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Brain-derived neurotrophic factor (BDNF) promotes adaptive plasticity within the spinal cord and mediates the beneficial effects of controllable stimulation

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Abstract

Brain-derived neurotrophic factor (BDNF) has been characterized as a potent modulator of neural plasticity in both the brain and spinal cord. The present experiments use an *in vivo* model system to demonstrate that training with controllable stimulation increases spinal BDNF expression and engages a BDNF-dependent process that promotes adaptive plasticity.

Spinally transected rats administered legshock whenever one hindlimb is extended (controllable stimulation) exhibit a progressive increase in flexion duration. This simple form of response-outcome (instrumental) learning is not observed when shock is given independent of leg position (uncontrollable stimulation). Uncontrollable electrical stimulation also induces a lasting effect that impairs learning for up to 48 hrs. Training with controllable shock can counter the adverse consequences of uncontrollable stimulation, to both prevent and reverse the learning deficit. Here it is shown that the protective and restorative effect of instrumental training depends on BDNF.

Cellular assays showed that controllable stimulation increased BDNF mRNA expression and protein within the lumbar spinal cord. These changes were associated with an increase in the BDNF receptor TrkB protein within the dorsal horn. Evidence is then presented that these changes play a functional role *in vivo*. Application of a BDNF inhibitor (TrkB-IgG) blocked the protective effect of instrumental training. Direct (intrathecal) application of BDNF substituted for instrumental training to block both the induction and expression of the learning deficit. Uncontrollable stimulation also induced an increase in mechanical reactivity (allodynia) and this too was prevented by BDNF. TrkB-IgG blocked the restorative effect of instrumental training and intrathecal BDNF substituted for training to reverse the deficit. Taken together, these findings outline a critical role for BDNF in mediating the beneficial effects of controllable stimulation on spinal plasticity.

Keywords

Brain-derived neurotrophic factor (BDNF); spinal cord; plasticity; instrumental learning

Research over the past 15 years has expanded the role of brain-derived neurotrophic factor (BDNF) from a key factor in neural development to a potent modulator of plasticity in the central nervous system. Within the brain, BDNF has been shown to regulate learning and memory in a number of paradigms. In hippocampal slices, BDNF promotes the development of long-term potentiation (LTP) (Kang & Schumann, 1995; Patterson et al., 1996). Behaviorally, BDNF is necessary for visuo-spatial and memory tasks, and BDNF expression has been associated with hippocampal-dependent contextual conditioning (Mizuno et al., 2000; Gomez-Pinilla et al., 2008; Greenwood et al., 2008).

BDNF has also been shown to potentiate plasticity in the spinal cord (Baker-Herman et al., 2004). BDNF can foster plasticity in cultured spinal neurons, and both the stimulation of BDNF release and the application of exogenous BDNF can lead to improved locomotor function following spinal cord injury (Ye & Houle, 1997; McTigue et al., 1998; Sharma, 2007; Zhou et al., 2008). Similarly, exercise-induced upregulation of BDNF mRNA is associated with functional recovery after spinal contusion injury (Gomez-Pinilla et al., 2001). Recent work in our laboratory suggests that behavioral control may be an important factor in regulating BDNF within the spinal cord (Gomez-Pinilla et al., 2007). The current experiments use a novel *in vivo* model of spinal learning to investigate the role of BDNF in the adaptive effects of instrumental training in the spinal cord. Rat subjects that have received a complete spinal transection at the 2nd thoracic vertebra are given electrical stimulation to a hindlimb whenever that limb is in an unflexed position. The stimulation elicits a flexion of the limb, and the shock is then terminated. Over time, the subject will learn to keep the limb in a flexed position in order to reduce shock exposure. In this way, these subjects are exhibiting a form of instrumental learning, in which a response (increased flexion) is associated with an outcome (reduced shock exposure). This model is unique because it allows researchers to study spinal plasticity in isolation from supraspinal input, while providing a behavioral and cellular context that can be extended to the treatment of spinal cord injury (Grau et al., 2004, 2006).

Stimuli applied below a spinal transection can have divergent effects on spinal neurons depending upon whether the stimulation is controllable (response-contingent) or uncontrollable (noncontingent). Uncontrollable stimulation induces a form of metaplasticity that impairs subsequent learning (Grau et al., 1998; Crown et al., 2002a; Ferguson et al., 2008). Controllable stimulation (instrumental training) has the opposite effect, enabling response modifications that reduce net exposure to noxious stimulation (Crown et al., 2001). Further, instrumental training can both prevent, and reverse, the learning impairment produced by uncontrollable stimulation (Crown & Grau, 2001). The present experiments show that the protective and restorative effects of controllable stimulation depend on BDNF. Using *in situ* hybridization and Western blotting, we show that controllable stimulation increases BDNF mRNA and protein expression within the lumbar spinal cord. Training also increases protein levels for the BDNF receptor TrkB within dorsal horn. Next, we show BDNF plays a functional role within the spinal cord *in vivo*. The BDNF inhibitor TrkB-IgG blocked the protective effect of instrumental training, and spinal (intrathecal) application of BDNF prevented the induction and expression of the learning deficit observed after uncontrollable stimulation. The restorative effect of instrumental training was also blocked by TrkB-IgG and intrathecal BDNF substituted for training to reverse the adverse effects of uncontrollable stimulation. A summary of the experimental strategy is provided in Figure 1.

1. EXPERIMENTAL PROCEDURES

1.1. Animals

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*. All experiments were carried out in accordance with NIH standards for the care and use of laboratory animals (NIH publications No. 80-23), and were approved by the University Laboratory Animal Care Committee at Texas A&M University. Every effort was made to minimize suffering and limit the number of animals used.

1.2. Surgery

All subjects received a spinal transection while anesthetized with isoflurane. The 2nd thoracic vertebra (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 laid 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 ortholetic 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°C). Supplemental 0.9% saline injections (2.5 mL, i.p.) were provided once daily to ensure proper hydration. Bladders were expressed twice daily and just before behavioral testing.

1.3. Instrumental learning apparatus and procedure

Instrumental training/testing was conducted while rats were loosely restrained in tubes (23.5 cm [length] × 8 cm [internal diameter]). Two slots in the tube, (5.6 cm [length] × 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. 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 a BRS/LVE (Laurel, MD) constant current (60Hz, AC) shock generator (Model SG-903) was attached to this wire. A 7-cm long, 0.46 mm diameter stainless steel contact electrode was secured to the foot between the second and third digits with a piece of porous tape. 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 contact electrode extended from the rear of the foot and was connected to a digital input monitored by a Macintosh computer. A plastic rectangular dish (11.5 cm [w] × 19 cm [l] × 5 cm [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. 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.

In order to standardize response parameters across subjects, we adjusted the intensity of electrical shock necessary to generate a 0.4 Newton flexion force for each subject. This

amount of force was found to be within an ideal range for subjects to be able to exhibit the learning response over time (Grau, et al., 2006). 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 40-cm 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. These data revealed a linear relation, which allowed us to convert voltage to force.

Upon determining the shock intensity that generated a 0.4 N flexion response for each subject, this intensity was subsequently used during the ensuing training/testing session.

To minimize lateral leg movements, a 20-cm piece of porous tape was wrapped around the leg and attached to a bar extending across the apparatus directly under the front panel of the restraining tube. The tape was adjusted so that it was taut enough to slightly extend the knee. Finally, three short (0.15 s) shock pulses, given at the intensity previously determined to elicit a 0.4 N flexion response, 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.

Recognizing that some experimental manipulations could potentially affect the subject's capacity to perform the target (flexion) response, we recorded two measures of baseline behavioral reactivity: the shock intensity required to elicit a flexion force of 0.4 N and the duration of the first shock-elicited flexion response. Independent ANOVAs showed that there were no group differences on either measure across all experiments, $F_s < 1.35$, $p < .05$.

Upon completion of this setup phase, the 30 min instrumental training/testing began. An instrumental (response-outcome) contingency was established by applying electrical stimulation (at the intensity previously determined to elicit a 0.4 N flexion force) to the tibialis anterior muscle each time the contact electrode touched the underlying salt solution. The shock stimulation typically elicited a flexion response, raising the contact electrode above the salt solution, which terminated the shock. The state of this circuit was sampled at a rate of 30 times/s.

1.4. Measures of instrumental learning and performance

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. As discussed elsewhere (Grau et al., 1998), a system capable of instrumental learning should exhibit an increase in response duration as a function of training. Because this measure avoids some interpretative problems that plagued earlier studies, and because we have shown that this measure is sensitive to a variety of manipulations known to impact learning (reviewed in Grau et al., 2006), it is used as our primary index of learning.

Prior work has also shown that treatments that disrupt learning do not normally interfere with the performance of a flexion response. Indeed, subjects that fail to learn (that do not

exhibit an increase in response duration) tend to exhibit the highest rates of responding (i.e., the greatest response number). This is important because it indicates that the failure to learn does not reflect a performance deficit. In the present experiments, we analyzed both response duration and response number and found the inverse relationship reported in prior studies. Because no unexpected results were obtained, we focus on our measure of learning—response duration.

1.5. Master-yoked learning paradigm

For histological (mRNA and protein) analysis of the differential effects of controllable versus uncontrollable shock on BDNF and TrkB (Experiments 1–3b), it was important to ensure that controllability was the only factor that was manipulated, and that the overall number, duration, and distribution of shocks was equated across groups. We addressed this issue using a master-yoke instrumental learning paradigm (Grau et al., 1998). Briefly, rats were set up for instrumental learning in pairs. Within each pair, one subject (the master) was given response contingent shock, wherein legshock was applied whenever the leg was extended and terminated when the leg was flexed (controllable shock). The second subject was experimentally yoked to the master rat and received shock at the same time, and for the same duration, as the master rat but independent of leg position (uncontrollable shock). A third group was setup in the same manner, but remained unshocked.

Because our aim was to compare the cellular consequences of controllable versus uncontrollable stimulation, we only included master-yoke pairs in which the master subject acquired the instrumental response (exhibiting a mean response duration greater than 20 s) did so in a gradual manner, assuring that the yoked subject received multiple (greater than 70) noncontingent legshocks. Twenty-five percent of master-yoked pairs that were tested failed to meet these criteria, and were subsequently excluded from analysis.

1.6. *In situ* hybridization

Tissue was collected for *in situ* hybridization in Experiment 1 immediately after master-yoked training. Subjects were overdosed with sodium pentobarbital (100 mg/kg) and then underwent intracardial perfusion with phosphate buffered saline (PBS) followed by 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer, pH 7.4. Following perfusion, spinal cords were extracted, post-fixed in PFA for 24 or 2 h for *in situ* hybridization and immunohistochemistry, respectively. Spinal cords were then cryoprotected in 20% sucrose dissolved in phosphate-buffered saline (PBS) for immunohistochemistry or PFA (for *in situ* hybridization) for 48 h, rapidly frozen on powdered dry ice and stored at -80°C until further processing. The spinal cord of each animal was sectioned (30 μm , coronal) through the lumbar region (L3–L5) using a freezing microtome. Sections were collected into cold paraformaldehyde and stored at 4°C .

All sections were processed in a single experiment to minimize interassay variability (for additional details, see Bizon et al., 2001). Briefly, free-floating sections of tissue were washed in 0.75% glycine in 0.1 M phosphate buffer, pH 7.2 (PB) and 0.1 M PB alone to remove excess fixative. Sections were permeabilized using proteinase K (1 mg/mL in 0.1 M Tris buffer containing 0.05% SDS) for 30 min at 37°C , acetylated in 0.25% acetic anhydride in 0.1 M triethanolamine, pH 8.0, and rinsed twice in 2X saline sodium citrate buffer (SSC; $1 \times \text{SSC} = 0.15 \text{ M}$ sodium chloride and 0.015 M sodium citrate, pH 7.0). Tissue was then hybridized for 42–44 h at 60°C in solution containing 50% formamide, $1 \times$ Denhardt's solution, 10% dextran sulphate, $4 \times \text{SSC}$, 0.25 mg/mL yeast tRNA, 0.3 mg/mL herring sperm DNA, 100 mM dithiothreitol (DTT) and ^{35}S -labelled BDNF cRNA at a final concentration of 1×10^7 CPM/mL. The BDNF cRNA was generated from PvuII-digested recombinant plasmid pR1112-8 using T3 RNA polymerase yielding a 540-base-length probe

with 384 bases complementary to the coding region of BDNF exon V-containing mRNA (Lauterborn et al., 1996). Following hybridization, sections were washed at 30 min intervals, twice in $4 \times \text{SSC}$, once in 50% formamide/ $2 \times \text{SSC}$ at 60°C and then treated with ribonuclease A (20 mg/mL in 10 mM Tris saline buffer containing 1 mM ethylenediaminetetraacetic acid) for 30 min at 37°C . Tissue sections were washed further in descending concentrations of SSC buffer containing 100 mM DTT to a final wash of $0.1 \times \text{SSC}$ and mounted onto Superfrost++ slides.

Air-dried sections were exposed along with ^{14}C -standards (American Radiolabelled Chemical, Inc., St. Louis, MO) for 24 h. Screens were scanned at high resolution of 25 microns (equivalent to approximate 1050 dots per inch) using a Typhoon Trio variable mode imager (Perkin Elmer, Waltham MA).

In situ hybridization was quantified by densitometric analysis using Densita imaging software (MBF Biosciences, Williston, VT). Hybridization densities were linearized and calibrated relative to the ^{14}C -labelled standards that were exposed along with tissue sections. For each subfield of the spinal cord section (i.e. dorsal and ventral horns and gracile fasciculus) individual values for hybridization signal intensity ($\mu\text{Ci/g}$ protein) were calculated for each rat as the average of multiple measurements from a minimum of six sections. Hybridization values from dorsal and ventral horn for each subject were then normalized to those obtained from the measurements taken from white matter of that animal (i.e., gracile fasciculus), an area where BDNF mRNA is not specially expressed. Indeed, BDNF cRNA hybridization values in this region were low and no differences were observed across groups ($F_{(2,15)} < 1.0$, $p > 0.05$). Mean hybridization data for each subject (presented as a % of the nonspecific hybridization) for each rat within each group were averaged to obtain a group mean $\pm \text{SEM}$ and these group means were used as the measure of regional BDNF mRNA abundance. The significance of group differences for dorsal and ventral horn was determined using a one-factor ANOVA with Fisher PSLD *post hoc* analyses.

1.7. Western blot

Tissue was collected for western blotting (Experiments 2 and 3a) immediately following master-yoked training. Subjects were anesthetized with pentobarbital (50mg/kg) and L3–L5 spinal segments were removed from all subjects. Total protein was extracted using the QIAzol lysis reagent protocol (Qiagen). Following determination of the protein concentration, using the Bradford Assay (BioRad), samples were diluted in Laemmli sample buffer and stored at -80°C . Equal amounts (30 μg) of total protein were subjected to SDS-PAGE using 12% Tris-HCl precast gels (Thermo Scientific) and then transferred to PVDF (for TrkB) or nitrocellulose (for BDNF) membranes (Millipore, Bedford, MA), which were subsequently blocked in 5% blotting grade milk (BioRad, Hercules, CA).

Blots were incubated in primary antibodies generated in rabbit for BDNF (1:100; #R-066–500– Novus Biologicals, Littleton, CO), both the full-length (145kDa) and truncated (95kDa) forms of the TrkB receptor (1:500; #07–225 - Upstate Cell Signaling, Temecula, CA) and β -actin (1:5,000; #Ab8227 – Abcam, Cambridge, MA), overnight at 4°C . Following a series of 3×10 minutes washes in Tris buffered saline with Tween (TBST) at room temperature, the blots were incubated in HRP-conjugated goat anti-rabbit secondary antibody (1:5,000; 31460, #JF11303808; Pierce, Rockford, IL) was added for 1 hour at room temperature. Following 3×10 minutes washes in TBST, the blots were developed with ECL (Pierce, Rockford, IL) and imaged with Fluorchem HD2 (Alpha Innotech, San Leandro, CA). Ratios of the densitometry intensity of BDNF, full-length and truncated TrkB to β -actin (loading control) were calculated, normalized to unshocked controls and averaged for animals within each group.

1.8. Immunohistochemistry

For immunohistochemistry (Experiment 3b), subjects underwent master-yoked training. They were then anesthetized with pentobarbital (50 mg/kg) and transcardially perfused with PBS followed by 4% PFA in PBS. The spinal cord was dissected and post-fixed in 4% PFA for 2 hours before being transferred to 30% sucrose for cryoprotection. Following at least 72 hours in sucrose, the lumbar (L3–5) spinal cord was cut into two 4 mm sections and frozen into a mold with optimal cutting temperature (OCT) compound. Twenty-micron cryostat (Leica) sections were prepared for immunohistochemistry. Master-yoked pairs were mounted on the same slides.

The spinal cord sections were first quenched in 0.6% H₂O₂ Tris buffered saline (TBS) then incubated in blocking solution (3% normal goat serum, 0.1% Triton X-100 in TBS) for 30 min. Sections were then incubated in rabbit anti-TrkB antibody (1:500; Novus Biological) overnight in blocking solution at room temperature. The sections were then incubated in biotinylated goat anti-rabbit secondary antibody (1:250; Vector Laboratories, Burlingame, CA) and immunoreactivity was detected with the avidin-biotin-peroxidase complex (ABC)-3,3'-diaminobenzidinetetra-hydrochloride technique. The number of TrkB positive cells was manually counted for the right and left superficial dorsal horn (SDH) and right and left ventral horn (VH) using Stereo Investigator software. The total number of cells per region were normalized to the region area and expressed as a ratio. A total of 5 spinal cord sections (L4/5) were averaged per animal.

For immunofluorescence, spinal cord sections were washed twice for 2 × 10 min in TBS, followed by 1 hr incubation in blocking solution. Sections were then incubated in either rabbit anti-TrkB antibody (1:500; NB) or a combination of anti-TrkB and mouse anti-NeuN (1:400; Millipore-Chemicon, Bedford, MA) in blocking solution at room temperature. Following incubation in the appropriate Alexa fluor-conjugated secondary antibodies (1:300, Invitrogen, Eugene, OR), the slides were mounted in Prolong Gold anti-fading mounting medium (Invitrogen, Eugene, OR). Control slides for both light and fluorescent immunohistochemistry were exposed to diluted normal goat serum instead of the primary antibody. Serial images were taken with a confocal microscope (Olympus BX61 with a Plan Apo N 60× 1.42NA oil objective) using Olympus Fluoview version 5.

1.9. Uncontrollable Shock

In Experiments 4,5,7 & 8, a computer program was used to deliver an intermittent tailshock schedule that emulated the pattern of uncontrollable stimulation given to a yoked subject in the master-yoked paradigm (Crown et al., 2002b). 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. Each shock was 80 ms in duration, delivered over the course of 6 minutes. The shocks were delivered intermittently in a randomized fashion between 0.2 and 3.8 seconds apart. This schedule allowed for 180 shocks to be presented, at an average of 2 seconds apart. To elicit tactile allodynia in Experiment 6, intermittent uncontrollable shock was delivered to the leg rather than tail, as described previously (Ferguson et al., 2006). Shock parameters were the same as used in tailshock, with the exception of shock intensity. This value was variable for each subject, and was determined by the intensity necessary to generate a 0.4 N flexion, as described in the instrumental learning procedure section (1.3).

1.10. Drug administration

For experiments involving an intrathecal injection (Experiments 4–8), a 25 cm polyethylene cannula (PE-10, VWR International, Bristol, CT) was 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. This placement was chosen as it has been shown that a number of other pharmacological agents have affected spinal learning when delivered in this fashion (Grau et al., 2006). The exposed end of the cannula was secured to the skin with cyanoacrylate. BDNF (Sigma-Aldrich, St. Louis, MO) was given at a dose of 0.04 $\mu\text{g}/\mu\text{L}$, dissolved in 10 μL of aCSF + 0.1% bovine serum albumin (BSA) vehicle (Gomez-Pinilla et al., 2007). Control subjects were given a 10 μL injection of this vehicle. The BDNF sequestering agent TrkB-IgG (R&D Systems, Minneapolis, MN) was also injected intrathecally, as described above. TrkB-IgG was given at a dose of 0.23 $\mu\text{g}/\mu\text{L}$, dissolved in 10 μL of PBS + 0.1% BSA vehicle. Control subjects were given a 10 μL injection of this vehicle. The opioid antagonist naltrexone (Sigma-Aldrich, St. Louis, MO) was administered intrathecally at a dose of 7 mg/mL dissolved in 10 μL saline vehicle. All subjects were given a 20 μL saline flush after each injection.

1.11. Test of tactile reactivity

Tactile reactivity was assessed in Experiment 6 using nylon von Frey filaments (Stoelting, Chicago, IL). Filaments of increasing tactile force were applied to the plantar surface of hindlimbs until a flexion response was observed. All subjects were tested twice on each leg for both baseline and postshock assessments of tactile reactivity threshold. Tactile data are reported using the linear monofilament number scale provided by the manufacturer: $\text{Intensity} = \log_{10}(10,000 \times \text{g})$.

1.12. Statistical Analyses

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

2. RESULTS

2.1. Experiment 1: Instrumental training increases BDNF mRNA expression in the dorsal and ventral horn

Using real-time quantitative RT-PCR, we have shown that controllable and uncontrollable stimulation have divergent effects on BDNF mRNA expression within the spinal cord; controllable stimulation increases BDNF mRNA expression and uncontrollable stimulation reduces expression (Gomez-Pinilla et al., 2007). The present experiment used *in situ* hybridization to explore the anatomical distribution of these effects within the lumbar spinal cord. Twenty-four hours after spinal transection, pairs of subjects were tested for instrumental learning according to the master/yoked learning paradigm ($n=6$). A comparison group was also included that was given an equivalent amount of restraint, but received no shock ($n=6$). Master subjects exhibited a mean response duration indicative of learning (46.06 ± 1.79 s), while the yoked subjects failed to learn (mean response duration, 4.93 ± 3.14 s). Analysis of variance revealed a significant difference between these groups, $F_{(1, 10)} = 107.78$, $p < .05$. Immediately following testing, subjects were anesthetized with pentobarbital (50 mg/kg) and spinal tissue was prepared for *in situ* hybridization as described above.

Levels of BDNF mRNA abundance following instrumental conditioning are shown in Figure 2. BDNF mRNA was primarily expressed by cells in the dorsal and ventral horns of spinal cord and expression was robustly increased in these regions in response to

controllable stimulation (Master) relative to uncontrollable stimulation (Yoked) and unshocked control subjects. Regional quantification of these data confirmed these observations. An ANOVA revealed BDNF mRNA abundance varied significantly by treatment group in both the dorsal and ventral horn (dorsal: $F_{(2,15)} = 6.63, p < .01$; ventral: $F_{(2,15)} = 5.59, p < .05$). *Post hoc* comparisons confirmed BDNF mRNA abundance in the master group compared to both yoked and unshocked groups ($p < .05$) in both regions with no significant differences in BDNF mRNA abundance observed between the latter groups in either dorsal or ventral horn ($p > .05$).

2.2. Experiment 2: Instrumental training increases spinal BDNF protein expression

Having established that training impacts BDNF mRNA expression, we then sought evidence that training affects BDNF protein levels. Twenty-four hours after spinal transection, pairs of subjects were tested for instrumental learning according to the master/yoked learning paradigm ($n=6$). A comparison group was also included that was given an equivalent amount of restraint, but received no shock ($n=4$). Master subjects exhibited a mean response duration indicative of learning (41.57 ± 3.99 s), while the yoked subjects failed to learn (mean response duration, 5.48 ± 3.14 s). Analysis of variance revealed a significant difference between these groups, $F_{(1, 10)} = 48.71, p < .05$. Immediately following testing, subjects were anesthetized and spinal tissue (L3-L5 segments) was prepared for Western blot analysis as described above.

Results from BDNF Western blot analysis are shown in Figure 3A. Master subjects exhibited higher levels of BDNF protein. While an overall ANOVA did not reveal a significant group difference, $F_{(2, 17)} = 1.62, p > .05$, there was a systematic difference between the master-yoke pairs across blots; master rats had higher BDNF protein levels than their yoked partner. A matched *t*-test confirmed that this difference was statistically significant, $t(7) = 3.00, p < .05$.

2.3.1. Experiment 3a: Instrumental training increases TrkB expression

The BDNF signaling cascade is first initiated by activation of the TrkB receptor. In the presence of BDNF, TrkB trafficking to the post-synaptic membrane is rapidly increased (Suzuki et al., 2004; Nagappan & Lu, 2005). This heightened TrkB expression is an essential component in BDNF-mediated plasticity. In order to explore the role of TrkB in spinal instrumental learning, we examined the expression of the TrkB receptor in response to instrumental training (controllable stimulation) and uncontrollable stimulation by Western blot analysis.

TrkB Westerns were performed using the same tissue used to assay BDNF protein levels ($n = 8$). TrkB protein levels are depicted in Figure 3B. Training with controllable shock (Master) increased protein expression of both the truncated form (TrkB 95) and full-length form (TrkB 145) of the TrkB receptor. Independent ANOVAs confirmed that these increases were significant, $F_s > 4.04, p < .05$. Further *post hoc* analyses of the group means revealed that TrkB 95 and TrkB 145 protein expression in master subjects was significantly higher than in yoked and unshocked subjects, $p < .05$.

2.3.2. Experiment 3b: Instrumental training increases TrkB expression in the dorsal horn

Immunohistochemistry was then conducted to examine how training affected the regional distribution of TrkB protein. Four master-yoke pairs, and an equal number of unshocked subjects were used. Master subjects exhibited a mean response duration indicative of learning (49.35 ± 3.73 s), while the yoked subjects failed to learn (mean response duration, 4.57 ± 2.92 s). Analysis of variance revealed a significant difference between these groups, $F_{(1, 6)} = 66.97, p < .05$. Immediately following testing, subjects were prepared for

immunohistochemistry as described above to analyze the regional distribution of TrkB expression. TrkB immunoreactivity was present throughout the lumbar spinal cord. The average number of cells/area ratio of the master and yoked animals was expressed relative to this ratio in the unshocked group, which was normalized to 100%.

As illustrated in Figure 4A, more TrkB positive cells were observed within the superficial dorsal horn of the master rats, relative to the yoked animals and unshocked controls. An ANOVA confirmed that TrkB expression varied across training condition, $F_{(2, 9)} = 7.54$, $p < .05$, and region (dorsal versus ventral), $F_{(1, 9)} = 242.69$, $p < .0001$. Most importantly, the interaction term showed that the impact of training depended upon region, $F_{(2, 9)} = 6.12$, $p < .05$. We also found that stimulation *per se* (collapsed across master and yoked subjects) produced slightly higher levels of expression in the ipsilateral cord (cell/area ratio; ipsilateral = 0.61 ± 0.01 ; contralateral = 0.58 ± 0.01), $F_{(1, 9)} = 6.24$, $p < .05$, but this factor did not interact with either training condition or region, all F s < 2.27 , $p > .05$. *Post hoc* comparisons of the overall means showed that master subjects exhibited higher levels of TrkB expression relative to both the yoked and unshocked groups, $p < .05$. No other differences were significant, $p > .05$. As Figure 4B shows, most of the TrkB labeling (green) was present in neurons (red) confirmed by double labeling with the neuronal marker NeuN.

Together, our cellular assays suggest that: 1) the impact of stimulation on BDNF function is modulated by instrumental control; 2) instrumental control produces an alteration in the TrkB receptor; and 3) these changes are most evident in the spinal dorsal horn.

2.4. Experiment 4: Endogenous BDNF is necessary for the protective effect of instrumental training

In a prior paper (Gomez-Piniella et al., 2007) we explored whether BDNF affects instrumental learning in spinally transected rats. Pretreatment with the BDNF inhibitor TrkB-IgG did not have a significant effect on initial learning. We then tested subjects on the contralateral leg, using a higher response criterion (an 8 mm, rather than 4 mm, electrode depth). As previously reported (Crown et al., 2002b), vehicle treated trained rats were able to learn, a form of positive transfer that suggests instrumental training *enables* subsequent learning. This enabling effect was blocked by pretreatment with TrkB-IgG.

Instrumental training also has a protective effect that blocks the induction of the learning deficit induced by exposure to uncontrollable stimulation (Crown & Grau, 2001). The present experiment examined whether this protective effect depends upon endogenous BDNF. We assessed this using the BDNF inhibitor TrkB-IgG; if BDNF is necessary for the protective effect of instrumental training, then inhibiting BDNF prior to instrumental training should attenuate the protective effect of instrumental training.

Twenty-four hours after complete transection, subjects ($n = 8$) were given a 10 μ L intrathecal injection of either vehicle (PBS + 0.1% BSA) or the BDNF inhibitor TrkB-IgG (0.32 μ g/ μ L), followed by a 20 μ L 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.

Both vehicle and drug-treated rats were able to learn. Mean response durations during training ranged from 37.68 sec $\pm$ 10.72 (vehicle-treated subjects) to 43.37 sec $\pm$ 6.95 (drug-treated subjects). An ANOVA confirmed that all groups exhibited a progressive increase in response duration over time, $F_{(29, 812)} = 14.02$, $p < 0.01$. 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, $F_{(1, 28)} < 1.0$, $p > .05$.

The testing phase is depicted in Figures 5A and 5B. 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). This protective effect was blocked by pretreatment with TrkB-IgG. An ANOVA revealed main effects of Drug, $F_{(1, 56)} = 6.54$, $p < .05$, Training, $F_{(1, 56)} = 6.26$, $p < .05$, and Shock, $F_{(1, 56)} = 47.94$, $p < 0.001$. The Drug X Shock and Training X Shock interactions were also significant, $F_s < 5.50$, $p < .05$. In addition, the ANOVA revealed a main effect of trials, as well as Trials X Shock and Trials X Training X Shock interactions, $F_s > 1.80$, $p < .05$. Finally, a four-way Trials X Drug X Training X Shock interaction approached significance, $F_{(29, 1624)} = 1.46$, $p = 0.056$. 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 significant, $F = 15.027$, $p < 0.001$. 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, $F_s > 4.45$, $p < .05$. *Post hoc* comparisons of group means revealed that all uncontrollably shocked subjects were significantly different from unshocked subjects, except for the vehicle-treated shocked subjects that received prior training, $p < .05$.

2.5. Experiment 5: BDNF given prior to uncontrollable shock blocks the induction of the learning deficit

We found that the BDNF inhibitor TrkB-IgG blocked the protective effect of instrumental training. If instrumental training prevents the induction of the learning deficit because it upregulates BDNF, then direct application of BDNF on to the spinal cord should substitute for instrumental training and prevent the deficit. Twenty-four hrs after complete transection, subjects ($n = 6$) received a 10 μ L intrathecal injection of either vehicle (aCSF + 0.1% BSA) or BDNF (0.04 μ g/ μ L) followed by a 20 μ L 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.

As depicted in Figure 6, vehicle and BDNF treated rats that were not given shock learned the instrumental relationship. Vehicle-treated rats that received uncontrollable shock exhibited a learning deficit, replicating previous findings. In contrast, subjects given BDNF prior to uncontrollable shock were able to learn. An ANOVA showed significant main effects of drug and shock, a Drug X Shock interaction, and a three-way interaction between trials, drug, and shock, $F_s < 16.33$, $p < .05$. *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, $p > .05$.

2.6. Experiment 6: BDNF given prior to uncontrollable shock attenuates shock-induced allodynia

Uncontrollable stimulation also produces a short-term increase in mechanical reactivity (allodynia) (Ferguson et al., 2006). Here we examined whether this effect is blocked by pretreatment with BDNF.

Twenty-four hours after complete transection, subjects ($n=8$) were given a 10 μ L intrathecal injection of either vehicle (aCSF + 0.1% BSA) or BDNF (0.04 μ g/ μ L) followed by a 20 μ L saline flush. Thirty-minutes later, one hindlimb was prepared for shock, and both hind paws

were subsequently assessed for baseline tactile reactivity using von Frey filaments as described above. Subjects then received 6 minutes of either uncontrollable shock or an equivalent period of restraint. Immediately following shock, tactile reactivity was again assessed for all subjects on both the ipsi- and contralateral hindlimbs.

There were no significant differences in baseline tactile thresholds between vehicle- and BDNF-treated groups, $p > .05$. All analyses were subsequently performed on the change between baseline and test. There was no significant difference between ipsi- and contralateral legs in each subject, and therefore all tactile reactivity data were collapsed across leg, $F_{(1, 56)} = 1.25$, $p > .05$. As expected, vehicle-treated subjects that did not receive shock exhibited very little change from baseline. In contrast, vehicle-treated subjects that received uncontrollable shock exhibited a marked decrease in paw withdrawal threshold, replicating prior findings (Ferguson et al., 2006). Interestingly, subjects that were administered BDNF showed no decrease in withdrawal threshold, regardless of whether they received uncontrollable shock (Figure 7). An ANOVA revealed a main effect of drug, $F_{(1, 28)} = 4.69$, $p < .05$, as well as a significant Drug X Shock interaction, $F_{(1, 28)} = 14.74$, $p < 0.01$. *Post hoc* analysis of the group means showed that the vehicle-treated group that received shock was significantly different from all other groups, $p < .05$. No other differences were significant, $p > .05$.

2.7. Experiment 7: Endogenous BDNF is necessary for the therapeutic effect of instrumental training

We have shown that instrumental training prior to uncontrollable stimulation blocks the induction of the learning deficit and that this effect depends on BDNF. Instrumental training can also have a restorative effect that can reverse the learning deficit after it has been induced (Crown & Grau, 2001). Demonstrating this is complicated by the fact that exposure to uncontrollable shock disrupts learning. To examine whether instrumental training can have a therapeutic effect, we need a way to encourage learning in previously shocked rats. This can be accomplished by administering the opiate antagonist naltrexone, which blocks the expression of the deficit and temporarily restores the capacity for learning (Joynes & Grau, 2004a).

Crown and Grau (2001) showed that instrumental training in the presence of naltrexone reverses the learning deficit and enables learning when subjects are tested 24 hours later. Importantly, this pharmacological manipulation does not reverse the deficit; it simply affords a short-lived window in which instrumental training can occur. Naltrexone given after uncontrollable shock (without instrumental training) has no effect on later testing, as these subjects continue to express the learning deficit. Here we examined whether the restorative effect of instrumental training depends on BDNF release. If BDNF is necessary for the therapeutic effect of instrumental training, then inhibiting endogenous BDNF will attenuate this effect.

Thirty rats were used in this experiment ($n = 6$). Twenty-four hours after complete transection, all subjects were given 6 minutes of uncontrollable shock, followed immediately by a 1 μ L intrathecal injection of naltrexone (7 mg/ml). The naltrexone was given to temporarily block the learning deficit and allow instrumental learning. Subjects then received either a 10 μ L injection of TrkB-IgG or vehicle (PBS + 0.1% BSA). Twenty minutes after injection, subjects were given instrumental training or nothing. Twenty-four hours later, all subjects were tested for instrumental learning. A comparison group was also included that was given uncontrollable shock and naltrexone prior to training as usual, but received TrkB-IgG immediately after training rather than before.

TrkB-IgG did not have a significant effect on the acquisition of instrumental learning in the training phase. The mean response duration for vehicle-treated subjects was 22.20 ± 1.92 seconds, and the mean response duration for TrkB-IgG-treated subjects was 12.03 ± 1.33 seconds. An ANOVA confirmed that all groups exhibited a progressive increase in response duration over time, $F_{(29, 290)} = 2.96, p < 0.01$. The main effect of drug treatment was not significant, $F_{(1, 10)} < 1.0, p > .05$. Likewise, the Drug X Time interaction was not significant, $F_{(29, 290)} = 1.12, p > .05$.

The testing phase is depicted in Figure 8A. As expected, subjects that did not receive prior training were unable to learn, and this was true regardless of drug treatment. Subjects that received prior training coupled with vehicle were able to learn, displaying the therapeutic effect of instrumental training. Importantly, those subjects that received prior training coupled with the BDNF inhibitor TrkB-IgG did not exhibit this therapeutic effect. An ANOVA revealed significant main effects of both drug and training, $F_s > 11.72, p < 0.01$. No other terms were statistically significant. There was also a main effect of trials, as well as significant two- and three-way interactions between trials, drug, and training conditions, $F_s < 2.70, p < 0.01$. *Post hoc* comparison of the means revealed that vehicle-treated subjects that received training differed significantly from all other groups, $p < .05$.

Although there were no significant training differences between groups, the group that received TrkB-IgG and naltrexone prior to training did have slightly lower response durations than we normally see. A similar pattern was found in a prior study (Gomez-Pinilla et al., 2007). In the present study, a TrkB-IgG-induced disruption in learning could potentially explain why the drug blocked the restorative effect of instrumental training. To address this possibility, an additional control group was included in which subjects were trained after receiving uncontrollable shock and naltrexone. TrkB-IgG was then administered immediately *following* training (Figure 8B). If the continued action of BDNF after instrumental training is essential to its restorative effect, TrkB-IgG given after training should also eliminate the beneficial effect of instrumental training. As expected, this group learned in the training phase (mean response duration, 30.63 ± 7.76) and did not differ from the other trained groups (mean response duration, 16.63 ± 5.43), $F_{(2,15)} = 1.26, p > .05$. At test, this comparison group (mean response duration, 27.31 ± 11.38) differed significantly from the vehicle pretrained group (mean response duration, 50.02 ± 4.01), $F_{(1,10)} = 5.02, p < .05$. These data confirm that the effect of TrkB-IgG was not due to its effect on training, and that the restorative effect of instrumental training depends on a continued action of BDNF after training.

2.8.1. Experiment 8a: BDNF given immediately after uncontrollable shock blocks the development of the learning deficit

We found that the restorative effect of instrumental training was blocked by pretreatment with the BDNF inhibitor TrkB-IgG. If BDNF plays a critical role, intrathecal application of BDNF should substitute for instrumental training and restore the capacity for learning. Further, if the lasting benefit of instrumental training depends on BDNF, administration of BDNF after uncontrollable stimulation should have a long-term therapeutic effect that is evident 24 hrs after drug treatment.

Twenty-four hours after complete transection, rats ($n = 6$) 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 ($0.04 \mu\text{g}/\mu\text{L}$). All injections were flushed with $20 \mu\text{L}$ saline. Twenty-four hours later, all subjects were tested for instrumental learning.

As expected, those subjects that did not receive uncontrollable shock were able to learn (Figure 9A). 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, $F_s < 15.9$, $p < .05$. 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, $p < .05$. There was also a main effect of trials and a significant Trials X Shock interaction $F_s < 3.50$, $p < .05$. No other comparisons were significant, $p > .05$.

2.8.2. Experiment 8b: BDNF given 24 hours after uncontrollable shock blocks the expression of the learning deficit

We have shown that intrathecal application of BDNF can block the induction of both the learning deficit and allodynia observed after uncontrollable shock. We next examined whether BDNF can block the expression of the deficit when given immediately prior to testing, 24 hrs after subjects have received uncontrollable stimulation.

Spinally transected rats ($n = 6$) were fitted with an intrathecal catheter at the time of surgery. Twenty-four hours after surgery, subjects 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 $\mu\text{g}/\mu\text{L}$) were then administered intrathecally, at a volume of 10 μL . Thirty-minutes after injection, all subjects were tested for instrumental learning.

As expected, unshocked subjects learned the instrumental task (Figure 9B). 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, $F_s < 9.6$, $p < .05$. 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, $p < .05$.

3. DISCUSSION

Stimulation can have divergent effects on spinal cord plasticity depending upon whether it occurs in a controllable, or uncontrollable, manner. Uncontrollable stimulation inhibits the acquisition of selective response modifications, impairs recovery after spinal cord injury, and induces allodynia (Grau et al., 1998; Grau et al., 2004; Ferguson et al., 2006). These effects are not observed if the stimulation is given in a controllable manner. Further, controllable stimulation can prevent, and reverse, the learning deficit observed after uncontrollable shock and counters signs of mechanical allodynia in a model of inflammatory pain (Crown & Grau, 2001; Ferguson et al., 2006; Hook et al., 2008). Based on prior work demonstrating that the neurotrophin BDNF can promote neural plasticity, and the observation that controllable stimulation enhances the expression of BDNF within the spinal cord, we hypothesized that BDNF could contribute to the protective effect of controllable stimulation. The present experiments provide additional biochemical evidence that training affects BDNF function and demonstrate that the neurotrophin impacts spinal plasticity *in vivo*. We have previously shown that controllable stimulation induces and increase in BDNF mRNA expression (Gomez-Pinilla et al., 2007). The current study expands on these findings, illustrating that BDNF mRNA expression after controllable stimulation is spread throughout the dorsal and ventral horns, suggesting the potential for BDNF to elicit adaptive alterations in both sensory and motor function as a function of instrumental training. Western blotting also showed that the expression of BDNF and TrkB protein expression was

enhanced by instrumental training. When applied intrathecally, BDNF was sufficient to prevent, and reverse, the adverse consequences of uncontrollable stimulation (both the learning deficit and allodynia). Further, inhibiting BDNF function using TrkB-IgG blocked the protective/restorative effects of instrumental training, demonstrating that BDNF plays an essential (necessary) role in mediating the beneficial effects of controllable stimulation.

3.1. BDNF and Spinal Plasticity

Much of what is known about BDNF-mediated spinal plasticity has grown out of research on spinal cord injury (Jakeman et al., 1998; Namiki et al., 2000; Jin et al., 2002). Cats receiving complete spinal transections can exhibit stepping behavior if they have been exposed to daily treadmill training (Lovely et al., 1986; Barbeau & Rossignol, 1987). Interestingly, Lemay and colleagues found that BDNF treatment could be substituted for training to produce an increase in locomotor performance that was commensurate with the functional improvements seen in trained subjects (Boyce et al., 2007). Likewise, Gomez-Pinilla et al. (2001) have shown that the beneficial effects of treadmill training are mediated by an upregulation of endogenous BDNF. They posited that it is the capacity for BDNF to regulate synaptic strength that is essential for locomotor recovery after spinal cord injury (Gomez-Pinilla et al., 2001; Ying et al., 2005). Studies examining spinally-mediated alteration in respiratory function have yielded additional evidence that BDNF promotes adaptive plasticity. Mitchell and colleagues found that phrenic long-term facilitation, a form of synaptic plasticity that is normally induced by intermittent hypoxia, can be elicited by BDNF alone, and is abolished when BDNF activity is inhibited (Baker-Herman et al., 2004). This BDNF-mediated facilitation of phrenic motor function has recently been shown to be an effective therapeutic avenue following cervical spinal cord injury (Vinit et al., 2009). The current study extends these earlier observations and helps to define the circumstances under which BDNF impacts spinal plasticity.

3.2 Possible Mechanisms of Action

BDNF has been shown to play a critical role in a number of forms of plasticity (Kang & Schumann, 1995; Patterson et al., 1996; Mizuno et al., 2000). Acute episodes of learning and memory in both the brain and spinal cord routinely induce rapid alterations in BDNF expression. In response to a conditioned taste aversion task, BDNF protein expression was significantly increased in the amygdala and insular cortex between 4 and 8 hours after completion of the task (Ma et al., 2011). In the spinal cord, plasticity induced by intermittent hypoxia has been shown to elicit significant increases in BDNF protein 60 min after training (Baker-Herman et al., 2004). Moreover, within minutes of activity-dependent release of BDNF from presynaptic terminals, a similarly rapid activation of TrkB receptors can occur (Aloyz et al. 1999). Considering our finding that BDNF-mediated spinal learning effects occurred quickly, these behavioral changes may reflect a local dendritic cleavage of the pro-form of BDNF into the mature form. This mechanism, mediated by the tissue plasminogen activator tPA, can occur quickly in response to neural activity, and has been previously implicated in underlying early BDNF-induced plasticity (Waterhouse & Xu, 2009). Therefore, although BDNF mRNA changes were seen throughout both the dorsal and ventral spinal cord, it is possible that the rapid changes in TrkB protein expression are in response to local BDNF cleavage, rather than BDNF synthesis.

The downstream effects of BDNF can vary widely depending upon the neurochemical/neurophysiological context. BDNF has been predominantly found to enhance glutamatergic transmission through an NMDA-receptor (NMDAR) mediated process (Levine, et al., 1998; Arvanian & Mendell, 2001; Garraway et al., 2003). We have previously shown that spinal learning is dependent on NMDAR activity, and have recently suggested that modulation of NMDAR function through an mGluR/PKC pathway mediates the inhibition of spinal

learning (Joynes et al., 2004b; Ferguson et al., 2008). As BDNF has been shown to regulate glutamatergic transmission by inducing alterations in NMDAR function, the restoration of adaptive spinal plasticity by BDNF may reflect a NMDAR-mediated compensatory mechanism that promotes future learning (Crozier et al., 2008; Gottmann et al., 2009).

To a lesser extent, BDNF has also been shown to modulate inhibitory signaling (Schinder et al., 2000; Wardle & Poo, 2003). The dual nature of BDNF's effects can be seen in the nociceptive literature (Merighi et al., 2008). While there is evidence that BDNF can enhance neuropathic pain, others have shown that BDNF treatment can reverse thermal hypersensitivity after a neuropathic pain state has been induced (Lever et al., 2003). It is interesting to note that, although we found an upregulation of TrkB activity in the superficial dorsal horn following controllable stimulation, BDNF treatment did not enhance nociceptive reactivity, and instead produced a marked attenuation of shock-induced mechanical allodynia. Elsewhere, we have suggested that uncontrollable stimulation enhances mechanical reactivity and interferes with spinal instrumental learning because it may produce a form of nociceptive plasticity that is akin to central sensitization (Ferguson et al., 2006; Grau, et al., 2006). Prior work has shown that alterations within the dorsal horn play a key role in the induction and maintenance of this effect (Woolf & Thompson, 1991). For BDNF to have a protective/restorative effect, it must counter these changes, and for this reason, the most robust alterations may occur within the dorsal horn. From this perspective, the increase in TrkB activity found in the dorsal horn is expected.

BDNF activity in the ventral horn is also important for spinal instrumental learning. We have previously shown that endogenous BDNF activity in the ventral horn is necessary in order for spinal instrumental training to confer a lasting facilitation of spinal learning (Gomez-Pinilla et al., 2007). Likewise, a number of other models of spinal plasticity have shown ventral BDNF mRNA to be elevated, particularly in response to locomotor training (Gomez-Pinilla et al., 2001; Macias et al., 2007). Likewise, as in previous studies of spinal plasticity involving locomotor circuits, we saw changes in ventral BDNF mRNA expression, but no significant increase after training in TrkB activity in the large motoneurons of the ventral horn (Macias et al., 2007).

That we saw BDNF mRNA changes in both the dorsal and ventral horns suggests that there may be a dual role for BDNF in promoting adaptive plasticity in this learning paradigm. While activity in the dorsal horn may protect against the nociceptive processes elicited by uncontrollable shock, ventral BDNF activity may modulate motor function, resulting in adaptations in the target response and ultimately improved spinal learning. Given that we saw no changes in TrkB expression in the ventral horn, these separate processes may occur on different timecourses, or reflect distinct neurotrophin/receptor dynamics.

Evidence suggests that BDNF cannot be narrowly defined as exclusively promoting excitatory or inhibitory action, but instead exerts an activity-dependent regulatory role that *normalizes* neurotransmission (Desai et al., 1999; Schinder et al., 2000). This interpretation is congruent with the role of BDNF in synaptic scaling, in which BDNF has been shown to play an essential role in the stabilization of excitatory cortical synapses following neural activity (Rutherford et al., 1998; Turrigiano, 2008). Recently, BDNF has been found to regulate activity-dependent scaling of inhibitory synapses as well (Swanwick et al., 2006). This neuroregulatory role can be seen behaviorally, as BDNF has been shown to normalize glutamatergic neurotransmission between the prefrontal cortex and nucleus accumbens following cocaine exposure, which in turn prevented further cocaine seeking (Berglind et al., 2009). In a similar manner, BDNF may be working in the spinal cord to counteract the barrage of excitatory input elicited by uncontrollable shock.

BDNF pretreatment has also been shown to provide protection against glutamatergic excitotoxicity by stabilizing calcium activity (Cheng & Mattson, 1994). Acting through the TrkB receptor, BDNF is known to increase the expression of the intracellular calcium binding protein calbindin, promoting calcium homeostasis following prolonged excitation (Ip et al., 1993; Fiumelli et al., 2000). Alternatively, BDNF has been shown to act at presynaptic terminals to increase the probability of GABA release, and this effect has been suggested to indirectly suppress overexcitation through GABAergic signaling (Cheng & Mattson, 1994; Pezet et al., 2002; Malcangio & Lessman, 2003). By normalizing neurotransmission, and by re-establishing a basal state that opposes the excitatory effects of uncontrollable stimulation, BDNF may promote a neural environment that is permissive for adaptive spinal plasticity. More generally, BDNF's action may best be conceived as having a homeostatic function that promotes selective neural adaptations by maintaining the neural network within an optimal operational range.

3.3 Conclusions

Research over the last two decades has suggested a new view of spinal function and shown that spinal mechanisms are not immutable, but instead modifiable by both environmental stimulation and brain-dependent processes. In response to environmental contingencies, brain mechanisms can induce lasting alterations in spinal reflexes (Wolpaw & Lee, 1989). Likewise, environmental input can induce a lasting change in spinal function. The most studied example is inflammation-induced central sensitization, an NMDAR-mediated memory-like effect (Woolf, 1984; Woolf & Thompson, 1991). Our work shows that NMDAR-dependent plasticity within the spinal cord is regulated by factors known to impact learning and memory, such as stimulus-stimulus (Pavlovian), and response-outcome (instrumental) relations (Grau et al., 1998; Joynes et al., 2004b). More recently, we have shown that spinal mechanisms are also sensitive to whether stimuli occur in a regular (predictable) or irregular (unpredictable) manner and that these two forms of stimulation have divergent effects; unpredictable shock inhibits instrumental learning and induces allodynia whereas shock applied at regular intervals engages a protective/restorative effect that promotes adaptive plasticity, and counters allodynia, through a BDNF-dependent process (Baumbauer et al., 2008; Baumbauer et al., 2009).

By linking these biological processes to lasting changes in behavior, we gain an attractive model of spinal plasticity with considerable clinical relevance. Behavioral training can promote locomotor function and the locomotor system is sensitive to instrumental relations (Barbeau & Rossignol, 1987; Edgerton et al., 1997; Hook & Grau, 2007). Interestingly, locomotor training enhances both BDNF function and instrumental learning (relative to stand trained subjects [Bigbee et al., 2007]). Finally, both training/exercise and BDNF can promote recovery after spinal cord injury (Gomez-Pinilla et al., 2001; Boyce et al. 2007). Our work suggests that, for behavioral training to have a beneficial effect, stimulation should be applied in a controllable (and/or predictable) manner and that this type of training benefits plasticity because it promotes BDNF/TrkB function.

Highlights

- We assess the role of BDNF in a model of spinal plasticity
- We show that controllable stimulation upregulates BDNF and TrkB expression
- We find that BDNF is necessary and sufficient for adaptive spinal plasticity

- We show that BDNF provides protection/therapy against a spinal learning deficit

Abbreviations

ANOVA	analysis of variance
BDNF	brain-derived neurotrophic factor
BSA	bovine serum albumin
LTP	long-term potentiation
PBS	phosphate-buffered saline
PFA	paraformaldehyde
SDH	superficial dorsal horn
T2	second thoracic vertebra
TBS	Trisbuffered saline
TrkB	tropomyosin-related kinase B
VH	ventral horn

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Cellular Assays (Exps. 1-3b)

Protection (Exps. 4-6)

Therapy (Exps. 7-8b)

Figure 1.

Summary of the experimental designs. To investigate the role of BDNF in the adaptive effect of controllable stimulation, experiments were broken into three categories: Cellular Assays, Protection, and Therapy. First, the differential expression of BDNF mRNA and protein following controllable and uncontrollable stimulation were assessed by *in situ* hybridization and Western blot respectively (Experiments 1 & 2), followed by assessment of the BDNF receptor TrkB by Western blot and immunohistochemistry (Experiments 3a & 3b). Second, the necessity for endogenous BDNF in the protective effect of controllable stimulation was tested (Experiment 4), followed by the sufficiency for exogenous BDNF to provide protection against uncontrollable stimulation (Experiments 5 & 6). Finally, the necessity for endogenous BDNF in the therapeutic effect of controllable stimulation was assessed (Experiment 7) followed by the sufficiency for exogenous BDNF to block the development and expression of the deficit induced by uncontrollable stimulation (Experiments 8a & 8b).

A BDNF *in situ* hybridization

Figure 2.

Controllable shock increases BDNF mRNA and protein expression. Pseudocolor images of BDNF cRNA hybridization in coronal sections through the L4 spinal cords of representative subjects for controllable shock (*Master*), uncontrollable shock (*Yoked*) and unshocked conditions. The scale bar indicates $\mu\text{Ci/g}$ protein with highest hybridization levels depicted in red. Note BDNF cRNA hybridization is largely restricted to the central gray (dorsal and ventral horn) and that controllable shock selectively increases BDNF mRNA abundance relative to uncontrollably shocked and unshocked groups. Analysis of BDNF mRNA following *in situ* hybridization indicated a significant elevation in both the dorsal and ventral horns of master relative to yoked and unshocked groups. Hybridization values from dorsal and ventral horn for each subject were normalized to those obtained from the measurements taken from white matter of that animal (i.e., gracile fasciculus), an area where BDNF mRNA is not specially expressed. Mean hybridization data for each subject (presented as a % of the nonspecific hybridization) for each rat within each group were averaged to obtain a group mean $\pm$ SEM and these group means were used as the measure of regional BDNF mRNA. See section 1.6 for statistical analyses. Scale bar represents 1 mm.

Figure 3.

Controllable shock increases protein expression of BDNF and TrkB. **(A)** Western blot analysis of BDNF protein. Controllable shock increases BDNF protein levels. Expression of spinal BDNF was assessed in subjects that received either controllable shock (*Master*), uncontrollable shock (*Yoked*), or unshocked. A matched *t*-test that compared each master/yoked pair revealed significantly higher levels of BDNF protein in master subjects (* $p < .05$). **(B)** Detection of truncated (TrkB 95) and full-length (TrkB 145) receptor protein by Western blot. Expression of the truncated and full length TrkB receptor was assessed in subjects that received either controllable shock (*Master*), uncontrollable shock (*Yoked*), or unshocked. Master subjects showed a significant increase in both receptor types in relation to yoked and unshocked controls (* $p < .05$, ** $p < .01$).

Figure 4.

Controllable shock increases neuronal TrkB expression. **(A)** Light immunohistochemistry shows increased TrkB immunolabeling in the dorsal horn of Master subjects (c; controllable shock) compared to both Yoked (b) and unshocked controls (a). Arrows indicate representative TrkB-labeled cells. Although TrkB was also present in the ventral horn of all groups, there was no difference in its expression. Right panel, immunohistochemical analysis of TrkB protein indicates a significant increase in TrkB expression in controllably shocked (Master) subjects, localized to the superficial dorsal horn (R= right, L= Left; SDH= superficial dorsal horn, VH= ventral horn, * $p < .05$). **(B)** BDNF receptor TrkB localization in controllably shocked subjects. Double immunolabeling with NeuN (a,b,c; red) and TrkB

antibodies (d,e,f; green) in the superficial dorsal horn (SDH), deeper dorsal horn area (DDH) and the ventral horn (VH) respectively, revealed that most TrkB expression was localized to neurons (g,h,i;merged yellow). To further illustrate TrkB/NeuN colocalization, the areas indicated by the white rectangles in the third column are enlarged in j, k, and l. Lower panel, representative diagram of spinal areas assessed. Shaded areas indicate subsets of dorsal and ventral horn in which cells were counted.

Figure 5.

BDNF inhibition with TrkB-IgG disrupts the protective effect of instrumental training. Left panels depict response duration over time, right panels show the mean response duration per group. **(A)** Testing session for vehicle-treated subjects. Subjects that received uncontrollable shock alone had significantly lower response durations when tested, indicating a shock-induced deficit, $*p < .05$. Subjects that received training prior to uncontrollable shock were able to learn at test, showing that prior training protects against the shock-induced deficit. **(B)** Testing session for TrkB-IgG-treated subjects. Subjects that received uncontrollable shock alone had significantly lower response durations than those that did not receive uncontrollable shock, $*p < .05$. In the presence of the BDNF inhibitor TrkB-IgG, prior training was unable to protect against the deficit induced by uncontrollable shock.

Figure 6.

Exogenous BDNF protects against the induction of the behavioral deficit. Left panel shows response duration over time, right panel shows mean response duration per group. Vehicle treated subjects that received uncontrollable shock had significantly lower response durations than all other groups, $*p < .05$. Treatment with exogenous BDNF prior to uncontrollable shock protected against the deficit, allowing subjects to learn when tested.

Figure 7.

BDNF protects against uncontrollable shock-induced tactile allodynia. Bars indicate positive (less responsive) or negative (more responsive) change in tactile reactivity threshold from baseline. Vehicle-treated subjects that received uncontrollable shock were more responsive to tactile stimulation (allodynic), as evidenced by change from baseline. In contrast, BDNF-treated subjects that received uncontrollable shock showed an attenuation of allodynia. $*p < .05$.

Figure 8.

BDNF inhibition with TrkB-IgG attenuates the therapeutic effect of training. Left panel shows response duration over time, right panel shows mean response duration per group. **(A)** Testing session. In vehicle-treated subjects, training after controllable stimulation reversed the deficit, allowing for learning when tested. In TrkB-IgG treated subjects, instrumental training did not reverse the deficit, $*p < .05$. **(B)** To rule out the possibility that TrkB-IgG blocked the therapeutic effect of training because it disrupted instrumental performance during behavioral therapy, a comparison group was included in which TrkB-IgG was given after training had occurred. When tested the next day, the response durations for this group were significantly lower than the vehicle-treated group, indicating that the inhibition of TrkB function after training blocked its therapeutic effect, $*p < .05$.

Figure 9.

(A) BDNF administration prevents the development of the behavioral deficit. Left panel shows response duration over time, right panel shows mean response duration per group. Subjects that received BDNF following uncontrollable shock had significantly higher response durations than vehicle-treated shocked controls, $*p < .05$. (B) BDNF administration prevents the expression of the behavioral deficit. Left panel shows response duration over time, right panel shows mean response duration per group. Subjects that received BDNF 24 hours after uncontrollable shock performed as well as unshocked controls, exhibiting significantly higher response durations than shocked controls, $*p < .05$.