TIMING IN THE ABSENCE OF SUPRASPINAL INPUT: EFFECTS OF TEMPORALLY REGULAR STIMULATION ON SPINAL PLASTICITY

A Dissertation

by

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ABSTRACT

Prior work has shown that spinal neurons are capable of discriminating between temporally regular and temporally irregular stimulation. These effects have been observed using an *in vivo* assay of spinal plasticity based on an instrumental learning task, in which response-contingent leg shock produces an increase in flexion duration. Exposure to temporally regular stimulation (fixed spaced stimulation; FT) promotes learning, and temporally irregular stimulation produces a learning deficit. The experiments in this dissertation were designed to test other properties of fixed spaced shock that promote spinal plasticity and the structure responsible for the FT effect.

Experiment 1 focused on the minimum number of stimulations necessary to re-establish the capacity to learn (a component of the "FT effect"), finding that180-360 shocks produced a learning deficit and that additional training (540-900 shocks) allowed learning. Experiment 2 found that shock number, not duration of exposure determined whether the FT effect emerged. Experiment 3 investigated if the FT effect emerges after shock was presented in two sessions separated by 24 hrs, and showed that two bouts of 360 shocks yielded the FT effect. Further, the initial bout of fixed spaced shock had a long-term benefit (Experiment 4).

The results of Experiment 5 suggested that omitting shocks from a train of FT stimulation has little effect on the benefit of fixed spaced shock treatment. Experiment 6 replicated this observation, showing that randomly deleting half of the shocks (from a

720 FT shock series) had no effect on learning. Further, this schedule also induces a lasting protective effect, blocking the learning deficit produced by variable spaced shock (Experiment 7).

To explore whether a central system or a peripheral filter mediates the FT effect, Experiment 8 challenged spinal neurons by phase shifting the relation between fixed spaced stimulation applied to two dermatomes. The FT effect only emerged when stimuli occurred in an alternating pattern across dermatomes, implying regularity is abstracted by a central system. Experiment 9 surgically isolated central pattern generator (L1-L2) from the portion of the spinal cord that mediates instrumental learning (L4-S2), finding that disrupting the connections between these two regions eliminated the FT effect.

DEDICATION

For my Father

ACKNOWLEDGEMENTS

I would like to thank Dr. James W. Grau for his patient guidance and support throughout the years, without his valuable perspective, encouragement and willingness to exchange ideas, this work would not be possible. I would like to thank my committee members, Drs. Michelle Hook, Rajesh Miranda and Mark Zoran, for their helpful feedback and enthusiasm. I would like to thank Drs. Kyle Baumbauer, J. Russell Huie and Kevin Hoy, my academic "big brothers" for the examples they have set for me, both personally and professionally. I would also like to express my gratitude to Dr. Sarah Woller, Kie Huang, Misty Strain, Sylvia Bernal, and Dr. Sandra Garraway who have never hesitated to lend a hand or an ear; thank you for your friendship and encouragement. As always, I must thank my parents, my family, and my friends for their countless efforts for me and love, which I carry always.

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CHAPTER I

INTRODUCTION

Spinal Plasticity: An Overview

The spinal cord has long been viewed as a simple conduit, relaying information from sensory input to the brain. However, research over the last century indicates that the spinal cord is not a simple conduit, but rather capable of changing and adapting to new environmental stimuli. This plasticity of the spinal cord, this ability to make long term modifications and adaptations to new afferent stimuli and capacity for learning, has been the focus of research on the spinal cord with an aim of promoting functional recovery after injury. Extensive work on spinal plasticity in the fields of pain processing, locomotor training after spinal cord injury, as well as learning (habituation, sensitization, Pavlovian conditioning and instrumental learning) has demonstrated the ability of spinal neurons to reorganize and adapt to new environmental stimuli (Groves & Thompson, 1970; Joynes & Grau, 1996; Grau, Barstow, & Joynes, 1998).

This dissertation will focus on behavioral learning and how adaptive plasticity is promoted by the presentation of regularly spaced electrical shock (fixed spaced [FT] shock; ISI: 2 s) as well as the parameters and conditions under which this effect occurs. I will first provide an overview of the model of spinal plasticity used in this dissertation and then review previous findings regarding the effects of regularly spaced electrical stimulation on spinal plasticity. I will then summarize the current literature investigating

the role of the central pattern generator, an endogenous oscillator within the spinal cord, in promoting spinal plasticity and recovery of function after spinal cord injury, and propose a hypothesis regarding its potential role in timing. Finally, the specific aims of the experiments in this dissertation will be outlined.

Spinal Instrumental Learning and the Effects of Peripheral Stimulation

Prior work by Grau et al. (2006; 2012) focused on the role of behavioral control in promoting spinal plasticity. When transected rats are given a leg shock whenever a hindleg is extended, subjects learn to maintain a leg flexion that minimizes shock exposure. However, when this response-outcome contingency is no longer relevant (Yoked subjects), subjects demonstrate a prolonged inability to acquire the instrumental learning response (learning deficit). Additionally, rats given variable stimulation prior to instrumental testing fail to learn (Grau, et al. 2006; 2012). Thus, controllability of peripheral stimulation is critical for adaptive spinal plasticity (instrumental learning) to occur within the spinal cord.

Interestingly, though uncontrollable in nature, when transected rats are given peripheral stimulation (tail or leg shock) on a fixed time (FT) schedule (ISI: 2 s), they exhibit normal learning when tested in the instrumental paradigm, whereas rats that receive peripheral stimulation on a variable time (VT) schedule (ISI: 0.2-3.8 s) fail to learn (Baumbauer et al., 2008). However, these effects are only observed after extended training. When 180 shocks were presented, both FT and VT shock produce a learning

deficit. However, when 900 shocks are given, only variable shock produces a learning deficit (Baumbauer et al., 2008).

Exposure to fixed spaced shock also has a protective effect. If sufficient fixed spaced shock (720 stimulations) is given it prevents the induction of the learning deficit by variable spaced shock, and this protective effect lasts up to 48 hrs (Baumbauer et al., 2009). Further, exposure to FT shock enables learning when subjects are tested with a more difficult learning task, using a higher response criterion (Baumbauer et al., 2009) . The protective/enabling effect of fixed spaced stimulation has been linked to the neurotrophin BDNF; pretreatment with BDNF prior to instrumental learning promotes adaptive plasticity (Huie et al., 2012), whereas the BDNF sequestering agent, TrkB-IgG, blocks the protective effect of fixed-spaced stimulation (Baumbauer et al., 2009). Other studies have linked the FT effect to NMDA receptor mediated plasticity and protein synthesis (Baumbauer et al., 2009).

We have also assessed the impact of fixed spaced stimulation and variable spaced stimulation on mechanical reactivity(Baumbauer et al., 2012). Prior studies have shown that treatments that induce neuropathic pain (e.g. peripheral inflammation) enhance mechanical reactivity (Ferguson et al., 2006). We have found that fixed spaced stimulation produces hyporeactivity and variable spaced stimulation produces hyperreactivity to tactile stimulation. Here too, the effects of fixed spaced shock depend on the number of shocks presented, as the effect of FT shock only emerged after extended training (900 stimulations; Baumbauer et al., 2012), which are similar to the results found on the impact of fixed spaced shock on instrumental learning. Baumbauer

et al. (2011) also tested whether FT stimulation affects the mechanical hyperreactivity and learning deficit caused by peripheral inflammation. Inflammation was induced by a subcutaneous injection of capsaicin (1%), the active ingredient in chili peppers, into the dorsal surface of the hindpaw. FT stimulation prevented and reversed the capsaicin induced allodynia and the learning deficit (Baumbauer & Grau, 2011; Baumbauer et al., 2012). Throughout this dissertation, this ability of FT stimulation to promote learning and protect against the induction of the deficit is referred to as the "FT effect."

Together, these data suggest that the FT effect may involve a form of learning about the temporal distribution of stimuli and that the FT stimulation both promotes adaptive plasticity but also produces a lasting, protective effect on that counters the learning deficit induced by VT stimulation (the "FT effect"). It is not currently known how spinal neurons discriminate between fixed and variable spaced stimulation to produce opposing behavioral effects. My thesis will explore this issue, providing evidence that a sense of time is derived from the central pattern generator (CPG), which organizes locomotor behaviors. In the next section, I provide an overview of the CPG with the aim of showing how this endogenous oscillator could contribute to spinal timing.

Spinal Plasticity and the CPG

Within the spinal cord, central pattern generators are defined as populations of neurons that are capable of sustained, rhythmic activity in the absence of any input,

descending or ascending. In an intact animal, the central pattern generator interacts with both descending pathways and afferent feedback to produce and modulate locomotor rhythms. This system of interactions between descending inputs, the central pattern generator and afferent inputs is what results in normal motor outputs such as walking, scratching and shaking. An implicit assumption within the field of recovery of function after spinal injury is that the restoration of the descending pathways is of primary importance for regaining normal motor outputs. This emphasis both excludes the importance of afferent feedback in eliciting and modulating locomotor movements, but also the capacity of intrinsic spinal circuits to adapt and contribute to recovery of locomotion after spinal cord injury. To find the most effective rehabilitation strategy, all three components must be considered and thoroughly investigated, especially the contribution of the locomotor circuits that are intact after spinal cord injury.

Early studies by Grillner (1973) found that after a complete spinal transection at the lowest thoracic segment (T13), hindlimb locomotion could be re-established on a treadmill after a few weeks of locomotor training in cats. This effect has since been replicated in adult cats and other species (Barbeau & Rossignol, 1987; Belanger et al. 1996; de Leon et al. 1998; Rossignol et al. 1996). In the cat, recovery of function is characterized by an initial lack of plantar placement of the paw and the presence of foot dragging of the hindlimbs on the treadmill. After a few weeks of treadmill training, cats with complete spinal transections display coordinated alternating hindlimb locomotion, weight supported stepping and consistent plantar placement of the paw. Additionally, electromyogram (EMG) recordings of the electrical activity in hindlimb muscles after

treadmill training indicate that the muscle activity is similar pre- and post-transection (Belanger et al. 1996), though the loss of particular descending pathways does seem to result in minor deficits in both extensor and flexor muscles.

After full spinal transection, sensory inputs are critically important for the regulation of normal locomotion. In treadmill training, early movements after transection are elicited by manual stimulation of the perineal region (Barbeau & Rossignol, 1987; Barriere et al., 2008). In the intact spinal system, the spinal cord receives rhythmic input from the sensory afferents and proprioceptive feedback, which reinforce and stabilize the output of the intrinsic networks and CPG. Most likely, the role of sensory feedback to spinal networks serves to control the timing of the different phases of the step cycle and also shape the pattern of activation of muscle groups by motoneurons. Further, the role of sensory feedback is critical for driving the excitation of motoneuron pools and thereby the long-term adaptations of locomotor patterns. After spinal injury, these functions are critical for the restoration of locomotion (for review, Hultborn & Nielsen, 2007).

Not only can spinalized cats recover locomotor hindlimb function during treadmill training, the locomotor output of spinalized cats can also adapt and change based on new environmental stimuli, such as an obstacle on the treadmill (Frossberg, Grillner & Rossignol, 1975). When tactile stimuli (obstacle) contact the dorsal surface of the paw during the swing phase, the entire hindlimb increases flexion to overcome the obstacle, lifting the leg above of the obstacle. Furthermore, when weak stimuli (simulating cutaneous tactile stimulation) or actual mechanical tactile stimulation was

applied to the dorsal surface of the paw during the extension phase, activation of the extension muscles was markedly increased (Frossberg, Grillner & Rossignol, 1975). Even more interestingly, in subsequent steps, the hyperflexion elicited by the cutaneous stimulation of the dorsal surface of the paw persists (Zhong et al., 2012) after the obstacle is removed, suggesting not only a memory of the obstacle, but a specific encoding of the timing of the obstacle presentation during the swing phase, and anticipatory response to avoid the obstacle (for review, Hodgson et al., 1994).

It is clear that retraining the injured spinal cord is beneficial to adaptive spinal plasticity (Barriere et al., 2008; van den Brand et al., 2012; de Leon et al., 1998). Though training is not critical for the re-expression of locomotion after partial lesion for cats and rats, it does greatly facilitate the course of locomotor recovery. This recovery of function is based largely on the plasticity of intrinsic spinal circuits and their interactions with sensory feedback (Barbeau & Rossignol, 1987; Belanger et al.,1988; Duysens & Van de Crommert, 1998; de Leon et al, 1998; Van de Crommert et al, 1998; Rossignol, 2006; Edgerton et al., 2004). For instance, Magnuson et al. (2005) found that the intact CPG facilitates recovery of function, observing a greater loss of locomotion after a contusion on the T13-L2 region when compared to the same injury at L3-L4. These results are attributed to the damage of the spinal CPG, which is thought to reside in the L1-L2 regions in the rat (Magnuson et al. 1999; 2005), demonstrating the importance of the plasticity of intrinsic spinal network in facilitating re-expression of locomotion.

CPG as a Clock

Elucidating the role of the CPG in spinal timing requires that I identify the elements needed for timing. For timing to occur in the central nervous system, three core elements are necessary: 1) a clock or pacemaker to mark the passage of time, 2) the ability of this clock to be entrained or influenced by environmental stimuli and 3) the flexibility of the clock to adapt and produce a change in behavior that is associated with a temporal property of the environmental stimuli. Though simple in its oscillatory mechanisms and significantly more limited in the range of its time scale than other brain structures involved in timing, the central pattern generator (CPG) in the spinal cord is a network of neurons that has the capacity to maintain rhythmic activity even in complete isolation from both descending control and ascending sensory input. The CPG is thought to be responsible for the alternating rhythmic motor outputs responsible for the timing of many motor actions, such as walking, scratching, and shaking. In other regions of the nervous system, a CPG is though to contribute to other rhythmic activities such as breathing and certain feeding behaviors (for review, Marder & Bucher, 2001; Kiehn, 2006; Frigon, 2012; Rossignol & Frigon, 2012; Hultborn & Nielsen, 2007). While it is known that environmental cues help maintain rhythmic activity and influencing the rhythmic output of the CPG is clear (Grillner et al., 1991; Griller & Zangger, 1979; Kiehn, 2006; Frigon, 2012; Rossignol & Frigon, 2011; Hultborn & Nielsen, 2007), it is not known whether this system can function in a more general way to provide a sense of time or tempo (i.e. a pace maker).

In summary, though the CPG is known to be a biological oscillator which is influenced by environmental stimuli and can adapt behaviors based on previous experience, it is yet to be determined if this biological oscillator can measure and produce a representation of the passage of time, and if that information is encoded, whether spinal networks can use temporal cues to adapt or control motor behaviors.

Specific Aims

When considering the basic behavioral effect of fixed spaced stimulation on performance in an instrumental learning task, three general questions arise: 1) Why does regular stimulation produce a beneficial effect and irregular stimulation produce a detrimental effect, 2) How does regular stimulation produce a beneficial effect in the spinal cord and 3) How does the spinal cord differentiate between the two types of stimulation. The focus of this dissertation is on how the spinal cord can detect the temporal relationship of fixed spaced stimulation and how fixed spaced stimulation can promote plasticity. In order to address the beneficial effects of fixed spaced shock, I must both address the temporal properties of fixed spaced shock that promote spinal plasticity as well as the subsequent endogenous mechanisms of spinal neurons engaged by fixed spaced shock. To address how the spinal cord can differentiate between the two schedules of stimulation, I will determine the role of the spinal endogenous oscillator (CPG) in processing the temporal regular stimulation. In the spinal cord, locomotion is organized and coordinated by the CPG, which is defined as a population of neurons that

is capable of sustained, rhythmic activity in the absence of any input, descending or ascending, in other words, a rhythmic oscillator (Brown, 1911; Marder & Bucher, 2001; Kiehn, 2006; Frigon, 2012; Rossignol & Frigon, 2011; Hultborn & Nielsen, 2007). Within the spinal cord, however, both descending and ascending inputs are necessary for appropriate locomotor function (for review, Rossignol & Frigon, 2011). Thus, I propose that through the oscillatory functions of the CPG, the spinal cord can process simple temporal cues.

I begin by examining circumstance under which spinal neurons encode temporal regularity (Chapter III). Next, I show that spinal timing is surprisingly impervious to missing stimuli (Chapter IV). Finally, I test whether the capacity for timing is mediated by a peripheral filter or a central system (Chapter V).

CHAPTER II

GENERAL METHOD

Subjects

Subjects for all experiments were male Sprague-Dawley rats obtained from Harlan (Houston, TX) that were approximately 100-120 days old, and between 300 and 400 g. All subjects were pair housed and maintained on a 12-hour light/dark cycle, with all behavioral testing performed during the light cycle. Food and water was available *ad libitum*.

Surgery

Subjects were anesthetized with isoflurane gas. Anesthesia was induced at 5% isoflurane and maintained at 2-3% isoflurane. Each subject's head was rendered immobile in a stereotaxic apparatus, and a small (5 X 4 X 2.5 cm) gauze pillow was placed under the subject's chest to provide support for respiration. An anterior to posterior incision over the second thoracic vertebrae (T2) was made and the tissue just rostral to T2 was cleared using rongeurs, and the cord exposed and cauterized. The remaining gap in the cord was filled with Gelfoam (Pharmacia Corp., Kalamazoo, MI) and the wound was closed with Michel clips (Fisher Scientific, Waltham, MA). Following closure of the wound, the surface of each leg was shaved for electrode

placement. Intraperitoneal injections (3 mL) of 0.9% saline solution were administered post-operatively to prevent dehydration. Following surgery, rats were placed in a temperature-controlled environment (25.5 °C) and monitored until awake. All rats were checked every six to eight hours during the 18-24 hr post-surgical period. During this time, hydration was maintained with supplemental injections of saline, and the rats' bladders and colons were expressed as needed.

Spinal transections were confirmed by observing the behavior of the subjects after they recovered to ensure that they exhibited paralysis below the level of the forepaws and did not exhibit any supraspinally-mediated pain responses to leg shock.

Apparatus

Instrumental Testing

Instrumental testing was conducted while subjects were loosely restrained in Plexiglas tubes. Both hindlegs were freely hanging over a salt bath (NaCl). Leg shock was delivered using a BRS/LVE (Laurel, MD) constant current (60 Hz, AC) shock generator (Model SG-903). Two electrodes placed above the tibalis anterior muscle were connected to a computer-controlled relay, which regulated the application of leg shock.

Leg shock was administered when the contact electrode touched the salt bath placed below the leg, completing a circuit monitored by a Macintosh computer, and delivering a shock to the tibialis anterior. The contact electrode was constructed of a 7 cm long, 0.46 diameter stainless steel rode, of which the last 2.5 cm of the electrode was

insulated using heat shrink tubing. A fine wire (0.01 sq mm [AWG] 20 cm) attached to the end of the stainless steel rod connected to the digital input monitored by the computer. The salt bath below the leg was placed approximately 7.5 cm below the restraining tube, and was composed of a NaCl solution with a drop of soap to reduce surface tension. A ground wire was connected to a 1 mm wide stainless steel rod, which was placed in the solution. The state of this circuit was sampled at a rate of 30 times/s.

Fixed Spaced or Variable Spaced Tailshock

All fixed spaced (ISI: 2 s) or variable spaced (ISI: 0.2 – 3.8 s) stimulation was administered to the tail through an electrode constructed from a modified fuse clip. The electrode was coated with Spectra electrode gel (Harvard Appartus, Holliston, MA) and secured with tape approximately 6 cm from the base of the tail. All subjects were loosely restrained in the Plexiglas tubes described above. A constant current 1.5 mA shock was delivered using a 660-V transformer and shock onset and offset were controlled by the computer.

Instrumental Learning Testing Procedure

Prior to testing, all subjects had their hindlimbs shaved for electrode placement. A wire electrode was then inserted through the skin over the distal portion of the tibialis anterior (1.5 cm from the plantar surface of the foot), and one lead from the generator was attached to this wire. Using a piece of surgical tape, a contact electrode was secured to the foot between the second and third digits. The shock generator was set to deliver a

0.4 mA shock, and the proximal portion of the tibialis anterior (approximately 1.7 cm proximal to the wire electrode) was probed with a 2.5-cm stainless steel pin attached to a shock lead to find a robust flexion response. The pin was then inserted 0.4 cm into the muscle and a strain gauge was utilized to verify that a single, intense (1.6 mA, 0.3 s) test shock can elicit at least a 0.8 N flexion force, and to determine the amount of shock necessary to elicit a 0.4 N flexion force.

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

Behavioral Measures

Three behavioral measures, response number, response duration and time in solution, were used to assess a subject's capacity to perform the instrumental response

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

To evaluate whether our experimental treatment affected baseline behavioral reactivity, we analyzed both the shock intensity required to elicit a flexion force of 0.4 N and the duration of the first shock-elicited flexion response. Independent ANOVAs showed that there were no group differences on either measure across all experiments, Fs < 2.58, $p < 0.05$.

Histology

To verify location of the knife cut transection, subjects were deeply anethestized by with pentobarbital (100 mg/kg; i.p.) and perfused intracardially with with 4% paraformaldehyde. A 1-cm long segment of the spinal cord rostral to the transection cut (at either T12 or L3) was collected for cryostat sectioning. The prepared spinal cord was sectioned coronally in 20-um-thick sections, and the first full section closest to the knife cut was collected. All sections were stained with cresyl violet and luxol fast blue for Nissl substance and myelin, respectively.

Statistics

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

CHAPTER III

PROPERTIES OF FIXED SPACED SHOCK

The first four experiments of this dissertation explored the stimulus conditions that support the fixed spaced shock effect. From prior studies, it is known that the fixed spaced shock effect is long lasting (over 48 hrs) and that it is observed after 900 shocks but not 180 shocks (Baumbauer et al., 2008). If only 180 fixed spaced stimulations are presented, fixed spaced shock produces a learning deficit, similar to variable spaced shock. The fact that extended training is needed to produce the FT effect, together with evidence that it depends on both the NMDA receptor and protein synthesis, suggests that the FT effect may involve a form of learning related to the temporal distribution of stimuli (Baumbauer et al., 2008; 2009). If it does, then other general properties attributed to learning should hold true for the FT effect. The experiments in this chapter explore this issue by examining (1) if there is a savings effect associated with fixed spaced shock and (2) the potential for stimulation spaced across time to have a more profound effect than stimulation massed across time, two properties associated with learning.

Experiment 1

The assessment of factors that affect the development of timing requires knowledge of some basic parametric variables such as the number of stimuli required to

produce the FT effect. I address this issue by testing the minimum number of FT shocks necessary for the FT effect to emerge.

Procedure

Experiment 1 used 40 rats (n=8). The design of this experiment is depicted in Figure 1. Twenty-four hours after complete transection of the second thoracic vertebra, subjects received 180, 360, 540, 720 or 900 fixed space stimulations to the tail. Immediately after, the impact of shock treatment on instrumental learning was tested.

<table>
<tr><td rowspan="5">Complete transection</td><td rowspan="5">24 hrs</td><td>180 FT shocks</td><td rowspan="5">Instrumental testing</td></tr>
<tr><td>360 FT shocks</td></tr>
<tr><td>540 FT shocks</td></tr>
<tr><td>720 FT shocks</td></tr>
<tr><td>900 FT shocks</td></tr>
</table>

Figure 1. Experimental design for Experiment 1.

Results

The effect of shock number on the primary index of learning (flexion duration) is depicted in Figure 2A. Subjects given 360 or fewer shocks exhibited a learning deficit, replicating previous results (Baumbauer et al., 2008). Subjects given 540 or more shocks were able to learn when tested with controllable shock (instrumental learning), suggesting that the beneficial effects of FT training emerges after 360 shocks. An ANOVA revealed a main effect of shock number F (4, 35) = 13.15, $p < .001$. Also, the Trial x Shock number interaction was significant, F (29, 1015) = 1.76, $p < .001$. *Post hoc* analysis determined that subjects that received 180 and 360 stimulations different from those subjects that received 540, 720 and 900 stimulations, $p < .05$.

As in past studies, subjects that failed to learn exhibited the highest rate of responding (Figure 2B). An ANOVA revealed a significant main effect of shock number on the number of responses made, F (4, 35) = 5.80, $p < .01$. It is important to clarify that this shows that the failure to exhibit increased response durations is not due to an inability to perform the response (leg flexion). Because all subsequent experiments yielded a similar, inverse, relationship between response duration and response number, only the former is reported.

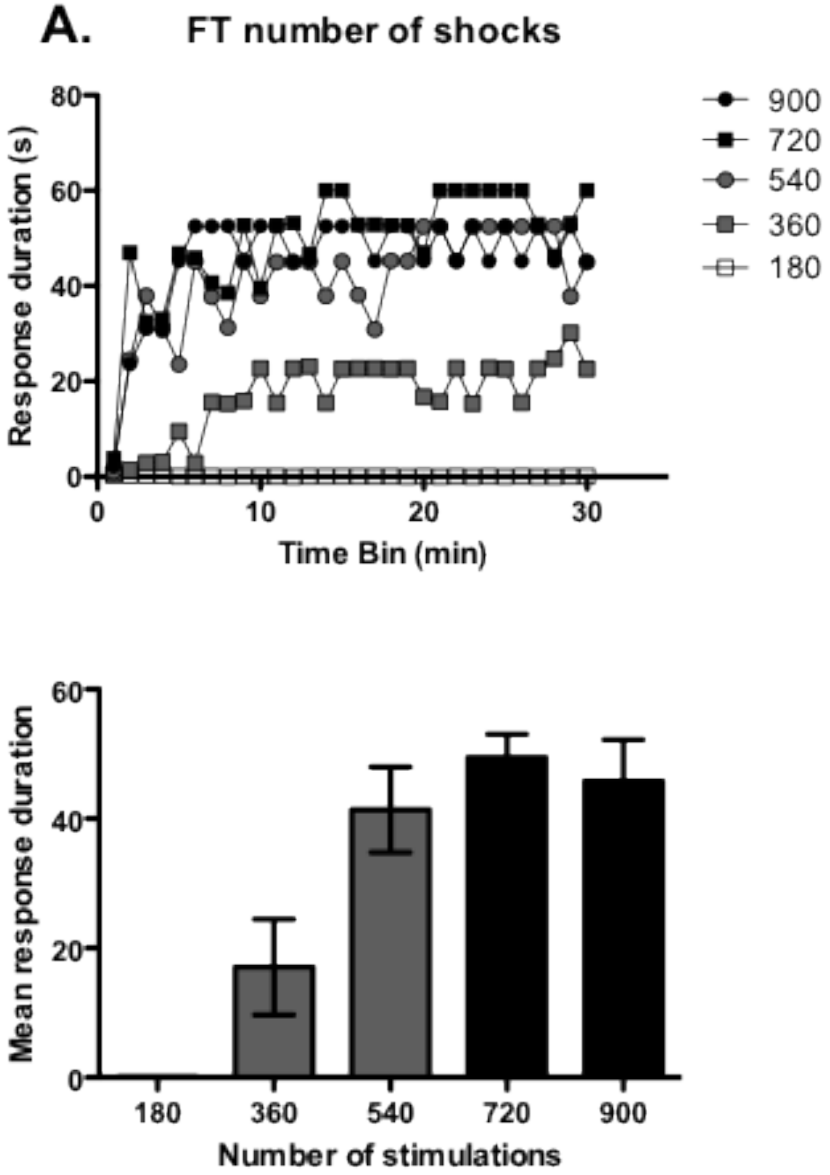

Figure 2. (A) Effect of number of stimulations on performance on an instrumental learning task. The top panel depicts response durations over time. The bottom panel depicts mean response durations for each group. Subjects that received 180 stimulations are shown in white, 360 and 540 in grey (square and circle, respectively), and 720 and 900 stimulations are shown in black (square and circle, respectively). Data indicate that 360 FT shocks or less produced a learning deficit.

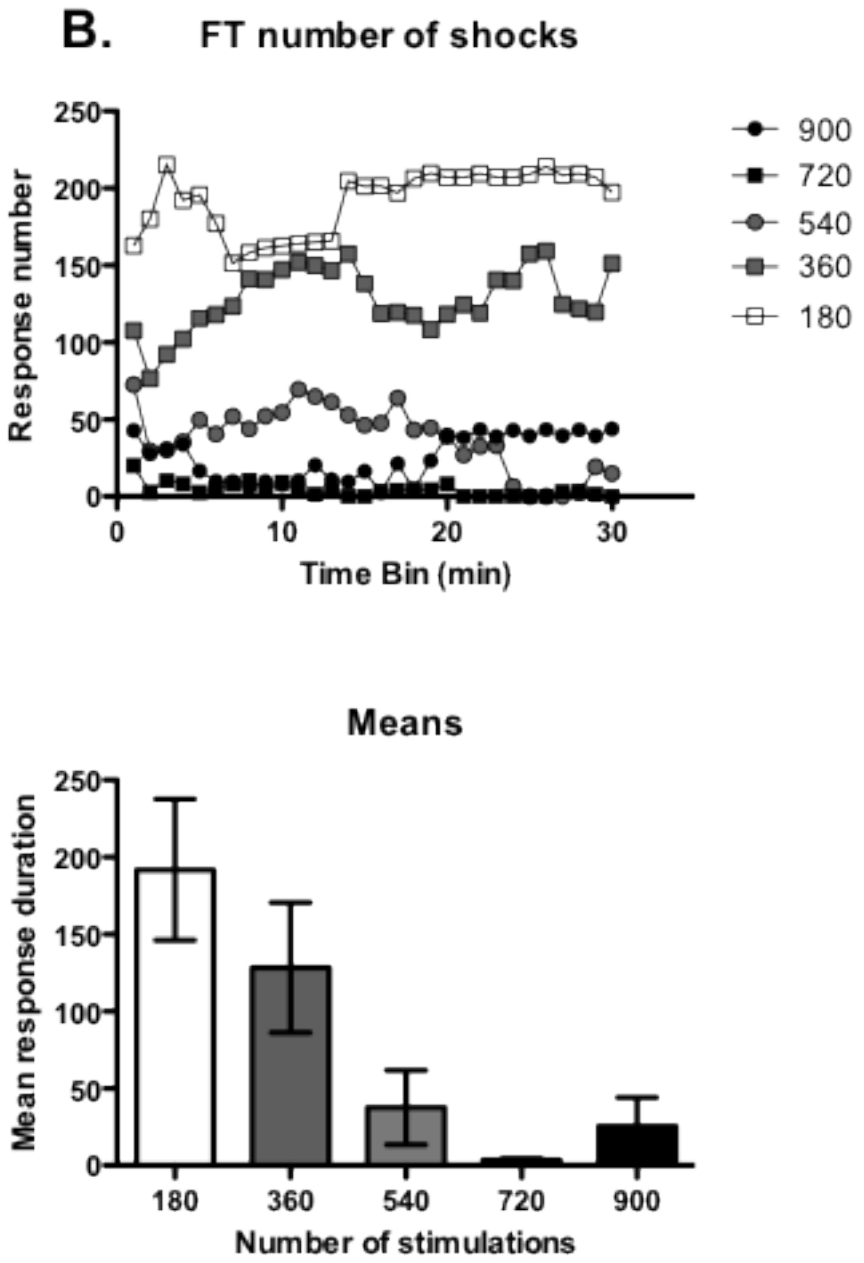

Figure 2. Continued. (B) Effect of number of FT stimulations on response number during instrumental learning. The top panel depicts number of responses over time. The bottom panel depict mean responses for each group. Subjects that received 180 stimulations are shown in white, 360 and 540 in grey (square and circle, respectively), and 720 and 900 stimulations are shown in black (square and circle, respectively).

Discussion

These data provide behavioral evidence for the importance of shock number and extended training for the FT effect to emerge. Subjects require at least 540 stimulations before the FT effect emerges, replicating previous results. Less stimulation produces a learning deficit. Thus, the minimum number of stimulations required to produce the FT effect is 540 stimulations. However, all the stimulations were presented at 0.5 Hz and the amount of time for each shock session was not equated. Experiment 2 explores whether it is the duration of shock exposure or the number of shocks that is critical.

Experiment 2

Experiment 1 showed that exposure to 720 fixed spaced shock re-established the capacity to learn in an instrumental task (a hallmark of what I refer to as the "FT effect"). However, in comparing treatments, one could argue that the critical factor is the duration of regular stimulation (> 18 mins), and not shock number (>540 stimulations). To address this possibility, I administered either 360 or 720 shocks for either 12 or 24 min and tested instrumental performance a day later to confirm shock treatment has a lasting effect.

Procedure

Experiment 2 used 32 subjects (n=8). The experimental design is depicted in Figure 3. After spinal transection, subjects received either 720 shocks at 1 Hz (12 min), 720 shocks at 0.5 Hz (24 min), 360 shocks at 0.5 Hz (12 min) or 360 shocks at 0.25 Hz

(24 min). Thus, duration of time was compared with shock number to determine the conditions under which the fixed spaced shock effect emerged. This yielded a 2x2 factorial design (session time x shock number). Twenty-four hours later, subjects were tested for instrumental learning.

<table>
<tr><td rowspan="4">Complete transection</td><td rowspan="4">24 hrs</td><td>720 for 12 min (1 Hz)</td><td rowspan="4">24 hrs</td><td rowspan="4">Instrumental testing</td></tr>
<tr><td>720 for 24 min (0.5 Hz)</td></tr>
<tr><td>360 for 12 min (0.5 Hz)</td></tr>
<tr><td>360 for 24 min (0.25 Hz)</td></tr>
</table>

Figure 3. Experimental design for Experiment 2.

Results

I found that the emergence of the FT effect is tied to the number of stimulations, not the session duration. Subjects that received 720 stimulations, regardless of the duration of time (12 or 24 min) were able to acquire the instrumental learning response. In contrast, subjects that received 360 stimulations, regardless of duration of stimulation, were unable to acquire the instrumental learning response (Figure 4). An ANOVA revealed a main effect of the number of stimulations, F (1, 28) = 14.79, $p < .001$. Neither the main effect of session duration was not significant, nor its interaction with number of stimulations was significant, Fs < 1.38, $p < 0.5$. *Post hoc* comparisons confirmed that the groups that received 360 stimulations were significantly different from the groups that received 720 stimulations, $p > .05$. No other effects were significant, $p < .05$.

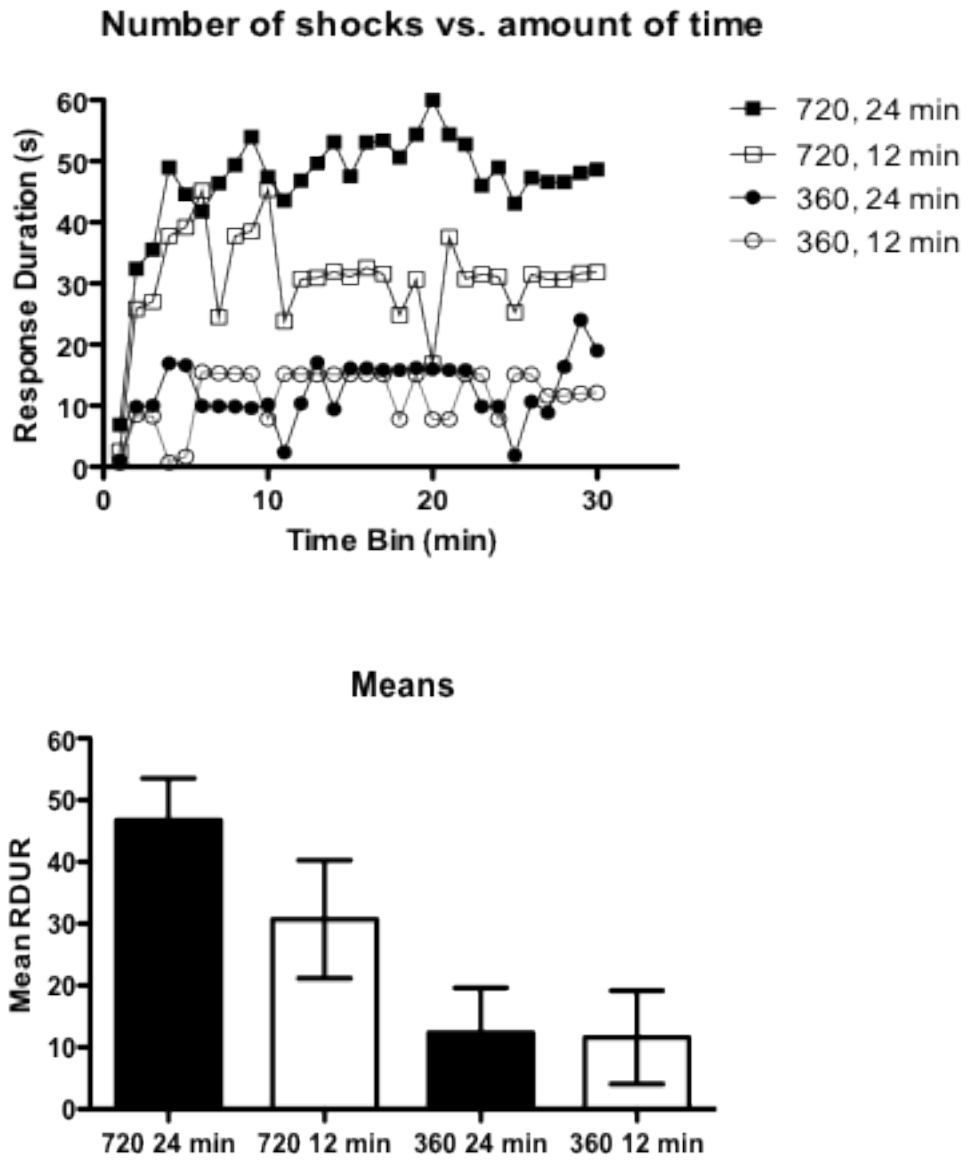

Figure 4. Effect of number of FT stimulations and session duration on performance on an instrumental learning task. Top panel depicts response durations over time. Bottom panel depicts mean response durations for each group, collapsed across time. Groups that received 720 stimulations are shown in squares and 360 stimulations are shown in circles. Groups with a session duration of 24 mins are shown in black, and session duration of 12 min are shown in white. Data indicate that subjects must receive 720 FT stimulations for the beneficial effect of fixed spaced shock to emerge.

Discussion

The results imply that shock number, and not session duration, is the critical factor for producing the FT effect. A common property of learning is that spaced stimulations yield better retention than stimulation given massed in short amounts of time. However, within the bounds tested, only shock number appeared to matter. Further work is needed to determine whether a trade-off emerges at a shorter duration of exposure.

Experiment 3

A hallmark of learning is the retention (memory) of information over time. For timing, a learning account assumes that a feature of the experience of fixed spaced stimulation is abstracted and stored. This could reflect either an index of regularity or the duration of the temporal interval that is abstracted and stored. However, in either case, if a temporal feature is abstracted and stored, there should be some savings across sessions. From Experiment 1, the minimum number of stimulations necessary for the FT effect to emerge is known. The current experiment examined if spinal neurons exhibit a form of savings across sessions by presenting two sessions of fixed spaced shock, separated by 24 hrs. Each session individually should induce the learning deficit. If there is savings across time, subjects that received shock on both day 1 and day 2 should be able to acquire the instrumental learning response.

Procedure

This experiment used 32 subjects and the experimental design is depicted below (Figure 5). The first group received 360 shocks on day 1 (n = 4), and the second group received 360 shocks on day 2 (n = 4). A third group (n=8) received 360 shocks on both days to the same dermatome (leg or leg), and a fourth group received 360 shocks on different dermatomes on both days (leg then tail; tail then leg). A fifth group received no shock prior to instrumental testing. Administration of shock was counterbalanced for dermatome. Subjects in the 720 Same group received stimulation to the same dermatome across days, and subjects in 720 Different group received stimulation to different dermatomes across days. All subjects were then tested for instrumental learning.

<table>
<tr><td rowspan="5">Complete transection</td><td rowspan="5">24 hrs</td><td>360 FT shock</td><td rowspan="5">24 hrs</td><td>360 Same</td><td rowspan="5">Instrumental testing</td></tr>
<tr><td>360 FT shock</td><td>360 Different</td></tr>
<tr><td>360 FT shock</td><td>No shock</td></tr>
<tr><td>No shock</td><td>360 shock</td></tr>
<tr><td>No shock</td><td>No shock</td></tr>
</table>

Figure 5. Experimental design for Experiment 3.

Results

Because the outcome of limiting the number of FT presentations have been extensively tested (Experiment 1 and 2), I collapsed 360 fixed shocks on day 1 (n=4) or day 2 (n=4) into one group, counterbalancing for day. An ANOVA revealed no difference between subjects that received 360 FT shocks on either day 1 or day 2, F (1, 6) = .44, $p > .05$. The effect of either 360 stimulations or 720 stimulations across two days is depicted in Figure 6. Regardless of locus of stimulation, subjects that receive a total of 720 stimulations across two sessions were able to learn, whereas subjects in the 360 Once group, which received only 360 stimulations in total, demonstrated a learning deficit (Figure 6). An ANOVA revealed a significant main effect of condition, and a significant interaction between shock condition and trial Fs > 5.23, $p < .05$. *Post hoc* comparisons of group means confirmed that the 360 Once group differed significantly from all other groups, $p < 0.5$. No differences approached significance, $p > .05$.

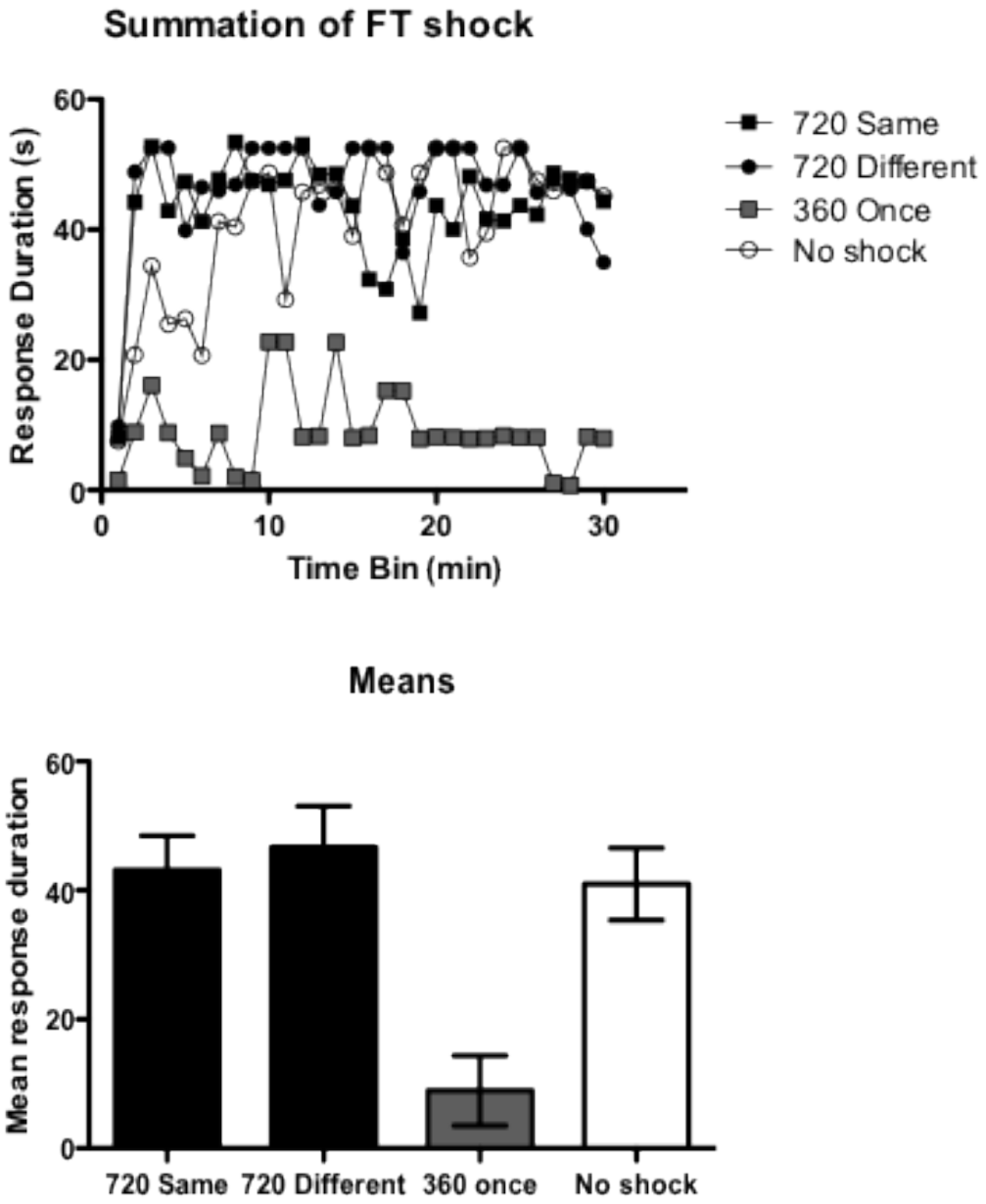

Figure 6. Effect of two sessions of FT stimulations on performance on an instrumental learning task. Top panel depicts response durations over time. Bottom panel depicts mean response durations for each group. Subjects that received 720 stimulations are shown in black, subjects that received 360 stimulations are in grey, and subjects that did not receive shock are in white. Subjects that received 720 stimulations to the same dermatome across two days are squares, and subjects that received 720 stimulations to different dermatomes across days are circles. Data indicate that subjects that receive 720 stimulations across two days are able to acquire the instrumental learning response.

Discussion

The results for Experiment 3 provide the first evidence of savings across time for the FT effect. Subjects that received only 360 stimulations across the two days demonstrated a learning deficit, replicating the results from Experiment 1. Further, subjects that received two sessions of FT shock across two days were able to acquire the instrumental learning response. Thus, these results suggest that the spinal cord is encoding a "memory" associated with each FT shock session and that the cues associated with FT shock are abstracted and stored, producing a beneficial effect that is retained over time.

Experiment 4

The results from Experiment 3 provide evidence of a savings effect, implying that the interval (2 s) of fixed spaced shock was somehow abstracted and stored, allowing the FT effect to emerge with continued training. If so, a savings effect should only emerge if the initial bout of stimulation is regular and has the same interval. I explore this issue by testing whether the emergence of the FT effect depends on subjects receiving the same type of stimulation across days. Indeed, prior work has shown that spinal systems are insensitive to whether the initial period (180 shocks) of stimulation is regular or irregular (Baumbauer et al., 2008). Perhaps longer periods of irregularity may be tolerated, allowing an FT effect to emerge when just a proportion (half) of the 720 shock sequence is given in a regular manner. To test whether the emergence of the FT

effect requires two bouts of regular stimulation, subjects were given 360 FT or VT shocks separated by 24 hrs.

Procedure

Experiment 4 was conducted in two replications and required 48 rats (n=12). The experimental design is depicted below (Figure 7). Subjects received 360 fixed shocks or 360 variable shocks. On day 2, subjects received either fixed shocks or 360 variable shocks. Administration of shock was counterbalanced for dermatome (leg or tail). Subjects were then immediately tested for instrumental learning.

<table>
<tr><td rowspan="4">Complete transection</td><td rowspan="4">24 hrs</td><td rowspan="2">360 FT shock</td><td rowspan="4">24 hrs</td><td>360 FT shock</td><td rowspan="4">Instrumental learning</td></tr>
<tr><td>360 VT shock</td></tr>
<tr><td rowspan="2">360 VT shock</td><td>360 FT shock</td></tr>
<tr><td>360 VT shock</td></tr>
</table>

Figure 7. Experimental design for Experiment 4.

Results

Subjects given two bouts of FT shock were able to acquire the instrumental learning response whereas subjects give two bouts of VT shock exhibited a learning deficit. Interestingly, the combination of VT and FT stimulation across two sessions did not yield a symmetrical effect; subjects given VT shock followed by FT shock yielded the expected learning deficit, but subjects that were given FT shock followed by VT shock were able to learn (see Figure 8). An ANOVA revealed a main effect of shock given on day 1, $F(1, 44) = 15.15$, $p < .001$, but not day two, $F(1, 44) = 1.74$, $p > .05$. There was, however, a significant interaction of Trial x Day 1 and Trial x Day 2, Fs >

1.86, $p < .05$. *Post hoc* comparisons showed that the VTVT group was significantly different than the FTFT and FTVT groups, and that the VTFT group was significantly different than the FTFT group but not the FTVT group, $p < .05$.

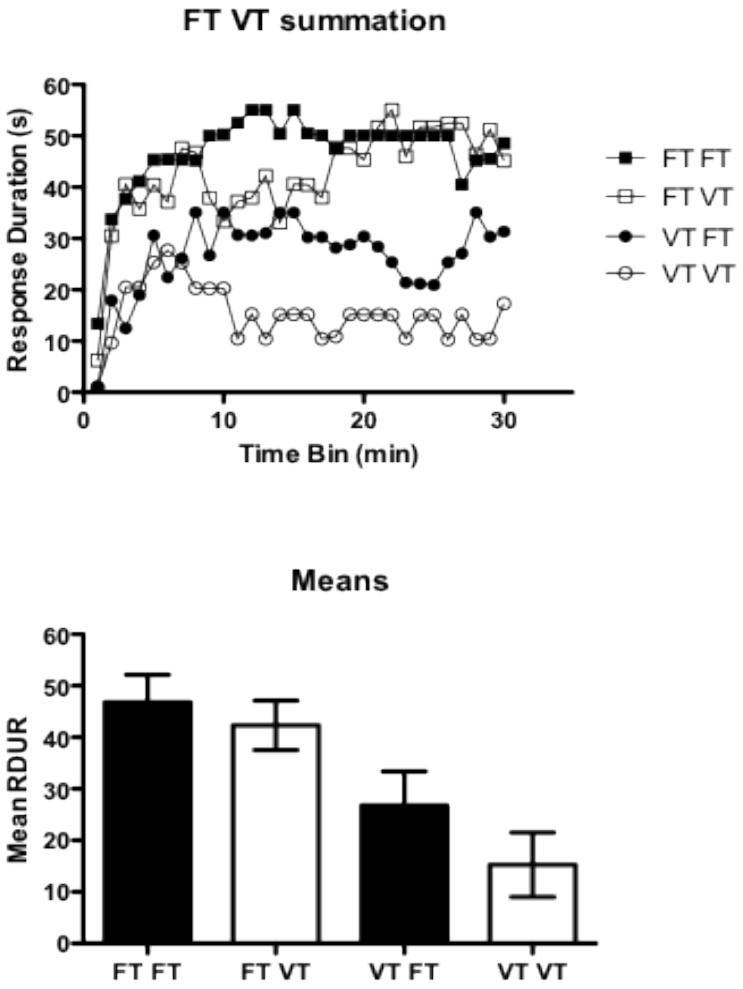

Figure 8. Effect of fixed or variable spaced stimulation across two days on performance on an instrumental learning task. The top panel depicts response durations over time. The bottom panel depicts mean response durations for each group, collapsed across time. Subjects that received FT stimulations on day 1 are shown in squares and VT stimulations on day 1 are shown in circles. Subjects that received FT stimulations on day 2 are shown in black, and subjects that received VT stimulation on day 2 are shown in white. I found that exposure to FT stimulation on day 1 promotes learning, regardless of subsequent shock.

Discussion

The present experiment examined whether the savings effect observed in Experiment 3 required two bouts of regular stimulation. As expected, subjects given FT shock on both days were able to learn in an instrumental learning task, replicating previous results. Also, the groups that received just VT shock across two days, or VT shock on day 1 and FT shock on day 2, displayed a learning deficit. What was unexpected is that an initial period of FT shock on day 1 was sufficient to minimize the adverse effect of VT shock on day 2. This implies that the initial training yielded some lasting benefit, that the initial training of FT shock appears to have biased the system toward "interpreting" subsequent stimulation as regular. Under these conditions, the system may treat shocks that occur around the expected duration (e.g. 2 s +/- 1) as regularly spaced stimulations, because they fall within acceptable limits. By "interpreting" these additional shocks as regular, the system may have received the number of repetitions necessary (> 540) for the FT effect to emerge.

CHAPTER IV

INTEGRATION OF FIXED SPACED STIMULATION

The previous experiments showed that spinal neurons are capable of summating stimulation across time and across dermatome (Experiment 3). These results suggest that spinal neurons are capable of integrating stimulation from two distinct dermatomes to produce FT effect, implying the role of a central structure in deriving temporal regularity. The current chapter was driven by the results of the next experiment, which was designed to explore whether the spinal cord is capable of integrating stimulation across dermatomes. The results yielded an unexpected outcome, suggesting that spinal systems may be able to derive regularity when half the FT stimulation were randomly omitted. This led me to further investigate the conditions under which the fixed spaced shock effect is observed and seek evidence that the fixed spaced shock effect is preserved when a large proportion of the stimuli are missing.

Experiment 5

The fifth experiment examined whether spinal neurons can integrate stimulation across distinct dermatomes, producing a beneficial FT effect by integrating two signals that are independently only semi-fixed. This was achieved by presenting 720 shocks at a regular interval, but randomly alternating the dermatome stimulated. My hypothesis was that a central system would integrate the stimulation across the two dermatomes,

yielding an FT effect. If, in contrast, regularity is derived by a peripheral filter, each signal should be processed locally as semi-fixed, which should cause a learning deficit.

Procedure

Experiment 5 used 24 subjects (n = 8). Twenty-four hours after spinal transection, one group of subjects (integrated) received 720 FT shocks with dermatome (leg or tail) randomly determined by a computer program. A second group (sequential) received the same shocks to the leg and the tail, but instead of an integrated presentation of shock to both dermatomes, the semi-fixed shocks were presented to the leg then the tail, or vice versa (see Figure 9). Thus, all subjects received a total of 720 stimulations. However, only the concurrent group received a physical stimulus at regular intervals (2 s; with the locus varying across time).

Results

The effect of a sequential or integrated shock schedule is depicted in Figure 10. Subjects given semi-fixed shock to one dermatome and then another (sequential) did not exhibit a learning deficit, even though the schedule of presentation is not actually fixed. An ANOVA revealed no significant effects between shock groups $F\ (2, 21) = 1.39$, $p > .05$. All subjects in this experiment were able to acquire the instrumental learning response.

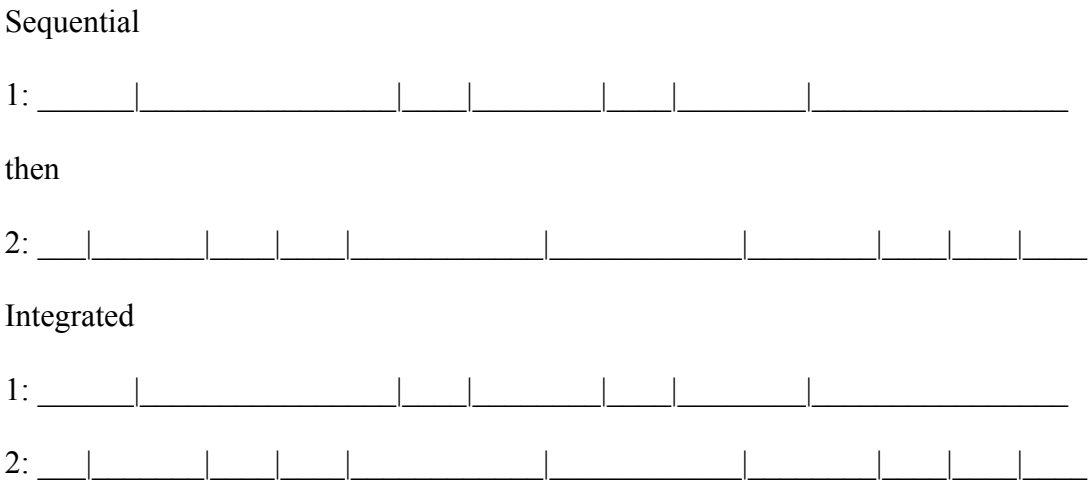

Figure 9. Depiction of the schedule of shocks presented in Experiment 5. The numbers "1" or "2" depict dermatome (leg or tail).

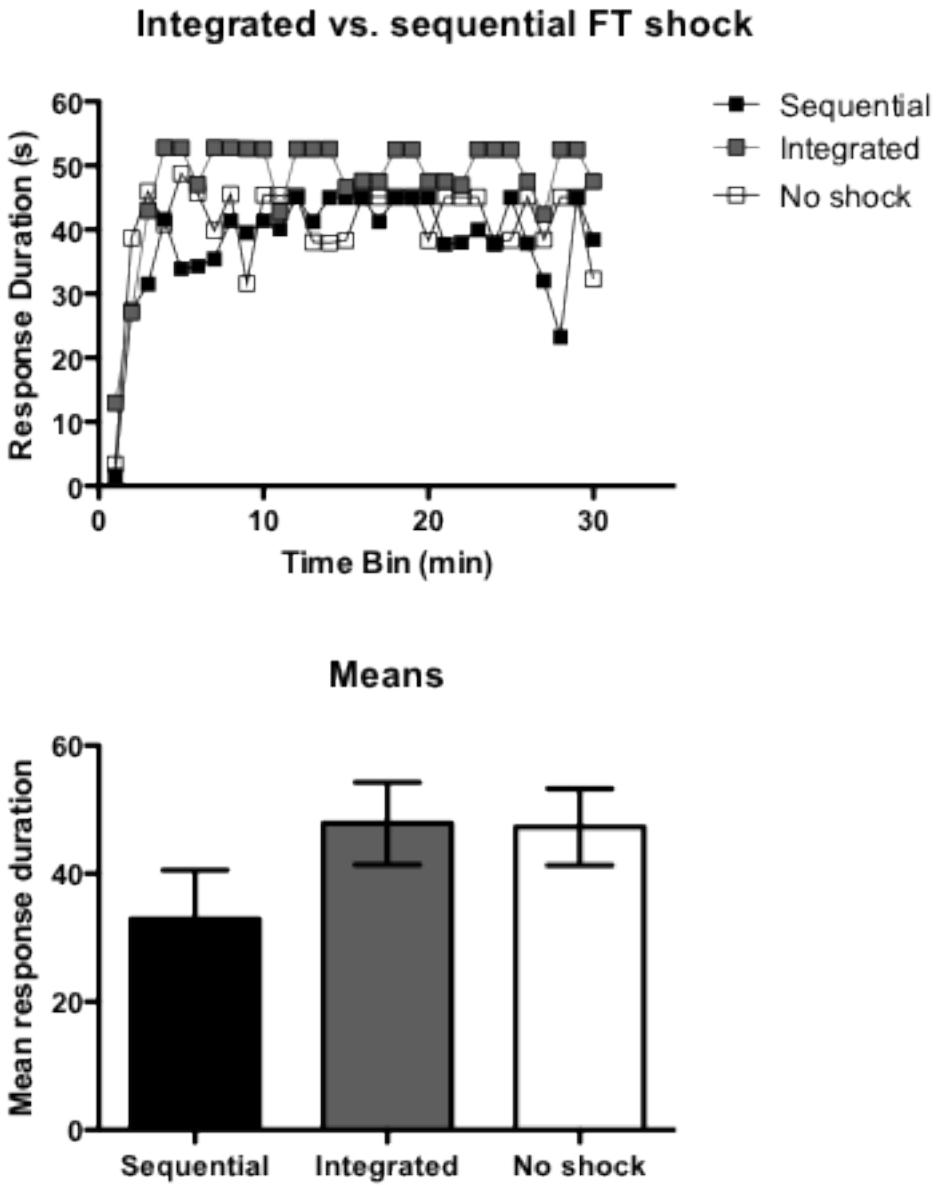

Figure 10. Effect of sequential, integrated or no shock on acquisition of an instrumental learning task. Top panel depicts response durations over time. Bottom panel depicts mean response durations for each group. The group that received sequential shock is shown in black, the group that received integrated shock is shown in grey, and the group that did not receive shock is shown in white. I found that semi-fixed shock did not induce a learning deficit, regardless of whether it was presented in an integrated or sequential manner.

Discussion

The results were suggest that spinal processes are capable of not only summating FT shock, but spinal processes can also "fill-in" missing stimuli from an FT schedule. However, both the sequential and integrated groups received 720 shocks, and as a result, there was ample exposure to the 2 s ISI, even though many shocks were missing. If the system can truly "fill-in" missing stimuli, a FT effect should still be observed when half the shocks from a 720 shock sequence are randomly omitted, resulting in roughly 360 shock presentations. Experiment 6 explores this possibility.

Experiment 6

One interpretation of the results from the previous experiment is that spinal mechanisms can "fill-in" missing stimuli. If spinal mechanisms do in fact "fill-in" missing stimuli, then randomly omitting half the shocks from a train of 720 stimulations should still yield a FT effect, even though only 360 shocks are actually presented – a shock number that normally produces a learning impairment (Experiment 1). However, without a positive control group to demonstrate the learning deficit, it was not clear whether or not these shock schedules yielded a FT effect or simply failed to impair learning in the instrumental task. The present experiment explored these issues by testing the impact of randomly omitting half the shocks (360 of 720) given on either a fixed or variable schedule. VT stimulation should induce learning and thereby provided a positive control.

Procedure

Experiment 6 used 40 rats (n=10). Subjects were presented with fixed or variable spaced stimulations at differing probabilities (1 or 0.5). Thus, the group with a probability of 1 received 720 fixed space stimulations at a frequency of 0.5 Hz. The group with a probability of 0.5 received approximately 360 stimulations at roughly 0.25 Hz. Immediately after, I tested whether these schedules of stimulation affected instrumental learning.

<table>
<tr><td rowspan="4">Complete transection</td><td rowspan="4">24 hrs</td><td>FT, p of 1</td><td rowspan="4">Instrumental testing</td></tr>
<tr><td>FT, p of 0.5</td></tr>
<tr><td>VT, p of 1</td></tr>
<tr><td>VT, p of 0.5</td></tr>
</table>

Figure 11. Experimental design for Experiment 6.

Results

Replicating previous results, subjects that received 100% variably spaced shock exhibited a learning deficit while the same number of shocks given in a regular manner did not (Figure 12). Both effects were still observed when half the shocks were randomly omitted, despite the decrease in shock number. An ANOVA revealed a significant main effect of shock schedule (FT or VT), $F\,(1, 36) = 26.41$, $p < .001$. The main effect of shock probability was not significant, $p > .05$. Additionally, the Trial x Shock schedule interaction was significant, $F\,(29, 1044) = 2.893$, $p < .001$.

Post hoc analysis of group means determined that subjects that received FT shock, regardless of probability, were significantly different from subjects that received VT shock, regardless of probability, $p < .05$.

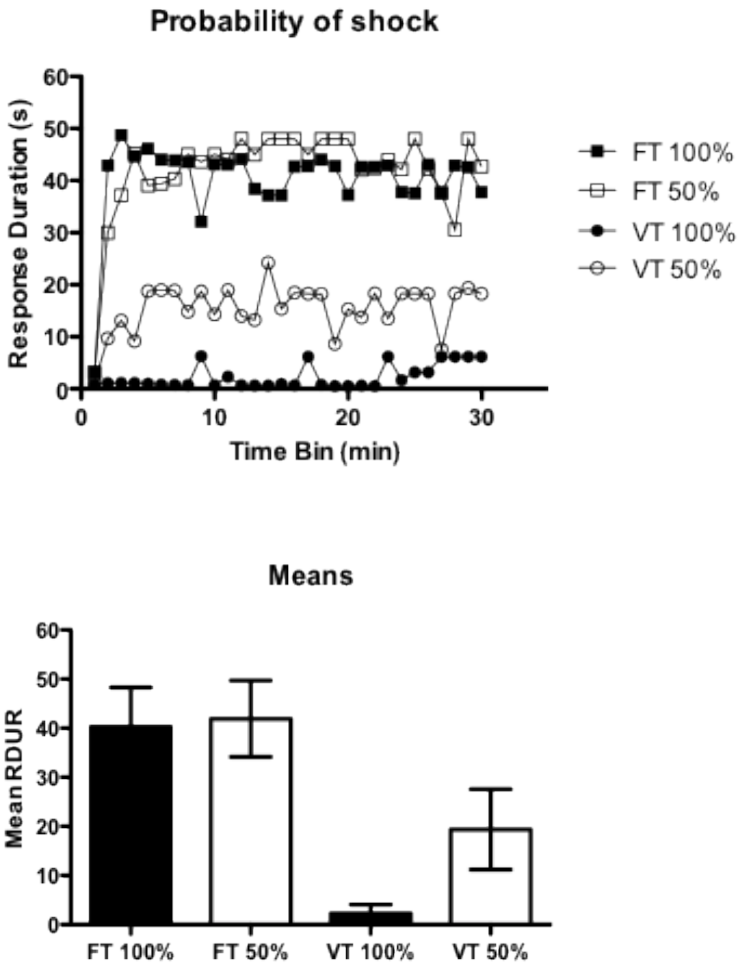

Figure 12. Effect of fixed or variable spaced stimulation and probability of presentation (50% or 100%) on performance on an instrumental learning task. Top panel depicts response durations over time. Bottom panel depicts mean response durations for each group, collapsed across time. Groups that received FT stimulations are shown in squares and VT stimulations are shown in circles. Groups with a probability of 100% are shown in black, and probability of 50% are shown in white. Data indicate that reducing the probability of shock for both FT and VT schedules did not impact the behavioral effects. Fixed spaced shock allowed learning, variable spaced shock produced the learning deficit.

Discussion

The results from Experiment 6 suggest that spinal neurons can "fill-in" the missing stimulation from the FT schedule, because even when only 360 (of 720) shocks are presented, subjects were still able to acquire the instrumental learning response. Further, our control group of VT shock at a p of 0.5 demonstrates that reducing the probability to 50% does not cause the stimulation parameters to become too spare, because variable shock presented at 50% is sufficient to produce the learning deficit. The fact that omitting some shocks from a variable schedule had little effect (compared to 100% VT shock) is not surprising, because the stimuli remain unpredictable. What is suprising is that the removing half of the shock from a train of FT stimulation, which introduces a level of unpredictability, had no effect.

Experiment 7

In addition to promoting spinal plasticity, fixed spaced shock (720 shocks at 0.5 Hz) prevents and reverses the induction of the learning deficit after subjects are exposed to VT shock (180 shocks). The previous experiment showed that subjects that receive fixed spaced shock at a probability of 50% are able to learn, one index of the FT effect. The current experiment examines whether a shock schedule with missing stimulation also induces a long-term protective effect that blocks the induction of the learning deficit by VT shock. I also extend the results of Experiment 6 by testing whether the system can support the omission of more stimuli (75% missing; p of 0.25).

Procedure

Experiment 7 used 40 subjects (n=10). The experimental design is depicted in Figure 13. Subjects received fixed spaced stimulation at a probability of 1, 0.5, 0.25 or 0 (no shock) one day after spinal transection at T2. Twenty-four hours later, all subjects were challenged with 180 VT shocks (0.2-3.8s). The impact of stimulation on instrumental learning was then tested.

<table>
<tr><td rowspan="4">Complete transection</td><td rowspan="4">24 hrs</td><td>FT, p of 1</td><td rowspan="4">24 hrs</td><td rowspan="4">VT shock</td><td rowspan="4">Instrumental testing</td></tr>
<tr><td>FT, p of 0.5</td></tr>
<tr><td>FT, p of 0.25</td></tr>
<tr><td>No shock</td></tr>
</table>

Figure 13. Experimental design for Experiment 7.

Results

As usual, subjects given 720 FT shocks (probability of 100%) were protected against the induction of the learning deficit produced by variable spaced shock. This same effect was observed when 50% but not 75% (p of 0.25) of the shocks were randomly omitted (Figure 14). An ANOVA revealed a significant main effect of shock, $F(3, 36) = 4.88$, $p < .01$. *Post hoc* analysis of group means revealed that subjects given 100% and 50% FT shock were significantly different than subjects that only received either 25% FT shock or no shock on day 1, $p < .05$.

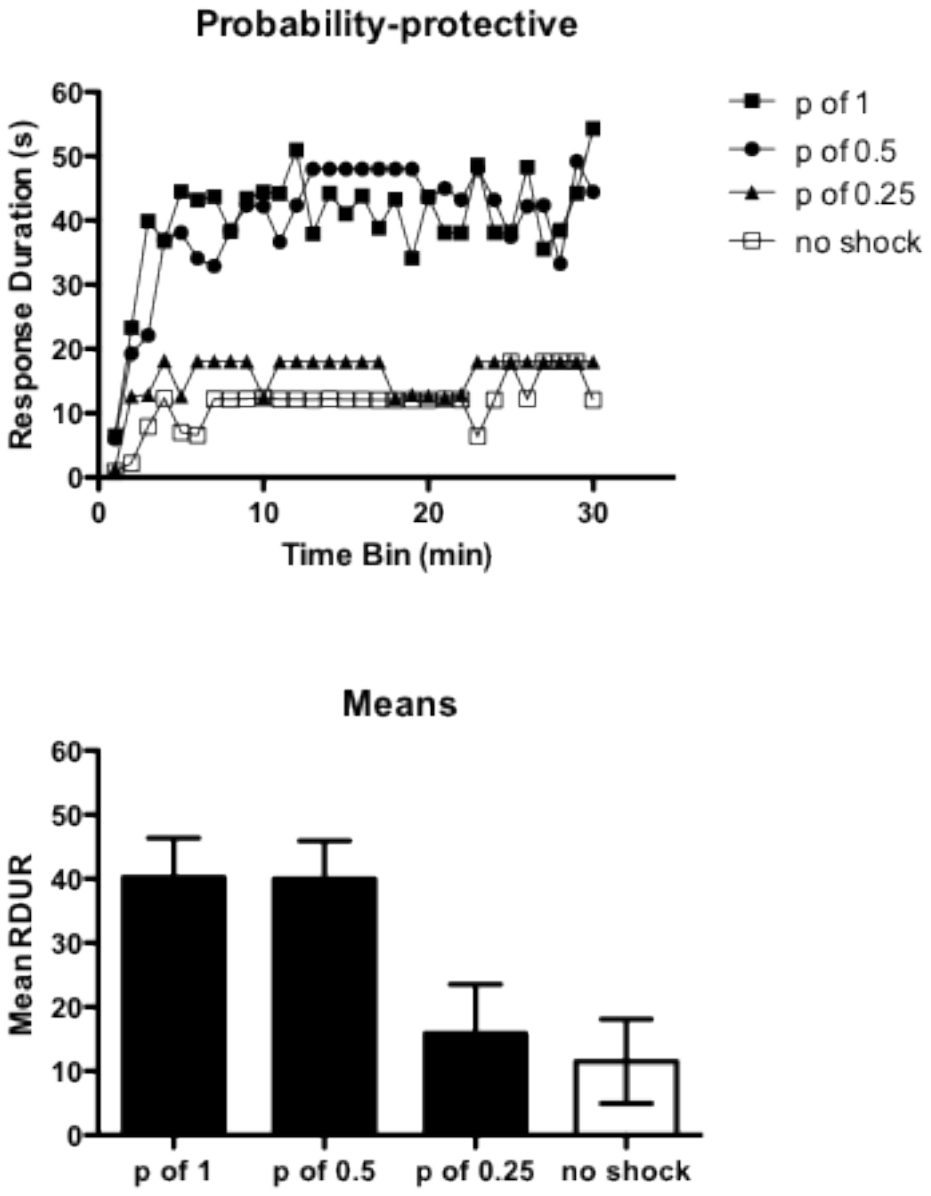

Figure 14. Effect of reducing the probability of FT shock presentation on the protective effect. The top panel depicts response durations over time. The bottom panel depicts mean response durations for each group. The groups that received FT shock prior to VT shock are shown in black, and the group that did not receive FT shock prior to VT shock is shown in white. Probability of shock is depicted as a square (100%), circle (50%), and triangle (25%). Data indicate that 100% and 50% FT shock was protective against the induction of the learning deficit.

Discussion

These results further demonstrate the basic effect of spinal neurons "filling-in" missing stimulation from a fixed spaced schedule. As previously found, subjects that received 720 FT shocks (p of 1) were protected against the induction of the learning deficit by VT shock. However, even though they are only presented with a total of 360 shocks, the p of 0.5 FT shock condition did not express a learning deficit and were protected against the induction of the learning deficit by VT shock. Together these findings suggest that spinal neurons can "fill-in" the missing stimulation when lower probabilities of shock are presented. However, when the presentation of stimulation becomes too scarce (p of 0.25), the protective potential of FT shock is no longer observed.

CHAPTER V

ROLE OF A CENTRAL SYSTEM IN THE FT EFFECT

There are two general mechanisms that could provide for a sense of time within the spinal cord. One is by means of a central system that discriminates regular and irregular stimulation independent of the locus of stimulation. The other is by means of a peripheral filter. For example, variable and fixed spaced stimulation might engage different fiber types, and the divergent effect might be due to a simple difference of fiber activation, rather than a difference in central processing. If fixed and variable spaced stimulation each activate distinct fiber pathways, then the difference in behavioral outcomes derives from the interaction of the two activated fiber pathways, and the pattern of shock is filtered through distinct fiber pathways before the afferent signal reaches a central locus (the spinal cord). In this case, an index of regularity (or possibly temporal interval) would be indicated by the subset of fiber activated. Additivity might then be achieved across dermatome by summating outputs. The key difference between these two accounts concerns the mechanism that underlies the "abstraction" of regularity. With a peripheral filter, this could emerge from the physiological properties of the different afferent fibers. In contrast, a central mechanism integrates stimulation across dermatomes and derives whether there is an overarching regularity between the two different dermatome inputs. The first experiment in this chapter aimed to elucidate the contribution of either a peripheral filter or a central processor to the development of the FT effect.

From the results of the first experiment in this chapter, it is clear that the spinal network (central processor) is critical to producing this timing dependent learning. These findings leave us asking both, "What structures within the spinal cord are necessary for timing to occur?" and "How does such a structure encode these temporal cues?" The focus of the second experiment in this chapter is whether the CPG contributes to the acquisition and maintenance of the FT effect on spinal learning, specifically if the CPG is the central structure processing fixed spaced shock, and if the CPG is necessary for the acquisition of the FT effect. To test this hypothesis, the spinal segments where the CPG is thought to reside must first be isolated. Previous studies suggest that the rostral lumbar enlargement plays an essential role in generating rhythmic behavior. Supporting this Magnuson et al. (2005) showed that contusion injuries centered on the rostral lumber cord (T13/L1, L2) had a greater impact on recovery of locomotor activity (stepping) than more caudal injuries, even though the caudal injuries lead to a greater loss of grey matter volume. Additionally, as Cazalets et al. (1995) demonstrated, when only the L1-L2 portion (lumbar enlargement) of the spinal cord of neonatal rats was exposed to a cocktail of 5-HT and NMDA, evoked rhythmic activity was recorded in all lumbar segments. Importantly, after 5-HT and NMDA application to L3-L6 (lower lumbar cord), the evoked rhythmic activity characteristic of the CPG was not observed, only tonic activity was observed (Cazalets et al., 1995).

Interestingly, the structures within the spinal cord responsible for instrumental learning seem to be located in the lower lumbar to upper sacral (L3-S2) region of the

spinal cord. Supporting this, an intrathecal lidocaine injection to the L3-S2 region disrupted the acquisition of the instrumental learning. Furthermore, when a dual transection was performed, the essential spinal network responsible for the acquisition of the instrumental learning response was between the L4 and S2 area of the spinal cord (Liu et al., 2005).

I hypothesized that the CPG acts like an internal clock, processing temporal cues related to stimulus regularity, and contributes to the beneficial plasticity after regular shock. Experiment 8 tested this hypothesis by examining whether surgically isolating the CPG from caudal segments eliminated the FT shock effect (Experiment 8).

Experiment 8

In seeking evidence for central integration in the previous chapter, I found evidence that spinal neurons are capable of inferring missing stimuli. However, what remains unclear is whether a peripheral filter or a central mechanism underlies this capacity. To address these alternative explanations, this experiment was designed to push the system in a different, more challenging way. I accomplished this by testing whether the phase relationship of stimulation given to the two dermatomes matters. If a peripheral filter account is at work it should not matter if concurrent stimulation is given in or out of phase. In contrast, if a central mechanism is responsible for the discrimination of fixed spaced versus variable spaced stimulation then it would follow that the two afferent stimuli must be given in phase to produce a FT effect.

Phase was manipulated in two ways. One way presented shock to two dermatomes in an alternating or simultaneous (concurrent) pattern (see Figure 16). If a peripheral mechanism is at work, both should yield a FT effect. If a central system abstracts regularity, then only the alternating pattern (coherent) should yield a FT effect. The second manipulation presented the phase relationship between shock to two dermatomes (e.g. the leg and tail) at slightly different frequencies (e.g. +/- 100 msec). Under these conditions, the stimuli begin in an alternating pattern, but the relationship is shifted slightly over time as the two schedules "rotate" in opposite directions (incoherent). Each dermatome receives regular stimulation, but the relationship across dermatomes is irregular. With this type of stimulation, a peripheral filter should yield a FT effect, but a central mechanism would not.

Procedure

Experiment 8 used a total of 48 rats (n=12). Spinally transected rats received FT shock to two dermatomes at 0.25 Hz, but with the phase relationship slowly rotated by 100 msec each cycle. For subjects in one group, the phase relationship rotated in the same direction (increasing or decreasing; counterbalanced), so that the alternating pattern was maintained across dermatomes (coherent). Subjects in a second group received the same number of shocks to each dermatome, but the phase relation was rotated in opposite directions (incoherent). Across dermatomes, this produced a varying temporal relation that grows longer and shorter across time. A third group (one dermatome: shifting) was included to verify that this increasing/decreasing pattern of

stimulation does not yield a FT effect when applied to a single dermatome. A fourth group received concurrent stimulation to the leg and the tail.

<table>
<tr><td rowspan="4">Complete transection</td><td rowspan="4">24 hrs</td><td>Two dermatomes: Coherent</td><td rowspan="4">Instrumental testing</td></tr>
<tr><td>Two dermatomes: Incoherent</td></tr>
<tr><td>Two dermatomes: Concurrent</td></tr>
<tr><td>One dermatome: Shifting</td></tr>
</table>

Figure 15. Experimental design for Experiment 8.

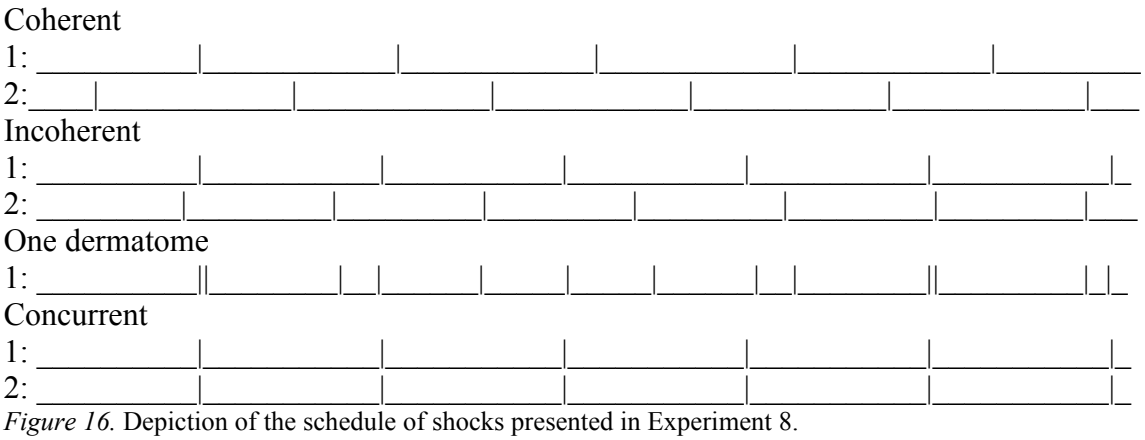

Figure 16. Depiction of the schedule of shocks presented in Experiment 8.

Results

When 720 stimulations were given in a coherent manner, alternating in a fixed spaced pattern between the leg and the tail, subjects were able to acquire the instrumental learning response. However, incoherent stimulation that shifted the phase relation across dermatomes produced a learning deficit. Concurrent stimulation to the leg and the tail and shifting stimulation presented to a single dermatome, also produced a learning deficit (see Figure 17). An ANOVA revealed a significant main effect of shock condition, $F\,(3, 44) = 5.48$, $p < .01$. *Post hoc* analysis of group means indicated that

subjects that received coherent stimulation were significantly different than subjects that received all other types of shock (incoherent, concurrent or shifting), $p < .05$.

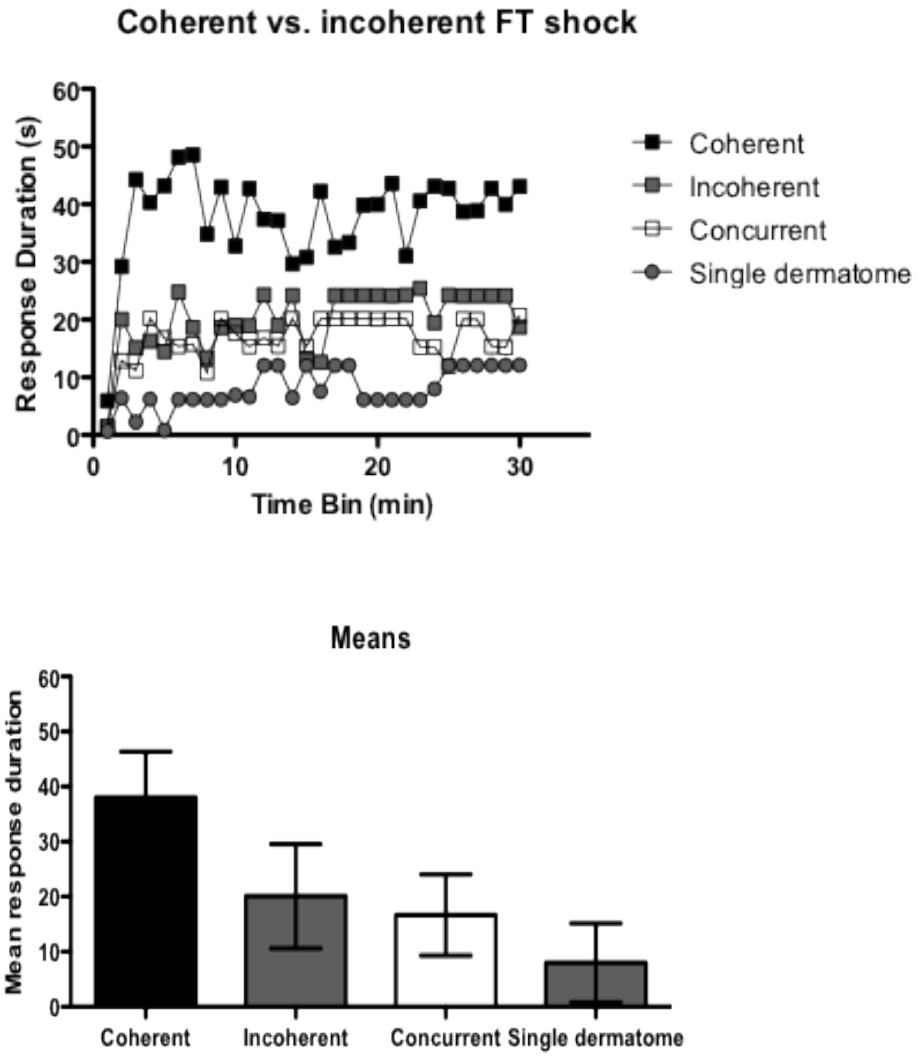

Figure 17. Effect of phase shifting FT stimulation on instrumental learning. The top panel depicts response durations over time, and the bottom panel depicts mean response durations for each group. The group that received coherent shock is shown black, the groups that received incoherent shock or single dermatome (shifting) shock are shown in grey, and the group that receive shock to two dermatomes concurrently is shown white. Incoherent shock is depicted as a square, and single dermatome shock are shown as circles. All stimulus conditions, except for coherent shock, produced a learning deficit.

Discussion

Only subjects that received coherent stimulation across two dermatomes were able to acquire the instrumental learning response. Subjects given incoherent stimulation, concurrent stimulation across two dermatomes, or shifting stimulation to one dermatome exhibited a learning deficit. It appears that 360 shocks to two dermatomes only induced a FT effect when the stimuli alternate in a regular manner. If a peripheral filter were responsible for the summation and integration effects observed in previous experiments, then both the incoherent stimulated subjects and the concurrent stimulated subjects should have yielded a FT shock effect that preserved the capacity for learning. The fact that this was not observed suggests that a central processor is involved in producing the fixed spaced effects. The next experiment aims to determine if that central processor is the CPG, a structure in the spinal cord known for controlling rhythmic behaviors.

Experiment 9

The results of Experiment 8 imply that a central processor underlies the abstraction of regularity. To examine whether the CPG plays a central role in producing the FT effect, I surgically separated the locus of the spinal learning (L3/L4) from the location of the CPG (L1/L2). If the CPG is involved in the FT effect, then a cut at L3 before FT stimulation should eliminate processing the fixed space shock as predictable. Under these circumstances, intermittent shock should induce a learning deficit.

Procedure

Subjects underwent a spinal transection rostral to T12 or at L3. The next day, they received 24 min of FT stimulation (720 shocks) or remained unshocked. They were then immediately tested in the instrumental learning paradigm.

<table>
<tr><td rowspan="2">Transection (T12)</td><td rowspan="4">24 hrs</td><td>FT shock</td><td rowspan="4">Instrumental testing</td></tr>
<tr><td>No shock</td></tr>
<tr><td rowspan="2">Transection (L3)</td><td>FT shock</td></tr>
<tr><td>No shock</td></tr>
</table>

Figure 18. Experimental design for Experiment 9.

Results

After a spinal transection at the T12 region of the spinal cord, rats were still able to acquire the instrumental learning response, with or without FT stimulation. Similarly, the ability to acquire the instrumental learning response was not disrupted by a cut at L3. However, in subjects that had a L3 transection, fixed spaced shock induced a learning deficit (Figure 17). Shock after a cut separating the rostrolumbar section (L1-L2) of the spinal cord from caudal sections (L3 and below) produced a learning deficit. An ANOVA revealed a significant main effect of shock as well as a significant interaction between cut and shock, $Fs > 4.37$, $p < .05$. *Post hoc* analysis revealed that subjects that received both a cut at L3 and FT shock were significantly different from all other groups, $p < .05$. No other groups were significantly different from each other, $p > .05$.

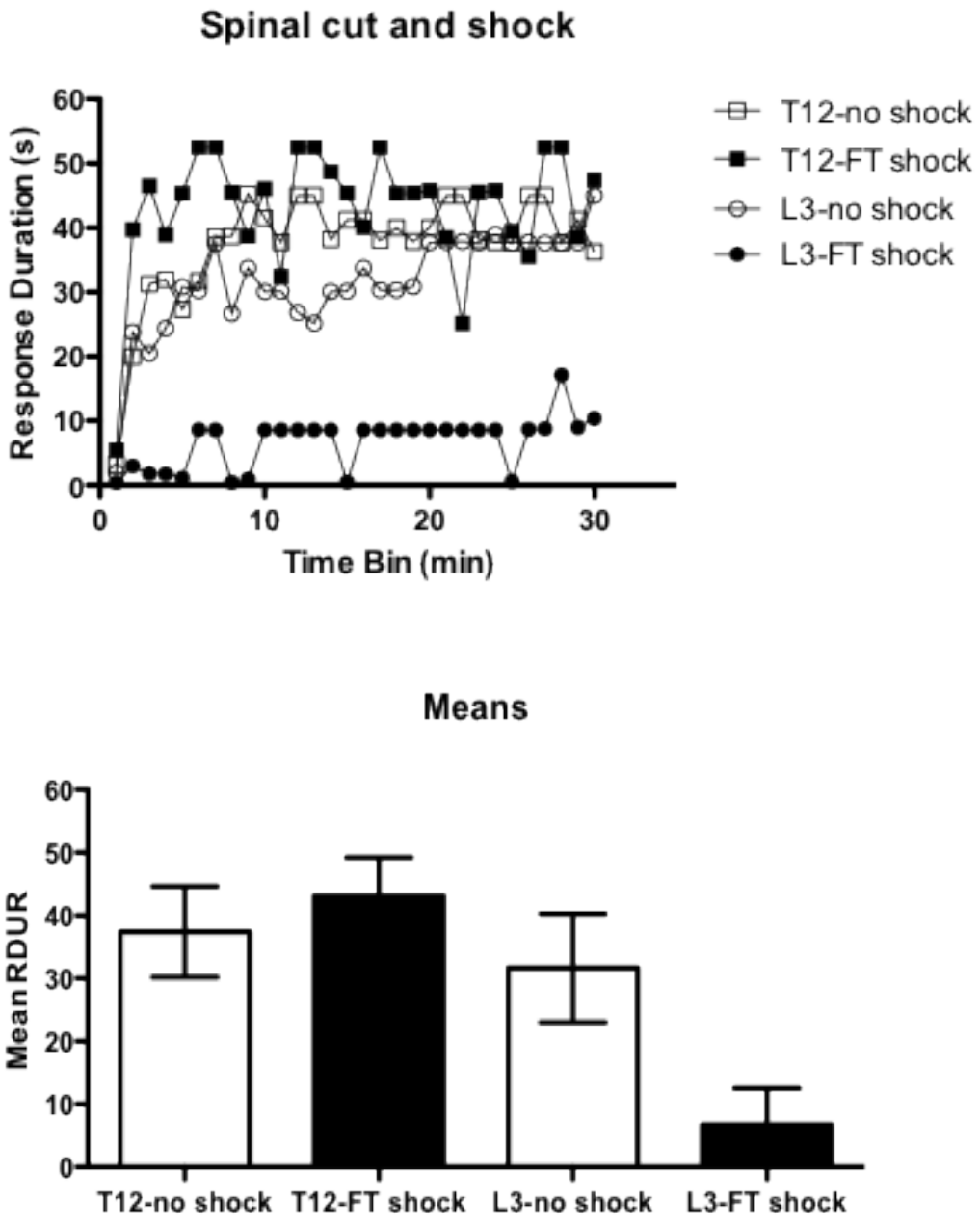

Figure 19. Effect of level of transection and fixed spaced shock on acquisition of an instrumental learning task. The top panel depicts response durations over time and the bottom panel depicts mean response durations for each group, collapsed across time. Subjects that received a transection at T12 are shown in squares and a transection at L3 are shown in circles. Subjects that received FT shock are shown in black, and subjects that received no shock are shown in white. FT stimulation induced a learning deficit in L3 transected rats.

Discussion

These results confirm that the L1 and L2 portions of the lumbar enlargement is not needed for instrumental learning. The L1-L2 tissue is, however, necessary for the production of the FT effect. Given prior studies linking this region of the spinal cord to the CPG, and the generation of rhythmic behavior, these results suggest this same system may contribute to other examples of spinal timing.

CHAPTER VI

GENERAL DISCUSSION AND SUMMARY

From these experiments, it is clear that spinal neurons are capable of much more than previously thought. Not only are spinal neurons capable of discriminating between temporally regular and temporally irregular stimulation, it seems that spinal neurons are also capable of summating fixed spaced stimulation across time as well as integrating fixed spaced stimulation across dermatomes. The experiments in this dissertation were designed to test two questions that arose regarding the FT effect from prior studies: first, if spinal neurons are actively learning about fixed spaced shock, what properties of fixed spaced shock are being learned; and second, where does the discrimination between FT and VT shock occur, peripherally or centrally.

Prior studies have shown that after a complete spinal transection at T2, training with fixed spaced stimulation (temporally regular; ISI: 2 s) has a beneficial impact on spinal plasticity as measured by performance on the instrumental learning task (Baumbauer et al., 2008). Subjects given 30 min (900 shocks) of fixed spaced stimulation were able to acquire the instrumental learning response (increased leg flexion). In contrast, variable spaced stimulation (temporally irregular; ISI: 0.2-3.8 s) produces a long lasting learning deficit, or inability to acquire the instrumental learning response (Baumbauer, et al. 2008). Importantly, fixed space stimulation allows not only for acquisition of the instrumental learning response, but also blocks and reverses the adverse effects of variable stimulation (learning deficit). Subjects given 30 min of fixed

spaced stimulation before or after 6 min of variable spaced stimulation were still able to acquire the instrumental learning response (Baumbauer, et al. 2009). Furthermore, prior administration of fixed spaced stimulation can block both capsaicin-induced allodynia as well as intermittent-shock induced allodynia (Baumbauer, et al. 2011). Thus, the benefits of temporally regular stimuli are apparent for both nociceptive processing and instrumental learning. These beneficial effects of fixed-spaced stimulation are eliminated by administration of protein synthesis inhibitor, cycloheximide, as well as NMDA receptor antagonist, MK801. Additionally, in subjects administered TrkB-IgG, an agent that sequesters endogenous brain-derived neurotrophic factor (BDNF; necessary for synaptic plasticity), the protective effects of fixed-spaced stimulation were blocked (Baumbauer, et al. 2009). Taken together, these data support the claim that an active process is engaged by the presentation of predictable stimuli and suggest that the beneficial aspects of fixed-spaced stimulation are learned.

The experiments in this dissertation built on these prior studies with the aim of detailing how fixed spaced stimulation produces beneficial outcomes for spinal plasticity. I first established how many presentations of fixed spaced shock are needed to induce the FT effect (Experiment 1). I found that 180 – 360 shocks yielded a learning deficit and that additional training (540 or more) attenuated this effect. These results agree with prior studies showing extended exposure to FT shock has a therapeutic effect that restores the capacity for learning. This restoration of plasticity is a key component of what I refer to as the "FT effect."

From Experiment 1, it was not clear whether the critical factor in producing the FT effect is shock number or duration of FT shock session. From the latter perspective, the beneficial effects of 540, 720 and 900 FT shocks emerge from the duration of shock session (18 min, 24 min and 30 min, respectively) rather than actual number of presentations. Conversely, 180 and 360 FT shock could have a detrimental effect because the stimuli occurred in a shorter period of time (6 and 12 min, respectively). To explore these alternatives, the impact of both shock session duration (12 and 24 min) and number of presentations (360 and 720 FT shocks) was tested (Experiment 2). I found that 720 shocks promote instrumental learning, regardless of the shock session duration. Further, 360 shocks impaired learning independent of session duration.

From Experiments 1 and 2, it is clear that 360 FT shocks produce the learning deficit. The next experiment (Experiment 3) explores whether the deficit produced by 360 FT shocks could be reversed by more FT shock 24 hrs after the initial shock was presented. This issue was of interest because, if additional shock could reverse the deficit produced by the earlier FT shock, it would suggest that a "memory" of shock presentation was encoded and that there was some "savings" across the two sessions. I found that when subjects were presented with two sessions of 360 stimulations, totaling 720 stimulations across two days, subjects were able to acquire the instrumental learning task. As found in Experiments 1 and 2, subjects that only received 360 stimulations demonstrated a learning deficit. Taken together with prior studies demonstrating the necessity of BDNF, NMDAR and protein synthesis for the production of the FT effect, these studies indicate that the beneficial effects of FT stimulation emerge from an active

process; that spinal neurons are learning about some property of fixed spaced shock and this process is how the beneficial effect of FT shock are engaged.

Experiment 4 provided a more detailed analysis of the savings effect. At issue is whether both bouts of stimulation must involve regular stimulation, as Experiment 3 tacitly assumes. To address this issue, subjects were given either FT or VT shock on days 1 and 2 (Experiment 4). As expected, two sessions of FT stimulation allowed acquisition of instrumental learning, whereas two bouts of variable shock produced a learning deficit. Surprisingly, when the temporal relation was switched across days, an asymmetrical effect was observed. Subjects that received VT shock on day 1 and FT shock on day 2 exhibited a learning deficit, whereas rats that received stimulation in the opposite order (FT then VT) did not. These results suggest that an index of regularity is encoded on day 1 that persists and biases future presentations of shock toward regularity. Further studies are necessary to clearly elucidate the meaning of these findings.

Experiment 3 both demonstrate a "memory"-like effect of FT stimulation, and showed that spinal neurons can integrate bouts of stimulation applied to distinct dermatomes (leg or tail). This finding led me to test whether or not the spinal cord can integrate peripheral stimulation on-line when the site of stimulation is randomly determined across trials. However, the initial experiment (Experiment 5) yielded a surprise. The results indicated that spinal neurons are capable of inferring missing stimulation from a fixed spaced schedule, because subjects that received a semi-integrated signal, where half of the FT shocks were randomly omitted, were able to learn. This led me to test whether systematically dropping the probability of shock

presentation would affect the emergence of the FT effect. I found that, not only can subjects that received fixed spaced shock presented at a 50% probability acquire the instrumental learning response (Experiment 6), this schedule has a lasting protective effect that blocks the induction of the deficit by variable spaced shock (Experiment 7).

Because spinal neurons seem to be able to "fill-in" missing stimulation (Experiments 5, 6 & 7), a more rigorous task was devised to test if a peripheral filter or a central processor mediated the FT effect. By phase shifting FT stimulation across two dermatomes, I was able to test the outcome of coherent shock, which was fixed spaced shock that perfectly alternated between the leg and the tail, and incoherent shock, in which leg stimulation was set at an ISI of 3.9 and tail stimulation was set at an ISI of 4.1 (or vice versa) causing a shifting pattern of stimulation, on spinal learning. Only subjects given coherent stimulation across the two dermatomes exhibited the FT effect; the other shock treatments produced a learning deficit, indicating the contribution of a central processor in mediating the FT effect.

Experiment 9 explored whether the tempo generator of CPG contributed to the FT effect. This was accomplished by surgically separating the spinal segments of the CPG (L1-L2) from the spinal segments responsible for instrumental learning (L4-L5). Subjects that received fixed spaced shock after a transection at L3 were unable to acquire the instrumental learning response, implying that the L3 transection eliminated the FT shock effect.

Taken together with previous findings, this dissertation revealed that spinal neurons are capable of learning about temporally regular stimulation can summate FT

shock across sessions, integrate FT shock across dermatomes, and infer missing FT shocks. Not only are spinal neurons capable of much more than relaying sensory information, spinal neurons are capable of promoting adaptive responses to new stimulation, and it seems that the CPG is critical to this process. In the remainder of this chapter, I will review studies that detail the oscillatory activity of the CPG, discuss the behavioral results of this dissertation in relation to current work on the CPG, as well as the implications of these results on promoting adaptive spinal plasticity and the recovery of function after spinal cord injury.

Rhythm of the CPG

This section will detail the cellular mechanisms necessary for both the excitatory rhythmic activity and the inhibitory component of the CPG and the interplay between the two components to produce coordinated movements.

For the characteristic expression of the rhythmic output of the central pattern generator an excitatory source is necessary. The activity of the vertebrate locomotor central pattern generator is mediate by both descending control of the release of glutamate by reticulospinal neurons and the intrinsic activity of the neurons of the CPG. Within the intact spinal cord, activation by descending ionotropic glutamatergic inputs rely on both AMPA and NMDA receptors (NMDAR) for synaptic transmission. Activation of AMPA receptors result in a rapid depolarization, which then facilitate NMDAR activation, by removing the Mg^{2+} block, which prevents anything from

entering the NMDAR. Once depolarization of the neuron leads to the removal of the Mg^{2+} block, positive feedback of the depolarization occurs, resulting in even more removals of the Mg^{2+} block. Though NMDAR have a slower response, NMDAR are critical to learning and memory, because once the Mg^{2+} block is removed, NMDA receptors are Ca^{2+} permeable. Ca^{2+}entry into the cytosol through NMDA receptors underlies the rhythmic oscillations of the CPG. After Ca^{2+} enters the cell via both the NMDAR and voltage gated calcium channels during the depolarization of the neuron, Ca^{2+} activated K+ channels are activated, resulting in a repolarization of the cell and the reinstatement of the Mg^{2+}block on NMDARs. This cycle is then continued by intracellular buffering of Ca^{2+} by removing Ca^{2+} from the cytosol, resulting the shutdown of the Ca^{2+} activated K+ channels. Though this process has been verified in mammals (Hochman et al., 1994), it is best characterized using the lamprey model (Wallen & Grillner, 1985; 1987). It is important to note that during activity dependent release of glutamate, both ionotropic (NMDA) and metabotropic glutamate receptors (mGluRs) are activated by glutamate. During locomotion then, Ca^{2+} does not just enter the cell via NMDARs but can also be released from internal stores via mGluR5 activation (Kettunen et al., 2002). Thus, the oscillatory rhythms seen by the CPG rely on not only the depolarizing conductances that result in Ca^{2+} influx, but also the termination of the depolarizing conductance by engaging Ca^{2+} activated K+ channels to hyperpolarize the cell.

In a recent study by Tazerart and colleagues (2008), the importance of the persistent sodium current (I_{NaP}) to the rhythm generating properties of the CPG is

examined. Though the pacemaker cells within the area of the CPG are typically characterized by intrinsic bursting properties and these intrinsic burst are a result of NMDAR activation and independent of sodium currents, when the I_{NaP} is blocked fictive locomotion is no longer expressed (Tazerart et al., 2008). This study demonstrates the importance of other cellular processes in maintaining the pacemaker oscillations of the spinal network in the absence of structures such as the calcium channel and NMDA receptor activation

Detailed extensively in the lamprey and Xenopus, the function of GABA and glycine in the CPG of mammalian vertebrates is less comprehensive. The inhibitory component of spinal function ($GABA_A$ and $GABA_B$) is activated by the CPG during locomotion in vertebrate models, and is an important aspect to the network of the CPG because it provides the reciprocal inhibition necessary for the alternating pattern of activation as well as coordinated stepping behaviors. As observed by Gossard and Rossignol (1990), $GABA_A$ and $GABA_B$ function are phase locked to locomotor and fictive locomotion cycles. When both $GABA_A$ and $GABA_B$ receptors are blocked, fictive locomotion in the lamprey is no longer expressed (Alford et al., 1991). Presynaptically, $GABA_A$ receptors inhibit activity by increasing chloride intracellular levels of in neurons. Postsynaptically, activation of $GABA_A$ receptors seems to be responsible for the termination of action, and GABA release by reticulospinal descending pathways engages this pathway. Activation of $GABA_B$ receptors postsynaptically seems to result in an inhibition of voltage-gated Ca^{2+} currents, which

alter the activation of Ca^{2+} activated K+ channels, which then markedly changes the locomotor burst frequencies of the CPG.

Timing and the CPG

From the experiments in Chapter V, it seems that a central system mediates the discrimination between fixed and variable spaced stimulation, that the CPG is necessary for the FT effect, and that the CPG is encoding some property of time or the temporal relationship between stimulation. In this section, I will detail the current literature on the central pattern generator and how the function and structure of the CPG relates to the results from these experiments. Experiments in Chapter IV revealed that the FT effect still emerges when 50% of the shocks are randomly omitted. This outcome is similar to a phenomenon observed in research using electrophysiological techniques on the central pattern generator. In a fictive locomotion preparation in which the mesencephalic locomotor region (MLR) of adult decerebrated cats was electrically stimulated after neuromuscular blockade of afferent pathways, the normally robust alternating flexor and extensor activity sometimes presents spontaneous failures of motoneuron activity. Early evidence of this phenomenon was found by Grillner and Zangger (1979), observing that the predictably alternating pattern between flexor and extensor of the CPG sometimes missed a burst of activity. If the knee extensor missed a burst, the missing activity of the extensor was paired with sustained knee flexor activity in the chronic spinalized, decerebrate cat. Additionally extensive work on these deletions has been conducted in

the turtle scratch reflex (for review, Stein, 2005). Called spontaneous deletions, deletions of activity were observed for both extensor and flexor motoneuron pools after electrical stimulation of the brainstem during fictive locomotion (Lafreniere-Roula & Mcrea, 2005). During a spontaneous deletion, the timing of the rhythmic activity was maintained, meaning the step-cycle period remained the same during the deletion. Even more interesting, when activity resumed after a deletion, it commonly occurred at an integer multiple of the pre-existing cycle period (Lafreniere-Roula & Mcrea, 2005). These results are particularly interesting to consider in the context of Experiments 6 and 7, where reducing the probability of presentation of fixed spaced shock still produced learning and the protective effect.

The last two experiments of this dissertation focus on the role of a central processor, the CPG, in mediating these FT effects (Experiment 8 & 9). Experiment 8 relied on an environmental manipulation to show that spinal neurons can abstract regularity across distinct dermatomes. These results also discounted the role of a peripheral filter in mediating the FT effect. Experiment 9 explored the necessity of the rostral lumbar cord (L1-L2) for the development of the beneficial effects of FT shock. My hypothesis was that, by surgically disconnecting the lower lumbosacral cord from the tempo generator in L1-L2, I could eliminate the FT effect, suggesting that the spinal cord gains a sense of timing or pattern of stimulation from the CPG. While the data support this conclusion, it must be acknowledged that some debate exists regarding the location of the CPG within the spinal cord. For instance, after complete transection at T12, Grillner and Zangger (1979) made a series of transections caudal to the transection

and found that as long as L5 was connected to the caudal lumbar cord, alternating activity was still observed. These were the first observations of a distributed CPG, spanning the entire lumbar region of the spinal cord. Additionally, the idea of a distributed CPG is supported by much of the work on scratch-like patterns of movement and outputs, with many researchers finding that L4-L5 segments are critical for the expression of the scratch output. Also, the L6-S1 region can also generate prolonged rhythmic outputs, though the more caudal segments are less excitable and the rostral segments show more excitability (for review, Kiehn & Kjaerulff; Kiehn, 2006). These results all seem to indicate that a rostrocaudal excitability gradient is important to the genesis of rhythmic activity, and that all segments of the lumbar cord are capable of generating the activity of the CPG, but some segments (rostral) are more excitable than others (caudal). The alternative view, assumed in Experiment 9, is that the CPG is localized to the L1-L2 region of the lumbar spinal cord. Supporting this, when only the L1-L2 portion (lumbar enlargement) of the spinal cord of neonatal rats was exposed to a cocktail of 5-HT and NMDA, evoked rhythmic activity was recorded in all lumbar segments. Localization of the lumbar enlargement was accomplished by building walls of Vaseline around the segment of interest, successfully preventing any diffusion of the bath application of drug cocktail. Importantly, after 5-HT and NMDA application to L3-L6 (lower lumbar cord), the evoked rhythmic activity characteristic of the CPG was not observed, only tonic activity was observed. The results and implications of this study are critical to consider because of the specific manner in which the drug cocktail typical to fictive locomotion studies was applied, localizing the areas that are pharmacologically

evoked, parsing the evoked rhythmic activity through 5-HT and NMDA in a specific area from the interactions between interneurons within all lumbar segments. Using a typical bath application of 5-HT and NMDA to all lumbar segments of the spinal cord, it is impossible to separate the evoked activity of all lumbar segments induced pharmacologically from the interactions between the interneurons of the lumbar and sacral segments and the disruption of a full transection. Additionally, in behavioral studies on the impact of the CPG on recovery of function, greater loss of locomotion (assessed by the BBB scale) after a contusion on the T13-L2 region was observed when compared to the loss of locomotion after a contusion injury at L3-L4. This difference in outcome was attributed to the damage of the spinal CPG, which is thought to reside in the L1-L2 regions in the rat (Magnuson et al. 1999; 2005). Independent of whether the CPG is a localized circuit or a distributed network, the rostral lumbar section of the spinal cord is necessary for the FT effect to emerge. I hypothesize that this is due to the contribution of the CPG which resides in the rostral lumbar section. Further studies are needed to demonstrate the sufficiency of the CPG in producing the beneficial effects of fixed spaced shock.

Role of the CPG in Promoting Adaptive Plasticity

The CPG is well established as a biological oscillator, producing self-sustained activity that is rhythmic in nature, though the exact structure for how the CPG then controls locomotion is currently debated. Many forms of environmental perturbations

(obstacles, immobilization, changes in load) that activate proprioceptive and afferent signals have been show to affect CPG output and cause adaptive changes. For instance, when an obstacle is placed on a treadmill during training after spinal cord injury, an increase in flexion is observed in all hindlimb muscles, and this increase is sustained across several subsequent steps. These studies indicate that the spinal CPG is not only capable of adapting to environmental changes, but that these environmental changes can cause changes in behavior based on a form of temporal processing. Additionally, the experiments in this dissertation would suggest that the CPG is necessary for temporal processing, but it is unclear how the contribution of the CPG results in instrumental learning.

Instrumental learning as a measure of spinal plasticity is a powerful behavioral tool. However, to understanding the effect of fixed spaced versus variable spaced stimulation on instrumental learning, we must consider the many factors that are important to the acquisition and maintenance of the instrumental learning. Since the initial study, demonstrating not only the plasticity of spinal neurons but also how controllable stimulation (response-contingent shock) can promote adaptive plasticity and how uncontrollable stimulation results in maladaptive plasticity, many studies have investigated the factors and mechanisms underlying the adaptive and maladaptive behavioral plasticity. For instance, Crown and Grau (2005) found that the descending serotonergic fibers had a protective effect on the adaptive plasticity of spinal neurons, observed by performance on the instrumental learning task as described above. Though uncontrollable stimulation (variable [intermittent] tailshock; similar to yoked

stimulation) produces an instrumental learning deficit, an intrathecal injection of a 5-HT agonist after uncontrollable stimulation blocked the expression of the learning deficit, and subjects were able to acquire the instrumental learning task (Crown & Grau, 2005). When intact subjects were given a micro-injection of a 5HT-IA antagonist (WAY 100635) and then given uncontrollable stimulation prior to testing on the instrumental learning apparatus, subjects were again unable to acquire the instrumental learning response. These findings indicate the protective role of the serotonergic system in the intact spinal cord because rats that received uncontrollable stimulation to the tail prior to spinal transection did not display a learning deficit (Crown & Grau, 2005). What remains unclear from these findings is the role of constitutively active serotonergic receptors on the acquisition of the instrumental learning response after spinal transection and if activating the CPG can also promote the serotonergic system. What is clear is that activity of the CPG is elicited by either pharmacological or electrical stimulation to the isolated spinal cord, and that serotonergic agonists are common to drug cocktails used to evoke this rhythmic activity.

Without afferent sensory feedback, the system cannot account for unexpected challenges or disturbances in the environment. With only a tonic descending drive from the brain, even with a central pattern generator, the behavior produced would be a poor caricature of walking, with little semblance to the coordinated and efficient locomotor function that is evident across animal species. Therefore, the role of peripheral sensory input to the central pattern generator is important to consider. As discussed earlier, it is clear from work in spinalized animals that descending inputs do not need to remain

intact for the initiation of locomotor activity. However, with sensory stimulation in the periphery, locomotor patterns (either walking or scratching or shaking) emerge. According to Pearson (1993), afferent feedback is considered to have three main functions on central pattern generator activity and thereby locomotion: first, to augment CPG activity; second, to control the timing of motor outputs for appropriate muscle drive; and third, to control the phase transition and avoid switching gait phases for stability and coordination during movement.

Studies focused on the role of afferent feedback in modifying CPG activity have found that though phase durations and transitions are controlled by the spinal CPG, inputs from peripheral mechanoreceptors can alter the timing of the pattern of motor activation (Frigon & Gossard, 2009; 2010). Further, when tactile stimuli (obstacle) contact with the dorsal surface of the paw during the swing phase, the entire hindlimb increases flexion to overcome the obstacle, lifting the leg above of the obstacle. When weak stimuli (simulating cutaneous tactile stimulation) or actual mechanical tactile stimulation was applied to the dorsal surface of the paw during the extension phase, activation of the extension muscles was markedly increased (Frossberg, Grillner & Rossignol, 1975). In subsequent steps, the hyperflexion elicited by the cutaneous stimulation of the dorsal surface of the paw persists after the obstacle is removed (Zhong et al., 2012), suggesting not only a memory of the obstacle, but a specific encoding of the timing of the obstacle presentation during the swing phase, and anticipatory response to avoid the obstacle (for review, Hodgson et al., 1994). Thus, the central pattern generator contributes to adaptive plasticity after spinal cord injury. The role of the CPG

in retraining locomotor function after spinal cord injury (SCI) is detailed in the next section.

Role of the CPG in Promoting Recovery of Function after SCI

As discussed in the introduction, much of the recent work focused on promoting recovery of function after SCI has aimed to promote the capacity of intrinsic spinal circuits to adapt and contribute to recovery of locomotion after spinal cord injury, focusing on the interplay between the afferent feedback from the peripheral nervous system to intact spinal circuits such as the CPG. Early studies have shown that even with a complete spinal transection, locomotion can be recovered by treadmill training, a result that has been replicated many times (Grillner, 1973; Belanger et al., 1996; deLeon et al., 1998; Guertin, 2009).

To examine the role of the CPG in recovery of function after incomplete spinal cord lesions, Barriere et al. (2008) used a dual-lesion paradigm in adult cats, in which an initial partial spinal lesion (T10-T11) was followed by a complete spinal transection at T13-L1. Thus, in the initial injury, the CPG is spared and is allowed to contribute to the recovery of locomotor function during treadmill training and the lasting changes induced by the CPG in promoting recovery of function after the initial injury can be revealed by observing how the hindlimb locomotion recovers after the transection. Treadmill running was assessed prior to any injury, and EMG and kinematic values were recorded in the intact state. After the initial, partial lesion to the T10-T11 region, subjects

displayed a hindlimb flaccid paresis on the same side as the lesion, or both sides, if the lesion was extensive. All subjects showed locomotor and weight-support deficits early in treadmill training after the partial lesions on one or both hindlimbs. Approximately half the subjects were left untrained on the treadmill and only periodically evaluated for locomotor recovery. The remaining subjects were trained on the treadmill for 20-30 min for 3-5 days per week. All subjects (trained and untrained) recovered voluntary quadrapedal locomotion, though the trained subjects had a faster recovery time. Then all subjects received a complete transection of T13-L1, and the time course of locomotor recovery was documented. It is important to note that recovery times after the initial injuries varied greatly between and within each group and that the complete transection was done whenever recovery of locomotion reached a plateau and not on the same day post-surgery. However, despite these differences in recovery process and subsequent second lesion, the expression of bilateral hindlimb movement as early as 24 hrs after complete transection in trained cats clearly indicates the role of the spinal CPG in inducing and facilitating behavioral changes and recovery of function after partial lesion/SCI, as well as the impact of treadmill training on promoting this plasticity and recovery in spinal neurons. Untrained cats were unable to fully recover hindlimb locomotion and displayed an asymmetry in stepping during the first 10 days after full spinal transection. Furthermore, with additional treadmill training, even the untrained cats were able to express normal, bilateral stepping activity after spinal transection (Barriere et al. 2008).

It is clear that retraining the injured spinal cord is beneficial to adaptive spinal plasticity, whether with epidural stimulation, treadmill training, or activity-based interventions (Barriere et al., 2008; van den Brand et al. 2012; de Leon et al., 1998). Though training is not critical for the re-expression of locomotion after partial lesion for cats and rats, it does greatly facilitate the course of recovery, resulting in better and quicker locomotor recovery. This recovery of function is based largely on the plasticity of intrinsic spinal circuits and their interactions with sensory feedback (Barbeau & Rossignol, 1987; Belanger et al.,1988; Duysens & Van de Crommert, 1998; de Leon et al, 1998; Van de Crommert et al, 1998; Rossignol, 2006; Edgerton et al., 2004). From these studies, it is clear that interactions between the CPG and sensory feedback from cutaneous stimulation are a useful tool in promoting plasticity in the spinal cord. My results suggest that how stimulation affects recovery will depend on whether it is presented in a regular or irregular manner. This work further suggests that regular stimulation may have a lasting beneficial effect that can promote adaptive plasticity and stimulate locomotor recovery and CPG activity.

Summary

Detecting the temporal relationship between events is a basic but critical function of the nervous system. Ranging from the molecular scale with synaptic coincidence detectors to the whole animal with conditioned behaviors, the intervals between events are encoded along with details from sensory afferents. Though the distribution of events

over time has a clear impact on how stimuli are encoded by the central nervous system, how the nervous system is able to abstract and store that information is not always clear. The vast range of the scales of time (from milliseconds to months) that the nervous system must encode requires that multiple mechanisms and multiple structures are in place to precisely and accurately encode the passage time (Ivry & Schlerg, 2008; Mauk & Buonomano, 2004). In instances of representation of time, not only do cyclical or diurnal behaviors become apparent, but also learned behaviors and subsequently anticipation of sensory stimuli. Thus, for behaviors such as anticipation based on the abstraction of time as an explicit cue, biological oscillators that record and encode the passage of time as well as the accessibility of that information to the system to interpret and in turn influence and adapt behaviors accordingly remain critically important (Ivry & Schlerg, 2008; Mauk & Buonomano, 2004; Herzog, 2007).

In summary, the network of spinal neurons responsible for the rhythmic alterations of locomotor patterns operates autonomously, but afferent and motor feedback can modulate the rhythmic outputs of these spinal neurons, indicating a limited entrainment capacity of spinal neurons. This entrainment of spinal neurons by sensory information from the periphery is clear from the control the timing of motor outputs and the control of phase transitions (swing to stance phases) that emerge as an interaction between the CPG and afferent feedback. Furthermore, the adaptive response of lifting the leg in anticipation of the obstacle during the swing phase demonstrates that this entrainment can lead to a change in behavior due to learning about environmental stimuli. It is clear is that the L1-L2 segments of the spinal cord are necessary to produce

the FT effects, and that this central structure in the spinal cord is capable of discriminating between temporally regular and temporally irregular stimulation. Further, this system allows for summation of shock sessions, integration of shock across dermatome, and inference of missing shock. The prospect of using predictable environmental stimuli promoting plasticity and potentially producing enhanced recovery of function after SCI is important to pursue and could lead to more effective methods of therapy after SCI.

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