive astrocytes. An abundance of hypertrophied astrocytes with large swollen processes were seen in the Injury + Vehicle groups, as compared with E2 treated. It should be noted, however, that hypertrophied astrocytes were also seen in the E2 groups, although not as prevalent as in the Injury + Vehicle groups.

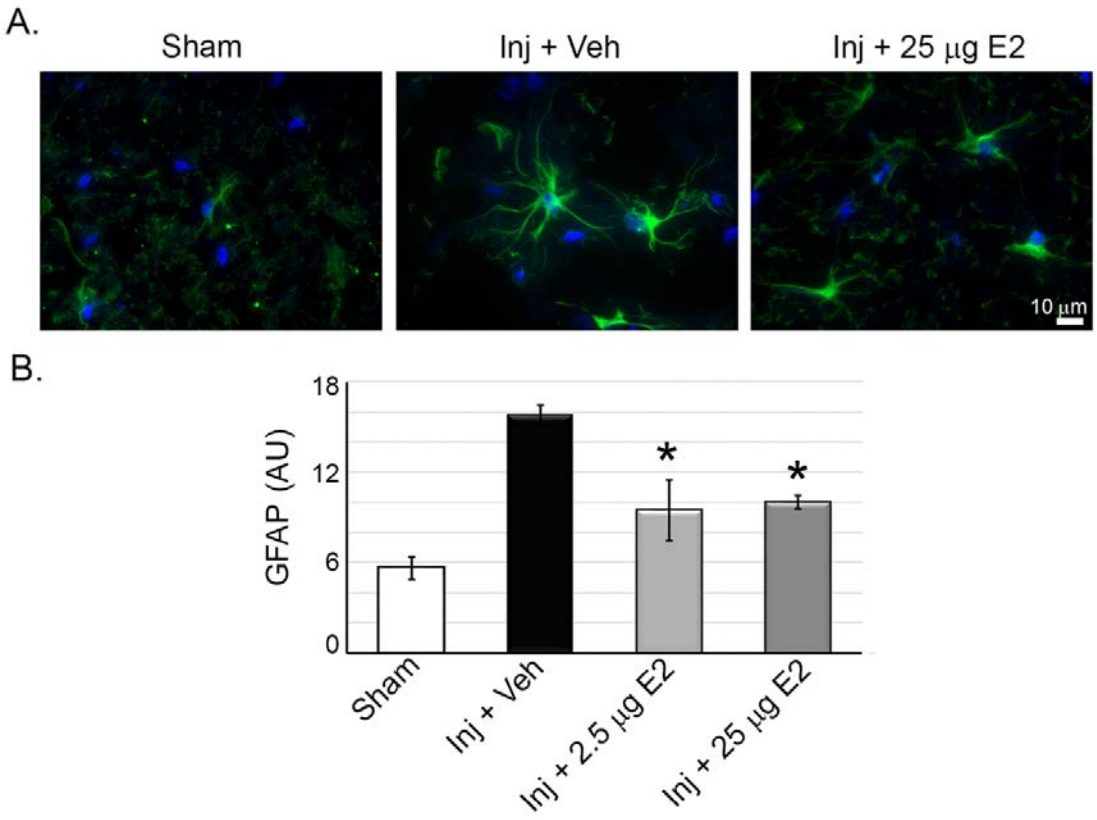


Figure 18. GFAP Immunofluorescent Staining (A). GFAP (green) and DAPI (blue) nuclear staining of astrocytes in the dorsal funinculus. Panel of representative images taken at 60x magnification. (B). Quantification of fluorescent staining intensity *p< 0.05 E2 group vs. Inj + Veh. n=3 per group

Immunofluorescence Analysis: S100β

S100β staining was increased in all Injury groups and found to be significantly decreased at the low dose of E2. The high dose of E2 trended to decrease S100β immunoreactivity. It is unclear as to why the high dose did not exert more of an effect on S100β.

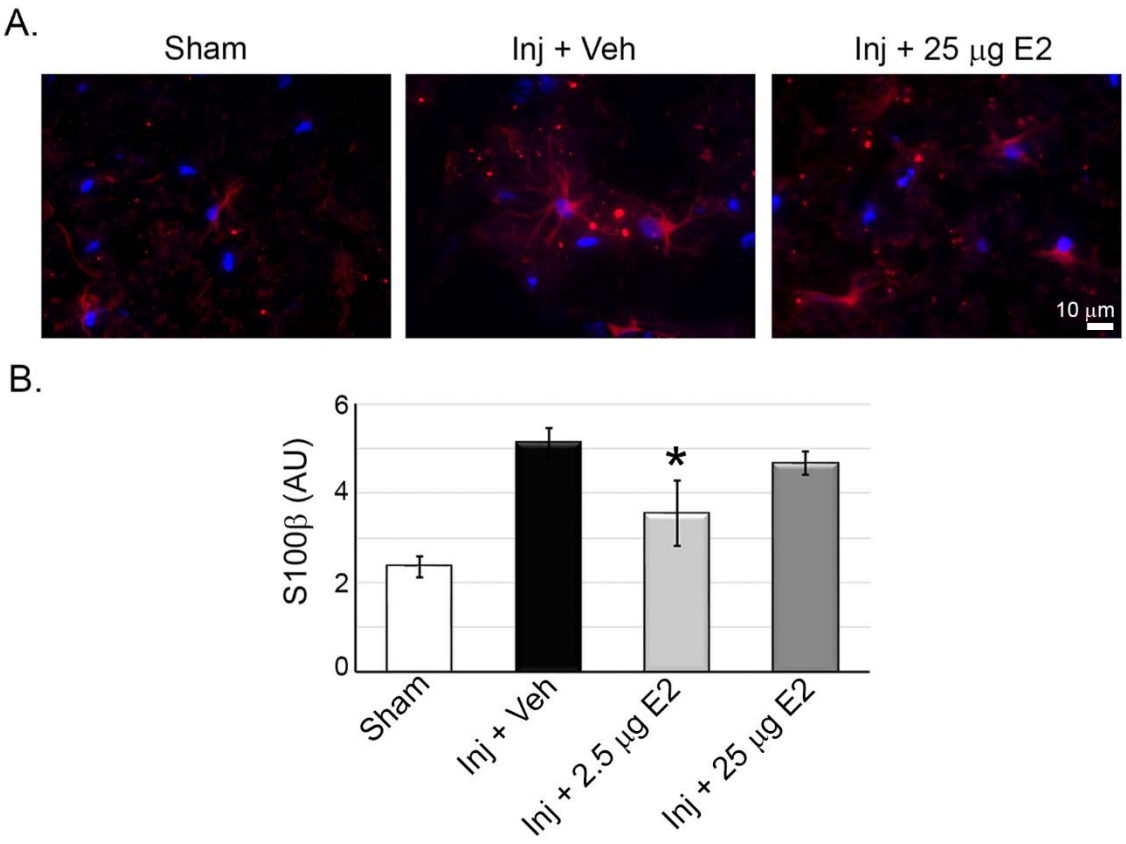


Figure 19. S100β Immunofluorescent Staining (A). S100β (Pink) and DAPI (blue) nuclear staining of astrocytes in the dorsal funinculus. Panel of representative images taken at 60x magnification (B). Quantification of fluorescent staining intensity *p< 0.05 E2 group vs. Inj + Veh. n=3 per group

Immunofluorescence Analysis: Iba-1

Staining of Iba-1 was increased in all Injury groups, and trended to be decreased with E2 treatment. The lack of significance in both groups may be attributed to high variance in the Injury + Vehicle group, and given this images were not captured.

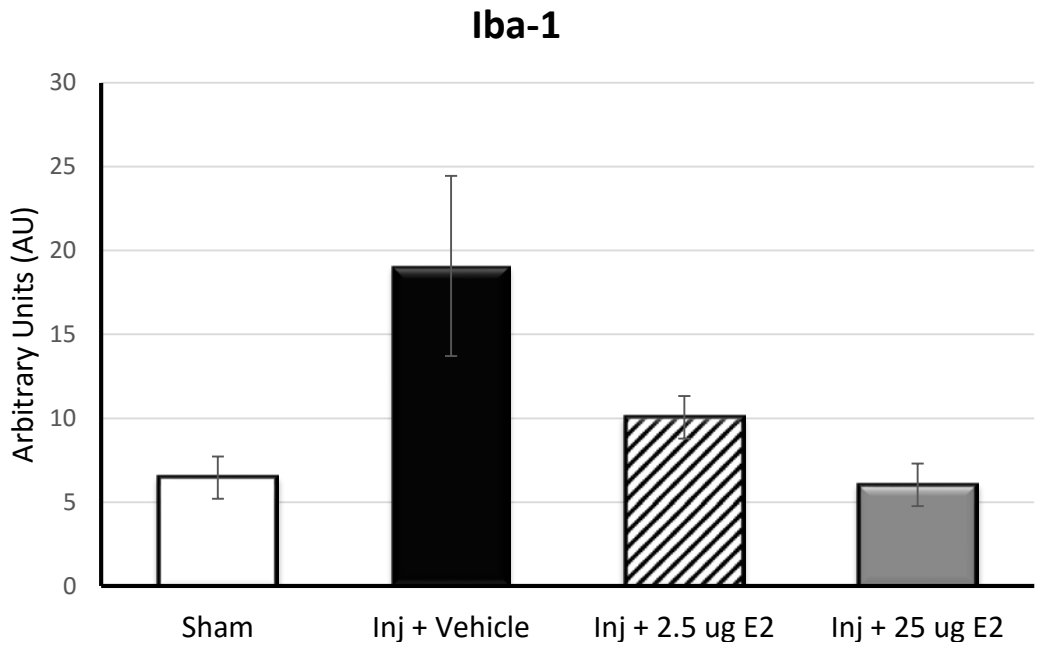


Figure 20. Immunofluorescence analysis of Iba-1. Iba-1, an activated microglia marker, shows elevation with Injury + Vehicle group, and trended for decrease with E2 treatment. n=3 per group

Immunofluorescence Analysis: ED-1

Staining of ED-1 was increased in the injured groups as compared with sham. Increased ED-1 positive cells are expected in lesion tissue at 48 hrs. E2 treatment at either dose significantly decreased ED-1 staining, indicating a decrease in either infiltrating macrophages or resident tissue macrophages.

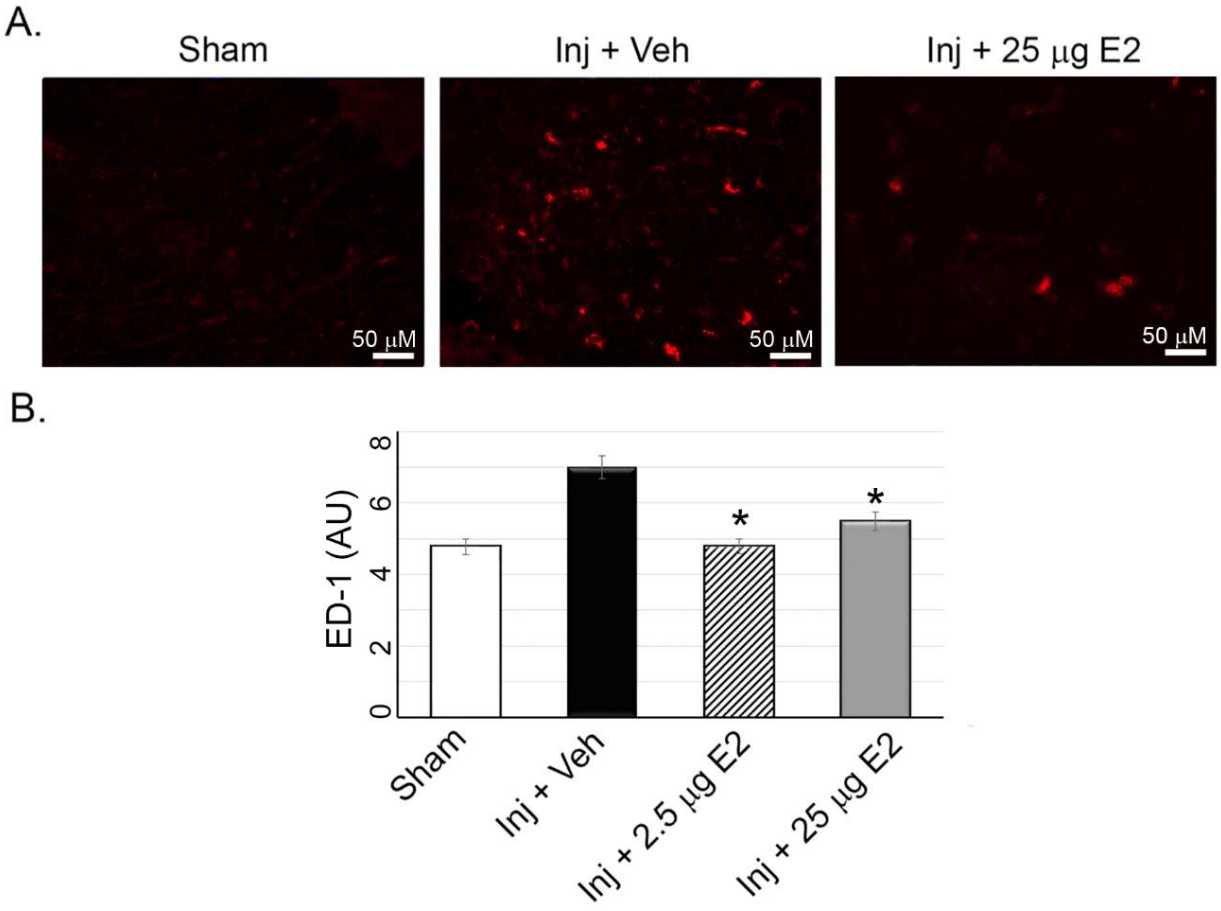


Figure 21. ED-1 Immunofluorescent Staining (A). ED-1 positive (red) macrophage staining in dorsal funinculus. (B). Immunofluorescence analysis of ED1. * $p<0.05$ E2 treated groups vs. Inj + Veh. n=3 per group

DISCUSSION:

Dose response time course studies measuring E2 levels in plasma revealed an increased concentration in the 25 μg dose group as compared to the 2.5 μg dose group. The maximum concentration was nearly 3X higher in the high dose group than the low at the Cmax (Figure 12). Given the data collected at 6 hrs suggested dose linearity, we had hypothesized dose linearity (10X difference in concentration) throughout this time course study. However, a range between 2x-3x increased E2 plasma concentration in the high dose group was observed. Also, it is important to note that at the 2.5 μg dose maximal plasma levels remained below those reported to occur in normal rat physiology (approximately 300pg/mL in pregnant rat) (Saddick 2014). Given that the 25 μg dose group showed supraphysiological E2 plasma concentration, this dose will no longer be investigated as the goal of this work is to maintain safe, physiologically relevant dosing of E2.

Dose administration studies highlighted the differences in plasma levels of E2 resulting from different dosing routes. IV dosing results in very high peak plasma exposure (1064 pg/mL, Figure 13). Arguably, this value may be much higher, as the first time point sampled was at 1HR. IV dosed drugs typically have a Cmax that occurs nearly instantaneously, as any drug that is predominately metabolized through the liver will undergo extensive first pass metabolism as the blood first clears the liver. Avoiding high E2 plasma

concentrations is critical in the treatment of SCI, in order to minimize risk of thromboembolism. Higher doses of E2 used for contraception have been shown to correlate with increased risk of stroke, and transdermal delivery of E2 confers lower risk of stroke due to lower maximal blood E2 concentration (Speroff 2010).

Intraperitoneal dosing of E2 in corn oil resulted in very low Cmax (324 pg/mL) and rapid clearance where levels dropped to physiological concentrations between 4 and 8 hours (Figure 13). Nanoparticle delivery of E2 resulted in moderate Cmax (500 pg/mL) and a slower clearance, as demonstrated by the plateau shape of the curve (Figure 13). Of particular note is the change in Tmax between dose groups. Both the IV and IP groups have a Tmax of 1 hr while the nanoparticle group has a Tmax of 4hrs (Figure 13). This highlights the potential of nanoparticles to slowly release E2, thereby allowing for extended plasma exposure while avoiding a high Cmax.

The glial cell response to injury has been shown to be modified by E2 treatment. The neurotrophin GDNF has been suggested in the literature to be affected by E2 (Chen, Yeh et al. 2010, Campos, Cristovao et al. 2012, Xu, Bi et al. 2013). Here, we report an upregulation of GDNF 48 hrs after E2 treatment (Figure 14). Using immunofluorescence we found GDNF positive cells in the dorsal funinculus of the spinal cord, in a mixed glial cell population. Increased GDNF production may contribute to neuronal survival since GDNF injected into lesioned spinal cord tissue was reported to result in increased neuronal survival through activation of mitogen-activated protein kinase pathway and upregulation

of the anti-apoptotic protein Bcl-2 (Cheng, Wu et al. 2002). Here, we also report an increase in Bcl-2 (Figure 17) that coincides with an increase in GDNF expression (Figure 14). A study has shown that administration of exogenous Bcl-2 resulted in increased neuronal survival (Nesic-Taylor, Cittelly et al. 2005). These data support the hypothesis that E2 is acting as a neuroprotectant through mechanisms including GDNF upregulation of Bcl-2.

Reactive gliosis has been described as "a constitutive, graded, mulit-staged, evolutionarily conserved defensive astroglial reaction" (Pekny, Wilhelmsson et al. 2014). The biological response is known to be both beneficial and maladaptive, especially in SCI where reactive gliosis causes glial scarring and prevention of regeneration of axons across the scar. However, the complete removal of reactive gliosis, as shown in various transgenic models, has been shown to be detrimental, as reviewed elsewhere (Sofroniew 2009). Thus, a modulation of gliosis may be preferential. As GFAP and S100β are two of the hallmarks of gliosis, both were examined in astrocytes in the dorsal funinculus of the caudal penumbra (Figures 18 & 19). Both markers were significantly decreased with E2 treatment, however, E2 treatment at either high or low dose did not completely restore levels to sham. E2 treatment therefore only dampened gliosis, potentially allowing for improved recovery. Activated astrocytes are one cell type that can produce the extracellular matrix protein chondroitin-sulfate proteoglycan (CSPG) that serves to inhibit axonal regeneration following SCI (Bradbury, Moon et al. 2002). If E2 treatment can

reduce astrogliosis, less CSPG will be deposited around the lesion and may result in greater regeneration of axons and improved functional recovery. This may be examined in a chronic model of SCI.

Glutamine synthase is another important marker of astrogliosis, and an indirect measure of neuronal glutamate signaling and excitotoxicity (Benton, Ross et al. 2000). This enzyme was significantly decreased with E2 treatment, as shown by both Western blot and representative immunofluorescent image (Figure 15). E2 treatment has been reported to upregulate glutamine synthase expression in the hippocampus of non-injured mice (Blutstein, Devidze et al. 2006). These findings suggest glia may be responsive to endocrine signaling control in neurotransmission, however, regulation in pathological states such as those we are investigating in SCI have not yet been reported. More than one potential mechanism may exist to explain these findings as E2 has also been reported to decrease glutamate induced excitotoxicity through interaction with the L-type voltage gated calcium channel in an *in vitro model (Sribnick, Del Re et al. 2009)*. Given this, E2 may be decreasing glutamine synthase by initially acting to decrease glutamate release from nearby neurons. Alternatively, as glial cells are known to express E2 receptors (Santagati, Melcangi et al. 1994) , the effect on glutamine synthase may be receptor mediated.

In addition to astrocytes, microglia, also contribute to reactive gliosis in SCI. Activated microglia are characterized by the cellular marker Iba-1, a calcium binding protein discussed previously (Ito, Imai et al. 1998), and by ED-1

(CD68), a classic macrophage marker. Immunostaining revealed increased Iba-1 and ED-1 staining in injury animals and decreased staining in E2 treated animals (Figure 20 & 21). ED-1 is a marker that will label both infiltrating monocytes expressing CD68 as macrophages as well as tissue resident macrophages such as microglia. MCP-1, as discussed above, is a chemokine that is released from damaged tissue to signal the infiltration of monocytes from the periphery. At 6hrs post injury E2 treatment significantly reduced MCP-1 levels in both CSF (Figure 8) and plasma (Figure 10). In the present study, E2 treatment significantly reduced ED-1 positive staining, suggesting that the early reduction of chemokines has resulted in less monocyte infiltration / macrophage activation.

Activated microglia may further contribute to local inflammation by release of factors such as COX-2 and iNOS. E2 treatment significantly decreased COX-2 expression in whole spinal cord lysates (Figure 16), and Western blot analysis (Data not shown) also revealed a non-significant decrease in iNOS expression. COX-2 has been shown to be expressed in rat microglial cells following nerve injury (Durrenberger, Facer et al. 2004). COX-2 expression has also been shown in neurons and epithelial cells (Schwab, Brechtel et al. 2000, Lau, Wong et al. 2014). Given multiple cell types may contribute to COX-2 levels in the injured spinal cord, the exact contribution of microglia derived COX-2 is yet unknown.

In summary, nanoparticle delivered E2 revealed a distinct plasma concentration profile from IV or IP dose routes. Nanoparticle dosing resulted in delayed Tmax and decreased Cmax with prolonged elevated plasma concentrations. This profile may prove to be more efficacious as it allows for E2 to exert neuroprotective effects for longer time. Additionally, as high Tmax is avoided, nanoparticle delivery may provide a safer dosing strategy if applied clinically.

Using an intermediate time model of 48 hrs the effects of E2 on the glial cell response can be examined. E2 exerted multiple effects on glial cells, including upregulation of GDNF, downregulation of the proinflammatory protein COX-2, decreased reactive gliosis (glutamine synthase, GFAP, S100β), and decreased microglia/macrophage activation (Iba-1 and ED-1). Taken together, these findings suggest that at the protein level E2 may be exerting neuroprotective effects through modification of the glial response. Reduction of the glial response in the intermediate time period maybe critical to lessening glial scarring in the chronic phase. Decreased astrogliosis has been shown to result in decreased CSPG (inhibitor of axonal regeneration) production and glial scarring (Li, Li et al. 2014). As numerous cell types express CSPG, oligodendrocyte precursor cells, macrophages, and other invading immune cells (McTigue, Tripathi et al. 2006) an approach that globally decreases astrogliosis as well as infiltrating immune cells is warranted. The data presented here, support the hypothesis that E2 may decrease glial scarring through modulating

infiltrating immune cells and glial response at the intermediate time phase. The question remains, however, if nanoparticle delivered E2 as a single low dose may drive improved locomotor functional recovery. These findings support the evaluation of this novel E2 dosing approach in a functional model of SCI.

CHAPTER FOUR

E2 NANOPARTICLES IN A FUNCTIONAL RECOVERY MODEL

Introduction

In Chapters two and three E2 treatment was shown to exert a number of biological effects in a peracute and intermediate model of SCI. The peracute model illustrated the potential for E2 to act as a rapid anti-inflammatory through modulation of cytokines in both systemic circulation as well as in spinal cord tissue. The intermediate model revealed the potential for E2 to exert extended biological effects after only a single administration through decreased astrogliosis as well as additional protein changes that suggest potential for enhanced recovery. While these early findings are promising, the goal of enhanced functional recovery remains. Hence, the focus of this specific aim will be evaluation of E2 nanoparticles in a functional recovery model.

To examine motor function, two techniques will be used - the Basso, Beattie, and Breshnahan (BBB) rating scale for locomotion, and the DigiGait platform that utilizes ventral plane videography paired with digitized image analysis. The BBB scale is a 21 point scale that scores locomotor function of rats in an open field and has become a standard endpoint in SCI models for nearly two decades (Basso, Beattie et al. 1995). The DigiGait platform is a relatively new technique whereby a video camera mounted below a transparent walking belt captures high resolution video of a rodent walking. Gait signals are generated through the measurement of paw area on the belt over time. A step cycle is

comprised of two phases, the stride and stance phase. These phases are further broken down into swing phase (time in which paw area is not in contact with the belt), propulsion phase (paw area decreasing over time) and braking phase (paw area increasing over time). These gait signals are analyzed for 27 different metrics of gait such as gait symmetry, hind paw area, swing to stance ratio, and hind paw rotation which have been reported in the rat SCI literature (Springer, Rao et al. 2010, Zhang, Bailey et al. 2015).

Functional recovery may also be assessed by neurogenic bladder recovery. Normal bladder voiding requires the coordination of relaxation of the external uretheral sphincter and contraction of the bladder detrusor under control of spinal circuitry (de Groat 1998). Following injury to the thoracic region of the spinal cord, the degree of impairment of bladder voiding is correlated with white matter loss in regions involving urination-related tracks (Pikov and Wrathall 2001). A study has shown that a positive correlation exists between severity of injury and functional deficits of the bladder in rats (Ozsoy, Ozsoy et al. 2012). Reduction of GFAP expression has been shown to improve neurogenic bladder recovery, as measured through spontaneous voiding restoration (Toyooka, Nawashiro et al. 2011). Given these findings, we hypothesize that E2 treatment may result in earlier recovery of spontaneous bladder voiding.

Pathological changes to the spinal cord will also be assessed through histological approaches. The formation of the wound cavity following SCI is one of the primary barriers to regeneration. This cavity is surrounded by fibrotic scar tissue, comprised of glial and fibrotic components such as fibronectin and CSPGs, and serves to prevent regeneration of axons across the lesion. Decreasing wound cavity size and preservation of surrounding white matter tracts would serve to lessen neurological impairment. To investigate how E2 treatment may modulate wound cavitation and white matter sparing, Luxol fast blue (LFB) stain for myelin will be conducted. Spinal cords will be collected and sectioned in a sagittal orientation, which will allow for a qualitative assessment of the wound cavity size as well as visualization of white matter sparing.

MATERIALS AND METHODS

Nanoparticles and Gel Plug Delivery System: Techniques will be the same as described in Chapter 2 with the addition of a 5.0 μg dose group.

Rat SCI Model & Bladder Function: Induction of injury and treatment with E2 nanoparticle will be conducted as described for the 6hr & 48 hr models, with the exception being time point for sacrifice will be extended to 28 days. Body weight will be taken weekly. Bladders will be manually expelled 2-4 times daily. The day in which an animal regains spontaneous voiding upon the first morning inspection

of the animals will be recorded. For sham group, n=5, Inj + Veh n=10, Inj + 2.5 μg E2 n=8, Inj + 5.0 μg E2 n=10.

Tissue Collection and Analysis: On Day 28 rats were deeply anesthetized under ketamine / xylazine IP injection and euthanized via decapitation. Spinal cord samples were dissected, and a 1.5 cm piece was collected and divided into 0.5 cm caudal, 0.5 cm lesion epicenter, 0.5 cm rostral to lesion. The tissue was placed into cassettes and then into 4% neutral buffered formalin for 72 hours at room temperature. Tissue was removed, paraffin embedded, and sectioned at 5 μM thickness. Slides were then processed for routine H&E staining. In brief, slides were (1). deparaffinized (2). Rehydrated (3). Hematoxylin staining 5 min (4). 1% Acid Alcohol 1 dip (5). 1% Ammonia Alcohol 10 dips (6). Eosin staining for 10 sec (7). EtOH (8). Xylene and coverslipped. Luxol fast blue (LFB) staining was conducted for detection of myelin. In brief, slides were (1). Deparaffinized (2). LFB stained at 60ºC for 2 hrs (3). EtOh (4). 0.05% lithium carbonate for 20 sec (5). Differentiated in 70% EtOH and then followed up with H&E staining.

Assessment of Locomotor Function: BBB Animals were assessed for locomotor function using the BBB scale beginning 24 hours after surgical induction and then once weekly for 4 weeks as previously described (Basso, Beattie et al. 1995). Scoring was performed by an experienced laboratory technician who was blinded to treatment group. Statistical differences were determined by a Student's T-test for each week.

DigiGait Analysis: On Day 28 animals were recorded on the DigiGait treadmill system. Briefly, animals were placed on the belt and allowed to acclimate for 2 minutes at a low speed, 5 cm/sec. The speed was then increased to 20 cm/sec and 1000 frames were captured, representing approximately 6 full step cycles. Videos were analyzed on the DigiGait Analysis software package (Mouse Specifics, Quincy MA) for 27 different indices of gait function.

In Vivo: Plasma and Spinal Cord Collection 48 hr profile study

A plasma and spinal cord profile study was conducted using the 5.0 μg E2 dose. Whole blood drawn via tail vein puncture was collected into EDTA containing tubes (final concentration EDTA 1.5 mg/mL). Samples were collected at 1, 2, 4, 8, 12, 24, and 48 hrs following drug administration. Animals were serially bled, n=3 per group, such that each animal underwent 6 tail vein bleeds and one final cardiac stick. Whole blood samples were spun at 10,000 RPM in a Sorvall Legend Micro21 bench top centrifuge, and plasma was collected and stored at -80°C. 17-β estradiol (E2) concentration was determined using a commercially available ELISA kit (Calbiotech Estradiol ELISA ES180S), and kit directions were followed exactly.

Cytokine Profiling: Plasma samples were collected at 24 and 48 hours from all animals on the 28 day study. Samples were stored at -80°C and then shipped on dry ice to Eve Technologies. Samples were run undiluted on the rat cytokine/chemokine 27-plex discovery assay.

RESULTS

Body Weight: Body weight measurements revealed similar changes between all injury groups. Animals lose between 5-10% body weight in the first week following surgery and then slowly regained the weight, ending the study with approximately a 20% weight gain from their starting weight.

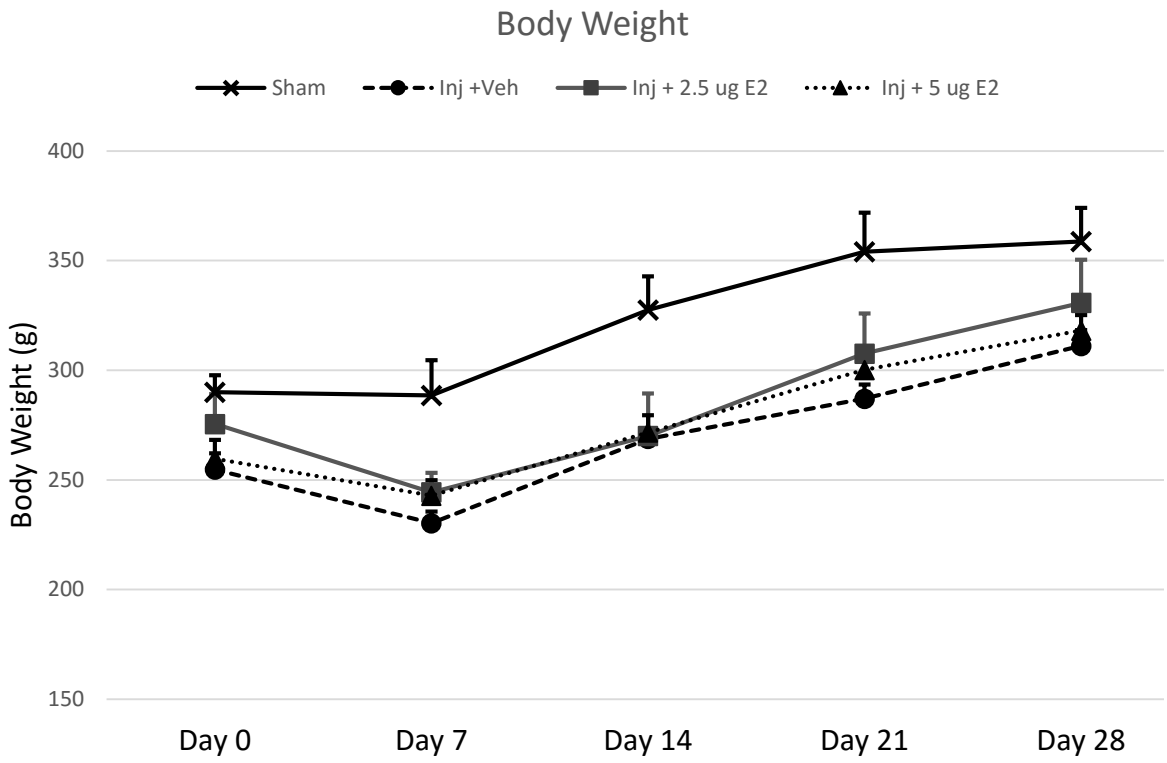


Figure 22. Body Weight 28 the Chronic Model Body weight changes in chronic 28 day SCI model. No significant changes in body weight between the injury groups. n=4 sham, n=8 Inj + 2.5 μg E2, n=10 Inj + Veh and Inj + 5.0 μg E2 groups

Bladder Function Recovery:

Bladder function recovery is defined here as the ability to spontaneously void the bladder. The day in which this behavior is restored indicates the animal no longer needs to be manually expressed. Both the Injury + Veh and Inj + 2.5 ug E2 groups took an average of 9.7 ± 0.6 days to spontaneously void bladders. The Injury + 5.0 ug E2 group took an average of 8 ± 0.5 days to spontaneously void bladders.

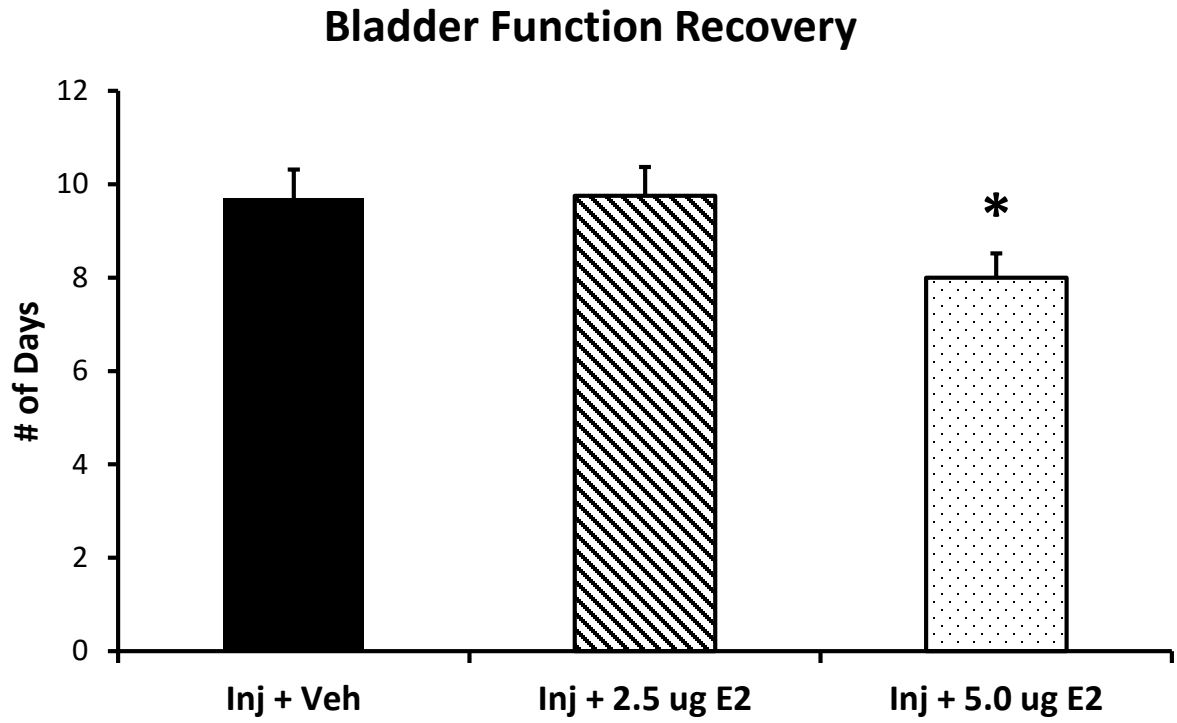


Figure 23. Bladder Function Recovery in the Chronic Model. Inj + Veh and Inj + 2.5 ug E2 groups took 9.7 days to recover spontaneous bladder voiding, while Inj + 6.0 ug E2 group took 8 days. * $p<0.05$ Inj + 5.0 ug E2 vs. Inj + Veh group

BBB Locomotor Recovery Assessment: BBB scoring revealed no difference between the Inj + Veh group and the Inj + 2.5 μg E2. There is significant improvement in BBB score on Day 7 between the Inj + Veh group and the Inj + 5.0 μg E2. There is a trend towards improved BBB score for days 14, 21, and 28.

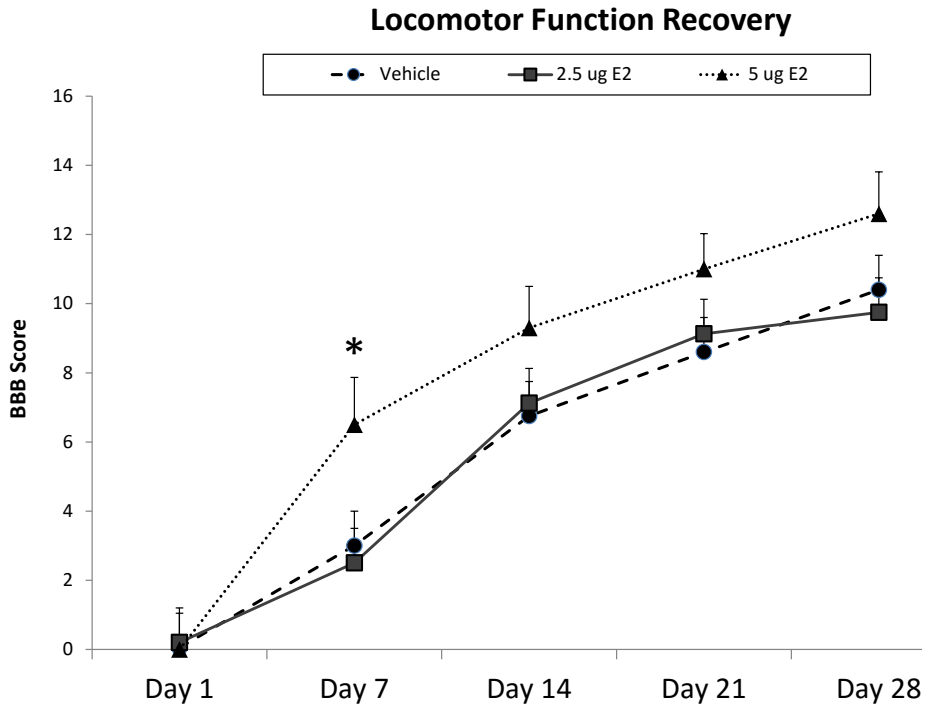


Figure 24. BBB Assessment in the Chronic Model. Significant improvement in BBB score is found in the Inj + 5.0 ug E2 group, as compared with Inj + Veh on Day 7. Sham animals, by criteria, are all scored a 21 at each time point and are therefore not displayed. * $p<0.05$ Inj + Veh vs. Inj + 5.0 ug E2. n=4 sham, n=8 Inj + 2.5 μg E2, n=10 Inj + Veh and Inj + 5.0 μg E2 groups

DigiGait Analysis: Stride Phase The stride phase of gait consists of three phases: propulsion, brake, and swing. Sham animals, representing normal gait, display a stride that is comprised of: 63% propulsion, 15% brake, and 23% swing phases. The Inj + Veh group had a stride phase comprised of 50% propulsion, 39% brake, and 11% swing phases. The brake phase or time in which the hindlimb is in contact with the belt is nearly three times as long in the injuried animals as in shams. This illustrates the inability of the animal to properly initiate movement rather the animal's hind limbs remain in contact on the belt without movement. The swing phase or phase in which hindlimb is not in contact with the belt, is 50% reduced in the injuried animals. Inj + 5.0 ug E2 animals showed a more normalized stride phase comprised of 50% propulsion, 30% brake, and 20% swing phases. E2 treated animals have a significant reduction in both brake and swing phases as compared with Inj + Veh groups, representing a more normalized gait.

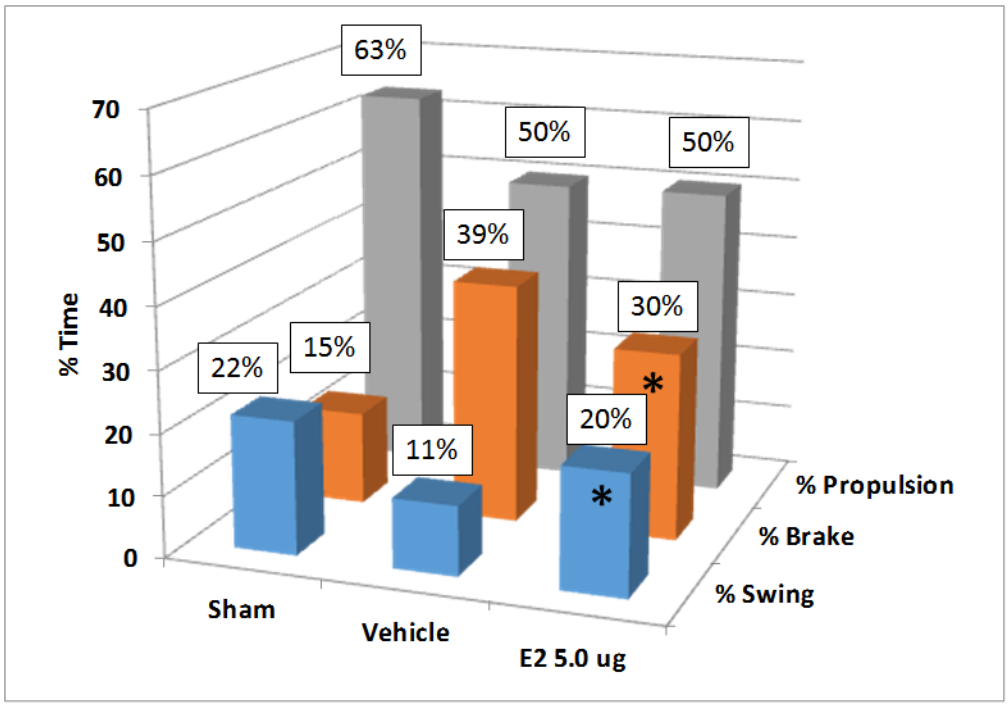


Figure 25. DigiGait Stride Analysis in the Chronic Model. DigiGait stride phase analysis shows the effect of SCI in the vehicle group with decreased propulsion and swing phases and increased brake phase as compared with sham controls. E2 treatment resulted in a significant decrease in both the brake and swing phases. * $p<0.05$ Vehicle vs. E2 5.0 μg E2 groups. n=4 sham, n=10 Inj + Veh and Inj + 5.0 μg E2 groups

DigiGait Analysis: Stance Phase The stance phase of gait is divided into two phases, the brake and the propulsion phase. Sham animals display a stance phase that is 81% propulsion and 19% brake. The propulsion phase is defined as decreasing paw area contact on the belt over time, such as the motion of a foot rocking from heel to lift off at the toe. Injury + vehicle animals display a stance phase that is 56% propulsion and 44% brake. This reduction in propulsion phase and increase in brake phase is reflective of their hind limb paralysis. E2 treatment significantly decreased the brake phase and trended towards increasing the propulsion phase (38% brake and 62% propulsion phase).

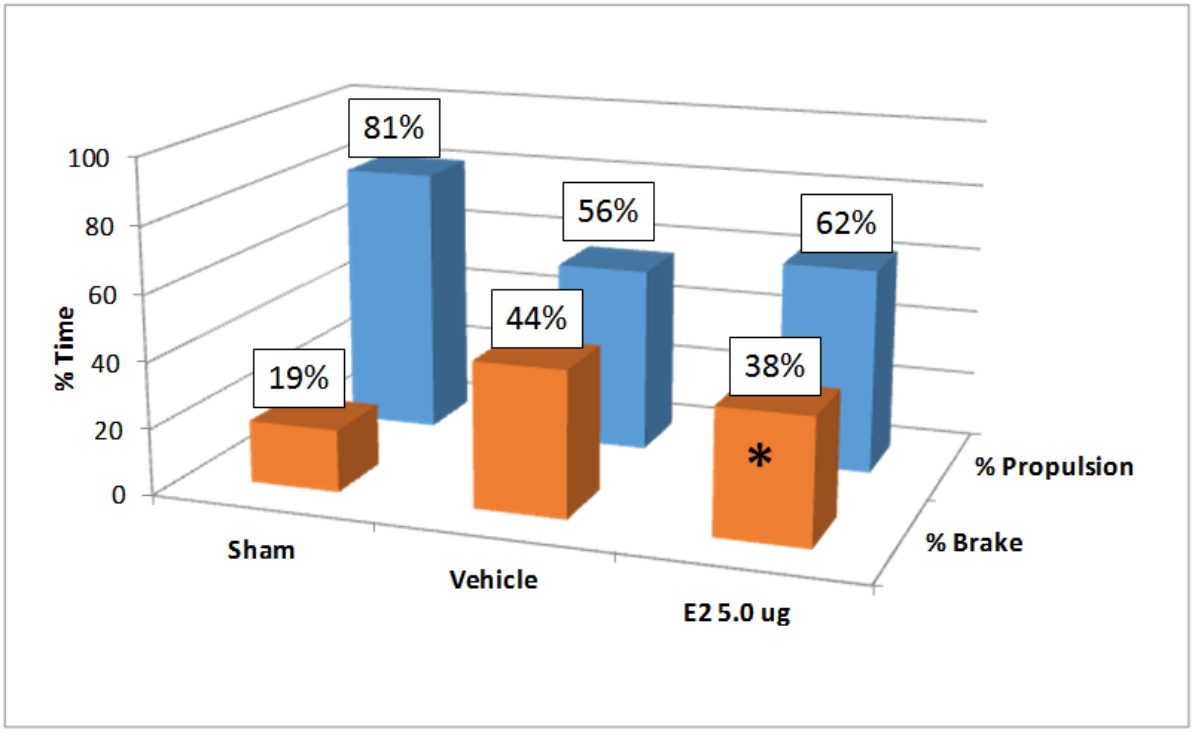


Figure 26. DigiGait Stance Analysis in the Chronic Model. DigiGait stance phase analysis shows the effect of SCI in the vehicle group with decreased propulsion and increased brake phase, as compared with sham controls. E2 treatment resulted in a significant decrease in the brake phase. * $p<0.05$ Vehicle vs. E2 5.0 μg E2 groups. n=4 sham, n=10 Inj + Veh and Inj + 5.0 μg E2 groups

DigiGait Analysis: Hind-limb shared stance, gait symmetry, and stride frequency

Additional parameters of gait were found to be significantly affected by E2 treatment. Hind-limb shared stance time is defined as the time that both hind paws are in contact with the belt. This measure may indicate balance, as an increased shared stance time is seen in unstable animals. Inj + Veh animals have an increased shared stance time, and E2 treatment significantly decreased shared stance time indicating a more stable posture. Gait symmetry is measured by forelimb step frequency to hindlimb step frequency ratio. In spinal cord injured animals, the loss of hindimb function results in a higher ratio, indicting more steps are being taken by the forelimbs. Treatment with E2 resulted in a significant decrease is the gait symmetry index, illustrating improved hindlimb function. Finally, stride frequency is number of strides taken per paw per second. Sham animals display a stide frequency of 1.5 ± 0.9 steps/s, Inj + Veh animals have a reduced stride frequency of 1.02 ± 0.08 steps/s. E2 treatment significantly increased stride frequency to 1.25 ± 0.05 steps/s.

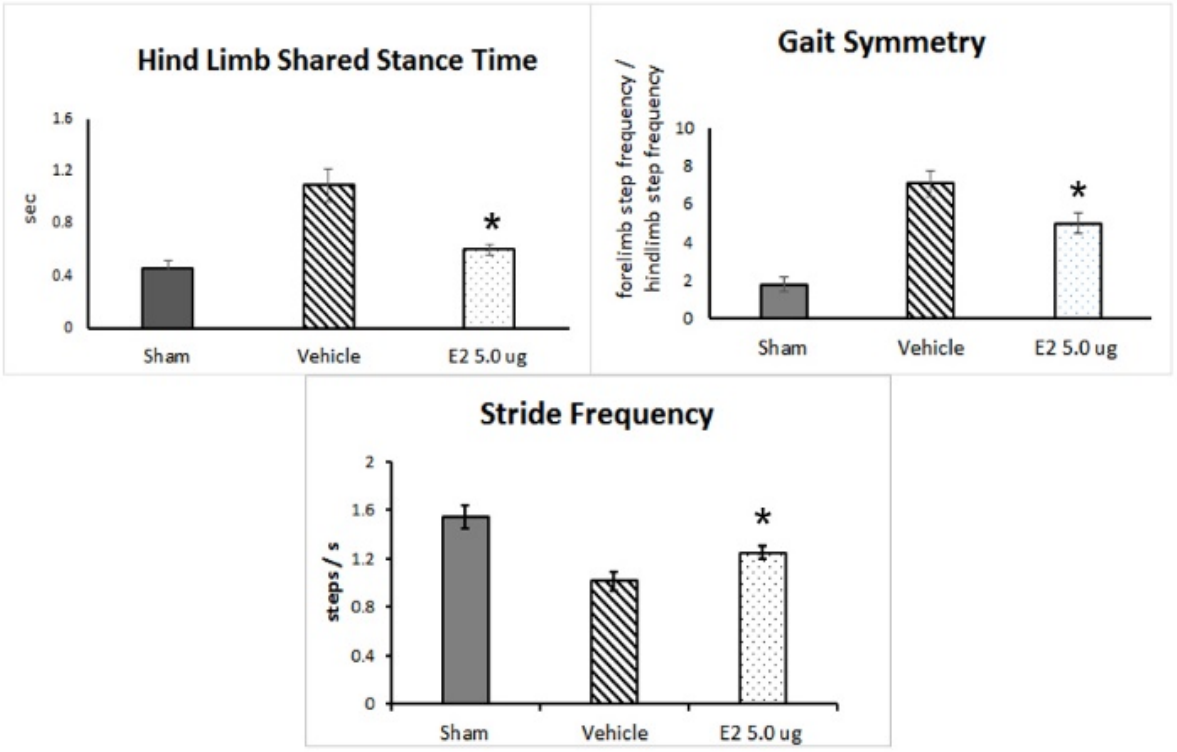


Figure 27. Additional DigiGait Analyses in the Chronic Model. DigiGait Parameters Affected by E2 Treatment. E2 treatment showed a significant change in hindlimb shared stance time, gait symmetry, and stride frequency as compared with vehicle treated animals. * $p<0.05$ E2 5.0 μg E2 vs. Vehicle. n=4 sham, n=10 Inj + Veh and Inj + 5.0 μg E2 groups

Histology: H&E and LFB Staining of Sagittal Spinal Cords Sagittal sectioning of spinal cords paired with H&E staining allows for visualization of entire lesion volume. Qualitatively, lesion volumes are reduced in the 5.0 μg dose group as compared with the vehicle group. LFB staining allows for visualization of white matter (blue) and gray matter (pink). Qualitative analysis reveals significant white matter sparing, particularly along the ascending/descending columns in the lateral portions of the spinal cord. Additionally, it can be seen that tissue integrity is enhanced in the E2 treated group

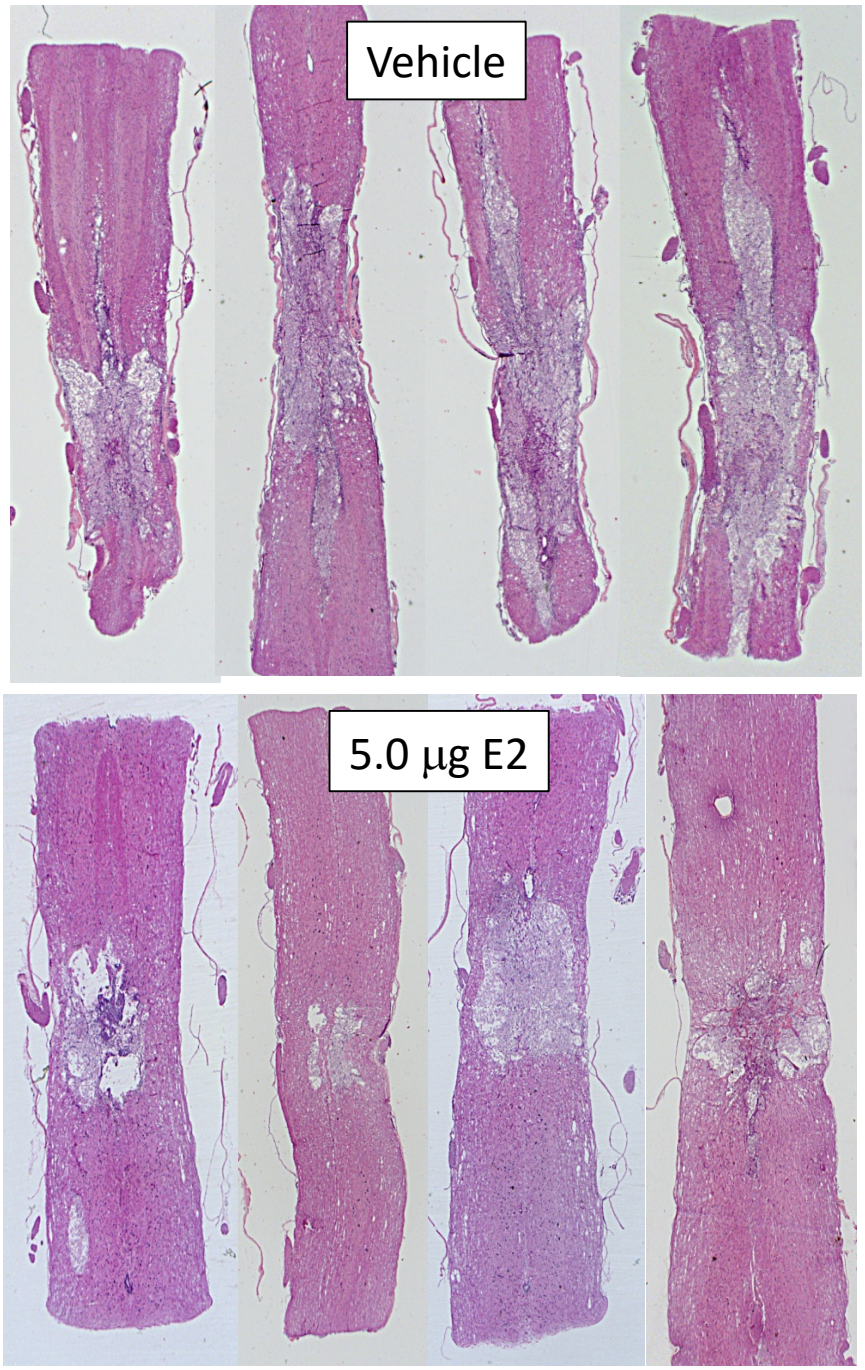


Figure 28. H&E Staining of Sagittal Spinal Cords. Images taken at 20X magnification. H&E staining reveal gross differences in lesion volume and tissue architecture between Vehicle and E2 treated groups. n=4 per group

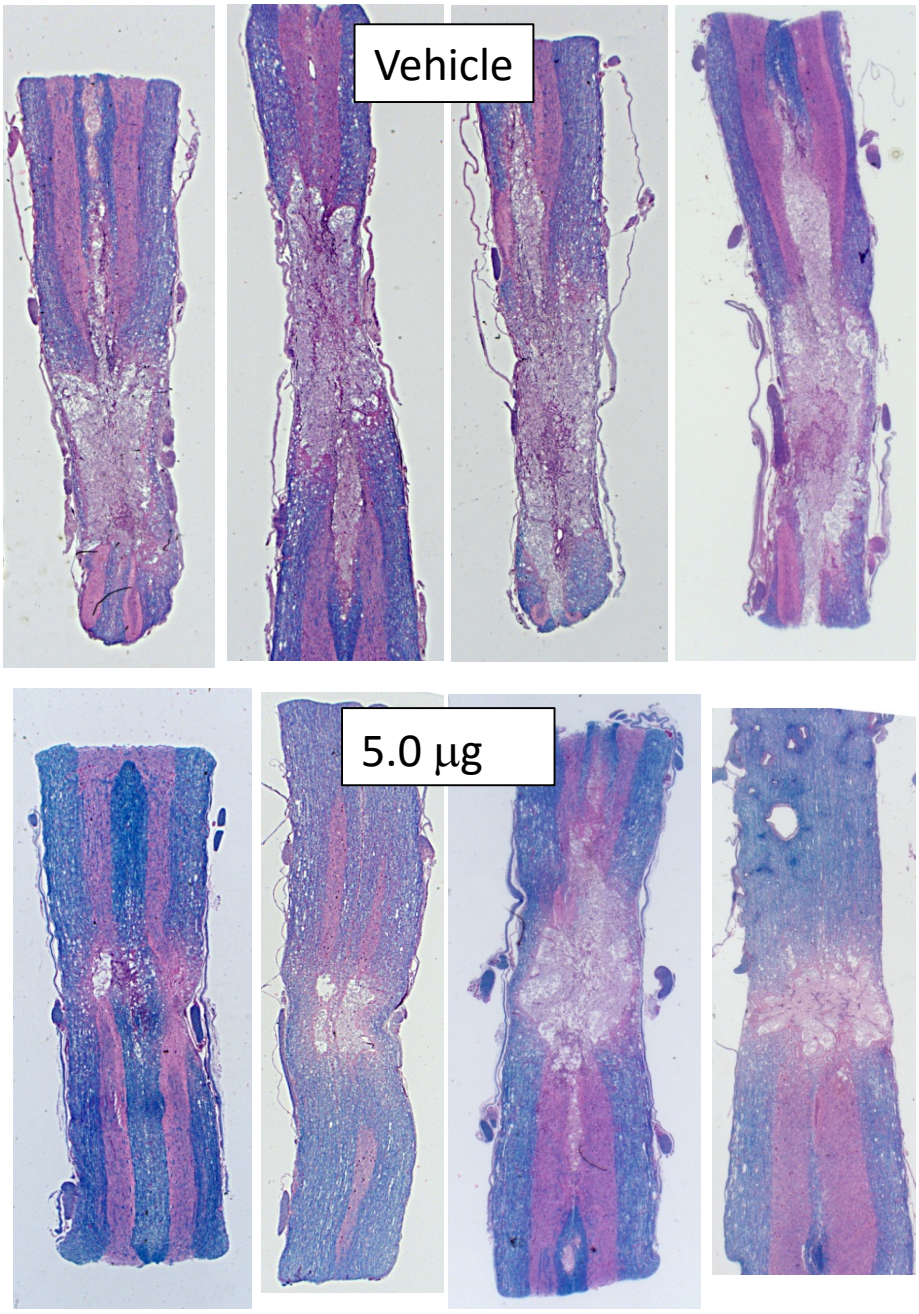


Figure 29. LFB Staining of Sagittal Spinal Cords. Images taken at 20X magnification. LFB staining reveal differences in white matter (stained blue) preservation between Vehicle and E2 treated groups. n=4 per group

E2 Plasma Concentration Profiling: 5.0 μg vs. 2.5 μg E2 Given the 2.5 μg dose was found to be ineffective for improvement of locomotor function, the dose was increased to 5.0 μg. A plasma profile study reveals the differences in exposures seen between the two groups. The Tmax for both groups was found to be 2 hours. The Cmax for the 2.5 μg dose was 172 pg/mL, and the Cmax for the 5.0 μg dose was 269 pg/mL. Besides this difference the plasma concentration curves appear to be very similar.

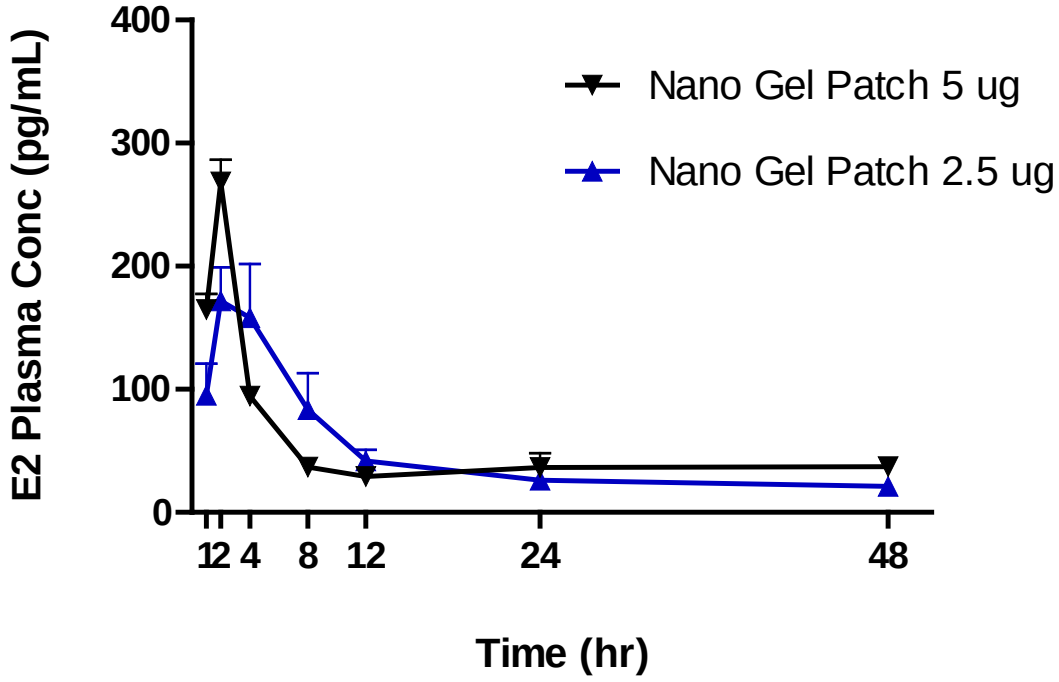


Figure 30. 5.0 μg E2 Plasma Concentration Time Course Experiment. Cmax for the 5.0 μg group was found to be 269 ± 18 pg/mL, and 172 ± 27 pg/mL for the 2.5 μg group. n=3 per group

Cytokine Analysis 24 & 48 Hr: At 24 hrs only one analyte, VEGF, was found to be statistically different. VEGF was 39% increased in the 5.0 μg E2 group as compared to a 4% decrease as seen in the vehicle group (25 pg/mL 5.0 μg E2,17 pg/mL vehicle group, 18 pg/mL Sham) (Figure 30). Other than VEGF no major differences between groups were seen in the plasma cytokine profiles examined. In general, all of the injured groups tended to have decreased cytokine/chemokine levels when compared to the sham group (Figure 31). At 48 hrs following injury, no statistically different chemokines/cytokines profiles were found between the E2 treated groups and vehicle. Although, it is interesting to note that overall the 5.0 μg E2 group trended to have increased cytokine/chemokine expression across the board (Figure 32).

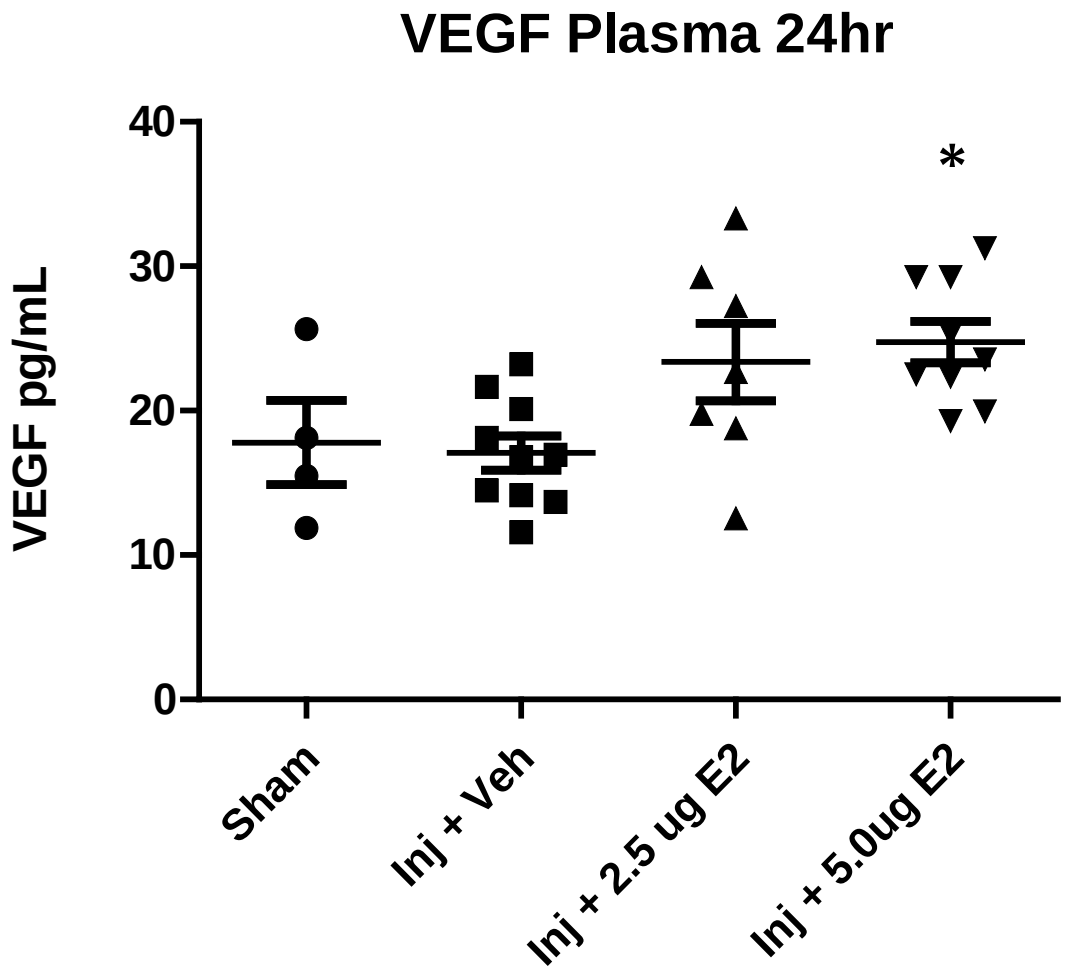


Figure 31. VEGF plasma levels at 24 hrs. Inj + 5.0 ug E2 group VEGF level (25 pg/mL ± 1.4) was significantly increased as compared with Inj + Veh (17 pg/mL ± 1.2) *p<0.05 Inj + 5.0 μg E2 vs. Inj + Veh. n=3 per group

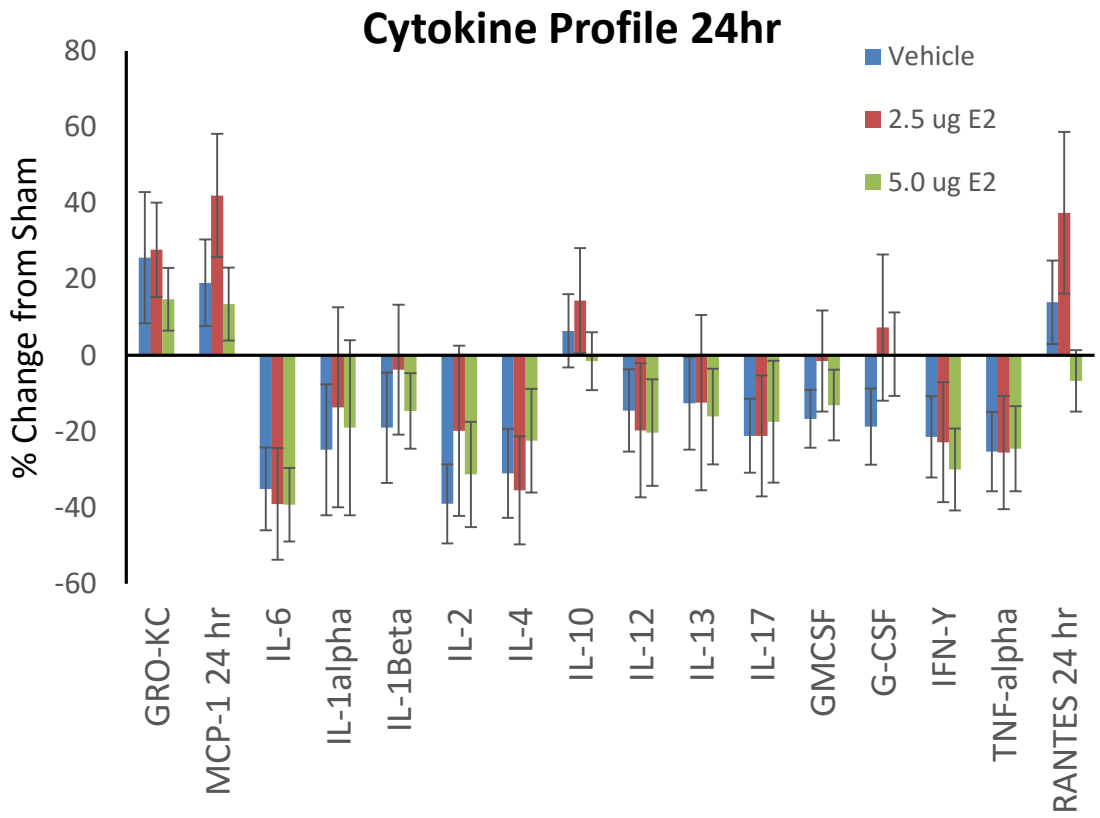


Figure 32. Plasma Cytokine/Chemokine Profile 24 hrs. Plasma taken from animals on 28 day study. Using the sham group as a control, the percentage change from sham group was calculated for 13 different plasma analytes at 24 hrs post injury/E2 treatment revealed a trend for all injured groups to display decreased cytokine/chemokine levels as compared to sham. n=10 vehicle, n=8 2.5 ug E2, n=10 5.0 ug E2.

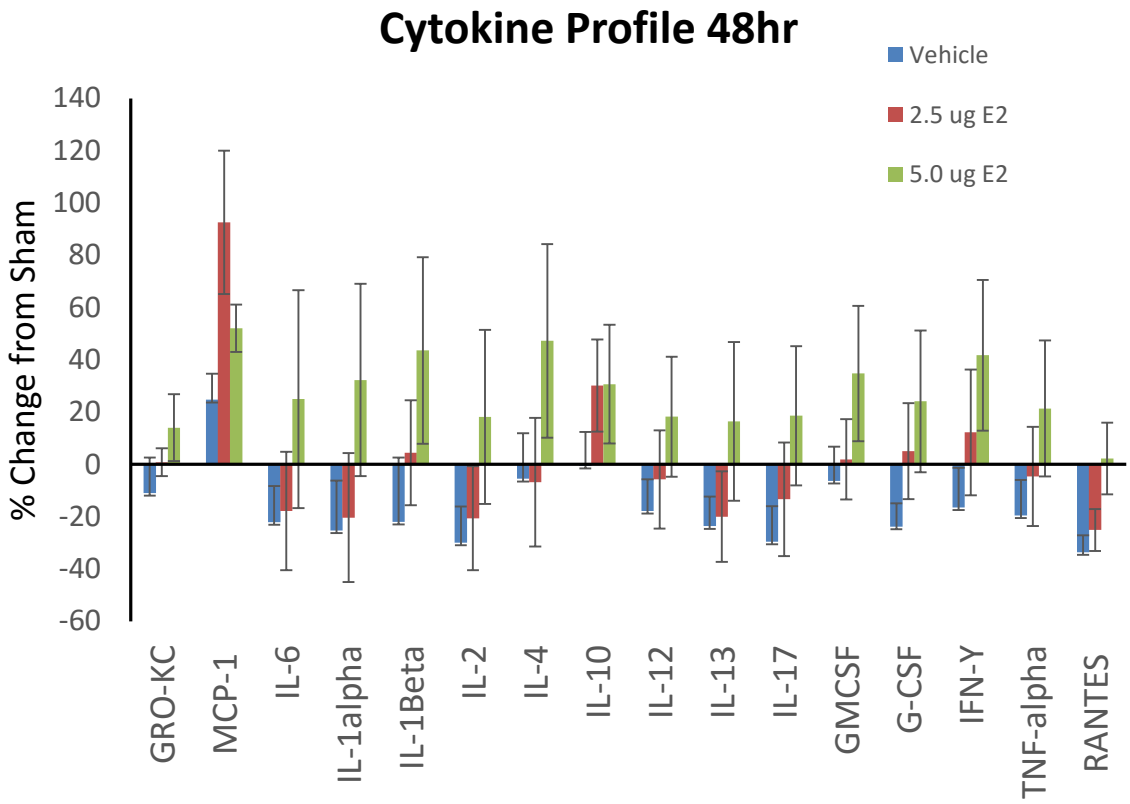


Figure 33. Plasma Cytokine/Chemokine Profile 48 hrs. Plasma taken from animals on 28 day study. Using the sham group as a control, the percentage change from sham group was calculated for 13 different plasma analytes. A global increase in cytokines/chemikines and the growth factor VEGF were seen at the 48hr time point in the 5.0 μg dose group. n=10 vehicle, n=8 2.5 ug E2, n=10 5.0 ug E2

Spinal Cord Tissue E2 Concentration 48 Hr: To continue to profile the release of E2 from nanoparticles, spinal cord tissue was analyzed for E2 content at the end of the plasma profiling study. The concentration of E2 was found to be 5.3 ± 0.7 pg/ug. Using the 6 hr tissue concentration study, the sham and vehicle groups were found to be under 2 pg/ug, representing the lowest detectible concentration on the ELISA format.

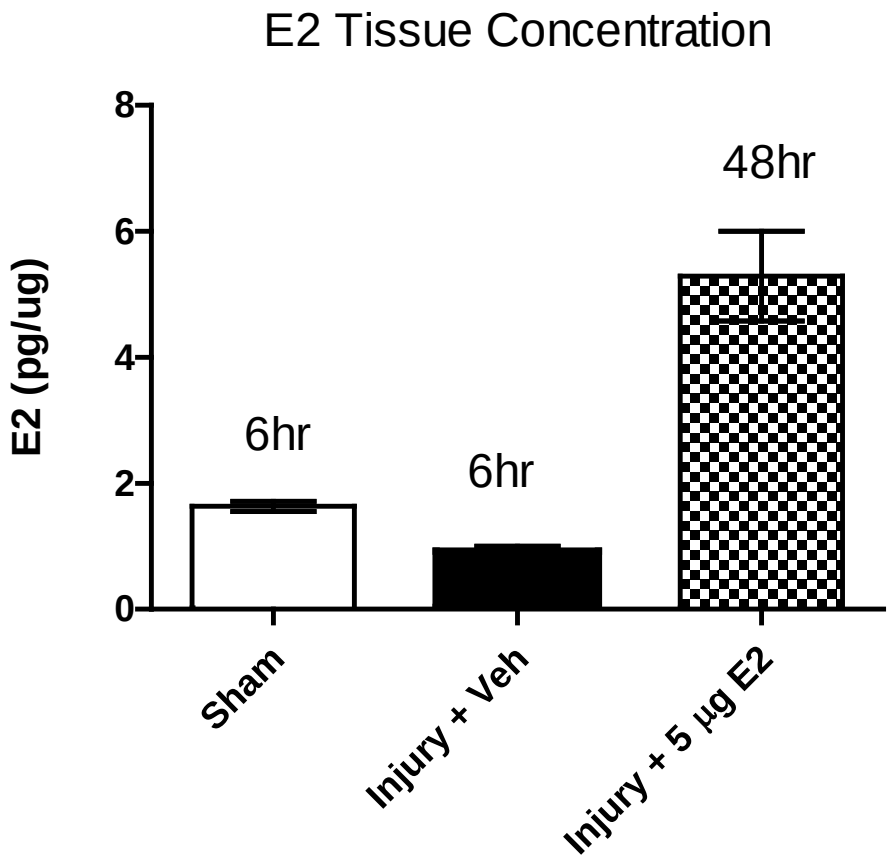


Figure 34. E2 Tissue Concentration 48 hrs. E2 concentration in lesioned spinal cord tissue determined via ELISA format. In the Inj + 5.0 μg group concentration at 48 hrs was found to be 5.3 ± 0.7 pg/ug. This value is approaching the normal range of E2 as seen in the sham and Inj + Veh group taken from the 6 hr study. n=3 all groups

DISCUSSION

The sub-acute and intermediate models provided data to support the hypothesis that the 2.5 μg dose of E2, delivered in nanoparticle embedded gel plugs, may be adequate to drive a functional improvement in the chronic model. However, we found that the 2.5 μg dose of E2 was sub-efficacious for driving a functional improvement. This finding is in agreement with the literature; to date, there have been no reports of locomotor function recovery below 50 μg. The lowest reported dose of E2, that has been shown to be efficacious for improvement of locomotor function is reported to be an approximate dose of 25 μg (100 μg/kg) given IV 15 min following injury and again 24 hrs following injury for a total dose of 50 μg (Hu, Sun et al. 2012). However, a single 50 μg subcutaneous pellet dosage was not found to be efficacious (Kachadroka, Hall et al. 2010). Our lab has found the lowest efficacious dose of E2, delivered for 7 days via osmotic pump, to be 10 μg/kg (data pending publication). Osmotic pumps deliver E2 at a constant rate that results in steady state levels, similar to pellet delivery. The differences between these studies may lie in the different dosing techniques, resulting in different plasma exposures. However, as neither paper reported E2 plasma levels, this cannot be determined.

Here, we report that the lowest efficacious dose of E2 was found to be 5.0 μg. This dose was found to decrease neurogenic bladder recovery time, increase BBB scores, improve various DigiGait parameters of gait, and

qualitatively spare white matter and decrease lesion volume in spinal cords. Differences in plasma E2 concentration profiles between the 2.5 μg and 5.0 μg dose groups reveal the only differences to be in the first two hours with a 50% increased Cmax (Figure 27), suggesting that there may be a minimal Cmax required to drive neuroprotection with E2. A formal pharmacokinetic study would be required to support this hypothesis, however. Tissue concentration at 48 hrs revealed only a modest increase in E2 concentration (approximately 5 times) as compared with non-treated, 5.3 pg/μg vs.0.9 pg/μg vehicle (Figure 31). This was an unexpected finding, as the 6 hr data revealed a tissue concentration of 162 pg/μg (Figure 6). E2 displays high affinity binding to the estrogen receptor β, with a dissociation constant of 0.6 nM (Kuiper, et al. 1997). Given its properties as a high affinity binder, even at low concentrations E2 may still be exerting biological effects. Again, a formal pharmacokinetic study would be required to test this hypothesis. Data presented here provide support for this hypothesis, as the single 5.0 μg dose of E2 revealed improvements in both locomotor function and spinal cord morphology. The single 5.0 μg dose is the lowest known efficacious dose, and these findings potentially highlight the advantage of using nanoparticle embedded gel plugs to focally deliver E2 and potential for clinical translation in SCI.

Functional recovery following SCI was monitored by both locomotor function as well as bladder function. Neurogenic bladder dysfunction has been found to be correlated with spinal cord injury severity (Ozsoy, Ozsoy et al. 2012).

Recovery of bladder function may therefore serve as a surrogate marker of injury severity. Here we report that the 5.0 μg dose significantly decreased the number of days required to reinstate bladder function (Figure 22). The recovery of bladder occurs between days 6-12, illustrating that the effects seen by E2 at the 6hr and 48hr time points translates into improved spinal reflex control in the chronic recovery period. Locomotor function was also improved, as seen in the BBB scores and DigiGait analyses (Figures 23-25). These findings are found in conjunction with histological analyses that qualitatively illustrate decreased lesion volume and increased white matter sparing (Figures 27 & 28). The differences seen in the lesion between vehicle and E2 treated groups may provide evidence to support hypothesis that E2 is exerting neuroprotective effects by decreasing secondary injury spread. Mechanisms contributing to secondary injury spread such as inflammation, apoptosis, and reactive gliosis are mitigated by E2 treatment, as shown in Chapter 3. The lesions seen in the E2 group are fairly compact with well-preserved tissue and well-defined borders, whereas the lesions in the vehicle groups are spreading and diffuse (Figures 27 & 28). These gross differences in lesion morphology highlight the potential for E2, when applied in the acute time period following injury, to spare white matter tracts and decrease secondary injury spread.

To further elucidate the neuroprotective mechanisms E2 exerts in SCI, plasma was profiled at the 24 and 48 hr timepoints for 27 different analytes (same profile as specific aim 1). Surprisingly, only one finding was statistically

significant. VEGF was significantly increased at 24 hrs in animals treated with 5.0 μg E2. This particular finding was not surprising, however, as E2 receptors have been reported to regulate VEGF gene transcription (Mueller, Vigne et al. 2000). Additionally, E2 receptors have also been shown to be involved in activation of one of the VEGF pathways (VEGF/Akt/NO) leading to enhanced angiogenesis through the VEGF receptor Kdr (Jesmin, Mowa et al. 2010). Therefore, increased VEGF circulating in the plasma, triggered by E2, may be beneficial by promoting angiogenesis in the lesioned spinal cord. The molecular mechanisms underlying VEGF stimulation of angiogenesis have been well studied and reviewed elsewhere (Evans 2015).

The panel of cytokines/chemokines at 24 hrs reveals that while the majority of the analytes are reduced when compared to sham levels, the two markers GRO-KC (human IL-8) and MCP-1 are elevated. This is consistent with the findings in Specific Aim 1 where these two markers were found to be specific to SCI and have been reported in the literature to be candidate biomarkers for human SCI severity (Kwon, Stammers et al. 2010). At 48 hrs the most striking trend is an overall increase in analytes in the 5.0 μg E2 dose group, although none of these trends were found to be statistically significant. The overall lack of robust cytokine regulation at 24 and 48 hrs post injury in both dose groups is found in parallel with a loss of plasma exposure (Figure 29). Plasma E2 levels nearly return to sham concentrations (15 pg/mL) by 48 hrs for the 2.5 μg E2 dose group (21 pg/mL. The 5.0 μg E2 group had slightly elevated plasma E2 levels at

48 hrs (37 pg/mL). Perhaps, the global increase in cytokine/chemokines in the 5.0 μg E2 dose group is a rebound response as a result of decreased plasma E2 exposure. Further studies are warranted to better understand if E2 is playing an active role in cytokine/chemokine response at this time point. Additionally, it should be noted that the cytokine/chemokine response in the spinal cord tissue was not investigated. Therefore, whether the plasma response is reflective of lesioned spinal cord tissue is not known at this time point.

These data provide evidence that when delivered focally, a single low dose of E2 may drive neuroprotection and improve locomotor recovery in a rat model. To date, this is the lowest reported efficacious dose of E2, and by monitoring plasma levels we have shown that by using the nanoparticle embedded gel delivery technique plasma levels remain in a physiologically relevant range. This novel approach may allow for a safe and efficacious drug treatment option for use in acute SCI individuals.

CHAPTER 5

CONCLUSION AND FUTURE DIRECTIONS

INTERPRETING THE RESULTS

E2 has been found to be a neuroprotectant in SCI by more than 10 groups to date (Elkabes and Nicot 2014). Given this plethora of data supporting the use of E2, the question of clinical translation remains. This dissertation examined a novel drug delivery technique, one that may allow for clinical application of this promising therapeutic. In addition to examining the hypothesis that a focal nanoparticle drug delivery may be a clinically viable approach, mechanistic investigations were also conducted to further uncover how E2 drives neuroprotection. In order to systematically address mechanistic questions, three models were used to examine signaling at peracute, intermediate, and chronic stages of SCI. In this concluding chapter, an attempt will be made to analyze the findings from each of these models in a broader sense and discuss clinical implications.

Studies using a peracute (6hrs following injury) model have shown here that E2 exerts a significant widespread anti-inflammatory effect. E2 was found to significantly decrease 15 analytes in the plasma compartment. As Chapter 2 has already discussed specific cytokines, this Chapter will place focus on a broader scale. These data provide evidence that E2 can rapidly regulate the innate immune response. E2 receptors α and β are expressed in cells comprising the innate immune

system (B cells, natural killer cells, monocytes) (Kovats 2015). These cells are responsible for the production and release of the proinflammatory cytokines into the plasma. Therefore, the hypothesis that E2 in circulation may be rapidly regulating numerous cell types of the innate immune system to downregulate cytokine production, thereby decreasing systemic inflammation is reasonable. For clinical translation these data also suggest the importance of using E2 very acutely following injury.

Moreover, E2 also exerted anti-inflammatory effects in the damaged spinal cord tissue. These changes were mirrored in CSF; however the mechanisms by which E2 may be exerting rapid anti-inflammatory effects are driven by different cell types in tissue. Spinal cord tissue consists of numerous cell types, including neurons, astrocytes, oligodendrocytes, and microglia. All of these cell types express E2 receptors, and all of these cell types have been reported to express cytokines to varying degrees. The widespread expression of E2 receptors makes distilling the protective effects down to a single or even few mechanistic pathways difficult. This complexity further highlights the true pleiotropic nature of E2 in an injury setting.

The peracute model was designed to allow for profiling of 3 biological compartments simultaneously. By doing so, we can gain tremendous insight into how the plasma or CSF reflect the tissue. The data generated illustrated that 26 of the 27 markers in plasma could not differentiate between sham and injured

animals. The one exception found was leptin. This is not altogether surprising as the laminectomy procedure itself is an invasive surgical approach. CSF, however, could differentiate between the sham and injured animals. The profile of cytokines/chemokines in the CSF most closely mirrored those seen in the lesioned tissue. These findings highlight the necessity of biomarkers to be sampled from CSF in the clinical setting. Currently, clinical trials rely on endpoints of functional recovery to determine efficacy, which require months to years to acquire. The addition of biomarkers, that could be sampled in the acute period following SCI from the CSF allowing for rapid identification of therapeutics capable of decreasing key cytokines/chemokines may greatly progress the field.

The lack of dose response in the peracute model between 25 and 2.5 μg doses also points towards a ceiling effect for E2. Essentially, the maximal reduction that E2 may achieve is seen at 2.5 μg dose of E2. Interestingly, as the 2.5 μg dose did not result in improved locomotor recovery, the important factor may be length of time during which E2 levels are high enough to drive maximal cytokine/chemokine reduction. These findings imply that longer, lower level plasma exposure to E2 may be most beneficial.

The intermediate model provided data to support the hypothesis that E2 may drive neuroprotection by decreasing astrogliosis. The bulk of previous literature reports have focused on the effects of E2 on neuronal survival. Hence, we focused on the glial cell response to E2 to potentially uncover novel therapeutic mechanisms. Decreased astrogliosis at the 48hr time point may be

linked to decreased cytokine/chemokine signaling at the 6 hr time point. Cytokines and chemokines serve as molecular triggers for activation of systemic and tissue immune cells, such as microglia and astrocytes. E2 treatment reduced 15 different cytokines/chemokines in plasma and 11 in tissue at the 6 hr time point. This translated into significantly decreased tissue astrogliosis at the 48 hr time point, further highlighting the importance of early treatment with an inhibitor capable of pan cytokine/chemokine modulation.

Finally, in the chronic model, a minimal dose to drive improvement in locomotor function was established. By using a focal nanoparticle drug delivery approach, we report the lowest efficacious dose known to date. Additionally, by reporting E2 levels in the plasma, a bench mark for future studies has been set. Data presented herein suggest that a high Cmax is not required. This data is vital for translation into clinical practice in regards to efficacy as well as safety. Retrospective observational studies have indicated that an increased risk for stroke occurs in women taking oral contraception (associated with high Cmax and rapid clearance) vs. transdermal (associated with low steady state levels) (Speroff 2010). These findings make it ill-considered to use a dosing technique that results in a high E2 Cmax in SCI individuals, who are already at greater risk for DVT. Rather, use a focal and sustained drug delivery technique, such as nanoparticles may be more appropriate.

FUTURE DIRECTIONS

Data from the intermediate model revealed that by 48 hrs both tissue and plasma concentration of E2 were near baseline levels. This finding was somewhat unexpected as *in vitro* data suggested E2 was released for 6 days. Regardless, the data reveal a need for a longer release nanoparticle formulation. The current work utilized one population of nanoparticles, composed of a set ratio of PLGA to PEG-PLA copolymer (10:2). By varying this ratio it is possible to change the properties of the nanoparticles, such as size and charge, thereby allowing for a different degradation rate. In future studies using a mixed population of nanoparticles, one for rapid release and another for slower release may be beneficial.

Continued work on acute biomarkers may better clarify the lack of efficacy seen in chronic model at 2.5 μg E2 vs. the efficacy with 5.0 μg E2. More specifically, how long do cytokines / chemokines need to be decreased? For these studies, time course models examining plasma, tissue, and CSF will be necessary. These data will provide profiles of cytokine/chemokine responses in both an efficacious and non-efficacious dose that can be applied clinically and may help determine clinical doses. For example, if the rat model illustrated that MCP-1 must be 80% reduced for a minimum of 48 hrs, then in the clinical situation CSF may be monitored and drug dosages adjusted accordingly.

The question remains of whether E2 may exert neuroprotection when dosed in the chronic time period. Given there is a large population of individuals

living with paralysis secondary to SCI, a need exists for treatment to potentially enhance motor function in the chronic stage. SCI individuals currently undergo extensive physical therapy, therefore, pairing exercise with E2 treatment in a chronic model may be clinically relevant. The hypothesis that E2 paired with forced exercise may improve locomotor recovery in a chronic model could be evaluated using a chronic model and a treadmill training system. To further study this hypothesis use E2 in conjunction with the scar dissolution drug Decorin, may result in further enhanced recovery.

Finally, nanoparticle drug delivery approach may allow for a number of drugs to be administered simultaneously. Pairing a cocktail approach with a rationally timed delivery system, i.e. nanoparticles designed for slow delivery loaded with drugs targeted to affect later occurring pathologies, may yield further improved outcomes. Using drugs that are off-patent and have already been investigated in SCI would offer the fastest approach for clinical application. One such combination of drugs that could be tested in a cocktail nanoparticle delivery approach could be E2 and Minocycline in quick release nanoparticles and Taxol (microtubule stabilizing agent for enhanced axonal regeneration) in slow release nanoparticles. Given the highly complex nature of SCI, it follows that a rational combination drug therapy approach may prove the most effective in finding successful treatment for this debilitating condition.

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