THE ROLE OF TUMOR NECROSIS FACTOR-ALPHA IN MALADAPTIVE SPINAL PLASTICITY

A Dissertation

by

JOHN RUSSELL HUIE

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

December 2010

Major Subject: Psychology

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Approved by:

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ABSTRACT

The Role of Tumor Necrosis Factor-Alpha in Maladaptive Spinal Plasticity.

(December 2010)

John Russell Huie, B.S; M.S., Texas A&M University

Chair of Advisory Committee: Dr. James W. Grau

Previous work has shown that the spinal cord is capable of supporting a simple form of instrumental learning. Subjects that receive controllable shock to an extended hind limb will increase the duration of limb flexion over time in order to reduce net shock exposure. Exposure to as little as 6 minutes of uncontrollable stimulation prior to instrumental testing can elicit a long-lasting learning deficit. Prior work has suggested that this deficit may reflect an overexcitation of spinal neurons akin to central sensitization, and that learning is inhibited by the saturation of plasticity. The experiments in this dissertation were designed to test the role of the cytokine tumor necrosis factor alpha (TNFα) in the induction and expression of the deficit. It is believed that the inflammatory properties of TNFα may mediate the excitatory processes that lead to maladaptive spinal functioning.

Experiments 1 and 2 tested the necessity of endogenous TNFα in the deficit produced by uncontrollable shock. These experiments showed that the inhibition of endogenous TNFα blocks both the induction and expression of the shock-induced deficit, suggesting a necessary role for TNFα in mediating the inhibition of spinal learning. Conversely, Experiment 3 was designed to test the sufficiency for TNFα in

producing a learning deficit. I found that treatment with exogenous TNFα undermined spinal learning in a dose-dependent fashion, whether given immediately, or 24 hours prior to testing. Experiment 4 demonstrated that the long-term TNFα-induced deficit is mediated by TNFα receptor activity, as a TNF inhibitor given prior to testing blocked the expression of this deficit.

As TNFα has been shown to be predominantly of glial origin, I next assessed the role that glia play in the TNFα-induced deficit. Experiment 5 showed that inhibiting glial metabolism prior to TNFα treatment blocked the capacity for TNFα to produce a long-term deficit. Experiment 6 assessed the potential for TNFα inhibition to block the deficit induced by lipopolysaccharide (LPS), an agent known to induce TNFα. TNFα has also been shown to drive neural excitation by increasing the trafficking of calcium-permeable AMPA receptors to the active zone of the post-synaptic bouton. Experiment 7 showed that selectively antagonizing these receptors prior to testing blocked the TNFα-induced deficit, suggesting a possible post-synaptic mechanism by which TNFα exerts its effects.

Finally, histological evidence was sought to reinforce the previous behavioral findings. Experiment 8 used quantitative RT-PCR to assess the differential expression of TNFα mRNA in uncontrollably shocked subjects as compared to those receiving controllable shock and no shock. To determine concentrations of TNFα protein, an ELISA was run in Experiment 9 comparing uncontrollably shocked subjects to unshocked controls.

DEDICATION

Every moment, and the labor and action therein, is dedicated to John Davis Huie. This work is no exception.

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CHAPTER I

INTRODUCTION

Spinal Plasticity: An Overview

The spinal cord is no longer viewed as a simple conduit. Instead, research over the past 70 years has broadened our understanding of the spinal cord, and has enlightened us to its potential for change, growth, and adaptation. In response to a barrage of central and peripheral input, the spinal cord is able not only to organize and relay signals, but to encode information and affect output. In this way, the spinal cord holds the capacity for learning and memory, once believed to be solely entrusted to the brain. The idea that the spinal cord is plastic, that it can react to environmental changes in a flexible manner that modifies future behavior, has been a hallmark of spinal cord research for the past half-century. Early work in spinal plasticity first demonstrated simple, single stimulus forms of learning such as habituation and sensitization, and later showed that the spinal cord could support more complex associations, including Pavlovian and instrumental learning (Fitzgerald & Thompson, 1967; Grau, Barstow & Joynes, 1998).

One of the main focuses of spinal plasticity research is the capacity for neural changes to elicit lasting beneficial effects in response to insult. This *adaptive* plasticity has been most notably studied in spinal cord injury paradigms (Reviewed in Fouad &

This dissertation follows the style and format of *Behavioral Neuroscience.*

Tse, 2008). Rossignol and colleagues (1999) first showed that cats with complete spinal transections will, over time, exhibit spontaneous locomotor recovery, suggesting an intraspinal modification of locomotor circuitry. Other studies revealed that locomotor treadmill training of spinalized cats potentiated this adaptive spinal response (Lovely, Gregor, Roy, & Edgerton, 1986; de Leon, Hodgson, Roy, & Edgerton, 1998). Although these examples most likely reflect long-term spinal reorganization, acute spinal plasticity has also been shown to occur. If spinalized cats that are trained on a treadmill are presented with an obstacle that hinders their gait, they will quickly learn to alter their step in order to avoid the obstacle (Forssberg, Grillner, & Rossignol, 1975).

In contrast to the promising potential for adaptive plasticity to protect spinal circuitry, the spinal cord also responds to neural insult in a manner that can drive further detriment to the system. This *maladaptive* spinal plasticity can lead to sustained pain states, excitotoxicity, and cell death. One of the most common and widely studied instances of maladaptive spinal plasticity is the phenomenon of central sensitization. Woolf demonstrated that following peripheral inflammation, the threshold for excitation in spinal neurons dropped substantially, and that this hyperexcitability was sustained long after the initial input (Woolf & Thompson, 1991). This form of nociceptive plasticity has been implicated as a mechanism by which neuropathic pain occurs (Latremoliere &Woolf, 2009). Maladaptive processes such as central sensitization produce long-lasting changes in synaptic efficacy that act to undermine the recovery of normal neurotransmission and spinal cord function. Thus, it is important to understand the cause and nature of these processes to combat their deleterious consequences.

This dissertation will investigate the neurobiology of maladaptive spinal plasticity. Using a behavioral model of spinal plasticity, the experiments in this dissertation have been designed to test the necessity and sufficiency of the inflammatory cytokine tumor necrosis factor alpha (TNFα) as it pertains to a spinal learning deficit. This introduction will provide an overview of the model of spinal plasticity that we have used, and highlight insights into maladaptive spinal plasticity that have been fostered using this paradigm. The biology and physiological effects of TNFα will then be discussed, and the link between spinal plasticity and TNFα will be drawn. Finally, the specific aims of this dissertation will be outlined.

Spinal Instrumental Learning

Research over the past 50 years has worked to test the limits of the types of learning the spinal cord can support. A series of experiments in the 1970's sought to determine if spinal neurons could encode simple response-outcome relations (Chopin & Bennett, 1975; Chopin & Buerger, 1975). Although Chopin reported positive results, the data were beset by a number of methodological issues (Church & Lerner, 1976). In a series of studies, Grau and colleagues addressed these concerns, and provided further evidence that the spinal cord could support instrumental learning(Grau, Barstow, & Joynes, 1998; Crown, Ferguson, Joynes, & Grau, 2002a; Reviewed in Grau, et al., 2006).

The instrumental learning task used by Grau and colleagues is a modification of a paradigm developed by Horridge in 1962. In this preparation, shock is delivered to the

tibialis anterior muscle of a spinalized rat each time a hind leg is in an unflexed position, and shock is terminated when the leg has flexed. Over time, the subject will learn to keep the leg in a flexed position that minimizes shock exposure. In this way, spinal neurons are capable of encoding the instrumental association between response (flexion) and outcome (shock).

This simple model of spinal plasticity has a number of distinct advantages. Other models of spinal plasticity that use behavioral outcome measures, such as the spinal contusion injury model, have difficulties isolating the role of intraspinal changes from supraspinal contributions. Conversely, completely isolating spinal neurons in cell culture preparations neglects the importance of a behavioral correlate. This spinal instrumental learning paradigm strikes an important balance between neurobiology and behavior by allowing for pharmacological and physiological manipulations of the isolated spinal cord, while also providing a direct behavioral measure of spinal plasticity. Further, the findings from this paradigm translate well to more naturalistic spinal cord injury preparations, providing clinical relevance to these pursuits (Grau, et al., 2004; Grau, et al., 2006).

The spinal instrumental learning paradigm also allows for testable predictions regarding adaptive and maladaptive forms of spinal plasticity. Instrumental training with *controllable shock* (in which shock delivery is contingent upon leg position) has been shown to yield a number of adaptive phenomena, including the facilitation of future learning and protection from processes that would normally undermine spinal learning.

In contrast, exposure to *uncontrollable shock*, in which shock is intermittently delivered regardless of leg position, can produce a long-lasting maladaptive learning deficit.

The Spinal Learning Deficit

The fact that uncontrollable stimulation can undermine adaptive spinal plasticity has had implications on the broader field of spinal cord injury research (Grau, et al., 2004; Grau & Hook, 2006). The administration of as little as 6 minutes of uncontrollable shock to the tail of a contused rat has been shown to exacerbate spinal tissue damage and undermine functional recovery (Grau, et al., 2004). For this reason, the shock-induced learning deficit, and the neurobiological mechanisms that underlie its induction and expression, have been a major focus of study for the past decade.

The spinal learning deficit was first observed using a master-yoked spinal learning preparation. In this setup, the master subject receives controllable shock, contingent upon their leg position. Each master subject is experimentally coupled to a yoked subject, who receives the same shock as the master, but regardless of leg position. In this way, the timing and number of shocks received by each subject is equivalent, and the only difference between subjects is controllability. After subjects are trained in this fashion, they are both tested with controllable shock. Interestingly, those that had been previously exposed to uncontrollable shock are unable to learn the association between leg position and shock. This form of maladaptive plasticity is very similar to the psychological phenomenon of *learned helplessness*. This comparison is important, in that it highlights a critical feature of this deficit: that uncontrollable shock *is* producing a

form of plasticity, but one that is maladaptive and undermines future learning. This notion that the capacity for plasticity itself can be shaped by experience has been referred to as *metaplasticity* (Ferguson, Bolding, et al., 2008).

In order to explore the conditions under which this deficit occurs, Crown and colleagues set out to isolate the shock parameters necessary to induce the deficit (Crown, et al., 2002a). They found that as little as 6 minutes of intermittent shock to the tail could induce a deficit that lasted up to 48 hours. It is believed that uncontrollable shock to the tail induces a deficit that is dependent upon spinal neurons, as it has been shown that an intrathecal injection of lidocaine was sufficient to block the induction of the shock-induced deficit (Joynes, Ferguson, Crown, Patton, & Grau, 2003).

Prior work has also sought to elucidate the neurobiological systems that are involved in the induction and expression of the deficit. Ferguson demonstrated a necessary role for the NMDA receptor in the deficit, showing that intrathecal administration of the NMDA receptor antagonist MK-801 blocked the induction of the deficit (Ferguson, Crown, & Grau, 2006). Further, it was shown that AMPA receptor activity was also necessary, and that overexpression of AMPA was sufficient to produce a lasting deficit (Hoy, Huie, & Grau, 2006; Hoy, Huie, Baumbauer, & Grau, 2007). Recently, Ferguson outlined a critical role for metabotropic glutamate receptors in the deficit as well (Ferguson, Bolding, et al., 2008). Other systems, including opioid and GABA activity have also been implicated in playing a role in the deficit (Ferguson, Washburn, Crown, & Grau, 2003; Washburn, Maultsby, Puga, & Grau, 2008). Interestingly, all of the systems that have been linked to the spinal learning deficit play

key roles in excitatory neurotransmission. This insight has led some to theorize that the deficit reflects a diffuse overexcitation that saturates spinal circuitry, blocking future learning (Ferguson, Crown, & Grau, 2006; Grau, et al., 2006). From this perspective, it can be suggested that the deficit produced by uncontrollable shock may be due to a central sensitization-like effect. In support of this view, uncontrollable shock has been shown to produce a behavioral phenomenon in which normally non-noxious stimuli elicit a pain response (*allodynia)*. Likewise, a number of pro-inflammatory neurochemical agents have been shown to effectively produce a spinal learning deficit, including capsaicin, carrageenan, and an NK1 agonist (Hook, Huie, & Grau, 2008; Ferguson, et al., 2006; Baumbauer, Young, Hoy, & Joynes, 2007). Thus, if the shock-induced deficit does reflect a form of overexcitation that disrupts future adaptive learning, then it will be important to investigate factors that are known to mediate such processes.

Tumor Necrosis Factor-Alpha

Tumor necrosis factor-alpha (TNFα) has been identified as a potent effector and key component in a host of neural processes. Although constitutively expressed on both glia and neurons, TNFα is most commonly synthesized and released from microglia and astrocytes in response to immune challenge. Following glial activation, TNFα can act directly on neurons, as well as in an autocrine fashion to induce glial synthesis of other cytokines, such as IL-1, IL-6, as well as the production and release of further TNFα (Wallach, et al., 1996; Kuno, et al., 2005; Ji & Suter, 2007).

Post-synaptically, TNFα has been shown to have wide-ranging and varied effects on neurons, from promoting cell survival and neuroprotection to excitotoxicity and apoptosis (Reviewed in Munoz-Fernandez & Fresno, 1998). TNFα binds to two receptor types, TNFR1 and TNFR2. Although their distinct roles have not been fully elucidated, it is generally believed that TNFR1 mediates pro-inflammatory and pro-apoptotic effects, while the lower affinity TNFR2 is responsible for the neuroprotective effects of TNFα. These varied functions reflect a complex intracellular signaling network, in which TNFα receptor activation can engage a number of distinct pathways (Chen & Goeddel, 2002; Ihnatko & Kubes, 2007). Given the many biological contexts in which TNFα functions, it is not surprising that TNFα has been implicated in a variety of pathologies, including Alzheimer's disease, ischemic stroke, Parkinson's disease, cancer, and multiple sclerosis (Tsukada, Miyagi, Matsuda, Yanagisawa, & Yone, 1991; Boka, et al., 1994; Hallenbeck, 2002).

TNFα and Plasticity

One of the main functions of TNFα is to affect change in neurotransmission and synaptic strength. TNFα has been shown to modulate ion currents, both directly by the coupling of TNFR1 with the NR1 subunit of the NMDA receptor, and indirectly through the downstream trafficking of further glutamate receptors. This capacity to affect changes in ion concentration and excitability has led many to investigate TNFα's effect on neural plasticity (Albensi & Mattson, 2000; Butler, O'Connor, & Moynagh, 2004). When given at high levels, TNFα has been shown to impair long-term potentiation

(LTP) in hippocampal slices (Cunningham, Murray, O'Neil, Lynch, O'Connor, 1996). Conversely, TNF receptor knockout mice show impaired hippocampal long-term depression (Albensi & Mattson, 2000). Behaviorally, TNFα overexpression in the hippocampus has been shown to impair spatial learning and memory (Golan, Levav, Mendelsohn, & Huleihel, 2004).

TNFα has also been shown to affect neural changes related to nociceptive signaling. Although shown to impair LTP in the hippocampus, exogenous TNFα administration in the dorsal horn of the spinal cord can induce LTP of C-fiber evoked field potentials (Liu, et al., 2007). Further data have shown TNFα to cause an increase in transient voltage-gated sodium channel currents on primary afferents, and this action has been suggested to underlie the induction and maintenance of neuropathic pain following nerve injury (Czeschik, Hagenacker, Schafers, & Busselberg, 2008).

In 2002, Beattie and colleagues demonstrated that TNFα plays a critical role in the regulation of AMPA receptor trafficking, which in turn dictates synaptic strength. Stellwagen and Malenka (2006) extended these findings when they observed that after neural activity falls, glia work to increase TNFα release in order to globally scale synapses up through AMPAR trafficking to the active region of the post-synaptic bouton (Turrigiano, 2008).

Although this excitatory role of TNFα provides a regulatory mechanism under normal conditions, the overexpression of TNFα in response to neural insult creates excitotoxic consequences that severely undermine adaptive plasticity (Perry, Collins, Wiener, Acton, & Go, 2001; Leonoudakis, Zhao, & Beattie, 2008). TNFα specifically

promotes the trafficking of AMPA receptors that lack the GluR2 subunit (Ogoshi, et al., 2005). Without this subunit, these AMPA receptors are calcium permeable (Hollmann, Hartley, & Heinemann, 1991). The overexpression of Glur2-lacking AMPARs has been shown to induce maladaptive synaptic plasticity in both the brain and spinal cord (Galan, Laird, & Cervero, 2004). By increasing the amount of Glur2-lacking AMPA receptors on the membrane, TNFα can induce excitability by setting a neural environment that is vulnerable to glutamate-mediated calcium influx. This mechanism has recently been implicated in the induction and maintenance of neuropathic pain following peripheral nerve injury, as well as undermining recovery of function following spinal contusion injury (Choi, Svensson, Koehrn, Bhuskute, & Sorkin, 2010; Ferguson, et al., 2008).

Specific Aims

This set of experiments is designed to assess the role of TNFα in the maladaptive effect of uncontrollable stimulation. The central hypothesis of this dissertation is that uncontrollable shock induces glial release of TNFα, and that TNFα acts post-synaptically to induce a long-lasting AMPAR-mediated overexcitation that undermines future adaptive plasticity. This study assessed behavioral, pharmacological, and histological outcomes to determine the role of TNFα in the spinal learning deficit.

From a behavioral perspective, if TNFα mediates the shock-induced deficit, then endogenous TNFα should be necessary in order for uncontrollable shock to elicit a deficit. A TNFα inhibitor was used in Experiments 1 and 2 to determine if the deficit would be induced and expressed in the absence of endogenous TNFα. Expanding on

these results, Experiment 3 tested whether exogenous TNFα would be sufficient to substitute for uncontrollable shock in order to produce both an acute and long-term deficit. Experiment 4 examined the nature of a long-term TNFα-induced deficit by investigating whether the expression of this deficit was TNFα mediated.

Next, the mechanisms by which TNFα exerts maladaptive effects were assessed. Prior work has shown endogenous TNFα release to be predominantly of glial origin. Additionally, it has been shown that TNFα can act on microglia in an autocrine fashion that causes further TNFα production and release. Thus, if the long-term TNFα-induced deficit is mediated by glial release of TNFα, then inhibiting glia should attenuate this deficit. This hypothesis was tested in Experiment 5, in which the glial metabolic inhibitor fluorocitrate was given prior to TNFα treatment. Experiment 6 tested whether TNF inhibition could block the expression of the deficit induced by lipopolysaccharide (LPS), an agent known to induce endogenous TNFα release.

I then assessed a possible post-synaptic effect of TNFα treatment. As mentioned previously, TNFα has been shown to act post-synaptically through the receptor TNFR1 to elicit trafficking of calcium permeable AMPA receptors to the membrane (Ogoshi, et al., 2005; Choi, et al., 2010). Experiment 7 assessed the possibility that this increase in calcium-permeable AMPA receptor populations to the cell surface is a TNFα-induced outcome that is necessary in order for TNFα to produce a deficit. Following TNFα treatment, an antagonist specific to calcium-permeable AMPA receptors was given to assess the role of this phenomenon in the spinal learning deficit.

Finally, histological techniques were used to assess the cellular evidence for the role of TNFα in the deficit. If TNFα mediates the deficit induced by uncontrollable shock, then it is expected that uncontrollable shock will cause an increase in endogenous TNFα populations. Experiment 7 used reverse transcriptase polymer chain reaction (RT-PCR) to assess whether uncontrollable shock upregulates TNFα mRNA production. Experiment 8 assessed levels of TNFα protein at three time points following uncontrollable shock in order to determine a timecourse by which TNFα expression occurs.

CHAPTER II

GENERAL METHOD

Subjects

Male Sprague-Dawley rats obtained from Harlan (Houston, TX) served as subjects. Rats were approximately 100-120 days old and weighed between 360 and 460 g. They were housed individually and maintained on a 12-hour light/dark cycle, with all behavioral testing performed during the light cycle. Food and water was available *ad libitum*.

Surgery

Subjects were anesthetized with isoflurane. The 2nd thoracic vertebrae (T2) was located by touch and a 2.5 cm anterior-posterior incision was made over T2. The tissue immediately rostral to T2 was cleared, exposing the spinal cord. A cautery was then used to transect the cord, and the cavity filled with Gelfoam (Harvard Apparatus, Holliston, MA). A 25cm polyethylene cannula (PE-10, VWR International, Bristol, CT) was subsequently threaded 9cm down the vertebral column, into the subarachnoid space between the dura and the white matter so that it lies on the dorsal surface of the spinal cord. The incision was closed using Michel clips (Fine Science Tools Foster, CA), and the exposed end of cannula tubing fixed to the skin with cyanoacrylate.

Immediately following surgery, subjects received an injection of 0.9% saline (2.5ml, i.p.). During recovery, the hindlimbs were maintained in a normal flexed position using a piece of porous orthaletic tape, wrapped gently around the rat's body.

The recovery period was 24 hours, throughout which the rats were housed in a temperature-regulated environment (25.5° C). Supplemental saline injections were provided to ensure proper hydration, and bladders expressed twice daily, and just before behavioral testing. Complete transection were confirmed by a) visually inspecting the cord during surgery, b) observing behavior following recovery, ensuring subjects exhibit paralysis caudal to the site of transection, and do not vocalize when shock is administered to the tail or hindpaw, c) examining the transection site postmortem in a randomly selected subset of subjects.

Apparatus

Instrumental Training and Testing

Instrumental testing was conducted while rats were loosely restrained in tubes (23.5 cm [length] x 8 cm [internal diameter]). Two slots in the tube, (5.6 cm [length] x 1.8 cm [width]), 4 cm apart, 1.5 cm from the end of the tube, allowed both hind legs to hang freely. To minimize the effects of upper body movement on leg position, a wire belt was secured to the rat's trunk within the tube. Leg shock was delivered using a BRS/LVE (Laurel, MD) constant current (60Hz, AC) shock generator (Model SG-903). Two electrodes inserted over the tibialis anterior muscle were connected to a computer-controlled relay that regulated the application of leg shock.

Leg position was monitored during testing using a contact electrode constructed from a 7 cm long, 0.46 mm diameter stainless steel rod taped to the foot. The last 2.5 cm of the electrode was insulated from the foot with heat shrink tubing. A fine wire (0.01 sq

mm [36 AWG] (20 cm) attached to the end of the rod extended from the rear of the foot and connected to a digital input monitored by a Macintosh computer. A plastic rectangular dish (11.5 [w] x 19 [l] x 5 [d]) containing a NaCl solution was placed approximately 7.5 cm below the restraining tube. A drop of soap was added to the solution to reduce surface tension. A ground wire was connected to a 1 mm wide stainless steel rod, which was placed in the solution. When the contact electrode attached to the rat's paw touched the solution, it completed the circuit monitored by the computer, delivering a shock to the tibialis anterior. The state of this circuit was sampled at a rate of 30 times/s.

Flexion force was measured by attaching a monofilament plastic line ("4 lb test" Stren, Dupont, Wilmington DE) to the rat's foot immediately behind the plantar protuberance. The 40 cm length of line passed through an eyelet attached to the apparatus directly under the paw, 16 cm beneath the base of the tube. The end of the line was attached to a strain gauge (Fort-1000, World Precision Instruments, New Haven, CT) fastened to a ring stand. After the line was connected to the rat's paw, the ring stand was positioned so that the line was taut, just barely registering on the gauge. The strain gauge was calibrated by determining the relationship between voltage and force in Newtons. These data revealed a linear relation, which allowed us to convert voltage to force.

Uncontrollable Tailshock

Uncontrollable shock was administered while rats were loosely restrained in

opaque black Plexiglas tubes that are 22 cm in length and 6.8 cm in diameter. A flat floor constructed from a sheet of black Plexiglas 5.5 cm wide was attached 5.3 cm below the top of the tube. Tailshock was delivered using an electrode constructed from a modified fuse clip. The electrode was coated with ECG gel (Harvard Apparatus, Holliston, MA) and secured with porous tape approximately 6 cm behind the base of the tail. Constant-current 1.5-mA shock was delivered using a 660-V transformer. A Macintosh computer controlled the onset and offset of shock.

Instrumental Learning Testing Procedure

All subjects were allowed to recover for 24 h following surgery and the hindlimbs were shaved and marked for electrode placement prior to testing. A wire electrode was then inserted through the skin over the distal portion of the tibialis anterior (1.5 cm from the plantar surface of the foot), and one lead from the generator was attached to this wire. A contact electrode was secured to the foot between the second and third digits with a piece of porous tape. The shock generator was set to deliver a 0.4 mA shock, and the proximal portion of the tibialis anterior (approximately 1.7 cm proximal to the wire electrode) was probed with a 2.5-cm stainless steel pin attached to a shock lead to find a robust flexion response. The pin was then inserted 0.4 cm into the muscle. A strain gauge was utilized to verify that a single, intense (1.6 mA, 0.3 s) test shock can elicit at least a 0.8 N flexion force, and to determine the amount of shock necessary to elicit a 0.4 N flexion force.

To minimize lateral leg movements, a 20 cm piece of porous tape was wrapped around the leg and attached to a bar extending across the apparatus directly under the front panel of the restraining tube. The tape was adjusted so that it was taut enough to slightly extend the knee. Finally, three short (0.15 s) shock pulses were applied and the level of the salt solution was adjusted so that the tip of the contact electrode (attached to the rat's foot) was submerged 4 mm below the surface. A rat's capacity to perform the instrumental response was then tested with exposure to 30 min of controllable shock. Whenever the rat's leg fell below the level of the salt solution, the electrodes delivered a shock to the tibialis anterior muscle causing the ankle to flex. Leg position was monitored using a Macintosh computer at a sampling rate of 30 Hz.

Behavioral Measures

Three behavioral measures, response number, response duration and time in solution, were used to assess a subject's capacity to perform the instrumental response (see Grau, et al., 1998). Performance was measured over time in 30 1-min time bins. The computer monitoring leg position recorded an increase in response number whenever the contact electrode was raised above the salt solution. Response duration was derived from time in solution and response number using the following equation: $\text{Response Duration}_i = (60\text{ s} - \text{time in solution}_i)/(\text{Response Number}_i + 1)$ where i is the current time bin.

To evaluate whether our experimental treatment affected baseline behavioral reactivity, we analyzed both the shock intensity required to elicit a flexion force of 0.4 N

and the duration of the first shock-elicited flexion response. Independent ANOVAs showed that there were no group differences on either measure across all experiments, Fs < 2.58, $p < 0.05$.

Histology

RT-PCR

Total RNA was isolated from tissues using the Trizol method (#15596-026 Invitrogen). Total RNA from each sample was purified with Qiagen's RNeasy MiniKit (#74104) and quantified by micro- fluidic separation (Agilent BioAnalyzer). Total RNA (1ug) was reverse-transcribed with the First-Strand cDNA Synthesis system for RT-PCR (#12371-09 Invitrogen) and SuperScript II Rnase H-Reverse Transcriptase (#18064-014 Invitrogen). Prior to real-time PCR analysis, forward and reverse primers for selected genes were analyzed using the Beacon Designer v.3 software (Premier BioSoft International). Primers were selected from a list of suggested primers only if the primer sequences cross intron-exon boundaries to eliminate secondary structure formation and amplification of genomic DNA. Selected primers were then optimized by a standard curve using serial dilutions of the template to determine the amplification efficiency of the PCR reaction. Single product formation was be verified by melt curve analysis. For real-time PCR, cDNA, forward and reverse primers, and BioRad's iQ Syber Green Supermix (#170-8882) were combined and analyzed with the iCycler MyiQ system from BioRad. Beta-actin served as the reference gene and the control condition served as the normalization control sample.

ELISA

TNFα levels were assessed using the TNFα immunoassay kit from R & D Systems (Minneapolis, MN). Briefly, tissue samples were homogenized in cold lysis buffer (phosphate-buffered saline, pH 7.4, with 1% Triton-X 100 and protease inhibitor cocktail). Supernatants were obtained by centrifugation (13,000g for 15 min at 2° C) and stored at –80° C until assays were conducted according to kit instructions. Absorbance was measured on a Victor 2 microplate reader (PerkinElmer) and TNF concentrations were normalized to total protein determined with the bicinchonic acid (BCA) method.

Statistics

All data was analyzed using repeated measures analysis of variance (ANOVA). An alpha value of .05 or below was considered significant. Differences between group means were assessed using Duncan's New Multiple Range *post hoc* tests when necessary.

CHAPTER III

ENDOGENOUS TNFα: BEHAVIORAL CONTRIBUTION TO THE SPINAL LEARNING DEFICIT

Experiment 1

The experiments in this dissertation were designed to test whether TNFα mediates the spinal learning deficit produced by uncontrollable shock. In order to comprehensively assess the behavioral role of TNFα in the spinal learning deficit, it is important to first test the necessity for endogenous TNFα in this phenomenon. Experiment 1 was designed to test whether uncontrollable shock can produce a deficit in the absence of functional endogenous TNFα. If TNFα is necessary in order for uncontrollable shock to cause a learning deficit, then inhibiting TNFα prior to uncontrollable shock should block the induction of this deficit.

Procedure

Experiment 1 used 32 rats (n=8). Twenty-four hours after complete transection, subjects received an intrathecal injection of either the soluble TNF receptor sTNFR1 (350 ng) or saline vehicle in 10 μL, followed by a 20 μL saline flush. Forty-five minutes later, subjects received either 6 minutes of uncontrollable shock or an equivalent period of unshocked restraint. All subjects were then immediately tested for instrumental learning.

Results

The effect of endogenous TNFα inhibition on the shock-induced deficit is depicted in Figure 1. As expected, vehicle-treated unshocked subjects were able to learn. Likewise, the TNF inhibitor sTNFR1 alone had no detrimental effect on the acquisition of the instrumental response. Vehicle-treated subjects that received uncontrollable shock exhibited a pronounced learning deficit. Interestingly, those subjects that received the TNFα inhibitor prior to uncontrollable shock were able to learn. An ANOVA did not reveal a significant main effect of drug or shock treatment, nor did it reveal a significant Drug X Shock interaction. There was, however, a main effect of trials, and a significant three-way interaction between trials, drug, and shock, $Fs > 1.54$, $p < .05$. This interaction indicates that learning (the change in flexion duration over time) is dependent on *both* shock and TNFα inhibitor treatment. No other interactions were significant, $p > .05$.

The number of responses made by each subject was also assessed (Figure 1B). On average, subjects that exhibited the learning deficit exhibited the highest rate of responding, while those that learned responded less frequently. The difference in total response number was assessed using an ANOVA, revealing main effects of drug, shock, and trials, as well as significant Drug X Shock, Trials X Drug, and Trials X Drug X Shock interactions and $Fs > 2.02$, $p < .05$. *Post hoc* analysis of group means showed that vehicle shocked subjects were significantly different from all other groups, $p < .05$. The higher response numbers for subjects that failed is due to the fact that these subjects had shorter response durations, and were therefore exposed to shock more often. This

finding is important, as it shows that the failure to exhibit increased response durations is not due to a lack of responding, and therefore not a performance deficit. Because all

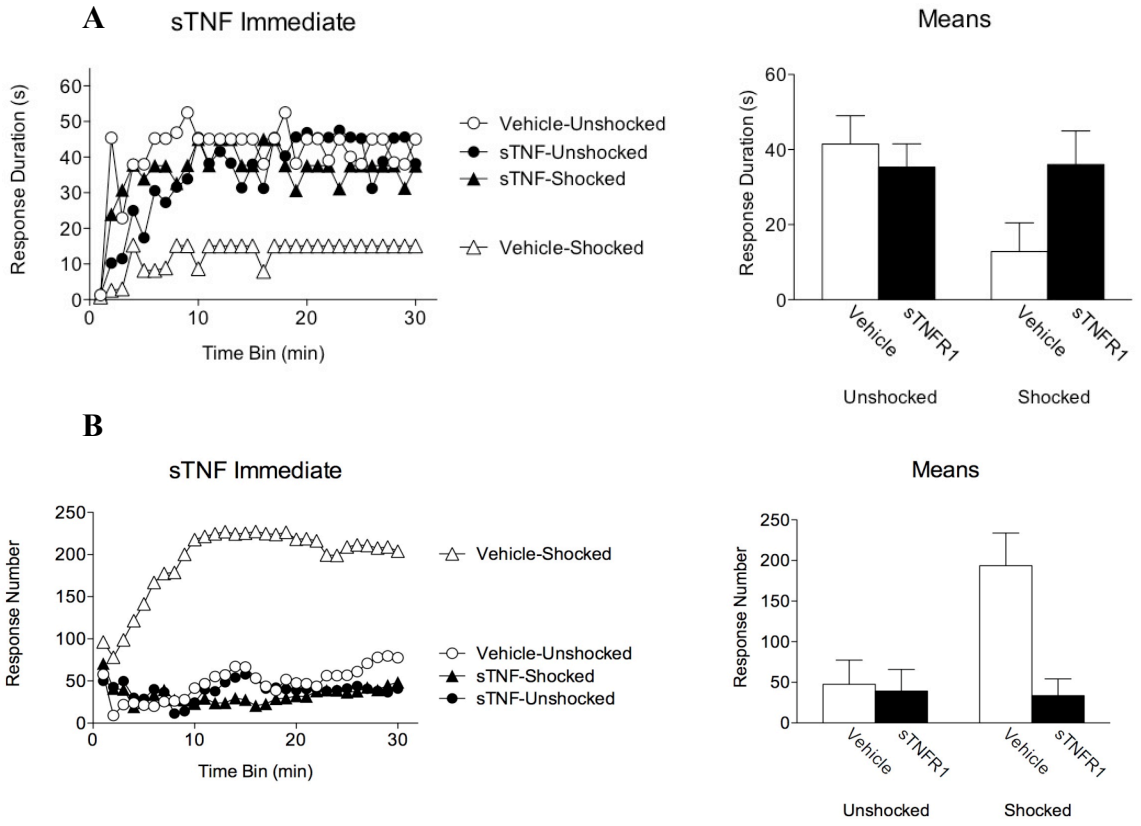

Figure 1. Effect of TNFα inhibition on the shock-induced deficit. Vehicle groups are shown in white, sTNFR1-treated groups in black. In the left panel, unshocked groups are represented with circles, shocked groups with triangles. A) Left panel depicts response durations over time. Right panel depicts mean response durations for each group, collapsed over time. Data indicate that sTNFR1 treatment blocks the shock-induced deficit. B) Left panel depicts response number over time, right panel depicts mean response number for each group. Data show that subjects who failed had higher response numbers, confirming that these subjects are not exhibiting a deficit in response performance.

subsequent experiments yielded a similar, inverse, relationship between response duration and response number, only the former is reported.

Discussion

These data provide behavioral evidence for the role of TNFα in the detrimental effects of uncontrollable shock. Blocking endogenous TNFα action prior to uncontrollable shock protected against the induction of the behavioral deficit, suggesting that TNFα receptor activation is necessary in order for the shock-induced deficit to occur.

Experiment 2

Experiment 1 demonstrated a necessity for endogenous TNFα in order for uncontrollable shock to produce a deficit immediately following stimulation. Expanding on that experiment, Experiment 2 is designed to further assess the role of TNFα in the deficit by examining the long-term effect of a TNFα inhibitor when given either prior to uncontrollable shock or prior to testing. In this way, the current experiment tests whether endogenous TNFα is necessary in order for uncontrollable shock to *induce* the deficit, as well as test the possibility that TNFα is necessary in order for the deficit to be *expressed* following uncontrollable shock.

<table>
<tr><td rowspan="2">Vehicle</td><td>Uncontrollable Shock</td><td rowspan="6">24 hr</td><td rowspan="2">Vehicle</td><td rowspan="6">Instrumental Testing</td></tr>
<tr><td>No Shock</td></tr>
<tr><td rowspan="2">sTNFR1</td><td>Uncontrollable Shock</td><td rowspan="2">Vehicle</td></tr>
<tr><td>No Shock</td></tr>
<tr><td rowspan="2">Vehicle</td><td>Uncontrollable Shock</td><td rowspan="2">sTNFR1</td></tr>
<tr><td>No Shock</td></tr>
</table>

Figure 2. Experimental design for experiment 2.

Procedure

Experiment 2 used 48 rats (n =8). The design of this experiment is depicted in Figure 2. To test the long-term effect of uncontrollable shock, two groups were given vehicle injections, followed 45 minutes later with either uncontrollable shock or none. Twenty-four hours later, subjects were given a second vehicle injection, followed by instrumental testing. To test the effect of TNFα inhibition on the induction of deficit, two groups were given sTNFR1, followed 45 minutes later with uncontrollable shock or none. Twenty-four hours later, subjects were given a vehicle injection, followed by instrumental testing. Finally, to test the effect of TNFα inhibition on the expression of the deficit, two groups were given vehicle injections, followed 45 minutes later with uncontrollable shock or none. Twenty-four hours later, subjects received an intrathecal injection of sTNFR1, followed by instrumental testing. This design allowed us to: a) verify that shock treatment inhibits learning 24 hours later, b) test whether sTNFR1

before shock blocks the induction of the deficit, and c) test whether sTNFR1 after shock blocks the expression of the deficit.

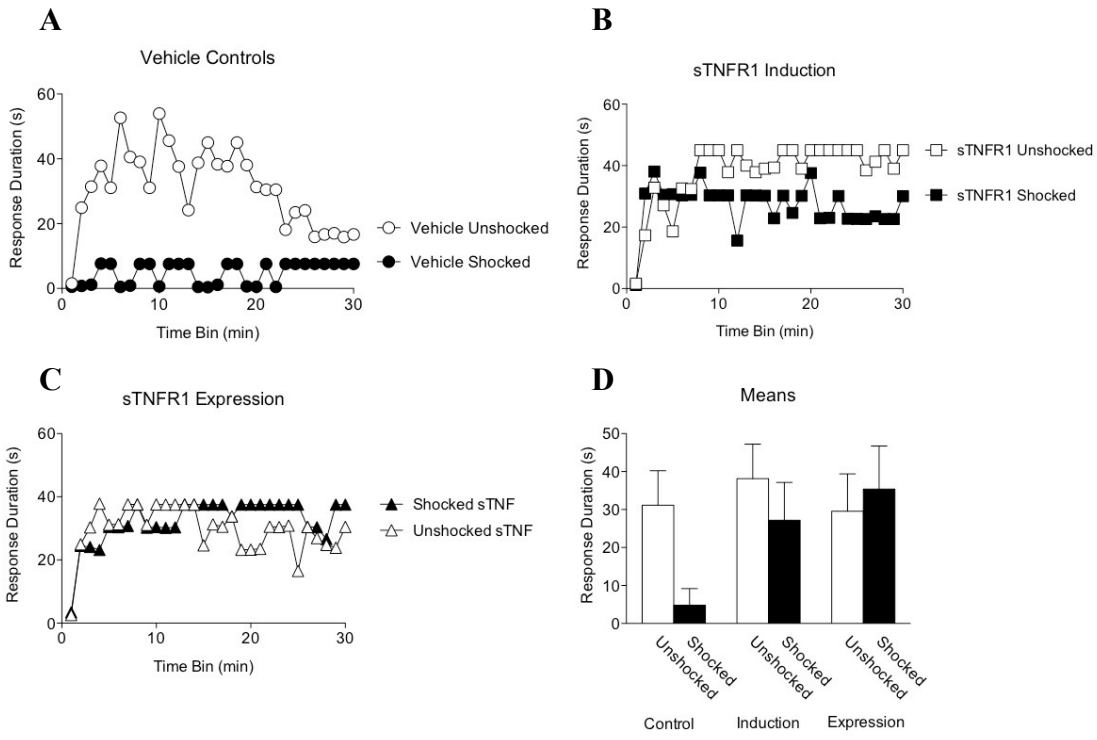

Figure 3. Effect of TNFα inhibition on the induction and expression of the shock-induced deficit. Unshocked groups are shown in white, shocked groups are shown in black. A) Response durations over time for subjects receiving vehicle only. B) Response durations over time for groups that received sTNFR1 prior to shock treatment. C) Response durations over time for groups receiving sTNFR1 prior to testing. D) Mean response durations for all groups collapsed over time. Data indicate that sTNFR1 treatment blocks the induction and expression of the shock-induced deficit.

Results

Induction. The design of this experiment allowed for the same shocked and unshocked vehicle-treated controls to be compared to both the induction and expression groups in the subsequent analyses. As expected, prior exposure to intermittent shock inhibited learning when subjects were tested 24 hours later (Figure 3A).

The effect of TNFα inhibition on the induction of the shock-induced deficit is depicted in Figure 3B. Administration of the TNFα inhibitor sTNFR1 alone had no detrimental effect on learning. Rats that received the TNFα inhibitor prior to uncontrollable shock were also able to learn. An ANOVA revealed main effects of drug, shock condition, and trials, $F > 3.45$, $p < .05$. Further, the Trials X Shock interaction and the Trials X Drug X Shock interaction were both significant, $F(29,812) > 2.38$, $p < .05$. *Post hoc* comparison of the group means confirmed that the vehicle shocked group differed significantly from all other groups, $p < .05$. No other effects were significant, $p > .05$.

Expression. The effect of TNFα inhibition on the expression of the shock-induced deficit is depicted in Figure 3C. As in the induction groups, those that received sTNFR1 alone had no impairment in learning. Surprisingly, those that received uncontrollable shock followed by sTNFR1 prior to testing were also able to learn. An ANOVA comparing these groups to the vehicle controls revealed a significant main effect of trials, as well as a significant Trials X Shock interaction, $F(29, 812) > 2.27$, $p < .01$. *Post hoc* comparison of the group means confirmed that the vehicle shocked group

differed significantly from all other groups, $p < .05$. No other effects were significant, $p > .05$.

Discussion

The protective effect of TNFα inhibition found in the induction groups of this experiment extends the findings from Experiment 1, in which sTNFR1 was previously shown to protect against the shock-induced deficit when subjects were tested immediately following shock. Here, this protective effect was evident when subjects were tested 24 hours after shock administration.

Beyond the protective effect of TNFα inhibition, this experiment also demonstrates that sTNFR1 can provide a therapeutic effect, blocking the expression of the shock-induced deficit when given 24 hours after uncontrollable shock. The capacity for TNFα inhibition to restore learning long after the deficit has been induced suggests that uncontrollable shock may not only cause TNFα release, but that this release may be sustained. That learning can be rescued by blocking TNFα receptor activity also suggests that the role for TNFα in the deficit is not a transient one. Rather than TNFα acting as a intermediary in a cascade that leads to an immutable intracellular modification, it appears that TNFα receptor activity may be a lynchpin of the mechanism that produces the shock-induced deficit, and sustained TNFα receptor activation is required in order for the deficit to be expressed.

CHAPTER IV

BEHAVIORAL IMPACT OF EXOGENOUS TNFα ON THE SPINAL LEARNING DEFICIT

Experiment 3

It has been shown that the overexpression of TNFα can lead to a robust increase in neural excitability that ultimately undermines learning (Leondoukis, et al., 2008). Similarly, a number of pharmacological agents that are known to induce the spinal learning deficit have been correlated with increased TNFα release (Cocchiara, et al., 1999; Hwang, Lee, Kim, Shim, & Hahm, 2008; Elliott, et al., 2009). The previous two experiments outlined a necessary role for TNFα in the shock-induced deficit, showing that the inhibition of endogenous TNFα can block the induction and expression of the deficit. In order to get a comprehensive view of the degree to which TNFα mediates the spinal learning deficit, it is also important to examine the sufficiency for TNFα in this phenomenon. Experiment 3 tests whether administration of exogenous TNFα can substitute for uncontrollable shock to produce a learning deficit.

Procedure

Experiment 3 used 48 rats (n =8). Twenty-four hours after complete transection, subjects received an intrathecal injection of one of three doses of TNFα (0, 600, or 6000 pg) in 10 µL, followed by a 20 µL saline flush. Subjects were then tested for instrumental learning either 45 minutes after injection, or 24 hours after injection.

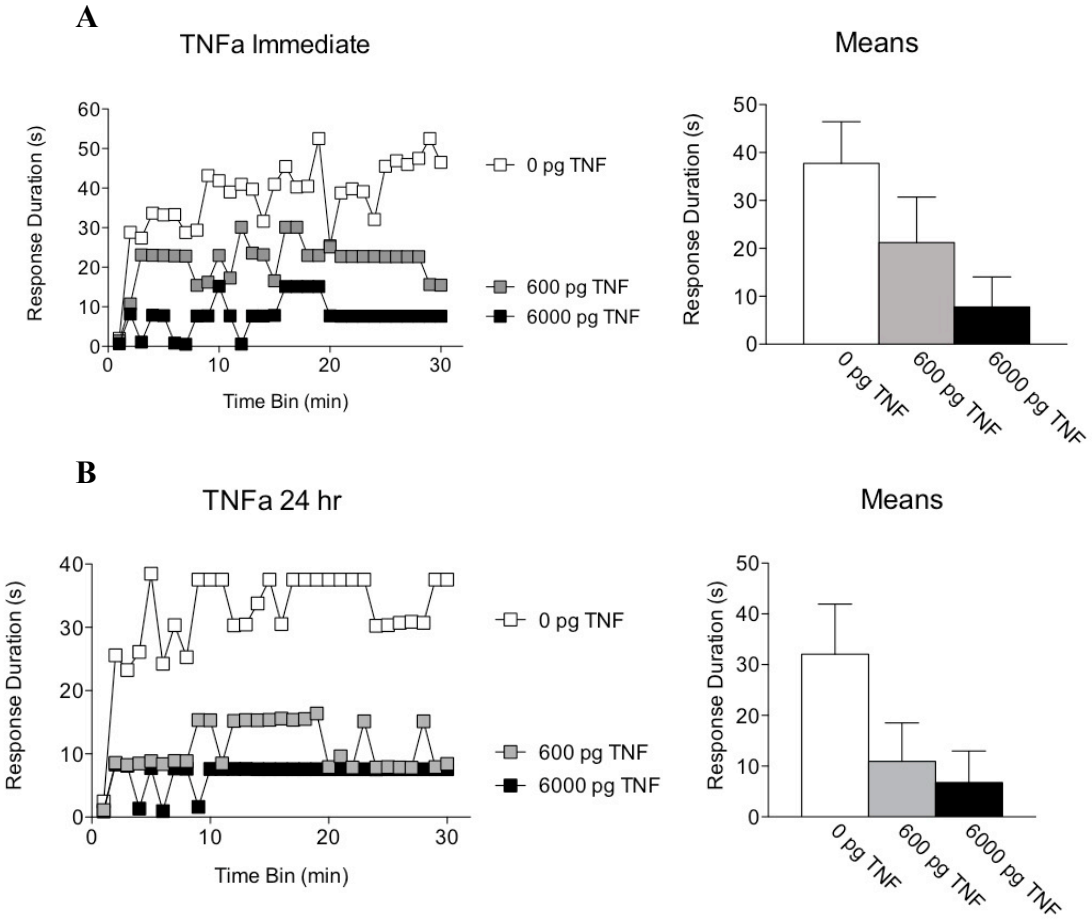

Figure 4. Immediate and long-term effect of exogenous TNFα treatment on instrumental learning. Left panels depict response durations over time. Right panels depict mean response durations for each group, collapsed over time. Vehicle-treated groups are shown in white, 600 pg dose in gray, 6000 pg dose in black. A) Response durations for groups receiving exogenous TNFα 45 minutes prior to testing. B) Response durations for groups receiving exogenous TNFα 24 hours prior to testing. Data indicate that exogenous TNFα undermines learning in a dose-dependent fashion, both immediately and 24 hours after treatment.

Results

The effect of exogenous TNFα treatment on instrumental learning is depicted in Figure 4. As expected, the subjects that were given the 0 ng dose (vehicle) were able to learn. All other groups failed to learn. An ANOVA revealed main effects of drug treatment and trials, as well as a significant Drug X Trials interaction, $Fs > 1.37, p < .05$. *Post hoc* analyses of the group means also showed a significant difference between the highest dose of TNFα and the lowest dose (vehicle), at both the immediate and 24 hour time points, $p < .05$. No other effects were significant, $p > .05$.

Discussion

The previous two experiments demonstrated that endogenous TNFα was necessary to produce a shock-induced deficit. Here we expand on those findings by showing that administration of TNFα is sufficient to produce both an acute and long-term deficit. The highest doses tested at either timepoint caused a deficit that is commensurate with the level of impairment seen in uncontrollably shocked subjects. This experiment lends further evidence that TNFα release and receptor activity may mediate the maladaptive behavioral effect of uncontrollable stimulation.

The finding that TNFα can produce a long-term deficit is especially interesting, because 24 hours should be long enough for the drug to clear he system. From this perspective, these data suggest that exogenous TNFα may engage other maladaptive processes that extend and sustain the period in which the deficit can be expressed. This idea is supported by others who have shown that high levels of TNFα can elicit a

persistent disruption in neural plasticity (Butler, O'Connor, & Moynagh, 2004). Similarly, Ji & Suter (2007) have also shown that TNFα treatment can produce a prolonged deficit in normal neural function, and have suggested an intriguing underlying mechanism. They have shown that TNFα, acting on microglia, can lead to the production and release of further TNFα, creating an autocrine feedback loop that sustains high levels of extracellular TNFα for an extended period of time. My next experiment explores whether this mechanism underlies the long-term effect of exogenous TNFα treatment on spinal learning.

Experiment 4

Prior work has shown that TNFα administration can lead to an increased expression of endogenous TNFα stores (Kuno, et al., 2005). Such sustained TNFα activity could mediate the long-term TNFα-induced learning deficit that was observed in Experiment 3. If sustained TNFα activity is responsible for the TNFα-induced deficit, then it would be expected that the expression of the deficit could be blocked by inhibiting TNFα prior to testing. Experiment 4 was designed to address this possibility, testing whether the long-term effect of TNFα treatment can be blocked by a TNFα-inhibitor prior to testing.

Procedure

This experiment used 48 rats (n =8). Twenty-four hours after complete transection, subjects received an intrathecal injection of TNFα (6000 pg) or vehicle in 10

μL, followed by a 20 μL saline flush. Twenty-fours later, subjects were given the TNFα inhibitor sTNFR1 or vehicle intrathecally (10 μL) 45 minutes prior to testing. Because pilot data indicated a 350 ng dose of sTNFR1 had a partial effect, I included two additional groups that were treated with a higher dose (700 ng) of sTNFR1 prior to testing.

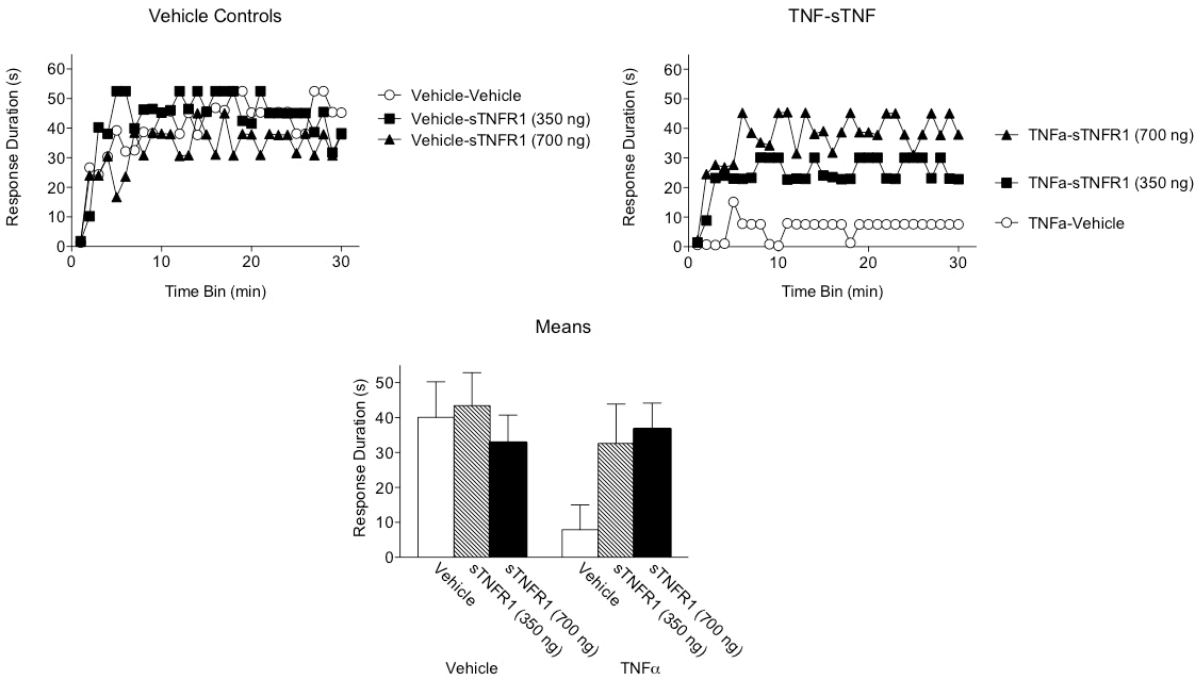

Figure 5. Effect of TNFα inhibition on the TNFα-induced deficit. Left panel shows response durations for groups that received vehicle injections prior to sTNFR1 treatment. Right panel shows response durations for groups that received TNFα treatment prior to sTNFR1 treatment. Bottom panel depicts mean response durations for all groups collapsed over time. Data indicate that the expression of the TNFα-induced deficit can be blocked by sTNFR1 treatment.

Results

The effect of TNFα inhibition on the TNFα-induced deficit is depicted in Figure 5. As expected, subjects that received only vehicle injections were able to learn. Vehicle-treated subjects that received sTNFR1, at either dose, also learned. Subjects given TNFα alone exhibited a learning deficit when tested 24 four hours later replicating the finding from Experiment 3. Interestingly, TNFα-treated subjects that were given sTNFR1 prior to testing were able to learn. An ANOVA revealed a significant main effect of both TNF treatment and trials, $F > 6.75$, $p < .05$. Although no other main effects or interactions were significant, the interaction between TNF treatment and sTNFR1 treatment approached significance ($F(2,42) = 3.13$, $p = .054$). To further explore this relationship trend analyses were run. These analyses revealed that the linear component of the Trials X TNF X sTNFR1 interaction was significant, $F = 10.42$, $p < .01$. The trend analyses also showed the quadratic component of the Trials X sTNFR1 interaction to be significant, $F = 5.81$, $p < .05$. Finally, a *post hoc* comparison of the group means revealed that the group that received TNFα alone differed significantly from all other groups, $p < .05$.

Discussion

As in Experiment 2, this experiment showed the therapeutic capacity of TNFα inhibition after a learning deficit has been induced. More importantly, it demonstrates that TNFα receptor activity is still required 24 hours after TNFα treatment in order for the deficit to be expressed. This finding lends further evidence to the possibility that

exogenous TNFα administration may elicit an increased expression of endogenous TNFα that outlasts the initial treatment.

CHAPTER V

MECHANISMS OF ACTION: GLIAL AND POST-SYNAPTIC EFFECTS OF TNFα

Experiment 5

In other preparations, the feedback loop that underlies the long-term effects of TNFα treatment depends on glial release of endogenous TNFα (Kuno, et al., 2005). Further, if TNFα underlies the long-term effect of uncontrollable shock, then the maintenance (memory) of that effect should depend on glial activity. Supporting this, Vichaya et al. (2009) have shown that the glial inhibitor fluorocitrate inhibits the long-term consequence of uncontrollable shock. Experiment 5 explores whether the administration of fluorocitrate prior to TNFα treatment will block its long-term effect on instrumental learning.

Procedure

This experiment used 24 rats (n =6). Twenty-four hours after complete transection, subjects received an intrathecal injection of either the glial inhibitor fluorocitrate (0.5 nmol) or vehicle in 10 μL, followed by a 20 μL saline flush. Forty-five minutes later, subjects received TNFα (6 ng) or vehicle, followed by a 20 μL flush. All subjects were then tested for instrumental learning 24 hours later.

Results

The effect of glial metabolic inhibition on the TNFα-induced learning deficit is shown in Figure 6. Subjects that only received vehicle treatment were able to learn as expected. Likewise, those subjects that received fluorocitrate alone also learned,

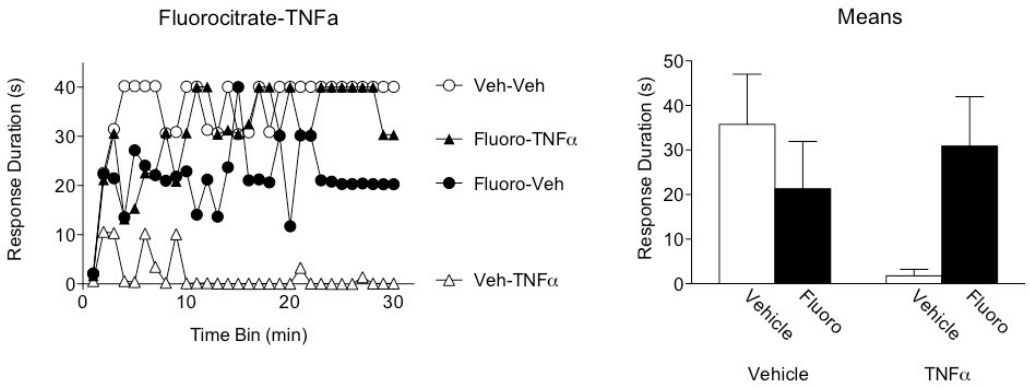

Figure 6. Effect of glial metabolic inhibition on the TNFα-induced deficit. Left panel depicts response durations over time. Right panel depicts mean response durations for each group, collapsed over time. Groups that received initial injections of vehicle are shown in white, those that received fluorocitrate are shown in black. For the second injection, groups that received vehicle are represented by circles, those that received TNFα are represented by triangles. Data indicate that fluorocitrate treatment prior to TNFα blocks the induction of the deficit.

replicating prior findings (Vichaya, Baumbauer, Carcoba, Grau, & Meagher, 2009). Subjects receiving TNFα alone exhibited a marked decrease in flexion duration, and this deficit was attenuated in subjects that received fluorocitrate prior to TNFα treatment. Although there were no significant main effects of TNFα or fluorocitrate ($F(1,20) < 1.99, p > .05$), an ANOVA revealed a significant interaction between drug treatments, $F(1,20) = 6.30, p < .05$. There was a significant main effect of trials, as well as a

significant interaction between trials, TNFα, and fluorocitrate treatment, $F(29, 580) > 1.62$, $p < .05$. *Post hoc* analysis of group means showed that subjects receiving TNFα alone differed significantly from those that received vehicle alone, as well as those who received fluorocitrate prior to TNFα treatment, $p < .05$. No other differences approached significance, $p > .05$.

Discussion

This experiment demonstrates the necessity for glial metabolism in order for TNFα treatment to produce a long-term spinal learning deficit. This finding is significant, in that it highlights a critical component of the maladaptive mechanism by which TNFα works to undermine learning. Taken together with the previous experiment, which showed that the long-term deficit requires continued TNF receptor activity, the current experiment provides further evidence that TNFα treatment may lead to sustained glial release of endogenous TNFα.

Experiment 6

The previous experiments have shown TNFα to be sufficient to produce a learning deficit, and that glial activation is necessary to produce this effect. In order to further understand the natural interaction between glial activation, TNFα release, and the expression of the deficit, it is important to investigate methods that may cause glial-mediated TNFα release. Administration of the potent microglial activator lipopolysaccharide (LPS) is known to cause an increase in TNFα protein levels in the

spinal cord (Shen, et al., 2008). It has also been previously shown that LPS is sufficient to produce a spinal learning deficit (Young, Baumbauer, Elliot, & Joynes, 2007). Experiment 6 is designed to test whether TNFα inhibition prior to testing is sufficient to block the LPS-mediated deficit.

Procedure

This experiment used 24 rats (n = 6). Twenty-four hours after complete transection, subjects were given an intrathecal injection of either 100 µg LPS in 10 µL saline or vehicle, followed by a 20 µL saline flush. Twenty-four hours later, subjects were administered an intrathecal injection of either sTNFR1 (350 ng) or saline vehicle in 10 µL, followed by a 20 µL saline flush. All subjects were then tested for instrumental learning.

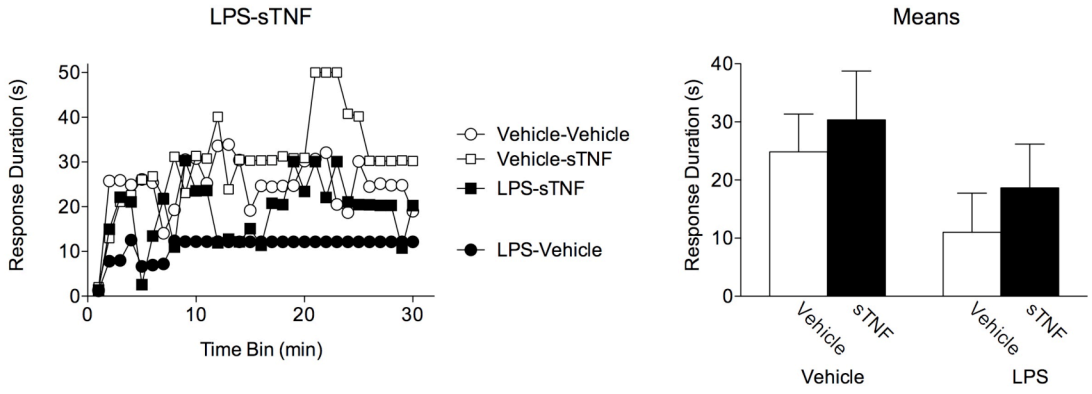

Figure 7. Effect of TNFα inhibition on the LPS-induced deficit. Left panel depicts response durations over time. Right panel depicts mean response durations for each group. Subjects that received an initial injection of vehicle are shown in white, subjects that received LPS are shown in black. Subjects that received vehicle as a second injection are represented by circles, subjects that received sTNFR1 are represented by squares. Data indicate that LPS produces a mild deficit, and sTNFR1 treatment has a slight therapeutic effect.

Results

The effect of TNF inhibition on the LPS-induced deficit is depicted in Figure 7. Subjects that received sTNFR1 or vehicle alone were able to learn. Rats receiving LPS alone had shorter response durations than all other groups, but did not exhibit as robust of a deficit as has been shown previously. Subjects in which TNFα was inhibited following LPS treatment had longer response durations than those receiving LPS alone, but were unable to learn as well as the controls. An ANOVA revealed a main effect of trials, $F(29, 551) = 2.64$, $p < .05$. None of the other main effects, or interactions, were significant, all $Fs < 1.71$, $p > .05$.

Discussion

Although TNFα inhibition caused LPS-treated rats to exhibit slightly higher response durations than rats treated with LPS alone, this effect is undermined by the fact that LPS did not produce a robust deficit. Likewise, the vehicle controls did not learn as well as those in previous experiments. Future work will be needed to assess whether a higher dose of LPS may be needed to produce a deficit, or whether a higher dose of TNFα inhibitor may be needed in order to see a greater difference between these two groups.

Experiment 7

Previous research has shown that neuronal TNFα receptor activation causes an increase in membrane trafficking of GluR2-lacking AMPA receptors, as well as GABA receptor endocytosis (Stellwagen, Beattie, Seo, & Malenka, 2005). Unlike AMPA receptors that express the GluR2 subunit, these receptors are calcium-permeable. This permeability allows for a substantial increase in postsynaptic excitability, and with sufficient stimulation, can lead to excitotoxicity. This phenomenon has recently been shown to underlie cell death following spinal cord injury (Ferguson, Christensen, et al., 2008). Experiment 6 was designed to assess the potential role of TNFα-mediated upregulation of Glur2-lacking AMPA receptors in driving the overexcitation that underlies the spinal learning deficit.

Procedure

Experiment 7 used 40 rats (n = 10). Twenty-four hours after complete transection, subjects were given an intrathecal injection of TNFα (60 pg) or vehicle in 10 µL, followed by a 20 µl saline flush. Twenty-four hours later, subjects were given an intrathecal injection of the GluR2-lacking AMPA receptor antagonist Naspm or vehicle in 10 µL, followed by a 20 µL saline flush. All subjects were then tested for instrumental learning.

Results

The effect of GluR2-lacking AMPAR antagonism on the TNFα-induced deficit is depicted in Figure 8. As expected, those subjects that received vehicle alone were able to learn, while those that received TNFα alone exhibited a learning deficit. Interestingly, those that were given Naspm prior to testing were able to learn, regardless of whether they had previously been given TNFα. An ANOVA revealed main effects of TNFα

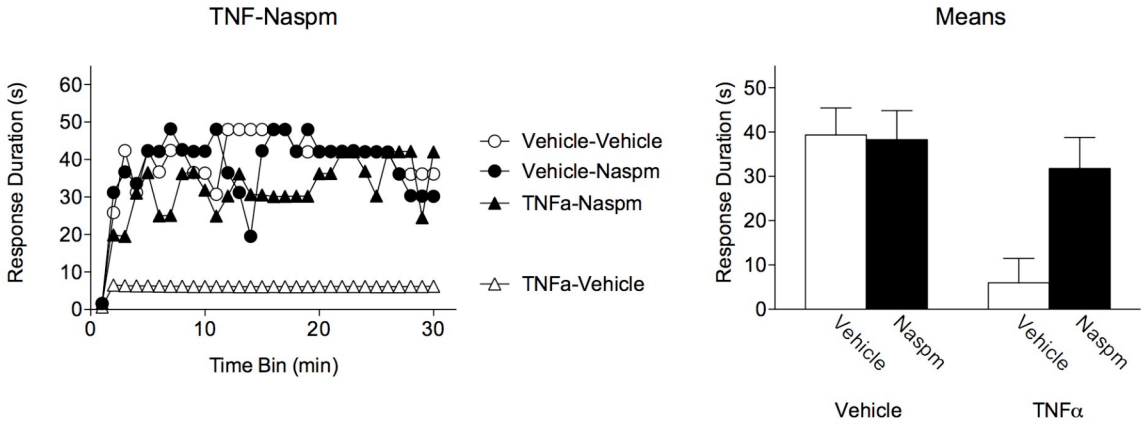

Figure 8. Effect of Glur2-lacking AMPA receptor antagonism on the TNFα-induced deficit. Left panel depicts response durations over time. Right panel depicts mean response durations for each group, collapsed over time. Groups that received an initial injection of vehicle are represented by circles, those that received TNFα are shown as triangles. For the second injection, groups that received vehicle are shown in white, groups that received Naspm are shown in black. Data indicate that Naspm treatment blocks the expression of the TNFα-induced deficit.

treatment and trials, Fs > 5.28, $p < .01$. Likewise, a significant interaction between TNFα and trials was observed, F (29, 1044) = 1.66, $p < .05$. The ANOVA did not show a significant three-way interaction between trials, TNFα, and sTNFR1, F(29, 1044) = 1.37, $p = .09$. Because this interaction approached significance, the relationship was further

explored using trend analyses. The trend analyses showed that the linear component of the Trials X TNFα X sTNFR1 interaction was significant, $F = 11.89$, $p < .05$. *Post hoc* analysis of the group means showed that those receiving TNFα alone were significantly different from all other groups, $p < .05$.

Discussion

This experiment showed a necessary role for calcium-permeable AMPA receptors in the expression of the TNFα-induced deficit. As these receptors have previously been shown to mediate overexcitation and excitotoxicity, this finding lends evidence to the notion that the deficit induced by TNFα may reflect a saturation of spinal plasticity. Further, the capacity for Naspm to block the expression of the deficit provides a unique therapeutic potential for the reinstatement of future plasticity.

CHAPTER VI

CELLULAR ASSESSMENT OF TNFα

Experiments 1-5 showed that pharmacological treatments that target TNFα, or its presumed downstream consequences, affect the capacity for spinal learning. These experiments suggest that the adverse effect of uncontrollable shock on instrumental learning is mediated by TNFα. My last two experiments used cellular assays to explore whether shock treatment impacts TNFα mRNA expression (Experiment 8), or protein levels (Experiment 9) within the spinal cord.

Experiment 8

As Experiments 1 and 2 showed that TNFα was necessary for the expression of the shock-induced deficit, it is expected that uncontrollable shock would cause an increase in TNFα production in the spinal cord. Experiment 8 utilizes quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) in order to assess levels of TNFα mRNA following controllable shock (master), uncontrollable shock (yoked), or no shock. As a positive control, I also assessed the expression of c-Fos, which should be increased by shock treatment.

Procedure

This experiment used 24 rats (n=8). Subjects were given either master training (controllable shock), yoked training (uncontrollable shock) or no shock over a 30 minute training session. This master/yoked paradigm ensures that the amount of shock received

within each master/yoked pair is equal. Equating shock within pairs is important, because it removes the variability of timing and number of shocks received within pairs, so that the only variable that is being tested is whether the shock is controllable or uncontrollable.

Following training, animals were anesthetized with pentobarbital (50 mg/kg), and 1 cm sections of L4-L5 spinal cords were removed and flash frozen at -80° C. Cord tissue was subsequently homogenized and prepared for RT-PCR as described in the general methods section.

Results

Previous work has shown that c-Fos expression is a reliable indicator of shock treatment, regardless of controllability. In order to first confirm that shock had an effect on the spinal tissue assayed, c-Fos expression was analyzed (Figure 9A). Both master and yoked groups showed an increase in c-Fos expression, and an ANOVA revealed a main effect of condition, $F(2, 21) = 6.73$, $p < .05$. *Post hoc* analysis of group means revealed that master and yoked groups were significantly different from unshocked controls, $p < .05$.

The effect of uncontrollable shock on TNF mRNA expression is depicted in Figure 9B. Yoked subjects that received uncontrollable shock were shown to have nearly a two-fold increase in TNFα mRNA expression as compared to unshocked controls. Though 7 of the 8 yoked rats exhibited higher TNFα mRNA expression than their

master partner, an overall ANOVA did not reveal any significant differences, $F(2, 21) = 1.07$, $p > .05$.

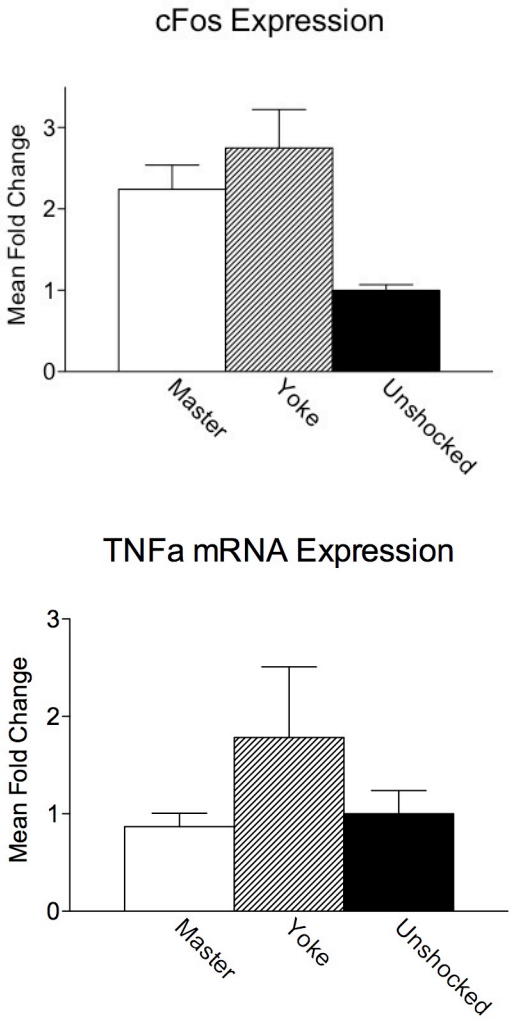

Figure 9. Effect of controllable and uncontrollable shock on the expression of TNFα mRNA. A) c-Fos mRNA expression in master, yoked, and unshocked groups. Upregulation of c-Fos expression was seen in both controllable (Master) and uncontrollable (Yoke) shock groups, as compared to unshocked controls, offering cellular confirmation of a shock effect. B) TNFα mRNA expression in master, yoked, and unshocked. Uncontrollably shocked (Yoke) subjects exhibited higher TNFα mRNA expression compared to master and unshocked subjects. Data expressed as mean fold change relative to unshocked controls.

Discussion

Reasoning that some variation was attributable to differences in early gene expression, we also performed an analysis of covariance, treating c-Fos expression as a covariate. As expected, the covariate did account for a significant proportion of the variance, $F(1, 20) = 25.83$, $p < .05$. More importantly, the analysis yielded a significant effect of treatment condition, $F(2, 20) = 3.65$, $p < .05$. These results provide preliminary evidence that uncontrollable stimulation engages TNFα mRNA expression within the spinal cord. However, while shock treatment had a robust effect on c-Fos expression, the observed differences in TNFα mRNA expression were less clear-cut and strongly linked to the level of early gene expression. Further work will be needed to clarify how shock treatment affects TNFα mRNA expression.

Experiment 9

Experiment 8 examined whether uncontrollable shock upregulated TNFα mRNA. While this finding suggests an increase in TNFα production, it is important examine TNFα protein expression in order to assure that there is an increase of TNFα in its active form following uncontrollable shock. Experiment 9 uses an enzyme-linked immunosorbent assay (ELISA) to assess the time-dependent changes in TNFα protein levels after uncontrollable shock.

Procedure

This experiment used 36 rats (n = 6). All Subjects were given either 6 minutes of uncontrollable shock to the tail or an equivalent period of restraint. Because controllable shock appears to have no effect on TNFα mRNA expression, this simplified design was used rather than a master/yoked paradigm. Also, because this design used shock to the tail rather than the hindlimb, the absolute shock intensity and number could be kept constant across all shocked subjects, which should reduce the variability observed in mRNA/protein expression.

Following shock, subjects were anesthetized with pentobarbital (50 mg/kg) and L4-L5 spinal cord segments were taken at one of three timepoints: Immediate, 6 hr, or 24 hr after shock administration. Cord tissue was subsequently homogenized and prepared for ELISA as described in the general methods section.

Results

The effect of uncontrollable shock on TNFα protein expression is depicted in Figure 10. Protein levels were based upon a standard curve that was run on the same plate with all spinal cord samples. These values were then normalized to each sample's relative total protein concentration, and this value is presented in the figure below. No change was seen in unshocked controls over time. In contrast, shock treatment appears to have produced a gradual increase in TNFα protein that emerges over time. Though the overall ANOVA did not yield a significant main effect or interaction, (Fs <

1.99, $p > .05$) trend analysis showed that the linear component of the interaction term approach significance, $F(1, 30) = 3.90$, $p = .06$.

To further explore the nature of this interaction, independent ANOVAs were performed at each time point. I found that shock had a significant impact at 24 hours [$F(1, 10) = 6.49$, $p < .05$], but not at 0 or 6 hours.

Discussion

Although no overall differences were seen between conditions, the significant difference between groups at 24 hours offers evidence that uncontrollable shock leads to long-term TNFα production, suggesting a mechanism by which uncontrollable shock may cause a long-term deficit.

Future assays may require a less dilute tissue sample in order to ensure that the TNFα protein levels observed are accurate. Due to the relatively insensitive nature of ELISA protein detection, more sensitive tests such as immunohistochemistry, may prove to be a better tool for investigating TNFα protein levels in the future.

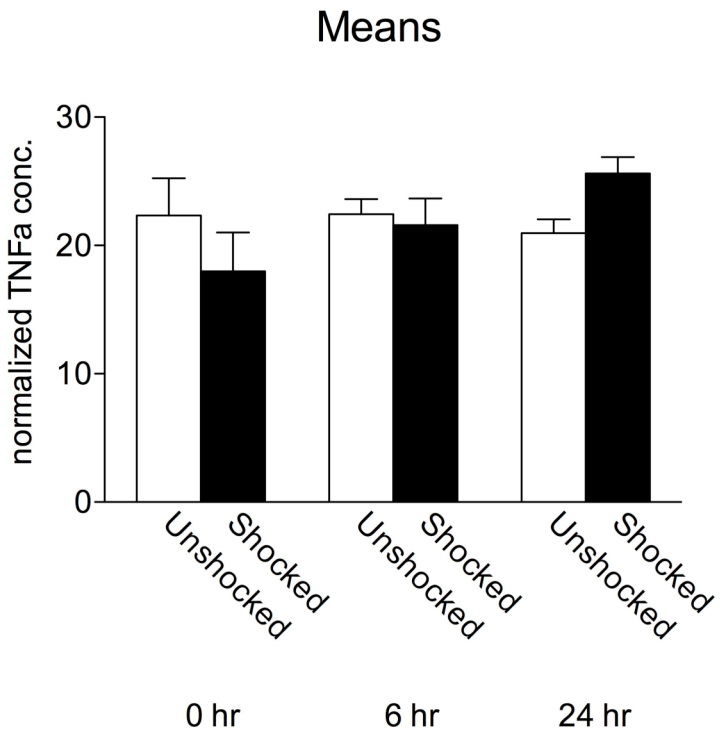

Figure 10. Effect of uncontrollable shock on TNFα protein expression. Group means are shown, depicting TNFα concentrations normalized to total protein concentrations per subject. Unshocked groups are shown in white, shocked groups in black. Data indicate neither time nor shock treatment affected TNFα concentration.

CHAPTER VII

GENERAL DISCUSSION AND SUMMARY

The experiments in this dissertation were designed to investigate the role of the cytokine tumor necrosis factor alpha (TNFα) in the inhibition of spinal instrumental learning. When assessing spinal plasticity using a spinal instrumental learning paradigm, we have previously found that uncontrollable stimulation produces a long-lasting learning deficit (Crown, et al., 2002a). This study tested whether the effect of uncontrollable stimulation was mediated by TNFα. Behaviorally, the learning deficit produced by uncontrollable shock is attenuated when endogenous TNFα is inhibited (Experiments 1 & 2). This effect is seen if TNFα is inhibited prior to uncontrollable shock, as well as if TNFα is inhibited prior to instrumental testing (24 hours after uncontrollable shock). Conversely, treatment with exogenous TNFα was shown to substitute for uncontrollable shock in eliciting a learning deficit (Experiment 3). This deficit was seen both immediately following and 24 hours after TNFα administration, and was dose-dependent.

The finding that exogenous TNFα produced a deficit that outlasted the initial drug treatment led us to investigate whether this long-term deficit was mediated by TNFα action, or if perhaps TNFα treatment induced a downstream neural modification that caused the long-term deficit. Experiment 4 showed that the long-term TNFα-induced deficit could be attenuated by TNFα receptor inhibition, suggesting the long-term deficit required sustained TNF receptor activity. Furthermore, the long-term effect of exogenous TNFα appears to be mediated by glial activation, as treatment with a glial

inhibitor attenuated the TNFα-induced deficit (Experiment 5). Experiment 6 tested the therapeutic effect of TNFα inhibition following treatment with LPS, which has been shown to induce TNFα release.

The post-synaptic effect of TNFα treatment was then assessed. TNFα has been shown to induce the trafficking of calcium-permeable AMPA receptors to the membrane, and it has been suggested that this mechanism plays a role in excitotoxicity and the saturation of spinal plasticity (Beattie, et al., 2002). Experiment 7 showed that selectively antagonizing calcium-permeable AMPA receptors prior to testing attenuated the long-term TNFα-induced deficit. Taken together, these findings outline a necessary and sufficient role for TNFα in the deleterious effect of uncontrollable stimulation, and suggest a critical role for TNFα in undermining adaptive spinal plasticity. Finally, I showed that uncontrollable shock can produce an upregulation of TNFα mRNA expression in the spinal cord (Experiment 8).

TNFα and the Deficit: Links to Central Sensitization

Work over the past decade has shown TNFα to be an important factor in glial-neuronal interactions. The capacity to induce changes in synaptic strength has made TNFα an attractive target as a potential mediator of synaptic plasticity (reviewed in Pickering, Cumiskey, & O'Connor, 2005; Park & Bowers, 2010). Stellwagen and Malenka (2006) outlined a constitutive role for TNFα in the global scaling of synaptic populations. They have shown that in response to a drop in neural activity, glial release of TNFα leads to an increase in excitation, which in turn brings the neural environment

back to homeostatic levels. Although this finding describes TNFα as being critical to regulating synaptic efficacy, homeostasis is a delicate balancing act. While appropriate levels of TNFα can act to normalize neurotransmission, overexpression of TNFα can lead to synaptic dysfunction. A number of studies have shown that the overexpression of TNFα (due either to pharmacological manipulation or pathological induction) can lead to excitotoxicity and disruption in plasticity (Hermann, Rogers, Bresnahan, & Beattie, 2001; Butler, O'Connor, & Moynagh, 2004). In slice preparations of both the CA1 and dentate gyrus regions of the hippocampus, bath application of pathophysiological levels of TNFα led to the inhibition of long-term potentiation, a form of synaptic plasticity believed to underlie learning and memory in these brain regions (Tancredi, et al., 1992; Cunningham, Murray, O'Neill, Lynch, O'Connor, 1996; Bliss & Collingridge, 1993). *In vivo* studies have confirmed these findings, showing that mice in which TNFα is overexpressed exhibit marked impairment in spatial learning using a water maze task (Aloe, et al., 1999).

Interestingly, high levels of TNFα in the spinal cord produce different effects than in the hippocampus. As opposed to inhibiting LTP in the hippocampus, overexpression of TNFα in dorsal horn neurons induces LTP (Liu, et al., 2007). This increase in excitatory synaptic strength has been most commonly found in primary afferents, which carry nociceptive sensory information (Sorkin, Xiao, Wagner, & Myers, 1997). Synaptic potentiation in the dorsal horn has been termed central sensitization, and has been suggested to be a mechanism for neuropathic pain (reviewed in Latremoliere & Woolf, 2009). Inducing central sensitization through a number of inflammatory models

has been shown to produce a marked increase in the expression of TNFα in both the peripheral and central nervous systems (Schafers, Geis, Svensson, Luo, & Sommer, 2003; Bao, Zhu, Elhassan, Wu, Xiao, & Zhu, 2001). TNFα has also been shown to induce behavioral symptoms of neuropathic pain (including allodynia and hyperalgesia) when given exogenously (Gao, et al., 2009; Youn, Wang, & Jeong, 2008). Conversely, inhibition of TNFα activity through genetic knockout of TNFα production and/or TNFα receptor antagonists have proven therapeutic against neuropathic pain (Mata, Hao, & Fink, 2008; Sommer, Schafers, Marziniak, & Toyka, 2001). Findings such as these have led many to consider TNFα an important mediator of enhanced nociceptive processing in the spinal cord, and have provided further evidence that TNFα contributes to maladaptive spinal plasticity (reviewed in Leung & Cahill, 2010).

Previous work from our lab has also implicated the kappa opioid system in the deficit produced by uncontrollable shock, showing a necessary and sufficient role of kappa opioid receptor activity in the spinal learning deficit (Joynes & Grau, 2004; Washburn, et al., 2008). Interestingly, kappa opioid receptor activation has also been shown to act on astrocytes to increase intracellular calcium concentrations, and could potentially lead to increased glial activation and TNFα release (Gurwell, et al., 1996; Stiene-Martin, Mattson, & Hauser, 1993; Shafer & Murphy, 1997). Further, others have shown direct immunomodulatory effects of kappa opioid ligands, as the endogenous opioid dynorphin has been shown to increase TNFα expression in the brain (Chao, et al., 1995). Because both TNFα and kappa opioid receptors have been shown to be essential

to the expression of the spinal learning deficit, there may be an important interaction between these systems that will need to be investigated in the future.

More recent findings from our lab have drawn a link between central sensitization and the spinal learning deficit, suggesting that uncontrollable stimulation induces a central sensitization-like effect in the spinal cord which undermines future learning (Ferguson, et al., 2006; Hook, Huie, & Grau, 2008). Experiments 1 and 2 showed that the deficit produced by uncontrollable shock requires TNFα. These findings mirror those that have shown TNFα to be necessary in the development of neuropathic pain. From this perspective, TNFα can be seen as an important neurobiological mediator by which uncontrollable shock undermines spinal learning, and provides further evidence that the deficit may reflect a change in synaptic strength that is akin to central sensitization.

Mechanisms of Action

The experiments in this dissertation provide evidence that the learning deficit produced by uncontrollable shock depends on TNFα. The following section will address the possible mechanisms by which 1) uncontrollable stimulation leads to TNFα production and release, 2) TNFα affects post-synaptic neurons to undermine spinal learning 3) TNFα mediates the long-term deficit.

Shock-Induced Production and Release of TNFα

Experiment 8 showed that uncontrollable peripheral shock can cause an upregulation of TNFα mRNA expression in the region of the spinal cord that is essential for instrumental learning. Given that uncontrollable shock has been previously shown to induce a central sensitization-like effect, these findings are not surprising. The role for TNFα in mediating inflammatory processes in the spinal cord has been well-defined. If we are to think of uncontrollable shock as working in a similar manner to other models of peripheral inflammation, then a central increase in TNFα production and release is to be expected. Intraplantar injection of inflammatory agents such as carrageenan and capsaicin induce robust increases in spinal TNFα levels, as do models of peripheral nerve injury including chronic constriction injury and spinal nerve ligation (DeLeo, Colburn, & Rickman, 1997; Schafers, Svensson, Sommer, & Sorkin, 2003). Following peripheral insult, a barrage of excitatory signaling from Aß and C fibers reaches primary afferents in the dorsal horn of the spinal cord. This heightened excitatory input sensitizes central neurons, leading to secondary hyperalgesia and ultimately the development of chronic neuropathic pain states. As the study of this phenomenon has grown, TNFα has emerged as a critical inflammatory mediator in both the peripheral and central nervous system. Inhibition of TNFα activity following peripheral nerve injury has been shown to alleviate injury-induced hyperalgesia (Sommer, Schafers, Marziniak, & Toyka, 2001). Likewise, inhibiting TNFα prior to peripheral injury provides a protection against the development of mechanical allodynia (Schafers, Svensson, Sommer, & Sorkin, 2003).

How might inflammatory signaling from the periphery (like uncontrollable shock) induce TNFα release in the CNS? The answer appears to lie in the activation of microglia and astrocytes. In response to nociceptive input, primary afferents innervating the spinal dorsal horn release neuromodulatory transmitters, including glutamate and substance P, and induce the release of adenosine triphosphate (ATP) and fractalkine from secondary spinal neurons (Moalem & Tracey, 2006). Fractalkine and ATP bind to the CXCR1 and P2X4 receptors, respectively, which are found on the glia surrounding neurons in the spinal cord. Activation of these receptors, as well as the substance P receptor NK1, induces a critical physiological and morphological change in microglia and astrocytes. Normally in a relatively quiescent, ramified state, receptor activity on the cell surface produces a shift to an amoeboid shape and sets in motion a flurry of intracellular activity, including the mobilization of intracellular calcium stores and downstream second messenger signaling (Watkins, Milligan, & Maier, 2001). Among the signaling cascade, mitogen-activated protein kinase p38 (p38 MAPK) has been shown to be essential (Tsuda, Mizokoshi, Shigemoto-Mogami, Koizumi, Inoue, 2004; Svensson, Schafers, Jones, Powell, & Sorkin, 2005). Activation of p38 leads to gene transcription that regulates the secretion of a number cytokines, including IL-1β, IL-6, and importantly, TNFα (Ji & Suter, 2007).

Given that uncontrollable shock has been shown to engage nociceptive pathways and induce central sensitization-like effects, it is likely that the shock-induced upregulation of TNFα mRNA observed in Experiment 8 is due to release from activated glia. This notion is further strengthened by the recent finding showing glial activation to

be necessary in order for uncontrollable shock to produce a spinal learning deficit (Vichaya, et al., 2009). Further work will be needed in order to confirm that shock-induced TNFα production and release is mediated by p38 MAPK. Although others have shown that inhibiting p38 in activated microglia attenuates TNFα expression, this work was done in microglial cell cultures (Bhat, Zhang, Lee, & Hogan, 1998). The difficulty in elucidating the role of microglial p38 in an *in vivo* model of spinal plasticity lies in the fact that p38 MAPK is also critical to neuronal function. Thus, a broad p38 MAPK inhibitor would alter both glial and neuronal p38 MAPK function.

TNFα Saturates Synaptic Plasticity

As discussed previously, the spinal learning deficit produced by uncontrollable shock is believed to reflect a form of overexcitation that saturates future plasticity. We have shown that uncontrollable shock produces an increase in TNFα mRNA expression (Experiment 8), and that TNFα is both necessary and sufficient to produce the deficit (Experiments 1-4). Further, we have also reviewed evidence for the role of TNFα in sensitizing spinal neurons. Together, these lines of evidence suggest that TNFα may mediate the shock-induced deficit. The following section will discuss the specific role of TNFα in modulating synaptic strength and provide a possible mechanism by which TNFα might elicit a saturation effect that undermines learning.

TNFα receptor (TNFR1) activation has been shown to increase neural excitability by directly, and indirectly, affecting ion channels through a number of distinct intracellular pathways. Using cultured hippocampal slices, Furukawa and

Mattson (1998) observed a significant increase in current through L-type calcium channels following long-term incubation with TNFα. This effect was dependent upon TNFR1 activation of the downstream transcription factor nuclear factor-kappa B (NF-kB). TNFα application has also been shown to rapidly enhance currents in tetrodotoxin-resistant Na+ channels, leading to acute mechanical sensitization (X. Jin & Gereau, 2006). Interestingly, this effect was mediated by a TNFR1-dependent phosphorylation of p38 MAPK. Coupled with the necessity for this kinase in the glial release of TNFα, this finding suggests a critical role for p38 MAPK in both the neural and glial effects of TNFα.

Despite having some direct effects on excitability through ion channels, TNFα has been more widely characterized by its capacity to indirectly affect synaptic strength by modulating glutamatergic neurotransmission. TNFα has been shown to induce an increase in the surface localization and phosphorylation of the NMDA receptor subunit NR1 (Wheeler et al., 2009). The phosphorylation of this subunit has been suggested as a mechanism by which NMDA receptor trafficking occurs (Swope, Moss, Raymond, Huganir, 1999). Furthermore, brief application of TNFα in this preparation was shown to increase NMDA-evoked calcium currents (Wheeler, et al., 2009). To date, an *in vivo* behavioral correlate for this effect has not been investigated.

In 2002, Beattie and colleagues demonstrated that glial TNFα act upon neuronal TNFR1 to increase trafficking of AMPA receptors to the post-synaptic membrane. Importantly, the AMPA receptors that were expressed were found to be lacking the GluR2 subunit, which normally prohibits the influx of calcium. As such, the TNFα-

induced AMPA receptor trafficking led to a increase in neuronal excitability. Stellwagen refined the view of this phenomenon by illustrating that TNFR1 acts upon the phosphotidylinositol 3-kinase (PI3) pathway to mediate the trafficking of calcium permeable AMPA receptors (Stellwagen & Malenka, 2006). Further, this effect has been recently shown to undermine neural function following spinal cord injury (Ferguson, et al., 2008). It was the well-defined nature of this effect that led us to hypothesize that TNFα saturates spinal plasticity through the increase in surface expression of calcium-permeable AMPA receptors. Experiment 6 showed that the selective inhibition of these AMPA receptors attenuates the TNFα-induced deficit. This finding provides substantial evidence for the possibility that this pathway mediates the detrimental effects of TNFα on spinal plasticity.

Glial TNF and the Long-Term Deficit

Experiment 3 showed that intrathecal TNFα treatment is sufficient to produce a spinal learning deficit when given immediately prior to testing, and interestingly, 24 hours prior to testing. This finding led me to investigate the mechanism by which this long-term effect of TNFα occurs. As discussed above, TNFα can engage a number of excitatory pathways that could be responsible for the long-term deficit. Surprisingly, I found in Experiment 4 that inhibiting TNFα receptor activity prior to testing blocked the long-term TNFα-induced deficit. This finding suggests that sustained TNFα receptor activity is necessary in order for the deficit to be expressed. From this perspective, TNFα can be thought to act in one of two ways: either the exogenous TNFα is

continuing to activate TNF receptors 24 hours after administration, or the administered TNFα is keeping receptors active by inducing the release of further endogenous TNFα stores. While acute TNF administration has not been shown to directly elicit sustained TNF receptor activation, the capacity for TNFα to stimulate the production and release of more TNFα has been well defined (reviewed in Ji & Suter, 2007; Kuno, et al., 2005). As stated before, TNFα is primarily produced by, and released from, astrocytes and microglia. Among the numerous receptors found on these glial cells are TNFα receptors. Thus, TNFα released from glial cells has the opportunity to bind to the same cell from which it came. Much like the ATP-binding P2X4 receptors, glial TNF receptors activate p38 MAPK pathway, inducing gene transcription and ultimately, the release of further TNFα. This autocrine function of TNFα produces a feed-forward loop in which TNFα protein levels, and corresponding neuronal TNF receptor activity, can be sustained for long periods of time (Yarilina, Park-Min, Antoniv, Hu, & Ivashkiv, 2008). This recursive pattern has been implicated as a mechanism for the perpetuation of inflammatory pain states (Kuno, et al., 2005).

In order for this effect to occur, a complex signaling cascade within glia must be engaged. Thus, if the long-term TNFα-induced deficit is mediated by such a mechanism, glial metabolism would be essential. In Experiment 5, we tested the necessity for glial metabolism by administering fluorocitrate, a glial inhibitor, prior to TNF treatment. We found that in order for TNF to produce a long-term deficit, glial metabolism was essential. Similarly, prior work showed fluorocitrate to block the long-term deficit induced by uncontrollable shock (Vichaya, et al., 2009). The convergence of these

findings provides an attractive link between uncontrollable shock, TNFα expression, and the long-term inhibition of adaptive spinal plasticity.

The Role of TNFα in the Deficit: A Molecular Model

When the results of the previous experiments are taken together, a comprehensive role of TNFα in undermining spinal plasticity begins to emerge. Figure 11 depicts a potential model of the way in which uncontrollable stimulation induces TNFα expression and the subsequent effects of TNFα on synaptic plasticity. Included in this model are the intracellular mechanisms for glial production of TNFα and the post-synaptic effects of TNFα on non-AMPA ion channels. Although these effects were not directly tested in the current experiments, a large body of work showing their importance in synaptic function suggests their possible involvement in the TNFα-induced deficit (Park & Bowers, 2010). This model is careful to remain neutral in the depiction of an activated glial cell, without choosing between microglia and astrocytes. Both cell types have been shown to produce and release TNFα upon activation (Lieberman, Pitha, Shin, & Shin, 1989; Chao, et al., 1992). Likewise, both astrocytes and microglia have been suggested to potentiate further cytokine release through autocrine mechanisms (Ji & Suter, 2007; Phulwani, Esen, Syed, & Kielian, 2008). Interestingly, others have also highlighted the importance of cross-talk between these cells, showing that the release of TNFα by activated astrocytes can be facilitated by concomitant microglial activation (Bezzi, et al., 2001). Therefore, it is unlikely that the role of glia in the TNFα-induced deficit is circumscribed to microglia or astrocytes alone.

Experiment 7 showed that GluR2-lacking AMPA receptors were necessary for the expression of the long-term TNFα-induced deficit, but their sufficiency in this effect is yet to be determined. As discussed previously, it is likely that TNFα engages a number of excitatory pathways, and thus the expression of the deficit may reflect a summation of these effects. A similar theory of the additive effect of TNFα has been proposed by Cumiskey and colleagues. They have shown inhibition of metabotropic glutamate receptors (mGluR) attenuates TNFα-induced impairment of LTP, and have suggested that the combined action of TNFα receptors and mGlu receptors on the p38 pathway is sufficient to increase intracellular calcium to a level that impairs plasticity (Cumiskey, Butler, Moynagh, & O' Connor, 2007; Pickering, Cumiskey, & O'Connor, 2005). This finding is especially interesting in light of the recent data showing a critical role for mGlu receptor activation in the spinal learning deficit induced by uncontrollable shock (Ferguson, Bolding, et al., 2008).

This model presents a simplification of a complex system, providing a representation of the key components of the system, rather than an exhaustive detailing of the intricacies therein. The model highlights the findings from the current experiments, while providing speculative connections based on work from other preparations. Further experiments will be necessary to test the extent to which these pathways are involved in the TNFα-induced deficit.

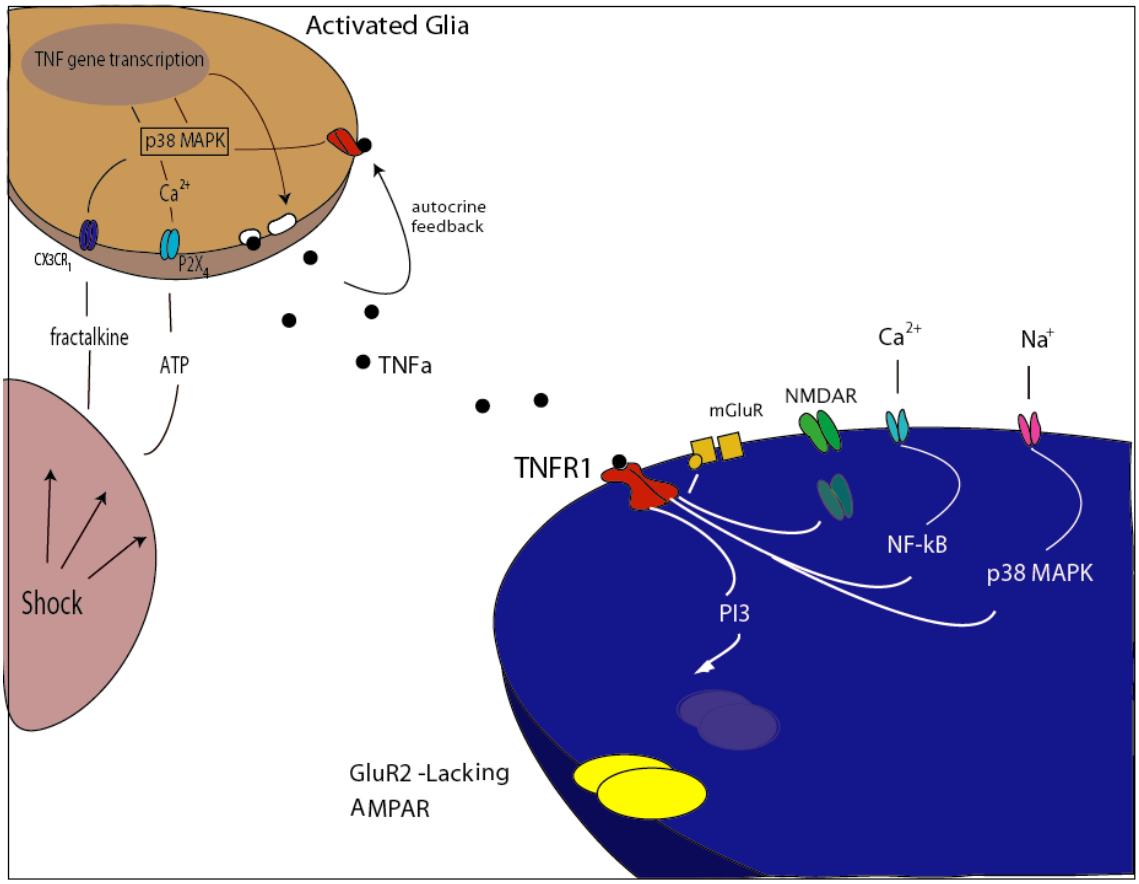

Figure 11. A model of the role of TNFα in the deficit. Following uncontrollable shock, a number of signaling molecules are released in the spinal cord, including fractalkine and ATP. These molecules bind to receptors on glia, causing an influx of calcium and the activation of the p38 MAPK pathway. This pathway engages gene transcription factors that mediate the production and release of TNFα. Once TNFα is released, it can bind to TNF receptors on the same cell from which it was released. This autocrine function leads back through the p38 MAPK pathway to induce further TNFα production and release. Post synaptically, TNFα binds to the TNFR1 receptor, which engages a number of distinct excitatory pathways, increasing trafficking of NMDA and AMPA receptors. It is proposed that the hyperexcitable state induced by sustained TNFα activity saturates plasticity, undermining future learning.

Clinical Implications

At normal physiological levels, TNFα has been shown to play an important role in regulating synaptic homeostasis. It is in response to neural insult or immune challenge

that TNFα overexpression can occur, causing an inflammatory response that may undermine proper neural functioning. The deleterious effect of TNFα can be seen in a number of pathologies in both the brain and spinal cord. Increased expression of TNFα has been shown in patients with Parkinson's disease, as well as in mouse models of Alzheimer's disease (Mogi, et al., 1994; Janelsins, et al., 2008). Although some have shown the link between TNFα in these diseases to be correlative, others have shown some direct effects of TNFα on the progression of the disease. Alzheimer's disease is characterized by the production of plaques that are believed to be derived from the expression of the peptide amyloid-beta (Aß). Administration of TNFα has been shown to increase the production of Aß (Luo, Wang, Cheng, Kuo, & Wolfe, 2004; Li, et al., 2004). Aß then stimulates the activation of microglia, leading to further TNFα release (J. J. Jin, Kim, Maxwell, Li, & Fukuchi, 2008). This feed-forward loop has been recently suggested to underlie the pathogenesis of the disease (Park & Bowers, 2010). As this mechanism is similar to the one proposed for the shock-induced increase in TNFα illustrated above, future work to determine the pathways involved in our model may have implications for the development of Alzheimer's disease.

Experiments 1, 2 and 4 showed that the inhibition of TNFα attenuates the deleterious effects of uncontrollable shock as well as prior TNFα exposure. Although designed primarily to test the necessity of TNFα receptor activity in the spinal learning deficit, these findings also outlined a role for TNFα inhibition as a therapeutic intervention. The inhibition of TNFα has recently gained attention as an important tool in fighting a number of inflammatory processes, and a TNFα inhibitors (infliximab,

etanercept, and adalimumab Humira) are currently indicated for the treatment of arthritis and psoriasis (Tobin & Kirby, 2005). Further research is expanding the role of TNFα inhibition as a therapy, as it has been shown that selectively ablating TNFα receptors can attenuate dopaminergic neurotoxicity, a major neural consequence believed to underlie the development of Parkinson's disease (Sriram, et al., 2002; McCoy, et al., 2006). Inhibition of TNFα receptor activity using the soluble TNFR1 has also shown promise in the fight against secondary damage following spinal cord injury. Ferguson and colleagues showed that inhibition of TNFα activity significantly reduced the trafficking of calcium-permeable AMPA receptors and attenuated subsequent excitotoxic cell death in the spinal cord (Ferguson, et al., 2008).

Summary and Future Directions

The experiments in this dissertation were designed to determine the role of TNFα in the spinal learning deficit. Cellular assays showed uncontrollable shock to increase TNFα mRNA expression in the spinal cord. Behavioral findings showed TNFα to be necessary in order for uncontrollable shock to produce a deficit, and to be sufficient to produce a deficit in lieu of shock treatment. The TNFα-induced deficit was shown to be long-lasting, and this long-term deficit required glial activation at the time of induction, as well as TNF receptor activation at the time of expression. Finally, the post-synaptic effects of TNFα were investigated, and the TNFα-induced expression of calcium-permeable AMPA receptors was shown to be necessary for the expression of the long-

term deficit. Together, these findings suggest a critical role for TNFα in producing an excitatory neural environment that saturates plasticity and undermines spinal learning.

In contrast to the deleterious effects of TNFα on spinal learning, a recent set of experiments has outlined a necessary and sufficient role for brain-derived neurotrophic factor (BDNF) in mediating the beneficial effects of spinal instrumental training (Huie, et al., 2007). Rather than induce a learning deficit, BDNF treatment can protect against the deleterious effects of uncontrollable shock, as well as rescue learning after the deficit has been induced. Interestingly, TNFα and BDNF have been shown to play opposite roles in synaptic scaling (Turrigiano, 2008). The opposing effects of these two agents suggests a possible constitutive balance between TNFα and BDNF, and tipping one or the other toward overexpression may be key in shifting spinal metaplasticity between adaptive and maladaptive outcomes. Future work will be needed to understand the dynamic between TNFα and BDNF. For example, can BDNF rescue the learning after the deficit has been induced by TNFα?

Other work has shown GABA receptor activation to be necessary and sufficient to produce a spinal learning deficit (Ferguson, et al., 2003). Interestingly, an increase in GABA receptors exocytosis has been observed following spinal cord injury (Ferguson, personal communication). Because injury has also been shown to increase TNFα levels, and both TNF and GABA have been shown to mediate the spinal learning deficit, there may be a critical interaction between these systems that will need to be investigated. If TNFα induces an increase in GABA receptor activity that contributes to the deficit, then

it would be of interest to determine whether treatment with a GABA antagonist could block the expression of the TNFα-induced deficit.

Future work will also be needed in order to fully elucidate the cellular mechanisms through which TNFα works to undermine spinal plasticity. Histological imaging of the colocalization of TNFα protein with microglia and astrocytes will provide much-needed information as to the relative roles of each cell type. Further, attention will need to be focused on intracellular signaling pathways, including p38 MAPK, NF-kB, and PI3 with the aim of identifying the potential mediators of TNFα overexpression and synaptic saturation. In understanding the role of TNFα in maladaptive spinal functioning, we can derive therapeutic potential and work to prevent or reverse these processes, reinstating a neural environment that is once again receptive to adaptive plasticity.

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