THE LOCALIZATION OF INSTRUMENTAL LEARNING WITHIN

THE SPINAL CORD

A Senior Honors Thesis

by

GRACE ALEXANDRA TSU-CHI LIU

Submitted to the Office of Honors Programs & Academic Scholarships
Texas A&M University
in partial fulfillment of the requirements of the

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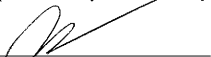
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ABSTRACT

The Localization of Instrumental Learning Within

The Spinal Cord. (April 2002)

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Spinal neurons of surgically transected rats can support a simple form of instrumental learning. Rats learn to maintain leg flexion as a response to shock. The present experiments localized the region of the spinal cord that mediates this learning. Experiment 1 used histological procedures to trace the fiber pathways that innervate the region of stimulation. The retrograde fluorescent tracer Fluoro-Gold was used to mark the neurons innervating the tibialis anterior muscle. Cell bodies were shown primarily in lamina IX of the lower lumbar segments. Experiment 2 used surgical transections at different spinal column levels to establish the segments involved in the learning. The surgical cuts established the boundary of tissue necessary for the learning. The lowest cut that supported learning was in L5 (lumbar segment 5).

ACKNOWLEDGEMENTS

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INTRODUCTION

The brain has been traditionally assumed as the location where learning or information storage occurs. Studies have shown that this is a limited view, and that spinal cord neurons are also capable of learning. Understanding the neurobiological mechanisms used by the nervous system to store information, specifically in the spinal cord, can lead to development of new treatments that can aid the recovery of function after spinal cord injury.

Spinal cord injury affects tens of thousands of new people per year in the United States, and spinal cord trauma involves 10% to 20% of all hospital neurotrauma admissions (van Dellen & Becker, 1991). Depending on the extent and level of injury, complete or partial paralysis can result. Injury to the cervical segments of the spinal cord can result in quadriplegia, while injury in the thoracic, lumbar or sacral segments result in paraplegia. Due to our detailed existing knowledge of its anatomy, the spinal cord offers a structurally simple and excellent neuronal system for the study of learning (Patterson & Grau, 2001).

Recent studies have shown that spinal cord plasticity (or learning) clearly exists. After a complete transection, the neural tissue can be viewed as a new spinal cord that needs to relearn motor tasks. There is a reorganization of the neural pathways below the injury, and motor performance especially in the lumbosacral region can be improved

This thesis follows the style and format of the journal *Behavioral Neuroscience.*

with training and exposure to repeated stepping and standing (Edgerton and others, 2001). The spinal cord is also capable of Pavlovian or classical conditioning—paired stimuli learning, and also instrumental conditioning or response-outcome learning (Joynes & Grau, 1996; Grau & Joynes, 2001). In instrumental learning, an organism makes a response in order to receive a particular outcome, or learns the relationship between a response and an environmental event (Domjan, 1998). This instrumental learning can occur in the spinal cord after it has been disconnected from the brain.

Instrumental learning in the spinal cord has been studied with surgically transected rats. Earlier studies have shown that rats with a transected spinal cord can "learn" to maintain their leg in a flexed position to keep from receiving leg-shock that is administered whenever the leg drops below a pre-specified criterion (Buerger & Chopin, 1976; Grau, Barstow, & Joynes, 1998). The procedure used involves a variation of Horridge's (1962) master-yoke paradigm. "Master rats" received reponse-contingent shock. Shock was applied whenever the leg was extended and turned off when the leg was lifted. Yoked control rats were shocked whenever "master" rats were shocked, receiving the same amount and duration of shock independent of leg position. Yoked rats possessed no control over whether or not they were shocked (non-contingent shock) and failed to learn. With the development of standardized procedures, spinally transected master rats were discovered to exhibit the same behavior as spinally intact rats, showing progressively longer flexion responses—learning to minimize shock exposure, while yoked rats still failed to learn (Grau et al., 1998). Master rats also showed positive transfer, quickly reacquiring the operant response of leg flexion while

yoked rats failed to learn to keep their leg up—a form of negative transfer, or behavioral

deficit that has properties similar to "learned helplessness" (Maier & Seligman, 1976).

Further studies established this learning as a spinal, centrally mediated phenomenon and

that spinal cord neurons are sensitive to response-outcome relations (Crown, Ferguson,

Joynes, & Grau, submitted).

The present study will use two methods to isolate the regions of the spinal cord

where instrumental learning occurs, one to trace the neural pathways involved and the

other to localize the longitudinal segment of spinal cord involved. The first experiment

traces the nerve fiber pathways that innervate the region of stimulation. Recent

neurological tracing advances provide more dye options for easier retrograde tracing of

spinal cord motor neurons in intact animals through injection in the muscle (Kobbert,

Apps, Bechmann, Lanciego, Mey & Thanos, 2000). Fluorescent tracers provide a

simple way to visualize neurons under fluorescent microscopy without complicated

staining and histochemical procedures (Vercelli, Repici, Barbossa & Grimaldi, 2000).

The retrograde fluorescent tracer Fluoro-Gold was selected for the localization of spinal

cord motor neurons involved in the leg flexion response. The pattern of marking will

likely vary as a function of time of transport and concentration across the lamina,

marking the cell bodies of the lower lumbosacral region and within the ventral horn of

the gray matter.

The second portion of the study will use surgical cuts of the cord at various levels

of the lumbar-sacral region to isolate the area of tissue necessary for learning. The cuts

will be combined with the previously established methods of behavioral measures for

instrumental learning in spinalized rats with the leg flexion-shock experiments (Grau et al., 1998). Since electrical stimulation to the tibialis anterior muscle elicits the flexion response, the ischiadic nerve comes from lumbar nerves IV to VI (Hebel & Stromberg, 1986). Given this and prior studies showing that the sciatic nerve is necessary for instrumental learning (Crown, et al., submitted), we hypothesize that the learning occurs in the lower segments of the lumbosacral enlargement.

GENERAL METHODS

All procedures described in these experiments have been reviewed and approved by the Texas A&M University Laboratory Animal Care Committee.

Subjects

The subjects were male Sprague-Dawley rats (*Rattus norvegicus*) obtained from Harlan (Houston, TX). The rats were approximately 100 days old, weighing (350-450g) and were individually housed with food and water continuously available. Rats were maintained on a twelve-hour light/dark cycle and were generally tested during the last six hours of the light cycle. Experiment 1 used three subjects for neural anatomical tracing (at Fluoro-Gold concentrations of 2 μl, 4 μl, 8 μl). Experiment 2 used four subjects per cell for the four spinal level conditions of L1, L3, L5 and S1.

Tracer Injection

Three subjects were used for the injection protocol. All injections were completed with Hamilton syringes with 25-gauge needles. Before injection of the fluorescent tracer, rats were anesthetized with pentobarbital (50 mg/kg, i.p.). Their hindlegs were shaved and probed for the region in the tibialis anterior muscle eliciting the most vigorous flexion response for the contact electrode, according to the established apparatus and behavioral procedure described in Grau et al. (1998). Rats were injected intramuscularly with 0.2 μl, 0.4 μl, or 0.8 μl of 2% Fluoro-Gold solution. One leg received an injection in the upper electrode placement site (pin), and the contralateral leg received an injection in the lower electrode placement site (wire). Rats were then

maintained in a temperature-controlled environment (~25.5°C) while recovering from anesthesia.

<u>Fixation, Sectioning, and Histology</u>

Approximately ten days after the tracer injection, rats were deeply anesthetized with pentobarbital (100 mg/kg, i.p.) and a thoracotomy was performed. Rats were perfused transcardially with 60 mL of phosphate buffered saline (PBS) followed by 160 mL of parafomaldehyde in 0.1 M saline buffer to fix the neural tissue. After perfusion, T8 to L3 of the spinal column was removed and postfixed in parafomaldehyde for 2 hours. The lumbosacral spinal cord was exposed by removing the skeletal muscles, and the isolated spinal tissue was stored in a 10% sucrose in PBS solution overnight in refrigeration. The lumbosacral enlargement was cut down to approximately 1.5 cm and embedded in a mold with mounting medium and frozen in liquid nitrogen for cryostat sectioning. The mold was removed and the cord sliced transversely into 25-μm-thick sections and placed serially on slides. The slides were then analyzed under fluorescence microscopy to localize the Fluoro-Gold marked cell bodies.

<u>Surgery</u>

In experiment 2, spinal transections were performed at spinal tissue L1, L3, L5 and S1 with four subjects per condition. Rats were anesthetized with pentobarbital (50 mg/kg, i.p.) and their backs shaved and swabbed with iodine in preparation for surgery. The rat's head was held in a stereotaxic instrument and a small gauze pillow (5 x 4 x 2.5 [height] cm) was placed under its chest to stabilize and position the rat's body for surgery. After the protuberance of the tenth/eleventh thoracic vertebra (T10/T11) was

localized by touch, an anterior-posterior incision was made. Next the tissue was cleared away from the respective vertebra to be removed to isolate the cord segment as referenced in Hebel et al. (1986): T12 for L1-L3, T13 for L3-L5, L1 for L5-S1. A dental drill was used to drill a hole for the raunciers to clear away the bone and vertebrae. The cord was transected with a scalpel cut, the remaining cavity filled with Oxycel (Parke-Davis), and the wound closed with Michel clips. Rats were immediately hydrated with 2.5 mL of 0.9% saline following surgery and maintained in a temperature-controlled environment (~25.5°C) for recovery. During recovery, the rat's rear legs were maintained in a normal flexed position by a piece of porous tape (Orthaletic, 1.3cm [width]) gently wrapped once around the rat's body. Subjects were given about 20 – 24 hrs to recover from surgery during which hydration was maintained and bladders were expressed at regular intervals.

Transections were confirmed by (a) inspecting the cord during the operation, (b) observing the behavior of the subjects after they recovered to insure that they exhibit paralysis of the hind limbs and did not vocalize to the leg shock, and (c) examining the spinal cord postmortem.

Apparatus

Rats were loosely restrained in dark enclosed tubes described in Grau et al. (1998) while instrumental training was conducted. The tubes were painted black and sealed in the front with two measured slots cut in the back, allowing the hind legs of the rat to hang freely. The midsection of the rat was gently secured with a wire "belt" to minimize the effects of upper body movements on leg position. Leg-shock was applied

by attaching one lead from a BRS/LVE constant current (60Hz, AC) shock generator (Model SG-903) to a stainless steel wire inserted though the rat skin over the tibia 1.5 cm from the tarsals. The other lead was attached to a 2.5 cm stainless steel pin that was inserted 0.4 cm into the tibialis anterior muscle 1.7 cm above the other electrode.

Limb position was monitored using an insulated contact electrode taped to the plantar surface of the rat's foot, just distal to the plantar protuberance. A fine wire attached to the proximal end of the contact electrode was connected to a digital input monitored by a Macintosh computer. The tip of the rod was positioned over a plastic dish under the restraining tube containing a NaCl solution with a drop of detergent to reduce surface tension. A ground wire was connected to a steel rod placed in the solution so that when the contact electrode attached to the rat's paw touched the solution, it completed the circuit monitored by the computer. The state of this circuit was sample at a rate of 30 times / sec.

Flexion force was measured by attaching a monofilament plastic line ("4 lb test" Stren, Dupont) to the rat's foot immediately behind the plantar protuberance. The 40 cm length of line was passed through an eyelet attached to the apparatus directly under the paw. The end of the line was attached to a strain gauge that was fastened to a ringstand. After the line was connected to the rat's paw, the ringstand was positioned so that the line was taut, just barely engaging the gauge. Shock intensity was adjusted to produce a flexion force of a predetermined 0.4 N. Then the strain gauge was removed from the rat's foot.
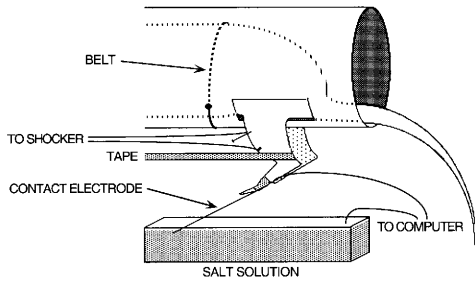
Figure 1. The apparatus for instrumental training

Behavioral Procedures

Sixteen rats were divided into four groups with four subjects per group. All behavioral procedures were started approximately 24 hours after surgery. The rear legs were shaved and marked for placement of the shock leads before the rats were placed in the restraining tubes. A stainless steel wire electrode was then inserted over the distal portion of the tibia before subjects were placed in the apparatus, and a contact electrode was taped to the paw to monitor leg position. Lateral leg movements and leg variability was minimized by loosely wrapping a 20 cm piece of porous tape (Orthaletic, 1.3 cm) around the leg and taped to a bar extending across the apparatus directed under the front panel of the restraining tube. The tape was adjusted so that it was taut enough to extend the joint between the tibia and femur. One lead from the shock generator was attached to the stainless steel wire inserted over the tibia. The shock generator was set to deliver a 0.1 mA shock and the region over the second mark was probed to find a site that elicited a vigorous flexion response. The pin was inserted perpendicular to the body into the tibialis anterior muscle. To check electrode placement, a single intense (1.6 mA) test shock of 0.3 s was applied to verify it provided a flexion response of at least 0.8 N. The shock intensity was then adjusted to produce a flexion force of 0.4 N with a 0.3-s shock, and the plastic line was removed. Three short 1.5-s pulse shocks were applied and the level of the salt solution adjusted so that the tip of the rod was submerged 4 mm below the surface.

Behavioral Measures

Three behavioral measures were used to monitor performance: time in solution, flexion number, and flexion duration (see Grau, et al., 1998, Figure 2). The computer recorded when the contact electrode touched the underlying solution (time in solution). Flexion number was increased by 1 whenever the electrode left the solution. The session was divided into 20 one-minute time bins to obtain a measure of performance over time.

Instrumental learning has previously been shown to be distinguishable from a reactive system that is insensitive to the reponse-reinforcer relation (Grau et al., 1998). A key difference concerns response duration; only the instrumental account anticipates that contingent shock will produce a progressive increase in response duration. Response duration was derived from time in solution and response number using the following equation: Response duration$_i$ = (60-s – time in solution$_i$) / (Response number$_i$ + 1) where i was the current time bin. To address the possibility that differences in response duration during testing reflect a loss of responding in the previously shocked rats, we also present flexion number.

Statistics

The results were analyzed using an analysis of variance (ANOVA). Post hoc comparisons were made using Duncan's New Multiple Range test. In all cases, a criterion of $p < .05$ was used to judge statistical significance.

RESULTS

Experiment 1

Transverse spinal cord segment pictures were adapted from Molander, Xu and Grant (1984) and used to orient and isolate the lumbosacral region (L1-L6, S1) of the spinal cord. Retrogradely-labelled cells were found to be distributed in throughout lamina IX of the transverse sections of the lumbosacral spinal cord. The concentrations of Fluoro-Gold (FG) labeling varied across spinal segment and along serial slices as depicted in figures 2 and 3. In spinal segments T13 through L2, cell bodies were sparsely labeled by FG, if at all in some slices. The lower lumbar segments: L3, L4, L5, L6 showed a higher proportion of motor neuron cell bodies than the upper lumbar segments. The 0.8μL concentration yielded better resolution than the 0.4 μL or the 0.2 μL injection amount. Figure 4 depicts a digital photograph of the spinal cord slice under fluorescence microscopy. FG marked cell bodies fluoresced a faint yellow and can be seen here as varying intensities of white splotches. The marked cell bodies were located in the superficial ventral horn, at the edge of the gray matter in lamina IX.
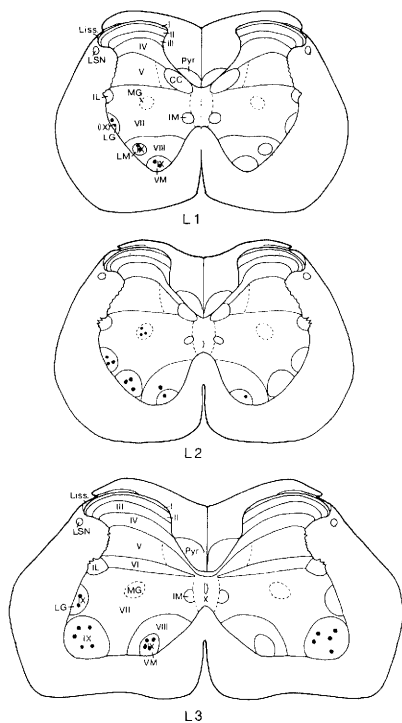
Figure 2. Spinal cord sections with FG cell bodies across L1 to L3, Spinal cord images were adapted from Molander et al., 1994.
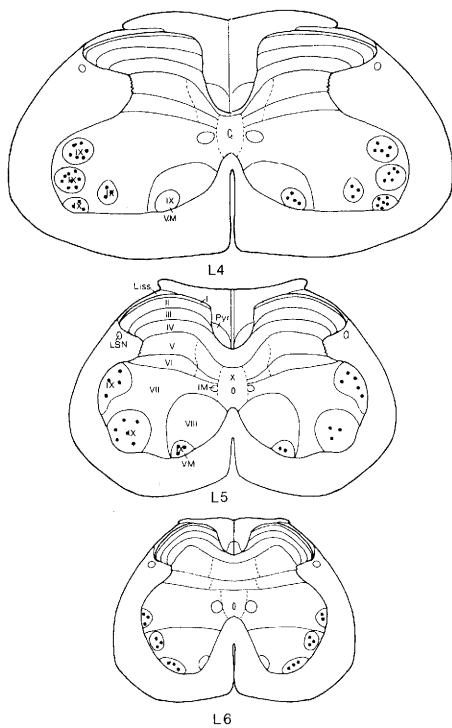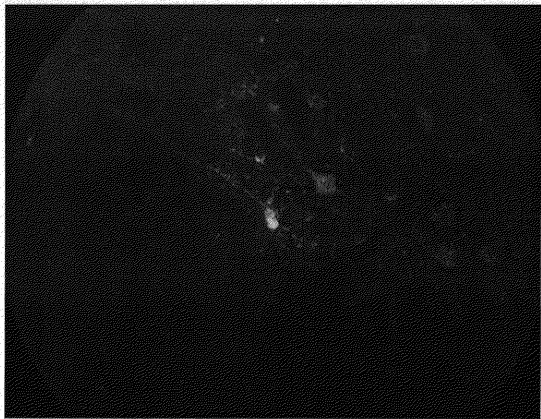
Figure 3. Spinal cord sections with FG cell bodies across L4 to L5.

Figure 3. Digital photograph of FG labeled motor neuron cell bodies in the ventral horn

Experiment 2

During behavioral training, most rats transected above L5 (at L1, L3 and L5) showed a progressive increase in response duration and longer average response duration during the 30-minute training period. Rats transected at the S1 condition failed to increase their response duration times and failed to learn. Rats at the L1 transection exhibited the longest response durations. An ANOVA revealed a significant effect of Spinal transection level (L1, L3, L5 and S1), Time, as well as Spinal level x Time interaction, all $Fs > 1.45$, $p < .01$. The mean flexion duration time ranged from 59.94 seconds (± 1.61) for L1, 39.018 seconds (± 2.58) for L3, and 32.89 seconds (± 2.6) for L5, while S1 had a flexion duration mean of 5.47 seconds (± 1.51). A Duncan New Multiple Range Post hoc test confirmed that the S1 transected group differed from the other three, $p < .05$. The other groups did not differ, $p > .05$.

As expected, rats that failed to learn exhibited a greater number of responses. An ANOVA revealed a significant effect over Time and also Time x Spinal Level interaction, all $Fs > 1.52$, $p < .005$. The main effect for transection level was not significant, $p > .05$. The number of responses decreased over time for all spinal levels. Response duration magnitude and response number over time are shown in Figure 4.
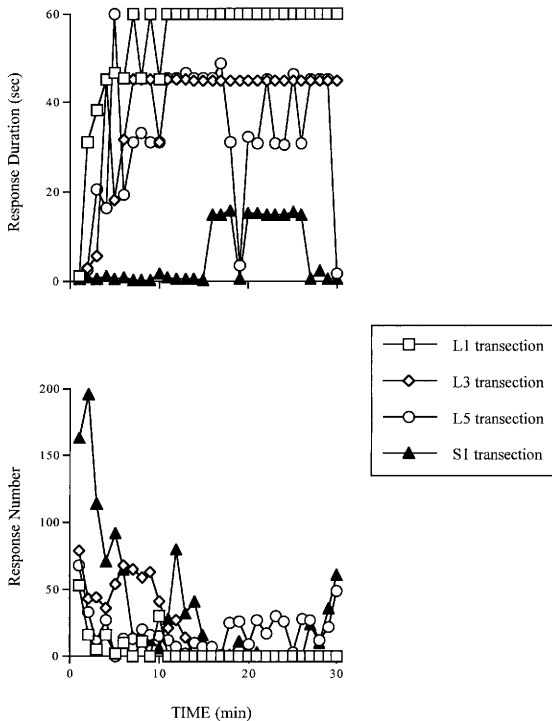
Figure 4. Behavioral results of Experiment 2: Response Duration and Response Number over Time.

DISCUSSION AND SUMMARY

It had been previously shown that spinalized rats exposed to response-contingent shock learn to maintain their leg in a flexed position, minimizing net shock exposure; the sciatic nerve was necessary for this learning, and it was centrally mediated by spinal cord neurons (Crown, et al, submitted). We expected the localization of learning to be in those regions innervated by ischiadic nerve somewhere around the lower half of the lumbosacral enlargement (L4-L6), but the possibility existed that the phenomenon existed in a different locale than anticipated.

Experiment 1 found that the retrograde fluorescent tracer Fluoro-Gold was effective in locating the motor neuron fibers of the tibialis anterior region stimulated, consistent with the function of lamina IX as the location of large motor cells in the ventral horn (Molander, et al, 1984). We were hoping for more influential data on spinal segments involved, but the tracing confirmed necessary retrograde transport time, spinal cord sections, and established procedures for more extensive tracing in the future. After the marking of motor cell bodies, we realized the need for a marker for ascending and descending tracts to visualize nerve axons in the dorsal horn. Potential investigations include the combined usage of different color fluorescent dyes and the tracing of axon pathways with the lipophilic carbocyanine tracer, DiI (Kobbert et al., 2000). This lays the groundwork for future neurochemical investigations.

The second experiment found that response-contingent shock fosters learning even when the L5 segment of the spinal cord was transected, which was lower than

expected. When S1 was cut, the subjects stopped learning, which established an upper boundary of L5, leaving a much smaller amount of tissue involved in the learning than previously thought. To continue to localize the minimal amount of tissue necessary for this instrumental learning, a lower boundary needs to be established. Future studies will use the L5 upper boundary and continue to make caudal transections to minimize the amount of tissue needed for the instrumental response.

Both learning and the deficit may depend on a common mechanism. The acquisition of learning being localized to a small region of the spinal cord is consistent with the selective modification view of instrumental learning. The consequences of non-contingent shock and central sensitization are diffuse—neurons do not learn anywhere, whereas the mechanism of learning is more specific—only neurons in a certain segment support the learning (Joynes, Ferguson, Crown, Patton, & Grau, submitted). The specific localization of learning within the spinal cord sets the foundation for more effective future studies of behavioral and neuronal mechanisms, such as isolating the cellular systems that mediate instrumental learning.

With the decreased amount of tissue to be analyzed, we anticipate performing more detailed biochemical assays such as running gene arrays on the few millimeters of spinal cord involved in instrumental learning. Biochemical markers of cellular activity found in c-fos and c-jun will also be explored. C-fos and c-jun are markers for immediate early genes, and studies have established that nociceptive (pain-causing) stimulation can induce immediate early gene expression within the spinal cord (Gu, Pan Cui, Klebuc, Shenaq & Liu, 1997; Huang & Simpson, 1999). C-fos and c-jun are

expressed within the spinal cord during nociceptive stimulation and after nerve injury (Gu et al., 1997; Huang & Simpson, 1999). C-fos functions dually in the cell, playing roles in gene-transcription and cell death. These cellular function proteins mark which neurons are engaged by contingent versus non-contingent shock and also aid in understanding mechanisms underlying some forms of nociceptive plasticity.

Behavioral, physiological, and pharmacological treatments that enable instrumental learning may promote the re-acquisition of stepping and other spinal movement. Given the prevalence of spinal cord injury, understanding the cellular components that induce, promote, and maintain spinal cord plasticity at the behavioral and neurobiological level holds great implications for clinical significance and recovery of function after injury.

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