THE ROLE OF BRAIN-DERIVED NEUROTROPHIN FACTOR (BDNF) IN SPINAL LEARNING

A Thesis

by

JOHN RUSSELL HUIE

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

MASTER OF SCIENCE

May 2008

Major Subject: Psychology

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Approved by:

Chair of Committee, James Grau
Committee Members, Michelle Hook
Rajesh Miranda
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ABSTRACT

The Role of Brain-Derived Neurotrophin Factor (BDNF) in Spinal Learning.

(May 2008)

John Russell Huie, B.S., Texas A&M University

Chair of Advisory Committee: Dr. James W. Grau

Previous research in our laboratory has shown that the spinal cord is capable of a simple form of instrumental learning. Spinally transected rats that receive controllable shock to an extended hindlimb exhibit a progressive increase in flexion duration that reduces net shock exposure. Subjects that receive uncontrollable shock, on the other hand, do not exhibit an increase in flexion duration, and are unable to produce this instrumental response even when they are later tested with controllable shock. This behavioral deficit can also be elicited by intermittent shock to the tail, and as little as 6 minutes of this shock is sufficient to produce a deficit that can last up to 48 hours as shown by Crown, Ferguson, Joynes, and Grau in 2002.

Instrumental training has been shown to provide a number of beneficial effects. The instrumental training regimen produces a lasting effect that enables learning when subjects are later tested with a more difficult response criterion. Similarly, instrumental training can provide protection against the deleterious effects of uncontrollable shock as shown by Crown and Grau in 2001. The present study aims to determine the role of

brain-derived neurotrophin factor (BDNF) in the beneficial effects of instrumental training.

Experiments 1 and 2 examined the role of BDNF in the facilitory effect of instrumental training. Through the inhibition of endogenous BDNF, Experiment 1 showed that BDNF is necessary for the facilitation effect. Experiment 2 demonstrated that exogenous BDNF can produce the facilitation effect in dose-dependent fashion.

Experiment 3 showed that the inhibition of BDNF attenuates the protective effect of instrumental training. Likewise, Experiment 4 showed that exogenous BDNF can substitute for instrumental training, and produce this protective effect. Experiment 5 showed that exogenous BDNF can block the development of the deficit when given immediately after uncontrollable shock. Experiment 6 showed that exogenous BDNF can block the expression of the deficit.

Taken together, these experiments outline a major role for BDNF in mediating the beneficial effects of instrumental learning in the rat spinal cord.

DEDICATION

The author would like to dedicate this thesis to his wife, Allison, for her constant love and inspiration, and his parents, John and Sarah, for their continued support.

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INTRODUCTION

In recent years the long-held view of the spinal cord as a simple and static conduit has been gradually replaced with the perception that spinal neurons are quite plastic. Spinal neurons have been shown to be sensitive to single stimulus learning (i.e. habituation, sensitization), and can support Pavlovian conditioning (Thompson & Spencer, 1966; Fitzgerald & Thompson, 1967). Previous research in our laboratory has shown that the spinal cord is also capable of a simple form of instrumental learning (Grau, Barstow, & Joynes, 1998). Spinally transected rats that receive controllable shock to an extended hindlimb exhibit a progressive increase in flexion duration that reduces net shock exposure. Subjects that receive uncontrollable shock (in which shock is delivered regardless of leg position) do not exhibit an increase in flexion duration and are unable to produce this instrumental response even when they are later tested with controllable shock. This behavioral deficit can also be elicited by intermittent shock to the tail and as little as 6 minutes of this shock is sufficient to produce a deficit that can last up to 48 hours (Crown, Ferguson, Joynes, & Grau, 2002).

In contrast to the deleterious effects of uncontrollable shock, instrumental training (controllable shock) has a number of beneficial effects. First, instrumental training produces a lasting effect that enables learning when subjects are later tested with a more difficult response criterion (Crown et al., 2002). This facilitory effect is preserved across contralateral transfer; that is, subjects are able to learn at a higher

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

criterion during testing even when tested on the previously untrained hindlimb. Instrumental training has also been shown to reverse the behavioral deficit induced by uncontrollable shock. Furthermore, instrumental training can protect against the behavioral deficit when given prior to uncontrollable shock (Crown & Grau, 2001).

Our laboratory has found that instrumental learning in the spinal cord reflects a form of NMDA receptor (NMDAR) mediated plasticity. Treatment with the NMDAR antagonists APV and MK-801 have been shown to block learning (Joynes & Grau, 2004; Ferguson, Crown & Grau, 2006). Further, antagonizing the NMDAR after the instrumental response was acquired reversed the consequence of learning. This finding suggests a role for the NMDA receptor in both the acquisition and maintenance of the instrumental response. This notion stands in contrast to other NMDAR-mediated phenomena, such as hippocampal LTP, in which NMDAR antagonism only affects acquisition.

Research indicates that NMDAR-mediated plasticity is modulated by a number of neurotrophins, such as brain derived neurotrophin factor (BDNF). BDNF has been shown to enhance the activity of NMDA receptors (Chen, Kitanishi, Ikeda, Matsuki & Yamada, 2007). Using hippocampal slices from adult rats, researchers have shown that exposure to BDNF induces a long lasting potentiation of afferent input to hippocampal pyramidal cells (Kang & Schumman, 1995). Similarly, in hippocampal slices of BDNF knockout mice, the ability to acquire LTP was abolished, and the subsequent bath administration of BDNF allowed for the reconstitution of LTP (Patterson, Abel, Deuel, Martin, Rose, & Kandel, 1996). In the spinal cord, exercise-induced upregulation of

BNDF mRNA has been found to foster recovery in subjects with spinal contusion injuries (Gomez-Pinilla, Ying, Opazo, Roy, & Edgerton, 2001). More recently, it has been shown that instrumental training (controllable shock) leads to the upregulation of BDNF mRNA expression, and uncontrollable shock down regulates the expression of BDNF mRNA (Gomez-Pinilla, Huie, Ying, Ferguson, Crown, Baumbauer, Edgerton, & Grau, 2007). Coupled with the finding that BDNF can have a modulatory effect on the NMDA receptor, these data suggest that BDNF plays an important role in instrumental learning.

The current experiments examine the hypothesis that the beneficial effects of instrumental training are mediated by BDNF release. Experiments 1 and 2 evaluate the necessity and sufficiency of BDNF in the facilitation effect of instrumental training. Experiments 3 and 4 evaluate the necessity and sufficiency of BDNF in the protective effect of instrumental training when given before uncontrollable shock. Finally, Experiments 5 and 6 examine the capacity for exogenous BDNF to block the expression of the behavioral deficit.

GENERAL METHOD

Subjects

Male Sprague-Dawley rats obtained from Harlan (Houston, TX) served as subjects. Rats were approximately 100-120 days old and weighed between 360 and 460 g. They were housed individually and maintained on a 12-hour light/dark cycle, with all behavioral testing performed during the light cycle. Food and water were available *ad libitum*.

Surgery

Subjects were anesthetized with isoflurane. The 2nd thoracic vertebrae (T2) was located by touch and a 2.5 cm anterior-posterior incision was made over T2. The tissue immediately rostral to T2 was cleared, exposing the spinal cord. A cautery device was then used to transect the cord, and the cavity was filled with Gelfoam (Harvard Apparatus, Holliston, MA). A 25 cm polyethylene cannula (PE-10, VWR International, Bristol, CT) was subsequently threaded 9 cm down the vertebral column, into the subarachnoid space between the dura and the white matter so that it lies on the dorsal surface of the spinal cord. The incision was closed using Michel clips (Fine Science Tools Foster, CA), and the exposed end of cannula tubing was fixed to the skin with cyanoacrylate.

Immediately following surgery, subjects received an injection of 0.9% saline (2.5ml, i.p.). During recovery, the hindlimbs were maintained in a normal flexed position using a piece of porous orthaletic tape, wrapped gently around the rat's body. The recovery period was 24 hours, throughout which the rats were housed in a temperature-regulated environment (25.5 ^{O} C). Supplemental saline injections were provided to ensure proper hydration. Bladders were expressed twice daily and just before behavioral testing. Complete transections were confirmed by a) visually inspecting the

cord during surgery, b) observing behavior following recovery, ensuring subjects exhibited paralysis caudal to the site of transection, and did not vocalize when shock is administered to the tail or hindpaw, c) examining the transection site postmortem in a randomly selected subset of subjects.

Preparation of Glass Microbeads

Fluorescent microbeads were coated with TrkB-IgG as described previously by Vaynman et al., 2003. Briefly, microbeads were coated with TrkB-IgG through passive absorbency by incubating overnight at 4^{O} C with a 1:5 mix of microbeads to TrkB-IgG (5 μg/μl in PBS with BSA) and cytC (100 ng/μl in sterile water). The following morning, this solution was centrifuged 14,000 x g for 30 minutes and the microbeads were resuspended in sterile water at a 10% concentration.

Histology

In order to localize the fluorescent beads, subjects were deeply anesthetized with 100 mg/kg of pentobarbital, i.p. Subjects were then perfused intracardially with 4% paraformaldehyde. A 1cm long segment of the spinal cord that included the L3-L5 region was taken and embedded in paraffin wax. The tissue was sectioned coronally in 20 *u*m-thick sections and every 10th slice was preserved for staining. All sections were stained with luxol fast blue. The beads were then localized using an Olympus BX60 fluorescent microscope.

Apparatus

Instrumental Training/Testing. Instrumental testing was conducted while rats were loosely restrained in tubes (23.5 cm [length] x 8 cm [internal diameter]). Two slots in the tube, (5.6 cm [length] x 1.8 cm [width]), 4 cm apart, 1.5 cm from the end of the tube, allowed both hind legs to hang freely. To minimize the effects of upper body movement on leg position, a wire belt was used to secure the rat's trunk within the tube.

Leg shock was delivered using a BRS/LVE (Laurel, MD) constant current (60Hz, AC) shock generator (Model SG-903). Two electrodes inserted over the tibialis anterior muscle were connected to a computer-controlled relay that regulated the application of leg shock.

Leg position was monitored during testing using a contact electrode constructed from a 7cm long, 0.46 mm diameter stainless steel rod taped to the foot. The last 2.5 cm of the electrode was insulated from the foot with heat shrink tubing. A fine wire (0.01 sq mm [36 AWG] (20 cm) attached to the end of the rod extended from the rear of the foot and was connected to a digital input monitored by a Macintosh computer. A plastic rectangular dish (11.5cm [w] x 19cm [l] x 5cm [d]) containing a NaCl solution was placed approximately 7.5 cm below the restraining tube. A drop of soap was added to the solution to reduce surface tension. A ground wire was connected to a 1mm wide stainless steel rod, which was placed in the solution. When the contact electrode attached to the rat's paw touched the solution, it completed the circuit monitored by the computer, delivering a shock to the tibialis anterior. The state of this circuit was sampled at a rate of 30 times/s.

Flexion force was measured by attaching a monofilament plastic line ("4 lb test" Stren, Dupont, Wilmington DE) to the rat's foot immediately behind the plantar protuberance. The 40cm length of line was threaded through an eyelet attached to the apparatus directly under the paw, 16 cm beneath the base of the tube. The end of the line was attached to a strain gauge (Fort-1000, World Precision Instruments, New Haven, CT) fastened to a ring stand. After the line was connected to the rat's paw, the ring stand was positioned so that the line was taut, just barely registering on the gauge. The strain gauge was calibrated by determining the relationship between voltage and force in Newtons. This data revealed a linear relation, which allowed us to convert

voltage to force.

Uncontrollable Tailshock. Uncontrollable shock was administered while rats were loosely restrained in opaque black Plexiglas tubes that were 22 cm in length and 6.8 cm in diameter. A flat floor constructed from a sheet of black Plexiglas 5.5 cm wide was attached 5.3 cm below the top of the tube. Tailshock was delivered using an electrode constructed from a modified fuse clip. The electrode was coated with ECG gel (Harvard Apparatus, Holliston, MA) and secured with porous tape approximately 6 cm behind the base of the tail. Constant-current 1.5-mA shock was delivered using a 660-V transformer. A Macintosh computer controlled the onset and offset of shock.

Instrumental Learning Testing Procedure. All subjects were allowed to recover for 24 h following surgery and the hindlimbs were shaved and marked for electrode placement prior to testing. 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. A contact electrode was secured to the foot between the second and third digits with a piece of porous tape. 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. The strain gauge was utilized to verify that a single, intense (1.6 mA, 0.3 s) test shock would elicit at least a 0.8 N flexion force, and to determine the shock intensity necessary to elicit a 0.4 N flexion force.

To minimize lateral leg movements, a 20cm 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. A rat's capacity to perform the instrumental response was then tested with exposure to 30 min of controllable shock. Whenever the rat's leg fell below the level of the salt solution, the electrodes delivered a shock to the tibialis anterior muscle causing the ankle to flex.

Behavioral Measures. Three behavioral measures were used to assess a subject's capacity to perform the instrumental response: response number, response duration and time in solution (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 left 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.

Statistics

All data were analyzed using repeated measures analysis of variance (ANOVA) with an *a priori* alpha value of .05 or below considered significant. Group differences of means were evaluated using Duncan's New Multiple Range *post hoc* tests.

EXPERIMENT 1*

Prior work has shown that instrumental training elevates BDNF mRNA levels in the lumbar-sacral region of the spinal cord (Gomez-Pinilla, Huie, Ying, Ferguson, Crown, Baumbauer, Edgerton, Grau, 2007). Further work has also shown that spinal instrumental training produces a lasting effect that enables learning when subjects are later tested with a higher response criterion (Grau, et al., 1998). Experiment 1 tested the effect of a BDNF inhibitor on instrumental training, and the resulting facilitory effect of instrumental training. If BDNF plays a role in instrumental learning or the enabling effect, then inhibiting the action of BDNF should have a disruptive effect.

Procedure

Experiment 1 used 24 subjects (n=6). At the time of surgery, the BDNF inhibitor TrkB-IgG or vehicle was stereotaxically injected bilaterally in the L4 region of the spinal cord, delivered on glass microbeads as described pre viously. Twenty-four hours later, subjects were set up for instrumental training on both hindlimbs. One hindlimb was set to be trained at a normal response criterion, while the contralateral leg was prepared with a higher response criterion. Half of the subjects in each drug condition then received 30 min of controllable shock on the pretrained leg (Pretrn). The remaining subjects received no pretraining (No Pretrn). At the end of this pretraining period, all subjects were tested for 30 min on the opposite leg with the high response criterion.

Results

Histological analyses revealed that the microbeads were concentrated along the bilateral injection tracts. Figure 1 illustrates the locus of the injection tips for subjects in

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* Reprinted with permission from "BDNF and learning: Evidence that instrumental training promotes learning within the spinal cord by up-regulating BDNF expression" by Gomez-Pinilla, Huie, Ying, Ferguson, Crown, Baumbauer, Grau & Edgerton, 2007. *Neuroscience,* 148, 893-906, Copyright [2007] by Elsevier Ltd.

each group. In general, they were localized in the L5 region of the spinal cord and penetrated the ventral gray matter.

In order to rule out any effects of baseline behavioral reactivity, the shock intensity required to produce a 0.4 N change in flexion force, as well as initial response duration, were analyzed. The mean shock intensity needed to elicit a 0.4 N change in flexion force ranged from 0.53 (+/- 0.05) to 0.65 (+/- .02) mA. Initial flexion durations ranged from 0.137 (+ 0.01) to 0.140 (+ 0.1) s. Independent ANOVAs confirmed that these differences were not significant, Fs < 1.14, $p > .05$.

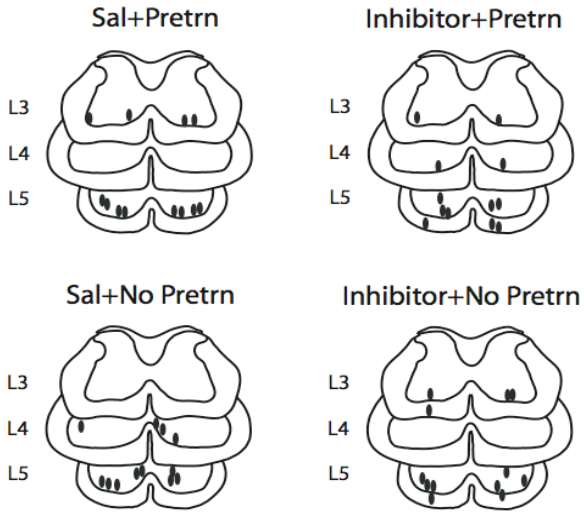

Figure 1. Localization of TrkB-IgG injection tip for each subject. Most injections were localized in the ventral horn of the L5 region of the spinal cord.

The effect of TrkB-IgG treatment on instrumental training is depicted in Figure 2. Both the vehicle treated and TrkB-IgG treated subjects exhibited a progressive increase in response duration, my index of learning. To measure learning in this task, response duration was assessed. An ANOVA revealed that both trained groups

exhibited a progressive increase in response duration over time, F(29, 290)= 1.52, $p <$ .05. This increase in response duration was independent of drug condition, F(29, 290) < 1.0, $p > .05$. The main effect of drug treatment did not approach statistical significance, F(1, 10) = 1.25, $p > .05$.

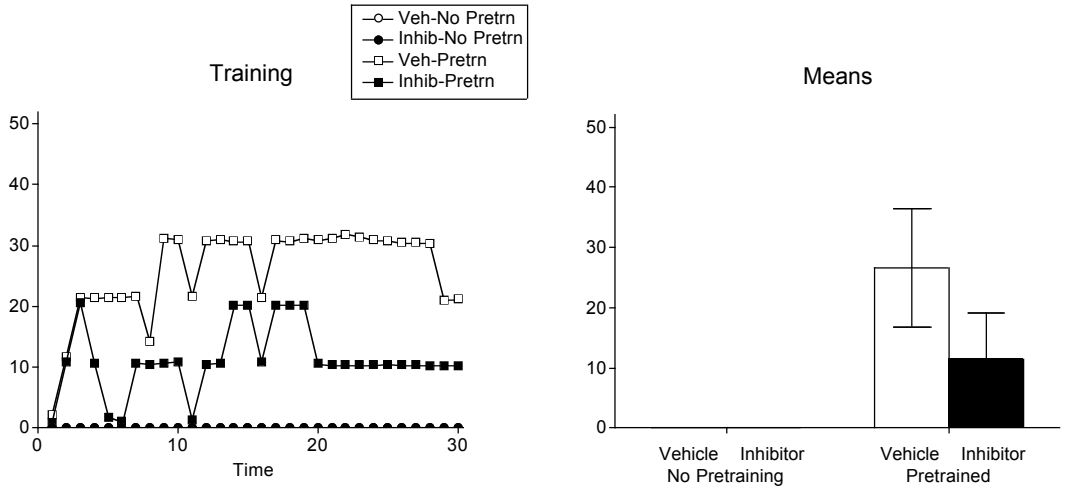

Figure 2. Effect of TrkB-IgG treatment on instrumental learning. Inhibiting BDNF does not attenuate instrumental learning.

In the testing phase, subjects that had not been pretrained failed as expected (Figure 3). As seen in previous studies, vehicle treated rats exhibited a facilitory effect, learning at a higher criterion after previous instrumental training. This facilitation effect was not seen in subjects that were injected with the BDNF inhibitor TrkB-IgG. An ANOVA confirmed that drug, pretraining, and the drug x pretraining interaction, were all statistically significant, all Fs > 6.06, $p < .05$. The drug x pretraining interaction indicates that test performance depended on both TrkB-IgG treatment and whether subjects received pretraining. No other differences approached significance, all Fs < 1.25, $p > .05$. *Post hoc* comparison of the group means confirmed that the vehicle treated

pretrained group differed from the other 3 ($p < .05$). No other differences approached significance, all Fs < 1.25, $p > .05$.

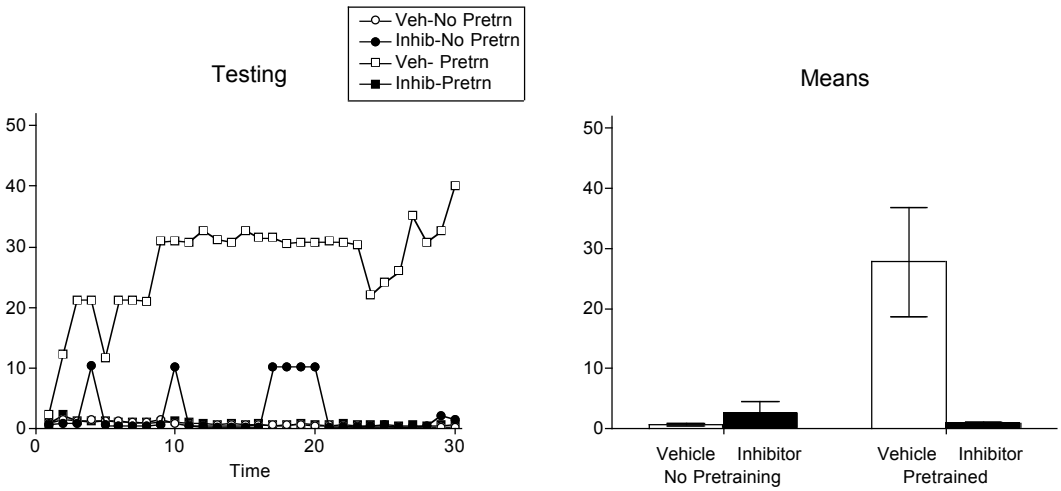

Figure 3. Effect of TrkB-IgG on instrumental testing. Inhibiting BDNF attenuates the facilitory effect of prior instrumental training.

It must be noted that in subjects that failed to learn, the failure was not due to a performance deficit. Although these subjects were unable to exhibit a progressive increase in response duration, they were still able to perform the target response. Mean response number over time is illustrated in Figure 4. Those that failed show a higher response number than those who learned, owing to the fact that longer response durations translate into a lower number of responses. A similar pattern for response number is observed throughout the subsequent experiments, therefore those data are not reported.

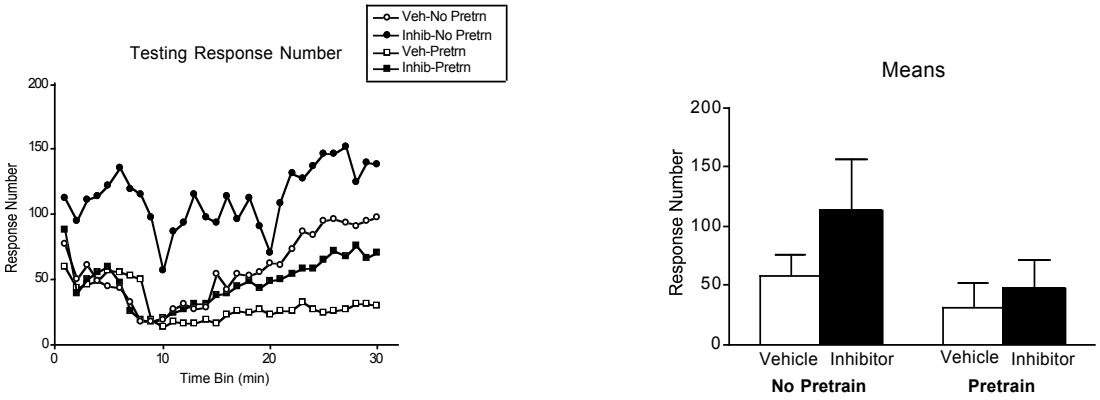

Figure 4. Effect of TrkB-IgG on instrumental testing. Response Number shows that those subjects that failed respond at a higher rate than those that learned.

Discussion

In summary, administration of the BDNF inhibitor TrkB-IgG did not significantly affect the initial acquisition of the instrumental response. Inhibiting BDNF did, however, block the facilitory effect of instrumental training (Gomez-Pinilla, et al., 2007) These results suggest that although BDNF may not be required for the acquisition of the instrumental response, it is necessary to induce the enabling effect of instrumental training.

EXPERIMENT 2

Experiment 1 verified that instrumental training enables learning when subjects are tested with a higher response criterion, and showed that this effect was blocked by TrkB-IgG. This suggests that BDNF plays a necessary role in the enabling effect. Experiment 2 assessed whether pretreatment with BDNF is sufficient to enable learning. If the enabling effect is mediated by BDNF, then the administration of exogenous BDNF should facilitate learning when subjects are tested with a higher criterion.

Procedure

In Experiment 2, 36 subjects (n=6) were used. Rats were placed in the apparatus (described above) and the cannula was threaded through an airhole in the tube for administration of drug. Subjects received a 10 *u*l intrathecal injection of BDNF in one of four doses (0.005, 0.01, 0.02, or .04 μg/μl), followed by a 20 μl saline flush. A control group received a 10 μl intrathecal injection of vehicle (aCSF +0.01% BSA), followed by a 20 μl saline flush. Each subject was tested in the instrumental learning paradigm (described above) 30 minutes after injection, using a higher criterion (solution depth of 8 mm, rather than 4 mm). A sixth group (Pretrained) was pretreated with the vehicle and then given 30 min of training with the usual (4 mm) response criterion. These subjects were then tested on the contralateral leg with the higher response criterion.

Results

As in Experiment 1, shock intensity and initial response duration for each subject were assessed in order to rule out any differences in baseline reactivity. The mean shock intensity needed to elicit a 0.4 N change in flexion force ranged from 0.67 (+/- 0.04) to 0.70 (+/- 0.03) mA. Initial flexion durations ranged from 0.06 (+/- 0.02) to 0.13 (+/-

0.00) s. Independent ANOVAs confirmed that these differences were not significant, Fs < 1.00, $p > 0.05$.

As expected, vehicle treated rats that had not been previously trained failed to learn when tested with a higher criterion (Figure 5). Subjects that had been previously trained (PreTrained) with a normal criterion were able to learn when tested on the contralateral leg with a higher criterion. Similarly, pretreatment with BDNF enabled learning in a dose-dependent fashion. An ANOVA revealed a significant effect of drug treatment F(4,25)=3.47, $p < .05$. *Post hoc* comparisons showed that subjects given doses of 0.01 μg/μl BDNF or more performed better than vehicle treated subjects ($p < .05$). An additional ANOVA was run to compare the pretrained controls to the untrained groups receiving either vehicle or 0.04 μg/μl BDNF. This analysis confirmed that pretreatment had a significant effect, F(2, 15)= 5.97, $p < .05$. *Post hoc* comparisons showed that the pretrained and BDNF treated groups differed from the vehicle treated untrained group ($p < .05$). No other differences were statistically significant.

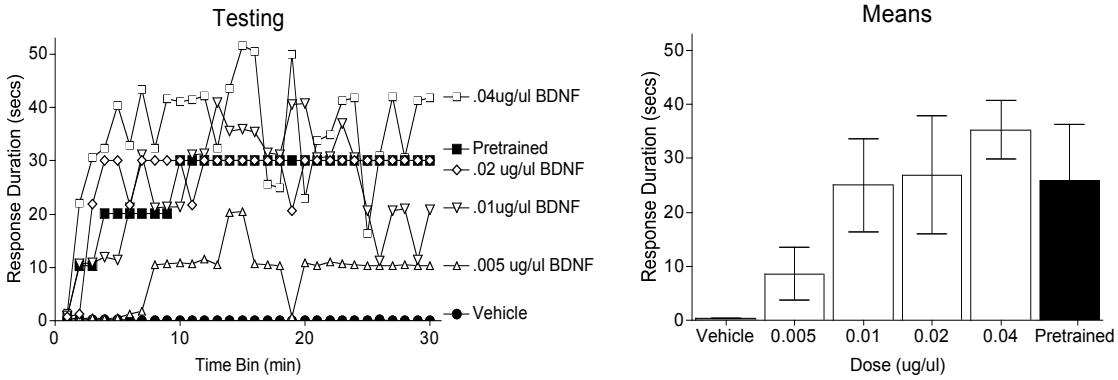

Figure 5. Effect of exogenous BDNF on learning with a higher criterion. BDNF administration facilitates learning in a dose-dependent fashion.

Discussion

In summary, BDNF pretreatment enables learning at a higher criterion. The results from Experiment 1 and 2 suggest that BDNF is both necessary and sufficient for this enabling effect.

EXPERIMENT 3

Prior work has shown that instrumental training can protect against the induction of the deficit (Crown & Grau, 2001). The current experiment was designed to test the necessity for BDNF as it pertains to the protective effect of instrumental training. If the protective effect of instrumental training is mediated by BDNF, then inhibiting BDNF before instrumental training should block this protective effect.

Procedure

The present experiment used 64 rats (n=8). Twenty-four hours after complete transection, subjects were given a 10 μl intrathecal injection of either vehicle (PBS + 0.1% BSA) or TrkB-IgG (0.32 μg/μl), followed by a 20 μl saline flush. Thirty minutes after injection, subjects received either instrumental training or nothing. Immediately following, subjects were given either 6 minutes of uncontrollable shock or nothing. Twenty-four hours later, all subjects were tested for instrumental learning.

Results

Shock intensity and initial response duration for each subject were assessed in order to rule out any differences in baseline reactivity. Mean shock intensity needed to elicit a 0.4 N change in flexion force ranged from 0.63 (+/- 0.03) to 0.69 (+/- 0.02)mA in the training phase, and from 0.61(+/- 0.05) to 0.66 (+/- 0.04) mA in the testing phase. Initial flexion durations ranged from 0.11 (+/- 0.01) to 0.13 (+/- 0.01) s in the training phase, and 0.11(+/- 0.02) to 0.13 (+/- 0.01) s in the testing phase. Independent ANOVAs confirmed that these differences were not significant, $Fs < 1.00$, $p > .05$.

The effect of TrkB-IgG on instrumental training is depicted in Figure 6. As seen in Experiment 1, TrkB-IgG had no effect on the acquisition of the instrumental response; both vehicle and drug treated rats were able to learn. An ANOVA confirmed that all groups exhibited a progressive increase in response duration over time, $F(29, 812) = 14.02$, $p < .01$. This increase in response duration was independent of drug and preshock condition, $F(29, 812) < 1.0$, $p > .05$. The main effects of drug treatment and preshock condition did not approach statistical significance, $F(1, 28) < 1.0$, $p > .05$.

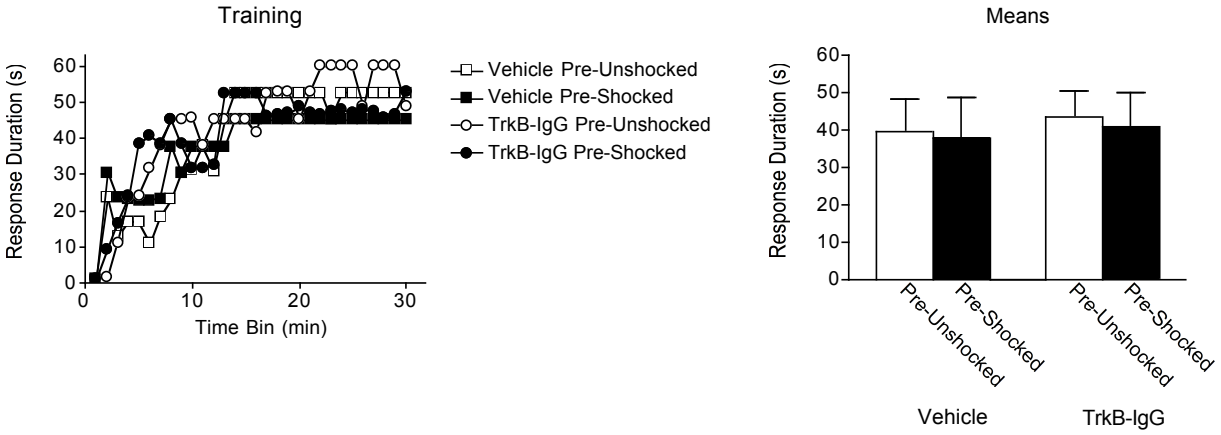

Figure 6. The effect of TrkB-IgG on instrumental training. Replicating findings from Experiment 1, the inhibition of BDNF does not affect acquisition of learning.

The testing phase is depicted in Figures 7 and 8. At test, vehicle and drug treated subjects that did not receive uncontrollable shock learned as expected. Likewise, vehicle and drug treated rats that did not receive training, but did receive uncontrollable shock, exhibited the learning deficit. Further, vehicle treated rats that received training followed by uncontrollable shock were able to learn, replicating the protective effect of instrumental training (Crown & Grau, 2001). TrkB-IgG treated rats that received training

followed by uncontrollable shock were unable to learn. An ANOVA revealed main effects of Drug, $F(1, 56) = 6.54$, $p < .05$, Training, $F(1, 56) = 6.26$, $p < .05$, and Shock, $F(1,56) = 47.94$, $p < .001$. The Drug X Shock and Training X Shock interactions were also significant, $Fs < 5.5$, $p < .05$. In addition, the ANOVA revealed a main effect of Trials, as well as Trials X Shock and Trials X Training X Shock interactions, $Fs > 1.8$, $p < .05$. A four-way Trials X Drug X Training X Shock interaction approached significance, $F (29, 1624) = 394.4$, $p = .056$. In order to further examine the nature of this interaction, trend analyses were run. These analyses revealed that the cubic component of the Trials X Drug X Training X Shock interaction was significiant, $F = 15.027$, $p < .001$. The trend analyses also revealed that the linear components of the Trials X Drug, Trials X Training, and Trials X Drug X Shock interactions were significant, $Fs > 4.45$, $p < .05$. *Post hoc* analysis of group means confirmed that TrkB-IgG treated subjects that were trained and given uncontrollable shock did not differ from groups that were not trained and given uncontrollable shock. Conversely, those given vehicle along with training and uncontrollable shock did not differ from those who were not given uncontrollable shock. No other group differences were significant.

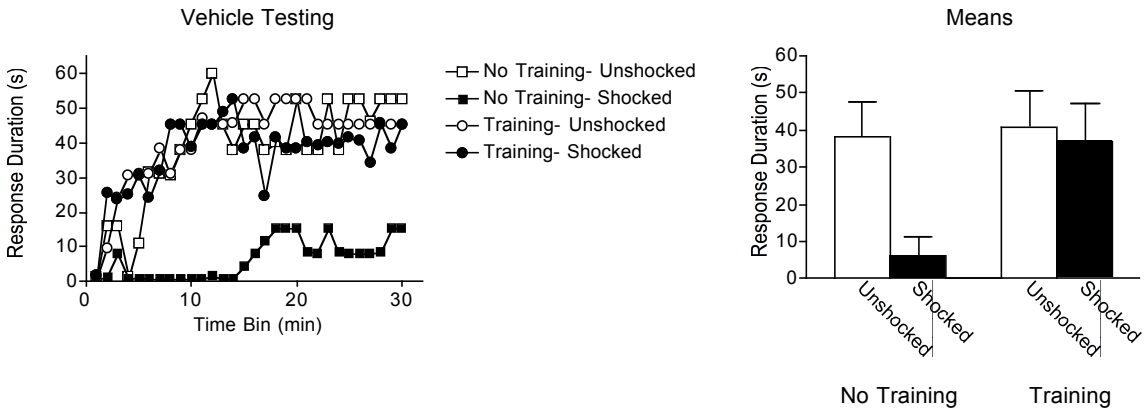

Figure 7. The effect of instrumental training on the induction of the behavioral deficit when given prior to uncontrollable shock. Instrumental training protects against the induction of the deficit in vehicle treated subjects.

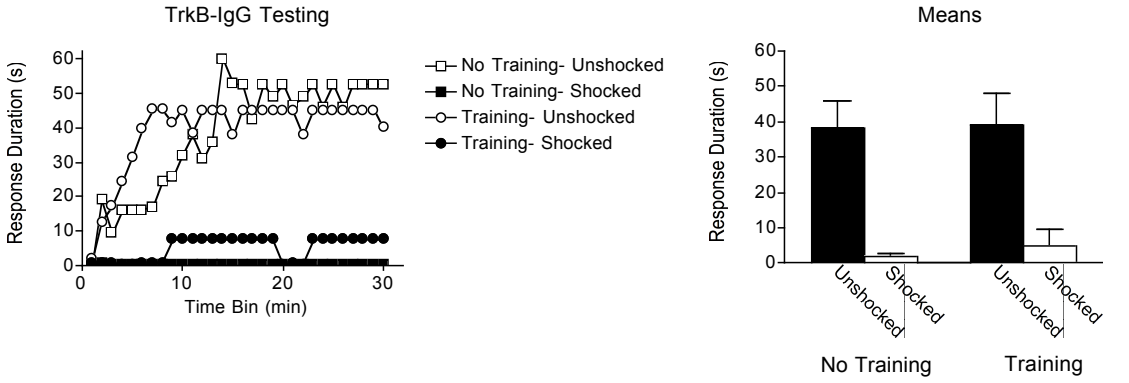

Figure 8. The effect of TrkB-IgG on the protective effect of instrumental training. Inhibiting BDNF attenuates the protective effect of instrumental training.

Discussion

As in past studies, instrumental training proved to be protective against the deficit. When BDNF was inhibited, instrumental training was not protective. These data show that BDNF plays a necessary role in the protective effect of instrumental learning.

EXPERIMENT 4

Building upon the finding form Experiment 3, Experiment 4 was designed to test whether BDNF treatment can provide protection from the learning deficit. If the protective effect of instrumental learning is mediated by BDNF, then BDNF treatment should substitute for instrumental training and have a protective effect.

Procedure

The current experiment used 24 rats (n=6). Twenty-four hours after complete transection, rats were placed in the apparatus and the cannula threaded through an airhole in the tube for administration of drug. Subjects received a 10 μl intrathecal injection of either vehicle (aCSF + 0.1% BSA) or BDNF (0.04 μg/μl) followed by a 20 μl saline flush. Thirty minutes after injection, subjects were given either 6 minutes of uncontrollable shock to the tail or no shock. Twenty-four hours later, all subjects were tested for instrumental learning.

Results

Shock intensity and initial response duration for each subject were assessed in order to rule out any differences in baseline reactivity. The mean shock intensity needed to elicit a 0.4 N change in flexion force ranged from 0.72 (+/- 0.03) to 0.55 (+/- 0.04) mA. Initial flexion durations ranged from 0.09 (+/- 0.01) to 0.15 (+/- 0.02) s. Independent ANOVAs confirmed that these differences were not significant, $Fs < 1.00$, $p > 0.05$.

As depicted in Figure 9, vehicle and BDNF treated rats that were not given shock learned the instrumental relationship as expected. Likewise, vehicle treated rats that did

receive uncontrollable shock exhibited a learning deficit, replicating previous findings. Subjects given BDNF prior to uncontrollable shock were able to learn. An ANOVA showed a significant main effect of Drug, $F(1, 20)= 5.54$, $p < .05$, as well as a main effect of Shock, $F(1, 20)= 16.25$, $p < .001$. Analysis also revealed a Drug x Shock interaction, $F(1, 20)= 16.33$, $p < .001$. Furthermore, a Trials x Drug x Shock three way interaction was observed, $F(29, 580) = 2.87$, $p < .001$. *Post hoc* comparison of group means confirmed that the vehicle shocked rats differed significantly from all other groups, $p < .05$. No other comparisons were significant, $p > .05$.

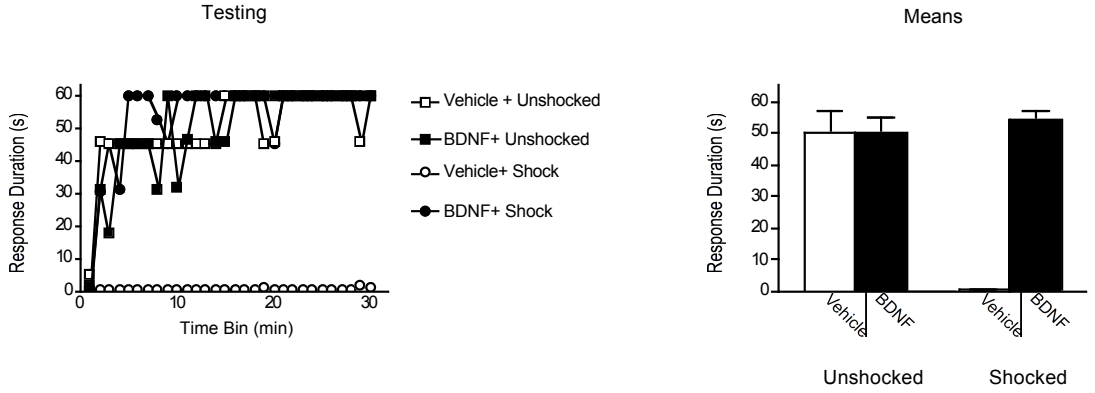

Figure 9. The effect of exogenous BDNF on the induction of the behavioral deficit elicited by uncontrollable shock. Administration of BDNF prior to uncontrollable shock blocks the induction of the deficit.

Discussion

These data reveal that BDNF can have a protective effect that blocks the detrimental effects of uncontrollable shock. This immunizing effect has also been produced by giving instrumental training prior to uncontrollable shock. The present finding suggests the possibility that BDNF and instrumental training may act on a similar mechanism to produce this protective effect.

EXPERIMENT 5

The previous experiment illustrated the capacity for BDNF to block the induction of the learning deficit when given before uncontrollable shock. Prior work has shown that the deficit reflects an intraspinal modification that depends on protein synthesis. This process appears to take time to develop, as evidenced by the capacity for a protein synthesis inhibitor (cycloheximide) to block the induction of the deficit even when given immediately after uncontrollable shock (Patton, Hook, Crown, Ferguson, & Grau, 2004). Given these data, the current experiment explores whether BDNF given after uncontrollable shock can inhibit the process that produces a lasting learning deficit.

Procedure

The current experiment used 24 rats (n=6). Twenty-four hours after complete transection, rats were given either 6 minutes of uncontrollable shock to the tail or nothing. Immediately following shock treatment or an equivalent period of restraint (unshocked), subjects were given an intrathecal injection of either vehicle (aCSF + 0.1% BSA) or BDNF (0.04 μg/μl). All injections were flushed with 20 μl saline. Twenty-four hours later, all subjects were tested for instrumental learning.

Results

As in previous experiments, shock intensity and initial response duration for each subject were assessed in order to rule out any differences in baseline reactivity. The mean shock intensity needed to elicit a 0.4 N change in flexion force ranged from 0.63 (+/- 0.04) to 0.73 (+/- 0.02) mA. Initial flexion durations ranged from 0.11 (+/- 0.01) to

0.12 (+/- 0.01) s. Independent ANOVAs confirmed that these differences were not significant, Fs < 1.00, $p > 0.05$.

As expected, those subjects that did not receive uncontrollable shock were able to learn (Figure 10). Shocked rats that were administered the vehicle exhibited a marked behavioral deficit. Conversely, subjects that received BDNF after shock were able to learn the instrumental response. An ANOVA revealed significant main effects of both drug and shock, Fs < 15.9, $p < .05$. Although no drug by shock interaction was observed, *post hoc* comparison of the group means showed that the vehicle shocked group differed from all other groups, $p < .05$.

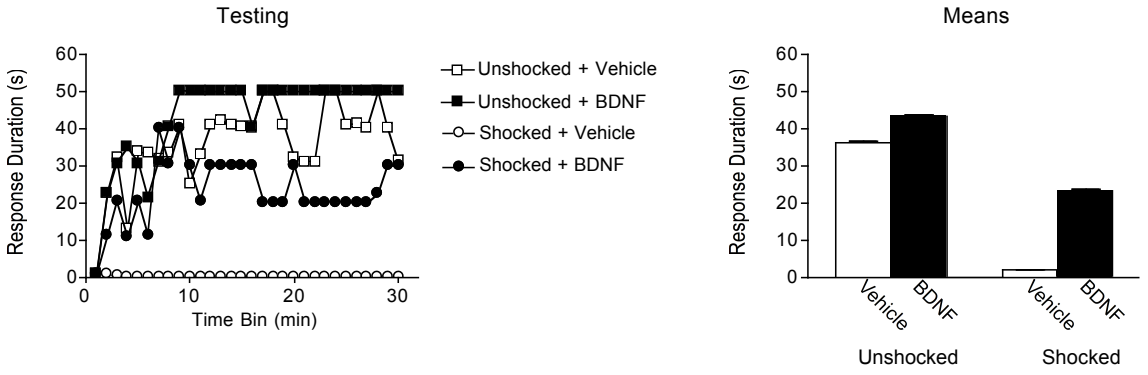

Figure 10. The effect of exogenous BDNF on the development of the behavioral deficit elicited by uncontrollable shock. Administration of BDNF immediately after uncontrollable shock blocks the development of the deficit.

Discussion

These data illustrate that BDNF can block the development of the deficit after the underlying neurochemical processes have been initiated, protecting the spinal cord's capacity for instrumental learning.

EXPERIMENT 6

Extending the findings of Experiment 5, this experiment was designed to assess the effect of BDNF when administered a day after the deficit has been induced. Allowing 24 hours to pass ensures that the intraspinal process that produces the deficit has fully developed. If BDNF has a restorative effect, BDNF treatment should reverse the deficit and block its expression at the time of testing.

Procedure

In this experiment, 32 rats (n=8) were used. Twenty-four hours after complete transection, rats were given either 6 minutes of uncontrollable shock to the tail or nothing. Subjects were then returned to their homecages for 23.5 hours. Injections of either vehicle (aCSF + 0.01% BSA) or BDNF (0.04 μg/μl) were then administered intrathecally, at a volume of 10 μl. All injections were followed by a 20 μl saline flush. Thirty-minutes after injection, all subjects were tested for instrumental learning.

Results

Shock intensity and initial response duration for each subject were once again assessed in order to rule out any differences in baseline reactivity. Mean shock intensity needed to elicit a 0.4 N change in flexion force ranged from 0.63 (+/- 0.04) to 0.67 (+/- 0.03) mA. Initial flexion durations ranged from 0.11 (+/- 0.01) to 0.18 (+/- 0.04) s. Independent ANOVAs confirmed that these differences were not significant, $Fs < 1.00$, $p > 0.05$.

As expected, subjects that received no shock learned the instrumental task (Figure 11). Rats that received shock and vehicle were unable to learn, reflecting the

behavioral deficit produced by uncontrollable shock. Administration of BDNF prior to testing blocked the expression of the deficit in previously shocked rats. An ANOVA revealed main effects of both Shock and Drug, Fs < 9.6, $p < .05$. Although there was no significant interaction between Shock and Drug, there was a three way interaction between Trials, Shock, and Drug, F (29, 812) = 1.53, $p < .05$. *Post hoc* comparison of the group means confirmed that the shocked vehicle treated group differed from the other 3 groups, $p < .05$.

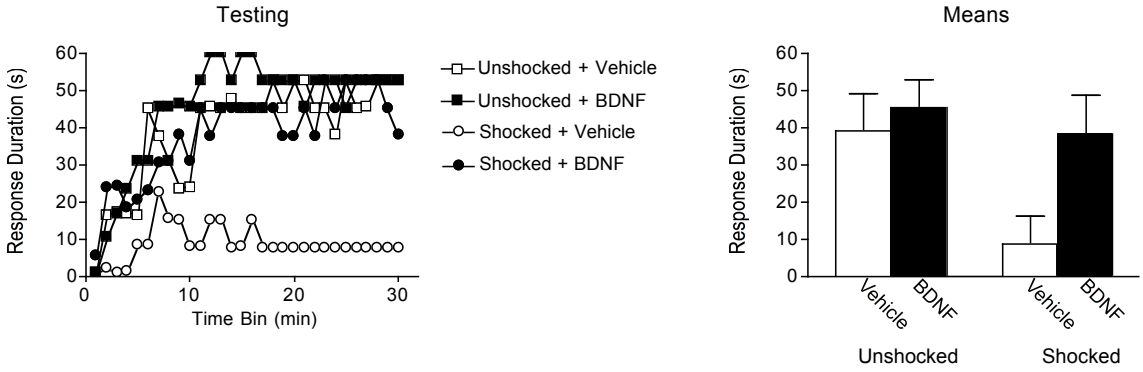

Figure 11. The effect of exogenous BDNF on the expression of the behavioral deficit. Administration of BDNF 24 hours after uncontrollable shock blocks the expression of the deficit.

Discussion

In summary, BDNF treatment blocked the expression of the behavioral deficit elicited by uncontrollable shock. In light of the previous two experiments, it appears that BDNF treatment can have both a protective and therapeutic effect against uncontrollable shock.

CONCLUSIONS

This study examined the role of BDNF in spinal instrumental learning. Previous research has found that BDNF is a component of instrumental learning, as subjects that receive instrumental training show an increase in BDNF mRNA expression. The experiments in this study were designed to characterize the degree to which BDNF is involved in instrumental learning, and whether BDNF might mediate the beneficial effects of instrumental training.

Experiment 1 tested the necessity for BDNF in the enabling effect of instrumental training. This enabling effect was demonstrated in control subjects, where rats that received instrumental training were able to later learn at a higher criterion. Subjects that were pretreated with the BDNF inhibitor TrkB-IgG were able to learn in the training phase, but did not exhibit the enabling effect when tested with a higher criterion. This finding demonstrates that BDNF is necessary for the enabling effect, and suggests that instrumental training enables learning through BDNF release. It also shows that, although instrumental training increases BDNF mRNA expression, BDNF may not be necessary for the acquisition of the instrumental response.

Experiment 2 demonstrated that administration of exogenous BDNF can sufficiently enable learning at a higher criterion. It appears that BDNF administration fosters an increase in performance that mimics the beneficial effects of instrumental training. The data from Experiments 1 and 2 suggest that BDNF enables learning, and is both necessary and sufficient for this effect. This finding complements previous research showing that exercise upregulates BDNF in lumbar spinal neurons, generally increasing

locomotor performance (Gomez-Pinilla, et al., 2001). Likewise, administration of exogenous BDNF has been shown to produce increases in locomotor performance that were behaviorally similar to the enhanced performance of those receiving prior training (Boyce, Tumolo, Fischer, Murray, & Lemay, 2007).

The next set of experiments was designed to examine what effect BDNF might have on the behavioral deficit elicited by uncontrollable shock. When given prior to uncontrollable shock, instrumental training has been shown to block the induction of the deficit (Crown, et al., 2002). If the protective effects of instrumental learning are mediated by BDNF, then inhibiting BDNF should attenuate this protective effect. Likewise, if BDNF mediates the beneficial effects of instrumental training, then administration of exogenous BDNF should be able to substitute for instrumental training.

In Experiment 3, vehicle treated subjects that received instrumental training prior to uncontrollable shock were able to learn when later tested, replicating prior research. Subjects in which BDNF was inhibited before instrumental training were able to learn in the training phase. This finding complements the earlier finding from Experiment 1, showing that BDNF is not required for intial learning. Interestingly, when these subjects were then given uncontrollable shock and later tested, they were unable to learn. The results from Experiment 3 showed that BDNF is necessary in order for instrumental training to have a protective effect against uncontrollable shock. To fully examine the role of BDNF in this effect, Experiment 4 tested the sufficiency of BDNF to protect against the detrimental effects of uncontrollable shock. As in Experiment 2,

exogenous BDNF was substituted for instrumental training to determine whether BDNF alone could produce the effect. Subjects given BDNF were able to learn, even after receiving 6 minutes of uncontrollable shock. Experiments 3 and 4 suggest that the protective effect of instrumental training is mediated by BDNF, and that BDNF is both necessary and sufficient to produce this effect.

The final two experiments were designed to examine the effect BDNF has on spinal learning after uncontrollable shock has been administered. Experiments 3 and 4 showed that BDNF is capable of blocking the behavioral deficit. Experiments 5 and 6 sought to elucidate the effect of BDNF on the consolidation and expression of the behavioral deficit.

Experiment 5 showed that BDNF, when given immediately after uncontrollable shock, can block the processes initiated by uncontrollable shock that would normally lead to a behavioral deficit. Experiment 6 illustrated the capacity for BDNF to block the expression of the behavioral deficit. BDNF was administered 24 hours after uncontrollable shock, and subjects were still able to learn. This window of time between uncontrollable shock and BDNF administration provides enough time for the effects of uncontrollable shock to have manifested.

BDNF Enables Learning

BDNF has been historically characterized by its capacity to promote neural generation and nerve growth. More recently, it has been shown to play a major role in a number of learning paradigms, across a number of neural systems. When applied to hippocampal slices from 12 day-old mice, BDNF can facilitate the induction of LTP in

cells that would otherwise be too young to do so (Figurov, Pozzo-Miller, Olafsson, Wang, & Lu, 1996). Likewise, the administration of endogenous BDNF can enable LTP in hippocampal slices of BDNF knockout mice (Patterson, et al., 1996).

Behavioral studies have also provdided substantial evidence for the role of BDNF in learning. Inhibiting BDNF has been shown to impair cognitive function and produce a marked deficit in the acquisition of conditioned fear (Gomez-Pinilla, et al., 2004; Rattiner, Davis, French, & Ressler, 2004). BDNF mRNA has been shown to be upregulated in the hippocampus following contextual learning (Chen, et al., 2007), as well as an increase in BDNF mRNA expression in the amygdala after fear conditioning (Rattiner et al., 2004). In 2001, Gomez-Pinilla showed that exercise upregulates BDNF in lumbar spinal neurons, generally increasing locomotor performance. Likewise, administration of exogenous BDNF has been shown to produce increases in locomotor performance that were behaviorally similar to the enhanced performance of those receiving prior training (Boyce, et al., 2007).

The present data build upon these prior findings, and provides new behavioral evidence for the role of BDNF in spinal learning. The inhibition of BDNF attenuates the protective effects of instrumental training. Conversely, the administration of exogenous BDNF enables learning in the spinal cord, and provides both protection and therapy against the deleterious effects of uncontrollable shock.

Possible Mechanisms of Action

The capacity for BDNF to enable learning is most likely due to the action of BDNF upon NMDAR functioning. The NMDAR has been well defined as mediating

synaptic plasticity, and a number of studies have shown that BDNF can modulate NDMAR activity (Arvanian & Mendell, 2001; Slack & Thompson, 2002; Garraway, Petruska, & Mendell, 2003). BDNF has been shown to enhance long-term potentiation in hippocampal cells by increasing NMDAR activity (Levine, Crozier, Black, & Plummer, 1998). Specifically, BDNF appears to alter the NR2B subunit of the NDMAR to elicit LTP in the hippocampus (Rosenblum, Dudai, & Richter-Levin, 1996; Black, 1999). Phosphorylation of the NR2B subunit leads to an increase in open probability for the NMDAR, ultimately allowing for postsynaptic potentiation (Rostas, Brent, Voss, Errington, Bliss, & Gurd, 1996). Experiments 1 & 2 showed that BDNF is necessary to produce the robust learning effect that is seen after testing with high criterion. Prior research suggests that this enabling effect may be due to the capacity for BDNF to increase phosphorylation of the NR2B subunit of the NMDAR.

The findings from Experiments 3-6 show that BDNF is necessary and sufficient to protect against the behavioral deficit. Findings suggest that the behavioral deficit elicitted by uncontrollable shock may reflect a form of long-term depression (LTD). Data from our lab has shown that the mGlu receptor agonist DHPG can induce the behavioral deficit. Other work has implied that DHPG induces LTD (Moult, Schnabel, Kilpatrick, Bashir, & Collingridge, 2002). Such work suggests that uncontrollable shock may act in a similar manner, inducing LTD and undermining spinal learning. Endogenous BDNF has been shown to prevent the induction of LTD in vivo, and exogenous BDNF administration has been shown to attenuate LTD in vitro (Akaneya, Tsumoto, Hatanka, 1996). A downstream role for phospholipase C has also been

implicated in this attenuation of LTD by BDNF (Ikegaya, Ishizaka, & Matsuki, 2002). If the behavioral deficit is due to a depression of the neural system, then BDNF may be counteracting this depression by TrkB receptor mediated activation of the PLC pathway. This PLC activation may provide adequate potentiation of the sytem, restoring the capacity for learning.

Previous research has shown that although instrumental learning appears to reflect a NMDAR mediated plasticity, it has also been shown that NMDAR antagonists such as MK-801 and APV can protect against the deficit when given prior to uncontrollable shock (Ferguson, Crown, & Grau, 2006). This has lead some to speculate that the detrimental effect of uncontrollable shock may be due to an overexcitation, or saturation, of the NMDA receptor, that is akin to central sensitization. If this is the case, then it is unlikely that the protective effect of BDNF is due solely to NR2B phosphorylation, since this would only further act to overexcite the cell. We may able to attribute the protective effect of BDNF to its unique action upon *other* NDMAR subunits. In cerebellar granule cells, BDNF has been shown to reduce expression of NR2A and NR2C subunits. This reduced expression has been implicated as a model for protection against glutamate neurotoxicity (Brandoli, Sanna, Bernardi, Follesa, Brooker, & Mocchetti, 1998).

How then, can one reconcile these two very different actions of BDNF? In one instance, BDNF increases excitation through activation of the NR2B subunit, whereas another instance sees BDNF blocking overexcitation by downregulating the NR2A and NR2C subunits. What could elicit these differential effects? The answer may lie within

the intracellular signaling pathway that leads from the TrkB receptor to the NMDAR. In 1999, Black suggested that environmental stimuli may play a role in the capacity for BDNF to produce the differential regulation of subunit phosphorylation. A noxious stimulus such as uncontrollable shock could have an effect on BDNF action. In prior research, CamKII and PKC have been implicated as essential in both the learning deficit and the downstream effects of BDNF (Baumbauer, Young, Hoy, France, & Joynes, 2006; Bolding, Hook, Ferguson, & Grau, 2003; Gardoni, Bellone, Cattabeni, & Di Luca, 2001). It is possible that uncontrollable shock may be activating similar excitatory pathways, and this competition could dictate the effect of BDNF on the NMDAR.

Clinical Implications & Future Studies

The current study was characterized by pharmacological manipulation and behavioral observation. To further elucidate the molecular mechanisms in which BDNF is involved, we will be taking a histological approach. Through the use of the ELISA procedure, we will work to quantify and localize endogenous BDNF protein levels after instrumental training, in an effort to better understand the role of BDNF in spinal learning. Additionally, Western blotting will be utilized to examine the intracellular cascade that is activated by BDNF release after instrumental training. We will assess the signaling pathway with which BDNF is historically associated, including the TrkB receptor, PKC, PLC, ERK, CREB, and NMDAR. To this end, the downstream processes elicited by BDNF release can be elucidated.

The findings from this and future works may have a number of broader implications for recovery of function following spinal injury. One of the hallmarks of the

spinal instrumental learning paradigm is that findings easily translate to more naturalistic paradigms. The principles learned within the isolated spinal cord can be applied to a contusion injury model. Future research will be directed towards the therapeutic effect of BDNF in the contused rat, and the mechanisms that underlying improvement of function. Likewise, study will be directed towards therapeutic interventions that promote endogenous BDNF release. In this way, work will continue to develop towards a clinically relevant end, in which the role and impact of BDNF on functional recovery after spinal injury can be realized.

Summary

This study assessed the role of BDNF in spinal instrumental learning. Blocking BDNF undermined the facilitory effect of instrumental training, while administration of exogenous BDNF was able to produce this facilitory effect, proving to be an effective substitute for instrumental training. These data suggest that the facilitory effect of instrumental learning appears to be mediated by BDNF.

Previous research has shown that instrumental training given before uncontrollable shock can protect against the behavioral deficit that is normally elicited by uncontrollable shock. Blocking BDNF attenuated the protective effect of instrumental training, and administration of exogenous BDNF was able to produce this protective effect, proving to be an effective substitute for instrumental training.

Other research has shown that in conjunction with pharmacological manipulation, instrumental training can block the development of the behavioral deficit when given after uncontrollable shock. When exogenous BDNF is given in place of

instrumental training immediately after uncontrollable shock, BDNF is similarly able to block the development of the deficit. Likewise, BDNF given 24 hours after uncontrollable shock is able to block the expression of the deficit.

Taken together, the current experiments provide a comprehensive study of the role of BDNF as it pertains to the beneficial effects of instrumental training. These data suggest that BDNF is both necessary and sufficent for the facilitation effect and the protective effect of instrumental training. Likewise, BDNF can be administered after uncontrollable shock to restore the capacity for instrumental learning.

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