THE EFFECTS OF BEHAVIORAL TRAINING ON SPINAL PLASTICITY AND ERK1/2 PHOSPHORYLATION, WITHIN THE INJURED SPINAL CORD

A Senior Scholars Thesis

by

ABBEY JEAN HUGHES

Submitted to the Office of Undergraduate Research
Texas A&M University
in partial fulfillment of the requirements for the designation as

UNDERGRADUATE RESEARCH SCHOLAR

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Major: Psychology

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Associate Dean for Undergraduate Research: Robert C. Webb

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ABSTRACT

The Effects of Behavioral Training on Spinal Plasticity and ERK1/2 Phosphorylation, Within the Injured Spinal Cord. (April 2009)

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Previous research has shown that even when communication between the brain and spinal cord has been eliminated, rats can learn a simple instrumental response. If rats receive shock to one hindlimb when it is extended (controllable shock), subjects will learn to maintain that leg in a flexed position to minimize shock exposure. Conversely, if shock is delivered irrespective of leg position (uncontrollable shock), subjects are unable to maintain the same response and exhibit a learning deficit when later tested with controllable shock. Recent studies have demonstrated that the adaptive modifications accompanying learning and the maladaptive consequences of uncontrollable nociceptive stimulation interact to influence behavioral outcomes. We have shown that the induction of inflammatory processes increases responding to noxious stimuli and undermines the capacity to learn. Behavioral training with controllable shock, on the other hand, protects against these effects, allowing subjects to learn. At the cellular level, the presentation of noxious stimuli increases nuclear

localization of phosphorylated (activated) extracellular signal-regulated kinases 1 and 2 (ERK1/2). Here, we explore the impact of instrumental training and inflammation on cytosolic ERK1/2 activation. Subjects received 30 min of instrumental training or subcutaneous hindpaw injections of 1% capsaicin (50 μL vol). The lumbar region of the spinal cord (L4-S2) was then collected 0, 3, and 24 hr later, and prepared for western immunoblot analysis. A group of untreated rats were included as controls. We found a time-dependent effect on cytosolic ERK1/2 phosphorylation, with the greatest levels observed 24 hr following treatment. We also found that instrumental training or capsaicin treatment downregulated cytosolic expression of phospho-ERK1 and phospho-ERK2. Taken together, these results suggest that instrumental training and inflammation engage similar signaling pathways. Further work will be integral for determining how the cellular consequences of instrumental training and inflammation interact to produce divergent behavioral outcomes.

ACKNOWLEDGMENTS

I would first and foremost like to thank my thesis advisors, Dr. Kyle M. Baumbauer and Dr. James W. Grau, for their continuous inspiration and patience throughout my undergraduate research career at Texas A&M University. I am truly grateful to Dr. Baumbauer for his countless hours of dedicated mentorship and invaluable support throughout every stage of this work. Through his own sincere commitment to the advancement of science in the fields of neuroscience and psychology, he has inspired me to pursue post-baccalaureate work and research in the psychological sciences. I wish to thank Dr. Grau for overseeing this thesis and providing continuous academic and financial support through grants from the National Institute of Mental Health (NIMH) and the National Institute of Neurological Disorders and Stroke (NINDS). These resources were essential to the completion of this thesis

I would also like to pay tribute to other members of the Grau Laboratory. I have greatly benefitted from the outstanding tuition and camaraderie from Dr. Michelle Hook, Dr. Sandra Garraway, Denise Puga, Russell Huie, Kevin Hoy, Sarah Woller, Milly Lee, Georgina Moreno, and Thomas Prentice.

Finally, words fail to express my appreciation and gratitude for my family. I thank my father, Eugene Hughes, and mother, Ina Hughes, for their unfailing dedication to my emotional and intellectual development, and their continued support of my education.

NOMENCLATURE

AMPA	α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate
BDNF	Brain Derived Neurotrophic Factor
Ca^{2+}	Calcium
CaMKII	Ca^{2+}/calmodulin-dependent Protein Kinase II
cAMP	Cyclic Adenosine Monophosphate
CNS	Central Nervous System
CREB	cAMP Response Element Binding Protein
ELISA	Enzyme-linked Immune Sorbent Assay
ERK	Extracellular Signal-regulated Kinase
FES	Functional Electrical Stimulation
GABA	Gamma-aminobutyric Acid
IDV	Integrated Density Value
IHC	Immunohistochemistry
L4-S2	Lumbar Region of the Spinal Cord
LPS	Lipopolysaccharide
LTP	Long-term Potentiation
NIH	National Institute of Health
NK1	Neurokinin 1
NSCISC	National Spinal Cord Injury Statistical Center
NMDA	N-methyl-D-aspartate

PCR	Polymerase Chain Reaction
PKA	Protein Kinase A
SCI	Spinal Cord Injury
T2	Second Thoracic Vertebrae
Trk B	Tyrosine Kinase B

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CHAPTER I

INTRODUCTION

An overview of spinal cord injury

According to the National Spinal Cord Injury Statistical Center (NSCISC; 2008), there are over 450,000 people in the United States living with a clinically disabling spinal cord injury (SCI). While the effects of SCI vary depending on the nature and location of the injury, people living with SCI often experience devastating physical, emotional, and social consequences. SCI damages the vital connections between the brain and periphery, resulting in impaired sensation and movement. In addition to paralysis, damage to these central relays can produce further adverse conditions, including bladder and bowel incontinence, sexual impotence, muscle spasticity, and severe neuropathic pain.

For most cases, SCI produces an extensive financial burden, including, but not limited to, the costs of hospitalization, rehabilitation, medical equipment, and income loss due to unemployment (NSCISC, 2008). It has been estimated that over the course of a lifetime, the cost of treatment and support for an injured individual can exceed $2.4 million (Berkowitz, O'Leary, Kruse, & Harvey, 1998). While the social costs of SCI remain

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This thesis follows the style of *Behavioral Neuroscience*.

difficult to quantify statistically, injured individuals suffer a markedly reduced quality of life, characterized by loss of social relationships and compromised family structure. Without even considering the personal costs and loss of employment productivity, the direct costs of SCI and its common secondary conditions amount to a national bill of $14.5 billion every year (Berkowitz et al., 1998).

Given these staggering figures, research on SCI has expanded considerably in recent years. Although the collaborative efforts of scientists and clinicians have allowed for improved therapeutic and rehabilitation strategies, these methods have achieved only modest success in improving the overall recovery and quality of life for people living with SCI. To go beyond current methods, we must now utilize a basic research approach to achieve a comprehensive understanding of the spinal cord and its inherent ability to control adaptive behavior.

Behavioral evidence for spinal plasticity

The study of SCI has a considerably long history, and, until recently, investigators have typically viewed the spinal cord as a static agent of the central nervous system (CNS), fixed after development. Under this presumption, subcortical systems can only demonstrate plasticity in the presence of supraspinal input, and therefore, as in the case of SCI, recovery cannot occur without restoring this vital connection. This ideology presents a particularly difficult obstacle in treating SCI, because recovery would then depend on a complete recreation of neural development and construction, currently an

impracticable feat. Fortunately, over the past century, research has shown that spinal neurons are indeed quite plastic, exhibiting changes in neural processes in response to various stimuli (Edgerton et al., 1992; Edgerton, Roy, de Leon, Tillakaratne, & Hodgson, 1997; Hodgson, Roy, de Leon, Dobkin, & Edgerton, 1994; Coderre, Katz, Vaccarino, & Melzack, 1993; Harkema et al., 1997). Remarkably, these neural modifications can occur in the complete absence of supraspinal input, indicating that spinal neurons alone can modify behavioral responses to environmental stimuli (Buerger & Chopin, 1976; Chopin & Buerger, 1976; Buerger & Fennessy, 1970; Grau, Salinas, Illich, & Meagher, 1990; Grau, Barstow, & Joynes, 1998; Grau et al., 2006). These effects demonstrate that there are inherent mechanisms within the spinal cord that support experience-dependent plasticity, indicative of learning and memory.

Previous work by a number of laboratories has shown that spinal neurons are capable of supporting a variety of forms of learning, such as habituation and sensitization (Groves & Thompson, 1970), and Pavlovian conditioning (Durkovic, 1975; Fitzgerald & Thompson, 1967; Grau et al., 1990; Joynes & Grau, 1996; Grau, 1987). Over the past decade, our laboratory has shown that spinal neurons can also recognize response-outcome (R-O or instrumental) relations (Grau et al., 1998). In a typical experiment, a master-yoke paradigm is used in which rats in one condition (master rats) are administered mild legshock whenever one hindlimb is extended (controllable shock). Rats treated in this manner will learn to maintain a flexed leg position that minimizes shock exposure. Rats in a second condition (yoked rats) are experimentally coupled to

master rats and receive shock whenever master rats receive shock (uncontrollable shock). Because yoked rats cannot control the timing of shock delivery, they cannot learn to maintain the same flexion response. When subjects are retested under common conditions using controllable shock, rats that previously received controllable shock training rapidly reacquire the prolonged flexion response, indicative of "memory", for the earlier training session (Grau et al., 1998). Conversely, rats previously treated with uncontrollable shock cannot acquire the target flexion response, and exhibit a learning deficit. Interestingly, this learning deficit is reminiscent of the learned helplessness effect seen in intact animals (Maier & Seligman, 1976). Taken together, these results demonstrate that the spinal cord can control behavior in the absence of supraspinal input.

Identifying neural mechanisms in spinal plasticity

At the functional level, controllable peripheral stimulation facilitates spinal learning following SCI, indicating an underlying adaptive plasticity at the neurobiological level (Ferguson, Crown, & Grau, 2006). Previous studies have shown that a variety of intracellular mechanisms contribute to N-methyl-D-aspartate (NMDA) receptor-mediated spinal plasticity. By examining key components in known signaling pathways, we can begin to identify the specific effects of behavioral training.

Following in the tradition of Hebbian principles (Hebb, 1949), we generally assume that instrumental learning results in the strengthening of synaptic connections by various chemical and structural processes within the neurons. Previous work has suggested that

learning engages a synaptic process, known as long-term potentiation (LTP), which is critically dependent on the action of the neurotransmitter glutamate on the NMDA receptor (Morris, 1989; Morris, Anderson, Lynch, & Baudry, 1986; Woolf & Thompson, 1991; Malenka, 2003; e.g., Grau et al., 2006). NMDA receptor activation by glutamate allows calcium (Ca^{2+}) ions to enter the cell, which then activate several downstream processes potentially involved in spinal plasticity. As illustrated in Figure 1, a variety of processes are involved in NMDA-mediated plasticity.

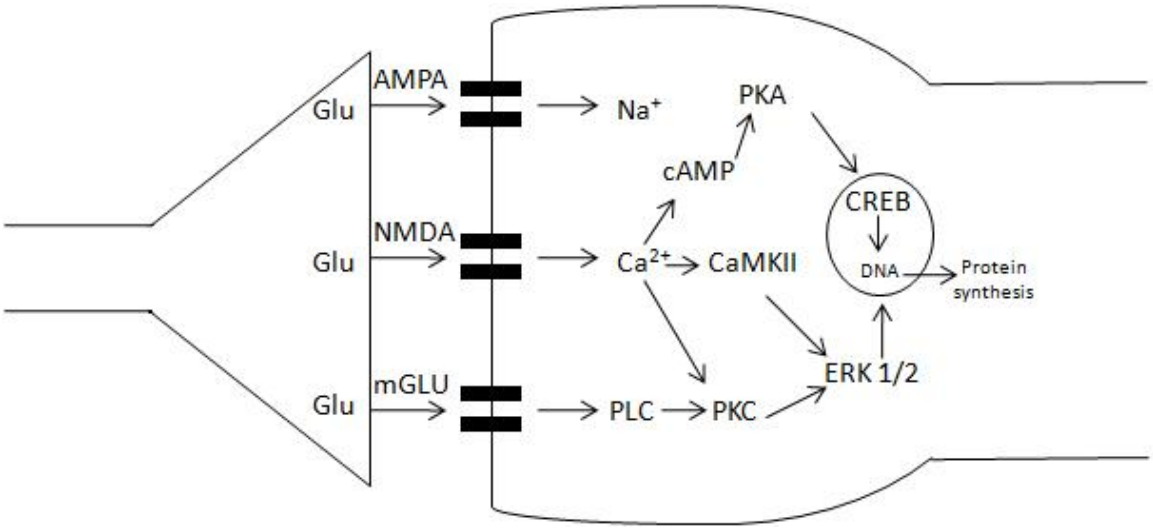

Figure 1. Cellular events that accompany learning and memory. Glutamate binds to the NMDA receptor, allowing an influx of calcium and activating several downstream components, including ERK 1/2 and CREB.

During NMDA receptor activation, Ca^{2+} serves as an important second messenger, initiating several constituent cellular processes (Figure 1). Ca^{2+} influx activates cyclic

adenosine monophosphate (cAMP), which subsequently phosphorylates (activates) protein kinase A (PKA), an early component of neural modulation. Calcium also activates protein kinase C (PKC) and Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII), which regulate the additional influx of extracellular Ca^{2+}, and extracellular signal-regulated kinases 1 and 2 (ERK1/2; Seger et al., 1991; Seger, & Krebs, 1995; Impey, Obrietan, & Storm, 1999). With repeated activity, phosphorylated ERK1/2 activates cAMP response element binding protein (CREB), which induces gene transcription, enables synthesis of new proteins, and promotes the release of glutamate (Impey et al., 1999; Byrne, Zwartjes, Jomayouni, Critz, & Eskin, 1993; Hawkins, Kandel, & Siegelbaum, 1993). Collectively, research has shown that activation of these signaling cascades and genetic transcription factors promotes synaptic restructuring, enhancing subsequent transmitter release and promoting neural function (Lever, Pezet, McMahon, & Malcangio, 2003; Tyler, Alonso, Bramham, & Pozzo-Miller, 2002).

Given the importance of NMDA receptor activation in the cellular events that accompany learning, our laboratory has examined its role in spinal learning and found that blocking NMDA receptor activation prevents the effects of controllable and uncontrollable shock on subsequent behavior (Joynes, Janjua, & Grau, 2004; Ferguson et al., 2006). Research has also shown that uncontrollable shock engages transcriptional hardware, as the deficit requires the synthesis of new proteins (Baumbauer, Young, Hoy, Abood, & Joynes, 2007a; Patton, Hook, Crown, Ferguson, & Grau, 2004). While we have been establishing a comprehensive picture of the cellular changes that accompany

learning and the deficit, much work is still needed. Therefore, the present study examines whether ERK phosphorylation plays a significant role in the effects of controllable and uncontrollable shock on spinally-mediated behavior.

Clinical implications for studies of spinal plasticity

By using learning as a dependent measure of spinal function, we have been able to increase our understanding of the physiological organization of the spinal cord. Moreover, the strides we have made in this realm may also have clinical relevance for understanding how therapeutic interventions, such as functional electrical stimulation (FES), promote recovery at a basic level (Hook & Grau, 2007). These innovations have the potential to promote recovery of function and ease degenerative conditions following SCI. With nearly one half million people in the United States suffering from SCI, and about 11,000 new injuries every year, our research aims to ameliorate the devastating effects of these injuries by learning the inherent capabilities of neural modifications and harnessing these mechanisms to foster recovery.

CHAPTER II

METHODS

Subjects

Male Sprague-Dawley rats were obtained from Harlan (Houston, TX). Rats were 90-110 days old and weighed approximately 350-400g at the time of spinal cord transection. Subjects were double-housed and maintained on a 12-hour light/dark cycle, with food and water readily available. All instrumental testing procedures were performed during the light cycle. All experiments were conducted in accordance with the National Institute of Health (NIH) requirements for the care and use of laboratory animals (NIH publications No. 80-23) and were approved by the University Laboratory Animal Care Committee at Texas A&M University.

Surgery

Subjects were anesthetized with 5% isoflurane gas, and maintained at a surgical plane of anesthesia with 2% isoflurane. Prior to surgery, fur over the thoracic region was shaved and the rat's head was immobilized in a sterotaxic apparatus. The second thoracic vertebrae (T2) was located by touch and a small anterior-posterior incision (2.5 cm) was made over T2. Tissue rostral to T2 was cleared to expose the cord. The spinal cord was then transected by cauterization, the cavity was filled with Gelfoam (Pharmacia Corporation, Kalamazoo, MI, USA), and the incision closed with Michel clips (Fisher Scientific, Waltham, MA, USA).

Following surgery, subjects immediately received an intraperitoneal injection (3 mL) of 0.9% saline to maintain hydration. Hindlimbs were gently wrapped using a piece of Orthaletic tape (30 cm [length] x 1.3 cm [width], Johnson & Johnson, New Brusnwick, NJ, USA) to maintain a fixed leg position and reduce potential injury during recovery. All rats were monitored during the 24 hr recovery period in a temperature-controlled environment (25.5 °C) and received supplemental injections of saline to ensure adequate hydration. Bladders and colons were expressed every 12 hr and just before instrumental testing. Spinal transections were confirmed by inspecting the cord during surgery, monitoring leg movement during recovery to ensure complete paralysis below the forelimbs, and postmortem inspection in a randomly selected subset of subjects.

Apparatus

During training and testing, all subjects were loosely restrained in Plexiglas tubes (23.5 cm [length] x 8 cm [internal diameter]). Figure 2 illustrates the instrumental training apparatus. Two slots were cut 4 cm apart and 1.5 cm from the end of the tube to allow hind legs to hang freely. A contact electrode (stainless steel, 7 cm) was taped to the plantar surface of the rat's foot (Orthaletic, 1.3 cm [width]; Johnson & Johnson, New Brunswick, NJ, USA). Heat-shrink tubing insulated the taped portion of the rod from the paw. To minimize upper body movement, a wire belt was used to secure the rat's midsection. To minimize lateral leg movement, an additional piece of Orthaletic tape was wrapped around the leg above the tarsus and attached to the restraining tube.

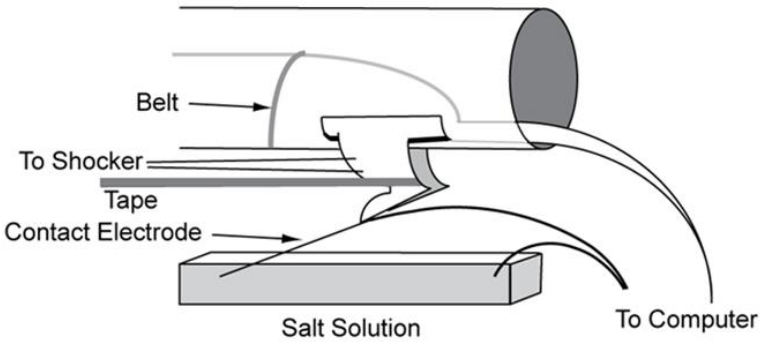

Figure 2. Instrumental training apparatus.

Leg shock (60 Hz, AC) was delivered by attaching one lead from a constant current shock generator (Model SG-903; BRS/LVE, Laurel, MD, USA) to a stainless steel wire (0.05 mm [30 AWG]) inserted through the skin over the tibia, 1.5 cm from the tarsals. A second lead was attached to a fine wire (0.01 mm [36 AWG], magnet wire single beldsol) inserted through the tibialis anterior muscle, 1.7 cm above the first electrode. A fine wire was attached to the contact electrode and connected to a digital input board that was monitored by a Macintosh G4 computer. A plastic rectangular dish containing a salt solution was placed approximately 7.5 cm below the restraining tube. When the contact electrode entered the salt solution, it completed a computer-monitored circuit, delivering shock to the tibialis anterior.

Shock intensity was adjusted to produce a 0.4N flexion response for all subjects, as described in Grau et al., 1998. Briefly, flexion force was measured before and after

training and testing by connecting a strain gauge to the rat's foot with a microfilament plastic line. The line (40 cm) was threaded through an eyelet attached to the apparatus directly beneath the paw, 16 cm under the base of the tube. The end of the line was attached to a strain gauge (Fort-1000, World Precision Instruments, New Haven, CT) fastened to a ring stand. The ring stand was then positioned so that the line was taut, only barely registering on the gauge. The gauge allowed us to determine a linear relationship between voltage and force in Newtons, and establish the necessary shock intensity needed to elicit a 0.4 N flexion response. After flexion force was set, the rat's paw and the saline solution were adjusted to allow the contact electrode to sit 4 mm under the surface of the solution.

Procedure

Instrumental training

After flexion force was determined, one-fourth of the subjects were administered 30 min of contingent shock (master rats). Master rats received shock whenever the rod used to monitor leg position entered the solution. Shock resulted in a flexion response, thereby removing the leg from the solution, breaking the closed circuit, and terminating shock. Another fourth were administered 30 min of non-contingent shock (yoked rats). Each yoked rat was experimentally coupled to a master rat so that shock was delivered to the yoked rat, irrespective of leg position, whenever its partner received shock. The remaining half of the subjects served as controls, half of which received no shock during

the 30-min training period, and the remaining half which received capsaicin injections and no shock training. Capsaicin was included as a positive control to ensure detection of our targets (Galan, Cervero, & Laird, 2003).

Behavioral measures

To assess the subject's ability to perform the instrumental response (leg flexion), training sessions were divided into 30 1-min time bins. The computer calculated the average flexion duration for each subject during each bin by using the following equation: Response Duration = (60 s – time in solution)/(Response Number + 1).

Tissue collection

Subjects were sacrificed with pentobarbital (100mg/kg) at multiple time points (0, 3, and 24 h) following instrumental training. After injection, spinal cord segments (L4-S2) were immediately removed and flash-frozen in liquid nitrogen. Tissue was homogenized in ice-cold lysis buffer consisting of 10 mM Tris, 200 mM sucrose, 1 mM EDTA, 1 µg/ml leupeptin, 1 µg/ml iodacetamide, 1 µg/ml phenanthroline, 1mM AEBSF, 1 mM FaF, 1 mM sodium orthovadate, pH 7.5. To maximize homogenization, tissues were passed through a 21-gauge syringe.

Homogenized tissues were then fractionated to obtain cytosolic extracts for western blot analysis. After differential centrifugation (16,110 x g, 15 min, 4 °C), the supernatant was collected for cytosolic target analysis. The remaining nuclear pellet was

resuspended in fresh lysis buffer, sonicated over ice 3 x 30 s, and collected for future nuclear target analysis.

Western blotting

Cellular targets were affinity purified using specific antibodies conjugated to protein A-beads (Pierce). Electrophoresis and transfer procedures were performed using the Mini-PROTEAN 3 Cell apparatus for western blotting (BioRad #165-3302). Proteins were loaded onto a fixed 12% polyacrylamide gel (25 μg protein per well, Thermo Scientific Precise Precast Protein Gel), along with a biotinylated protein ladder (Cell Signaling 7727), and a molecular weight marker (Pierce 26681). Proteins were separated electrophoretically (150V, 40 min) in a Tris-HEPES-SDS running buffer (Pierce 28398) and transferred, on ice, to a nitrocellulose membrane in a methanol-containing buffer (25 mM Tris, 192 mM glycine, 20% methanol). Westerns for phosphorylated ERK1/2 were transferred at 40 volts constant for 90 min. After transfer, the membrane was blocked for 1 hr (5% BSA [Sigma Aldrich] in TBST [0.1% Tween-20 in TBS]). The ladder was then cut from the blot and incubated overnight in fresh blocking buffer. The remaining portions were incubated overnight (4 °C) in blocking buffer and Anti-phospho-specific antibody (Anti-phospho-MAP Kinase 1/2 [ERK1/2], 1:500, Millipore #07-467). Blots were then washed 3 x 15 min in TBST and immediately incubated for 1 hr in TBST with HRP-conjugated biotinylated goat-anti-rabbit antibody (1:10000, Vector Laboratories BA-1000). The ladder portion of the blot was simultaneously incubated in purified HRP-conjugated streptavidin (1:10000, Fisher Scientific #21124). After washing again

to remove excess antibody (3 x 15 min in TBST), blots were then incubated for 1 min in chemiluminescence substrate (Pierce ECL Western Blotting Substrate #32106) and imaged for band detection (FluorChem, Alpha Innotech).

Blots for phospho-ERK1/2 were then stripped in 1.0 M glycine 3 x 20 min, washed 3 x 15 min in TBST, and re-blocked in blocking buffer for 30 min. Ladders were stored in TBST and were not stripped. Blots were re-probed and incubated overnight (4 °C) with antibody for β-tubulin as a loading control (Anti-β-Tubulin [clone AA2], 1:500, Millipore #05-661). Blots were washed, re-probed with HRP-conjugated biotinylated goat-anti-mouse secondary antibody (1:10000, Vector Laboratories BA-2001), and reimaged using the same procedure described for the pre-stripped blots.

Densitometry

Bands were imaged using Spot Densitometry Analysis software (AlphaView, Alpha Innotech). Individual bands of equal area were selected and assigned an integrated density value (IDV) that corrected for background levels. The corrected IDV, which served as the primary measure of protein levels, was calculated for each band using the following equation: $IDV = Area_{Band} \times (Average_{Band} - Average_{Background})$.

Statistics

All data were analyzed using a mixed-design analysis of variance (ANOVA). Differences between groups were assessed using Tukey's *post hoc* test. For band

densities, values that were not representative of the data were not included in analysis. For all analyses, an alpha value of 0.05 or below was used to determine significance.

CHAPTER III

RESULTS

Experiment 1: Impact of instrumental training and capsaicin treatment on ERK1 phosphorylation

Previous research has shown that even in the absence of supraspinal input, rats can learn a simple instrumental response (Grau et al., 1998). At the cellular level, research has shown that NMDA receptor activation is critical for learning and induction of the learning deficit (Durkovic & Prokowich, 1998; Joynes et al., 2004; Ferguson et al., 2006; Grau et al., 2006). ERK1/2 is an intracellular signaling protein that, once activated following NMDA receptor activation, promotes gene transcription and modulates NMDA receptor function (Impey et al., 1999; Lever et al., 2003; Tyler et al., 2002). Given the role of the NMDA receptor in learning and the deficit, and the relationship between NMDA receptor activation and ERK1/2 activation, the current experiment was designed examine the effect of behavioral training on ERK1 phosphorylation.

Procedure

Seventy-two rats (n=6) received spinal transections and were randomly assigned to one of four experimental conditions: controllable shock (master), uncontrollable shock (yoked), no shock (unshocked), or subcutaneous hindpaw capsaicin injection (1%; 50 μL vol). Capsaicin-treated rats served as positive controls for phospho-ERK1/2 activation

(Ji, Baba, Brenner, & Woolf, 1999; Galan et al., 2003). To examine the effect of shock and capsaicin administration on phospho-ERK1 protein levels, spinal cords were collected 0, 3, or 24 hr after treatment, and were prepared for western immunoblot analysis as described earlier. A protein assay revealed adequate protein levels for western blot analysis of 48 subjects (n=4). Blots were probed with Anti-phospo-ERK1, stripped, and re-probed with Anti-ERK1 and β-tubulin. Phospho-ERK1 levels were measured by computing band densities and normalizing phospho-ERK1 IDVs to β-tubulin IDVs.

Results: Instrumental learning

Master rats exhibited progressively longer flexion durations, while yoked and unshocked rats did not (Figure 3). An ANOVA yielded significant main effects of Treatment Condition, $F(2, 51) = 146.45$, $p < .001$, and Time, $F(29, 1479) = 2.51$, $p < .001$, as well as a significant Treatment Condition X Time interaction, $F(58, 1479) = 2.53$, $p < .001$. Post hoc comparisons of group means confirmed that master rats maintained significantly longer flexion durations compared to yoked and unshocked rats ($p < .05$). No other statistical effects approached significance, all Fs < 2.06, $p > .05$.

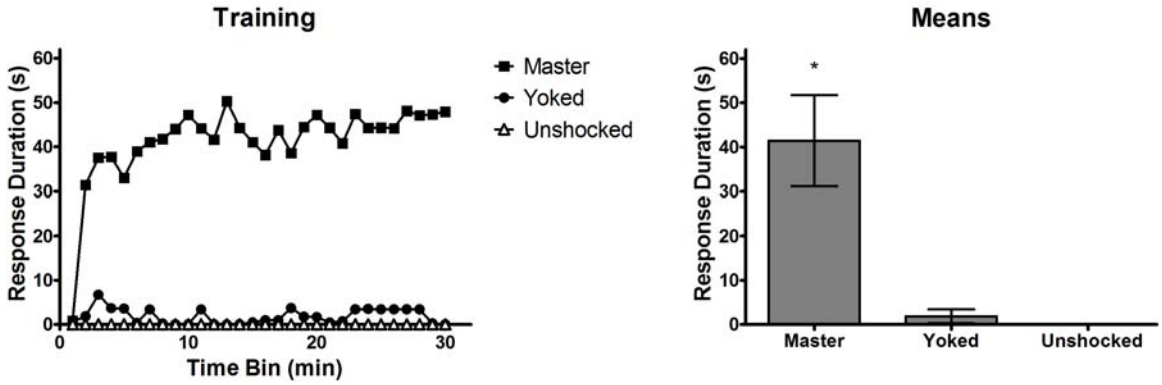

Figure 3. Effect of shock treatment on instrumental performance. (Left) Response durations across time; (Right) Average response durations collapsed across trials. Rats that received controllable shock exhibited progressively longer flexion durations over time, while rats that received uncontrollable or no stimulation did not. The asterisk indicates a statistically significant difference ($p < .05$), and error bars indicate ± SEM ($n = 6$ subjects per group).

Results: ERK1 phosphorylation

Western blot analyses revealed a time-dependent effect on ERK1 phosphorylation across all conditions (Figure 4.) Tissues analyzed 24 hr following treatment exhibited greater levels of ERK1 phosphorylation than tissues collected at 0 hr and 3 hr. An ANOVA revealed a significant main effect of Time, $F(2, 45) = 8.74$, $p < .01$. Post hoc analyses of group means demonstrated that tissues collected at 24 hr had significantly greater phospho-ERK1 IDVs relative to tissues collected at 0 and 3 hr following treatment ($p < .05$). No other effects approached significance, all Fs < 1.69, $p > .05$.

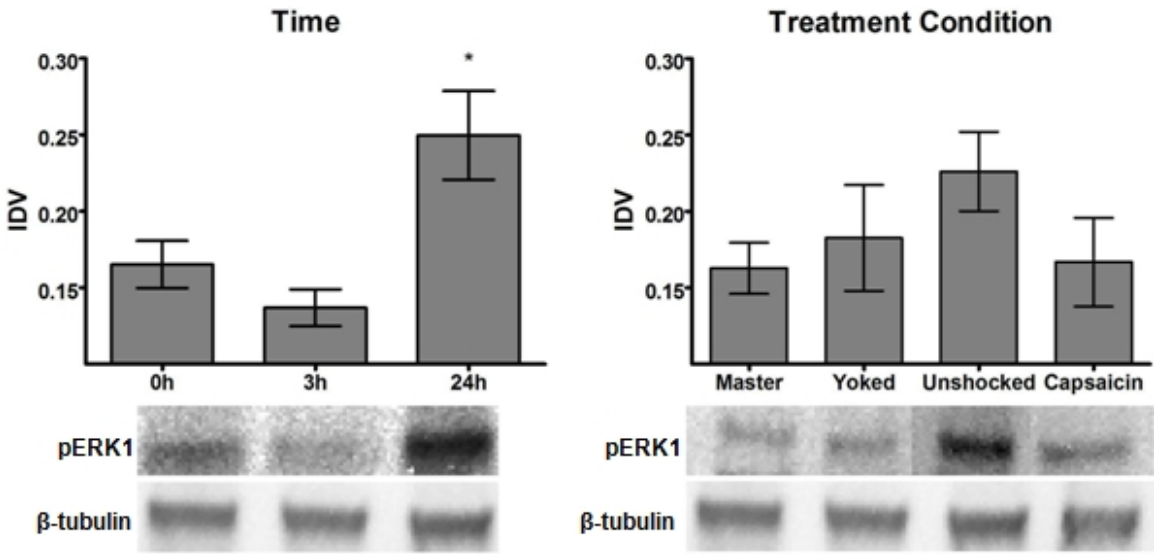

Figure 4. Effect of shock or capsaicin treatment on ERK1 phosphorylation. (Left) IDVs and representative western blot bands for time collapsed across treatment conditions; (Right) IDVs and representative western blot bands for treatment conditions collapsed across time. Phospho-ERK1 protein levels were significantly greater at 24 hr relative to 0 hr and 3 hr. The asterisk indicates a statistically significant difference ($p < .05$), and error bars indicate ± SEM (n = 4 subjects per group).

Experiment 2: Impact of instrumental training and capsaicin treatment on ERK2 phosphorylation

Experiment 1 demonstrated a time-dependent increase of phospho-ERK1 following instrumental training or capsaicin treatment. Research has shown that the ERK1 and ERK2 show similar patterns of activation (Impey et al., 1999; Seger et al., 1991; Seger & Krebs, 1995). Therefore, the current experiment was designed to assess the effect of behavioral training and capsaicin treatment on phospho-ERK2.

Procedure

Tissues were prepared as described in Experiment 1. ERK2 phosphorylation was measured by computing band densities and normalizing phospho-ERK2 IDVs to β-tubulin IDVs.

Results: ERK2 phosphorylation

ERK2 phosphorylation changed as a function of time and treatment condition (Figure 5). An ANOVA revealed significant main effects of Time, $F(2, 45)=11.87$, $p < .05$, and Treatment Condition, $F(3, 44) = 3.11$, $p < .05$. No other effects approached statistical significance, all Fs < 1.21, $p > .05$. Post hoc comparisons of group means revealed that tissues analyzed 24 hr following treatment exhibited significantly greater IDV levels of phospho-ERK2 than tissues collected at 0 hr and 3 hr. Additional post hoc analysis also revealed that unshocked controls had significantly greater phospho-ERK2 levels relative to shock- or capsaicin- treated subjects ($p < .05$).

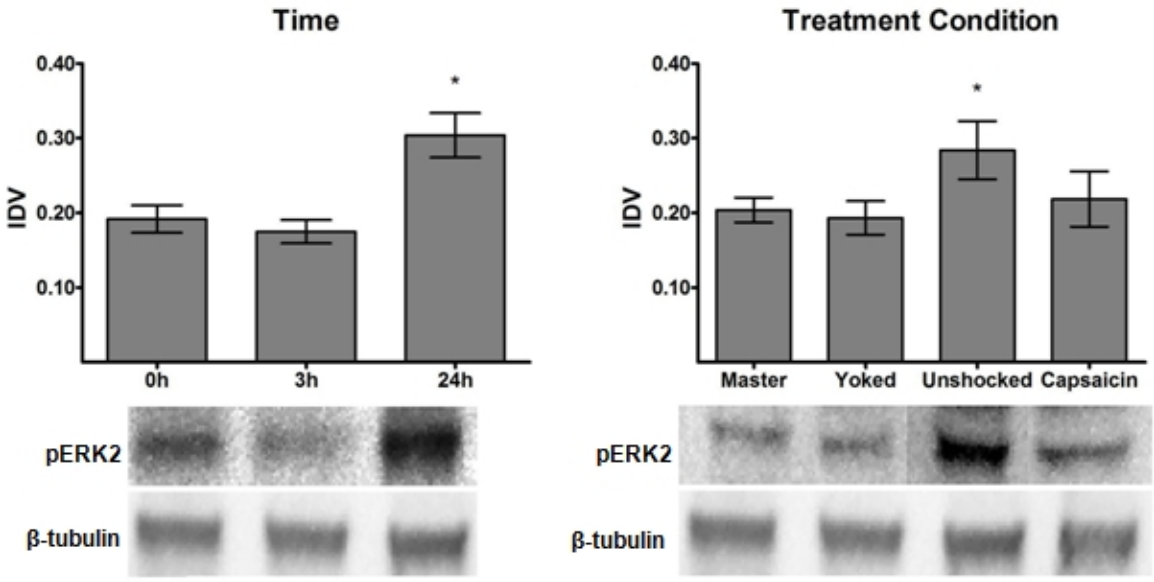

Figure 5. Effect of shock or capsaicin treatment on ERK2 phosphorylation. (Left) IDVs and representative western blot bands for time collapsed across treatment conditions; (Right) IDVs and representative western blot bands for treatment conditions collapsed across time. Phospho-ERK2 protein levels were significantly greater at 24 hr relative to 0 hr and 3 hr. Unshocked controls exhibited significantly greater phospho-ERK2 protein levels relative to shock- or capsaicin-treated groups. Asterisks indicate statistically significant differences ($p < .05$), and error bars indicate ± SEM ($n = 4$ subjects per group).

CHAPTER IV

GENERAL DISCUSSION AND SUMMARY

The nervous system integrates essential information about the environment, enabling organisms to respond to and learn from sensory experiences. Without neuronal plasticity (variations in synaptic structure and function), organisms would be unable to adapt to their changing environment. Plasticity can occur at all levels of the neural axis, including the spinal cord. Research has shown that even in the absence of supraspinal input, spinal neuron activity can increase (sensitize) or decrease (habituate) in response to various environmental stimuli (Groves & Thompson, 1970; Thompson & Spencer, 1966), and organisms can modify their responses based on previous exposure (R-O or instrumental learning; e. g., Grau et al., 1998). Moreover, repeated exposure to stimulation can have long-lasting effects on synaptic activity that are believed to underlie learning and nociception, suggesting that these processes share common spinal pathways (Mendell, 1984; Walters, 1994; Impey et al., 1999, Baumbauer, Young, & Joynes, under review; Ji et al., 1999). Interestingly, these two phenomena engage related intracellular pathways, including phosphorylation of ERK1/2 (Impey et al., 1999; Adams & Sweatt, 2002; Ji et al., 1999; Lever et al., 2003; Galan et al., 2003). Previous work has shown that ERK1/2 phosphorylation plays a substantial role in synaptic plasticity, and has been implicated in learning, memory and nociceptive processing (Adams & Sweatt, 2002; Ji, Kohno, Moore, & Woolf, 2003). The present experiments

were designed to assess ERK1/2 phosphorylation following instrumental training or capsaicin treatment.

Experiment 1 examined the impact of instrumental training or capsaicin treatment (models of learning and nociception) on cytosolic ERK1 phosphorylation. I replicated earlier effects and demonstrated that rats given controllable shock learned, while rats that received uncontrollable shock did not. I also showed that treatment with shock or capsaicin caused a reduction in ERK1 phosphorylation within 3 hr of treatment, and that levels returned to baseline 24 hr later. Given that ERK2 phosphorylation is observed concurrently with ERK1 phosphorylation (Seger et al., 1991), Experiment 2 used similar methods to explore the role of cytosolic phospho-ERK2 following instrumental training or capsaicin treatment. As in Experiment 1, I observed a downregulation in cytosolic phospho-ERK2 levels within 3 hr of treatment, and a return to baseline within 24 hr. I also showed that shock or capsaicin treatment caused a significant reduction in levels of phospho-ERK2. Collectively, these results provide evidence that learning and inflammation result in similar patterns of ERK1/2 activation.

Behavioral effects following instrumental training

Over the past decade, our laboratory has focused on characterizing the behavioral effects associated with controllable and uncontrollable shock treatments. Grau et al. (1998) showed that rats trained with controllable shock (master) exhibit a progressive increase in flexion duration during training. When later tested with controllable stimulation,

previously trained master rats can reacquire the flexion response more rapidly than previously unshocked controls, a phenomenon indicative of savings, or "memory," in the spinal cord (Grau et al., 1998). Additionally, when tested with a higher response criterion, previously trained master rats can acquire the response, while previously untrained rats cannot (Crown, Ferguson, Joynes, & Grau, 2002a). These results suggest that controllable shock engages a beneficial form of plasticity that persists beyond the training period.

Contrary to the beneficial effect of controllable stimulation, uncontrollable stimulation appears to have a detrimental effect on learning. Subjects trained with uncontrollable legshock do not exhibit the same increase in flexion duration during training. When later tested with controllable shock, subjects previously trained with uncontrollable shock still cannot acquire the target response. Interestingly, this effect is not limited to legshock. Other research has shown that uncontrollable intermittent shock delivered to the tail or sciatic nerve also impairs learning (Crown, Ferguson, Joynes, & Grau, 2002b; Baumbauer et al., 2008). Collectively, these results demonstrate the general capacity of uncontrollable shock to undermine spinal function.

Work in our laboratory has suggested that the divergent behavioral outcomes observed following controllable and uncontrollable shock result from oppositional forms of plasticity that are mutually inhibitory (Grau et al., 2006). If this is true, then inducing one effect should counter the effects of the other. Supporting this notion, Crown and

Grau (2001) showed administering controllable shock prior to uncontrollable shock protects against induction of the learning deficit. Crown et al. (2002b) also examined the ability of controllable stimulation to reverse the effects of uncontrollable shock. Because uncontrollable shock produces a deficit that inhibits learning, an additional manipulation was required that allowed subjects to learn. To accomplish this, Crown et al. (2002b) administered intrathecal naltrexone, an opioid antagonist that blocks the expression of the deficit. Under these conditions, controllable shock administration was able to reverse the deficit produced by uncontrollable shock. These findings demonstrate the mutually inhibitory nature of each shock regime, and further suggest that by encouraging the spinal cord's inherent, adaptive mechanisms, controllable stimulation can serve as an effective form of behavioral therapy, promoting healthy spinal function and reducing the maladaptive consequences of uncontrollable nociception.

Central sensitization and maladaptive pain processing

Under most circumstances, nociception serves to alert an organism to noxious environmental stimuli, thereby serving an adaptive function. However, research has shown that spinal cord injury or inflammation can induce a maladaptive form of spinal plasticity that results in neuropathic pain. The mechanism responsible for this change in neuronal behavior is known as central sensitization. Once produced, central sensitization causes an increase in neuronal excitability that results in increased nociceptive responding to noxious (hyperalgesia) and innocuous (allodynia) stimuli

(Willis, 2001a, b). Interestingly, inflammation and peripheral injury also appear to have detrimental effects on learning. Administration of the inflammatory agent lipopolysaccharide (LPS) produces a robust immune/inflammatory response that undermines learning (Young, Baumbauer, Elliott, & Joynes, 2007a). Hook, Huie, and Grau (2008) also demonstrated that prior treatment with the inflammatory agent capsaicin dose-dependently inhibits learning. Moreover, Young, Baumbauer, Elliott, and Joynes (2007b) demonstrated that hindpaw injury in neonatal rats undermines learning when subjects are later tested as adults. These results suggest that central sensitization and the learning deficit are linked. Supporting this notion, Ferguson et al. (2006) showed that uncontrollable shock produces allodynia, a behavioral symptom associated with central sensitization. If the deficit and central sensitization are similar phenomena, then pretreating subjects with controllable shock should protect the spinal cord against deficit induction. Indeed, Hook et al. (2008) demonstrated that prior treatment with controllable shock prevents the allodynia associated with capsaicin administration and protects against induction of the capsaicin-induced learning deficit. Taken together these results suggest that uncontrollable stimulation and peripheral inflammation engage common mechanisms that negatively impact spinal function, and that controllable stimulation can foster adaptive plasticity.

Neural mechanisms

Recent work in our laboratory has started to explore the neurobiological underpinnings of learning and the deficit. Given that noxious information is initially processed in the

spinal cord, (Melzack & Wall, 1965; Ji et al., 2003), and that electrical shock engages a nociceptive response, we have examined learning using results obtained from models of nociceptive function as a directing factor. Using this approach, we have focused on the NMDA receptor and its associated signaling cascades.

NMDA receptor-mediated plasticity

Previous research has shown that NMDA receptor activation plays a critical role in central sensitization and nociceptive processing. Specifically, targeted mutations and pharmacological blockade of the NMDA receptor prevent central sensitization (Woolf & Thompson, 1991; Coderre et al., 1993; Xu, Zhang, Hokfelt, & Wiesenfeld-Hallin, 2005; Willis, 2001a, b; Zhang, Wu, Lei, Fang, & Willis, 2005). Experiments from our laboratory have shown that the NMDA receptor also plays a critical role in both spinal learning and the deficit (Ferguson et al., 2006; Grau et al., 2006). For example, Joynes et al. (2004) showed that blocking the NMDA receptor with the antagonist APV dose-dependently undermines the ability of subjects trained with controllable shock to acquire the target flexion response. Moreover, when APV is administered following acquisition of the prolonged flexion response, the ability to maintain that response is undermined. We have also shown that blocking the NMDA receptor prior to uncontrollable shock administration prevents induction of the learning deficit, allowing subjects to learn at test (Ferguson et al., 2006). These results suggest that NMDA receptor activation is important for multiple forms of spinal plasticity. To further understand how controllable and uncontrollable shock affect subsequent behavior, we have begun to examine more

closely the cellular structures and mechanisms underlying learning, focusing on intracellular signaling pathways downstream of NMDA receptor activation.

NMDA-dependent cellular mechanisms

Activation of NMDA receptors results in an influx of Ca^{2+}, which then triggers several intracellular signaling cascades, including PKA, PKC, CaMKII, and ERK1/2 (Roberson et al., 1999; Kawasaki et al., 2004). These kinases have been shown to play a role in enhancing neural reactivity, postsynaptic function, and learning in intact animals (Silva, Stevens, Tonegawa, & Wang, 1992; Yang et al., 2004; Ji et al., 2003; Fang, Wu, Lin, & Willis, 2002, 2003). Our work has shown that application of uncontrollable shock increases PKC activity, and that blocking PKC activity prevents the induction of the learning deficit (Bolding, Hook, Ferguson, & Grau, 2003; Ferguson et al., 2008), suggesting that PKC activation undermines learning. We have also shown that controllable stimulation increases CaMKII mRNA expression, while uncontrollable stimulation decreases expression (Gómez-Pinilla et al., 2007). Moreover, inhibiting CaMKII undermines the enabling effect of controllable shock and can prevent the learning deficit observed in subjects given uncontrollable shock, suggesting that CaMKII activation is important for maintaining the long term modifications involved in learning and the deficit (Gómez-Pinilla et al., 2007; Baumbauer et al., 2007a).

We extend our previous work by examining the impact of shock treatment on changes in ERK1/2 phosphorylation. A number of studies have suggested an important role for

ERK1/2 phosphorylation in learning, memory, and pain processing (Seger & Krebs, 1995; Impey et al., 1999; Sweatt, 2001; Galan et al., 2003; Ji et al., 2003). Following NMDA receptor activation, PKC, PKA, and CaMKII promote activation of ERK1/2 (Impey et al., 1999). ERK1/2 is then translocated from the cytosol to the nucleus, where it enhances gene transcription, receptor trafficking, protein synthesis, and possibly modulates phosphorylation of the NMDA receptor (Ji et al., 2003). Given that activated ERK1/2 translocates from the cytosol to the nucleus, we expected subjects given shock or capsaicin treatment to exhibit decreased cytosolic levels of phospho-ERK1/2. Supporting our hypothesis, the present experiments showed that phospho-ERK1/2 was decreased in the cytosol immediately following treatment, suggesting that ERK1/2 may play a role in spinal instrumental learning.

ERK1/2 activation: adaptive or maladaptive?

Given the divergent effects of controllable and uncontrollable stimulation on behavior and NMDA receptor-dependent kinase activity (e. g., PKC, CaMKII), we expected ERK1/2 to exhibit divergent patterns of phosphorylation as well. Contrary to our expectations, shock condition did not differentially impact ERK1/2 phosphorylation. These surprising results have raised some interesting questions about the role of ERK1/2 in learning and the deficit. Of particular interest is determining the specific cellular pathways that lead to ERK1/2 phosphorylation following controllable or uncontrollable shock. Because the current experiments examined isolated ERK1/2 protein levels, it is difficult to determine whether ERK1/2 was activated by common cellular events.

Several studies have addressed this issue, speculating that while controllable and uncontrollable stimulation engage related forms of NMDA receptor-dependent plasticity, there may be additional, response-specific cellular processes that, when activated concomitantly with the NMDA receptor, enable mutually exclusive neural modifications that either support or undermine learning (e. g., Grau et al., 2006). Under this premise, controllable shock activates one or a collection of processes with the NMDA receptor, and these co-occurring events foster an adaptive form of plasticity that supports learning. Conversely, uncontrollable shock activates additional processes with the NMDA receptor, and these events result in a maladaptive form of plasticity that undermines learning. Recent work has supported this explanation, suggesting that Substance P and brain-derived neurotrophic factor (BDNF) divergently modulate NMDA receptor-mediated learning (Baumbauer, Young, Hoy, & Joynes, 2007b, c; Gómez-Pinilla et al., 2007).

During electrical stimulation or inflammation, sensory fibers release increased quantities of glutamate and the neuropeptide Substance P, which binds to the NMDA and neurokinin 1 (NK) receptors, respectively. Once activated, NK1receptors promote increased Ca^{2+} influx, kinase activity, and glutamate release (Carlton, Zhou, & Coggeshall, 1998; Kangrga & Randic, 1990; Okano, Kuraishi, & Satoh, 1998; Werry, Liu, & Bennett, 2006). With prolonged stimulation, Substance P release contributes to the hyperexcitable state associated with central sensitization (Willis, 2001a, b, 2002). Given the link between central sensitization and the learning deficit, subsequent work

has suggested that Substance P has a detrimental effect on learning. For example, Baumbauer et al. (2007c) showed that simulating Substance P release with an NK1 agonist undermines the ability of subjects trained with controllable shock to acquire the target flexion response. Additionally, Baumbauer et al. (2007b) showed that blocking the NK1 receptor prior to uncontrollable shock administration prevents induction of the learning deficit, allowing subjects to learn at test. Taken together, these results suggest that uncontrollable nociceptive stimulation results in concurrent NMDA receptor activation and SP release that, together, promote a diffuse state of neural excitability that results in maladaptive pain processing and the learning deficit (Grau et al., 2006; Ferguson et al., 2006; Hook et al. 2008; Baumbauer et al., 2007b, c, under review).

Contrary to uncontrollable stimulation, controllable stimulation appears to engage cellular events that counter the deleterious effects of Substance P. During controllable stimulation, sensory fibers release increased quantities of glutamate and the neurotrophin BDNF, which binds to the NMDA and tyrosine kinase B(Trk B) receptors, respectively (Gómez-Pinilla et al.,2007). Once activated, Trk B receptors activate and enhance CaMKII function, supporting our earlier findings that controllable stimulation increases CaMKII mRNA expression. BDNF release also leads to increased release of the neurotransmitter gamma-aminobutyric acid (GABA) (Kuczewski et al., 2008; Sivakumaran, Mohajerani, & Cherubini, 2009). Given that GABA has been established as an inhibitory agent that reduces neural excitability, Baumbauer et al. (under review) suggest that BDNF-induced GABA release may counter the maladaptive of Substance P

on neural excitability, and, therefore, reduce the negative impact of Substance P on learning. Supporting the notion, Huie, Hoy, Miranda, and Grau (2007) demonstrated that intrathecal administration of BDNF fosters learning and blocks induction of the learning deficit. Taken together, these results suggest that controllable shock engages concurrent NMDA receptor activation and BDNF release that enable pathway-specific modifications, countering the diffuse state of Substance P-induced excitation and promoting adaptive plasticity.

If learning and the deficit rely on modulators that coincide with NMDA-dependent processes, as the Substance P and BDNF data suggest, then it is possible that ERK1/2 serves as a common signaling pathway for learning and the deficit. The present results support this hypothesis, suggesting that ERK1/2 activation plays a critical role in the downstream consequences of NMDA receptor activation, and that these effects may or may not be modulated by additional cellular processes such as Substance P and BDNF. Future studies will help correlate ERK1/2 activity with these proposed modulators and provide a more detailed picture of the ERK1/2 signaling cascade and its precise role in learning and the deficit.

Limitations and future directions

While our methodology incorporated adequate procedures for detecting ERK1/2 phosphorylation, several factors limited the scope of this project. For example, given the impact of controllable and uncontrollable stimulation on behavior, one might expect to

observe differences in ERK1/2 phosphorylation based on shock treatment. One reason we did not observe any differences may be that our tissue samples were comprised of whole spinal cords, which included sensory fibers that innervate the dorsal horn, as well as motor neurons found in the ventral horn. Given that our research focuses on plasticity in the sensory regions of the cord, the protein in our samples may have been diluted by ventral motor neurons, limiting our ability to resolve subtle differences in phosopho-ERK1/2 expression. To overcome this limitation, ongoing studies are examining fractionated dorsal horn regions for optimal protein detection in sensory neurons.

Additionally, this study only examined cytosolic fractions for ERK1/2 phosphorylation. While research has shown that once activated, ERK1/2 translocates to the nucleus, and the present results demonstrated a decrease in cytosolic ERK1/2 expression, we can only assume that ERK1/2 was translocated to the nucleus. Ongoing studies with fractionated dorsal horn sections will allow us to correlate changes in cytosolic and nuclear ERK1/2 activation, and, therefore, provide more conclusive evidence that ERK1/2 differentially phosphorylates and translocates to the nucleus following controllable and uncontrollable shock.

Finally, while western immunoblotting provides one technique for analyzing ERK1/2 activation, further studies are needed to achieve a more complete picture of this cellular signaling mechanism. Additional cellular analysis methods, such as enzyme-linked immunosorbent assays (ELISAs), polymerase chain reaction (PCR),

immunohistochemistry (IHC), and *in situ* hybridization, may offer greater resolution than western immunoblotting, allowing us to determine the magnitude and location of ERK1/2 activation following treatment.

If the methods described above elucidate the impact of instrumental training on ERK1/2 activation in dorsal horn neurons, some interesting questions can be raised. On one hand, if controllable shock produces significantly different levels of ERK1/2 than uncontrollable shock, we can begin to characterize divergent cellular pathways that underlie learning and the deficit. For example, if uncontrollable shock results greater phosphorylation, we can speculate that ERK1/2 plays a role in the deficit. In this case, pharmacological manipulations of ERK1/2 could provide additional support for the role of ERK1/2 in the learning and the deficit. On the other hand, if controllable and uncontrollable shock results in comparable phosphorylation levels, ERK1/2 may play an indirect, complex role in learning and the deficit. If this is the case, we must then consider additional cellular mechanisms that may modulate the ERK signaling pathway to produce the divergent behavioral outcomes.

Supporting this notion, research has shown that cellular events downstream of ERK1/2 may play important roles in learning and nociceptive processing (Ji et al., 2003). Once translocated to the nucleus, ERK1/2 activates CREB, which regulates gene expression and protein synthesis (Roberson et al., 1999; Sweatt, 2001). Within the learning literature, inhibiting protein synthesis reverses the learning deficit, suggesting that

CREB may play an important role in stabilizing neural modifications needed to maintain learning and the deficit (Gómez-Pinilla et al., 2007; Baumbauer et al., under review). In addition to protein synthesis, CREB activation promotes the trafficking of α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate (AMPA) receptors to the postsynaptic membrane, increasing postsynaptic glutamate sensitivity, and therefore, neural excitability (Perkinton, Sihra, & Williams, 1999). Given the role of CREB in neural plasticity, future studies could extend previous work to determine the impact of controllable and uncontrollable stimulation on CREB activation and expand our understanding of the divergent cellular mechanisms underlying learning and the deficit (Grau et al., 2006; Baumbauer et al., under review; Gómez-Pinilla et al., 2007).

In addition to examining established components of the ERK signaling cascade and its downstream constituent processes, we may also want to explore neurotrophins that modulate NMDA receptor activity. Future studies could examine how BDNF impacts downstream cellular processes following NMDA receptor activation. Given that the beneficial effects of controllable stimulation selectively cause BDNF release, exploring the downstream events activated concurrently with BDNF may help isolate the cellular events specific to learning.

Clinical implications: A neural-functionalist approach

Given the nature of the injury, SCI initially damages the vital sensory and motor neuronal networks that allow organisms to perceive and navigate their environment.

Without the important sensory experience of pain or adequate locomotive function, SCI can then initiate a cascade of secondary processes, including inflammation, ischemia (insufficient blood supply), and necrosis/apoptosis (cell death). Often more damaging than the initial injury, this secondary phase engages neural destructive processes, exacerbating tissue damage and undermining recovery (NSCISC, 2008). Given these deleterious consequences, work in our laboratory has largely been guided by the phenomena associated with secondary injury. If we can identify the mechanisms responsible for these maladaptive forms of plasticity, the conditions in which they come into play, and the underlying biological processes, we can achieve a better understanding of secondary injury and offer treatments that attenuate destructive processes and foster recovery (Grau & Joynes, 2005).

Our instrumental training paradigm provides several clinical implications for examining SCI and subsequent processes. For example, we have shown that inflammation, a common secondary condition of SCI, produces allodynia and undermines learning (Hook et al., 2008). Conversely, training with controllable stimulation reduces allodynia, and enables learning (Hook et al., 2008), suggesting that learning can protect against the maladaptive consequences of injury. Consequently, identifying the conditions and manipulations that support spinal learning, such as controllable stimulation, may help to develop new treatments that protect against conditions of secondary injury, and foster recovery from SCI (Baumbauer, under review). Interestingly, controllable stimulation is currently used as a form of therapy in clinical settings to encourage walking and other

forms of locomotion. One training paradigm, FES, delivers shock to the peroneal nerve during the swing phase of the gate, initiating a flexion response that encourages stepping (Creasey et al. 2004). Importantly, the beneficial effects of FES training can continue even after electrical stimulation has stopped, paralleling the effects of controllable stimulation in our model of instrumental training. These results further suggest a link between learning and recovery of function following injury. While FES therapies have had some success in human and animal models (Hook & Grau, 2007), additional work is needed to thoroughly delineate the parameters that encourage recovery from those that undermine it. This work will be integral for the development of successful behavioral treatments for SCI.

In addition to behavioral therapy, our research also provides clinically relevant findings for pharmacological therapy. Administering drugs that prevent maladaptive plasticity and encourage adaptive forms of plasticity may help reduce central sensitization following SCI, facilitate recovery of function, and provide an effective treatment of neuropathic pain. For example, Hook et al. (2008) established that the opioid antagonist naltrexone can protect against the deleterious effects of uncontrollable shock and capsaicin administration, suggesting that naltrexone treatment provides an effective form of pharmacological therapy for SCI. Collectively, we maintain that our instrumental learning paradigm provides an effective model for studying spinal plasticity, and that this work will help answer important questions about learning and pain processing, particularly as these phenomena relate to SCI.

Summary

In summary, this study assessed the impact of controllable stimulation, uncontrollable stimulation, and inflammation on cytosolic ERK1/2 phosphorylation within the spinal cord. Across treatment conditions, cytosolic phospho-ERK1/2 was downregulated immediately following treatment, suggesting a translocation of phospho-ERK1/2. Additionally, nociceptive stimulation significantly downregulated cytosolic phospho-ERK2 levels relative to unshocked controls. The present results suggest that controllable shock instrumental training and inflammation produce similar effects on ERK1/2 phosphorylation levels. However, other research suggests that despite having similar effects on ERK1/2 phosphorylation, learning and the deficit engage divergent underlying pathways. Additional work is needed to characterize other signaling pathways and downstream consequences of ERK1/2 activation. Further work is also needed to determine how these cellular processes interact to facilitate or inhibit recovery of function after spinal cord injury.

REFERENCES

Adams, J. P., & Sweatt, J. D. (2002). Molecular psychology: Roles for the ERK MAP kinase cascade in memory. *Annual Review of Pharmacology & Toxicology*, 42, 135–163.

Baumbauer, K. M., Hoy, K. C., Huie, J. R., Hughes, A. J., Woller, S. A., Puga, D. A., et al. (2008). Timing in the absence of supraspinal input I: Variable, but not fixed, spaced stimulation of the sciatic nerve undermines spinally-mediated instrumental learning. *Neuroscience*, 115, 1030-1047.

Baumbauer, K. M., Young, E. E., Hoy, K. C., Abood, A., & Joynes, R. L. (2007a). Administration of a Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) inhibitor reverses the noncontingent shock learning deficit observed in spinal rats. *Behavioral Neuroscience*, 121, 570-578.

Baumbauer, K. M., Young, E. E., Hoy, K. C., Joynes, R. L. (2007b). Intrathecal administration of neurokinin 1 and neurokinin 2 receptor antagonists undermines the savings effect in spinal rats seen in an instrumental learning paradigm. *Behavioral Neuroscience*, 121. 186-199.

Baumbauer, K. M., Young, E. E., Hoy, K. C., Joynes, R.L. (2007c). Neurokinin receptors modulate the impact of uncontrollable stimulation on adaptive spinal plasticity. *Behavioral Neuroscience*, 121, 1082-1094.

Baumbauer, K. M., Young, E. E., & Joynes, R. L. (under review). Pain and learning in a spinal system: Contradictory outcomes from common origins. *Brain Research Reviews*.

Berkowitz, M., O'Leary, P., Kruse, D., & Harvey, C. (1998). *Spinal cord injury: An analysis of medical and social costs*. New York: Demos Medical Publishing Inc.

Bolding, K. A., Hook, M. A., Ferguson, A. R., Grau, J. W. (2003). The general protein kinase C inhibitor, bisindolylmaleimide-I, dose-dependently prevents a shock-induced behavioral deficit in spinalized rats. In *2003 Abstract Viewer/Itinerary Planner*. Washington, DC: Society for Neuroscience.

Buerger, A. A., & Chopin, S. F. (1976). Instrumental avoidance conditioning in spinal vertebrates. *Advances in Psychobiology*, 3, 437-161.

Buerger, A. A., & Fennessy, A. (1970). Long-term alteration of leg position due to shock avoidance by spinal rats. *Nature*, 225, 751-752.

Byrne, J. H., Zwartjes, R., Homayouni, R., Critz, S., & Eskin, A. (1993). Roles of second messenger pathways in neuronal plasticity and in learning and memory: Insights gained from *Aplysia*. *Advances in Second Messenger & Phosphoprotein Research Journal*, 27, 47-108.

Carlton, S. M., Zhou, S., Coggeshall, R. E. (1998). Evidence for the interaction of glutamate and NK1 receptors in the periphery. *Brain Research Bulletin*, 790, 160-169.

Chopin, S. F., & Buerger, A. A. (1976). Instrumental avoidance conditioning in the spinal rat. *Brain Research Bulletin*, 1, 177-183.

Coderre, T. J., Katz, J., Vaccarino, A. L., & Melzack, R. (1993). Contribution of central neuroplasticity to pathological pain: Review of clinical and experimental evidence. *Pain*, 52, 259-285.

Creasey, G. H., Ho, C. H., Triolo, R. J., Gater, D. R., DiMarco, A. F., Bogie, K. M., et al. (2004). Clinical applications of electrical stimulation after spinal cord injury. *The Journal of Spinal Cord Medicine*, 27, 65-375.

Crown, E. D., Ferguson, A. R., Joynes, R. L., Grau, J. W. (2002a). Instrumental learning within the spinal cord: II. Evidence for central meditation. *Physiology & Behavior*, 77, 259-267.

Crown, E.D., Ferguson, A.R., Joynes, R.L., Grau, J.W. (2002b). Instrumental learning within the spinal cord: IV. Induction and retention of the behavioral deficit observed after noncontingent shock. *Behavioral Neuroscience*, 116, 1032-1051.

Crown, E. D., & Grau, J. W. (2001). Preserving and restoring behavioral potential within the spinal cord using an instrumental paradigm. *Journal of Neurophysiology*, 86, 845-855.

Durkovic, R. G. (1975). Classical conditioning, sensitization, and habituation in the spinal cat. *Physiology & Behavior*, 14, 297-304.

Durkovic, R. G., & Prokowich, L. J. (1998). D-2-Amino-5-phosphonovalerate, an NMDA receptor antagonist, blocks induction of associative long-term potentiation of the flexion reflex in spinal cat. *Neuroscience Letters*, 257, 162–164.

Edgerton, V. R., Roy, R. R., de Leon, R. D., Tillakaratne, N., & Hodgson, J. A. (1997). Does motor learning occur in the spinal cord? *Neuroscientist*, 3, 287-294.

Edgerton, V. R., Roy, R. R, Hodgson, J. A., Prober, R. J., de Guzman, C.P., & de Leon, R. D. (1992). Potential of adult mammalian lumbosacral spinal cord to execute and acquire improved locomotion in the absence of supraspinal input. *Journal of Neurotrauma*, 9, S119–S128.

Fang, L., Wu, J., Lin, Q., & Willis, W. D. (2002). Calcium-calmodulin-dependent protein kinase II contributes to spinal cord central sensitization. *Journal of Neuroscience*, 22, 4196-4204.

Fang, L., Wu, J., Lin, Q., & Willis, W. D. (2003). Protein kinases regulate the phosphorylation of the GluR1 subunit of AMPA receptors of spinal cord in rats following noxious stimulation. *Molecular Brain Research*, 118, 160-165.

Ferguson, A. R., Bolding, K. A., Huie, J. R., Hook, M. A., Santillano, D. R., Miranda, R. C., et al. (2008). Group I metabotropic glutamate receptors control metaplasticity of spinal cord learning through a protein kinase c-dependent mechanism. *The Journal of Neuroscience*, 28(46), 11939-11949.

Ferguson, A. R., Crown, E. D., & Grau, J.W. (2006). Nociceptive plasticity inhibits adaptive learning in the spinal cord. *Neuroscience*, 141, 421–431.

Fitzgerald, L. A., & Thompson, R. F. (1967). Classical conditioning of the hindlimb flexion reflex in the acute spinal cat. *Psychonomic Science*, 47, 345-351.

Galan, A., Cervero, F., & Laird, J. M. A. (2003). Extracellular signaling-regulated kinase-1 and -2 (ERK 1/2) mediate referred hyperalgesia in a murine model of visceral pain. *Molecular Brain Research*, 116, 126-134.

Gómez-Pinilla, F., Huie, J. R., Ying, Z., Ferguson, A., Crown, E. D., Baumbauer, K. M., et al. (2007). BDNF and learning: Evidence that instrumental training promotes learning within the spinal cord by up-regulating BDNF expression. *Neuroscience*, 148, 893-906.

Grau, J. W. (1987). The central representation of an aversive event maintains the opioid and nonopioid forms of analgesia. *Behavioral Neuroscience*, 101, 272-288.

Grau, J. W., Barstow, D. G., & Joynes, R. L. (1998). Instrumental learning within the spinal cord: I. Behavioral properties. *Behavioral Neuroscience*, 112, 1366-1386.

Grau, J. W., Crown, E. D., Ferguson, A. R., Washburn, S. N., Hook, M. A., & Miranda, R. C. (2006). Instrumental learning within the spinal cord: Underlying mechanisms and implications for recovery after injury. *Behavioral and Cognitive Neuroscience Reviews*, 5, 191–239.

Grau, J. W., & Joynes, R. L. (2005). A neural-functionalist approach to learning. *International Journal of Comparative Psychology*, 18, 1-22.

Grau, J. W., Salinas, J. A., Illich, P. A., & Meagher, M. W. (1990). Associative learning and memory for an antinociceptive response in the spinalized rat. *Behavioral Neuroscience*, 104, 489-494.

Groves, P. M., & Thompson, R. F. (1970). Habituation: A dual-process theory. *Psychological Review*, 77, 419-450.

Harkema, S. J., Hurley, S. L., Patel, U. K., Requejo, P. S., Dobkin, B. H., & Edgerton, V. R. (1997). Human lumbosacral spinal cord interprets loading during stepping. *Journal of Neurophysiology*, 77(2), 797-811.

Hawkins, R. D., Kandel, E. R., & Siegelbaum, S. (1993). Learning to modulate transmitter release: Themes and variations in synaptic plasticity. *Annual Review of Neuroscience*, 16, 625-665.

Hebb, D. O. (1949). *The organization of behavior*. New York: Wiley Interscience.

Hodgson, J. A., Roy, R. R., de Leon, R., Dobkin, B., & Edgerton, V. R. (1994). Can the mammalian lumbar spinal cord learn a motor task? *Medicine and Science in Sports and Exercise*, 26, 1491-1497.

Hook, M. A., & Grau, J. W. (2007). An animal model of functional electrical stimulation: Evidence that the central nervous system modulates the consequences of training. *Spinal Cord*, 45, 702-712.

Hook, M. A., Huie, J. R, & Grau, J. W. (2008). Peripheral inflammation undermines the plasticity of the isolated spinal cord. *Behavioral Neuroscience*, 122:233–249.

Huie, J. R., Hoy, K. C., Miranda, R. A., & Grau, J. W. (2007). Instrumental learning in the spinalized rat: A therapeutic effect of BDNF. In *2007 Abstract Viewer/Itinerary Planner*, Washington, DC: Society for Neuroscience.

Impey, S., Obrietan, K., & Storm, D. R. (1999). Making new connections: Role of ERK/MAP kinase signaling in neuronal plasticity. *Neuron*, 23, 11-14.

Ji, R. R., Baba, H., Brenner, G. J., & Woolf, C. J. (1999). Nociceptive-specific activation of ERK in spinal neurons contributes to pain hypersensitivity. *Nature Neuroscience*, 2, 1114-1119.

Ji, R. R., Kohno, T., Moore, K. A., & Woolf, C. J. (2003). Central sensitization and LTP: do pain and memory share similar mechanisms? *Trends in Neuroscience*, 26(12), 696-705.

Joynes, R. L., & Grau, J. W. (1996). Mechanisms of Pavlovian conditioning: The role of protection from habituation in spinal conditioning. *Behavioral Neumscience*, 110, 1375-1387.

Joynes, R. L., Janjua, K. R., & Grau, J. W. (2004). Instrumental learning within the spinal cord: VI. Disruption of learning by the NMDA antagonist APV. *Behavioural Brain Research*, 154, 431-438.

Kangrga, I., & Randic, M. (1990). Tachykinins and calcitonin gene-related peptide enhance release of endogenous glutamate and aspartate from the rat spinal dorsal horn slide. *Journal of Neuroscience*, 10, 2026-2038.

Kawasaki, Y., Kohno, T., Zhuang, Z. Y., Brenner, G. J., Wang, H., Van Der Meer, C., et al. (2004). Ionotropic and metabotropic receptors, protein kinase A, protein kinase C, and Src contribute to C-fiber-induced ERK activation and cAMP response element binding protein phosphorylation in dorsal horn neurons, leading to central sensitization. *Journal of Neuroscience*, 24, 8310-8321.

Kuczewski, N., Langlois, A., Fiorentino, H., Bonnet, S., Marissal, T., Diabira, D., et al. (2008). Spontaneous glutamatergic activity induces a BDNF-dependent potentiation of GABAergic synapses in the newborn rat hippocampus. *Journal of Physiology*, 586, 5119-5128.

Lever, I. J., Pezet, S., McMahon, S. B., & Malcangio, M. (2003). The signaling components of sensory fiber transmission involved in the activation of ERK MAP kinase in the mouse dorsal horn. *Molecular & Cellular Neuroscience*, 24, 259-270.

Maier, S. F., & Seligman, M. E. P. (1976). Learned helplessness: Theory and evidence. *Experimental Psychology*, 105, 3-46.

Malenka, R. C. (2003). The long-term potential of LTP. *Nature Reviews Neuroscience*, 4(11), 923-926.

Melzack, R., & Wall, P. D. (1965). Pain mechanisms: A new theory. *Science*, 50, 971-979.

Mendell, L. M. (1984). Modifiability of spinal synapses. *Physiological Reviews*, 64, 260–324.

Morris, R. G. M. (1989). Synaptic plasticity and learning: Selective impairment of learning and blockade of long-term potentiation in vivo by the N-methyl-D-aspartate receptor antagonist, AP5. *Journal of Neuroscience, 9,* 3040-3057.

Morris, R. G. M., Anderson, E., Lynch, G., & Baudry, M. (1986). Selective impairment of learning and blockade of long-term potentiation by an N-methyl-D-aspartate receptor antagonist, AP5. *Nature*, 319, 774-776.

National Spinal Cord Injury Statistical Center (NSCISC). (2008). *Spinal cord injury: Facts and figures at a glance*. Retrieved December 1, 2008, from University of Alabama at Birmingham, NSCISC Website: http://www.spinalcord.uab.edu

Okano, K., Kuraishi, Y., & Satoh, M. (1998). Involvement of spinal substance P and excitatory amino acids in inflammatory hyperalgesia in rats. *Japanese Journal of Pharmacology*, 76, 15-22.

Patton, B. C., Hook, M. A., Crown, E. D., Ferguson, A. R., & Grau, J. W. (2004). The behavioral deficit observed following noncontingent shock in spinalized rats is prevented by the protein synthesis inhibitor cycloheximide. *Behavioral Neuroscience*, 118, 653-658.

Perkinton M. S., Sihra T. S. & Williams R. J. (1999). Ca(21)-permeable AMPA receptors induce phosphorylation of cAMP response element-binding protein through a phosphatidylinositol 3-kinase-dependent stimulation of the mitogen-activated protein kinase signaling cascade in neurons. *Journal of Neuroscience*, 19, 5861-5874.

Roberson, E. D., English, J. D., Adams, J. P., Selcher, J. C., Kondratick, C., & Sweatt, J. D. (1999). The mitogen-activated protein kinase cascade couples PKA and PKC to cAMP response element binding protein phosphorylation in area CA1 of hippocampus. *Journal of Neuroscience*, 19, 4337–4348.

Seger, R., Ahn, N. G., Boulton, T. G., Yancopoulos, G. D., Panayotatos, N., Radziejewska, E., et al. (1991). Microtubule-associated protein 2 kinases, ERK1 and ERK2, undergo autophosphorylation on both tyrosine and threonine residues: implications for their mechanism of activation. *Proceedings of the National Academy of Sciences of the United States of America*, 88(14), 6142-6146.

Seger, R., & Krebs, E. G. (1995). The MAPK signaling cascade. *The Federation of American Societies for Experimental Biology Journal*, 9, 726-735.

Silva, A. J., Stevens, C. G., Tonegawa, S., & Wang, Y (1992). Deficient hippocampal long-term potentiation in calcium-calmodulin kinase II mutant mice. *Science*, 257, 201-206.

Sivakumaran, S., Mohajerani, M. H., & Cherubini, E. (2009). At immature mossy-fiber CA3 synapses, correlated presynaptic activity persistently enhances GABA release and network excitability via BDNF and camp-dependent PKA. *Journal of Neuroscience*, 29, 2637-2647.

Sweatt, J. D. (2001). The neuronal MAP kinase cascade: a biochemical signal integration system subserving synaptic plasticity and memory. *Journal of Neurochemistry*, 76, 1-10.

Thompson, R. F. & Spencer, W. A. (1966). Habituation: A model phenomenon for the study of neuronal substrates of behavior. *Psychological Review*, 73, 16-43.

Tyler, W. J., Alonso, M., Bramham, C. R., & Pozzo-Miller, L. D. (2002). From acquisition to consolidation: On the role of brain-derived neurotrophic factor signaling in hippocampal-dependent learning. *Learning & Memory*, 9(5), 224-237.

Walters, E. T. (1994). Injury-related behavior and neuronal plasticity: An evolutionary perspective on sensitization, hyperalgesia, and analgesia. *International Review of Neurobiology*, 36, 325-427.

Werry, E. L., Liu, G. J., & Bennett, M. R. (2006). Glutamate-stimulated ATP release from spinal cord astrocytes is potentiated by substance P. *Journal of Neurochemistry*, 99, 924-936

Willis, W. D. (2001a). Role of neurotransmitters in sensitization of pain responses. *Annals of the New York Academy of the Sciences, 933,* 142–156.

Willis, W.D. (2001b). Mechanisms of central sensitization of nociceptive dorsal horn neurons. In M. M. Patterson & J. W. Grau (Eds.), *Spinal cord plasticity: Alterations in reflex function* (pp. 127-162). Boston: Kluwer Academic.

Willis, W.D. (2002). Long-term potentiation in spinothalamic neurons. *Brain Research Reviews*, 40, 202-214.

Woolf, C. J., & Thompson, S. W. N. (1991). The induction and maintenance of central sensitization is dependent on N-methyl-D-aspartic acid receptor activation: Implications for the treatment of post-injury pain hypersensitivity states. *Pain*, 44, 293-299.

Xu, X. J., Zhang, X., Hokfelt, T., Wiesenfeld-Hallin Z. (2005). Plasticity in spinal nociception after peripheral nerve section: Reduced effectiveness of the NMDA receptor antagonist MK-801 in blocking wind-up and central sensitization of the flexor reflex. *Brain Research*, 670, 342-346.

Yang, H.-W., Hu, X.-D., Zhang, H.-M., Xin, W.-J., Li, M.-T., Zhang, T., et al. (2004). Roles of CaMKII, PKA, and PKC in the induction and maintenance of LTP of C-fiber evoked field potentials in rat spinal dorsal horn. *Journal of Neurophysiology*, 91, 1122–1133.

Young, E. E., Baumbauer, K. M., Elliott, A. E., Joynes, R. L. (2007a). Lipopolysaccharide induces a spinal learning deficit that is blocked by IL-1 receptor antagonism. *Brain Behavior & Immunity*, 21, 748-757.

Young, E. E., Baumbauer, K. M., Elliott, A. E., Joynes, R. L. (2007b). Neonatal hind-paw injury disrupts acquisition of an instrumental response in adult spinal rats. *Behavioral Neuroscience*, 121, 1095-1100.

Zhang, X., Wu, J., Lei, Y., Fang, L., & Willis, W.D. (2005). Protein phosphatase modulates the phosphorylation of spinal cord NMDA receptors in rats following intradermal injection of capsaicin. *Brain Research. Molecular Brain Research*, 138, 264-272.

Zhou, D., Fei, M., Shen, Q., Cheng, C., Wang, Y., Zhao, J., et al. (2008). Phosphorylation of extracellular signal-regulated kinases 1/2 predominately enhanced in the microglia of the rat spinal cord following lipopolysaccharide injection. *Cellular & Molecular Neurobiology*, 28(6), 867-874.

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