

THE IMPACT OF GLIAL INHIBITION ON THE SPINAL INSTRUMENTAL
LEARNING PARADIGM

A Thesis

by

ELISABETH GOOD VICHAYA

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

MASTER OF SCIENCE

August 2007

Major Subject: Psychology

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Approved by:

Chair of Committee, Mary W. Meagher

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ABSTRACT

The Impact of Glial Inhibition on the Spinal

Instrumental Learning Paradigm. (August 2007)

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Chair of Advisory Committee: Dr. Mary W. Meagher

Although neural plasticity has traditionally been studied within the brain, evidence indicates that the spinal cord is quite plastic as well. Spinal neurons can even support a simple form of instrumental learning (Grau et al., 1998), as indicated by spinally transected rats' ability to exhibit an increase in hind limb flexion duration when limb extension is associated with shock (controllable shock). If limb extension is not associated with shock (uncontrollable shock), a learning deficit develops. Recent research indicates that other forms of plasticity, such as long-term potentiation and central sensitization, do not depend on neural activity alone, but also on glial cells. I examined whether glial cells are also necessary in spinal instrumental learning and the learning deficit. Therefore, two glial inhibitors were selected: minocycline and fluorocitrate. To examine the role of glial cells in spinal instrumental learning, rats received intrathecal minocycline, fluorocitrate, or saline prior to testing with 30-minutes of controllable leg-shock.

Results indicate that both drugs dose-dependently reduced acquisition, with higher doses resulting in shorter response durations. Once the response was acquired, fluorocitrate did not alter response maintenance. This suggests that glial cells are involved in the acquisition, but not the maintenance, of spinal learning. To examine the

role of glial cells in the spinal learning deficit rats were given intrathecal minocycline, fluorocitrate, or saline prior to testing with 6-minutes of uncontrollable tail shock or no shock. Twenty-four hours later all rats were tested with 30-minutes of controllable leg-shock. Results indicated the learning deficit induced by uncontrollable shock was prevented by prior administration of fluorocitrate. Minocycline did not prevent the deficit; moreover, it appears that even in the absence of shock, minocycline caused a learning deficit. Overall, this data indicate that glial cells are necessary for the acquisition of spinal instrumental learning and the learning deficit. Furthermore, it provides further evidence for the role of glial cells in plasticity.

DEDICATION

To my father, mother, and husband.

ACKNOWLEDGEMENTS

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INTRODUCTION

Neural plasticity is the ability of the central nervous system (CNS) to reorganize neural pathways based on experience, development, or injury. This process underlies learning and memory as well as recovery of function after neurological injury and the development of pathological pain. Although neural plasticity has traditionally been studied within the brain, evidence indicates that the spinal cord is quite plastic as well. It has been shown to be capable of habituation and sensitization, as well as Pavlovian and instrumental conditioning (Egger, 1978; Grau et al., 1998; Joynes & Grau, 1996).

Instrumental learning involves a change in behavior based on a response-outcome contingency. The rat spinal instrumental learning paradigm was developed by Grau and colleagues (1998) and provides a well-characterized model of plasticity. In this model a spinally transected rat demonstrates an increase in response duration when leg extension is associated with shock. This is termed controllable shock, because a behavioral response determines the onset and offset of the shock.

More specifically, a rat is transected at the second thoracic vertebrate (T2). After a 24 h recovery period, rats are tested with 30 min of controllable shock to the tibialis anterior muscle whenever their leg falls below a set criterion. The shock produces a reflexive upward flexion of the leg, which terminates the shock. The training session is divided into 30 1-min bins. Over time rats exhibit increased response durations, which minimizes shock exposure and indicates that subjects are learning the relationship

This thesis follows the style of *Behavioral Neuroscience*.

between shock and leg position (Grau et al., 1998). Furthermore, learning this relationship on one leg enhances learning on the contralateral leg.

Yoked controls verify that these effects are due to learning a response-outcome contingency and not merely shock exposure. Yoked subjects were exposed to the identical shock regimen, however it was administered in a manner that they could not control. The yoked subject was coupled to a subject being trained with controllable shock (master subject), in that both subjects received a shock when the master subject's leg fell below criterion. In this design master subjects show a progressive increase in response duration, while the yoked subjects do not exhibit this increase (Grau et al., 1998). Furthermore, if the yoked rats were later tested with controllable shock on the same or contralateral leg they exhibit a learning deficit. This deficit lasts up to 48 hours and can be induced by as little as 6-min of uncontrollable leg or tail shock (Crown et al., 2002a).

The aforementioned research provides compelling evidence that a rat can learn the relationship between a response and an outcome in the absence of brain mediated activity and the contralateral transfer observed suggested that this learning is in fact spinally mediated. This observation was further established by interrupting communication between the periphery and the spinal cord, through cutting the sciatic nerve (the primary source of innervation from the region of the leg shock), which prevented both learning and the deficit (Crown et al., 2003b; Joynes et al., 2003). Furthermore, intrathecal administration of lidocaine (a Na²⁺ channel blocker), which

prevents neuronal action potentials, blocked spinal instrumental learning and protected against the development of the learning deficit (Crown et al., 2003b; Joynes et al., 2003).

Given that spinal cord neurons mediate spinal instrumental learning, this paradigm provides a model of neural plasticity, and understanding the mechanisms that underlie learning in this paradigm may provide insight into the mechanisms of plasticity in general. Furthermore, it provides valuable information on spinal plasticity, which is clinically important for developing therapies and interventions for spinal cord injuries and the prevention and treatment of pathological pain.

Glial Cells

It is well established that plasticity is neurally mediated, however, recent research suggests that non-neural, or glial, cells within the CNS may play an important modulatory role. Glial cells are located in all regions of the CNS and vastly outnumber neurons. There are many types with a wide variety of functions.

For example, oligodendrocytes myelinate axons to enhance the speed of signal propagation. Ependymal cells form the epithelial lining of the CNS. Radial glia function both as the scaffolding upon which neurons migrate and as neural progenitors in the developing nervous system. In the mature CNS, characteristic radial glial cells are only retained in the cerebellum, Bergmann glia, and the retina, Müller cells.

Astrocytes are the most abundant glial cell type and have characteristic star-shaped morphology. Due to their highly branched processes that intertwine with neurons, they are well positioned to provide structural support to neurons. They are essential in the regulation of extracellular and synaptic ion concentrations, particularly

glutamate and potassium. Astrocytes also metabolically support neurons, providing both lactate and glutamine (glutamate precursors) to neurons. They have end feet that encircle endothelial cells and form part of the blood brain barrier (BBB). When astrocytes encounter transmitters such as adenosine triphosphate (ATP), cytokines, and glial derived proteins, they increase their activation state and become more branched. They respond to increases in intracellular Ca^{2+} by releasing glutamate and ATP (Haydon, 2001; Jourdain et al., 2007). Additionally, Ca^{2+} waves can propagate through astrocyte networks via gap junctions, allowing their impact to be wide reaching. They also release D-serine, reactive oxygen species (ROS), cytokines, and NO, although it is not known if these are released in a Ca^{2+} dependent fashion.

Finally, microglia, which make up about 15 percent of the cells in the CNS, are specialized macrophages that serve to protect neurons. In their basal state they are highly branched and constantly sample from their environment. If they encounter a triggering substance, such as viruses, bacteria, or inflammatory products, their activation state heightens and they move toward the source of activation. They can also begin to release a variety of transmitters, such as proinflammatory cytokines (interleukin [IL]-1, IL-6, and tumor necrosis factor-alpha [TNF- α]), nitric oxide (NO), D-serine, and brain-derived neurotrophic factor (BDNF).

For many of the products that are released by glial cell there are corresponding receptors on neurons. Additionally, glia can respond to neurotransmitters, such as glutamate and ATP. Therefore, it has been shown that they are able to communicate with neurons in a bi-directional fashion (Allen & Barres, 2005; Araque et al., 2001; Fields &

Stevens-Graham, 2002; Haydon, 2001; Lalo et al, 2006; Todd et al., 2006; Vernadakis, 1996), which makes their location near the synapse noteworthy. Traditionally the synapse has been viewed as the junction where two neurons communicate. Synaptic plasticity is an enhancement or depression of this communication. However, recently it has been proposed that the synapse should be viewed as tripartite, including not only the pre-synaptic and postsynaptic neurons but also the synaptically associated glial cells (Araque et al., 1999).

Glial Cells in Synaptic Plasticity

Given that glial cells are able to communicate with neurons it has become important to consider their role in synaptic plasticity. A recent study has even demonstrated that astrocyte-released glutamate can enhance synaptic strength by increasing glutamate release from the pre-synaptic neuron (Jourdain et al., 2007). It appears that this may be one mechanism by which glia are involved in long-term potentiation (LTP). LTP is a persistent increase in synaptic strength due to high-frequency activation. It is considered the major mechanism underlying learning and memory, processes that appear to be glially modulated (Gibbs et al., 2006a and 2006b; Hertz et al., 1996; Hydén & Egyházi, 1963; O'Dowd et al., 1994; Tanaka et al., 2006).

The induction of LTP is the result of Ca^{2+} increases in the postsynaptic neuron. This usually occurs in an N-methyl d-aspartate (NMDA) receptor mediated fashion. The process begins with the pre-synaptic neuron's release of glutamate. This glutamate activates both alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and NMDA receptors. The AMPA channels open quickly and allow ions such as Na^{2+} to

begin depolarizing the postsynaptic neuron. The NMDA receptor, however, is a ligand-gated channel that is blocked with a Mg^{2+} ion even after it has been stimulated by glutamate. The Mg^{2+} ion can be removed in response to high levels of depolarization and stimulation from a co-agonist (e.g., glycine or D-serine) at the strychnine insensitive glycine recognition site. When the NMDA channel is open, Ca^{2+} ions are allowed to enter the postsynaptic terminal and begin a cascade of events that can potentiate (LTP) or depress (LTD) synaptic communication.

One example of the involvement of glial cells in LTP is that stimulation of the sciatic nerve that usually results in LTP, results in LTD in the presence of a glial metabolic inhibitor (Ma & Zhao, 2002). Furthermore, glial transmitters have been shown to influence the development of LTP as well. For example, increased proinflammatory cytokines has been associated with reductions in LTP (Griffin et al., 2006; Kelly et al., 2003). Although high levels of inflammation inhibit learning, low levels may be necessary for learning. For example, Brennan and colleagues (2003) demonstrated that low doses of IL-1 β enhance learning, and Bohme and colleagues (1993) demonstrated that inhibiting NO synthase impairs spatial learning. Exogenous NO also contributes to LTP in hippocampal slices (Bohme et al., 1991; Lu et al., 1999; Malen and Chapman, 1997; Zhuo et al., 1993) and blocking glially released NO disrupts the development of LTP (Ikeda & Murase, 2004).

Additionally, D-serine, another glial cell product, has also been shown to be important in LTP. It is synthesized from L-serine in microglia and synaptically associated astrocytes particularly in regions with NMDA receptors (Diamond, 2006;

Schell et al., 1995; Wu et al., 2004; Yang et al., 2003). D-serine functions as a potent co-agonist at the NMDA strychnine-insensitive glycine recognition site, which allows it to regulate LTP and other forms of NMDA receptor mediated plasticity (Mothet et al., 2000; Oliet and Mothet, 2006; Schell et al., 1997; Wolosker et al., 1999, 2006). Research suggests that D-serine works in conjunction with glutamate; glutamate activates AMPA receptors on glial cells containing D-serine, which causes D-serine to be released so that glutamate and D-serine can act together on the postsynaptic NMDA receptors (Mustafa et al., 2004).

The involvement of glial cells has not only been noted in other forms of plasticity, such as central sensitization. Central sensitization is a process during which nociceptive neurons of the spinal cord dorsal horn exhibit amplified responses due to repeated stimulation. This process appears to underlie the development of chronic pathological pain conditions. Meller and colleagues (1994) were among the first to suggest a role for glia in pain. They showed that glial inhibitors attenuated inflammation induced thermal and mechanical hyperalgesia. It was then shown that the development of HIV-1 gp120 (Milligan et al., 2001) and formalin (Watkins et al., 1997) induced pain is prevented by glial cell inhibitors. Glial inhibitors can also prevent and reverse mirror image pain (Milligan et al., 2003) and a spinal nerve transection model of neuropathic pain (Sweitzer et al., 2001; Takeda et al., 2004). Not only is glial cell activation necessary for the development and maintenance of pathological pain states, it has been shown that injections of activated microglia are sufficient to set off a cascade of events that leads to the development of pain (Narita et al., 2006).

Aims of This Study

Like LTP and central sensitization, spinal instrumental learning and the learning deficit are NMDA mediated phenomena (Ferguson et al., 2006; Joynes et al., 2004). Additionally, many processes that affect pain processing also affect spinal instrumental learning and the deficit. For example, the stimulation used to induce the learning deficit also enhances reactivity to mechanical stimulation (Ferguson et al., 2006). Additionally, inflammatory substances, such as capsaicin and carrageenan, produce pain and learning deficits (Hook et al., 2005; Ferguson et al., 2006). Similarly, early peripheral injury leads to life-long enhanced pain responsivity and spinal learning deficits (Young et al., 2007).

Further evidence indicates that stimuli that prevent or disrupt pathological pain states often protect against the learning deficit. For example, blocking the activity of substance P, a neurotransmitter involved in the transmission of pain impulses, by antagonizing the substance P neurokinin (NK-1) receptor, also blocks the development of the spinal instrumental learning deficit (Baumbauer et al., 2005). Additionally, agonists of serotonin and the 5HT serotonin receptor block pathological pain and the spinal instrumental learning deficit (Crown & Grau, 2005).

Due to the involvement of glial cells in other forms of related neural plasticity the following series of experiments was designed to evaluate the necessity of glial cells in spinal instrumental learning and the learning deficit. Two drugs were selected to pharmacologically block glial cells: minocycline and fluorocitrate. Minocycline is a tetracycline that crosses the BBB and has glial inhibitory and anti-inflammatory effects (Elewa et al., 2006; Lee et al., 2004; Yrjanheikki et al., 1999) completely unrelated to its

antimicrobial effects. Research indicates that minocycline exerts its effects through blocking p38 MAP kinase activity (Hua et al., 2005, Tikka et al., 2001). Because p38 MAP kinase is primarily observed in microglia (Hua et al., 2005), it is thought to directly block microglia; however, evidence indicates that other forms of glia are also be affected (e.g., Miller et al., 2006; Ryu et al., 2004).

Fluorocitrate is a suicide substance for aconitase, a necessary product of the tricarboxylic acid (TCA) cycle. By inhibiting the TCA cycle, energy dependent transmitter up-take and release is inhibited. The selectivity of this drug comes from its preferential uptake by glial cells. At low doses its actions are both selective and reversible (Paulson et al., 1987).

In Experiments 1 and 2 the effect of minocycline on spinal learning and the learning deficit was explored. Results indicate that minocycline disrupts learning in a dose dependent fashion, but did not protect against the development of the learning deficit. Rather, minocycline appeared to cause a learning deficit even in the absence of shock, indicating that it has long lasting inhibitory effects on learning. In Experiments 3, 4, and 5 examined the effects of fluorocitrate on the acquisition and maintenance of learning and the induction of the deficit were explored. Fluorocitrate also disrupted learning in a dose dependent fashion. It did not, however, disrupt the maintenance of already acquired learning. Additionally, fluorocitrate prevented the development of the learning deficit. As a whole, this data demonstrates that glial cells modulate spinal instrumental learning and the learning deficit, which provides further evidence for the role of glial cells in neural plasticity.

GENERAL METHOD

Subjects

Male Sprague-Dawley rats obtained from Harlan (Houston, TX) were used in all experiments. At the time of testing, animals were approximately 100-120 days old and weighed between 350 and 410 g. They were individually housed on a 12 h light-dark cycle with food and water available ad libitum. All procedures were performed during the light portion of their cycle and were approved by the Texas A&M University Laboratory Animal Care Committee.

Transection and Cannula Placement

Subjects were anesthetized with 2% isofluorine gas, except in experiment three where 50 mg/kg of pentobarbital was used. Once the animal was no longer pain responsive, the shoulder area was shaved and sterilized with iodine; the hind legs were also shaved in preparation for instrumental testing. The protuberance of the 2nd thoracic vertebra (T2) was localized by touch and an approximately 1.5 cm long anterior-posterior incision was made. The tissue above T2 was cleared to expose the spinal cord. Then the cord was transected by cauterization, and the cavity was filled with Gelfoam (Harvard Apparatus, Holliston, MA). The cannula, consisting of polyethylene tubing (PE-10, VWR International, Bristol, CT) cut to 25cm and fitted with a stainless steel wire (0.09 mm diameter; Small Parts Inc., Miami Lakes, FL), was inserted 9 cm down the dorsal surface of the cord via the subarachnoid space. The cannula was secured to with glue to the anterior portion of the incision, and the incision was closed with Michel

Clips (Fine Science Tools, Foster City, CA). The wire guide was then removed from the cannula.

Post-Surgical Care

Immediately following surgery, rats were given a 5 mL intraperitoneal (ip) injection of 0.9% sterile saline. In order to prevent damage, their hind legs were secured to their body in a natural flexed position with porous tape (Ortholetic 1.3 cm width). The rats recovered in a temperature-controlled room (26.7°C) with food and water available *ad libitum*. Because the surgery causes loss of bladder function, rats were expressed twice a day and immediately prior to any behavioral testing. Additionally, they were supplemented with 5 mL of saline, ip, every 8 to 12 h. Upon completion of all necessary testing, rats were euthanized with 100 mg/kg of pentobarbital.

Controllable Leg Shock Apparatus and Procedure

Spinal instrumental learning was assessed using controllable leg shock. The rat was loosely restrained in an opaque black Plexiglas tube (8 cm diameter x 23.5 cm length; as described by Grau et al., 1998). Notches in the tube allowed subjects hind limbs to dangle. The leg used for testing was counterbalanced across subjects.

The shock generator (BRS/LVE Model SG-903, Laurel, MD) was used to administer shock. Two electrodes, one inserted in the skin and another into the tibialis anterior muscle, were connected to a computer-controlled relay that regulated the administration of shock. The first electrode consisted of a stainless steel wire inserted through the rats skin over the tibia, 1.5 cm above the tarsals. A lead from the generator was attached to this wire. The pin for the second electrode was inserted 1.7 cm above the

first. To insert the pin the shock generator was set to deliver a 0.4 mA shock and the area was probed with a barbed 2.5 cm stainless steel pin. Once a point that produced a robust flexion response was located, the pin was inserted 0.4 cm into the muscle. A lead was also attached to this pin.

Limb position was monitored using a contact electrode consisting of a 7-cm 0.46 mm diameter stainless steel rod. The proximal end was insulated with 2.5 cm of heat shrink tubing and was attached to a fine wire (0.01 sq mm [36 AWG], [1] x 20 cm) connected to a digital input monitored by a Macintosh computer. The proximal end of the electrode was secured with porous tape to the plantar surface of the foot just distal to the plantar protuberance. The limb was stabilized by wrapping a piece of porous tape (about 20 cm in length) around the leg and by attaching it directly under the restraint tube. The shock intensity was then adjusted in order to produce a flexion force of 0.4 N with a 0.3-s shock. To measure flexion force, a plastic line (4 lb test Stren; Dupont, Wilmington, DE) was connected to the rat's foot immediately behind the planter protuberance. The line passed through an eyelet directly under the foot of the rat and attached to a strain gauge (Fort-1000, World Precision Instruments, New Haven, CT) fastened to a ring stand. The ring stand was positioned to make the line was taut and register a low gauge. The reading on the gauge was monitored while a shock was administered. Given that the relationship between the voltage registering on the gauge and force in newtons (N) was previously determined, the shock voltage could be adjusted to produced a 0.4 N force.

Once the necessary voltage was determined, the line was removed and the flexion force apparatus was put aside. A dish of NaCl solution (with a drop of detergent to reduce surface tension) was placed below the contact electrode. Three short (0.15 s) shock pulses were then delivered and the water depth was adjusted so that the tip of the contact electrode, which is attached to the rat's foot, was submerged 4 mm below the surface of the solution. A ground wire was placed in the solution.

During testing each time the contact electrode touched the solution the circuit was completed and a shock was administered to the tibialis anterior muscle. The shock would cause the foot to be drawn out of the solution, which terminated the shock. A computer monitored the state of the circuit at a rate of 30 Hz.

Uncontrollable Tail Shock

Uncontrollable tail shock was administered while the rats were loosely restrained in opaque black Plexiglas tubes (8 cm diameter x 23.5 cm length). Shock was delivered to the tail using an electrode constructed from a modified fuse clip. The clip was coated with electrocardiogram (ECG) gel (Harvard Apparatus, Holliston, MA) and secured 6 cm behind the base of tail with porous tape. A 660-V transformer administered constant-current 1.5 mA shocks on a computer-controlled random schedule. The shock duration was 0.08 s and there was an inter-shock-interval between 0.2 and 3.8 s. The procedure lasted for 6 min.

Behavioral Measures

During testing with controllable shock, three behavioral measures were collected: time in solution, response number, and response duration. These measures were used to assess the capacity of the rat to perform an instrumental response. Performance on these measures was taken in 1-min bins for the duration of the controllable shock. Response number increased whenever the contact electrode left the solution. Response duration was derived from the time in solution during a bin and the response number during that time using the following equation: Response Duration = $(60 \text{ s} - \text{time in solution}) / (\text{response number} + 1)$.

Drug Administration

Fluorocitrate (Sigma-Aldrich, F9634) was dissolved in a saline vehicle at concentrations of 0.0078, 0.0625, 0.5, and 4.0 nmol per 1 μl of saline. The drug or an equal volume of vehicle was administered into the implanted cannula and flushed through with 20 μl of vehicle. Minocycline (Sigma-Aldrich, M9511) was dissolved in saline vehicle at concentration of 6.25, 25, or 100 μg per 10 μl of saline. The drug or an equal volume of vehicle was administered into the implanted cannula and flushed through with 20 μl of vehicle.

Statistics

The effect of experimental treatment was analyzed using mixed analysis of variance (ANOVA). Additionally, baseline shock thresholds were analyzed with one-way or two-way ANOVAs, as appropriate. When necessary, a Tukey's honestly significant difference (HSD) *post hoc* test was performed. To clarify statistical analysis a trend analysis of linear contrasts was also performed. For all analysis, an alpha value equal to or less than 0.05 was considered significant.

EXPERIMENT 1: EFFECT OF MINOCYCLINE ON SPINAL INSTRUMENTAL LEARNING

Minocycline is associated with glial cell inhibition through the p38 MAP kinase pathway. This inhibition is associated with anti-inflammatory effects, which have been shown to enhance recovery after spinal cord injuries (Lee et al., 2003; Stirling et al., 2004; Teng et al., 2004; Wells et al., 2003), reduce pathological pain (Hua et al., 2005; Ledebor et al., 2005; Mika et al., 2007), and improve neurodegenerative disease outcomes (Chen et al., 2000; Du et al., 2001; Ryu et al., 2004; Wu et al., 2002). It is also known that minocycline has been associated with attenuated inflammation induced spatial learning deficits (Fan et al., 2007; Liu et al., 2007) and inhibited LTP (Wang et al., 2004). Other research, however, suggests that proper glial cell function is necessary for synaptic plasticity (Gibbs et al., 2006a & 2006b; Hertz et al., 1996; O'Dowd et al., 1994). Therefore, this study proposes to explore the impact of minocycline on spinal instrumental learning.

Method

Rats were transected at T2 and a catheter was implanted 9 cm below site of transection. After a 24-h recovery period, rats were given 10 μ L intrathecal infusions of minocycline (6.25, 25, or 100 μ g) or vehicle. After 20-min, rats were given 30-min of controllable shock.

Results

Figure 1 depicts the effects of minocycline on baseline behavioral reactivity. To examine whether drug treatment affects baseline behavioral reactivity, the shock

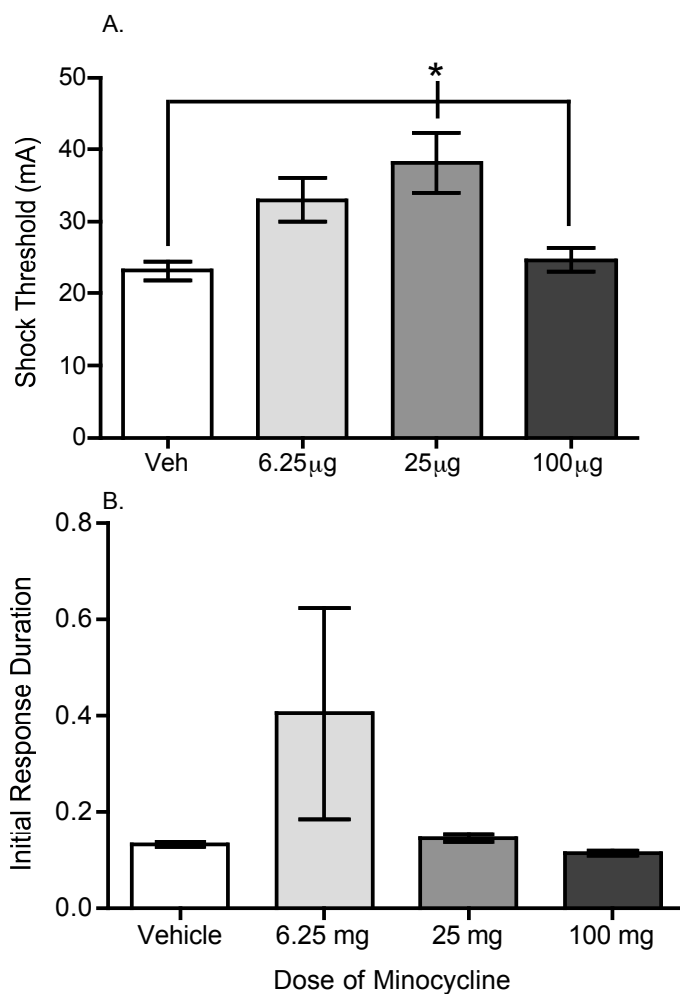


Figure 1. The effect of minocycline on behavioral reactivity. Transected subjects received minocycline (6.25, 25, 100 µg) or vehicle 20-min before testing with 30-min of controllable shock. To determine whether minocycline affects a subject's ability to perform the behavioral response, we examined the effect of minocycline on the shock threshold required to produce a 0.4 mA flexion force (A) and on initial response duration (B). An asterisk (*) indicates that Tukey *post hoc* indicated that there was a statistically significant difference ($p < .05$).

intensity required to produce a 0.4 N change in flexion force prior to controllable shock and the initial response duration were analyzed. A one-way ANOVA indicated that there was an effect of dose, $F(3,23) = 6.33, p < .05$, (Figure 1A). A Tukey *post hoc* revealed that the 25 μg dose was significantly different from the vehicle and the 100 μg dose ($p < .05$). However, an analysis of initial flexion duration, which measures the initial behavioral response to the shock, revealed that there was no difference between the drug treatment groups, $F(3,23) = 1.56, p > .05$ (Figure 1B). To verify that differences in flexion force do not explain the differences reported below, flexion force was used as a covariate for the subsequent analyses.

The impact of minocycline on spinal learning is shown in Figure 2. Subjects given the vehicle alone exhibited a progressive increase in flexion duration across the 30 min of training. This learning was blocked in a dose dependent fashion by minocycline. A repeated measures analysis of covariance (ANCOVA) of response duration controlling for flexion force was performed. The analysis revealed significant main effects of time, $F(29,551) = 1.77, p < .05$, and dose, $F(3,19) = 3.62, p < .05$, indicating that flexion duration increased over time and decreased with increasing doses of minocycline. These main effects were qualified by a significant interaction between dose and time, $F(87,551) = 1.92, p < .05$, which indicates that the change in performance observed over time was attenuated by minocycline in a dose-dependent manner (Figure 2A). A Tukey *post hoc* test indicated that there was a statistically significant difference between the 100 μg group and the vehicle group (Figure 2B).

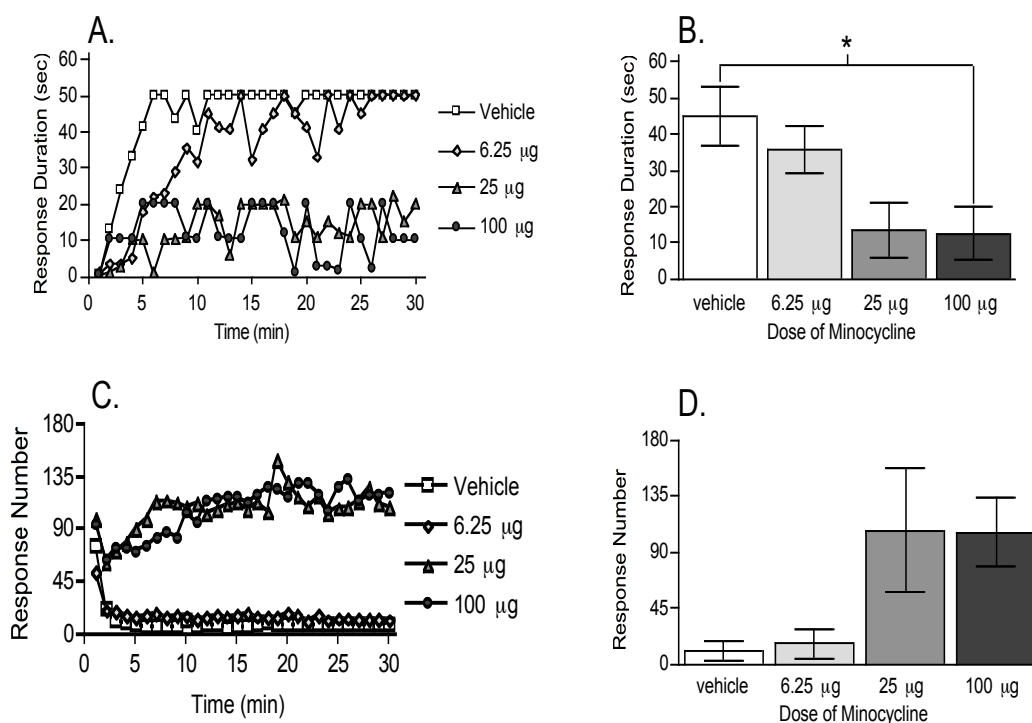


Figure 2. The effect of minocycline on instrumental learning and performance. Transected subjects received minocycline (6.25, 25, 100 μg) or vehicle 20-min before testing with 30-min of controllable shock. Minocycline affected learning in a dose-dependent fashion. Changes in response duration across time (A) and mean (\pm SEM) response durations are depicted (B). Furthermore, changes in response number across time (C) and mean (\pm SEM) response numbers (D) were analyzed. An asterisk (*) indicates that Tukey *post hoc* indicated that there was a statistically significant difference ($p < .05$).

There was no main effect of flexion force, $F(1, 19) = 2.14$, $p > .05$, nor an interaction between time and flexion force $F(29, 551) < 1.0$, $p > .05$.

The impact of minocycline on spinal learning is shown in Figure 2. Subjects given the vehicle alone exhibited a progressive increase in flexion duration across the 30 min of training. This learning was blocked in a dose dependent fashion by minocycline. A repeated measures analysis of covariance (ANCOVA) of response duration controlling for flexion force was performed. The analysis revealed significant main effects of time, $F(29, 551) = 1.77$, $p < .05$, and dose, $F(3, 19) = 3.62$, $p < .05$, indicating that flexion duration increased over time and decreased with increasing doses of minocycline. These main effects were qualified by a significant interaction between dose and time, $F(87, 551) = 1.92$, $p < .05$, which indicates that the change in performance observed over time was attenuated by minocycline in a dose dependent manner (Figure 2A). A Tukey *post hoc* test indicated that there was a statistically significant difference between the 100 μg group and the vehicle group (Figure 2B). There was no main effect of flexion force, $F(1, 19) = 2.14$, $p > .05$, nor an interaction between time and flexion force $F(29, 551) < 1.0$, $p > .05$.

The performance differences observed were not due to the drug treatments disrupting the subject's capacity to make the targeted response. The drug treated groups actually exhibit a higher rate of responding, while the vehicle treated group progressively made fewer responses, as they learned the task (Figure 2C). A repeated measures ANCOVA of response numbers revealed a significant effect of dose, $F(3, 19) = 3.47$, $p < .05$, while time, flexion force, and the interactions were non-significant, all

$F_s < 1.2$, $p_s > .05$. Given that there was no indication that drug administration affected a subject's capacity to perform the behavioral response, these data are not reported for subsequent experiments.

Discussion

Minocycline disrupted spinal instrumental learning in a dose dependent fashion. The vehicle treated group progressively acquired and maintained the instrumental response, while higher doses of minocycline caused greater impairments. These findings suggest that glial cells play an essential role in spinal instrumental learning.

EXPERIMENT 2: EFFECT OF MINOCYCLINE ON THE SPINAL INSTRUMENTAL LEARNING DEFICIT

Exposure to uncontrollable shock causes subsequent learning deficits. This deficit can be prevented by many substances that prevent pathological pain, such as NK-1 receptor antagonists and 5HT agonists (Baumbauer et al., 2005; Crown & Grau, 2005). Given that glial cell inhibition can prevent pathological pain (Hains & Waxman, 2006; Hua et al., 2005; Lan et al., 2007; Ledebuer et al., 2005; Mika et al., 2007; Obata et al., 2006; Qin et al., 2006), this experiment sought to determine if it would also prevent the spinal instrumental learning deficit.

Method

Rats were transected at T2 and a catheter was implanted 9 cm below site of transection. After a 24-h recovery period, rats were given 10 μ L intrathecal infusions of minocycline (25 or 100 μ g) or vehicle. After 20-min, rats were given 6-min of uncontrollable shock or no shock. Twenty-four hours later all of the rats received 30-min of controllable shock.

Results

To verify that the drug did not impact baseline behavioral reactivity, the initial shock intensity required to produce a 0.4 N change in flexion force and initial response duration were analyzed. ANOVAs indicated that there was no effect of drug on required flexion force, $F(2, 29) < 1.0, p > .05$, nor on initial response duration, $F(2,30) = 1.24, p > .05$. Additionally, pre-treatment with uncontrollable shock did not affect required flexion force, $F(1,29) = 2.14, p > .05$, nor initial response duration, $F(1,30) < 1.0, p > .05$.

Figure 3 depicts the effects of minocycline on the learning deficit induced by 6 min of uncontrollable tail shock. As usual, vehicle treated unshocked rats exhibited a progressive increase in flexion duration, while vehicle treated shocked rats exhibited a learning deficit (Figure 3A). Pre-treatment with minocycline does not appear to prevent the development of the learning deficit; rather minocycline, even in the absence of shock, produced learning deficits (Figure 3B and 3C). These impressions were verified by a repeated measure ANOVA. There was a main effect of time, $F(29,870) = 6.30, p < .001$, and shock, $F(1,30) = 11.87, p < .05$, indicating that flexion duration increased over time and decreased due to shock. The time by shock interaction also reached significance, $F(29,870) = 2.72, p < .05$, and indicates that unshocked subjects exhibit a progressive increase in flexion duration that is not observed in the uncontrollably shocked subjects. Given that the drug by shock interaction is non-significant, $F(2,30) < 1.0, p > .05$, we can conclude that minocycline did not prevent the acquisition of the spinal instrumental learning deficit.

A time by shock by drug interaction, $F(58,870) = 1.4, p < .05$, was also noted. This three-way interaction indicates that drug doses moderated the performance differences observed over time between the unshocked and shocked groups. A trend analysis was performed on just the unshocked groups to further elucidate the effect of dose. This analysis indicated that there was a significant dose by time linear contrast, $F(29,435) = 1.56, p < .05$, suggesting that minocycline inhibited learning in the absence of shock even 24 h after injection.

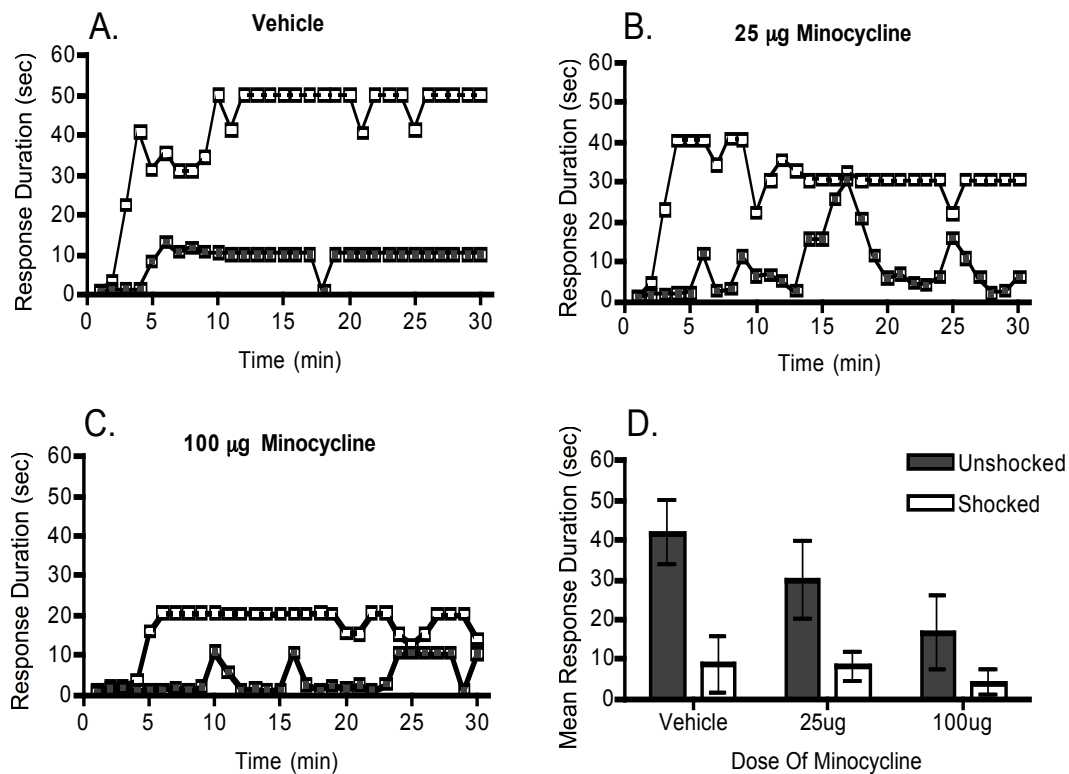


Figure 3. The effect of minocycline on the development of the instrumental learning deficit. Transected subjects received minocycline (25 or 100 µg) or vehicle 20-min before uncontrollable shock or no shock. Rats were tested with controllable shock 24 hours later. Uncontrollable shock caused a learning deficit that minocycline did not protect against. Changes in response duration across time during controllable shock in subjects injected with vehicle (A), 25 µg of minocycline (B), and 100 µg of minocycline (C) prior to uncontrollable or no shock are depicted. Mean (\pm SEM) response durations for all groups are also depicted (D).

Discussion

As expected a learning deficit was produced by prior exposure to uncontrollable shock; however, prior administration of minocycline did not prevent this deficit. Not only did minocycline fail to protect against the development of the learning deficit, a trend analysis revealed that minocycline alone causes a deficit in learning even in the absence of uncontrollable shock. This indicates that the effects of minocycline are long lasting. Normally, the half-life of minocycline is 11 to 17 hours in health human volunteers; given that spinal injury can disrupts drug clearance it may be even longer in our subjects (Cruz-Antonio et al., 2006; García-López et al., 2006). More research examining the time course for minocycline in this model is necessary to determine if the failure to quickly clear the drug is occurring. Given that this minocycline data is difficult to interpret, future studies will focus on the effects of fluorocitrate, a shorter acting glial inhibitor, on the spinal instrumental learning paradigm.

EXPERIMENT 3: THE EFFECT OF FLUOROCITRATE ON SPINAL INSTRUMENTAL LEARNING

Results from Experiment 1 indicated that glial cells were involved in spinal instrumental learning, therefore, this experiment sought to provide converging evidence for this finding through the use of a different glial cell inhibitor: fluorocitrate.

Fluorocitrate is a product of fluoroacetate, which has a short half-life of approximately 2 hours (Ponde et al., 2007). It disrupts the TCA cell cycle and prevents energy dependent uptake and release. Additionally, fluorocitrate has previously been shown disrupt learning and memory consolidation (Gibbs et al., 2006; Hertz et al., 1996; O'Dowd et al., 1994).

Method

Rats were transected at T2 and a catheter was implanted 9 cm below site of transection. After a 24-h recovery period, rats were given 1 μ L intrathecal infusions of fluorocitrate (0.0078, 0.0625, 0.5, and 4.0 nmol) or vehicle. After 20-min, rats were given 30-min of controllable shock.

Results

To evaluate whether drug treatment affected baseline behavioral reactivity, the initial shock intensity required to produce a 0.4 N change in flexion force and the initial response duration were analyzed. ANOVAs indicated that there was no effect of drug dose on shock intensity required, $F(4,24) < 1.0, p > .05$, nor on initial response duration, $F(4,25) = 1.33, p > .05$. Therefore, it can safely be concluded that fluorocitrate doses not disrupt behavioral reactivity.

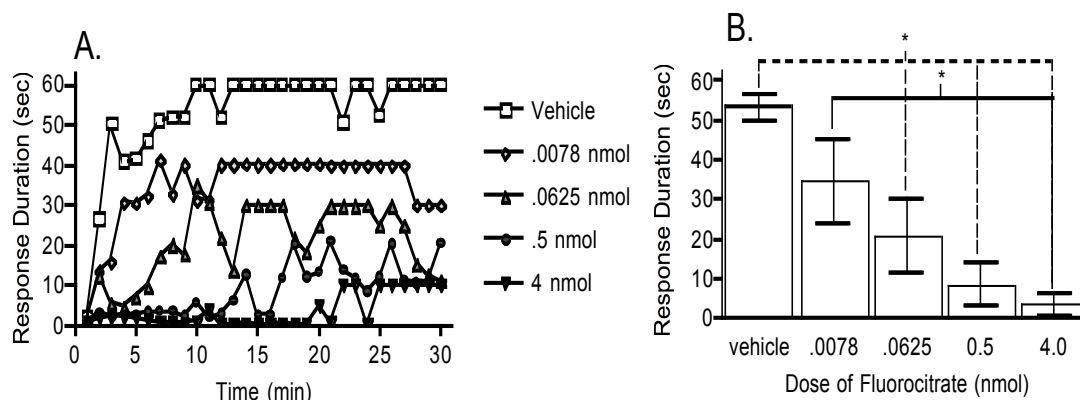


Figure 4. The effect of fluorocitrate on instrumental learning and performance. Transected subjects received fluorocitrate (0.0078, 0.0625, 0.5, or 4.0 nmol) or vehicle 20-min before testing with 30-min of controllable shock. Fluorocitrate affected learning in a dose-dependent fashion. Changes in response duration across time differed based on group (A) and mean (\pm SEM) response durations (B) are depicted. An asterisk (*) indicates that Tukey post hoc indicated that there was a statistically significant difference ($p < .05$).

The effects of fluorocitrate on spinal learning and performance are depicted in Figure 4. Subjects given the vehicle alone exhibited a progressive increase in flexion duration across the 30-min of training. This learning was blocked in a dose dependent fashion by fluorocitrate. A repeated measure ANOVA of response duration was performed. It revealed that there was a significant main effects of time, $F(29,725) = 6.77, p < .00$, and dose, $F(4,25) = 9.21, p < .001$, indicating that flexion duration increased over time and decreased with increasing doses of fluorocitrate. These main effects were qualified by a significant interaction between dose and time, $F(116,725) = 1.34, p < .05$, which indicates that the change in performance observed over time was attenuated by fluorocitrate in a dose dependent manner (Figure 4A). A Tukey *post hoc* test indicated that there was a statistically significant difference between the vehicle group and the 4.0 nmol, 0.5 nmol, and the 0.0625 nmol groups. Additionally, there was a significant difference between the 0.0078 nmol and the 4.0 nmol groups (Figure 4B).

Discussion

Fluorocitrate disrupted spinal instrumental learning in a dose dependent fashion. As expected, the vehicle group progressively increased their response durations indicating that they learned the relationship between shock and leg position. However, the fluorocitrate disrupted learning in a dose dependent fashion. This experiment in conjunction with Experiment 1 provide converging lines of evidence indicating that glial cells are necessary for the acquisition of spinal instrumental learning.

EXPERIMENT 4: THE EFFECT OF FLUOROCITRATE ON THE MAINTENANCE OF SPINAL INSTRUMENTAL LEARNING

Previous research indicates that some manipulations, such as antagonizing NMDA receptors, affect the maintenance of spinal instrumental learning (Joynes et al., 2004). Furthermore, there is evidence that glial cells can inhibit the maintenance of pathological pain states (Hansson, 2006; Milligan et al., 2003; Zhuang et al., 2006). Therefore, this experiment seeks to evaluate the role of glial cells in the maintenance of spinal instrumental learning. In this procedure subjects are tested with spinal instrumental learning for 60 min. The drug is administered part way through testing, to see if the drug will disrupt the maintenance of a previously learned behavior.

Method

Rats were transected at T2 and a catheter was implanted 9 cm below site of transection. After a 24-h recovery period, rats were secured into the controllable shock apparatus. Subjects received 60-min of controllable shock. After 20-min of testing they received 1 μ L intrathecal infusions of fluorocitrate (0.5 nmol) or vehicle. The last 30-min of controllable shock is considered the testing phase and is used for statistical analysis. Another experiment was run concurrently to verify the potency of the drug on the acquisition of spinal instrumental learning. Rats in this experiment were also transected at T2 and implanted with a catheter. Twenty-four hours later they were restrained in the controllable shock apparatus for 60-min. After 20-min they received a 1 μ L intrathecal infusions of fluorocitrate (0.5 nmol) or vehicle and 10 min after the injection they received 30-min of controllable shock.

Results

The initial shock intensity required to produce a 0.4 N change in flexion force and initial response duration were analyzed at the onset of restraint to ensure there were not differences in behavioral reactivity. ANOVAs indicated that there were no differences on shock threshold required to produce a 0.4 mA flexion force, $F(3,28) < 1.0, p > .05$, nor on initial response duration, $F(3,28) < 1.0, p > .05$. These findings suggest that fluorocitrate does not disrupt initial behavioral reactivity.

The impact of fluorocitrate on the maintenance of spinal learning is depicted in Figure 5a and 5b. Prior to drug administration, both groups exhibited a progressive increase in response duration and demonstrated learning by the 20-min. Drug or vehicle administration did not alter the maintenance of the behavioral responses, as indicated by a non-significant effect during the testing phase for time, $F(29,406) = 1.06, p > .05$, drug, $F(1,14) < 1.0, p > .05$, and time by drug, $F(29,406) < 1.0, p > .05$.

Figures 5c and 5d depict the results from the control experiment. It is clear that this dose of fluorocitrate disrupted the acquisition of spinal learning, which demonstrates that the lack of effect was not related to the drug's potency. A repeated measure ANOVA confirmed that there was a significant main effect of time, $F(29,406) = 3.88, p < .001$, and dose, $F(1,14) = 8.38, p < .05$, indicating that flexion duration increased over time and decreased with drug administration. The interaction between time and dose also reached significance, $F(29,406) = 1.70, p < .05$ and a Tukey *post hoc* indicated that there was a significant difference between subjects pre-treated vehicle and fluorocitrate, as observed in Experiment 3.

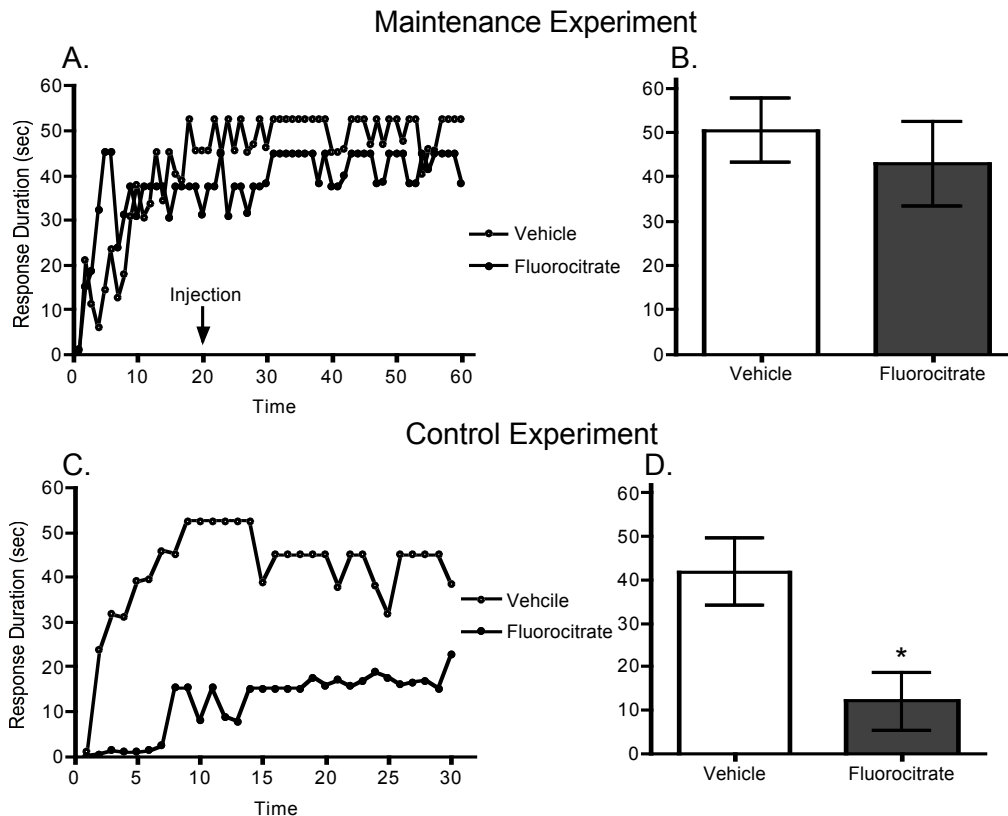


Figure 5. The effect of fluorocitrate on the maintenance of spinal instrumental learning. In the maintenance experiment transected subjects received fluorocitrate (0.5 nmol) or vehicle 20-min into a 60-min session of controllable shock. Fluorocitrate did not disrupt maintenance of the instrumental response. In the control experiment transected subjects received fluorocitrate (0.5 nmol) or vehicle 10-min prior to testing with 30 min of controllable shock. This verified the potency of the drug. Changes in response duration did not differ between the groups (A) and mean (\pm SEM) response durations for the last 30-min of testing (B) are depicted. Changes in response duration between the groups (C) indicate that the drug continues to disrupt acquisition of spinal instrumental learning. Mean (\pm SEM) response durations for 30-min of testing (D) are depicted. An asterisk (*) indicates that Tukey *post hoc* indicated that there was a statistically significant difference ($p < .05$).

Discussion

This data suggests that glial cells are not necessary for the maintenance of spinal instrumental learning. Subject's ability to maintain the learned behavioral response was not disrupted by administration of fluorocitrate, which was shown here, and in Experiment 3, to block the acquisition of that behavioral response. Given that prior research indicates that different mechanisms may mediate the acquisition and maintenance of different forms of plasticity (Ma & Woolf, 1995; Milligan et al., 2003), such a finding is not surprising.

EXPERIMENT 5: THE EFFECT OF FLUOROCITRATE ON THE SPINAL INSTRUMENTAL LEARNING DEFICIT

In Experiment 2 minocycline failed to block the development of the learning deficit, however, the unanticipated long-lasting effects of minocycline caused this data to be difficult to interpret. Therefore, this experiment proposes to examine the effects of glial cell inhibition on spinal instrumental learning through the use of fluorocitrate. Fluorocitrate not only blocks glial inflammatory products and activation but also energy dependent transmitter uptake and release.

Method

Rats were transected at T2 and a catheter was implanted 9 cm below site of transection. After a 24-h recovery period, rats were given 1 μ L intrathecal infusions of fluorocitrate (0.5 nmol) or vehicle. After 20-min, rats were given 6-min of uncontrollable shock or no shock. Twenty-four h later all of the rats received 30-min of controllable shock.

Results

To verify that fluorocitrate did not impact baseline behavioral reactivity, the shock intensity required to produce a 0.4 N change in flexion force prior to controllable shock and the initial response duration were analyzed. An ANOVA indicated that there was no effect of drug on required flexion force, $F(1, 20) < 1.0, p > .05$, nor on initial response duration, $F(1,20) = 1.97, p > .05$. Additionally, pre-treatment with uncontrollable shock did not affect required flexion force, $F(1,20) < 1.0, p > .05$, nor did it affect initial response duration, $F(1,20) < 1.0, p > .05$.

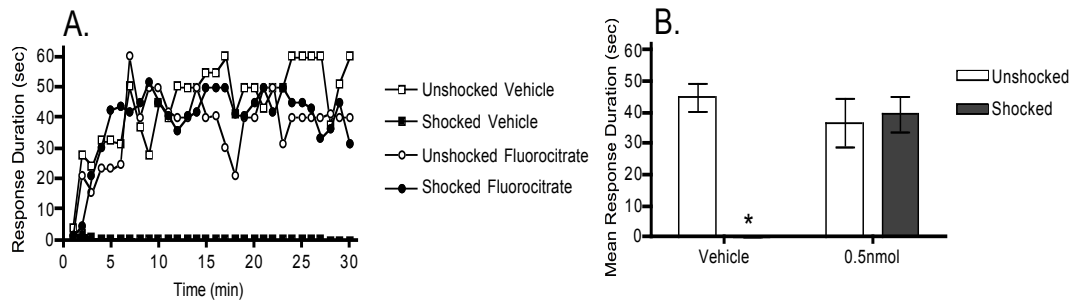


Figure 6. The effect of fluorocitrate on the development of the instrumental learning deficit. Transected subjects received fluorocitrate (0.5 nmol) or vehicle 20-min before uncontrollable shock or no shock. Rats were tested with controllable shock 24 h later. Uncontrollable shock caused a learning deficit that was prevented by administration of fluorocitrate. Changes in response duration across time (A) and mean (\pm SEM) response durations for all groups (B) are depicted. An asterisk (*) indicates that Tukey *post hoc* indicated that there was a statistically significant difference ($p < .05$).

Figure 6 depicts the impact of fluorocitrate on the development of the instrumental learning deficit. As usual, vehicle treated unshocked rats exhibited a progressive increase in flexion duration, while vehicle treated shocked rats exhibited a learning deficit (Figure 3A). However, subjects that received fluorocitrate prior to the onset of uncontrollable shock also progressively acquired and maintained the instrumental response (Figure 6A). These impressions were verified by repeated measures ANOVA. There was a main effect of time, $F(29,580) = 5.40, p < .001$, shock, $F(1,20) = 13.05, p < .01$, and drug, $F(1,20) = 7.21, p < .05$, indicating that flexion duration increased over time, decreased with shock, and increased with drug administration. The time by shock interaction, $F(29,580) = 1.50, p < .05$, indicated that the unshocked subjects improved over time, while shock disrupted performance. The shock by drug interaction, $F(1,20) = 16.42, p < .01$, indicated that pre-treatment with fluorocitrate protected against the effect of the shock. A Tukey *post hoc* test indicated that the uncontrollably shocked vehicle group performed significantly worse than all other groups and that there was no difference between the shocked and unshocked fluorocitrate treated subjects (Figure 5B).

Discussion

As anticipated, a learning deficit was produced by prior exposure of uncontrollable shock. This deficit was prevented by inhibiting glial cells with fluorocitrate prior to the onset of uncontrollable shock. Prior research indicates that fluorocitrate inhibits glial cell metabolism (Clarke & Nicklas, 1970; Hassel et al., 1992; Paulsen et al., 1987; Swanson & Graham, 1994). This suggests that energy dependent

glial cell functions are involved in the development of the spinal instrumental learning deficit.

GENERAL DISCUSSION AND CONCLUSIONS

Spinally transected rats exposed to controllable shock can learn to maintain a flexed leg position when leg extension is associated with shock. Additionally, uncontrollable shock causes a learning deficit to develop. Previous research has shown that both learning and the learning deficit in this paradigm depend upon spinal cord neurons (Crown et al., 2002b; Joynes et al., 2003) and are mediated by NMDA receptors (Ferguson et al., 2006; Joynes et al., 2004). The results from the present experiments suggest that glial cells are also involved in spinal instrumental learning and the learning deficit.

Experiment 1 indicated that administration of minocycline disrupts learning in a dose dependent fashion. While Experiment 2 suggested that minocycline does not prevent the acquisition of the learning deficit; rather, it appears to have long lasting disruptive effects on learning that persists for at least 24 h. Experiment 3 indicated that fluorocitrate also disrupts learning in a dose dependent fashion; however, Experiment 4 indicated it did not disrupt the maintenance of previously acquired learning. Additionally, Experiment 5 suggests that fluorocitrate can protect against the development of the learning deficit when administered prior to uncontrollable shock.

Given that both drug manipulations affected the acquisition of spinal instrumental learning they provide converging evidence for a role of glial cells in the acquisition of spinal instrumental learning. Additionally, fluorocitrate, which disrupts all energy dependent uptake and release, did not disrupt the maintenance of learning; this suggests that the maintenance of spinal instrumental learning occurs primarily at the

neuronal and not glial level. Furthermore, given that fluorocitrate protected against the learning deficit, while minocycline did not, may suggest that the mechanism by which the deficit was blocked may be due to a glial transmitter blocked by fluorocitrate and not known to be blocked by minocycline (e.g., glutamate or glutamine release). However, given the unanticipated long-lasting effects of minocycline the minocycline data is difficult to interpret. Overall, these results provide further evidence for the role of glial cells in spinal plasticity.

However, demonstrating the involvement of glial cells in spinal plasticity opens more questions than it answers. The next step is to begin to understand the mechanisms by which glial cells mediate plasticity. Previously minocycline treatment has been associated with enhanced learning and LTP in pathological conditions (Choi et al., 2007; Fan et al., 2007; Liu et al., 2007); it appears that this occurs through reducing inflammation. However, it has also been shown that there are optimal levels of inflammatory products, such that too high or too low of levels are inhibitory (Bohme et al., 1993 and 1991; Brennan et al., 2003; Lu et al., 1999; Malen and Chapman, 1997; Zhuo et al., 1993). Therefore, it is possible that minocycline treatment during spinal instrumental learning reduced inflammatory products (such as, IL-1 β and NO) to levels deleterious to learning.

The long-lasting effects of minocycline may merely be an effect of a slowed drug metabolism. Minocycline has a relatively long half-life, 11 to 17 hours in healthy human volunteers. Given that these rats are spinally transected, it is possible that the half-life is even longer for them (Cruz-Antonio et al., 2006; García-López et al., 2006). If this is the

case, minocycline may be at clinically significant levels even 24 h after drug administration. Therefore, the unshocked subjects in the deficit experiment may have merely been a delayed replication of the learning experiment.

In order to begin to understand the effect of minocycline on the spinal instrumental learning paradigm, it would be important to test how the model responds to other anti-inflammatory treatments, such as IL-10. This would allow us to decipher minocycline's anti-inflammatory effects from its other effects. If such a treatment demonstrated that inflammation can at least partially explain the effects of minocycline on spinal instrumental learning, more targeted inflammatory products could be considered (e.g., glial NO inhibitors, IL-6 and IL-18 antagonists) in order to further elucidate the mechanisms by which glial modulate neural plasticity.

Additionally, given that minocycline is thought to exert its effects through inhibiting p38 MAP kinase, a p38 antagonist would provide another method to understand the impact of minocycline on spinal instrumental learning paradigm. Furthermore, fractalkine could be used to increase glial cell activation state, specifically microglia activation. Fractalkine is released by neurons and, to a lesser extent, by astrocytes, and its receptors are expressed exclusively by microglia (Milligan et al., 2004; Verge et al., 2004). Inhibiting fractalkine or its receptor would inhibit neural-glia communication, decrease microglia activation, and provide more information about the role of glia in spinal instrumental learning.

Another explanation that must be consider is the possibility that minocycline caused neuronal damage in the spinal instrumental learning paradigm. Although many

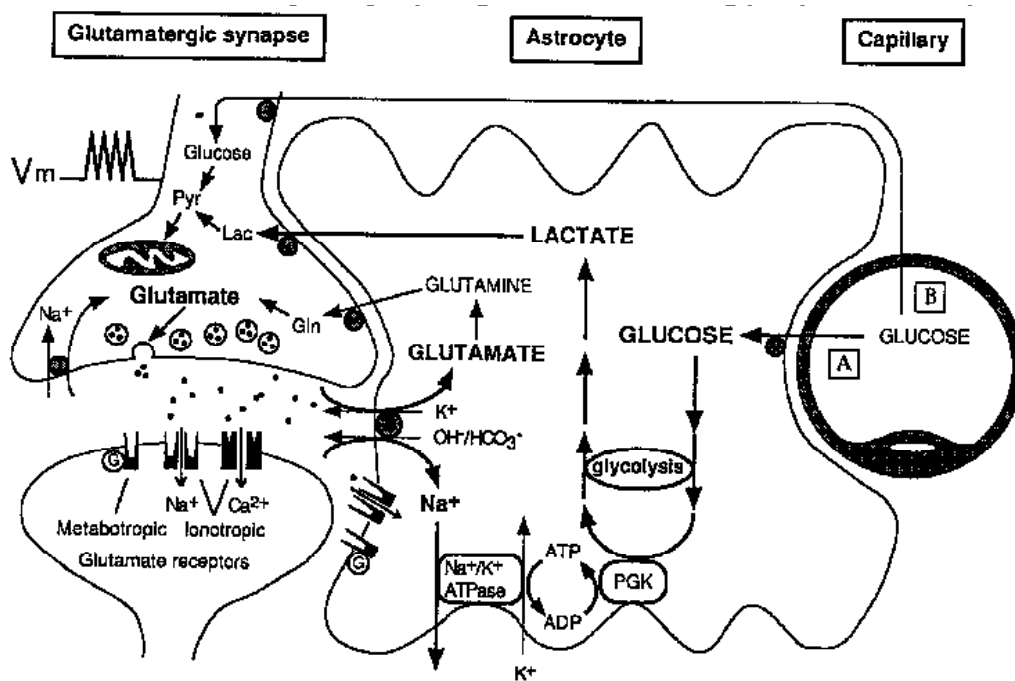
studies have shown clinically relevant, positive findings through the use of minocycline, there are a few that indicate that minocycline, in some situations, can lead to neuronal damage. For example, Yang and colleagues (2003) used the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) model of Parkinson's disease in mice and showed that although minocycline treatment inhibited microglia activation it also led to significantly more damage to dopaminergic neurons. Additionally, Diguett and colleagues (2004) used the MPTP model in cynomolgus monkeys; those receiving minocycline showed greater behavioral impairment and more severe neuronal cell loss in the dorsal striatum. However, in the same study they showed that low doses of minocycline blocked neurotoxicity in a mouse model of Huntington's disease. This research on minocycline suggests that its effects may vary across species, model, route of administration, and dose. However, further research and histological analysis would be necessary to determine if minocycline is causing neuronal damage in the spinal instrumental learning model.

Fluorocitrate not only disrupted learning, but also prevented the acquisition of the spinal instrumental learning deficit. This may be due to its wider set of actions; it does not merely inhibit glial transmitter release, but shuts down energy dependent uptake and release. Therefore, it does not only inhibit proinflammatory cytokines and NO, it also inhibits glial cell metabolic support to neurons and regulation of extracellular ion concentrations. Any one or combination of these effects may be necessary for spinal instrumental learning and the learning deficit.

For example, fluorocitrate may disrupt glial cell metabolic support to neurons. There is glutamate-glutamine cycle (refer to figure 7) between glial cells and neurons. Glial cells take up glutamate released into the synaptic cleft. Within the glial cell glutamate can be converted to glutamine, released, and taken up by the pre-synaptic neuron. Within the neuron, glutamine is converted into glutamate and released in response. This is a major pathway for the replenishment of neuronal glutamate (Bröer and Brookes, 2001). Therefore, a reduction in glutamine production in response to fluorocitrate may lead to a depletion of pre-synaptic glutamate and a suppression of neurotransmission. Another possible mechanism is astrocytes released glutamate. Recent research indicates that glutamate released by astrocytes can modulate synaptic strength by acting upon the pre-synaptic neuron (Jourdain et al., 2007).

D-serine is another transmitter that ought to be considered. It acts as a co-agonist at the NMDA strychnine-insensitive glycine recognition site and regulates NMDA receptor mediated neurotransmission (Wolosker et al., 1999, 2006). Previous research suggests that it plays an important role in regulating neural plasticity in the brain (Mothet et al., 2000; Olier and Mothet, 2006); however, it is unknown if it also plays a role in spinal plasticity.

Another mechanism that needs to be considered is the impact of fluorocitrate on extracellular ion concentrations. Given that fluorocitrate inhibits energy dependent uptake, it may also compromise glial cell regulation of the extracellular ion concentration. This may cause excessive ions to build up in the synaptic cleft, which



From Tsacopoulos and Magistretti, 1996, *J. Neuroscience* 16:877-885.

Figure 7. The glutamate-glutamine cell cycle, from Tsacopoulos and Magistretti, 1996. Glutamate released from the pre-synaptic neuron not only affects the post-synaptic neuron, but also is taken up by synaptically associated astrocytes. In the astrocyte it is converted into either glutamine or lactate in order to re-supply the pre-synaptic neuron. The astrocyte can also release glutamate, which has been shown to enhance synaptic communication. These processes are disrupted by fluorocitrate administration.

would cause the post-synaptic cell to become highly stimulated and prevent learning. However, evidence from Keyser and Pellmar (1994) suggest that this is not occurring, because they showed that the synapse actually became less activated in response to fluorocitrate administration.

In summary, the findings from the present studies indicate that glial cells play an important modulatory role in spinal instrumental learning and the learning deficit, which adds to the growing body of evidence indicating that glial cells modulate neural plasticity. Additionally, this data indicates that the spinal instrumental learning paradigm provides a good model to continue to explore the mechanisms by which glial cells modulate neural plasticity. Future work is necessary to further elucidate the cellular and neurochemical mechanisms by which glial cells modulate neural plasticity.

Understanding these mechanisms would not only contribute to our understanding of learning, but would also have many clinical implications. For example, further research may allow us to identify a mechanism that would prevent the development of the learning deficit without impacting the acquisition or maintenance of learning, which would provide a novel drug discovery target for pathological pain and recovery of function after a spinal cord injury.

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