NEUROPATHIC PAIN AND THE INHIBITION OF LEARNING WITHIN THE SPINAL CORD

A Dissertation

by

ADAM RICHARD FERGUSON

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

May 2004

Major Subject: Psychology

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Approved as to style and content by:

_____	_____
James W. Grau (Chair of Committee)	Mary W. Meagher (Member)
_____	_____
C. Jane Welsh (Member)	Rajesh C. Miranda (Member)

_____
W. Steve Rholes
(Head of Department)

May 2004

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ABSTRACT

Neuropathic Pain and the Inhibition of Learning Within the Spinal Cord.

(May 2004)

Adam Richard Ferguson, B.A., Southwestern University;

M.S., Texas A&M University

Chair of Advisory Committee: Dr. James W. Grau

Prior work from our laboratory has shown that the spinal cord is capable of supporting a simple form of instrumental (response-outcome) learning. In a typical experiment, animals are given a spinal transection at the second thoracic vertebra, and tested 24 h after surgery. If animals are given shock when their leg is in a resting position (controllable shock), they quickly learn to maintain the leg in a flexed position, thereby minimizing shock exposure. Animals exposed to shock that is independent of leg position (uncontrollable shock) fail to learn. This learning deficit can be induced by as little as 6 minutes of shock to either limb or to the tail, and lasts for up to 48 h.

The aim of this dissertation was to explore whether the deficit shares behavioral features and pharmacological mechanisms similar to those involved in the induction of neuropathic pain. Work within the pain literature has identified a spinal hyperexcitability that is induced by intense stimulation of pain fibers. This phenomenon, known as central sensitization, is characterized by an

increase in tactile reactivity (allodynia) that can be induced by shock or peripheral inflammation. Pharmacological findings have revealed that central sensitization depends on the activation of the N-methyl-D-aspartate (NMDA) and group I metabotropic glutamate receptors (mGluRs). Experiment 1 showed that uncontrollable shock induces a tactile allodynia similar to that observed in central sensitization. Experiment 2 showed that peripheral inflammation caused by a subcutaneous injection of formalin generates a dose-dependent deficit. Experiment 3 indicated that the formalin-induced deficit was observed 24 h after delivery of the stimulus. Experiments 4-8 revealed that the NMDA and group I mGluRs are involved in the deficit. The NMDA receptor was found to be necessary (Experiment 4), but only sufficient to induce a deficit at neurotoxic doses (Experiment 5). Both of the group I mGluRs (subtypes, mGluR1 and mGluR5) were found to be necessary (Experiments 6 & 7). A general group I mGluR agonist summated with a subthreshold intensity of shock to produce a robust deficit (Experiment 8), suggesting shock and mGluR activation produce a deficit through a common mechanism.

ACKNOWLEDGMENTS

I would like to thank my mentor, Jim Grau for his intellectual and financial support. Without his contribution, this work would not have been possible. I would also like to thank my committee members, Mary W. Meagher, C. Jane Welsh, and Rajesh C. Miranda for their intellectual contributions to this dissertation. My gratitude goes out to my friends and family. Specific thanks are due to my immediate family who have always been understanding when I have needed to work. Several members of the Graulab were essential in helping me complete this dissertation. Eric D. Crown, Michelle A. Hook, Kevin A. Bolding, and J. Russell Huie all made a direct contribution. Special thanks are due to other members of the laboratory including Anne C. Bopp, Stephanie N. Washburn, Brianne C. Patton, and Grace T. Liu for their intellectual support. Finally, I would like to thank Jessica M. Stern for her constant support and patience as I completed this work. This dissertation is dedicated to musicians past and present who have kept me sane and made me better at what I do.

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CHAPTER I

INTRODUCTION

Overview

The spinal cord has often been considered a conduit for information traveling to and from the brain that organizes simple reflexes, but is otherwise incapable of modifying behavior. Challenging this perspective, work spanning almost a century suggests that the spinal cord is capable of significant plasticity, and, may be capable of learning to walk again after injury (for reviews see Patterson & Grau, 2001). However recent advances have shown that this plasticity is fragile, and if not protected, may be lost. The loss of plasticity can result from stimulation of peripheral sensory fibers, and may be associated with impaired recovery after injury. This makes the troubling prediction that uncontrolled sensory input into the cord could compromise recovery.

My dissertation explores the mechanisms by which peripheral stimulation limits plasticity. By characterizing behavioral and molecular mechanisms I hope to shed light on the conditions that lead to losses. Throughout the course of this work I will seek to describe behavioral features of the phenomenon and characterize the forms of stimulation that lead to a loss of plasticity.

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

I also explore some of the pharmacological mechanisms, with the hope that an understanding of mechanism will lead to drug therapies that can protect plasticity after injury.

In the introduction I will review what is known about plasticity within the spinal cord. I will first address the issue of plasticity within pain pathways and evidence for learning within the spinal cord. I will then review the behavioral and pharmacological links among the different forms of spinal plasticity. I will focus on molecular mechanisms that are important for the experiments contained within the dissertation. I will close with the specific aims of the current experiments.

Plasticity Within Pain Pathways

Prior to the 1960s the scientific community assumed that spinal cord neurons simply relayed incoming pain signals to the brain (for review, see Melzack & Wall, 1965). This position changed when Melzack and Wall (1965) proposed that the spinal cord plays a critical role in pain processing. Their 'gate control theory' of pain posited that the superficial portion of the spinal dorsal horn received input from specialized peripheral pain afferents (nociceptors) and modulated this incoming signal, allowing amplification or diminution. This theory was one of the first formal statements that spinal plasticity may be involved in the central transmission of incoming noxious (nociceptive) signals.

Wind-up

The year after Melzack and Wall proposed the gate control theory, Mendell (1966) provided a potential mechanism of plasticity within the spinal cord. He showed that repeated stimulation of C-fibers (a class of unmyelinated nociceptor) leads to a summation of slow synaptic potentials, yielding a prolonged after-discharge in the postsynaptic neuron. This phenomenon known as 'wind-up' provided a potential electrophysiological mechanism by which peripheral injury could lead to plastic changes within the spinal cord. Subsequent work has shown that wind-up depends on excitatory transmission within the spinal cord and that the brain can inhibit the development of wind-up through a neural pathway that descends through the dorsolateral funniculus (DLF; Herrero, Laird, & Lopez-Garcia, 2000; Hillman & Wall, 1969). Spinal transection (spinalization) disrupts this inhibition facilitating wind-up (Gozariu, Bragard, Willer, & Le Bars, 1997). This sensitization depends on the excitatory amino acid (EAA) glutamate (for review, see Herrero et al., 2000).

Central sensitization

Subsequent studies showed that persistent increases in neural excitation within the spinal cord are observed after tissue damage, and may lead to enhanced pain (Woolf, 1983; Coderre & Melzack, 1985). In these landmark studies, peripheral tissue damage was shown to produce a persistent hyperexcitability within spinal cord neurons (Woolf, 1983; Coderre & Melzack,

1985). This effect, known as central sensitization, has subsequently been shown to be mediated by excitatory amino acids (Woolf & Thompson, 1991). Although, it is tempting to consider central sensitization as equivalent to wind up, there does not appear to be a simple isomorphism (Woolf, 1996). First, central sensitization can occur in the absence of wind-up (Woolf, 1996). Second, only central sensitization seems to induce a phenomenon analogous to long-term potentiation (Willis, 2001b). Long-term potentiation (LTP) is an electrophysiological phenomenon involving an activity-induced enhancement of synaptic transmission (Bliss & Lomo, 1973). After induction of LTP the threshold for activation of postsynaptic neurons is greatly lowered, resulting in hyperexcitability. Unlike wind-up, which causes a only a temporary change in excitability, LTP can last for several hours, and in some cases, days (Woolf, 1996). It is known that activation of C-fibers and A-delta fibers (a small myelinated nociceptor) can lead to the induction of LTP in neurons within the superficial laminae of the spinal cord (Sandkuhler & Liu, 1998). This hyperexcitability is more easily evoked in the transected than the intact spinal cord, again suggesting that descending fibers in the intact cord regulate the induction of spinal LTP (Liu, Morton, Azkue, Zimmermann, & Sandkuhler, 1998).

Of the two forms of plasticity within pain pathways that I have discussed, central sensitization seems to have the greater clinical relevance. Central sensitization manifests behaviorally as enhanced tactile (allodynia) and thermal

(hyperalgesia) reactivity both ipsilateral and contralateral to an injury (Woolf, 1983; Coderre & Melzack, 1985). Windup, on the other hand, does not clearly correlate with hyperalgesia or allodynia (Herrero et al., 2000). As a consequence in my dissertation I will focus on central sensitization as the exemplar of plasticity within pain pathways.

Learning and Memory in the Spinal Cord

The traditional perspective in the psychological literature has been that learning is a capacity unique to the brain. However, a literature dating back almost 40 years has shown that the spinal cord is capable of demonstrating several different forms of learning, including single stimulus learning, Pavlovian conditioning, and instrumental conditioning. These conclusions are based on findings generated through the rigorous use of formal learning paradigms, a feature required to discount alternative explanations.

Single stimulus learning

Single stimulus learning involves an incremental change in response magnitude as a consequence of repeated exposure to a stimulus. This change can take the form of a response increment (sensitization) or a response decrement (habituation). Sherrington (1906) first reported that the spinal cord was capable of demonstrating habituation. Subsequent work has replicated this finding and shown that the spinal cord demonstrates sensitization as well (Groves & Thompson, 1970; Thompson & Spencer, 1966).

Pavlovian conditioning

In addition to demonstrating single stimulus learning, the spinal cord is capable of encoding relationships among stimuli. This form of learning, known as Pavlovian conditioning, was first noted in the spinal cord by Shurrager and Culler (1940). Subsequent work has revealed that the spinal cord can display a number of Pavlovian phenomena, including extinction, latent inhibition, and overshadowing (Durkovich & Damianopoulos, 1986; Fitzgerald & Thompson, 1967; Joynes & Grau, 1996; Patterson, Cegavske, & Thompson, 1973).

Instrumental conditioning

The spinal cord appears to be able to alter its behavior to meet the demands of an environmental outcome. Response-outcome learning is formally referred to as instrumental conditioning (Domjan, 1998). Early work suggested that a spinally transected (spinalized) animal, and even a headless cockroach, was capable of learning to hold its leg in a flexed position (response) if extending the leg resulted in shock (outcome; Chopin & Bennett, 1975; Chopin & Buerger, 1975; 1976; Horridge, 1962). This original work met with controversy and was ultimately discounted when it did not address feasible alternative hypotheses (Church & Lerner 1976). However, recent work has addressed the criticisms raised by Church and Lerner (1976), and provided compelling evidence that the spinal cord can support instrumental learning (Crown, Ferguson, Joynes, & Grau, 2002b; Grau, Barstow, & Joynes, 1998). Further evidence for instrumental

learning can be found in studies of spinally-mediated locomotion. Research has shown that spinalized animals can learn to step after treadmill training and can modify this stepping to clear an obstacle (Edgerton, et al., 1997; Hodgson, Roy, de Leon, Dobkin, & Edgerton, 1994).

Our paradigm is a modification of the Horridge procedure originally used to explore learning in cockroaches (Horridge, 1962). Subjects are given shock to a hind leg if the leg falls below a preset criterion. In the presence of this controllable shock, spinalized animals are capable of learning to maintain the leg in a flexed position (response) thereby minimizing shock exposure (outcome).

Exposure to shock that occurs independent of leg position (uncontrollable shock) can produce a behavioral deficit that prevents future instrumental learning (Crown & Grau, 2001; Grau et al., 1998; Joynes, Ferguson, Crown, Patton & Grau, 2003). This deficit can be induced by just 6 min of shock to the leg or tail, and lasts for well over 24 h (Crown, Ferguson, Joynes, & Grau, 2002a). The deficit shows transfer to the contralateral leg, and shock to the tail generates a deficit on both legs (Joynes et al., 2003). In addition, the deficit can be prevented with intrathecal blockade of neurotransmission using lidocaine (Joynes et al., 2003). Together these data indicate that the deficit depends on a change within the central nervous system.

To the extent that recovery of function after spinal cord injury involves learning within remaining spinal circuits, one might suspect that uncontrollable

shock would have a similar negative impact on recovery. Indeed, we have recently found that uncontrollable shock impairs recovery of function in a rat model of spinal contusion injury (Grau, Garcia, Ferguson, Crown, & Miranda, 2001). This recovery deficit was evident across multiple measures of recovery including locomotion, bladder function, and sensory function (Grau et al., submitted).

Despite the fact that uncontrollable shock has deleterious effects on spinal learning and recovery of function after injury, the clinical relevance of the deficit is not clear. At the heart of this issue is whether or not shock mimics the effects of a more naturalistic stimulus. If the findings do not extend to naturalistic stimuli, then the clinical relevance of these effects is limited to treatment regimes that involve shock exposure after injury. Several recent reports have suggested that functional electrical stimulation (FES) can lead to gains in function after injury (Barbeau, Ladouceur, Mirbagheri, & Kearney, 2002; Steers, Wind, Jones, & Edlich, 2002; Wilder, Jones, Wind, & Edlich, 2002). Presumably, if FES is delivered in an uncontrollable manner (i.e. stimulation is delivered independent of leg position), then FES should impair rather than improve recovery of function. There is little data available that explicitly examines the impact of uncontrollable FES. However, recent work suggests that proper timing of FES is essential for inhibition of dysfunctional reflexes during locomotion in patients with spinal cord

injury, suggesting that FES has greater efficacy if delivered in a position-specific (i.e., controllable) manner (Barbeau et al., 2002; Fung & Barbeau, 1994).

Although, our uncontrollable shock effects may have some clinical implications for FES, these implications would only extend to a small subset of the individuals with spinal cord injuries (those undergoing FES therapy). On the other hand, if uncontrollable shock mimics the effects of naturalistic stimulation, then the clinical relevance is profound and wide ranging. If, for example, shock negatively impacts learning and recovery by activating nociceptors, then this has implications for the treatment of pain after spinal cord injury. Such an effect would imply that peripheral tissue damage could permanently undermine plasticity and recovery of function after spinal cord injury.

Links Between Central Sensitization and the Deficit

If peripheral injury were to undermine instrumental plasticity, then there should be parallels between the deficit and central sensitization. Present data suggests that these links may exist.

Behavioral links

At the present time there are only a few pieces of evidence for behavioral similarities between central sensitization and the deficit. The first is that spinal LTP and the deficit can both be induced by shock exposure (Grau et al., 1998; Sandkuhler & Liu, 1998). In addition, both phenomena are more easily induced in spinally transected than intact animals (Liu, Morton, Azkue, Zimmermann, &

Sandkuhler, 1998; Grau & Crown, 2000). Finally central sensitization and the deficit show similar anatomical features. Both phenomena transfer to the contralateral leg, and both can be prevented by lesioning the sciatic nerve prior to stimulation of the hindpaw (Coderre & Melzack, 1985; Joynes et al., 2003; Woolf, 1983).

Pharmacological links

Prior pharmacological studies suggest central sensitization and the deficit may be linked. One of the most common models of peripheral injury involves experimental induction of peripheral inflammation with an injection of an irritant (LeBars, Gozariu, & Cadden, 2001). Under inflammatory conditions, there is an upregulation of several neurotransmitters including GABA and kappa opioids (Castro-Lopez, Tavares, Tolle, & Coimbra, 1994; Dubner & Ruda, 1992). We have recently shown that these systems are also affected by uncontrollable shock (Ferguson, Washburn, Crown, & Grau, 2003; Joynes & Grau, in press). Antagonism of the $GABA_A$ receptor blocks both the induction and the expression of the deficit (Ferguson et al., 2003). Moreover a kappa opioid antagonist reverses the deficit if given at the time of testing (Joynes & Grau, in press). These same systems have also been implicated in injury-induced sensitization. Intraspinal administration of a $GABA_A$ antagonist can reduce the release of excitatory amino acids (EAA) and excitability in spinal cord neurons caused by peripheral inflammation (Sluka et al., 1994; Weng, Laird, Cervero, &

Schouenborg, 1998). This reduction in EAA levels is associated with a commensurate decrease in tactile and heat hypersensitivity (Sluka et al., 1993; 1994). These findings indicate that GABA-mediated changes in the spinal cord contribute to tactile hyperreactivity (allodynia) after peripheral injury. Central sensitization is also associated with an increase in spinal levels of the endogenous kappa opioid dynorphin (for review see Dubner & Ruda, 1992), and intrathecal injections of dynorphin A induces a hyperexcitability in spinal neurons that can be reversed with antagonism of EAA receptors (Vanderah et al., 1996). Together these findings suggest that uncontrollable shock and central sensitization may involve similar pharmacological mechanisms.

Another similarity between the deficit and central sensitization involves the role of descending systems. As mentioned previously, research indicates that uncontrollable shock does not induce a deficit if administered prior to spinal transection (Grau & Crown, 2000). Subsequent work has shown that this protection depends on fibers contained within the dorsolateral funniculus (Crown, Ferguson, Dhruv, Patton, & Grau, 2001). Because the dorsolateral funniculus (DLF) is a major source of descending inhibition within the spinal cord, these findings imply that the deficit is normally blocked by inhibitory tone. A significant portion of the DLF-mediated inhibition is serotonergic and noradrenergic in nature. Work has shown that replacing serotonin and norepinephrine after injury prevents the deficit (Crown et al., 2001; Crown &

Grau, 2002). In addition, application of a selective serotonin 5-$HT1_A$ receptor antagonist before uncontrollable shock in an intact animal allows induction of the deficit in a manner similar to a DLF lesion (Crown & Grau, 2002). This suggests that serotonergic fibers contained within the DLF normally confer a protective inhibitory tone that prevents the induction of the deficit. By extension, this suggests that uncontrollable shock may induce overexcitation. Research suggests that fibers also protect the spinal cord against induction of wind-up (Hillman & Wall, 1969). To the extent that windup can lead to central sensitization, this data provides another link between the deficit and central sensitization (Woolf, 1996).

The common theme from our pharmacology findings is that all of the systems that have been implicated in the deficit modulate excitatory transmission within the spinal cord. Activation of both $GABA_A$ and kappa opioid receptors is associated with elevated levels of excitatory amino acids under certain experimental conditions (Dubner & Ruda, 1992; Sluka et al., 1994). On the other hand, serotonin has been shown to reduce excitatory transmission within the dorsal horn of the spinal cord (el-Yassir, Fleetwood-Walker, Mitchell, 1988). Therefore, it seems likely that excitatory neurotransmitters within the spinal cord would have a large impact upon the deficit.

The role of the excitatory amino acid (EAA) glutamate in central sensitization is well established. Reviewing this evidence requires an

understanding of the mechanisms of glutamatergic transmission within the spinal cord. Two major classes of glutamate receptors have been identified in the central nervous system (Fundytus, 2001). The ionotropic glutamate receptors (iGluRs) are coupled to transmembrane ion channels whereas the metabotropic glutamate receptors (mGluRs) are coupled to intracellular second messenger systems. The iGluRs consist of receptors that respond to □-amino-3hydroxy-5methylisoxazole-4-propionic acid (AMPA) and/or kainate and receptors that respond to N-methyl-D-aspartate (NMDA). The AMPA/kainate receptors function as typical ionotropic receptors, undergoing a conformational shift upon glutamate binding. This opens the ion channel allowing influx of Na^+ ions and efflux of K^+. The net effect of AMPA/kainate activation is depolarization of the cell. Binding of glutamate to the NMDA receptor does not normally affect membrane potential because the pore of the channel is blocked by a Mg^{++} ion. However, depolarization of the cell dislodges this Mg^{++} block, allowing ions to pass through the channel with subsequent glutamate binding. The NMDA channel is permeable to Ca^{++} in addition to Na^+ and K^+. Interestingly, intracellular changes associated with NMDA activation and subsequent Ca^{++} influx have been implicated in the induction of LTP (Regehr & Tank, 1990).

Group I mGluRs (subtypes mGluR1 and mGluR5) have also been implicated in changes in spinal neuronal excitability observed after peripheral inflammation (Neugebauer, Chen, & Willis, 1999). These receptors are coupled

to phospatidyl-inositol hydrolysis, and their activation leads to increases in intracellular Ca^{++} and activation of protein kinase C (Fundytus, 2001). It has been suggested that group I mGluRs can cause long term changes in spinal neurons and contribute to the induction of central sensitization (Neugebauer et al., 1999).

Both the iGluRs and the group I mGluRs have been implicated in nociception after peripheral injury. The inflammatory phase of formalin pain can be blocked by intrathecal administration of an NMDA antagonist (Coderre, 2001; Yamamoto & Yaksh, 1992), and administration of NMDA itself can exacerbate inflammatory pain (Woolf & Thompson, 1991). Several recent papers (e.g., Benquet, Gee, Gerber, 2002; Dang, Naeem, Walker, Bowery, & Urban, 2002; Lan et al., 2001) have found that NMDA receptor activity can be modulated by the group I mGluRs. Antagonists to the group I mGluRs have been found to reduce NMDA currents (Dang et al., 2002). Moreover, mGluR agonists have been found to enhance NMDA currents (Benquet et al., 2002) and increase NMDA receptor trafficking (Lan et al., 2001). These receptors also play a role in modulating spinal neuronal excitation in inflammatory pain. Intraspinal infusion of group I metabotropic receptor agonists have been found to cause spontaneous nociceptive behaviors (Fisher & Coderre, 1996a). In addition group I mGluR agonists have been found to enhance, and antagonists to reduce, the central sensitization induced by peripheral injection of the irritant capsaicin (Neugebauer

et al., 1999). Similar results have also been found with other inflammatory agents such as formalin (Fisher & Coderre, 1996b; Karim, Wang, & Gereau, 2001), and carrageenan (Zhang, Lu, Chen, & Westlund, 2002). Interestingly, some of the increases in activity due to group I mGluRs can be blocked with NMDA antagonism, suggesting that the two systems are closely interconnected (Fisher & Coderre, 1996a; 1996b).

Specific Aims

The experiments contained within my dissertation examine the links between central sensitization and the deficit. The overarching hypothesis of this work is that the deficit produced by uncontrollable shock engages mechanistic changes that mimic those evoked by peripheral injury. The driving assumption is that links between the instrumental deficit and peripheral injury should be evident both behaviorally and pharmacologically.

If uncontrollable shock engages nociceptive systems in a manner reminiscent of peripheral injury, then shock should produce hyperexcitability within the spinal cord. This central sensitization-like change should manifest behaviorally in the form of tactile allodynia. Conversely, if the deficit depends on nociceptive activation, then a naturalistic peripheral injury should undermine instrumental learning within the spinal cord. The first 3 experiments of my dissertation explored these possibilities. Experiment 1 tested whether 6 mins of uncontrollable shock to the hindleg induced a tactile allodynia on the ipsilateral

or contralateral leg. Experiment 2 examined whether peripheral injection of the inflammatory agent formalin generated a dose-dependent behavioral deficit 20 mins after formalin administration. Experiment 3 extended the findings of experiment 2 by testing whether, like uncontrollable shock, formalin produced a deficit at 24 h after exposure to the stimulus. The remaining 5 experiments used pharmacological manipulations to explore whether the deficit shares molecular features consistent with inflammation-induced changes.

If the deficit in instrumental performance relies on mechanisms that are similar to central sensitization, then it should be modulated by the NMDA and group I mGluR systems. Experiments 4-8 formally explored the roles of these receptors. Experiment 4 used intrathecal administration of an antagonist prior to 6 mins of uncontrollable tailshock to assess whether the NMDA receptor was *necessary* for the induction of the behavioral deficit in spinal animals. Experiment 5 tested the converse issue, whether intrathecal NMDA administration was *sufficient* to induce the deficit. Experiments 6 & 7 explored whether the group I mGluRs (mGluR1 & mGluR5, respectively) were *necessary* for the induction of the deficit produced by uncontrollable shock. Finally, Experiment 8 explored whether a general agonist of the group I mGluRs was *sufficient* to induce the deficit.

CHAPTER II

GENERAL METHOD

Subjects

The subjects were male Sprague-Dawley rats obtained from Harlan (Houston, Texas). The rats were 100-120 days old and weighed between 400 and 460 g. Subjects were individually-housed, maintained on a 12-hr light-dark cycle, and given *ad libitum* access to food and water.

Surgery and Intrathecal Cannulization

In preparation for surgery, subjects were pretreated with a dose of atropine (40 mg/kg). After approximately 5 mins animals were anesthetized using pentobarbital (50 mg/kg, i.p.), and their backs were shaved and cleaned with iodine. To maintain hydration during the surgery, animals were given a preoperative injection of warm 0.9% saline (2.5 ml, i.p.). For the spinal transection surgery, each subject was placed in a stereotaxic instrument and a small gauze “pillow" was placed under its chest to stabilize the animal. The second thoracic vertebra (T2) was localized by touch, and an anterior-posterior incision was made. The tissue in front of T2 was cleared away and the spinal cord was transected using a cautery device. The void produced by the transection was filled with Oxycel (Parke-Davis, Morristown, NJ) to limit post-surgical bleeding. In experiments requiring an intrathecal cannula (Experiments 4-8) a segment of polyurethane tubing (25 cm; PE-10) fitted with 0.23 mm

(diameter) stainless steel wire (SWGX-090, Small Parts Inc., Miami Lakes, FL) was inserted 9 cm caudally into the subarachnoid space between the dura and the spinal cord. The exposed end of tubing was secured to the adjacent tissue using cyanoacrylate. The wire was then gently pulled from the tubing and the wound was closed using Michel clips (Fine Science Tools, Foster City, CA).

After spinalization, subjects were hydrated with an i.p. injection of 2.5 ml of warm 0.9 % saline and placed in temperature-controlled environment (approximately 25.5 ºC). To prevent injury to the hind limbs during recovery, the rear legs of spinalized animals were maintained in a normal flexed position by two pieces of porous tape (Orthaletic, 1.3 cm [width]) gently wrapped once around the rat's body.

Spinal transections were confirmed by (a) inspecting the cord during the operation, (b) observing the behavior of the subjects after recovery to ensure that they exhibited paralysis below the level of the forepaws and did not vocalize to the leg shock, and (c) examining the spinal cord post-mortem in a randomly selected subset of the subjects.

Apparatus

Uncontrollable tail shock was delivered in Plexiglas restraint tubes (King, Joynes, Meagher, & Grau, 1996). Briefly, the tubes were 22 cm in length and 6.8 cm in diameter. A 5.5 cm wide sheet of Plexiglas served as the floor of the tube (upon which the subjects lay during uncontrollable shock). Uncontrollable shock

was applied using a 660-V AC transformer with a large series resistance. In Experiments 4-8 uncontrollable shock was delivered through electrodes constructed from a modified fuse clip. The metal leads of the fuse clip were coated with electrode gel and taped to the subject's tail approximately 6 cm from the base prior to the delivery of uncontrollable shock. In Experiment 1, subjects received uncontrollable shock to the leg. In Experiments 2-8 testing with contingent leg shock in spinalized animals was conducted using an apparatus similar to that used in previous studies from our laboratory (e.g. Grau et al., 1998). Briefly, rats were loosely restrained in Plexiglas tubes (20.0 cm [length] x 7.0 cm [internal diameter]; see Figure 1). Two slots (6.0 cm [length] x 1.7cm [width]) were cut in the sides and base of tube, allowing both hind legs to hang freely. Shock was delivered using a BRS/LVE shock generator (Model SG-903). Leg shock was applied by attaching one lead from the shock generator to a wire inserted through the skin over the tibia 1.5 cm from the tarsals. The other lead was attached to a 2.5 cm stainless steel pin that was inserted 0.4 cm into the tibialis anterior muscles 1.7 cm above the other electrode.

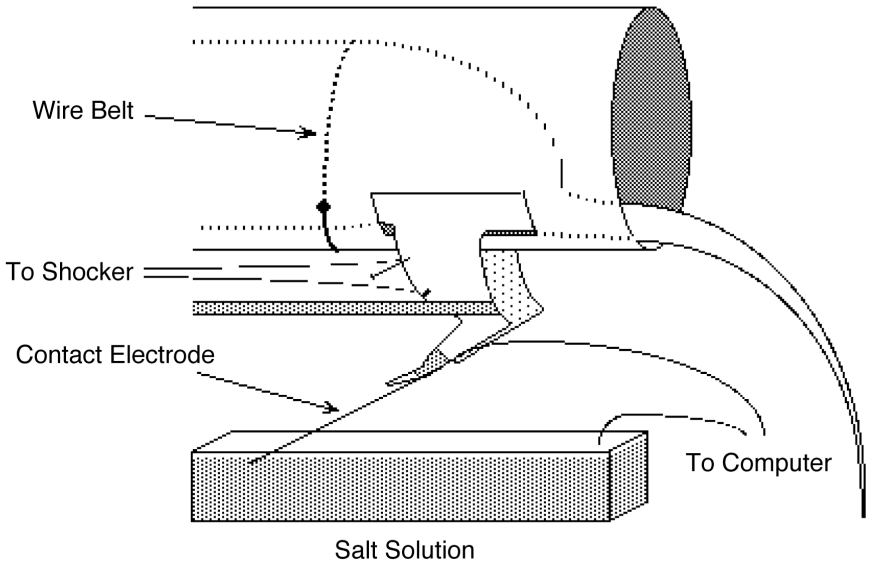


Figure 1. Apparatus used to assess instrumental performance in spinalized rats. Animals were placed in a plexiglas restraint tubes and a contact electrode was attached to the hindpaw. When the contact electrode touched the underlying salt solution this completed a circuit monitored by a computer, and resulted in delivery of shock to the tibialis anterior (modified from Grau et al., 1998, Figure 1).

Leg position was monitored using a contact electrode constructed from a 7 cm 0.018" stainless steel rod that was 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]) was attached to the end of the rod. This wire (20 cm) extended from the rear of the foot and was connected to a digital input monitored by a Macintosh computer. The rod was taped to the plantar surface of the rat's foot with approximately 8 cm of porous tape (Ortholetic, 1.3 cm, Johnson and Johnson, Arlington, TX) with the end positioned directly in front of the plantar protuberance. 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 stainless steel rod that 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. 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) to the rat's foot immediately behind the plantar protuberance. The 40 cm length of line was 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 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 activating the gauge. The strain gauge had previously been calibrated by determining the relationship between voltage and force in Newtons. The data revealed a linear relation that allowed us to convert voltage to force. Shock intensity was adjusted to produce a flexion force of a fixed value. The strain gauge was then removed from the rat's foot.

General Procedure

A summary of the designs for the proposed experiments is provided in Figure 2. In Experiments 4-8 drug was delivered intrathecally immediately before the administration of uncontrollable shock. Intrathecal administration was performed using a 10 μl Hamilton syringe that was inserted into to the exposed end of the intrathecal cannula. To deliver uncontrollable tail shock, an electrode was attached to the tail and subjects were given 6 mins of AC shock (1.5 mA, 2 s average interstimulus interval). In Experiments 2-8 subjects were tested with contingent leg shock at varying time points after uncontrollable shock exposure. Prior to testing the subject's leg was shaved and marked for placement of the shock leads. A wire electrode was then inserted over the tibia at the distal mark and the rats were placed in restraining tubes. Next the contact electrode used to monitor leg position was taped to the paw. To minimize lateral leg movements, a

20 cm piece of porous tape (Ortholetic, 1.3 cm) was wrapped around the leg and taped to a bar extending across the apparatus directly under the front panel of restraining tube. The tape was adjusted so that it was taut enough to slightly extend the knee. One lead from the shock generator was attached to the stainless steel wire inserted over the tibia. The shock generator was set to deliver a 0.1 mA shock and the region over the second mark was probed to find a site that elicits a vigorous flexion response. The pin was then inserted perpendicular to the body into the tibialis anterior muscle. The shock intensity necessary to induce a 0.4 N flexion response was obtained for each hind leg using the strain gauge described in the Apparatus section. To set the criterion for learning, three short (0.15-s) shock pulses were applied and the level of the salt solution adjusted so that the tip of the rod was submerged 4 mm below the surface. Subjects were tested for instrumental learning with 30 mins of contingent shock.

Experiment		Induction			
1.		Noncontingent legshock (0 or 0.4 mA)	→	Tactile test	
2.		Formalin Dose (0, 5, 10, 15%)	→	Test	
3.		Formalin Injection			Test
4.	MK-801 → (0, 1, 10, 100 nmol)	Noncontingent tailshock (0 or 1.5 mA)			Test
5.	NMDA → (0, 1, 10, 100 nmol)	Noncontingent tailshock (0 or 1.0 mA)			Test
6.	CPCCOEt → (0, 1, 10, 100 nmol)	Noncontingent tailshock (0 or 1.5 mA)			Test
7.	MPEP → (0, 1, 10, 100 nmol)	Noncontingent tailshock (0 or 1.5 mA)			Test
8.	DHPG → (0, 1, 10, 100 nmol)	Noncontingent tailshock (0 or 1.5 mA)			Test
	Day 1 (24 hrs after spinal transection)				**Day 2**

Figure 2. Experimental designs for Experiments 1-8. Experiments 1-3 focus on the behavioral correlates between central sensitization and the effects of uncontrollable shock. Experiments 4-8 explore the role of the glutamate ionotropic (Experiments 4 & 5) NMDA receptor, and group 1 metabotropic receptors (Experiments 6-8) in the deficit produced by uncontrollable shock.

Behavioral Measures

Instrumental learning was assessed using three behavioral outputs: Time in solution, flexion number, and flexion duration (see Figure 3). The computer recorded the amount of time that the contact electrode was in contact with the solution (Time in solution). Whenever the electrode left the solution, the number of flexion responses was increased by 1 (Flexion number). To observe learning across trials, the training session was divided into 30, 1 min training bins. From time in solution and flexion number, we derived flexion duration using the following equation:

$$\text{Flexion duration}_i = (60 - \text{Time in solution}_i)/(\text{Flexion number}_i + 1),$$ where i was the current training bin.

Statistics

The results were analyzed using analyses of variance (ANOVA), analyses of covariance (ANCOVA) and trend analysis to determine the impact of experimental treatments over time. Group differences were evaluated using post hoc tests when appropriate.

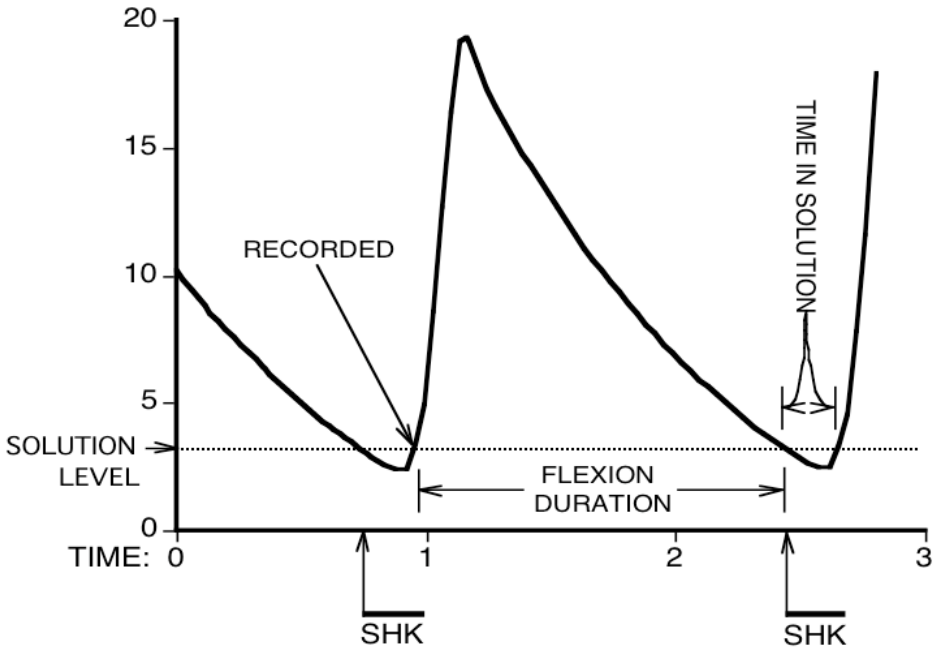


Figure 3. Response measures used in the instrumental paradigm. The solid line reflects the position of the contact electrode and the dashed line, the surface of the solution. The y-axis represents vertical position and the x-axis, time (adapted from Grau et al., 1998, Figure 3).

CHAPTER III

BEHAVIORAL LINKS BETWEEN PERIPHERAL INJURY AND THE DEFICIT

Experiments 1-3 explored the behavioral correlates between the deficit produced by uncontrollable shock and the consequences of peripheral injury. The rationale was that, if such correlates exist, then shock and peripheral injury should have similar behavioral consequences. To test this hypothesis Experiment 1 examined whether shock induces a tactile allodynia. Conversely, Experiments 2 and 3 examined whether formalin-induced peripheral injury produced a behavioral deficit.

Experiment 1

Experiment 1 examined whether uncontrollable shock produces heightened tactile reactivity (allodynia), a behavioral feature consistent with central sensitization (for review see Willis, 2001a). Subjects were tested for allodynia with a tactile stimulus immediately after uncontrollable shock. If uncontrollable shock produces a state reminiscent of central sensitization, then animals should show heightened reactivity to tactile stimulation after shock exposure.

Method

Spinalized rats (N = 20) were placed in loose restraint tubes and secured as described in the General Methods. After a 5 min acclimation period baseline tactile reactivity was established using von Frey stimuli. These stimuli consist of

polymer monofilaments of differing diameters. When flexed against the skin, each filament delivers a standard force of a known intensity. During testing subjects were stimulated serially with increasing von Frey filament forces until the stimulation elicited a flexion response. Prior to shock, subjects were tested twice on both the ipsilateral (shocked) and contralateral leg in a counterbalanced ABBA order. Subjects were then given 6 mins of uncontrollable shock to one leg or an equivalent period of tube restraint. Immediately after shock exposure subjects were again tested with von Frey stimuli twice on both the ipsilateral and contralateral leg in a counterbalanced ABBA order. This resulted in the two tests for a given leg being separated by approximately 2 mins.

Results

The impact of shock on the change from baseline tactile reactivity is depicted in Figure 4. Shocked rats showed lower tactile thresholds relative to baseline whereas unshocked subjects had slightly elevated thresholds. Confirming this, an ANOVA on the change from baseline revealed a significant Shock X Time (baseline vs. postshock) interaction, $F(1, 18) = 8.30$, $p < .01$. No

other main effects or interactions reached significance, all Fs < 4.41, p > .05. To explore the impact of shock on tactile reactivity during the two post shock tests, I used a 2 (shocked vs. unshocked) x 2 (ipsilateral vs. contralateral leg) x 2 (test 1 vs. test 2) mixed design. This mixed ANOVA revealed a significant main effect of shock, $F(1, 18) = 8.30$, $p < .01$. In addition the main effect of time (test 1 vs. test 2) reached significance $F(1, 18) = 5.06$, $p < .05$. Neither the main effects of leg, nor any of the interaction terms, reached significance, all Fs < 4.41, p > .05.

Summary

The findings indicate that uncontrollable shock induces a bilateral tactile allodynia in spinalized rats. It should be noted that subjects tended to have greater mechanical reactivity on the first von Frey test after the experimental manipulation. However the failure to detect a significant interaction of shock and time suggests that the shock induced allodynia occurred at both time points.

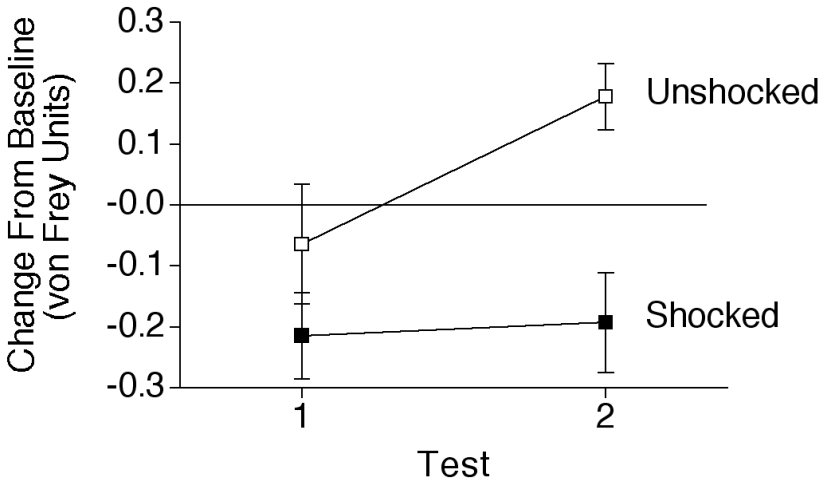


Figure 4. The impact of uncontrollable shock on tactile reactivity over the two tests. The testing time points were separated by a 2 mins for each leg.

Experiment 2

Experiment 1 examined whether uncontrollable shock induces allodynia, a behavioral measure of central sensitization (Dubner & Ruda, 1992; Kenshalo, Leonard, Chung, & Willis, 1982; Woolf, 1983). Experiment 2 examined the converse issue—whether an inflammatory agent that is known to induce allodynia and hyperalgesia can induce a deficit in instrumental performance. Within the pain literature the most common model of inflammation consists of an intracutaneous injection of a dilute solution of formalin (Lebars, Gozariu, & Cadden, 2001). This manipulation produces a well-documented sensitization of spinal neurons (Coderre, 2001) that can be blocked by both N-methyl-D-aspartate (NMDA; Coderre & Melzack, 1992; Yamamoto & Yaksh, 1992) and group I metabotropic glutamate receptor antagonists (Fisher & Coderre, 1996b). If the deficit depends on a central sensitization-like effect, formalin should induce a deficit that resembles the effects of uncontrollable shock.

Method

Spinalized subjects (N = 24) were given a single 50 μl subcutaneous injection of formalin in 1 of 4 concentrations (0, 5, 10, or 15%) into the dorsal surface of one hindpaw, yielding a one-way (formalin concentration) design (n = 6). These doses are consistent with those used in prior studies (for reviews see Coderre, 2001; Tjolsen, Berg, Hunskaar, Rosland, & Hole, 1992). Subjects were tested for instrumental learning with contingent shock on the contralateral leg 20

min after injection of formalin.

Results

To elucidate whether the experimental manipulations had a direct impact on response performance that could confound the conclusions about learning, I analyzed the duration of the first response and the shock intensity necessary to induce a 0.4 N change in flexion force. The mean durations for the first response (± *SE*) ranged from 0.13 (± 0.01) to 0.15 (± 0.01) s. The mean shock intensity necessary to elicit a 0.4 N flexion force ranged from 0.50 (± 0.04) to 0.53 (± 0.05) mA. Independent one-way ANOVAs failed to yield a significant effect of formalin on either duration of the initial duration or shock intensity, both Fs < 3.10, $p > .05$.

The impact of formalin on response duration and response number over time is depicted in Figure 5. Saline treated animals showed increases in response duration over time (Figure 5, top left panel). Formalin prevented this learning in a dose-dependent manner. An ANOVA on response duration revealed a significant main effect of Dose, $F(3, 20) = 6.81$, $p < .01$. In addition, there was a significant Dose X Time interaction, $F(87, 580) = 1.37$, $p < .05$.

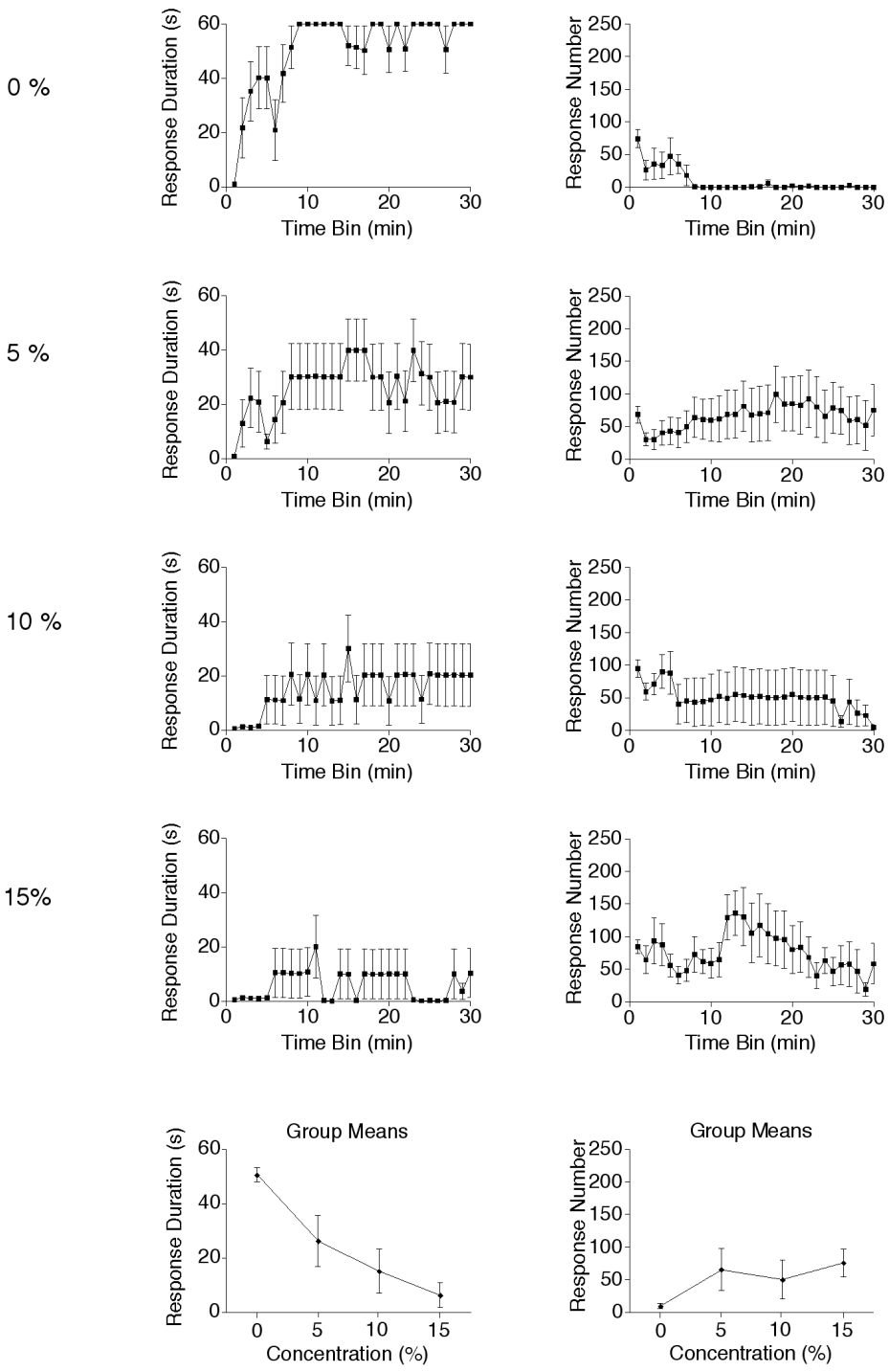


Figure 5. The impact of a single subcutaneous injection of formalin on instrumental performance when tested on the contralateral limb. The left panels depict response duration and right panels, response number. Dose of formalin increases from top to bottom panels.

The impact of formalin on response number over time is graphically depicted in the right panels of Figure 5. An ANOVA on response number yielded a significant main effect of time, $F(29, 580) = 1.67$, $p < .05$. Neither the main effect of dose nor the Dose X Time interaction reached significance, both Fs < 1.32, $p > .05$.

Summary

The analyses suggest that formalin produced a deficit in instrumental behavior when animals were tested 20 mins after formalin injection. Like the effects of uncontrollable shock (Joynes, Ferguson, Crown, Patton, & Grau, 2003), the formalin-induced deficit transferred to the contralateral leg, suggesting that the effect was centrally-mediated. It should be noted that others have argued that the two highest doses used in the present study (10 and 15%) produce a persistent peripheral sensitization in addition to central sensitization (Coderre, 2001). This suggests that the current effects may, in part, be due to a peripheral change. However, given that the animals were tested for instrumental performance on the contralateral leg, it is not clear to what extent such peripheral changes would influence learning. Indeed, in the original arguments for a central mechanism in pain modulation relied heavily on the finding that injury induces a hyperreactivity on the contralateral limb (Coderre & Melzack, 1985; Woolf, 1983). If we apply a similar argument to the present findings, the

evidence suggests that the formalin-induced deficit represents a change occurring within the spinal cord.

Experiment 3

Experiment 2 revealed that formalin can produce a deficit in instrumental performance on the contralateral leg in a dose-dependent manner. This finding suggests that formalin may alter spinal plasticity in a manner similar to uncontrollable shock. Experiment 3 sought to examine whether the loss of instrumental plasticity induced by formalin and uncontrollable shock follow a similar time course. Prior work has revealed that 6 mins of uncontrollable shock to the tail generates a deficit that lasts at least 24 h (Crown et al., 2002a). To test whether the same is true for formalin, subjects were tested on the contralateral limb 24 h after injection.

Method

Spinalized subjects (N = 12) were given a single 50 μl subcutaneous injection (0 or 15% formalin) into the dorsal surface of one hindpaw. Subjects were tested 24 h later with contingent shock.

Results

To examine whether the experimental manipulations affected response performance, I analyzed the duration of the first response and the shock intensity necessary to induce a 0.4 N change in flexion force. The mean durations for the first response ($\pm$ *SE*) were 0.14 ($\pm$ 0.01) and 0.16 ($\pm$ 0.02) s for

the saline and formalin groups, respectively. A one-way ANOVA showed that the effect of formalin was not significant, $F(1, 10) < 4.96$, $p > .05$.

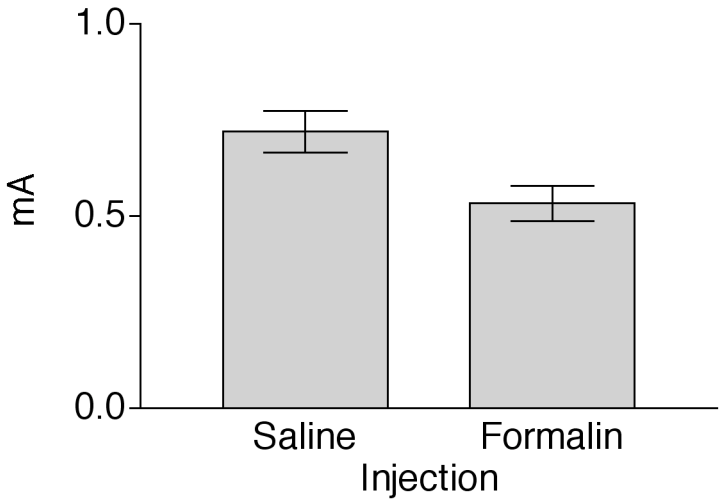


Figure 6. The impact of formalin injection 24 hours before testing on the shock intensity necessary to elicit a 0.4 N change at the time of testing. Formalin was delivered at concentration of 15%.

The mean shock intensities necessary to elicit a 0.4 N change in flexion force are depicted in Figure 6. The formalin-treated subjects required a slightly lower intensity of shock to demonstrate a 0.4 N change in flexion force than the saline treated subjects. A one-way ANOVA revealed a significant main effect of formalin dose, $F(1, 10) = 5.86$, $p < .05$. To control for differences among groups that may be due to this *a priori* difference in response performance, mean shock intensity was entered as a covariate in subsequent analyses of response duration and response number.

The impact of formalin treatment on response duration and response number are depicted in Figure 7. Saline control animals showed an increase in response duration over the 30 min testing interval. Animals that had received formalin 24 h prior to testing failed to demonstrate this learning. A 2 (formalin dose) x 30 (time) mixed ANCOVA on response durations over time indicated that the shock intensity necessary to elicit a 0.4 N change in flexion force was a significant covariate, $F(1, 9) = 6.95$, $p < .05$. After controlling for shock intensity, there was still a significant effect of formalin dose, $F(1, 9) = 48.25$, $p < .0001$. The main effect of time reached significance, $F(29, 261) = 1.82$, $p < .01$. In addition there were significant interactions of Time X Shock Intensity and Time X Dose, both Fs > 1.46, $p < .05$.

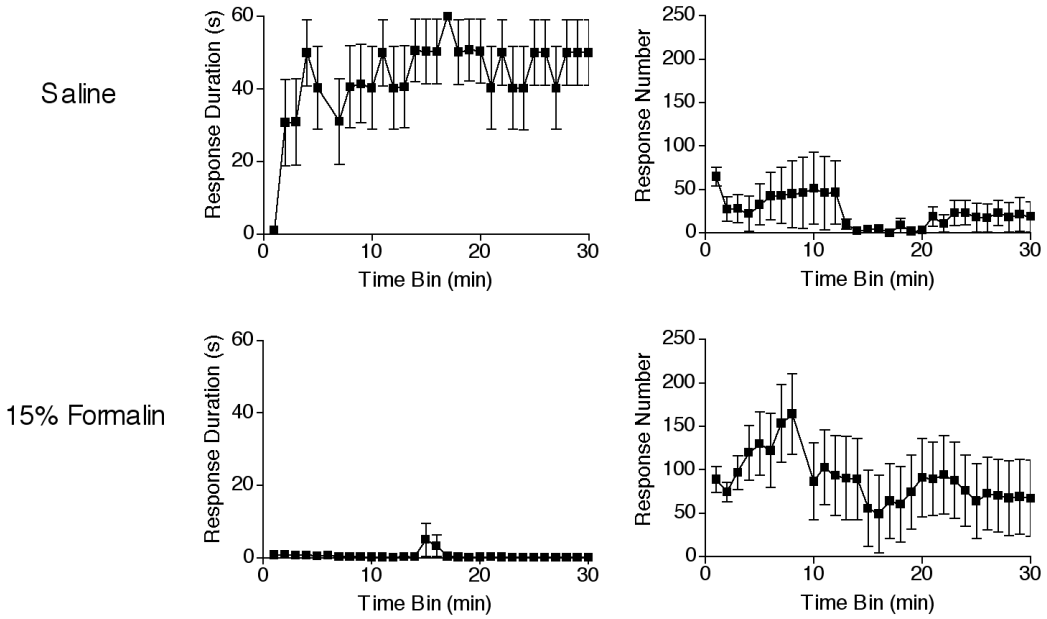


Figure 7. The impact of formalin on learning 24 h after delivery. The left panels depict response duration and right panels, response number. Top panels depict the impact of saline and the bottom panels the impact of 15% formalin injected into the dorsum of the hindpaw.

As depicted in Figure 7 (left panels), saline control animals appeared to make fewer responses over the testing interval than the formalin-treated animals. However it should be noted that this difference was not significant at □ = .05. An ANCOVA indicated that the shock intensity necessary to elicit a 0.4 N change in flexion force was not a significant covariate, $F(1,9) < 5.12$, $p > .05$. As a consequence shock intensity was excluded in subsequent analyses. A mixed ANOVA yielded a marginally significant main effect of formalin dose, $F(1, 10) = 3.27$, $p = .10$. However neither the main effect of time, nor the Time X Formalin Dose interaction reached significance, all Fs < 1.46, $p > .05$.

Summary

The results indicate that, like shock, formalin induced a deficit in instrumental behavior when animals were tested on the contralateral leg at 24 h after injection. Moreover, the animals that received formalin also required less shock to elicit a 0.4 N change in flexion force (Figure 6). This lowered threshold for eliciting the response could be interpreted as a kind of hyperalgesia. This suggests that the formalin-induced deficit may be associated with a central sensitization 24 h after formalin exposure. This is an unusual finding given the prior literature. Formalin has been traditionally found to induce nociceptive behaviors and spinal hyperexcitability that wanes by 40 mins after injection (Tjolsen el al., 1992). To my knowledge no papers have examined the impact of

formalin on nociceptive behaviors at 24 h. Although researchers have shown changes in microglia several days after formalin injection, indicating that inflammatory processes do not end within 40 mins of injection (Fu, Light, Matsushima, & Maixner, 1999). The present results extend the findings of Experiment 2, suggesting that formalin-induced inflammation produces a long-term change in behavioral potential. The long-term nature of this effect is similar to the deficit produced by uncontrollable shock (Crown et al., 2002a). However, further work is needed to determine if, like the effect of shock, the formalin-induced deficit wanes after 48 h.

Together, Experiments 1-3 suggest that uncontrollable shock engages nociceptive systems, and that a more naturalistic source of nociception induces a behavioral deficit. These findings imply that nociception can undermine behavioral plasticity in the spinal cord.

CHAPTER IV

ROLE OF THE NMDA SYSTEM IN THE DEFICIT

Experiments 1-3 explored the similarities among the behavioral effects of uncontrollable shock and inflammation. Experiment 1 showed that uncontrollable shock induced tactile allodynia, suggesting that uncontrollable shock has behavioral sequelae that are similar to inflammation. Conversely, Experiments 2 & 3 showed that inflammation can produce a deficit that mimics the effects of uncontrollable shock. The remaining experiments in this dissertation examined whether the deficit induced by uncontrollable shock engages some of the same pharmacological systems that are commonly implicated in inflammation-induced central sensitization. Glutamatergic systems were of specific interest in the present work. Experiments 4 and 5 explored the impact of the NMDA receptor on the deficit. Experiment 4 examined whether blocking the NMDA receptor with an intrathecal antagonist can prevent induction of the deficit. Experiment 5 tested whether administration of NMDA agonist can induce a deficit.

Experiment 4

Experiment 4 tested the necessity of the N-methyl-D-aspartate (NMDA) receptor for the induction of the deficit, using the noncompetitive NMDA antagonist MK-801. If the NMDA receptor is necessary for the development of the behavioral deficit, MK-801 should block induction in a dose-dependent manner. Prior work has shown that the NMDA receptor is necessary for the

acquisition of the instrumental response in our paradigm (Joynes, Janjua, & Grau, in press). As a consequence, all testing for instrumental learning was performed 24 h after drug exposure. It should be noted that the deficit has been found to last for over 48 h, so the ability to detect the deficit in the saline controls should be unaffected at the time of testing.

Method

Spinalized subjects (N = 64) were given MK-801 (Tocris, Ellisville, MO) at one of 4 doses (0.0, 0.1 1.0, or 10.0 nmol) dissolved in 1.0 μl of 0.9% saline. The drug was administered over 30 s followed by a 20 μl flush over the course of 2 min. Immediately after drug administration subjects were placed in Plexiglas tubes and given uncontrollable shock to the tail as described in the General Method section. The experimental manipulations produced a 4 (dose) x 2 (shock) factorial design (n = 8). The next day (24 h later) subjects were prepared as described in the General Method, and tested for instrumental learning with 30 min of response-contingent leg shock. The leg on which the subjects receive instrumental training was counterbalanced across subjects within each experimental condition.

Results

To confirm that the experimental manipulations did not have a direct impact on response performance, I examined the duration of the initial response and the shock intensity necessary to induce a 0.4 N change in flexion force. The

mean duration (± *SE*) of the first response ranged from 0.13 (± 0.01) to 0.24 (± 0.05) s across groups. The mean shock intensities (± *SE*) necessary to elicit a flexion force of 0.4 N ranged from 0.42 (± 0.02) to 0.48 (± .03) mA. Independent ANOVAs failed to yield any significant main effects or interactions on either measure, all Fs < 2.76, p > .05.

The effects of MK-801 and shock on response duration and response number are graphically depicted in Figure 8. Unshocked animals that were given saline showed an increase in response duration over the 30 min testing interval (Figure 8, top left panel). Shocked animals did not learn to increase response duration, the typical shock-induced deficit in instrumental behavior. Intrathecal MK-801 administered prior to uncontrollable shock blocked the induction of the deficit in a dose-dependent manner (Figure 8, left panels).

Confirming these impressions, an ANOVA on response duration yielded significant main effects of dose and shock as well as a significant Dose X Shock interaction, all Fs > 2.76, p < .05. There was a significant main effect of time, $F(29, 1624) = 15.19$, p < .05. In addition the interactions of Time X Dose and Time X Shock were both significant, both Fs > 1.46, p < .05. The Time X Dose X Shock three-way interaction was marginally significant, $F(87, 1624) = 1.26$, p = .057.

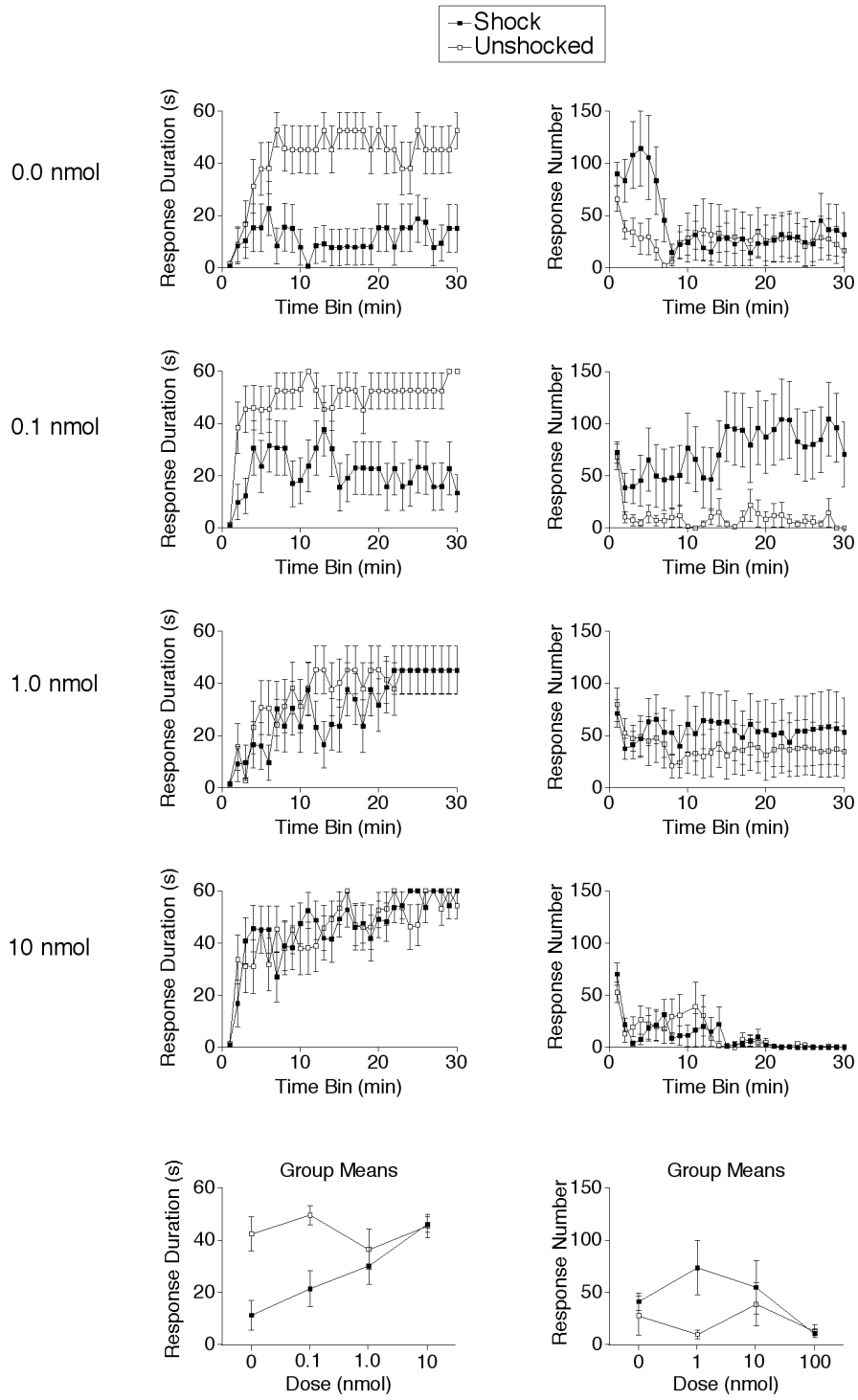


Figure 8. The impact of MK-801 on the deficit produced by uncontrollable shock. Response Duration is depicted in the left panels. Response number is shown in the right panels. Dose of MK-801 increases from top to bottom.

The experimental manipulations appeared to have a nonmonotonic effect on response number. Subjects in the saline shocked group failed to learn and habituated to the shock, resulting in low response numbers over time (Figure 8, top right). Conversely, animals that learned tended to maintain their leg in a flexed position, thereby decreasing response numbers over time (Figure 8, right panels). The highest response numbers were seen in shocked animals that were given the low dose of MK-801 (0.1 nmol). Although animals that received this dose never learned to fully maintain their leg in a flexed position, the drug appeared to prevent the habituation during testing that was seen in the saline-treated controls. An ANOVA on the response duration data revealed a significant main effect of time, $F(29, 1624) = 3.55$, $p < .001$. In addition, both the interaction of Time X Dose and the 3-way interaction of Time X Dose X Shock reached significance, both Fs > 1.32, $p < .001$. Trend analysis was used to examine the degree to which the effects were monotonic in nature. The trend analysis revealed significant linear trends (monotonic) for the main effect of time, the Time X Dose interaction, and the Time X Dose X Shock 3-way interaction, all Fs > 10.8, $p < .001$. In addition there were significant quadratic trends (1 inflection) for the main effect of time as well as the Time X Dose X Shock 3-way interaction, both Fs > 10.8, $p < .001$. No other terms showed significant linear or quadratic trends, all Fs < 2.60, $p > .05$.

Summary

The analyses indicate that NMDA antagonism with MK-801 dose dependently blocked the induction of the instrumental deficit produced by uncontrollable shock. This effect was seen at 24 after uncontrollable shock, suggesting that MK-801 was protective even after having cleared the system. The protective effect of MK-801 on the deficit is similar to its impact on the central sensitization produced by formalin inflammation (Coderre & Melzack, 1992). Intrathecal administration of MK-801 has been found to block formalin induced central sensitization at doses similar to those used in the present study (Coderre & Melzack, 1992). Although, the effects of MK-801 are typically measured shortly after peripheral injury (e.g., Coderre & Melzack, 1992), there is some precedent for the current finding that MK-801 has a protective effect on the spinal cord after the drug has cleared the system (Munglani et al., 1999). Research has shown that preemptive MK-801 can block long term changes in pain reactivity caused by peripheral nerve injury (Munglani et al., 1999). Indeed, this protective effect was observed on allodynia scores 28 days after injury (Munglani et al., 1999). These findings suggest that the deficit shares pharmacological features that are consistent with central sensitization.

Experiment 5

Experiment 4 revealed that activation of the NMDA receptor is *necessary* for the induction of the shock-induced deficit in instrumental performance. Experiment 5 examined the converse issue—whether NMDA activation is *sufficient* to induce a deficit similar to the one produced by uncontrollable shock. I examined this issue by assessing the impact of an NMDA agonist given alone or in combination with uncontrollable shock delivered at an intensity that is subthreshold for inducing a strong deficit. Figure 9 depicts the potential outcomes. The first possibility is that the drug has no effect. This finding, in conjunction with Experiment 4, would imply that the NMDA receptor is necessary, but that engaging this link in the molecular chain is not in of itself sufficient to induce the deficit or influence its development. The second option is that NMDA plays a modulatory role, enhancing the level of excitation produced by uncontrollable shock, but having little effect by itself (Figure 9, mechanism 2). A third possibility is that NMDA activation and shock could both inhibit plasticity, but do so through different mechanisms (Figure 9, mechanism 3). This predicts that an NMDA agonist would induce a deficit, but the magnitude of this effect would be unaltered by shock treatment (neither additivity nor synergy). Additivity of drug and shock is the final potential mechanism (Figure 9, mechanism 4). In this case, a low dose of NMDA and a low intensity of shock should each yield a partial effect, and act together to produce a robust deficit.

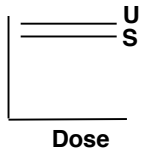
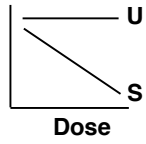
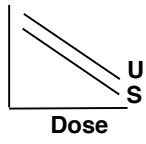
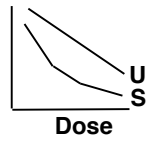
Mechanism	Pattern of Results	Graph
1. No impact of drug	Drug → No effect Weak shock → Small effect Weak shock + Drug → Small effect	
2. Modulation	Drug → No effect Weak shock → Small effect Weak shock + Drug → Deficit	
3. Distinct mechanism	Drug → Deficit Weak shock → Small effect Weak shock + Drug → no additivity	
4. Additivity	Drug → Small effect Weak shock → Small effect Weak shock + Drug → Deficit	

Figure 9. Potential alternative mechanisms by which a glutamate agonist may have an impact on instrumental learning within the spinal cord. Each mechanism anticipates a different pattern of results. Mechanisms 2 & 4 predict a Drug X Shock interaction. Mechanisms 1 & 3 predict no interaction. Mechanism 1 would yield no effect of drug or shock. Mechanism 3 would yield a main effect of drug alone. Graphical depictions of each of these patterns of results are shown on the right. The y-axes represent response duration and the x-axes represent dose. Abbreviations: U, Unshocked; S, Shocked

To test these different alternatives, the present experiment used a shock intensity that did not generate a robust deficit on its own. Prior work has shown that 6 min of uncontrollable shock at an intensity of 1.0 mA produces only a weak deficit (Crown et al., 2002a; Ferguson et al., 2003). Harnessing this subthreshold shock intensity and a range of NMDA doses, Experiment 5 was sensitive to any additive or modulatory effects.

Method

Spinalized subjects (N = 48) received NMDA (Tocris, Ellisville, MO) in one of 4 doses (0.0, 1.0, 10.0, or 100 nmol) dissolved in 15 μl of saline. The drug was delivered over the course of 3 min followed by a 10 μl saline flush over 2 min. Immediately after drug delivery subjects were placed in Plexiglas tubes and given 6 mins of uncontrollable shock (1.0 mA) to the tail or an equivalent period of tube restraint. The experimental manipulations yielded a 2 (shock) x 4 (dose) design (n = 6). Prior work has shown that 1.0 mA of uncontrollable shock is not sufficient to induce a reliable deficit (Crown et al., 2002a; Ferguson et al., 2003). Twenty-four hours later subjects were prepared as described in the General Method and tested with 30 mins of contingent leg shock.

Results

To examine whether shock or NMDA had a lasting effect on performance of the flexion response, I analyzed the duration of the initial response and the shock intensity necessary to induced a 0.4 N change in flexion force. The mean

duration of the initial response (± *SE*) ranged from 0.12 (± 0.02) to 0.16 (± 0.01). The mean shock intensity necessary to elicit a flexion force of 0.4 N (± *SE*) ranged from 0.61 (± 0.05) to 0.50 (± 0.06) mA. Independent ANOVAs failed to reveal any significant main effects or interactions, all Fs < 2.84, p > .05.

The impact of shock and NMDA administration on instrumental performance is depicted in Figure 10. Animals that were given saline showed normal learning when tested with controllable shock 24 h later (Figure 10, top left). Prior exposure to 1.0 mA of uncontrollable shock appeared to have little impact on this learning. Animals that received the highest dose of NMDA appeared to have a deficit at the time of testing that was evidenced by low response durations and high numbers of responses (Figure 10, bottom panels). Animals that received 100 nmol of NMDA and 1.0 mA of shock appeared to make fewer responses than subjects that received 100 nmol of NMDA alone (Figure 10, bottom right).

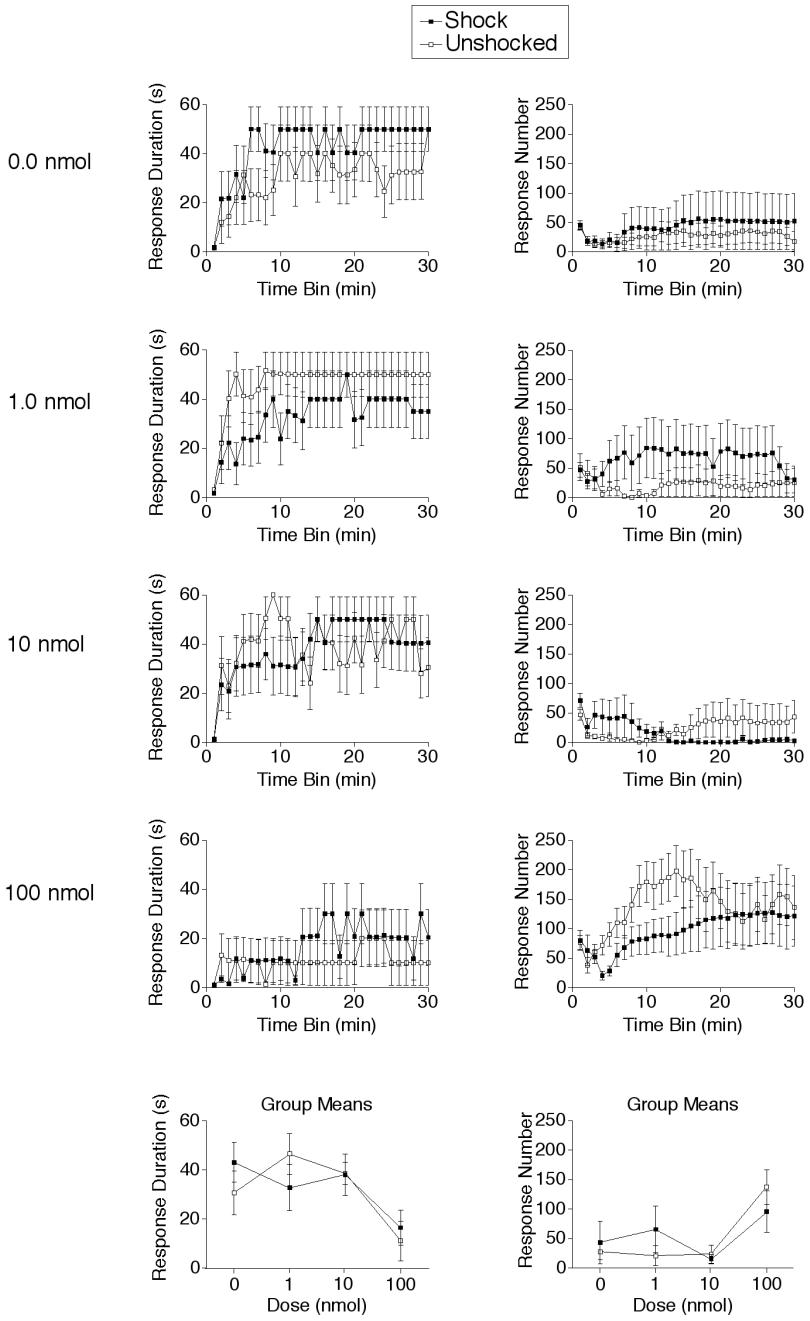


Figure 10. The impact of NMDA and subthreshold shock (1.0 mA) on instrumental performance 24 h later. Response duration data is displayed in right panels and response number, in left panels. Dose of NMDA increases from top to bottom panels.

An ANOVA on response duration data revealed a significant main effect of NMDA dose, $F(3, 40) = 3.94$, $p < .05$. A Duncan's post hoc revealed that the 100 nmol was significantly different from all other doses, $p < .05$. In addition there was a significant main effect of time, $F(29, 1160) = 10.62$, $p < .001$. No other main effects or interactions reached significance, all $Fs < 1.46$, $p > .05$. Trend analysis revealed significant linear trends for time and the Time X Shock interaction, both $Fs > 3.84$, $p < .05$. In addition there was a significant quadratic trend for time, $F(1, 1160) = 97.15$, $p < .001$. No other components reached significance, all $Fs < 3.84$, $p > .05$

An ANOVA on response number revealed a significant main effect of NMDA dose, $F(3, 40) = 4.30$, $p < .05$. The main effect of time also reached significance, $F(29, 1160) = 2.39$, $p < .01$. The Time X Dose interaction and the 3-way Time X Dose X Shock interaction both reached significance, both $Fs \geq 1.27$, $p \leq .05$. This significant 3-way interaction indicates that at the highest dose, unshocked animals made more responses than shocked animals. In all other conditions the shocked animals made more responses than the unshocked. No other main effects or interactions reached significance, all $Fs < 1.27$, $p > .05$. Trend analysis revealed significant linear trends for time, Time X Dose, and Time X Dose X Shock, all $Fs > 3.84$, $p < .05$. In addition there were significant quadratic trends for the effect of time, Time X Dose, and Time X Dose X Shock, all $Fs > 3.84$, $p < .05$. Examination of Figure 10 suggests that the 3-way

interaction reached significance for the linear trend because at the 100 nmol dose, unshocked animals made more responses than shocked animals. The opposite pattern was observed at the other doses. The quadratic trend was significant because the unshocked animals had an inverted U shaped pattern over time at the 100 nmol dose. No other components reached significance, all Fs < 3.84, $p > .05$

Summary

The analyses revealed that the highest dose of NMDA caused a deficit in both shocked and unshocked animals. The only indication of an interaction between NMDA and shock occurred with the highest dose of NMDA, and was only evident on the response number measure. Because both learning and a failure to learn can result in a low number of responses, a significant interaction on response number is difficult to interpret.

Did NMDA induce a deficit through the same mechanism as uncontrollable shock? At intermediate doses, there was little indication of additivity or synergy. A significant deficit was only observed after the highest dose of the agonist. At this dose, the inhibition of learning could be related to a drug-induced excitotoxicity. This could lead to cell death in the circuits necessary for learning. Supporting this hypothesis, the highest dose was within a range used in other studies to induce excitotoxic lesions within the brain (e.g. Strauss, Maisonnette, Coimbra, Zangrossi, 2003). A Medline search failed to produce any

papers that directly examined the impact of NMDA administration on spinal cord excitotoxicity *in vivo*. However, recent *in vitro work* suggests that NMDA doses that are much lower than those used in the present study can cause excitotoxicity in the spinal cord dorsal horn neurons (Annis & Vaughn, 1998). In addition, after contusion injury, NMDA administration at a dose of 100 nmol has been found to enhance injury-induced functional loss, presumably through excitotoxic damage (Faden & Simon, 1988). Because shock can also lead to cell death within the spinal cord (Liu et al., 2003), and because even subthreshold shocks could lead to greater excitatory amino acid release, it is surprising that the two effects did not interact. One possibility is that the neurons most sensitive to NMDA-mediated cell death are not involved in the acquisition of the instrumental response. The corollary to this is that, if uncontrollable shock induces a deficit because it increases cell death, this cell loss must involve a different populations of cells. A follow-up study will be needed to determine how these manipulations affect cell survival.

Despite the similar impact of NMDA and uncontrollable shock on cell death, the present data do not appear to support the hypothesis that NMDA and shock induce the deficit in the same manner. As shown in Figure 9, if NMDA and shock shared a common mechanism, then there should have been additivity between shock and NMDA (Figure 9, mechanism 4). Given the lack of additivity, the present findings are more consistent with the hypothesis that NMDA induced

a deficit through a distinct mechanism (Figure 9, mechanism 3).

CHAPTER V

ROLE OF GROUP I mGluRs IN THE DEFICIT

The findings from Experiments 4 and 5 implicate the NMDA receptor in the induction of the deficit. Experiments 4 suggested that the ionotropic NMDA receptor is necessary for the induction of the deficit. Although NMDA appeared to be sufficient to induce a deficit at the highest dose (Experiment 5), the failure to show summation with shock implies that the NMDA effect is qualitatively different from shock-induced deficit. It is possible that sufficiency for the shock-induced deficit resides in a different receptor population that exerts an indirect effect on NMDA receptor function. Such a mechanism would be consistent with the necessity but lack of sufficiency of the NMDA receptor. Metabotropic glutamate receptors are potential candidates for this type of role. Recent work suggests that metabotropic glutamate receptors (mGluRs) have a modulatory impact on ionotropic glutamate signaling (for review see Bruno et al., 2001). The group I mGluRs (subtypes mGluR1 and mGluR5) appear to be primarily excitatory, and are capable of producing long-lasting changes in glutamate transmission within the spinal cord after inflammatory pain (Fisher & Coderre, 1996a; 1996b; Karim et al., 2001) or injury (Mills, Johnson & Hulsebosch, 2002). The remaining experiments in this dissertation explore the role of these receptors in the induction of the deficit. Experiments 6 examined whether antagonism of the mGluR1 and mGluR5, respectively, can prevent induction of

the deficit. Experiment 8 tested whether activation of both group I mGluRs would induce a deficit or lower the threshold for induction.

Experiment 6

The present experiment tested whether the group I metabotropic glutamate receptor mGluR1 is necessary for the induction of the deficit by using the selective noncompetitive antagonist 7-(hydroxy-imino)cycloproa[b]chromen-1a-carboxylate ethyl ester (CPCCOEt). This drug has been found to reduce central sensitization after peripheral nociception (Neugebauer et al., 1999).

Method

Spinalized subjects (N = 48) were given CPCCOEt (Tocris, Ellisville, MO) in one of 4 doses (0.0, 1.0, 10.0, or 100.0 nmol) dissolved in 3 μl of 0.9% saline. Drug was administered slowly over 2 min, followed by a 20 μl saline flush over the span of 4 min. Immediately after drug administration, subjects were given 6 mins of uncontrollable shock (1.5 mA), yielding a 4 (dose) x 2 (shock) factorial design (n = 6). Twenty-four hours later subjects were prepared as described in the General Method and tested with 30 mins of contingent shock.

Results

Before examining the impact of the experimental manipulations on learning, it was necessary to analyze their direct impact on response performance. The mean duration of the initial response (± *SE*) ranged from 0.14 (± 0.01) to 0.16 (± 0.01) and the mean shock intensity necessary to elicit a 0.4 N change in flexion force ranged from 0.66 (± 0.07) to 0.76 (± 0.06) mA. Independent ANOVAs failed to reveal any significant main effects or interactions on either measure, all Fs < 2.84, $p > .05$.

The impact of CPCCOEt on the behavioral deficit induced by 1.5 mA of uncontrollable shock is shown in Figure 11. Animals that received saline and no shock learned to hold their leg in a flexed position, thereby increasing response duration and decreasing response number. However, animals that received saline and uncontrollable shock failed to learn (Figure 11, top panels). Intrathecal administration of CPCCOEt prior to shock exposure blocked the induction of the learning deficit in a dose-dependent manner.

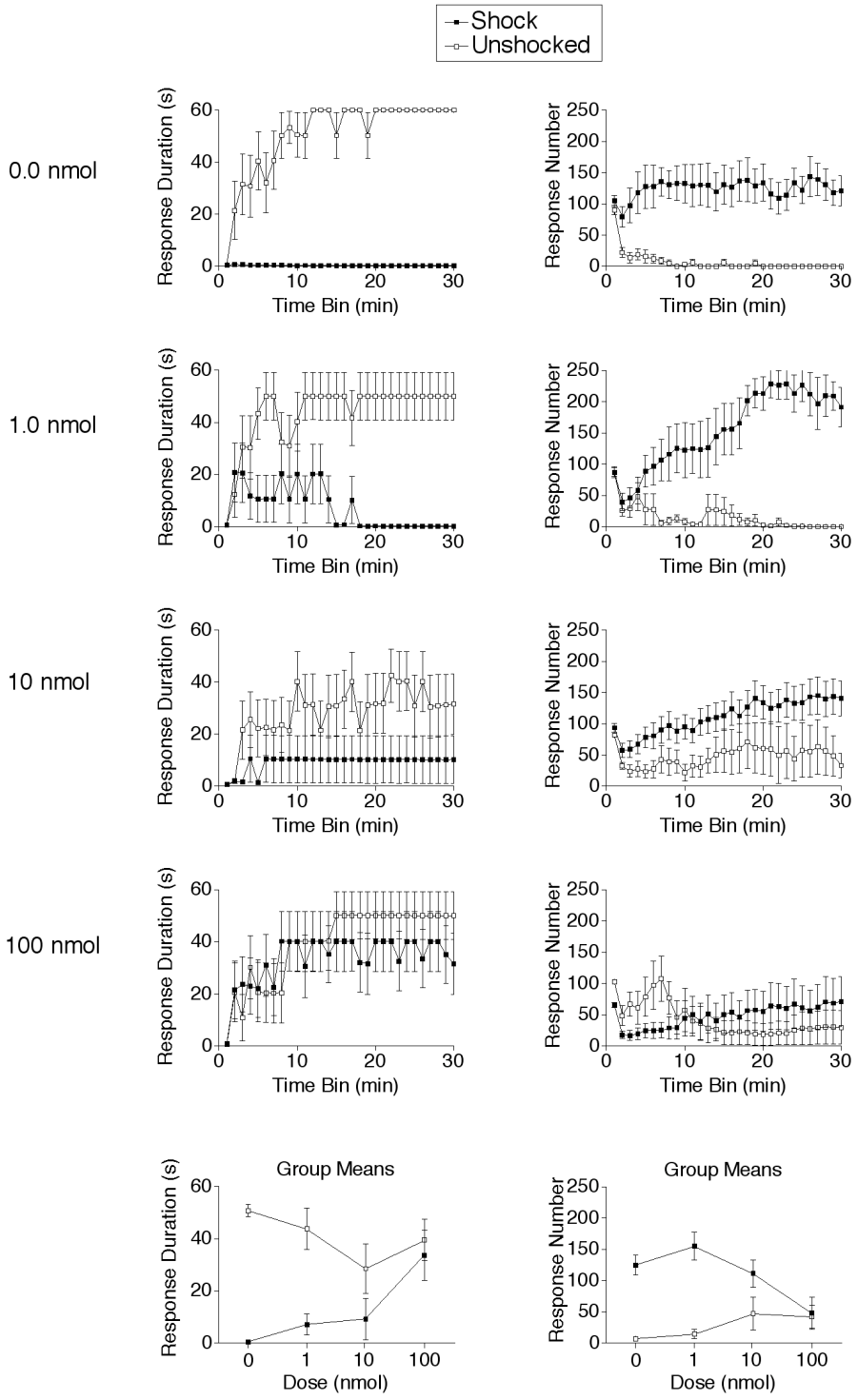


Figure 11. The impact of CPCCOEt on the deficit produced by uncontrollable shock. Response duration is shown in left panels and response number in right panels. Dose of CPCCOEt increases from top to bottom.

An ANOVA on response duration revealed a significant main effect of shock, $F(1, 40) = 26.67$, $p < .0001$. In addition, there was a significant Shock X Dose interaction, $F(3, 40) = 3.21$, $p < .05$. The Dose X Time interaction also reached significance, $F(87, 1160) = 1.31$, $p < .05$. No other main effects or interactions reached significance, all Fs < 1.30, $p > .05$. To clarify the nature of the effects over time, trend analyses were performed. These analyses revealed significant linear trends for the main effect of time, the Time X Shock interaction, the Time X Dose interaction, and the Time X Shock X Dose 3-way interaction, all Fs > 10.8, $p < .001$. In addition there were significant quadratic trends (1 inflection) for the Time X Shock and Time X Shock X Dose 3-way interaction, both Fs > 3.78, $p < .01$. No other terms reached significance, all Fs < 2.60, $p > .05$.

An ANOVA on response number revealed a significant main effect of shock, $F(1, 40) = 30.34$, $p < .001$. In addition there was a significant Shock X Dose interaction, $F(3, 40) = 4.03$, $p < .05$. The main effect of time, the Time X Shock interaction, the Time X Dose interaction, as well as the Time X Dose X Shock 3-way interaction all reached significance, all Fs > 1.32, $p < .05$.

Summary

The data indicated that the mGluR1 antagonist CPCCOEt given prior to the uncontrollable shock exposure blocked the induction of the deficit seen at the time of testing 24 h later. This suggests that the mGluR1 subtype of the

group I metabotropic glutamate receptors is necessary for the induction of the deficit.

Experiment 7

Experiment 6 found that the group I mGlu receptor mGluR1 is necessary for the induction of the deficit. Several authors have reported dissociations between the roles of mGluR1 and the other group I mGlu receptor, mGluR5. For example, intrathecal mGluR1 but not mGluR5 antagonists have been shown to reduce cold hypersensitivity after nerve injury (Fisher, Lefebvre, & Coderre, 2002). Moreover, mGluR1 and mGluR5 have been shown to have divergent effects on spinal electrophysiology, locomotor activity, and recovery of function after spinal cord injury (Kettunen, Hess, & El Manira, 2003; El Manira, Kettunen, Hess, & Krieger, 2002; Mills, Johnson, & Hulsebosch, 2002). However, long term potentiation in some brain regions appears to depend on combined activation of both mGluR1 and mGluR5 (Gubellini et al., 2003), and both receptor subtypes have been implicated in inflammatory pain (Karim, Wang, & Gereau, 2001). Given the literature, it is difficult to anticipate the role of the mGluR5 receptor on the behavioral deficit induced by uncontrollable shock based on the positive finding from Experiment 6. Experiment 7 directly tests whether mGluR5 is also necessary for the induction of the deficit. The selective noncompetitive mGluR5 antagonist 2-methyl-6-(phenylethylnyl)pyridine (MPEP) was used to test whether blockade of the receptor prevents induction of the

deficit.

Method

Spinalized subjects (N = 64) were given MPEP (Tocris, Ellisville, MO) in one of 4 doses (0.0, 1.0, 10, or 100 nmol) dissolved in 3 μl of 0.9% saline. The drug was administered slowly over 2 min, followed by a 20 μl saline flush over the span of 4 min. Immediately after drug administration, subjects were given 6 mins of uncontrollable shock (1.5 mA) to the tail in a 4 (dose) x 2 (shock) factorial design (n = 8). Twenty-four hours later subjects were prepared as described in the General Method and given 30 mins of contingent shock to test for instrumental learning.

Results

To elucidate the direct impact of the experimental manipulations on response performance I analyzed the duration of the initial response and the shock intensity necessary to induce a 0.4 N change in flexion force. The duration

of the initial response (± *SE*) ranged from 0.14 (± 0.01) to 0.18 (± 0.02) and the shock intensity necessary to elicit a 0.4 N change in flexion force (± *SE*) ranged from 0.57 (± 0.06) to 0.67 (± 0.04). Independent ANOVAs failed to reveal any significant main effects or interactions, all Fs < 4.0, $p > .05$.

The impact of MPEP on response duration and response number is depicted in Figure 12. Saline-treated animals that were unshocked learned to maintain their leg in a flexed position over time, thereby increasing response duration and decreasing response number. Animals that were given saline and uncontrollable shock failed to show this learning (Figure 12, top panels). MPEP blocked the learning deficit in a dose dependent manner (Figure 12, bottom panels).

An ANOVA on response duration revealed a significant main effect of shock, $F(1, 56) = 4.57$, $p < .05$. Both the main effect of shock and the Shock X Dose interaction reached significance, both $F(3, 56) > 2.76$, $p < .05$. In addition, the main effect of time, $F(29, 1624) = 13.30$, $p < .001$ reached significance. No

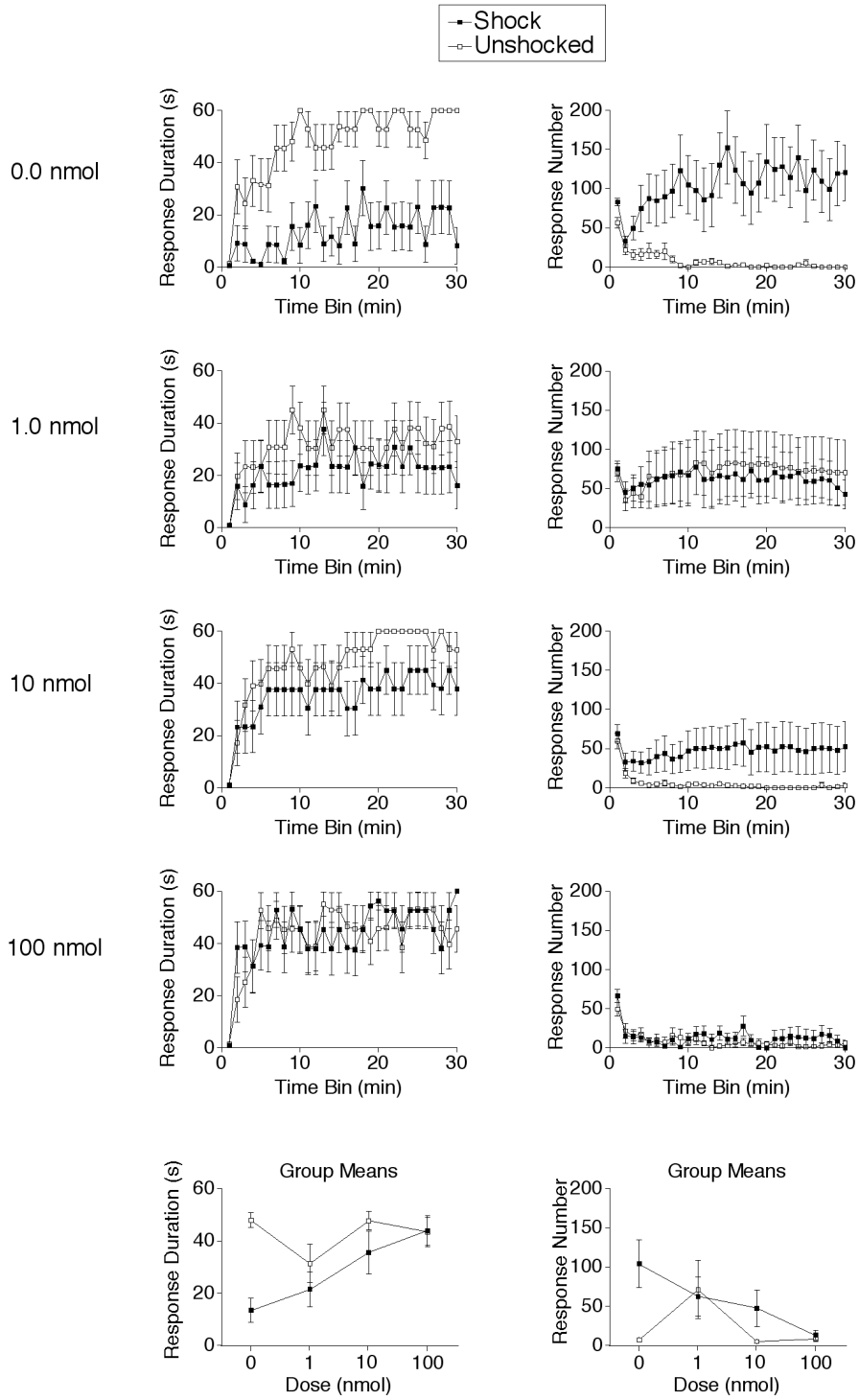


Figure 12. The impact of the mGluR5 antagonist MPEP on the deficit produced by uncontrollable shock. Left panels depict response duration and right panels, response number at each dose of drug. Dose increases from top to bottom.

other main effects or interactions were significant, all Fs < 1.32, p > .05. Trend analyses on the response duration data revealed significant linear trends for the main effect of time as well as the Time X Shock and Time X Dose interactions, all Fs > 3.84, p < .05. In addition, there were significant quadratic trends for the main effect of time and the Time X Shock interaction, both Fs > 3.84, p < .05. No other trends reached significance, all Fs < 3.84, p > .05.

An ANOVA on response number revealed a significant main effect of shock, $F(1, 56) = 25.5$, $p < .01$. Neither the main effect of Dose nor the Dose X Shock interaction reached significance, both Fs < 2.76, p > .05. The main effect of time reached significance, $F(29, 1624) = 2.13$, $p < .001$. No other main effects or interactions reached significance, all Fs < 1.32, p > .05. Trend analyses revealed a significant linear and quadratic trends for the Time X Shock, Time X Dose, and Time X Shock X Dose interactions, all Fs > 3.84, p < .05.

Summary

The analyses revealed that the mGluR5 antagonists MPEP protected against the deleterious effects of uncontrollable shock on instrumental performance in spinalized animals. Together with results from Experiment 6, the present findings suggest that both of the group I metabotropic glutamate receptor subtypes (mGluR1 and mGluR5) are necessary for the induction of the behavioral deficit with uncontrollable shock. This functional similarity is reminiscent of the shared impact of mGluR1 and mGluR5 antagonists on

inflammatory pain and LTP within the hippocampus (Gubellini et al., 2003, Karim et al., 2001).

The findings from Experiments 4, 6, & 7 suggest that glutamate transmission is necessary for induction of the deficit. These findings are consistent with prior findings implicating excitatory transmission in the deficit (Crown & Grau, 2002; Ferguson et al., 2003; Joynes & Grau, in press). We have previously found that the kappa opioid receptor is necessary for the expression of the deficit (Joynes and Grau, in press). In addition, the $GABA_A$ receptor is necessary for both the induction and expression of the deficit (Ferguson et al., 2003). Although both of these neurotransmitter systems have traditionally been associated with inhibition, research suggests that under inflammatory conditions they may both contribute to excitation within the spinal cord (Dubner and Ruda, 1992; Sluka et al., 1993; 1994). We have also found that pharmacological manipulations that reduce excitation within the spinal cord inhibit the induction of the deficit (Crown & Grau, 2002). These convergent findings suggest that overexcitation is necessary for induction and expression of the deficit.

Experiment 8

Although several pharmacological manipulations indicate that excitation is necessary for the induction and maintenance of the deficit, the issue of sufficiency has been more elusive (Experiment 5; Ferguson et al., 2003). Experiment 5 failed to show summation of NMDA and a subthreshold amount of shock. However, the interactions between group I mGluRs and NMDA suggest that the mGluR agonist could produce sufficiency even if NMDA did not. Group I mGluRs are proposed to produce effects through a long-lasting enhancement of NMDA transmission (Benquet et al., 2002; Dang et al., 2002; Lan et al., 2001). Given that the deficit lasts for over 48 h, it seems plausible that the long term changes induced by mGluRs may be sufficient for induction, however a more temporary activation of NMDA with a direct agonist may be insufficient. Experiment 8 tests whether a general group I mGluR agonist that activates both mGluR1 and mGluR5 is sufficient to induce the deficit. As discussed in the introduction to Experiment 5, sufficiency could manifest as a direct effect of drug dose or a summation with a subthreshold shock intensity (see Figure 9). The present study tests the alternative mechanisms by which an agonist might induce a deficit by using subthreshold intensity of shock. In addition, a wide range of doses of the general group 1 metabotropic glutamate agonist 3,5-dihydroxyphenylglycine (DHPG) were used to allow for the detection of modulation (Figure 9, mechanism 2) or additivity (Figure 9, mechanism 4).

Method

Spinal subjects (N = 64) were given intrathecal administration of DHPG (Tocris, Ellisville, MO) in one of four doses (0.0, 1.0, 10.0, or 100 nmol) dissolved in 3 *µ*l of 0.9% saline. Drug was administered over the span of 2 min followed by a 20 *µ*l saline flush over the span of 4 min. Immediately after drug delivery, subjects were placed in Plexiglas tubes and given 6 mins of 1.0 mA of uncontrollable shock to the tail or an equivalent period of tube restraint, producing a 2 (shock) x 4 (dose) design (n = 8).Twenty-four hours later subjects were prepared as described in the General Method and tested with 30 mins of contingent leg shock.

Results

To determine whether DHPG or shock had a direct impact on response performance I analyzed the duration of the initial response and the shock intensity necessary to elicit a 0.4 N change in flexion force. The mean duration of the initial response ($\pm$ *SE*) ranged from 0.14 ($\pm$ 0.01) to 0.17 ($\pm$ 0.01) and the mean shock intensity necessary to elicit a 0.4 N change in flexion force ranged from 0.53 ($\pm$ 0.04) to 0.73 ($\pm$ 0.08) mA. Independent ANOVAs failed to reveal any significant main effects or interactions on either measure, all Fs < 2.76, p > .05.

The impact of shock and DHPG on response duration and response number is shown in Figure 13. Subjects that were given saline and no shock

learned to maintain their leg in a flexed position over the testing period (Figure 13, top left panel). DHPG appeared to prevent this learning at every dose examined. The dose of shock (1.0 mA) used in the present experiment had a slight impact on response duration over time that was apparent in all but the highest dose of drug. The group means collapsed across time are depicted in the bottom panels of Figure 13.

An ANOVA on response duration revealed a significant main effect of drug, $F(3, 56) = 3.02$, $p < .05$. In addition, the main effect of time and the Time X Shock interaction both reached significance, both Fs > 1.99, $p < .001$. No other main effects or interactions reached significance. Trend analysis revealed significant linear trends of the main effect of time and the Time X Shock, Time X Drug, and Time X Shock X Drug interactions, all Fs > 2.60, $p < .05$. Examination of the graphs in Figure 13 (left panel) suggested that the 3-way interaction was significant because the shocked animals that were given drug tended to perform more poorly over time than the shocked animals given 0.0 nmol. In addition there were significant quadratic trends for the main effect of time, the Time X Drug interaction, and the Time X Shock interaction, all Fs > 2.60, $p < .05$. No other terms reached significance, all Fs < 2.60, $p > .05$.

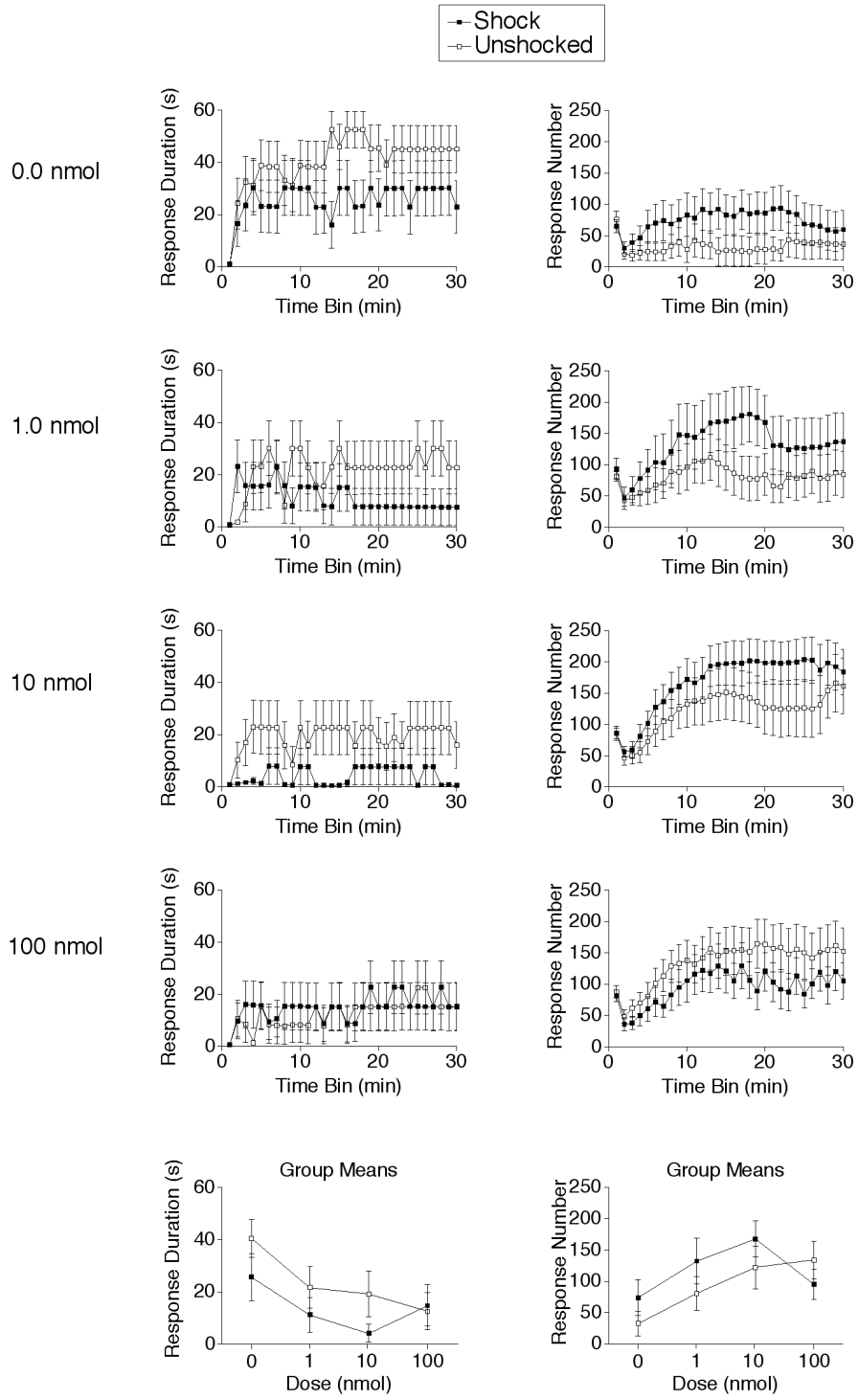


Figure 13. The impact of DHPG on response duration (left panels) and response number (right panels) 24 h after drug administration. Dose increases from top to bottom.

An ANOVA on response number revealed a significant main effect of drug, $F(3, 56) = 3.02$, $p < .05$. The main effect of time and the Time X Shock interaction also both reached significance, both Fs > 1.70, $p < .01$. No other main effects or interactions reached significance. Trend analysis on response number revealed a significant linear trend for the main effect of time as well as the Time X Drug, Time X Shock and Time X Drug X Shock interactions, all Fs > 2.60, $p < .05$. Examination of the graphs in Figure 13 suggested that the 3-way interaction reached significance because shocked animals that received the 10 nmol dose made more responses than the 0.0 nmol group. There was also a significant quadratic trend for the main effect of time, $F(1, 1624) = 154.7$, $p < .001$. In addition, the quadratic trends for the Time X Drug, Time X Shock, and the Time X Drug X Shock interactions all reached significance, all Fs > 2.60, $p < .05$. Figure 13 suggested that the 3-way interaction reached significance because the shocked animals that were given 1.0 nmol showed a nonmonotonic pattern for response numbers over time (Figure, 12, second panel from the top right). No other terms reached significance, all Fs < 2.60, $p > .05$.

Summary

The findings from Experiment 8 revealed that the group 1 metabotropic glutamate agonist DHPG produced a behavioral deficit that was apparent 24 h after drug administration. In addition there was a hint of summation with shock, however this summation was most clear at the lower doses, and appeared to be

partially reversed at the highest dose of drug (Figure 13, bottom panels). This pattern of results may be consistent with other work that suggests that DHPG produces LTP at low does but LTD at high doses (Tan, Hori, & Carpenter, 2003; Wisniewski & Car, 2002). This possibility will be addressed in greater detail in the General Discussion. Together with the findings from Experiments 6 and 7, the data suggest that the group 1 metabotropic glutamate receptors are both *necessary* and *sufficient* for the induction of a deficit in instrumental performance in spinalized rats.

CHAPTER VI

GENERAL DISCUSSION

The experiments in the present dissertation were designed to explore whether the loss in instrumental performance observed after uncontrollable shock relies on mechanisms similar to those invoked by peripheral inflammation. The findings revealed that the deficit was associated with a bilateral tactile allodynia (Experiment 1) and that a long-lasting deficit could be induced with peripheral administration of formalin (Experiments 2 & 3). Both the NMDA (Experiment 4) and the group I mGluR systems (Experiments 6 & 7) were found to be necessary for the induction of the deficit with uncontrollable tailshock. Agonists of both NMDA (Experiment 5) and group I mGluRs (Experiment 8) were found to be sufficient to induce a deficit in learning 24 h after drug delivery. However, the NMDA effect only occurred at the highest dose, and did not summate with a subthreshold intensity of uncontrollable shock. These findings were interpreted as evidence that NMDA and shock did not produce a deficit through a common mechanism. The group I mGluR agonist produced a deficit at much lower dose and showed summation with shock, leading to the conclusion that uncontrollable shock may induce a deficit through activation of group I mGluRs.

The results bolster the links between the consequences of peripheral inflammation and uncontrollable shock. Both NMDA and metabotropic glutamate

systems have been shown to be involved in nociceptive processing and inflammation (Bordi & Ugolini, 2000; Coderre & Melzack, 1992; Fisher & Coderre, 1996a; 1996b; Fisher, Lefebvre, & Coderre, 2002; Fundytus, Osborne, Henry, Coderre, & Dray, 2002; Karim, Wang & Gereau, 2001; Neugebauer, Chen, & Willis, 1999; Mills et al. 2002; Stanfa & Dickenson, 1998; Zhang, Lu, Chen, & Westlund, 2002). Intrathecal administration of NMDA has been shown to induce spontaneous nociceptive behaviors and hyperalgesia (Raigorodsky & Urca, 1987; Kolhekar, Meller, & Gebhart, 1993). Conversely, administration of an NMDA antagonist reduces the nociceptive responses to inflammation caused by formalin, carrageenan, and complete Freund's adjuvant (Coderre & Melzack, 1992; Coderre & Van Empel, 1994; Ren, Williams, Hylden, Ruda, & Dubner, 1992). More recent data suggests that activation of group I mGluRs can induce spontaneous nociceptive behaviors as well (Fisher & Coderre, 1996a). Moreover, administration group I mGluRs has been found to reduce nociceptive responses in number of paradigms including sciatic nerve ligation (Fisher et al., 2002), inflammation caused by carrageenan/kaolin or formalin (Karim, Wang, & Gereau, 2001; Stanfa & Dickenson, 1998; Zhang, Lu, Chen, & Westlund, 2002), and intradermal capsaicin administration (Neugebauer, Chen, & Willis, 1999).

It is interesting that the behavioral deficit and nociceptive processing show such similar pharmacological mechanisms and behavioral features. The present data are consistent with the hypothesis that the deficit may be a

consequence of inappropriate nociceptive activity within the spinal cord that limits the capacity for subsequent learning. In the following sections, I review some potential mechanisms that could account for the present results. Throughout the course of this discussion I will: 1) address issues related to the mechanisms of neuropathic pain, 2) explore plausible physiological underpinning of the behavioral deficit, 3) propose a putative molecular model of the deficit, 4) review the implications of the present findings, and 5) discuss future directions.

Links to Central Sensitization

A well established consequence of intense and/or prolonged nociceptive stimulation is increased sensitivity of neurons in the dorsal horn of the spinal cord (Willis, 2001a; Woolf, 1983). This process, known as central sensitization, can be induced by a number of manipulations including thermal injury (Coderre & Melzack, 1985; Woolf, 1983), inflammation (Coderre, Vaccarino, & Melzack, 1990), capsaicin-induced activation of primary afferent nociceptors (C-fibers; Dougherty & Willis, 1992), nerve injury (Tabo, Jinks Eisele, & Carstens, 1999), and electrical stimulation (Wall & Woolf, 1984). The present section reviews the evidence linking the deficit to the process of central sensitization. It should be noted that the extent to which these links exist determines the clinical relevance of the present findings. If the deficit and central sensitization are shown to be isomorphic processes, than this implies that nociception can undermine plasticity within the spinal cord.

Central sensitization can manifest behaviorally as heightened tactile reactivity (allodynia; Woolf, 1983; Woolf & Wall, 1986), and appears to be dependent on activation of the NMDA receptor (Woolf & Thompson, 1991). The present finding that uncontrollable shock induces a tactile allodynia (Experiment 1) suggests that our uncontrollable shock regimen may invoke a central sensitization-like mechanism. This hypothesis is bolstered by the observations that formalin inflammation can induce a deficit in instrumental performance (Experiments 2 & 3), and that delivery of an NMDA antagonist blocks the induction of the deficit (Experiment 4). Moreover, previous work suggests that the deficit induced by a different inflammatory agent, carrageenan, mimics the timecourse of the central sensitization and excitatory amino acid release induced by this substance (Ferguson, Crown, Washburn, Miranda, & Grau, 2001; Sluka, Willis, & Westlund, 1994; Xu Elfvin, Wiesenfeld-Hallin, 1995).

The role of metabotropic glutamate receptors in central sensitization is a relatively new area of inquiry (Bordi & Ugolini, 2000; Fisher & Coderre, 1996a; 1996b; Fisher et al., 2002; Fundytus, Osborne, Henry, Coderre, & Dray, 2002; Neugebauer et al., 1999). However, recent data make a compelling case for the role of the group I mGluRs in the induction of neuropathic pain in several models of central sensitization. For example, Fisher & Coderre (1996b) found that intrathecal administration of a group I mGluR antagonist reduced the number of nociceptive behaviors generated by a 2.5% formalin solution. Conversely, 2 to

20 nmol of DHPG summated with a 1.0% solution of formalin, increasing the number of nociceptive behaviors during the late phase of inflammation. This effect was long lasting, developed slowly over testing and remained for the duration of testing (90 min). Together these data suggest that the central sensitization induced by formalin relies on a group I mGluR mechanism. Other work suggests that selective antagonists for the mGluR1 and mGluR5 receptor can reduce tactile allodynia in nerve injury and joint inflammation preparations (Fisher et al., 2002; Zhang et al., 2002). Direct electrophysiological recordings from spinothalamic tract neurons confirm the role of group I mGluRs in central sensitization (Neugebauer et al., 1999). Neugebauer et al. (1999) found that the general mGluR agonist DHPG enhances capsaicin-induced central sensitization of spinothalamic tract neurons. However, at high doses DHPG appeared to reduce central sensitization. Surprisingly, an agonist selective for the mGluR5 receptor appeared to have an inhibitory action. Delivery of an mGluR1 selective antagonist appeared to have no effect on the inhibitory effects of high doses of DHPG. Together these findings indicate that high doses of DHPG may have inhibitory action through activation of the mGluR5 receptors.

The present findings that the relatively selective mGluR1 antagonist CPCCOEt prevented the induction of the shock-induced deficit (Experiment 6) are consistent with the hypothesis that the deficit involves a central sensitization-like mechanism. Likewise the dose-response function observed with DHPG

mimics the impact of this drug on central sensitization. At the highest dose (100 nmol) there appeared to be a partial recovery of response durations in the shocked animals (Figure 13, bottom left), a finding consistent with the nonmonotonic effects of DHPG on central sensitization (Neugebauer et al., 1999). That said, the finding that the selective mGluR5 antagonist MPEP was protective (Experiment 6) presents a potential problem for a central sensitization-based mechanism.

There have been mixed reports about the impact of mGluR5 drugs on nociceptive processing. Some authors have found little evidence that mGluR5 activation induces nociceptive behaviors or contributes to central sensitization (Fisher and Coderre, 1996a; Neugebauer et al., 1999). However, others have argued that spinal mGluR5 receptors are involved in processing of acute nociceptive stimuli such as shock (Bordi & Ugolini, 2000). The present finding that mGluR5 antagonism with MPEP prevents the induction of the deficit may be due to an inhibition of acute nociceptive processing. The current studies are not capable of distinguishing an acute inhibition from a more long term impact. If the deficit relies on a central sensitization-like mechanism blocking acute nociceptive transmission would still be expected to prevent its induction if the drug is given prior to shock exposure.

However, it should be noted that glutamate systems are important in the maintenance phase of central sensitization as well as the induction phase (Woolf

& Thompson, 1991). If mGluR5 is only involved in acute nociceptive processing, and the deficit depends on a central sensitization-like mechanism, then the drug should not be protective if given after uncontrollable shock. In contrast, because the mGluR1 receptors have been more heavily implicated in central sensitization, the mGluR1 antagonist CPCCOEt might be expected to have an impact before or after shock exposure. Intrathecal delivery of an antisense oligonucleotide knock-down of mGluR1 has recently been found to reverse tactile allodynia after inflammation, suggesting that mGluR1 may be involved in the maintenance of central sensitization (Fundytus et al., 2002). Whether a similar post hoc manipulation using mGluR1 and mGluR5 antagonists would reverse the deficit is an interesting empirical question that should be addressed in future studies.

Long Term Potentiation vs. Long Term Depression

It has been argued that central sensitization depends on long-term potentiation (LTP), an electrophysiological phenomenon commonly associated with learning and memory within the brain (Willis, 2001a). This would seem to indicate that LTP might be involved in the deficit as well. However, the glutamate systems implicated in the present experiments have also been shown to be involved in the induction of LTP and an opposing form of synaptic plasticity, long term depression (LTD; Cho & Bashir, 2002; Gubellini et al., 2003; Harris, Ganong, & Cotman, 1984; Mulkey & Malenka, 1992; Wilsch, Behnisch, Jager,

Reymann, & Balschun, 1998). This fact makes clear statements about the electrophysiological underpinnings of the deficit difficult to make. Indeed, it seems possible that the deficit reflects a maladaptive form of synaptic plasticity that depends on either LTP or LTD. The present section explores whether the deficit is more likely to involve one of these processes over the other. By bootstrapping the present findings to past work, it may be possible to reach a conclusion with some degree of confidence.

Prior work indicates that NMDA activation is necessary for the induction of both forms of synaptic plasticity within the hippocampus (Harris, Ganong, & Cotman, 1984; Mulkey & Malenka, 1992). Hippocampal activation of group I metabotropic glutamate receptors has also been implicated in both LTP and LTD (Bortolotto, Fitzjohn & Collingridge, 1999; Cho & Bashir, 2002; Wilsch et al., 1998). These findings do little to elucidate the electrophysiological underpinnings of the deficit. However, an in-depth examination of prior work may shed some light on the issue. In the following paragraphs I will review the literature on the role of glutamate systems in LTP and LTD in an attempt to decipher the more plausible electrophysiological mechanism.

The relationship between the group I mGluRs and LTP is a complex one (see Figure 14, Wilsch et al., 1998). A rise in intracellular Ca^{++} levels is necessary for induction of LTP. This increase in Ca^{++} can be generated through an influx of Ca^{++} through membrane channels or through the liberation of

intracellular stores. The former occurs via activation of L-type (voltage-gated) and ionotropic NMDA channels. The latter can be produced through activation of group I mGluRs. In the presence of high levels of stimulation, the combined Ca^{++} influx through L-type (voltage-gated) and NMDA channels is sufficient to yield LTP. In this case, blockade of group I mGluRs has little impact on LTP induction. However, in the event of low stimulation, the Ca^{++} influx is insufficient. Under these conditions, activation of mGluRs can liberate intracellular Ca^{++} stores and thereby push the Ca^{++} levels into the range necessary for LTP induction. Such a mechanism would anticipate summation of shock and mGluR activation to induce LTP. If the deficit relies on induction of LTP, then there should be summation of the mGluR agonist DHPG and shock. The findings from Experiment 8 support this idea (see Figure 13). However, it should be noted that at the highest dose (100 nmol) this summation reversed (Figure 13, bottom left panel).

If we assume that the deficit relies on LTP, the present pattern of results would seem to suggest that there was a reversal of DHPG-induced LTP at the highest dose. Similar findings have been seen in the central sensitization and learning literatures (Bortolotto et al., 1999; Neugebauer et al., 1999; Wisniewski & Car, 2002). It has been argued that DHPG induces LTP at low doses and LTD at higher doses (Wisniewski & Car, 2002). Moreover, recent work suggests that the LTD induced by DHPG shows no summation with shock induced LTD in the

hippocampus, suggesting that it relies on an independent mechanism (Palmer, Irving, Seabrook, Jane, & Collingridge, 1997). Again, this is consistent with the lack of summation seen at the highest dose of DHPG. Together, these data seem to support the hypothesis that DHPG induced LTP at the lower doses, and this LTP summated with shock exposure to yield a more pronounced deficit. At the highest dose this LTP was counteracted by a DHPG-induced induction of LTD. This resulted in higher response durations in the 100 nmol group and a reversal of the summation with shock. If the deficit were to depend on LTD then one might expect a reverse pattern—a summation of shock and DHPG that increases with dose. In the context of prior work, the present mGluR data appear to favor LTP over LTD as the mechanism of the deficit.

The finding that the NMDA antagonist MK-801 prevents the induction of the deficit provides additional insight into the mechanism of the deficit. Recent data suggests that the shared role of NMDA in the opposing processes of LTP and LTD results from differences in subunit configuration of the NMDA receptor (Hrabetova et al., 2000). The NMDA receptor is comprised of receptor 1 subunits (NR1) and combinations of the NR2 glutamate binding subunits (named NR2A-D). Antagonism of the NR2A/B subunits reduced LTP but had little impact on LTD at the same doses (Hrabetova et al., 2000). An antagonist that has more equivalent affinity for NR2A/B and NR2C/D impaired LTP as well as LTD. This implies that NR2A/B is necessary for LTP induction whereas LTD depends on

NR2C/D (Hrabetova et al., 2000). If the deficit depends on LTP then antagonizing the NR2A/B subunit should prevent the deficit. On the other hand if an LTD-based mechanism underlies the deficit, then NR2A/B should have little effect. Authors have found that MK-801 has a greater affinity for the NR2A/B receptor than the NR2C/D receptor (Buller et al., 1994). In accordance with this, researchers have argued that MK-801 more reliably blocks LTP than LTD (Hrabetova et al., 2000). If the deficit relies on LTD, then MK-801 should not have been protective in Experiment 4. These positive findings are more consistent with the hypothesis that uncontrollable shock induces LTP in the spinal cord. Together, the findings that DHPG induced a nonmonotonic dose-response function (Experiment 8), and that MK-801 was protective, provide converging support for an LTP rather than an LTD-based mechanism.

The findings that uncontrollable shock induces a tactile allodynia provides further evidence that the deficit may be associated with LTP rather than LTD. Work by Sandkuhler and colleagues suggests that electrical stimulation of A-delta primary afferents induces LTD in C-fiber evoked field potentials in the spinal cord (Liu, Morton, Azkue, Zimmermann, & Sandkuhler, 1998). This LTD was manifested as a depression of C-fiber response amplitude and as a depotentiation of earlier LTP, suggesting that the LTD could have a hypoalgesic impact. Moreover, after spinalization the same stimulation induces LTP rather than LTD (Liu et al., 1998). This suggests that the threshold for the induction of

LTP is lowered after spinal transection and stimulation below C-fiber intensity may cause hyperalgesia under these conditions. In the present study, I used spinalized animals. As a consequence, the uncontrollable electrical stimulation is more likely to have produced LTP than LTD. The observation that uncontrollable shock induces a tactile allodynia rather than a depression of reflex function is consistent with this idea.

In summary, several lines of research provide converging support for the hypothesis that uncontrollable shock may engage LTP within the spinal cord. It should be noted that the most direct way to test this hypothesis involves electrophysiology. Despite the absence of this crucial data, the pharmacological and behavioral data in the present dissertation point toward an LTP-based mechanism.

Loss of Plasticity and Cell Death

It is not clear how LTP would prevent instrumental learning in our paradigm. One possibility is that the deficit represents an overexcitation within the spinal cord that leads to excitotoxicity and cell death. The present section examines the merits and weaknesses of this hypothesis. In the section that follows I will discuss findings of cell death after injury and their potential involvement in the deficit.

Several laboratories have shown that excitotoxicity can lead to cell death after spinal cord injury (Beattie, Farooqui, & Bresnahan, 2000; Lu, Ashwell,

Waite, 2000; Lowrie & Lawson, 2000). Injury is associated with an increase in group I metabotropic glutamate receptors in the spinal cord (Mills, Fullwood, & Hulsebosch, 2001; Mills & Hulsebosch, 2002). This upregulation may have functional significance because group I mGluRs are implicated in sensory and motor dysfunction as well as cell death after spinal cord injury (Mills, Johnson, & Hulsebosch, 2002). Nociceptive stimulation may also contribute to cell death. Ligation of the sciatic nerve has recently been shown to increase cell death in the dorsal horn (Sugimoto, Bennett, & Kajander, 1990). Because nerve ligation also induces an allodynia (Tabo et al., 1999) these data suggest that the induction of central sensitization may contribute to cell death after injury.

The hypothesis that uncontrollable shock produces the deficit through the induction of cell death is easily tested with histological analyses and molecular assays for cell death markers. Indeed, recent work from our laboratory suggests that uncontrollable shock increases levels of several markers of cell death within the spinal cord (Liu et al., 2003). In addition, we have shown that uncontrollable shock increases tissue loss after a contusion injury, a finding that is consistent with a shock-induced enhancement of cell death (Grau et al., submitted).

Is the deficit produced by uncontrollable shock a direct effect of cell death or are both cell death and the deficit a consequence of a third process? Despite positive findings of cell death, the data appear to favor the latter perspective. The deficit produced by 6 mins of uncontrollable shock appears to passively

wane after 48 h (Crown et al., 2002a). It is not clear how such a rapid restoration of plasticity could occur if the cells involved in learning have died. In addition, the deficit is seen immediately after shock exposure, but the shocked-induced increase in cell death is not significant until 24 h after shock exposure (Liu et al., 2003). It should be noted that the molecular markers examined in the aforementioned study preferentially label cells that are undergoing programmed cell death (apoptosis). It is possible that uncontrollable shock also induces a more rapid form of cell death, necrosis.

Even if uncontrollable shock induces necrosis it is not clear how a necrosis-induced deficit would reverse after 48 h. Is this enough time for compensatory plasticity? Recent work suggests that there is increased sprouting and arborization of A-delta, A-beta, and C-fibers in the superficial spinal laminae after spinal cord transection (Wong, Atkinson, & Weaver, 2000). However, this is seen at 14 days after transection. Data on synaptic changes in the dorsal horn after spinal cord transection indicate that at 3 days after injury there is a loss of synaptic density followed by a compensatory increase in arborization by 2 weeks after injury (Llewellyn-Smith & Weaver, 2001). These data suggest that the restoration of instrumental plasticity that is seen after 48h hours post-shock is not likely to involve compensatory sprouting.

However, the timecourse of the deficit is consistent with the turn-over of glutamate receptor populations. Grossman et al. (2001) have found that

changes in the AMPA receptor subunit levels can be detected by 24 h after injury (Grossman, Rosenberg, & Wrathall, 2001). A similarly rapid increase was seen for expression of the NR1 and NR2A receptor subunits of the NMDA receptor after spinal cord injury (Grossman, Wolfe, Yasuda, & Wrathall, 2000). This is significant given that the NR2A subunit has been found to be involved in the induction of LTP (Hrabetova et al., 2000). It is possible that uncontrollable shock causes an upregulation of NR2A that leads to LTP induction. This excitatory activity could lead to both the deficit and cell death. Supporting this idea prior research has shown that NMDA activation is involved in cell death after spinal cord injury (Faden & Simon, 1988). To elucidate whether the deficit relies on NMDA receptor upregulation requires assaying subunit expression in the spinal cord at multiple timepoints after uncontrollable shock. If the hypothesis is correct, uncontrollable shock should cause an upregulation of NR2A levels that lasts for 48 h after shock.

A key component of this hypothesis is that that LTP somehow limits subsequent plasticity. This perspective seems counterintuitive given the wealth of data suggesting that LTP is involved in learning within the brain (for review, see Goosens & Maren, 2002). However, the learning literature also provides a potential mechanism by which LTP might yield the deficit. Researchers have found that there is a critical window of excitability that promotes learning (McNaughton, Barnes, Rao, Baldwin, & Rasmussen, 1986; Moser, Krobert,

Moser, & Morris, 1998). If LTP is saturated in the dentate gyrus then a deficit in hippocampal-dependent learning occurs (Moser et al., 1998). It is possible that a similar electrophysiological state underlies the deficit. If this were the case, then manipulations that reduce excitability should reduce the deficit. The finding that glutamate antagonists prevent the induction of the deficit (Experiments 4, 6, and 7), provide some evidence that inhibiting excitatory transmission restores plasticity.

Molecular Model of the Deficit

Developing a molecular model of the deficit would help integrate the present findings, and provide a framework for future studies. In the present section I will focus on the mechanisms by which shock might induce a deficit. To the extent to which the model is correct, we may be able to begin conceptualizing therapeutic manipulations that can protect spinal cord plasticity from the deleterious effects of nociceptive stimulation. My objective is to develop an account that allows activation of mGluRs and shock, but not NMDA, to lead to LTP saturation and the deficit. In addition to these features, a viable molecular model must also allow NMDA and mGluR antagonism to prevent induction of the deficit.

If the deficit depends on an LTP-mediated overexcitation, then activation of NMDA receptors should have lowered the threshold for induction of the deficit in the present study. Yet this was not observed (Experiment 5). If we consider

this finding in isolation, it would seem to issue a devastating blow the LTP-based hypothesis. However, the mGluR findings may provide evidence for an LTP-based mechanism in which NMDA is not sufficient. The dissociation between the effects of low doses of NMDA (Experiment 5) and low doses of DHPG (Experiment 8) suggests that the group I mGluRs have a more protracted effect than activation of the ionotropic NMDA receptor. Yet the finding that the NMDA antagonist MK-801 prevents induction of the deficit (Experiment 4) suggests that NMDA still plays a critical role. These data indicate that the NMDA receptor is necessary (Experiment 4), whereas the group I mGluRs are both necessary (Experiment 6 & 7) and sufficient (Experiment 8) to induce the deficit. Perhaps the mGluRs affect instrumental plasticity through an NMDA-mediated mechanism. Both mGluR1 and mGluR5 have been found to influence NMDA function (Allen, Vicini, & Faden, 2001; Benquet et al., 2002; Blaabjerg, Fang, Zimmer, & Baskys, 2003; Bruno et al., 2001; Fisher & Coderre, 1996a; 1996b; Lan et al., 2001). It has been argued that mGluR1 enhances excitation by inhibiting GABA release whereas mGluR5 has a direct impact on NMDA function through receptor coupling (Bruno et al., 2001; Mills, Xu, McAdoo, & Hulsebosch, 2001).

However, other data suggests that both group I mGluRs have direct effects on NMDA function through intracellular signaling (Benquet et al., 2002). Both group I mGluRs contribute to depolarization by activating G-proteins and

phospholipase C (PLC), leading to production of inositol triphophate (IP3) and diacylglycerol (DAG; see Figure 14). This results in an IP3-induced liberation of intracellular Ca^{++} stores and a DAG-induced activation of protein kinase C (see Bruno et al., 2001 for review). Intracellular Ca^{++} activates calcium-calmodulin kinase II (CamKII), a step that is necessary for the induction of LTP (Bliss & Collingridge, 1993). In addition, PKC activation contributes to LTP induction through activation of the non-receptor tyrosine kinase, cell adhesion kinase □/proline-rich tyrosine kinase 2 (CAK□/Pyk2). Activation of CAK□/Pyk2 results in activation of Src kinase, which in turn leads to enhanced NMDA function and LTP induction (Lu et al., 1999; Huang et al., 2001). Activation of mGluR1 can also enhance NMDA function through a different pathway that involves G-protein-independent activation of Src Kinase (Benquet et al., 2002). Together these data provide a mechanism by which an group I mGluR agonist could induce an NMDA dependent deficit.

Additional evidence comes from findings that, through activation of these pathways, the group I mGluRs may have a more protracted impact on NMDA function than a direct NMDA agonist alone. Assuming that the deficit involves a long term NMDA-dependent LTP saturation, this mechanism could account for the present findings. In keeping with this perspective, recent work suggests that a long lasting form of LTP cannot be induced in mGluR5 knock out mice. Furthermore, activation of mGluR1 has recently been found to cause a rapid

increase in NMDA receptor trafficking (Lan et al., 2001). Interestingly, this rapid exocytosis is preferential for the NR1 and NR2A subunits of the NMDA receptor, and is associated with LTP induction (Lan et al., 2001). This suggests that activation of group I mGluRs can not only enhance NMDA function directly, but can also increase the number of surface receptors on the post-synaptic membrane.

Group I mGluRs have also been implicated in AMPA activation (Bruno et al., 2001). This may involve phophorylation of the GluR2 subunit of the AMPA receptor thereby increasing the calcium permeability of the receptor (Bruno et al., 2001). An additional mechanism by which mGluRs are implicated in AMPA function involves subunit trafficking through Ca^{++}-induced activation of CamKII and protein kinase A (PKA; Bruno et al., 2001; Rongo, 2002). Together these findings provide evidence that group I mGluRs may enhance LTP not only by affecting NMDA, but by affecting AMPA function as well.

If the deficit depends on a long term saturation of excitation within the neurons responsible for performing the instrumental learning, then it seems plausible that transient activation of ionotropic glutamate receptors might not be sufficient. However, given the long-term impact of group I mGluRs on NMDA and AMPA receptor function, it is reasonable to think that activation of group I mGluRs could produce the deficit.

Figure 14 presents a putative molecular model of the deficit. The model assumes that there is a basal level of glutamate release in the spinal cord after transection. This basal level is insufficient to induce the deficit. However, with shock exposure the level of glutamate is increased leading to significant activation of AMPA, NMDA, and group I mGluRs. The activation of mGluRs induces a cascade of intracellular events that ultimately leads to upregulation of AMPA and NMDA receptors at the post synaptic membrane. After this process has been induced, the post-synaptic cell is hyperexcitable and basal levels of glutamate can maintain LTP saturation. This leads to a loss of plasticity and expression of the instrumental deficit.

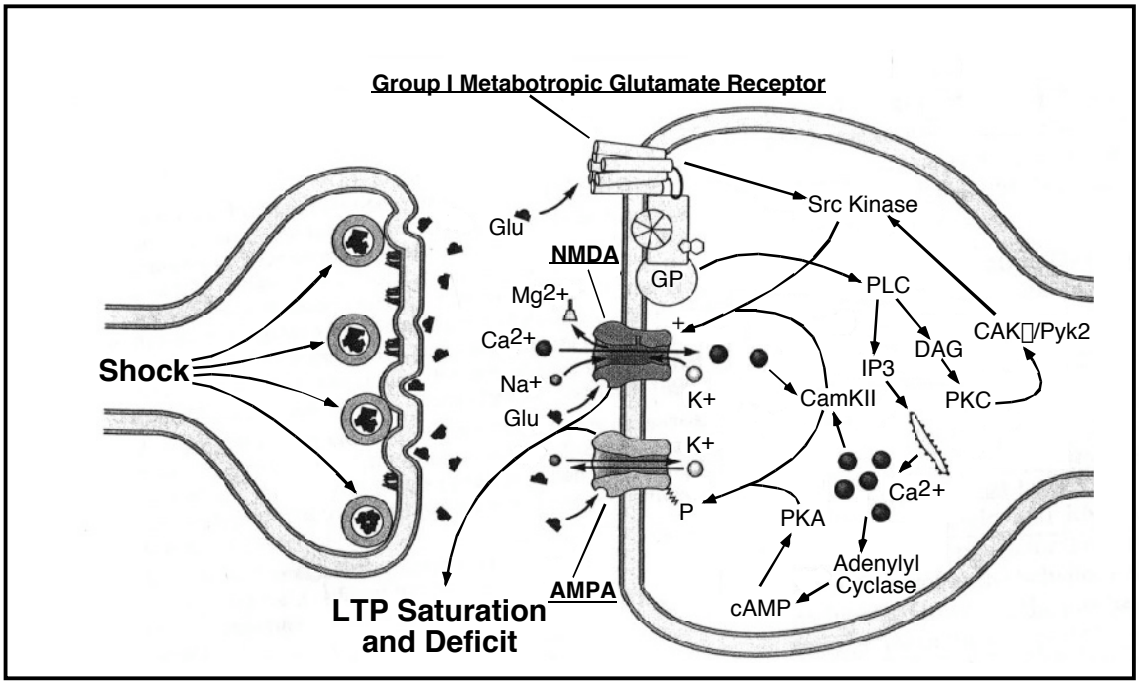


Figure 14. Proposed molecular model of the deficit. Shock causes excessive glutamate (Glu) release. This activates the group I metabotropic, and the NMDA and AMPA ionotropic receptors. Activation of the mGluR induces activation of Src kinase through G-protein dependent and G-protein independent pathways. Src kinase causes a long lasting enhancement of NMDA function. Further enhancement is a consequence of NMDA and AMPA receptor upregulation caused by calcium calmodulin kinase II (CamKII) and protein kinase A (PKA). Both PKA and CamKII are activated by a rise in intracellular Ca^{++}, a consequence of activation of the G-protein-linked pathway of the mGluR. Abbreviations: PLC, Phospholipase C; DAG, diacylglycerol; PKC, protein kinase C; CAK□/Pyk2, cell adhesion kinase □/proline rich tyrosine kinase 2; IP3, inositol triphosphate; cAMP, cyclic adenosine monophophate; GP, G-protein.

This model suggests several interesting predictions. First, it provides a mechanism by which activation of group I mGluRs would be sufficient whereas NMDA and AMPA would not. Assuming that activation of NMDA or AMPA only leads to transient increases in excitability, the deficit induced by these receptors would not be expressed 24 h after drug delivery. The present data are consistent with this mechanism, because NMDA did not appear to produce a deficit at non-excitotoxic doses (Experiment 5). Whether the same is true for AMPA has not been tested. On the other hand, activation of group I mGluRs with DHPG was found to be sufficient to induce the deficit, presumably through induction of intracellular cascades. A second prediction is that interfering with intracellular signaling should prevent induction of the deficit. A recent study performed in our laboratory suggests that intrathecal administration of a PKC inhibitor undermines induction of the deficit, a finding that supports a role for PKC in the deficit (Bolding, Hook, Ferguson, & Grau, 2003). A third prediction is that the deficit depends on activation of Src kinase through both G-protein-dependent and G-protein independent mechanisms. This has yet to be tested. Finally, the model suggests that both PKA and CamKII may play a role in the deficit. Supporting this, preliminary findings suggest that PKA is involved in the deficit (R. Joynes, personal communication). It should be noted that this is only one small branch of the extensive cascade of intracellular events presented in the present molecular model. As a consequence it is not clear to what extent this work will continue to

yield positive findings. It seems that manipulation of Src kinase is likely to produce the most significant, replicable effects given what is currently known about intracellular mechanisms. Indeed, it is possible that Src kinase functions as the locus of memory for the deficit, a molecular engram of sorts. Further studies should be performed to assess this possibility.

One potential weakness of the model is that it is not immediately clear how NMDA antagonism would prevent the deficit. This problem can be addressed by positing that Ca^{++} influx through the NMDA (and to a lesser extent, AMPA) receptors after activation of mGluRs contributes to the intracellular cascades that lead to long term changes in receptor density. This is plausible since both CamKII and PKA are sensitive to increases in intracellular Ca^{++}. Such a mechanism is consistent with findings that the impact of group I mGluRs on formalin-induced central sensitization can be blocked by MK-801 (Fisher & Coderre, 1996b). It is possible that AMPA would, in a like manner, be necessary but not sufficient to induce the deficit. The present study does not address the role of AMPA. Further work is required to test its involvement in the deficit.

Another weakness of the model is that, in its current form, it only represents a monosynaptic change. This suggests that the learning and the deficit occur within a single reflex pathway. Yet, all of the changes reported in the present dissertation occur at remote synapses. For example, both the shock-induced and the formalin-induced deficits transfer to the contralateral leg

(Experiments 2 & 3; Joynes et al., 2003). Moreover, tailshock was used to induce the deficit in performance of the instrumental leg flexion response in Experiments 4-8. These data indicate that the changes involved in the deficit are likely polysynaptic. That said, the problem of oversimplification is not unique to the model proposed in Figure 14. Similar monosynaptic models have been proposed to account for neuropathic pain, cell death and even learning in the brain (e.g. Bruno et al., 2001; Cho & Bashir, 2002; Fundytus, 2001). All of these phenomena involve large scale changes that are likely polysynaptic in nature. In the case of both the deficit and these other phenomena it is possible to conceptualize changes in remote synapses as a consequence of diffuse activation of presynaptic fibers. The postsynaptic mechanism would remain the same, and the model would remain viable. To date, we have little conclusive data about whether the deficit reflects a presynaptic or post-synaptic change. The present data appear more consistent with the latter. However, the possibility that presynaptic changes are also engaged cannot be discounted.

Neurons vs. Glia

Glial activation is one potential mechanism by which remote synapses could be affected by uncontrollable shock, yielding a global deficit. In recent years it has become clear that glia contribute to neuronal signaling, and may actually play a role in information processing within the nervous system (Banati, 2002; Fields & Stevens-Graham, 2002; Perea & Araque, 2002; Robertson,

2002). Two types of glia, astrocytes and microglia, have been implicated in neuropathic pain states (Banati, 2002; DeLeo & Yezierski, 2001; Watkins & Maier, 2000). Microglia act as the resident macrophages within the brain serving to perform 'general house keeping' functions within the central nervous system under normal circumstances (DeLeo & Yezierski, 2001). However, after injury microglia become activated, and may contribute to hyperalgesia and secondary injury mechanisms (Banati, 2002; DeLeo & Yezierski, 2001; Watkins & Maier, 2000). This is illustrated by findings that elevations in OX-42, a marker of activated microglia, correlates with allodynia on both the ipsilateral and contralateral side of a ligated nerve (Hunt, Winkelstein, Rutkowski, Weinstein, & DeLeo, 2001). In addition, thermal hyperalgesia produced by nerve ligation, can be limited by the anti-inflammatory cytokine interleukin-10 (IL-10; Wagner, Janjigian, & Myers, 1998). This reduction in hyperalgesia was associated with lower levels of endoneural macrophages (Wagner et al., 1998). Microglia and some of the cytokines they produce (e.g. TNF□) are also implicated in cell death after spinal cord injury (Beattie, Farooqui, & Bresnahan, 2000). Finally, microglia have been found to release glutamate, and may thereby affect excitability within the central nervous system. Given this literature, it is possible that uncontrollable shock causes microglial activation. This activation could contribute to overexcitation within the spinal cord and play a role in the induction of the deficit. Our laboratory intends to dedicate resources to this issue in the future.

Astrocytes are also attractive candidates for a role in the deficit. These cells are intimately involved in synaptic transmission, ensheathing synapses and even regulating neurotransmitter levels in the synaptic cleft (for review see Fields & Stevens-Graham, 2002). Astrocytes also have glutamate receptors and are responsible for the majority of glutamate uptake that occurs in the central nervous system (Anderson & Swanson, 2000). Binding of glutamate to AMPA receptors and mGluRs on astrocytes can cause influx of Ca^{++} and liberation of internal Ca^{++} stores (Vesce, Bezzi, & Volterra, 1999). Although astrocytes do not show propagation of Na^{+} mediated action potentials, they do appear to show a form of excitation through Ca^{++} oscillations (Aguado, Espinosa-Parrilla, Carmona, & Soriano, 2002; Vesce et al., 1999; Perea & Araque, 2002; Fields et al., 2002). Astrocytic excitation occurs through propagation of Ca^{++} waves and can result in astrocytic release of glutamate at nearby synapses (Aguado et al., 2002). Moreover, astrocytes communicate to one another through gap junctions, and Ca^{++} oscillations in one astrocyte can cause spreading activation in the form of Ca^{++} waves in nearby astrocytes (Aguado et al., 2002). Through this mechanism, neurotransmission at one synapse can cause astrocytic glutamate release at distal synapses (Aguado et al., 2002; Perea & Araque, 2002). Indeed recent evidence suggests Ca^{++} signaling in astrocytes can affect neuronal excitation through an NMDA mediated mechanism (Parri, Gould, & Crunelli, 2001).

An intriguing element of astrocytic excitability is that it appears to be plastic (Perea & Araque, 2002). The pattern of neuronal activity determines the astrocytic response, with high levels of stimulation producing astrocytic excitation and lower levels having little effect (Perea & Araque, 2002). In addition, the astrocytic Ca^{++} response to synaptic glutamate becomes sensitized as a consequence of prior activity (Pasti, Volterra, Pozzan, & Carmignoto, 1997). It is not difficult to imagine how such a mechanism might be involved in the propagation of overexcitation in the central nervous system.

Since the deficit appears to rely on glutamatergic transmission, it seems plausible that astrocytes are involved in its induction. Indeed an astrocytic mechanism would account nicely for the diffuse nature of the deficit. If we consider the role of astrocyte in glutamate signaling, the results of the present dissertation are not inconsistent with an astrocyte based mechanism. Figure 15 shows

a modification of the molecular model to allow for astrocytic modulation of the deficit. This ‘tripartite synapse’ (Robertson, 2002), consisting of pre and post synaptic neurons and the regulating astrocyte, retains all of the features of the model in Figure 14. However Figure 15 has the additional benefit of allowing the system to affect, and respond to, remote synapses through astrocytic activation. The basic mechanistic features still rely heavily on changes in the postsynaptic membrane, however the model assumes that the initiating release of glutamate comes from two sources—the presynaptic neuron and the astrocyte. Shock could affect both sources of glutamate release by directly impacting the presynaptic neuron, and by activating other neurons that lead to Ca^{++} signaling in adjacent astrocytes. It is assumed that Ca^{++} oscillations in nearby astrocytes could, through gap junctions, lead to activation of the local astrocyte.

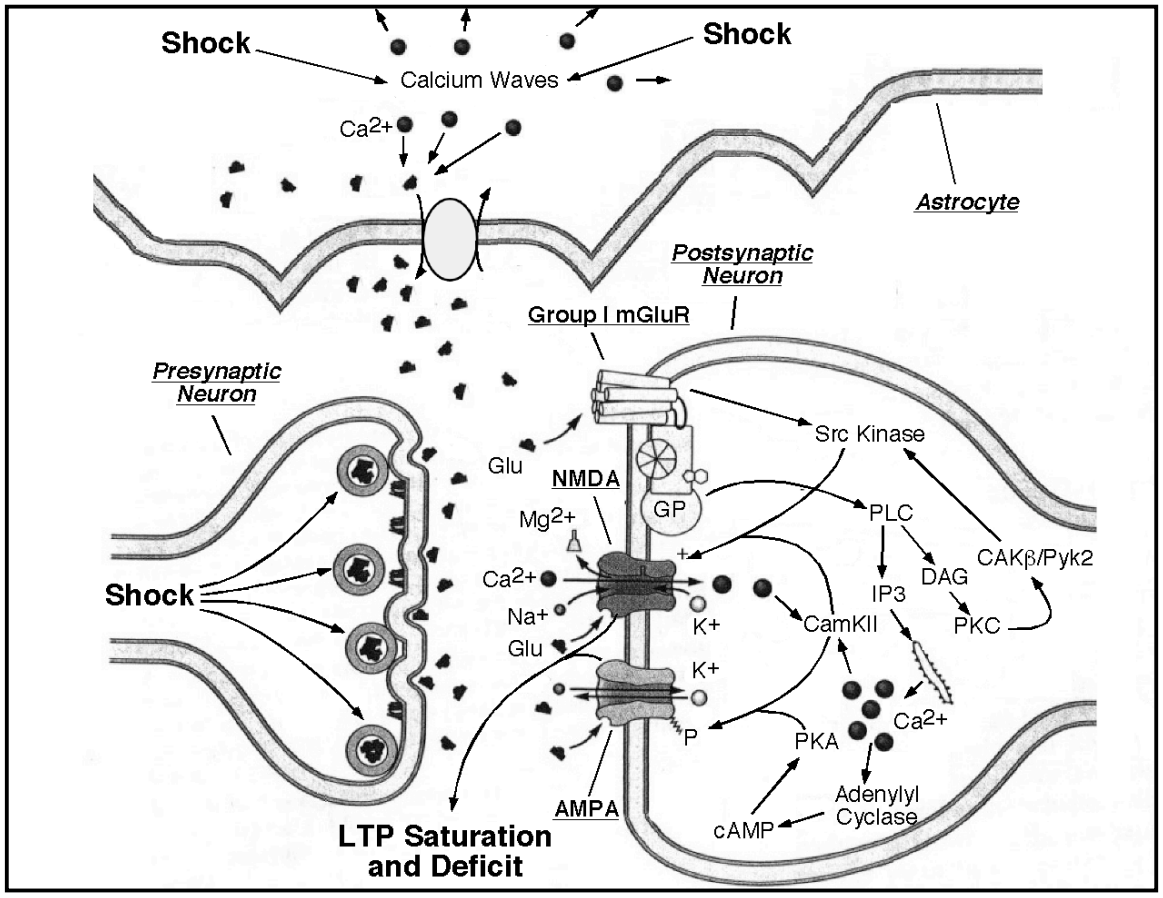


Figure 15. A molecular model illustrating the potential role of astrocytes in the induction of the deficit. Shock is proposed to influence glutamate release through two mechanisms. It has a direct and diffuse impact on glutamate release from presynaptic neurons. Consequent glutamate release occurring at other synapses causes an induction of Ca^{++} waves in the adjacent astrocytes. Through gap junctions, excitation in these distal astrocytes influences Ca^{++} levels in the proximal astrocyte. This leads to a astrocytic release of glutamate into the synapse and further activation of the post-synaptic neuron. See text for more details.

It should be noted that this model still allows for the finding that direct activation of group I mGluRs is sufficient for induction of the deficit. Recent evidence suggests that there is a basal level of activity within astrocytic networks (Aguado et al., 2002). This astrocytic activity is mirrored by NMDA-dependent activity within neuronal networks (Atuado et al., 2002). This indicates that there is a basal level of glutamate release that results from spontaneous activity in astrocytic networks. The model in Figure 15 assumes that this basal level of glutamate is insufficient to induce the deficit by itself. However, with the addition of the mGluR agonist DHPG, the response of the post synaptic neuron to glutamate would be enhanced. This could lower the threshold for the induction of the deficit, and now basal levels would be sufficient.

Although this conceptualization of the deficit emphasizes the impact of mGluR activation on the postsynaptic membrane, there is also a possibility that DHPG enhances astrocytic activation directly. Recent data suggest that DHPG enhances calcium signaling in astrocytes, suggesting that group I mGluRs are present on astrocytes (see Wisnieski & Car, 2002 for review). Moreover, group I mGluRs on astrocytes are thought to contribute to white matter injury after SCI (Agrawal, Theriault, & Fehlings, 1998). The direct effect of DHPG on astrocytes could have contributed to the drug-induced deficit seen in Experiment 8. In addition, shock-induced activation of group I mGluRs on both the post synaptic

neuron and adjacent astrocytes could have contribute to the elevation in cell death that we have recently found results from shock exposure (Liu et al., 2003).

A direct test of the role of astrocytic excitation in the deficit would be difficult at the present time. Much of the work examining Ca^{++} signaling in astrocyte has been performed *in vitro* using Ca^{++} sensitive fluorescent dyes (e.g. fura-2; Aguado et al., 2002). However, there are methods by which we could quantify the number of activated astrocytes. Glial fibrillary acidic protein (GFAP) immunoreactivity has been shown to increase when astrocytes are undergoing morphological changes after spinal cord injury (Hadley & Goshgarian, 1997). However, the present molecular model (Figure 15) does not require astrocytes to undergo morphological change to play a role in the deficit. As a consequence, a null finding with respect to GFAP would not necessary negate astrocytic involvement in the deficit. The true test of the hypothesis requires imaging calcium oscillations in astrocytes *in vivo*. Several Medline searches failed to suggest a means by which this could be accomplished. It appears that this may be beyond the limits of current technology. Perhaps in the future, there will be a way to perform this study.

Implications for Pain Management in Spinal Cord Injury

The molecular models presented in the preceding section suggest several points of intervention that could undermine or prevent the induction of the deficit. Such interventions could become important if the deficit is shown to be related to

neuropathic pain after spinal cord injury. The findings from this dissertation provide convergent evidence that nociceptive systems are involved in the loss of plasticity in a rat model of spinal transection injury. Do these findings extend to other forms of injury? Do they extend beyond rats and have implications for humans with spinal cord injuries? The present section deals with these issues. In the paragraphs that follow I will review what is known about pain after spinal cord injury. Through the course of this review I will discuss how the current findings may inform recovery in human patients.

There is variability in the rate and level to which patients with spinal cord injury recover. The variables that dictate these differences remain largely unknown. The present data suggest that one critical variable of interest might be nociceptive input into the spinal cord. The findings suggest that peripheral injury, like the effect of uncontrollable shock, has a deleterious impact on intrinsic behavioral plasticity within the spinal cord. In the context of prior findings that uncontrollable shock retards recovery of function after a spinal contusion injury (Grau et al., submitted), the present findings suggest that peripheral nociception may have an impact on recovery after spinal cord injury. Moreover, this potential impairment in recovery may involve changes in the spinal cord, and could occur even in the absence of conscious perception of pain. The present medical practice is to treat pain only if the patient provides evidence of pain (J. Baggett, personal communication). In the absence of the affective experience of pain, it is

unlikely that physicians would choose to reduce the nociceptive input into the spinal cord.

How commonly are spinal cord injuries associated with peripheral injuries? Unfortunately several Medline searches have failed to produce any reports that explicitly state the prevalence of comorbid peripheral injuries after traumatic spinal cord injury. Yet most spinal cord injuries result from traumatic events such as automobile accidents, indicating that peripheral damage is also likely (Marino, Ditunno, Donovan, & Maynard, 1999; Sekhon & Fehlings, 2001). One of the early diagnostic features of spinal cord injury is a lack of sensation from the periphery, so it is likely that spinal cord injury patients with peripheral damage would not report pain from the periphery (Benzel & Larson, 1986; Chehrazi, Wagner, Collins, & Freeman, 1981; Frankel, 1969). This suggests that it is plausible, if not common, that spinal cord injury patients come into the emergency room with peripheral injuries and receive no treatment for the nociceptive afferent barrage reaching the spinal cord. The present findings suggest that these patients may have greater impairment of function and less recovery over time. To my knowledge there are no papers to date that report on peripheral damage as a prognostic indicator of recovery in spinal cord injured patients. This is an important issue that needs to be addressed through a study of epidemiology.

It is known that spinal cord injury is commonly associated with neuropathic pain (Yezierski, 1996). Indeed, prior studies have reported that anywhere from 47-98% (mean = 66%) of patients with spinal cord injury develop neuropathic pain (Yezierski, 1996). At the present time, it is not clear to what extent peripheral injury is involved in induction of these pain syndromes. However, it is known that glutamatergic transmission is involved in the induction of pain after spinal cord injury (Agrawal et al., 1998; Allen et al., 2001; Bennett, Everhart, & Hulsebosch, 2000; Gomez-Pinilla, Tram, Cotman, & Nieto-Sampedro, 1989; Mills, Johnson, & Hulsebosch, 2002). These same changes have also been implicated in loss of function after injury, suggesting a commonality between loss of function and the onset of neuropathic pain (Mills et al., 2002). Given the impact of nociceptive stimulation on glutamate systems, it seems possible that peripheral stimulation could contribute to both the induction of neuropathic pain and the loss of plasticity after injury. Additional support for this hypothesis is provided by a recent report that electroacupucture delivered in acutely injured patients shortly after arrival to the emergency room improves sensory and motor function at 1 year post injury (Wong et al., 2003). To my knowledge, there are no studies that have explicitly examined whether neuropathic pain is associated with lower motor performance after spinal cord injury.

The present findings suggest clear links between peripheral nociception and the loss of plasticity. Do these findings extend to the induction of central pain after injury as well? If this were the case, then individuals with spinal cord injury who are also subjected to nociceptive stimulation should have a greater probability of developing central pain. Supporting this, we have recently found that rats with spinal contusion injuries have higher incidence of autophagia after injury (Grau et al., submitted). In the past, this excessive grooming behavior has been interpreted as evidence of neuropathic pain (Yezierski, Liu, Ruenes, Kajander, & Brewer, 1998).

The present findings indicate that interfering with pain transmission may lead to greater recovery of function after injury. It may also have the added benefit of reducing the prevalence of neuropathic pain in spinal cord injury. The implication is that nociception should be treated even if the patient does not consciously experience pain, because much of the damage to plasticity and sensory function may occur at the level of the spinal cord. This runs precisely counter to the present clinical practice. Nociception after spinal cord injury may be an instance in which the professional doctrine to 'treat the patient, not the disease' may need to be abandoned and replaced with the perspective that one must 'preemptively treat the pain to treat the patient'.

As part of a therapeutic regimen to preemptively treat pain in spinal cord injury, administration of an NMDA or group I mGluR antagonist would likely yield

benefits. The latter may be more desirable because it is both more likely to be causally related to losses in plasticity (Experiment 8), and less likely to yield unwanted side effects (Bruno et al., 2001). Interfering with the intracellular cascades invoked by group I mGluRs is also a promising avenue for therapeutic intervention.

CHAPTER VII

SUMMARY AND FUTURE DIRECTIONS

My original objective with this dissertation was to explore the mechanisms by which peripheral stimulation can affect plasticity within the spinal cord. The experiments revealed that nociceptive transmission can undermine learning within the spinal cord. This impact of nociception on plasticity was recapitulated in both behavioral and pharmacological findings. The converging pattern of results allowed me to propose a testable molecular model that could provide additional insight into the nature of plasticity in the spinal cord.

However, significant work remains before we can be confident about the specific links between nociception and spinal plasticity. In future studies it would be interesting to test the veracity of the proposed molecular model by selectively manipulating specific components. Perhaps through experimentation the model can be honed to better reflect the true nature of spinal plasticity. In addition, significant work is required to better characterize the nature of the loss of plasticity caused by peripheral inflammation. Although I have argued that peripheral inflammation produces losses in spinal plasticity through the same mechanism as electrical stimulation (Experiment 3), there is only limited empirical evidence to support this claim. A better characterization of the temporal features, the pharmacological mechanisms, and the clinical validity of

this effect are essential. Through further work we may be able to clarify our assertions about the true impact of nociception on spinal plasticity.

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VITA

Adam Richard Ferguson

Address: Department of Psychology
Texas A&M University
College Station, TX 77843
(979) 862-4852

Education: Ph.D. Texas A&M University, 2004
M.S. Texas A&M University, 2000
Major: Psychology
B.A. Southwestern University, 1998
Major: Psychology; Minor: Biology

Selected Publications:

Meagher, M. W., Ferguson, A. R., Crown, E. D., McLemore, S., King, T. E., Sieve, A. N., & Grau, J. W. (2001). Shock induced hyperalgesia: IV Generality. *Journal of Experimental* Psychology: Animal Behavior Processes, *27*, 219-238.

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