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Implications of poly(*N*-isopropylacrylamide)-*g*-poly(ethylene glycol) with codissolved brain-derived neurotrophic factor injectable scaffold on motor function recovery rate following cervical dorsolateral funiculotomy in the rat:

Laboratory investigation

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Abstract

Object—In a follow-up study to their prior work, the authors evaluated a novel delivery system for a previously established treatment for spinal cord injury (SCI), based on a poly(*N*-isopropylacrylamide) (PNIPAAm), lightly cross-linked with a polyethylene glycol (PEG) injectable scaffold. The primary aim of this work was to assess the recovery of both spontaneous and skilled forelimb function following a cervical dorsolateral funiculotomy in the rat. This injury ablates the rubrospinal tract (RST) but spares the dorsal and ventral corticospinal tract and can severely impair reaching and grasping abilities.

Methods—Animals received an implant of either PNIPAAm-*g*-PEG or PNIPAAm-*g*-PEG + brain-derived neurotrophic factor (BDNF). The single-pellet reach-to-grasp task and the staircase-reaching task were used to assess skilled motor function associated with reaching and grasping abilities, and the cylinder task was used to assess spontaneous motor function, both before and after injury.

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Disclosure

The authors report no conflict of interest concerning the materials or methods used in this study or the findings specified in this paper.

Author contributions to the study and manuscript preparation include the following. Conception and design: Grous, Vernengo, Himes, Shumsky. Acquisition of data: Grous, Vernengo, Jin, Himes. Analysis and interpretation of data: Grous, Jin, Himes, Shumsky.

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Results—Because BDNF can stimulate regenerating RST axons, the authors showed that animals receiving an implant of PNIPAAm-*g*-PEG with codissolved BDNF had an increased recovery rate of fine motor function when compared with a control group (PNIPAAm-*g*-PEG only) on both a staircase-reaching task at 4 and 8 weeks post-SCI and on a single-pellet reach-to-grasp task at 5 weeks post-SCI. In addition, spontaneous motor function, as measured in the cylinder test, recovered to preinjury values in animals receiving PNIPAAm-*g*-PEG + BDNF. Fluorescence immunochemistry indicated the presence of both regenerating axons and BDA-labeled fibers growing up to or within the host-graft interface in animals receiving PNIPAAm-*g*-PEG + BDNF.

Conclusions—Based on their results, the authors suggest that BDNF delivered by the scaffold promoted the growth of RST axons into the lesion, which may have contributed in part to the increased recovery rate.

Keywords

hydrogel; spinal cord injury; reaching; recovery of function; rubrospinal tract; rat

THE inability of the CNS to regenerate damaged axons after SCI²⁴ typically results in permanent functional loss.^{15,33,35} In particular, cervical SCI typically results in tetraplegia²⁶ and can severely impair reaching and grasping abilities. Injured people consider regaining arm and hand function as the highest priority for improving their quality of life.^{2,8} While the corticospinal tract is the primary descending tract involved in the control of skilled limb movements,¹³ focal ablations of this tract do not prevent many skilled forelimb-reaching behaviors, such as directed aiming, transport of the forelimb, and grasping in rats.^{28,41} Another descending system that contributes to skilled reaching is the RST in the dorsolateral funiculus, which is of particular interest in this study. When studying reach-to-grasp behavior, prior research found that only lesions to the dorsolateral funiculus, involving the RST, produced a persistent deficit in reaching success by impairing the ability to flex the digits during the reach-to-grasp task.²⁸ It was also found that neuronal activity in the red nucleus in rats during a reach-to-grasp task correlated with the distal movements associated with the grasp.¹⁴ Ablation studies of the red nucleus have also confirmed its role in the control of fine forelimb/forepaw movements in the rat.^{40,42}

Current therapeutic strategies that have emerged for SCI treatment include the use of synthetic scaffolds or matrices for delivery of neurotrophic factors or cellular transplants to promote neuronal regeneration and functional recovery.^{23,25} Neurotrophic factors have been shown to promote neural survival and axon regeneration. For example, neurotrophin-3 (NT-3) or BDNF delivery to the spinal cord promotes axon growth and plasticity.^{5,10,26,27,30,34,37} Of particular interest, BDNF delivery to the spinal cord promotes regeneration of RST axons.^{18,19} Additionally, our prior in vitro work using a synthetic scaffold based on PNIPAAm-*g*-PEG indicated that codissolved BDNF is released in bioactive form from PNIPAAm-*g*-PEG with a minimal burst, followed by a linear release for 4 weeks.⁶ Our recent in vivo research using PNIPAAm-*g*-PEG showed that the hydrogel is biocompatible, can be successfully injected into an injury site and gelled in situ to fill and remain in a spinal cord lesion, without contributing to an injury-related inflammatory response.⁷ The LCST of PNIPAAm-*g*-PEG of approximately 32°C allows it to be injected as

a viscous liquid at room temperature and solidify in situ without the use of toxic monomers or cross-linkers.³⁸

Thus, this work uses a combination of strategies to investigate the role of the RST in forelimb behavior. Since our prior work indicated that PNIPAAm-*g*-PEG is permissive to axonal growth with the addition of BDNF,⁷ we hypothesized that a BDNF-induced enhancement in the RST response would promote growth of RST axons into and beyond a hydrogel implant, aiding in accelerated functional recovery following a cervical lesion. To assess this, we combined an implant of PNIPAAm-*g*-PEG copolymer with codissolved BDNF to aid in the promotion of RST axon regeneration following a cervical dorsolateral funiculotomy. In particular, this study examines both spontaneous and skilled forelimb motor function using various behavioral tests deemed most useful and efficient for this lesion model.³² The results of this study examine the relationships and differences between outcomes of the various behavioral tests to identify improvements in forelimb motor function associated with the addition of BDNF.

Methods

Animal Subjects

Eighteen of 20 adult female Sprague-Dawley rats (weighing 225–250 g, Taconic) that demonstrated either a consistent right or consistent left forelimb preference when reaching for single pellets during a 3-week training session prior to injury were included in this study. The rats received a lesion to the cervical dorsolateral funiculus ipsilateral to their forelimb reaching preference. Animals were housed in pairs under a 12-hour light/dark cycle (lights on at 7:00 a.m.) and were maintained on a food-restricted diet of 12–15 g of standard rat chow per day per animal, resulting in animals achieving approximately 90% of the free-feeding body weight. Animals were weighed daily to ensure health, and 1 week preand postlesion surgery were allowed free access to food. Food restriction was resumed prior to Week 1 behavioral testing. All surgical and behavioral procedures were approved by the Drexel University College of Medicine's Institutional Animal Care and Use Committee and followed National Institutes of Health Guidelines for working with animals.

Behavioral Training and Testing

Behavioral testing was performed daily from 9:00 a.m. to 1:00 p.m. The single-pellet reach-to-grasp task and the staircase-reaching task were used to assess skilled motor function associated with reaching and grasping abilities, and the cylinder task was used to assess spontaneous motor function. Tests were scored in this blinded experiment by 2 trained observers who had achieved > 95% interrater reliability with the laboratory standard.

Single-Pellet Reach-to-Grasp Test

Prior to surgery, all animals were trained on a single-pellet reach-to-grasp task for 3 weeks in a manner similar to that of Whishaw and various coauthors,^{40–42} McKenna and Whishaw,²⁰ and later by Stackhouse et al.³² The same handlers conducted training and testing 5 days per week between 9:00 a.m. and 12:00 p.m. The Plexiglas reaching chamber was 45 cm deep × 40 cm tall × 12.5 cm wide, with a 1-cm wide slit cut out and centered on

the 12.5-cm wide front wall, as previously described.³² On the outside of the front wall, a 3.2-cm deep × 12.5-cm wide shelf was mounted, so that the top surface was 4 cm above the chamber floor. The shelf contained 2 food pellet wells, centered to each edge of the slit and 1.5 cm away from the outside front wall.³² The rats were trained to reach with the dominant forelimb through the slit to grasp and consume a chocolate food pellet (45 mg, Bioserv) that was placed in the contralateral food well. Training was performed through successive approximation. First, crushed pellets were placed at the shelf so the animal associated the shelf with food. Then a pellet was dropped into the back of the chamber whenever the animal approached the front of the chamber to encourage shuttling from back to front before reaching for the pellet on the shelf. Shuttling after a reach creates a natural separation between reaches and encourages consistency in how the animal orients and initiates itself to perform the next reach.⁴² The animal was considered to be successfully trained when it reached with the dominant forelimb with a success rate of 50% from a total of 20 reach attempts.^{40,42}

Quantitative assessment of the single-pellet reach-to-grasp task was performed from 20 reach attempts in the reaching chamber, as described above. From the video data, 2 raters scored the reach attempts as successful or unsuccessful. A successful reach was defined as a reach during which that rat made contact with the food pellet and grasped it to remove it from the pellet well on the platform. A reach during which the animal “raked” the pellet from the well into the chamber and did not grasp the pellet with digit flexion was not counted as a successful reach. The percentage of successful reaches was calculated as follows: (number of successful reaches/number of total reach attempts) × 100%. This value was averaged between the 2 raters.

Qualitative components of the single-pellet reach-to-grasp task were scored using a movement rating scale,⁴² which assesses 10 different components of a reach. Each component was given a score of 2 if the movement appeared normal, 1 if the movement appeared abnormal but recognizable, or 0 if the movement was absent or was compensated for by moving other parts of the body. The ratings from each component of the reach were summed to get a deficit score from each trial (20 = no deficit; 0 = maximum deficit). The 10 components of the reach are 1) limb lift: the forelimb is lifted from the floor by the upper arm and the digits are brought to the body’s midline; 2) digits close: as the forelimb is lifted, the digits are semi-flexed and the paw is supinated so that the palm faces the midline of the body; 3) aim: the elbow is adducted by the upper arm so that the forearm and digits are aligned with the body’s midline and the paw is held just under the mouth; 4) limb advance: the head is lifted and the limb is advanced above and beyond the pellet; 5) digits open: the digits are extended and opened as the limb is advanced; 6) pronation: the elbow is abducted by the upper arm and the paw is pronated over the food; 7) grasp: as the palmar pads touch the food, the digits close around it, and closure can occur before or during paw withdrawal; 8) supination I: as the paw is withdrawn, it is dorsiflexed and supinated 90° by a movement around the wrist and by adduction of the elbow; 9) supination II: as the rat sits back on its haunches, the paw is supinated by a further 90° and is ventroflexed so that the food is brought up to its mouth; and 10) release: the digits are opened and the food is transferred to the mouth. Three reaches from each animal were scored from video replay by 2 raters and were averaged regardless of the animal’s reaching success. The single-pellet reach-to-grasp

function was assessed and scored when the animals were trained on the task prior to SCI, and at 1, 2, 5, and 8 weeks postinjury.

Staircase-Reaching Test

The staircase-reaching test was done to examine reach-to-grasp performance in a context that minimizes shoulder function against gravity.^{4,21} As previously discussed by Stackhouse et al.,³² the staircase-reaching test was used because the shelf of the single-pellet reaching apparatus was 4.0 cm above the floor and therefore requires control of shoulder muscles to lift and aim the forelimb against gravity to permit contact with the pellet. The staircase-reaching test measures reaching function largely independent of shoulder control, which may be impaired after dorsolateral funiculotomy, if the injury also disrupts the ventral gray matter at the C3–4 spinal cord level, containing the motor neuron pool for shoulder musculature. The animals were trained to reach and grasp up to 21 chocolate pellets located in the animals' right or left staircase, depending on the dominant paw, daily for 2 weeks prior the surgery. The week prior to injury, the animals performed the task (two 15-minute sessions/day for 5 days), and the best 3 sessions were averaged to obtain the measure of pellet retrieval.⁴ Any pellets that were retrieved by the animals' tongues were not counted toward the total. Postinjury testing was performed similarly at Weeks 1, 4, and 8.

Cylinder Test

To assess spontaneous gross forelimb motor function, animals were placed in a Plexiglas cylinder (17.8 cm diameter × 35.5 cm height) for 3 minutes at a time, as previously described.³² When placed in the cylinder, animals spontaneously rear and contact the walls with their forepaws. A mirror was placed behind the cylinder to observe the forelimbs irrespective of the position of the animal. The number of forepaw contacts with the wall of the cylinder was scored from video replay. Contacts with the dominant (impaired limb), nondominant, and both simultaneous forepaws were counted and expressed as a percentage of the total placements. The percentage use of the dominant plus both forepaws were added to reflect the full usage of the impaired limb.^{29,31,32} The animals performed this test prior to SCI, 2–3 days postinjury, and at 1, 2, 4, 6, and 8 weeks during the recovery period.

PNIPAAm-*g*-PEG Copolymer Synthesis and Preparation for In Vivo Implantation

The PNIPAAm-*g*-PEG copolymer was prepared as described previously.³⁸ Briefly, *N*-isopropylacrylamide (NIPAAm) monomer was polymerized in methanol in the presence of PEG (8000 g/mol) dimethacrylate at a molar ratio of NIPAAm monomer units to PEG blocks of 1000:1. This ratio was chosen because it produced highly elastic PNIPAAm-*g*-PEG hydrogels at physiological temperature, with suitable volume retention over time and minimum liquid viscosity in water below the LCST due to the limited cross-link density. After synthesis and purification, PNIPAAm-*g*-PEG was dissolved in DMEM medium (Gibco, Invitrogen) and was steam-sterilized prior to implantation. Scaffold-only control animals 1 (*n* = 9) received an implant of 10 wt% PNIPAAm-*g*-PEG copolymer alone. For scaffold + BDNF animals (*n* = 9), BDNF (PeproTech) was reconstituted and codissolved with 12 wt% PNIPAAm-*g*-PEG, with a final BDNF concentration of 0.05 µg/µl. Our prior in vitro work indicated that codissolved BDNF at a concentration of 0.05 µg/µl is released in bioactive form from PNIPAAm-*g*-PEG hydrogels with a minimal burst, followed by a linear

release for at least 4 weeks.⁶ The addition of BDNF diluted the PNIPAAm-*g*-PEG solution to 10 wt%. This concentration was chosen because it is the lowest concentration that produced a solid gel above the LCST of PNIPAAm, and this concentration was also used in our previous in vivo study.⁷ All solutions were kept on ice prior to implantation.

The group receiving an implant of only the scaffold was chosen as our control for 2 main reasons. First, prior research has studied this lesion model with other implants or with no implant at all and received similar results to our study.³² Since our scaffold implant has been shown to be comparable to other current commercially available scaffolds,⁷ we chose our scaffold as the control group. Second, due to the availability of animals remaining on our grant, we did not have enough animals remaining to perform this experiment with a second control group, which would have ideally received just the dorsolateral funiculotomy without any implant.

Lesion Model and Animal Surgery

A cervical unilateral dorsolateral funiculotomy was chosen for the injury in this study because of the known effects of this injury on the reach-to-grasp function in the rat.³² The side of the surgery was chosen so as to injure the forepaw the animal preferred using in presurgical reaching tests. Briefly, animals were anesthetized using 4% isoflurane in oxygen (1 L/min), and anesthesia was maintained during surgery with 2.5% isoflurane in oxygen. The skin was cleaned and treated with Xenodine antiseptic surgical scrub. Once the animal reached a surgical plane of anesthesia (did not respond to toe pinch or corneal stimulation) a midline incision was made through the muscle and skin, and these layers were retracted. A partial laminectomy of the C3–4 vertebrae was performed to expose 1 spinal cord segment. The spinal cord midline and the dorsal root entry zone were identified, and the dura mater was opened. A shallow (~ 1-mm-deep) rostral-caudal incision was made in the spinal cord starting at the dorsal root entry zone of C-3 and extending to the C-4 dorsal root entry zone (~ 2–3 mm in length). Gentle aspiration in combination with microscissors was used to create a shallow cavity and extend the lesion laterally. This lesion completely disrupts the dorsolateral funiculus, which contains the RST, and partially involves the ipsilateral lateral funiculus and gray matter but leaves the dorsal columns and ventral funiculus intact. Sutures were placed in the dura on both sides of the lesion but were not tightened. After achieving hemostasis in the spinal cord lesion, approximately 5 µl of PNIPAAm-*g*-PEG (scaffold-only controls, n = 9) or PNIPAAm-*g*-PEG loaded with BDNF (scaffold + BDNF, n = 9) copolymer solution was injected directly into the cavity using a positive displacement pipette. The dural sutures were tightened to close the lesion and keep the solution within the lesion site. The muscle layers were then sutured and the skin was stapled closed. Early evidence of autophagia was controlled and discouraged during the 8 weeks of behavioral testing post-SCI by application of New-Skin or by Chew Guard spray.

Due to time constraints, the surgeries were performed and the scaffolds were placed immediately. As such, we were not able to perform any functional assessment prior to scaffold placement to ensure that the injuries were comparable. To address this, once animals were killed and tissues were processed, selected horizontally sliced tissue sections

from the lesion were processed for glial scar immunocytochemistry to measure the lesion area for each animal, using ImageJ software (National Institutes of Health).

Anterograde BDA Labeling

After 8 weeks of behavioral testing, the 18 remaining animals received BDA injections into the red nucleus. The injection of BDA into the red nucleus labels RST axons, which were interrupted upon creation of the lesion during the dorsolateral funiculotomy. Animals were anesthetized with isoflurane as described above and were positioned in a stereotactic apparatus. The brain was exposed by a hole drilled through the skull at the coordinates 5.9 mm caudal to the bregma, 0.7 mm either left or right of the midline (depending on whether each animal previously received a left or right dorsolateral funiculotomy). At a depth of 7 mm below the dura, 10% BDA (2 μ l, Molecular Probes) was injected slowly into the left or right red nucleus using a 26-gauge needle attached to a 10- μ l Hamilton syringe. The needle was left in place for another 2 minutes and was gradually withdrawn over 2 minutes. Animals survived for 2 weeks after BDA injections. However, 2 of the 18 animals (1 animal from each group) had to be killed during this period, due to autophagia of the contralateral hindlimb digits, leaving 8 animals in each group.

Tissue Collection for Histological Analysis

Two weeks after BDA injections and after 10 weeks of study, the rats were killed with an overdose of Euthasol (Webster Veterinary) and were transcardially perfused with 500 ml of ice-cold 4% paraformaldehyde. The spinal cord from each animal was dissected and postfixed in 4% paraformaldehyde for 3 days, followed by cryoprotection in 30% sucrose (Fisher Scientific) in 0.1 M phosphate buffer pH 7.4 for 5 days, both maintained at 4°C. The spinal cords were then embedded in M-1 media (Thermo Shandon, Inc.), and fast frozen on dry ice. Tissue blocks were sectioned and collected on gelatin-coated glass slides prior to staining. The C1–2 spinal cord was sliced in cross-sections to assess axon-tracing intensity. The C3–6 spinal cord tissue sections, containing the lesion, were sliced horizontally, to assess axon sprouting within the lesion.

Histology and Fluorescent Immunocytochemistry

To evaluate the presence of axons and astrocytes/glia scar formation, selected horizontal sections were stained with either of the following primary antibodies: mouse RT-97 for neurofilaments (1:1000 dilution, Developmental Studies Hybridoma Bank) or rabbit anti-GFAP (1:2000 dilution, Millipore). Briefly, slides were washed in PBS with 0.2% Triton X-100, and were incubated in 10% goat serum (Invitrogen) in PBS for 1 hour at room temperature to block nonspecific staining. Sections were incubated in their primary antibody diluted in PBS with 2% goat serum in a humidified chamber overnight. The tissue sections were then washed in PBS, followed by incubation in the dark for 2 hours at room temperature in a corresponding secondary fluorescent antibody. All secondary antibodies (Jackson Laboratories) were used at 1:400 dilution in PBS with 2% goat serum and were conjugated to either Rhodamine Red-X or fluorescein isothiocyanate. To evaluate the presence of BDA-labeled axons, selected sections from C1–2 and horizontal sections through the lesion block were washed and incubated in 10% goat serum in PBS to block nonspecific staining as described previously but were then incubated in Texas Red–

conjugated Streptavidin (1:400 dilution, Jackson Laboratories). For all 3 protocols, after incubation, the sections were again rinsed with PBS and coverslipped with Vectashield mounting medium containing DAPI (Vector Laboratories) and stored flat at 4°C until imaged using a Leica DM 5500B Microscope (Leica Microsystems) with Slidebook software (Intelligent Imaging Innovations).

In addition, selected horizontally sliced tissue sections from the lesion were processed and labeled with GFAP to evaluate the lesion size and location, using an ABC elite kit. Briefly, slides were washed in PBS with 0.2% Triton X-100 and were incubated in rabbit anti-GFAP primary antibody (1:2000 dilution, Millipore) diluted in PBS with 5% goat serum in a humidified chamber overnight. Tissue sections were then washed in PBS, followed by incubation for 2 hours in secondary antibody conjugated to biotin (1:200 dilution, Jackson). Slides were again washed in PBS, followed by incubation in ABC reagent for 2 hours. Slides were incubated for 20 minutes in SIGMAFAST 3,3'-diaminobenzidine tablets (DAB, Sigma), rinsed, dehydrated, and coverslipped.

To quantify the amount of glial scar formation and the lesion size in both groups, selected tissue sections were processed for glial scar immunocytochemistry and were measured and averaged for each group using ImageJ software. The total area (in mm²) of the lesion was measured, as well as the GFAP-positive area surrounding the lesions, indicative of glial scar formation. These measurements were taken on 5 tissue sections at a distance of 20 µm apart per animal. In addition, BDA-labeled fibers that grew up to or into the host-graft interface or crossed a line 500 µm rostral to the host-graft interface were measured in terms of mean length of growth and the percentage of graft length occupied by regenerating axons by using ImageJ software.

Statistical Analysis

After the 8 weeks of behavioral testing, 2-way ANOVA, with time taken as the repeated measure, was conducted for each behavioral outcome measure. If an interaction was found to be significant, post hoc t-tests (unpaired, 2 tailed) were performed to identify where the differences were. Significance levels were set to 0.05 for all comparisons. Means and standard errors were used for graphical representation of the data.

Sections of horizontal tissue processed for glial scar immunocytochemistry to evaluate the lesion area were measured and averaged for each group using ImageJ software. The means of the 2 groups of animals were analyzed using 1-way ANOVA. Significance levels were set to 0.05 for all comparisons.

Results

Single-Pellet Reach-to-Grasp Test

Dominant forelimb grasp function was assessed using the single-pellet reach-to-grasp test, which requires the animal to accurately aim and retrieve a single pellet from a platform. Prior to SCI, scaffold-only control animals (PNIPAAm-*g*-PEG only) and scaffold + BDNF animals (PNIPAAm-*g*-PEG + BDNF) were able to retrieve the pellet approximately 50% of the time, as shown in Fig. 1. One week after SCI, the percentage of reaching successes

decreased considerably, to approximately 20%. There was a significant interaction between the groups over time after injury [$F(3,64) = 8.47$, $p < 0.01$]. While successful single-pellet retrieval did not fully recover over time, post hoc t-tests revealed an accelerated recovery in the percentage reaching success to 26% in scaffold + BDNF animals at 5 weeks after SCI ($t = 2.13$, $p < 0.01$).

When examining the 10 individual movement components of the reach, a significant interaction between groups was found in the pronation [$F(2,48) = 4.40$, $p < 0.01$] and the grasp [$F(2,48) = 13.32$, $p < 0.001$] components over time following SCI (Fig. 2). Post hoc t-tests revealed that the pronation ($t = 2.13$, $p < 0.01$) and grasp ($t = 2.26$, $p < 0.001$) components recovered at an accelerated rate in scaffold + BDNF animals compared with scaffold only control animals at 5 weeks after SCI. To add to our findings, we noticed that the pronation movement was performed abnormally in both scaffold-only controls and scaffold + BDNF animals (mean 0.86 and 1.08 at 5 weeks post-SCI, respectively), but that the movement was more recognizable and often led to a successful grasp of the pellet, in scaffold + BDNF animals.

Staircase-Reaching Test

The dominant forelimb grasp function was also assessed using the staircase-reaching test, as shown in Fig. 3. Prior to SCI, animals from both groups were able to retrieve about 16 (76.2%) of 21 pellets from the staircase apparatus. There was a significant interaction between groups over time after injury [$F(2,48) = 5.43$, $p < 0.001$]. Post hoc t-tests revealed that pellet retrieval recovered at a more accelerated rate in scaffold + BDNF animals than in scaffold-only controls at 4 weeks ($t = 2.23$, $p < 0.001$) and 8 weeks ($t = 2.13$, $p < 0.001$) after SCI.

Forepaw Contacts With a Cylinder

Spontaneous forelimb motor function, as assessed by the number of dominant forepaw contacts with a cylinder, is shown in Fig. 4. Prior to cervical SCI, the animals used their dominant forepaw to contact the cylinder surface (alone or both forepaws simultaneously) during vertical explorations. There was a significant interaction between groups over time following injury [$F(5,96) = 3.94$, $p < 0.001$]. This was due to a decrease in the number of dominant forelimb contacts with the cylinder immediately after SCI, with a gradual increase through Week 4 postinjury. In scaffold-only control animals, between Weeks 4 and 8 postinjury, forelimb usage recovered and plateaued at a lower rate than preinjury baseline. However, in scaffold + BDNF animals, forelimb contacts recovered with an increasing rate through Week 8, with a plateau that was not different from preinjury baseline. Post hoc t-tests revealed that dominant forepaw contacts in scaffold + BDNF animals increased significantly more than scaffold only control animals at 4 weeks ($t = 2.32$, $p < 0.01$), 6 weeks ($t = 2.11$, $p < 0.001$), and 8 weeks ($t = 2.31$, $p < 0.001$) post-SCI.

Evaluation of Lesion Size and Glial Scar Formation

To quantify the amount of glial scar formation and the lesion size in both groups, selected tissue sections were processed for GFAP immunocytochemistry and were measured and averaged for each group using ImageJ software. The total area (in mm^2) of the lesion was

measured, as well as the GFAP-positive area surrounding the lesions (Fig. 5), indicative of glial scar formation. In scaffold-only control animals, the total area of the lesions measured $11.1 \pm 1.28 \text{ mm}^2$. Similarly, in scaffold + BDNF animals, the total area of the lesion measured $10.9 \pm 1.42 \text{ mm}^2$. The GFAP-positive area surrounding the lesions, indicative of reactive astrocyte and glial scar formation, measured $15.2 \pm 3.23 \text{ mm}^2$ and $14.5 \pm 2.20 \text{ mm}^2$ for scaffold-only controls and scaffold + BDNF, respectively. There was no significant difference found between the groups when comparing the total lesion areas ($t = 2.14$, $p = 0.82$) or the GFAP-positive area surrounding the lesions ($t = 2.18$, $p = 0.59$). Glial scar formation was assessed for GFAP labeling to determine if both groups had a comparable presence of scar formation. Consistent with the results of our prior work,⁷ by visual inspection, scaffold-only controls and scaffold + BDNF animals have a comparable presence of reactive astrocytes and glial scar formation around the lesions, as labeled by GFAP fluorescence immunohistochemical staining (Fig. 6A and C).

RT-97– and BDA-Labeled Axons

To verify that PNIPAAm-*g*-PEG is permissive to host axonal growth with the addition of BDNF, as we determined in our prior work,⁷ slides were stained with RT-97, which highlights neurofilaments and axons. It is important to note that while the hydrogel alone supports axonal growth, the hydrogel with the addition of BDNF supports more robust axonal growth, fostering regeneration through the graft and even back into the host spinal cord.⁷ The images in Fig. 6B (scaffold-only controls) and Fig. 6D (scaffold + BDNF) are tissue sections stained with RT-97. The hydrogel, with the addition of BDNF (Fig. 6D), promotes more robust neurofilament growth within the injury site, verifying the results from our prior study, that PNIPAAm-*g*-PEG with the addition of BDNF fosters axon regeneration.

To visualize the regrowth of RST axons, BDA was injected into the right or left red nucleus, depending on whether each animal previously received a left or right dorsolateral funiculotomy. The presence of BDA-labeled axons in C1–2 cross-sectioned tissue were seen in 15 of the 16 animals (data not shown). Labeling was seen in 8 of 8 scaffold-only control animals and in 7 of 8 scaffold + BDNF animals. Additionally, BDA-labeled fibers that grew nearby or into the host-graft interface or crossed a line 500 μm rostral to the host-graft interface were measured in terms of mean length of growth and the percentage of graft length occupied by regenerating axons, using ImageJ software (Fig. 7). In the control group, labeled RST axons were not seen at the host-graft interface, and labeled RST axons were not seen within 500 μm rostral of the interface. In comparison, RST axons were seen growing within 500 μm rostral of the host-graft interface in 6 (85.7%) of 7 animals in scaffold + BDNF animals. In 5 animals (71.4%), BDA-labeled axons extended as close as $212 \pm 51 \mu\text{m}$ to the rostral host-graft interface (Fig. 7 upper). In 1 animal, BDA-labeled axons grew across the host-graft interface (Fig. 7 lower), but most axons failed to extend into the graft for long distances. Those axons that grew beyond the host-graft interface reached a mean length of $286 \pm 158 \mu\text{m}$, which represented 13.1% of the total length of the graft. The maximum length of ingrowth by RST axons represented 21.5% of the total graft length, measured from the rostral end. Our results indicate that the addition of BDNF to the PNIPAAm-*g*-PEG scaffold may have increased the number and length of RST axons near and into the graft and also may have prevented axon retraction, which was seen in scaffold-only control animals.

However, none of the animals in either group showed RST axon regeneration entirely through the graft and into the spinal cord caudal to the injury.

Discussion

In this study, we compared the rate of recovery of forelimb reaching function following a cervical SCI in animals receiving scaffold implants with and without BDNF. Scaffold-only control animals performed similarly to operated control animals from our previous study³² on all behavioral tasks. While the lesion produced visible deficits in reaching performance on both the single-pellet and staircase tests, scaffold + BDNF animals showed an accelerated recovery in pellet retrieval over the 8-week study, compared with scaffold-only control animals. While full recovery of function was not achieved, this improvement in scaffold + BDNF animals is a significant outcome in the hopes of eventually fostering recovery of function.

Using the qualitative reaching score in the single-pellet reach test, we identified 2 phases (pronation and grasp) of the reach that showed a modest but significant improvement in scaffold + BDNF animals 5 weeks post-SCI. The movement was more recognizable and often led to a successful grasp of the pellet in scaffold + BDNF animals. Anderson et al.³ found that a persistent deficit in the grasp phase consisting of a lack of active digit flexion despite making pellet contact with the palmar surface of the forepaw often prevented successful pellet retrieval. While this was the case in scaffold-only control animals in our study, as the grasp phase was either absent or completely abnormal, some scaffold + BDNF animals were able to not only make contact with the pellet but were also able to grasp the pellet, with an abnormal but recognizable movement. These animals also had a noticeable improvement in digit flexion during this phase, which allowed them to make a more successful attempt at grasping the pellet. While these improvements were significant in leading to the eventual reach-to-grasp recovery of function, these improvements were not sufficient to increase the overall percent of successful reaches, since many of the other components that make up a reach did not show significant improvement during the duration of this study.

It is difficult to assign the movement deficits and improvements to a particular ascending or descending system, because this lesion interrupts the RST,¹⁹ the lateral corticospinal fibers,³⁹ and some parts of the reticulospinal tract.¹² However, prior studies have implicated the role of the red nucleus in the control of fine forelimb movements in the rat and have also shown that the movement components of single-pellet reaching affected by red nucleus ablations are the aim, advance, pronation, grasp, and supination phases.^{40,41} Based on these studies and on our improvements shown in the pronation and grasp phases, we suggest that BDNF promotes RST sprouting up to and within the host-graft interface, which may have contributed to the improvements seen in the rate of recovery of forelimb reach-to-grasp function. We hypothesize that the rats were not able to show a complete recovery of function since the RST sprouting did not completely bridge the entire lesion, entering the surrounding distal spinal cord. In the rat, previous anatomical data have shown that some rubrospinal fibers do make direct projections onto motor neurons of the extensor and flexor digitorum and the flexor and abductor digiti,¹⁷ and electrophysiological data support that the red

nucleus is active during reach.^{9,14} We can also suggest that, at the least, BDNF stimulated the activity of spared, nonfunctional connections in the injured cord. Another contributing factor to the improvements in the rate of recovery is the possibility that the RST axon terminals at the interface may have reconnected or formed synaptic connections with motor neurons or with cells that migrated into the interface.

The dorsolateral funiculotomy produced only minor deficits in spontaneous forelimb function that were only seen early after the lesion (2–7 days) in both groups. As seen in the cylinder test, in scaffold-only control animals between Weeks 4 and 8 post-SCI, function did not recover to baseline values. Conversely, in scaffold + BDNF animals, recovery continued to improve between 4 and 8 weeks post-SCI, without significant variation from pre-SCI values. This lack of a forelimb use deficit in the cylinder test is in contrast to prior work involving a C3–4 partial hemisection model^{19,29,31,36} but is consistent with results produced in Stackhouse et al.,³² which were attributed to a smaller lesion that spares gray matter. We can hypothesize that since gray matter is spared, sprouting of interneuronal pathways may contribute to this particular recovery. It is possible that interneurons in the rostral cervical spinal cord that still receive RST input after the injury could be stimulated by the BDNF released from the graft, thus establishing connections with more caudal interneurons or directly with the motor neurons that control the muscles of reaching and grasp. While prior studies have noted that this lesion does not impair forelimb usage in cylinder exploration²² and that spontaneous motor function is only modestly disrupted following damage to the dorsolateral funiculus,³² our results showed an observable improvement in forelimb usage in scaffold + BDNF animals at Weeks 4, 6, and 8 post-SCI when compared with scaffold-only control animals. Rats that favor their nonimpaired forelimb during cylinder touches are the result of unilateral RST damage,¹⁹ which was consistent with our study. However, by Weeks 6 and 8, scaffold + BDNF animals returned to using the injured (dominant) forepaw or both forepaws simultaneously the majority of the time, suggesting that spontaneous motor function in rats could in part be attributed to RST axon sprouting, as well as to sprouting of interneuronal pathways. While BDA injections indicated the presence of sprouted RST axons up to or into the host-graft interface in animals containing PNIPAAm-*g*-PEG + BDNF implants (scaffold + BDNF), it is also possible that the corticospinal tract axons sprouted into denervated regions of the spinal cord and was thus responsible for some of the improvement in associated motor functions, since the corticospinal tract and RST are interrelated. However, to support our theory, it must be noted that RST activity is related to individual digit movements,¹⁶ not just conjoint or grouped movements of the digits.¹¹ Also, proximal-distal limb representations occur in the red nucleus in a proportion similar to that in the motor cortex.¹¹ Future studies using a longer survival period postsurgery may help promote growth of RST across the entire lesion. This would hopefully lead to full functional recovery in animals receiving BDNF.

Conclusions

This study used a previously established treatment for SCI, based on PNIPAAm-*g*-PEG injectable scaffold, to evaluate recovery of reach-to-grasp function following a cervical dorsolateral funiculotomy, which damages the RST. We examined the relationships and differences between outcomes of the various behavioral tests to identify improvements in

forelimb motor function associated with the addition of BDNF. Based on the concept that the application of a specific neurotrophin can stimulate regenerating axons, we showed that animals receiving a graft of PNIPAAm-*g*-PEG with codissolved BDNF had an increased recovery rate of motor function when compared with the control. In particular, animals receiving BDNF recovered at a faster rate on both the staircase-reaching and the single-pellet reach-to-grasp tasks. Additionally, 8 weeks post-SCI, animals' gross spontaneous motor function, as measured in the cylinder test, recovered to preinjury values. We also showed the presence of both regenerating axons and BDA-labeled RST fibers within the lesion in scaffold + BDNF animals. Although full recovery of function was not seen, this unique study is a step in the right direction toward eventual SCI recovery.

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Abbreviations used in this paper

BDA	biotinylated dextran amine
BDNF	brain-derived neurotrophic factor
LCST	lower critical solution temperature
PBS	phosphate-buffered saline
PEG	polyethylene glycol
PNIPAAm	poly(<i>N</i> -isopropylacrylamide)
RST	rubrospinal tract
SCI	spinal cord injury

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Fig. 1.

The single-pellet reach-to-grasp task detected an accelerated improvement in percent reaching success in animals receiving scaffold + BDNF grafts (*dark gray*) compared with animals receiving a scaffold only control graft (*light gray*) 5 weeks postinjury. Immediately following SCI, both groups had fewer successful reaches from baseline values. *Whiskers* indicate SE. Significant post hoc t-tests, ** $p < 0.01$.

Fig. 2.

The pronation and grasp components of a reach revealed an accelerated recovery rate in animals receiving scaffold + BDNF grafts compared with scaffold-only controls 5 weeks post-SCI. **Upper:** Graph comparing the pronation phase between scaffold-only controls (*light gray*) and scaffold + BDNF (*dark gray*) animals. **Lower:** Graph comparing the grasp phase between groups. 0 = absent movement; 1 = abnormal movement; 2 = normal movement. Significant post hoc t-tests, $**p < 0.01$ for pronation; $***p < 0.001$ for grasp.

Fig. 3.

The staircase-reaching test detected a significant increase in the number of pellets retrieved by animals receiving scaffold + BDNF grafts (*dark gray*) compared with scaffold-only controls (*light gray*) at 4 and 8 weeks after SCI. Significant post hoc t-tests, *** $p < 0.001$.

Fig. 4.

Usage of the injured (dominant) forelimb in the cylinder showed a significant increase in dominant forelimb contacts in animals receiving scaffold + BDNF grafts (*dark gray*), compared with scaffold-only controls (*light gray*), at 4, 6, and 8 weeks following SCI. Significant post hoc t-tests, ** $p < 0.01$; *** $p < 0.001$.

Fig. 5.

Scaffold-only controls and scaffold + BDNF animals did not have a significant difference in lesion size ($p = 0.82$) or a significant difference in the amount of glial scar formation around the lesions ($p = 0.59$). **Left:** An example of scaffold + BDNF tissue processed for GFAP immunocytochemistry is shown at low magnification to help visualize the lesion size and location of the lesion within the host tissue. **Right:** A higher-magnification image of the same section is shown to better evaluate the presence of astrocytes. Bar = 1 mm.

Fig. 6.

Scaffold-only controls and scaffold + BDNF animals show a similar presence of glial scar formation; scaffold + BDNF is permissive to host axonal growth. GFAP (*green*) stains astrocytes around the graft site, and RT-97 labels host axons. DAPI labeling (*blue*) was used to identify cell nuclei. **A and C:** GFAP staining for scaffold-only controls and scaffold + BDNF, respectively. **B and D:** Images obtained at a higher magnification, showing RT-97 labeling for scaffold-only controls and scaffold + BDNF, respectively. *Asterisks* indicate the specific area of the lesion that was taken at a higher magnification in B and D. Bar = 100 μ m.

Fig. 7. BDA-labeled RST axons grow within 500 μm rostral to the scaffold + BDNF host/graft interface. **Upper:** BDA-labeled axons that grew to within $212 \pm 51 \mu\text{m}$ of the rostral host-graft interface. *Arrows* indicate the direction of RST axon growth. **Lower:** In contrast, BDA-labeled axons do not approach the scaffold-only controls host/graft interface. DAPI labeling (*blue*) was used to identify cell nuclei. Bar = 100 μm .