HHS Public Access

Author manuscript

IEEE Trans Neural Syst Rehabil Eng. Author manuscript; available in PMC 2021 November 06.

Published in final edited form as:

IEEE Trans Neural Syst Rehabil Eng. 2020 November ; 28(11): 2459–2467. doi:10.1109/TNSRE.2020.3027393.

Intrathecal Delivery of BDNF into the Lumbar Cistern Re-Engages Locomotor Stepping after Spinal Cord Injury

Francesca Marchionne,

Bioengineering Department, Temple University, Philadelphia, PA 19122 USA. She is now with iMotions Inc, Boston, MA 02111 USA

Alexander J. Krupka,

Bioengineering Department, Temple University, Philadelphia, PA 19122 USA. He is now with the Department of Natural Science, DeSales University, Center Valley, PA 18034 USA

George M. Smith,

Shriners Hospitals Pediatric Research Center, Temple University, Philadelphia, PA 19140 USA

Michel A. Lemay

Bioengineering Department, Temple University, Philadelphia, PA 19122 USA

Abstract

Delivery of neurotrophins to the spinal injury site via cellular transplants or viral vectors administration has been shown to promote recovery of locomotion in the absence of locomotor training in adult spinalized animals. These delivery methods involved risks of secondary injury to the cord and do not allow for precise and controlled dosing making them unsuitable for clinical applications. The present study was aimed at evaluating the locomotor recovery efficacy and safety of the neurotrophin BDNF delivered intrathecally to the lumbar locomotor centers using an implantable and programmable infusion mini-pump. Results showed that BDNF treated spinal cats recovered weight-bearing plantar stepping at all velocities tested (0.3-0.8 m/s). Spinal cats treated with saline did not recover stepping ability, especially at higher velocities, and dragged their hind paws on the treadmill. Histological evaluation showed minimal catheter associated trauma and tissue inflammation, underlining that intrathecal delivery by an implantable/programmable pump is a safe and effective method for delivery of a controlled BDNF dosage; it poses minimal risks to the cord and is clinically translational.

Keywords

animal model; intrathecal; locomotion; neurotrophin; spinal cord injury

I. INTRODUCTION

SPINAL cord injury (SCI) leads to a loss of motor and sensory functions below the level of the lesion. Nonetheless, the intrinsic circuitry below the injury, in particular the locomotor circuitry, remains functional after complete isolation [4]. This is evidenced by the possibility of re-engaging the lumbar locomotor circuits through treadmill training to produce functional stepping in animal models of SCI [5]–[8]. Rehabilitative strategies using body weight supported training (BWST) are used clinically to improve stepping in spinal cord injured individuals. However, significant improvements have been observed only in patients with incomplete deficits (American Spinal Injury Association (ASIA) C or D) [9]–[11]. Therefore, there is a need to identify different approaches to restore the functionality of the spinal circuitry. Neurotrophic factors, such as brain-derived neurotrophic factors (BDNF) and neurotrophin-3 (NT-3), are potential locomotion promoters as they are upregulated by intense exercise in both the spinal cord and hindlimb muscles [12] and associated with locomotor recovery [13]. We use delivery of neurotrophins to the spinal injury site to promote locomotor recovery comparable that observed with treadmill training, or supplement the recovery obtained with training [3]. Transplantation of fibroblasts engineered to express BDNF and/or NT-3 at the injury site of adult spinal cats can independently promote recovery of stepping in untrained animals, and the recovery is maintained up to 12 weeks after grafting [3], [14], [15].

Chronic intrathecal infusion of BDNF to the injury site through implantable osmotic pump was investigated as an axonal regeneration approach and found to enhance locomotor behavior [13]. Intrathecal delivery minimizes the risks of damage to the spinal cord as the associated surgery does not involve debriding scar tissue to create a pocket for the graft, or injections into the cord. The present work extends on the previous studies with intrathecal delivery by introducing the use of a programmable and implantable mini-pump for delivery of BDNF to the lumbar spinal cord, instead of at the injury site, in cats with complete thoracic SCI. Our results show that chronic intrathecal BDNF delivery promotes recovery of stepping ability in untrained spinalized cats as effectively as locomotor training and/or neurotrophin delivery at the injury site without causing additional damage to the cord. This delivery method is already largely accepted in clinical practice [16]–[18], overcomes the drawbacks associated with our previously used delivery methods, and is potentially clinically translational.

II. METHODS

A. Experimental Design

Fourteen adult female domestic shorthair cats (Liberty Research Inc. Waverly, NY) were used for this study (weight: 2.2–3.6 Kg). All animal care and procedures were performed according to the National Institute of Health guidelines, as well as to the USDA regulations for the use of felines in research and were approved by the Institutional Animal Care and Use Committee of Temple University (protocol 4272, initial approval 03/04/2016).

Animals were acclimated to walk on a motorized treadmill at a range of velocities between 0.3 and 0.8 m/s for up to 20 minutes/day for 3–8 weeks prior to spinal transection. Once

acclimated, locomotor kinematics were collected, and the animals were spinalized. In eight cats, a 50 ng/day dose of BDNF was delivered intrathecally to the lumbar spinal cord for 35 days post-transection (post-Tx) through a programmable mini-pump implanted subdermally. The remaining six animals underwent spinal transection and catheter/pump implant as well, but the pump's reservoir was filled with 0.9% NaCl and those animals served as controls. Kinematic evaluation was conducted before, 3 and 5 weeks after injury/pump implant. Time points post-injury were chosen based on previous results with cellular delivery of neurotrophins into the transection cavity [14]. In six animals, four BDNF treated and two controls, Ground Reaction Forces (GRFs) were measured 5 weeks post-Tx during locomotion trials at 0.4 m/s to assess the animals' weight support ability. Animals were euthanized 5 weeks after injury and histological analysis was conducted.

B. Surgical Procedure: Spinal Transection and Pump Implant

Both spinal transection and pump implant took place during the same surgery, performed under aseptic conditions. Anesthesia was induced/maintained using isoflurane inhalation, and a midline incision was made between the T9 and L1 level. Muscles were detached from the dorsal aspect of the spinal column using a surgical spatula. Rongeurs were used to make a narrow laminectomy at the junction of the T11-T12 vertebrae. The dura was carefully opened and the spinal cord was severed between the T11-T12 roots with microscissor, leaving a 3-5 mm gap in the cord. The dura was sutured closed, then the muscles and finally the skin incision.

A second incision was then made between the L7 and S2 spinal segments. A catheter (32G, ReCathCo 220EO) was implanted within the lumbar cistern through the dura mater at the L7/S1 junction. The cannula was inserted subdurally and run rostrally until reaching approximately the L3 spinal segment level in two cats (BDNF-L3), while it was pushed until the L7 level in the other six cats (BDNF-L7). The catheter was then connected to a mini-pump (iPrecio SMP-200, MISZU, Tokyo) implanted subdermally. The pump was programmed to deliver a constant flow rate of 2 $\mu\text{L}/\text{h}$. The pump was filled with BDNF (Catalog#: H00000627-P02, Abnova) diluted into 0.9% NaCl saline at a concentration of 1 $\mu\text{g}/\text{mL}$, thus delivering 50 ng/day of BDNF to the treated animal. Control animals underwent spinal transection and pump implant as well, but the pump was filled with 0.9% NaCl. The catheter was run subdurally to the L3 spinal segment in three of those animals and to the L7 spinal segment in the remaining three. The pump was refilled approximately every 15 days after implantation via percutaneous access to the pump's refill port.

C. Locomotor Training and Kinematic Data Collection

Cats were acclimated to a motorized treadmill through 20 minutes/day sessions of quadrupedal walking at a range of speed between 0.3 and 0.8 m/s prior to spinalization. The treadmill was enclosed in a Plexiglass frame to minimize lateral movements of the animals during training, and food rewards were given to entice the animal to walk forward. After transection, the forelimbs were kept stationary on a Plexiglas platform elevated 1 cm above the treadmill and a Plexiglas divider was placed between the hindlimbs to avoid scissoring of the limbs during bipedal hindlimb locomotion.

Locomotion was recorded using the Vicon motion capture system (Vicon Motion System Ltd., Oxford, UK). Three infrared cameras collected the positions of 11 reflective markers placed on the cat skin at the level of the ischium, femoral head, knee joint, lateral malleolus, metatarsophalangeal joint and the tip of the digits on the right hindlimb as well as on the humeral head, elbow joint lateral epicondyle, metacarpophalangeal joint and the tip of the digits on the right forelimb. Fig. 1 shows the location of the reflective markers. After injury, the experimenter (MAL) held the cat at the base of the tail to provide balance support during locomotor sessions and perineal stimulation was delivered to maximize locomotor output in each of the animals [3], [14], [15]. Only perineal stimulation sufficient to initiate/maintain stepping was applied, as excessive stimulation can interrupt stepping or cause aberrant patterns. Actual treadmill recording time was approximately 1 minute at each speed, a time duration that has shown no training effects in previous studies in our laboratory [3]. Animal's gait kinematics were evaluated, and the animal was considered able to step when capable of executing 10 consecutive plantar weight bearing steps after injury at the speed tested, and all the speeds below.

D. Kinematic Analysis

Horizontal and vertical components of each joint marker were exported and processed using custom Matlab (Mathworks, Natick, MA) scripts. The slippage of the knee and elbow markers was corrected by triangulating their positions from the neighboring markers and bone segment lengths, obtained post-mortem [19]. Quantitative analysis of the stepping parameters was conducted with IgorPro (Wavemetrics, Lake Oswego, OR) and then exported to SPSS (SPSS Inc., Chicago, IL) for statistical evaluation.

Marker locations were used to calculate a number of kinematic parameters, which included: stance length, swing height, Da (horizontal displacement of the foot relative to hip at toe-down), Dp (horizontal displacement of the foot relative to hip at liftoff), and hindlimb joint angles excursion. Values of stance length, swing height, Da, Dp were normalized to pre-transection values.

Kinematic parameters were exported to SPSS (IBM Corp., Armonk NY) for statistical analysis. The average values of each parameter per trial (i.e. about 60 sec of walking) was calculated in order to reduce the number of data points. To evaluate the effects of BDNF delivery on the amount of locomotor recovery, linear mixed models with the kinematic parameters (stance length, swing height, etc.) as the dependent variable and speed (0.4 & 0.8 m/s) and time post-implant (3 & 5 wks) as repeated measure factors were used to evaluate the effects of those factors on the kinematic parameters. The estimated marginal means (EMMs) of the dependent variables were then compared to 100% (i.e. pre-transection level). Linear mixed models were also used to evaluate the effects of time (before, 3 and 5 weeks after injury; time as a repeated measure factor) on joints angles for the BDNF group. Post-hoc pairwise comparisons with Bonferroni correction between the means at the different times were then conducted.

E. Ground Reaction Forces Data Collection and Analysis

Ground reaction forces (GRFs) were collected in six cats (two Controls and four BDNFs) during locomotion trials 5 weeks post-transection using an instrumented split belt treadmill where each belt motor assembly (left and right) was mounted on a Kistler force plate (Kistler Instrument Corp., Winterhur, SUI) [20]. The vertical components of the GRFs (Fz) were evaluated during locomotion at 0.4 m/s to estimate the animals' ability to weight support. Stepping during locomotion trials was initiated, and when necessary maintained, via perineal stimulation. Forces applied to the tail were minimized during those trials and the animal was allowed to drop as low as possible while maintaining the locomotor cycle. The vertical force components under the right and left hind paws were evaluated. Data were collected at 960 Hz and processed in Matlab (The Mathworks, Natick, MA) using customized scripts.

Values of the vertical component of the force (Fz) in Volts were first converted into Newton using the manufacturer's conversion factor (5 mV/N). A Butterworth filter with cut-off frequency of 10 Hz was applied to remove vibration associated noise from the locomotion data. The peak vertical forces were evaluated and averaged for each trial and then normalized for the cats' weight at the time of the recording session. For the BDNF cats, we set a threshold of 2 N/kg, in order to keep only valid peaks not biased by the animal's position being re-adjusted on the treadmill. The value of the threshold was lowered to 1 N/kg for Control cats, since this group showed only few or no force peaks higher than 2 N/kg. A linear mixed model with average peak vertical force per trial as the dependent variable and group (BDNF or Control), side (left or right) and the group*side interaction as factors (with side and trial as repeated measure variables and cat as the grouping variable) was used to evaluate the effects of group on the peak vertical GRF. According to results on able-bodied cats showing that the hindlimbs support about 40% of the body weight during locomotion [21], [22], the vertical force necessary for full weight support is about 4 N/kg (normalized to the animal's mass) [20].

F. Tissue Preparation and Histological Procedures

At the conclusion of the experiment, animals were euthanized and intracardially perfused with 0.9% cold saline flush followed by cold 4% paraformaldehyde in phosphate buffer. After fixation of the tissue, the thoracic and lumbar spinal cord were carefully retrieved and post-fixed in 4% paraformaldehyde. After 2-3 weeks, the tissue was then cryoprotected in 30% sucrose solution for at least one week. The spinal cord was then divided into small segments, usually < 1 cm, embedded in M-1 matrix medium, and frozen at -80°C . Frozen tissues were cut into 40 μm thick horizontal sections on a cryostat, mounted on charged glass slides and kept at -20°C until immunohistochemistry and staining were performed.

To investigate the potential trauma caused by catheter insertion, we stained for Glial Fibrillary Acidic Protein (GFAP). Staining was performed on eight animals: five BDNF (two with L3 delivery and three with L7 delivery) and three Control (two with L3 delivery and one with L7 delivery) on horizontal sections taken from the catheter entry zone's segment (L7/S1) to the segment where the tip of the catheter was found post-mortem. Since the glial scar formed at the lesion site after SCI is populated with reactive astrocytes which undergo

morphological changes and increase synthesis of GFAP, we stained the transection site of four cats (three BDNF and one Control) to serve as reference.

Horizontal sections were left at room temperature for 10-15 minutes and tissue was surrounded with hydrophobic pen. Sections were rehydrated with three 5-minutes rinses in PBS and blocked with 5% NGS in dilution buffer for one hour at room temperature. The dilution buffer included Tris-HCl pH 7.2 (25 mM), NaCl (300 mM), 0.3% Triton X-100, BSA (0.5 mg/ml) and 0.01% Thimerosal diluted in 100 ml PBS. Tissues were then incubated in primary antibody in buffer solution overnight at 4° C (1:500, Polyclonal rabbit antibody Anti-GFAP, Dako). The day after, sections were rinsed with one 2-minute wash, followed by four 5-minutes washes in PBS and incubated in secondary antibody diluted in buffer solution for two hours at room temperature in the dark (1:200, Alexa Fluor 590 conjugated AffiniPure Goat Anti-rabbit IgG (H+L)). Sections were rinsed as previously described and coverslipped with a glycerol-based liquid mounting medium (ProLong Diamond Antifade Mountant, ThermoFisher Scientific). Mounted slides were observed under fluorescence microscope and compared with stained slides from the transection area.

Lesion completeness was visually confirmed in all cats and histologically verified using Nissl-myelin staining in a subset of animals. The Luxol Fast Blue Stain (Myelin Stain) from Abcam was used to identify the basic neuronal structure in the spinal cord. Staining was performed on five cats and the chosen sections were distributed over different depth levels of the spinal cord in order to evaluate lesion completeness over the entire thickness of the cord.

III. RESULTS

A. Overall Kinematic Observations

Fig. 2 illustrates the kinematics of the right hindlimb for one exemplar step during locomotion before and after injury for one animal in each group. BDNF treated animals recovered plantar weight-bearing stepping, while saline treated animals stepped mostly on the dorsal aspect of the paw. Five BDNF cats were capable of executing 10 consecutive plantar weight-bearing steps at 0.4 m/s as early as 3 weeks post-Tx; and among them, three had recovered stepping abilities for the full range of velocities tested (0.3-0.8 m/s) by that time. By 5 weeks, five could step at all the velocities tested, one could step at up to 0.5 m/s and two did not step at any walking speed. The pump's reservoir leaked subdermally in one of those two animals, leading to pump encapsulation. In the second cat, histology analysis did not show BDNF accumulation meaningfully higher than background level within the cord's tissue, suggesting that either the delivered protein was not active or did not infuse intrathecally.

Although two Control cats could perform some stepping at slow velocities (0.3-0.4 m/s), all but one never executed 10 consecutive weight-bearing plantar steps at any of the speeds tested and showed mostly dorsal foot placement during stance. Overall, the proportion of animals who recovered stepping ability up to at least 0.4 m/s at 5 weeks was significantly higher in BDNF treated animal (6/7 (excluding leaking pump case) *versus* 1/6 in Control (Fischer's Exact Test, $p = .016$).

Lesion completeness was confirmed by Nissl-myelin stain of the transection site in the one Control cat that could step so the recovery can not be attributed to a partial injury. Despite recent claims of spontaneous locomotor recovery in spinal cats [23], we [3], [14], and others [2], [7], [8], have never observed such a level of spontaneous recovery in untrained spinalized cats, and the animal was considered an outlier and excluded from further analysis.

B. Kinematic Parameters

Kinematic parameters were evaluated in the six BDNF treated cats that could step at up to 0.5 m/s by 5 weeks post-Tx via the use of linear mixed models. In BDNF treated cats, time post-Tx had a significant effect on stance length ($p = .002$), swing height ($p = .015$), Da ($p = .002$) and Dp ($p = .049$) while speed and the speed*time interaction term had no effect ($p > .05$, range .094-.7390). BDNF treated cats showed post-Tx values of stance length and swing height comparable to pre-transection levels. EMMs of the stance length and swing height indices for the BDNF cats are shown in Figs. 3A and B for the 3 and 5 weeks post-injury time points while walking at medium (0.4 m/s) or maximum velocity (0.8 m/s). Controls were incapable of reaching the minimum of 10 consecutive plantar steps and, thus, statistical evaluation was not performed on this group. In contrast, BDNF treated animals recovered $75 \pm 18\%$ (mean $\pm 95\%$ CI (confidence interval)) of their stance length when walking at medium speed (0.4 m/s) 5 weeks post-Tx. Similarly, they recovered $78 \pm 18\%$ of pre-transection values at the maximum walking speed tested (0.8 m/s), but in both cases the values were significantly lower than the pre-transection values (95% CI of the EMMs did not include 100%). Swing height index post-Tx was $88 \pm 18\%$ of pre-transection value while stepping at 0.4 m/s and $73 \pm 20\%$ of pre-transection value while stepping at 0.8 m/s. Animals recovered swing height to pre-transection value when walking at 0.4 m/s, but they showed a statistically lower swing height when walking at 0.8 m/s (EMM 95% CI did not include 100%). When compared to previous studies with treadmill training or cellular delivery at the transection site [1–3, 14], our results suggest that intrathecal delivery of BDNF to the lumbar spinal cord is as efficacious as training or BDNF cellular delivery at the injury site at promoting the recovery of locomotor behavior in untrained spinalized cats.

Data for Da and Dp indices are shown in Figs. 3C and D. All BDNF treated cats showed decreased Da and increased Dp 3 weeks post-injury. As noted by Belanger et al. in treadmill trained animals [1], these changes suggest that the paw tended to be placed more posterior to the hip as well as not go as far forward after SCI. Da remained significantly lower even at 5 weeks post-Tx, while Dp decreased over time but was never significantly higher than the pre-transection values (EMMs 95% CI always included 100%).

Stepping parameters (stance length, swing height, Da, Dp) were, thus, reduced following injury, but recovered with BDNF treatment to values comparable to the ones obtained with treadmill training [1], [2] or cellular delivery [3].

C. Hindlimb Joint Angles

Post-Tx joint angles were compared to pre-transection values to assess recovery in BDNF treated animals. Pre-, 3 and 5 weeks post-Tx joint angles for the hip, knee and ankle joints for walking speed of 0.4 m/s are shown in Fig. 4. Linear mixed models with time (pre-, 3

and 5 weeks post-Tx) as repeated factor, cat as grouping variable, and angle parameter as the dependent variable indicated a significant effect of time (p between .000-.03) on every joint angle parameters but the ankle angle minimum value ($p = .292$). Post-hoc pairwise comparisons between joint angles with Bonferroni correction showed a statistically significant reduction of the hip and knee joint range, maximal and minimal angles following transection, and a significant increase in the range and maximal angle of the ankle joint. The decrease in minimal hip angle was only significant at 5 weeks post-Tx but the other changes were significant at both 3 and 5 weeks post-Tx. Although statistically significant, the differences were relatively small and the 95% CI of the EMMs overlapped in most cases as seen in Fig 4. Only hip and ankle joints maximal angles at 5 weeks, and the knee maximal angle at both time points had EMMs that did not overlap with their pre-injury values.

D. Vertical Ground Reaction Forces Improved With BDNF Treatment

GRFs analysis showed better weight bearing capability for BDNF treated animals compared to Controls after SCI. Fig. 5 shows the profiles of the normalized vertical component of the GRFs (F_z) under the right paw for one representative cat of the Control (Fig. 5A) and BDNF (Fig. 5B) groups. All BDNF treated cats showed bouts of locomotion where the normalized peak vertical forces were equal or greater than 4 N/kg (see Fig. 5B) on certain steps, demonstrating that intrathecal delivery of BDNF restores full weight support on certain steps. On the other hand, no Control cats showed normalized peak vertical ground reaction forces higher than 2.5 N/kg on any steps. Linear mixed model with average peak F_z per trial as the dependent variable and group (BDNF or Control), side (left or right) and the group*side interaction as factors (with side and trial as repeated measure variables and cat as the grouping variable) showed only a significant effect for group ($p = .015$) on the peak vertical GRF. Post-hoc comparison of mean peak F_z showed a significantly lower ground reaction forces in the Control group ($p = .015$). The estimated marginal means of the right and left peak vertical GRFs were lower than the theoretically established minimum of 4 N/kg necessary for full weight-bearing capability on every step (BDNF Group: avg right F_z : 3.3 ± 0.5 N/kg; avg left F_z : 2.9 ± 0.5 N/kg; EMM $\pm$ 95% CI).

E. Histological Analysis

After sacrifice, the cord was retrieved from each animal. Gross examination of the spinal cord did not show any damage due to catheter insertion. The GFAP staining confirmed this result, underlining the safety of intrathecal BDNF delivery through implantable mini-pump. We compared the catheter insertion area, L7 for the BDNFs-L7 group and from L7 to L3 for the BDNFs-L3 group, to the transection site, which served as our control. As expected, high GFAP expression, was found in the tissue surrounding the transection lesion. No significant accumulation above background levels was observed at the cannula insertion area as well as in the cord from the entry point to the site of BDNF delivery, indicating minimal catheter-associated trauma (Fig. 6).

Transection sections were stained with Nissl-myelin to assess the completeness of the injury and potential growth across the lesion. As previously shown, [3], [14], no axonal regeneration was detected across the lesion, indicating that the lesion was complete.

IV. DISCUSSION

This is the first study to report locomotor recovery using intrathecal delivery of BDNF to the lumbar spinal cord via a programmable and implantable mini-pump in a feline spinal cord transection injury model.

The major findings of this study are 1) intrathecal delivery of BDNF to the lumbar spinal cord promotes the recovery of locomotor behavior in untrained spinalized cats that is 2) similar or better than the recovery obtained with cellular delivery at the transection site, 3) delivery via implantable and programmable mini-pump is a safe method for complete spinal cord injury treatment, posing minimal risk to the animals and their spinal cord.

A. Advantages of Intrathecal BDNF Delivery via Implantable Mini-Pump

This study showed that BDNF treated cats could step at all range of velocities tested (0.3-0.8 m/s), as early as 3 weeks post injury, and that stepping was maintained for the entire duration of the experiment (5 weeks post-Tx). Intrathecal delivery of BDNF was as effective as delivery via cellular grafts or treadmill training alone in our model at restoring locomotor functions. This was underlined by the similar or better recovery of stance length and swing height compared to data previously reported with NTF grafts implanted at the injury site or locomotor training [1]–[3], [14].

Kinematic analysis shows similar gait recovery with BDNF or treadmill training in spinalized cats. The analysis of the GRFs also showed results similar to trained animals in terms of weight support [20] and superior to non-treated animals. The BDNF treated cats were capable of performing some full weight-bearing stepping during locomotion at medium speed (0.4 m/s). The statistically significant differences with the minimal force required for full weight bearing in able-bodied cats (4 N/kg) may in part be due to a different weight distribution between fore- and hind-limbs in spinal cats. The hindlimbs weight loss resulting from muscles atrophy may reduce the minimum force necessary for full weight bearing, allowing BDNF treated cats to achieve full weight support even at lower forces. control cats that received saline were incapable of plantar weight-bearing stepping on a consistent basis.

While delivery of neurotrophin producing grafts promoted locomotor recovery, it required an invasive surgery that produces additional damage to the spinal cord and the neurotrophin delivery level is neither constant nor controlled. Those drawbacks represent significant obstacles for clinical application. The features of the micro infusion pump, such as being programmable, subdermally implantable and percutaneously refillable, minimize the number of invasive surgeries and allow delivery of a constant and controlled dosage of BDNF, making this study a viable clinical therapy. Fibroblasts engineered to produce BDNF produced different levels of BDNF, all leading to similar levels of locomotor performances [3], but their production was downgraded by 5 weeks post-grafting [14]. Those results suggest that the dose range effective at promoting locomotor recovery may be relatively large and that delivery may not need to be continuous. In this study, the use of an implantable and programmable mini-pump allowed delivery of a controlled dosage and the determination of a minimum effective dosage of BDNF necessary to elicit locomotor recovery. This is important for defining standardized protocols for SCI treatment. We

delivered a significantly lower dosage of protein (50 ng/day) compared to previous studies (150 μ g/day in [13]), which most likely eliminated the synchronous hindlimb activity observed with viral delivery where dosage is difficult to control [24], [25]. Future studies covering a greater range of dosage may uncover a dosing range that would lead to recovery of stepping at all speeds tested in all BDNF treated animals, yet avoid spastic-like hindlimb movements. This dosage may be species-specific but it would still provide valuable information about potential clinical outcomes if BDNF treatment is attempted in humans.

B. Implantable and Programmable Mini-Pump Is a Safe Method for Intrathecal BDNF Delivery

Prior results suggests that intrathecal BDNF delivery in humans would be a safe approach that could obtain FDA approval for clinical tests. Chronic intrathecal infusion has been proven safe and effective for Baclofen delivery for spasticity treatment [17] and morphine delivery for pain treatment [18] in clinical trials. Mechanical complications (catheter or pump issues [26]–[28]; similar to our leakage problem in one cat) have been observed in a number of cases and required revisions but can be reduced with appropriate techniques and post-surgical management [29]. Lumbar intrathecal pump delivery has also been used in Phase I/II clinical trials for BDNF delivery in ALS patients receiving a range of dosing: 25, 60, 150, 400, and 1000 μ g/day [16]. Patients reported events of minor severity due to pump/catheter implant that mainly consisted of post-surgical headache and local pain [16]. In line with these results in humans, gross examination of the animals' spinal cord retrieved at 5 weeks post-injury showed no damage to the cord or roots. Delivery to the lower lumbar cisterna is minimally invasive and the additional intrathecal space at that level reduces the risk of potential damage to the spinal cord. GFAP stain confirmed the visual and microscopic examination results, showing minimal trauma and inflammation due to catheter insertion and establishing the safety of the delivery method.

C. Regeneration Is an Unlikely Mechanism for Locomotor Recovery

As previously reported in [3], [14], [15], Nissl-myelin stain showed no axonal regeneration across the lesion cavity, confirming that locomotor recovery is not associated with axonal regeneration. Prior reports on delivery of BDNF to the spinal injury site concentrated on the effects of neurotrophins on axonal growth and sprouting to promote recovery of function [13], [30]–[32]. These studies showed the benefits of exogenously applied neurotrophic factors to axonal regrowth in mature neurons [33], [34] but while increased axonal regrowth was seen within the lesion and the cord caudal to the lesion, few axons extended beyond the injury site [13], [35]. In our study, since locomotor recovery occurred as early as 3 weeks after injury and was not associated with any axonal sprouting across the lesion site, regeneration of descending axons is an unlikely mechanism for recovery in BDNF treated cats. We instead propose that locomotor recovery can be attributed to the BDNF-induced plasticity within the lumbar spinal cord. We can speculate that the mechanism by which this class of NTFs induces recovery may be similar to the mechanism by which BWS training promotes locomotor activity. As previously shown by Gomez-Pinilla et al., exercise induces up-regulation of BDNF mRNA in the lumbar spinal neurons and skeletal muscles of complete spinalized rats [12]. In particular, studies have shown that, unlike other classes of neurotrophins, BDNF is released in an activity-dependent manner and exercise intensity

modulates serum BDNF concentrations [36], [37]. It is likely that exogenous delivery of BDNF mirrors the increase in BDNF that results from training and exercise, activating the same mechanisms. Neurotrophins have been shown to contribute to the efficiency of synaptic transmission [38], and produce an increase in the expression levels of molecules involved in neurotransmission [25] when delivered to the spinal cord. Changes in spinal synaptic transmission following training have been directly implicated in recovery of stepping [39], [40] and simulation studies with a basic neuromechanical model of the cat limb have shown that increases in synaptic strength like the ones seen with delivery of neurotrophins or locomotor training are sufficient to restore locomotor capabilities [41], [42]. Furthermore, electrophysiological studies conducted by Mendell's group showed that chronic BDNF or NT-3 delivery quickly enhances functional connectivity to motoneuron in the neonatal rat, and it does it as early as after 6 hours of treatment [43]–[45]. Thus, the strengthening of synaptic connections between the interneurons of the lumbar spinal cord remains a likely potential mechanism by which NTFs restore locomotion in animal models of complete SCI.

Acknowledgments

This work was supported in part by The National Institute of Neurological Disorders and Stroke Grants NS074406 and NS11605.

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Fig. 1.

Experimental setup: Location of the reflective markers and diagram of the kinematic parameters evaluated. Markers were placed on the cat over the humeral head (A), the elbow joint lateral epicondyle (B), the lateral carpal joint (C), the metacarpophalangeal joint (D) and the tip of the digits (E) on the right forelimb, as well as on the level of the ischium (F), femoral head (G), knee joint (H), lateral malleolus (I), metatarsophalangeal joint (J) and the tip of the digits (K) on the right hindlimb. Some kinematic parameters are also represented: stance length, swing height, hip height, D_a , D_p and hip, knee and ankle angles.

Fig. 2.

Kinematics of the right hindlimb: Stick figures represent the stance and swing phase of one step for a representative cat from each group pre-transection and 5 weeks post-injury at 0.4 m/s. The arrow indicates the direction of forward progress. Control cats showed dorsal foot placement during stance, while most BDNF treated cats were able to execute plantar weight bearing stepping at all the velocities tested (0.3-0.8 m/s).

Fig. 3.

A) and B) show the EMMs $\pm$ 95% CI of the stance length and swing height indices 3- and 5-weeks post-Tx at medium (0.4 m/s, black bars) and maximum (0.8 m/s, grey bars) walking velocity for the BDNF group (N = 6 animals for 0.4 m/s walking speed at 5 weeks; N = 5, 0.4 m/s at 3 weeks & 0.8 m/s at 5 weeks; N = 3, 0.8 m/s at 3 weeks). The dashed black and grey lines represent values obtained with treadmill training alone (BWST) [1], [2] and with neurotrophins cellular delivery (NTF) [3] respectively. C) and D) show the EMMs $\pm$ 95% CI for the Da and Dp indices for the BDNF group at the same time points and walking speeds. The dotted black line indicates pre-Tx performance (100%) in all panels. For most parameters (except Dp) the EMM 95% CIs post-Tx did not overlap with the pre-Tx levels, indicative of a remaining deficit, but were similar or above the levels obtained with BWST or NTF.

Fig. 4.

EMMs of the hip, knee and ankle joints angles for BDNF (N = 6 for Pre-Tx and 5 wks, N = 5 for 3 wks) cats during walking at 0.4 m/s for all time points: pre-transection (Pre-Tx), 3, and 5 weeks after injury. The black bars represent the hip, knee and ankle angles range, the light grey bars represent the joints maximum angle and the dark grey bars show the values of the minimum joints angle. All values are reported as $EMM \pm 95\%CI$. Linear mixed models with the angular parameter as dependent variable and time as a repeated measure factor showed a significant effect of time for all the parameters except the ankle joint minimum

angle. Post-hoc EMM pairwise comparisons (all time points) with Bonferroni correction showed a number of significant differences with pre-Tx values for the post-Tx joint angle parameters (indicated by * along with the p-value) but no significant differences between angular parameters at 3 and 5 weeks post-Tx.

Fig. 5.

Profiles of the normalized vertical GRFs of the right hindlimb over multiple steps in an exemplar 10 seconds period for one representative cat of each group, Control A) and BDNF B). Although the average peak vertical forces during a trial were lower than 4 N/kg (dotted horizontal line), BDNF treated cats were capable of full weight bearing stepping over some of the stepping period. On the other hand, Control cats did not display any ability to full weight support during the trial, with peaks of the vertical forces lower than 2.5 N/kg. C) shows the EMMs $\pm$ 95%CI of the peak vertical forces normalized for the animals' weight at

the moment of the recordings under the right and left paws. The black bars refer to BDNF treated cats ($N = 4$) while the grey bars to the Control group ($N = 2$ that could execute some plantar steps). The dotted black line represents the minimum required vertical force needed for full hindlimb weight support (about 4 N/kg). BDNF treated cats showed significantly higher weight support when compared to Control cats (see text for details) although the 95% CI EMMs of the peak vertical forces overlapped for the left limb (lack of overlap for the right limb is indicated by joining bars).

Fig. 6.

GFAP expression at the lesion site A) and at the catheter insertion area B): Staining was performed on eight animals, 5 BDNF (2 with L3 delivery and 3 with L7 delivery) and 3 Controls (two with L3 delivery and one with L7 delivery) on horizontal sections taken from the catheter entry zone's segment (L7/S1) to the segment where the tip of the catheter was found post-mortem. We also stained the transection site of four cats (3 BDNF and 1 Control) to serve as control/reference. A) The staining at the lesion site of BDNF-treated cat, going from rostral (R) to caudal (C) of the lesion, indicates glial reactivity of the tissue surrounding the injury. Image at higher magnification show enlarged GFAP positive cells. B) Catheter insertion area of a BDNF cat. The catheter was found on the ventrolateral aspect of the cord in the L7 segment (yellow arrow). We did not find any evidence (indentation or staining) on the cord of the exact location of the catheter tip, indicating that no damage to the cord was caused by catheter insertion. GFAP staining confirmed this visual observation as minimal to no evidence of over background GFAP levels occurs at the catheter insertion area. In (B) the higher red expression observed at the spinal cord border (white arrows) are astrocyte along the spinal cord pia, which often show higher levels of GFAP staining. This edge staining pattern was observed along the entire length of the spinal cord. Higher

magnification image on the left shows stronger GFAP staining within the astrocytes along the pial margin.

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