BDNF FACILITATES INSTRUMENTAL LEARNING AND SPINAL PLASTICITY *IN VIVO*

An Undergraduate Research Scholars Thesis

by

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ABSTRACT

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Prior work has shown that the neurotrophin, brain-derived neurotrophic factor (BDNF), fosters adaptive plasticity within the spinal cord. To be clinically useful, BDNF must be applied over extended periods of time (days to weeks). The present proposal evaluated whether this can be accomplished using a hydrogel containing BDNF. Using *in vitro* procedures, a hydrogel was developed that slowly releases BDNF over a one-week period. We tested the effectiveness of this hydrogel *in vivo*. The impact of drug treatment was assessed by testing its effect on mechanical reactivity and spinal learning. Spinally transected rats had the BDNF-containing hydrogel applied over the lumbosacral spinal cord. Subjects were tested 3-24 hrs later. Prior work has shown that BDNF enhances learning (adaptive plasticity) without affecting mechanical reactivity (a form of maladaptive plasticity indicative of enhanced pain). I found that BDNF applied using a hydrogel fostered adaptive plastic without affecting mechanical reactivity. This work provides the foundation for future studies designed to test drug effectiveness after a contusion injury.

ACKNOWLEDGEMENTS

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NOMENCLATURE

BDNF	Brain Derived Neurotrophic Factor
SCI	Spinal Cord Injury
T2	Second Thoracic Vertebra
L3	Third Lumbar Vertebra
S1	First Sacral Vertebra
i.p.	Intraperitoneal Injection
HA	Hyaluronic Acid
MC	Methylcellulose
LTP	Long Term Potentiation

CHAPTER I

INTRODUCTION

Neurotrophins are a group of proteins associated with growth, development and functioning of neurons. The current proposal focuses on the neurotrophin brain-derived neurotrophic factor (BDNF), which is found in the central nervous system and periphery and is associated with neural growth. BDNF is responsible for survival of existing neurons as well as the development and differentiation of new neurons. BDNF is also responsible for regulation of learning and memory within the brain and encourages the development of long-term potentiation (LTP) (Kang & Schumann, 1995). BDNF plays a vital role in spatial memory (Mizuno et al., 2000). BDNF is vital to normal nerve growth and mice born unable to produce BDNF suffer from brain defects and die shortly after birth (Enfors, 1995). BDNF has also been shown to promote neural plasticity within the brain (Cohen, 2012.). Here, I explore a procedure designed to apply BDNF over extended periods with the aim of fostering adaptive plasticity within the spinal cord.

Prior work has shown that acute treatment with BDNF can foster adaptive plasticity within the spinal cord (Huie et al., 2012). With the use of an *in vivo* model system it was demonstrated that training with controllable stimulation (shock that is response dependent) increases spinal BDNF expression and engages a BDNF-dependent process that promotes adaptive plasticity as evidenced by increased BDNF mRNA expression and protein within the lumbar spinal cord and the appearance of a learning deficit (failure to maintain leg flexion to avoid shock) when a BDNF inhibitor (TrkB-IgG) was applied (Gómez-Pinilla et al., 2007, Huie et al., 2012). Direct (intrathecal) application of BDNF substituted for instrumental training (administration of

electrical shock to the hind leg whenever the leg is in an extended position) to block both the induction and expression of the learning deficit (Huie et. al, 2012). The ability of BDNF to prevent and reverse a typically apparent learning deficit without adverse effects suggests that application of BDNF after a spinal injury could promote recovery. Of specific concern, at higher doses BDNF can sometimes enhance reactivity to mechanical stimulation (Cejas et al., 2000; Coull et al, 2005) causing subjects to exhibit a nociceptive (pain) reflex to moderate cutaneous stimulation (a form of maladaptive plasticity known as allodynia). However, Huie et al. (2012) found BDNF prevented shock-induced allodynia in spinally transected rats. Therefore, BDNF is a prime candidate to promote adaptive plasticity within the spinal cord but in large doses BDNF overpromotes plasticity causing an adverse effect in the form of allodynia.

Researchers have also explored the possibility that BDNF treatment can foster recovery after a spinal contusion injury. While a transection cuts the spinal tissue, a contusion injury provides a bruising that better emulates a typical human injury. While studies examining the impact of BDNF on spinal function after injury have reported some positive effects, such as enhanced neuron excitability, promoting recovery of locomotor function, difficulties have been encountered, including increased spasticity and heightened pain (Boyce et al., 2012). Also of particular concern, BDNF is extremely expensive ($50,000 per a gram). Moreover, to be clinically useful, the neurotrophin must be applied for extended periods of time (days to weeks) at a concentration that does not have an adverse effect. To address these limitations, a targeted drug delivery platform that allows drug delivery to the injured site is needed. Ideally this drug delivery platform would be biodegradable.

Bioengineered hydrogels may provide a useful technique for the local administration of BDNF over extended durations. Many hydrogels have been developed with the goal of direct drug delivery. Natural hydrogels are preferable for their similarity to the living tissue in which they are to be injected. Hyaluronic Acid (HA) is a commonly used natural material that can be highly tailored, allowing for the controlled release of drug depending on how it is encapsulated. The ability to modify the release profile of the hydrogel allows for a better evaluation of the effect of the drug of interest. Previous work has shown that HA and methyl cellulose (MC) can be used as an intrathecal delivery (Gupta et al., 2006; Kang et al., 2009).

My project evaluated whether BDNF can be applied using a hydrogel that slowly releases BDNF at a concentration that should foster adaptive plasticity without inducing behavioral signs of allodynia. Previous studies delivering BDNF showed positive results but also had adverse effects, we sought to determine whether a hydrogel could be used as a platform to acutely administer BDNF to the injury site without negative effects. Working with bioengineers at the University of Florida at Gainesville, FL, a hydrogel composed of HA and MC was developed that slowly releases BDNF *in vitro*. The HAMC hydrogel was shown to slowly deliver encapsulated BDNF *in vitro,* with all encapsulated BDNF released after 16.5 hours.

My study explored whether a BDNF-containing hydrogel could be used to promote adaptive plasticity within the spinal cord by the facilitation of learning of an instrumental task at a criterion in which naïve rats fail to learn. This was accomplished using the spinal learning procedure described by Huie et al. (2012), in which spinally transected rats are tested using an instrumental learning procedure. Rats that have undergone a thoracic spinal transection received

leg shock whenever one hind limb is extended. Over time, subjects learn to maintain the shocked leg in a flexed position that minimizes net shock exposure. Huie et al. showed that a low dose of BDNF fosters learning in this paradigm without inducing signs of allodynia. My aim was to test whether the drug is effective when applied using a hydrogel. A BDNF containing hydrogel was applied over the lumbosacral spinal cord at the time of surgery as this area has shown to be the locus of learning (Joynes et al., 2004). The onset and duration of drug treatment was then evaluated from from 3-24 hrs after surgery to establish the effectiveness of BDNF gel *in vivo*. We expect to find that at a higher response criterion BDNF will facilitate learning for the instrumental learning task indicating its ability to promote plasticity and be potentially used for recovery of locomotor function after spinal cord injury (SCI).

CHAPTER II

METHODS

Subjects

Subjects were adult male Sprague-Dawley acquired from Harlan labs in Houston, TX. All subjects were approximately 100-120 days old and weighed between 350-450 g. The subjects were housed in individual cages and were kept on a 12 hour light and dark cycle. All behavioral testing was performed during the light period. Food and water were available *ad libitum*. This 4 (time) by 2 (drug+gel or gel only) design required 48 Sprague-Dawley rats (n= 6).

Surgery

Surgery was performed with isoflurane as the anesthetic. The subjects' head was immobilized by a stereotaxic apparatus. A small gauze pillow was placed under the subject's chest to provide support for breathing. The second thoracic vertebra (T2) was located by feel and an incision made anterior to posterior. The tissue above T2 was cleared away using rongeurs until the spinal cord was visible. Subjects were spinally transected at T2 using a cautery tool. Closure of the incision site was done using Michel clips (Fine Science Tools, Foster, CA). Another anterior-posterior incision was made above the L3/S1 region. Using rongeurs the tissue surrounding the vertebrae was cleared. A laminectomy, in which vertebrae is removed leaving the spinal cord exposed, was performed over the L3/S1 area. Thermally set BDNF hydrogel or gel containing no BDNF was placed over the space in the L3/S1 area (Figure 1).

Figure 1. Procedure for HAMC Hydrogel Application onto the Spinal Cord.

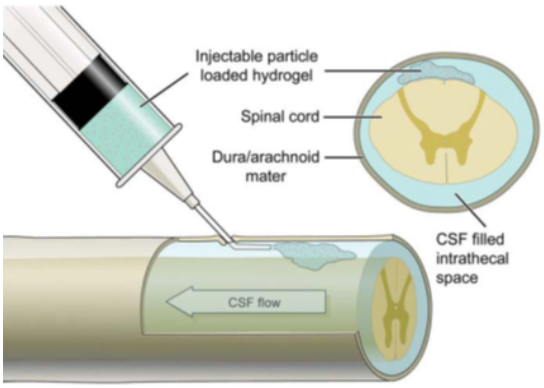

Figure 1. Procedure for HAMC Hydrogel Application onto the Spinal Cord. Method of delivering BDNF into the intrathecal space using an injectable HA-based hydrogel. Figure adapted from (Baumann et al 2009).

The incision site was closed with Michel Clips. Subjects then had each leg shaved for electrode placement. Subjects were given a 3 mL i.p. injection of 0.9% saline solution to maintain hydration. Subjects' legs were kept in a fixed position immediately after surgery until testing with the assistance of porous orthaletic tape, gently wrapped around the rat's body. Subjects were placed in a temperature controlled environment until testing occurred. Subjects' bladders were expressed as needed and immediately before testing.

Mechanical Reactivity

Prior to instrumental testing, mechanical reactivity was assessed using von Frey stimuli. Subjects were placed in a Plexiglas tube with hind legs exposed and freely hanging. Sensitization was examined by the pressing of Von Frey filaments into the mid plantar portion of the hind paw. Filaments were presented with increasing intensity until a flexion response was produced. The same procedure was used after instrumental testing was performed.

Instrumental Learning Apparatus and Procedure

Independent groups were tested 3, 6, 12, and 24 hours after surgery. Subjects then received 30 minutes of testing in our instrumental learning paradigm with the underlying saline solution level set to a high (8mm) response criterion (for a detailed description of the test apparatus and procedure see figure below as well as Huie et al., 2012). Flexion duration was recorded by the time in which the electrode did not make contact with the solution.

Figure 2. Instrumental Learning Paradigm.

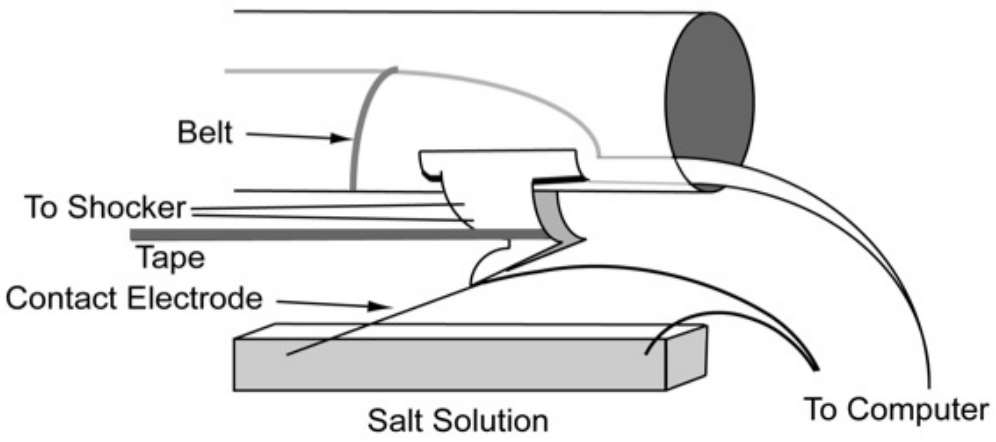

Figure 2. Instrumental Learning Paradigm. The apparatus used to evaluate instrumental learning (from Grau et al., 1998).

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

RESULTS

Subjects underwent 30 min of testing with a high response criterion at which naïve rats typically fail. In this paradigm, learning brings about an increase in flexion duration that minimizes net shock exposure. When tested with a high response criterion, vehicle treated rats typically fail to learn (and do not exhibit an increase in flexion duration). The key question is whether the BDNF-containing hydrogel would allow learning. As shown in Figure 3A, vehicle treated rats failed to learn when tested 3, 6, 12, and 24 hrs after surgery. BDNF-treated rats showed superior learning and this effect was most evident 12 hrs after treatment (Figure 3B). Statistical analyses confirmed that the main effects of time point and drug on response number were significant, all $F(3,87)= 3.42$, $p < .05$. The BDNF gel 12 hour time point performed significantly better for the instrumental learning task ($p< .05$). A post-hoc comparison of response duration means across groups showed that 12-hour BDNF gel ($M= 40.65$ $SD= 26.86$) differed significantly from the 12-hour gel only group ($M= 3.24$, $SD= 11.06$). No other group differences were significant ($p> .05$).

Figure 3A. Learning with Gel Only

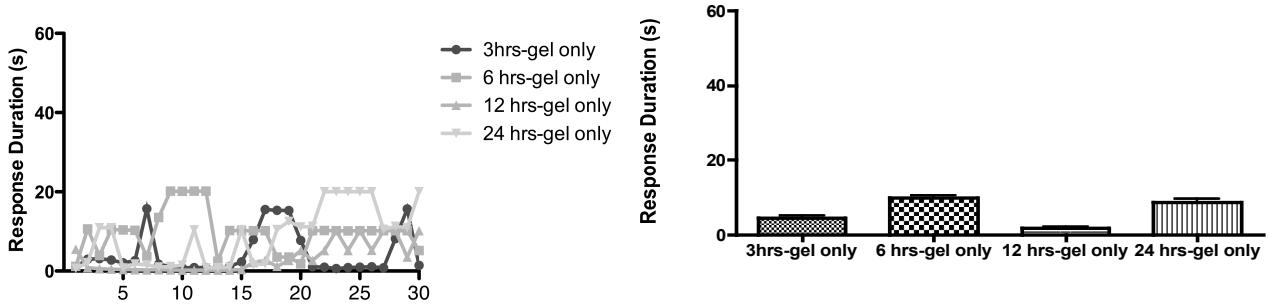

Figure 3B. Learning with BDNF Gel

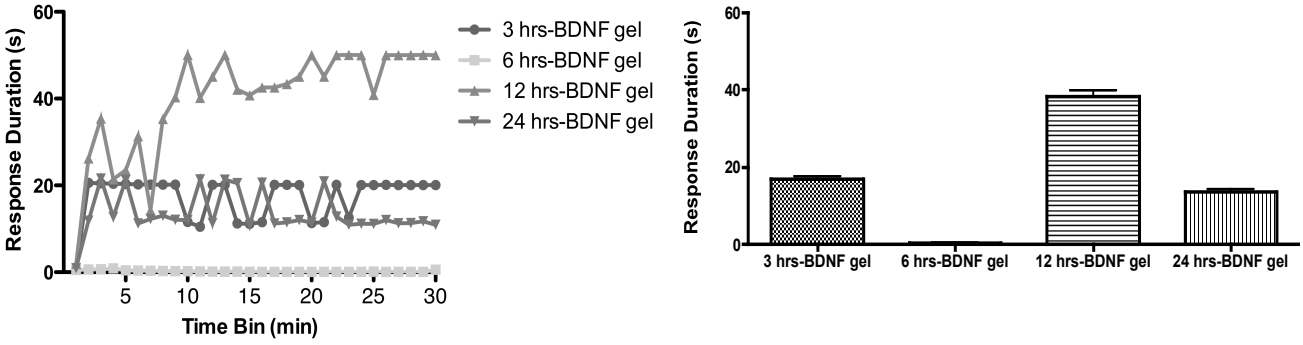

Figure 3. Response Duration for Instrumental Learning Task after BDNF Application. (A) Gel administered without BDNF did not facilitate learning. (B) Facilitation of learning was greatest at 12 hours after BDNF application.

A failure to learn could, potentially, reflect a performance deficit. To address this possibility, I also examined response number. Under normal circumstances, subjects that fail to learn make the highest number of responses; the repeatedly experience a flexion-eliciting shock, but this shock fails to produce an increase in flexion duration. This was observed in the present experiment (see Figure 4), where I found that subjects given just gel are able to respond but do not maintain this flexion (Figure 4A). BDNF- treated rats demonstrated lower response numbers indicating flexion occurred and was maintained longer than their control counterparts. Statistical analyses confirmed that response number for the 12-hour BDNF group was significantly different with all $F(3,87)=.77$, $p<.05$. The 12-hour BDNF gel group displayed a significantly lower response number ($M= 64.73$, $SD= 97.73$) than the 12-hour gel only group ($M= 87.08$, $SD= 94.73$).

Figure 4A. Response Number with Gel Only

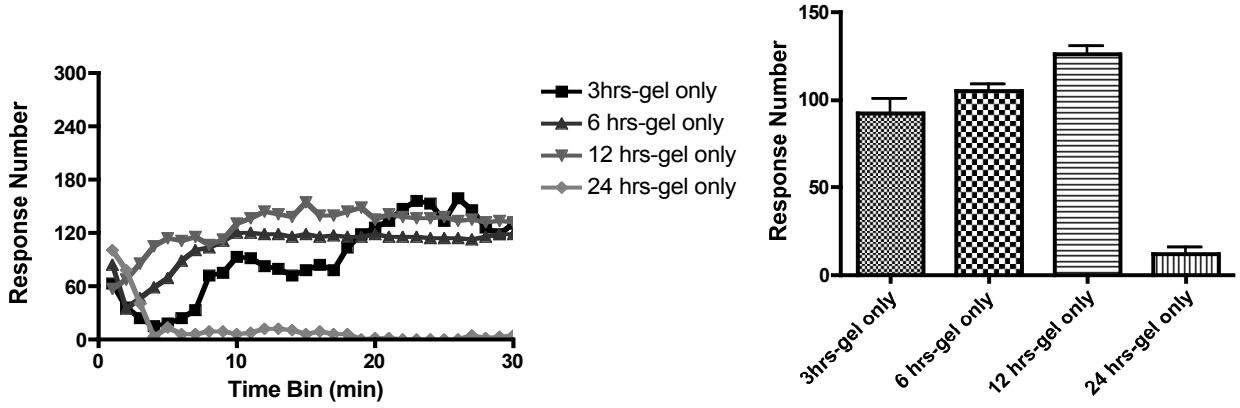

Figure 4B. Response Number with BDNF

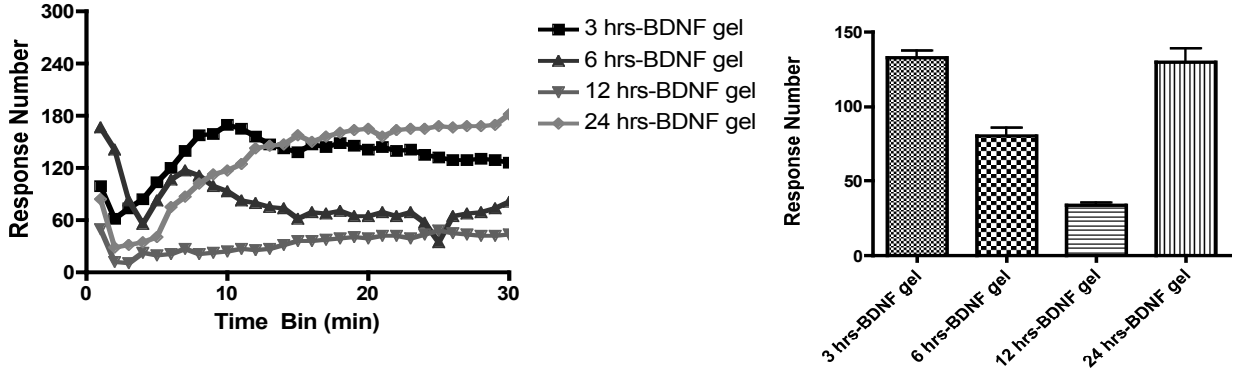

Figure 4. Response Number for Instrumental Learning Task after BDNF Application. (A) The flexion response was observed in the gel only group but this response was not maintained as demonstrated by the higher numbers (B) BDNF gel facilitated learning at 12 hours as demonstrated by lower response number indicative of maintained flexion.

The impact of drug treatment on mechanical reactivity is illustrated in Figure 5. I found that drug had no effect between treatment groups. An ANOVA demonstrated a main effect of time on mechanical reactivity with a statistically significant difference between the 6 hour groups and all other time points, all $F(1,3)= 1.03$, $p < .05$. Within subjects there was no change from baseline before and after instrumental testing. No other comparisons were significant ($p > .05$).

Figure 5. Mechanical Reactivity Threshold

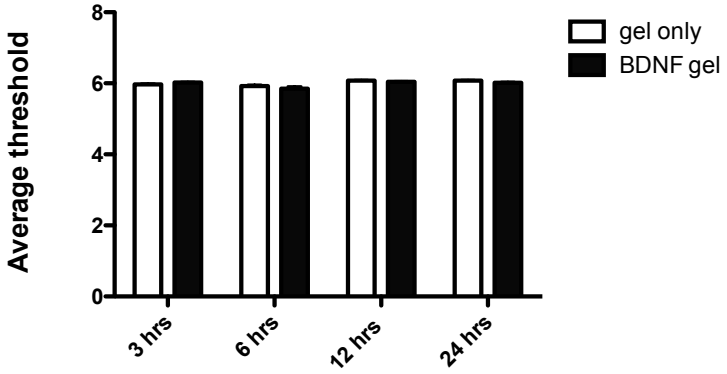

Figure 5. Effect of BDNF Application on mechanical reactivity. There was no significant difference in mechanical reactivity between treatment groups.

CHAPTER IV

DISCUSSION

Spinally transected rats who received acutely administered BDNF hydrogel displayed enhanced learning, as demonstrated by the increased flexion duration under a higher response criterion for the instrumental learning paradigm. The greatest increase in learning occurred at the twelve hour time point but the hydrogel was still visible 24 hours after application. BDNF has been shown to assist in recovery of function after spinal cord injury. The effect of BDNF was apparent at the 12 hours post application but nonexistent at 24 hours post application. Although the hydrogel was able to positively affect learning, this effect did not occur as quickly as we anticipated as demonstrated by unapparent difference between the BDNF and gel only groups at the 3 hour, 6 hour and 24-hour mark. The slow onset and short duration of the drug effect are both concerns that future studies will potentially address by manipulating concentrations of BDNF as well as adjusting the manner in which BDNF is encapsulated.

BDNF facilitated learning as indicated by the increase in flexion duration. Coinciding with these findings response number decreased in the group in which facilitation occurred (12 hour BDNF gel). If learning is occurring response number decreases because the ability to maintain flexion is indicative of the ability to acquire the learning task. These findings are supported by previous work which established that BDNF can be used to promote adaptive plasticity after SCI (Vinit et al., 2009).

Coinciding with previous studies done by Huie et.al 2012 the dosage of .4µg BDNF administered did not induce mechanical allodynia. This is consistent with other recent work from our laboratory indicating that BDNF has an anti-allodynic effect in spinally transected rats. The hydrogel BDNF was able to deliver the benefits seen without adverse effect. BDNF's ability to increase neuron excitability and promote plasticity makes it a protein of interest for future studies trying to examine drug therapies for injury within the nervous system, specifically SCI. The ability to directly administer drug to the site of injury minimizes potential possible negative effect to surrounding tissues or systems and also allows for targeted, concentrated drug delivery.

Further research will look at drug effectiveness over a longer time frame, as well as the effect of high and low concentrations of BDNF on learning. Future studies will try to establish a more effective means of eliciting the drug effect at a time point sooner than 12 hours. To be clinically useful BDNF should be applied over extended periods of time (days to weeks), so future studies will look at how to elicit this drug effect sooner and how to maintain this effect for much longer duration. They will do so by varying encapsulation techniques to explore how to prolong drug delivery so that the effect is maintained for a longer period of time.

The long-term goal is to establish an effective treatment drug delivery platform to be used after a contusion injury to help enhance recovery of locomotor function. We hope to identify an ideal drug concentration to accomplish this and will do so by testing several concentrations of BDNF. By the end of the BDNF hydrogel experiments, we hope to determine an effective and efficient way to combine BDNF and behavioral training to improve recovery after SCI. Ultimately these findings will provide the foundation for an injectable drug delivery platform that would allow for

targeted, long-term release of therapeutics to the affected tissue, circumventing the blood brain barrier, allowing for unrestricted drug delivery.

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