BDNF guides neural stem cell-derived axons to ventral interneurons and motor neurons after spinal cord injury

Yuanyuan Li, Amanda Tran, Lori Graham, John Brock, Mark H. Tuszynski, Paul Lu

Department of Neurosciences, University of California, San Diego, La Jolla, CA 92093, USA
Veterans Administration Medical Center, San Diego, CA 92161, USA

ABSTRACT

Neural stem cells (NSCs) implanted into sites of spinal cord injury (SCI) extend very large numbers of new axons over very long distances caudal to the lesion site, and support partial functional recovery. Newly extending graft axons distribute throughout host gray and white matter caudal to the injury. We hypothesized that provision of trophic gradients caudal to the injury would provide neurotrophic guidance to newly extending graft-derived axons to specific intermediate and ventral host gray matter regions, thereby potentially further improving neural relay formation. Immunodeficient rats underwent C5 lateral hemisection lesions, following by implants of human NSC grafts two weeks later. After an additional two weeks, animals received injections of AAV2-BDNF expressing vectors three spinal segments (9 mm) caudal to the lesion in host ventral and intermediate gray matter. After 2 months additional survival, we found a striking, 5.5-fold increase in the density of human axons innervating host ventral gray matter (P < 0.05) and 2.7-fold increase in intermediate gray matter (P < 0.01). Moreover, stem cell-derived axons formed a substantially greater number of putative synaptic connections with host motor neurons (P < 0.01). Thus, trophic guidance is an effective means of enhancing and guiding neural stem cell axon growth after SCI and will be used in future experiments to determine whether neural relay formation and functional outcomes can be improved.

1. Introduction

Neural stem cells (NSCs) and neural progenitor cells (NPCs) implanted into sites of spinal cord injury (SCI) extend extremely large numbers of axons (>10,000) over distances up to 50 mm caudal to a site of SCI, forming synapses with denervated host neurons (Lu et al., 2017; Lu et al., 2012b; Lu et al., 2014; Rosenzweig et al., 2018). In turn, host axons regenerate into NSC grafts driven to a spinal cord identity (Bonner et al., 2011; Kadoya et al., 2016), forming synaptic connections. These implants result in partial, significant recovery of function after SCI in both rodents and primates (Kadoya et al., 2016; Kobayashi et al., 2012; Koffler et al., 2019; Lu et al., 2017; Lu et al., 2012b; Rosenzweig et al., 2018; Salewski et al., 2015; Weinger et al., 2013).

In this experiment we explored the hypothesis that trophic gradients established by viral vector delivery of brain-derived neurotrophic factor (BDNF) would guide newly extending stem cell axons to intermediate and ventral gray matter to enhance connectivity with caudal host motor systems. We chose BDNF because it exerts crucial roles in axon guidance during development (Cohen-Cory et al., 2010; Kowianski et al., 2018; Phillips, 2017) and because its receptor, trkB, is broadly expressed by neurons of the spinal cord (Friens et al., 1992). Moreover, BDNF over-expression has previously been shown to increase adult axonal growth after SCI (Bonner et al., 2010; Charsar et al., 2019; Lu et al., 2012a; Ramer et al., 2007).

Adult rats underwent C5 lateral hemisection lesions and stem cell grafting two weeks later. One month after injury, AAV2 vectors expressing full-length BDNF were injected 3 spinal cord segments (9 mm) caudal to the graft/lesion site. When examined three months after injury, BDNF expression enhanced the number of graft-derived axons penetrating into caudal host intermediate and ventral gray matter 5.5-fold (P < 0.05), without affecting axon density in non-targeted, dorsal host gray matter (P = 0.16). These ventral axons resulted in a >20-fold increase in the number of putative synaptic contacts onto host motor neurons (P < 0.01). We conclude that administration of trophic
2. Methods

2.1. Experimental design

A total of $N = 8$ immunodeficient rats were subjects of this study and underwent C5 lateral hemisection lesions (Fig. 1A). 2 animals died after SCI due to complications of injury. Two weeks later, spinal cords were re-exposed and neural stem cells (H9-NSCs) derived from the human H9 embryonic stem cell line (Lu et al., 2017) were implanted into the lesion site. After an additional two weeks, 3 animals received injections of AAV2-BDNF into host intermediate and ventral gray matter at C8 while 3 control animals received injections of AAV2-GFP into host intermediate and ventral gray matter at C8 (Fig. 1A). After an additional 2-month survival period, histological analyses were performed.

2.2. Human neural stem cell (NSC) culture

The H9-NSC line was purchased from Aruna Biomedical and Invitrogen (Thermo Fisher Scientific, catalog N7800–100). H9-NSCs were cultured as a monolayer on a CELLstart coated flask (Thermo Fisher Scientific) in NSC serum-free medium (Thermo Fisher Scientific, catalog A1050901) with B27 supplement and FGF-2 (R&D Systems, catalog 2547-FG-025) and EGF (R&D Systems, catalog 236-EG-200) (Lu et al., 2014). To enable the tracking of transplanted human NSCs in vivo, we transduced proliferating H9-NSCs with lentiviral vectors expressing red fluorescent protein (RFP) under the cytomegalovirus/β-actin hybrid promoter (CAG) (Taylor et al., 2006). Briefly, 10 μl of lentiviral solution at a titer $3 \times 10^8$ infectious unit (IU) was added to NSCs cultured in 6-well plates at approximately 70% confluence, using a multiplicity of...
infection (MOI) of approximately 10. The medium was changed after 24 h and RFP-expressing cells were enriched by fluorescence activated cell sorting (FACS). Approximately 95% of cells obtained by FACS expressed RFP.

2.3. Measurement of trkB expression by neural stem cells

We measured expression of trkB in neural stem cells using RNAseq. Total RNA was collected using the RNeasy mini kit from both NSCs and their parent embryonic stem cell line (ESC) (Kumamaru et al., 2018). RNA integrity was examined with the Agilent Bioanalyzer 2000 (Agilent, Santa Clara, CA). TrueSeq stranded mRNA-seq libraries were prepared from 1 μg of total RNA (Illumina mRNA-seq kit, RS-122-2103) and sequenced on an Illumina HiSeq 2500 at the IGM Genomics Center, UCSD, generating 18.4 million single-end 100-base reads per sample on average (range, 13.8–35.6 million reads). Reads were mapped to human genome GRCh38 using the STAR spliced read aligner with default parameters. Between 88.5% and 92.3% (average: 90.0%) of the reads mapped uniquely to the human genome. Total counts of read fragments aligned to candidate gene regions were derived with the HTSeq program (http://htseq.readthedocs.io) using the refSeq gene model (GRCh38) as a reference and used as a basis for the quantification of gene expression. Relative RNA quantities were compared in the NSC and ESC samples. 3 replicates of ESC and NSC samples were quantified.

2.4. Animals

A total of 8 adult female athymic, T cell-deficient rats (Harlan Laboratories) weighing 180-200 g were subjected to right side unilateral C5 hemisection (CSHX). All experiments were conducted in strict accordance with NIH laboratory animal care and safety guidelines. All animals’ procedures were approved by the Institutional Animal Care and Use Committee of the Department of Veterans Affairs (VA) San Diego Healthcare System. The animals were deeply anesthetized using a combination (2 ml/kg) of ketamine (25 mg/ml), xylazine (1.3 mg/ml), and acepromazine (0.25 mg/ml) under aseptic conditions. The adult rats received a right C5 hemisection as described previously (Lu et al., 2012a; Lu et al., 2014). Briefly, following C5 dorsal laminectomy, the dura was cut longitudinally and retracted. A 1.5-mm-long block of the right spinal cord was excised using a combination of iridectomy scissors and micro-aspiration, with visual verification to ensure complete transection ventrally, medially, and laterally.

2.5. Transplantation surgeries and BDNF delivery

Two subjects died one day after C5 hemisection, leaving a total of 3 animals in the AAV2-BDNF group and 3 animals in the AAV2-GFP control group. Two weeks later, cultured human neural stem cells at passage 10 were trypsinized, washed with PBS, and resuspended at a concentration of 200,000 cells/μl in fibrinogen and thrombin mixed with growth factor cocktail as described previously (Lu et al., 2012b; Lu et al., 2014). The growth factor cocktail consisted of BDNF (50 μg/ml), FGF2 (10 μg/ml), VEGF (10 μg/ml) and an anti-apoptosis small molecule, MDL28170 (50 μM). 4 μl of the human neural stem cell mixture in fibrin and growth factor cocktail were micro-injected into the sub-acute lesion cavity using a pulled glass micropipette with an inner diameter of 40 μm, connected to a PicoSpritzer II (General Valve, Fairfield, NJ) (Fig. 1A). Cells were injected into 6 sites
encompassing the lesioned hemicord: two side-by-side injections were made at the center of the lesion cavity, spaced 0.5 mm apart (in the mediolateral plane); two side-by-side injections were made 0.5 mm rostral to the center of the lesion site, spaced 0.5 mm apart (in the mediolateral plane); and two side-by-side injections were made 0.5 mm caudal to the center of the lesion site, spaced 0.5 mm apart (in the mediolateral plane) (Lu et al., 2014).

Animals then went on to receive injections of either AAV2-BDNF or AAV2-GFP into the host spinal cord below the lesion two weeks later. AAV2 vectors expressing full length human BDNF under the chicken beta-actin promoter (CAG) promoter (UNC Vector Core) at a titer of $1 \times 10^{13} \text{vg/ml}$ were injected into three animals, while control subjects received AAV2-GFP-expressing vectors at the same titer. Vector was injected into the right hemicord at the C8 spinal cord segment, targeting the host intermediate gray zone and ventral gray matter neurons (coordinates, 0.7 mm lateral to midline; depth, 1–1.5 mm) two weeks after cell grafting (4 weeks post-SCI; $n=3$) (Fig. 1A). 500 nl of AAV2 were injected into each of 3 sites centered at C8 in the rostrocaudal dimension at 0.5 mm intervals. Three grafted subjects received AAV2-BDNF and three grafted control subjects received AAV2-GFP. Animals survived additional 2 months post BDNF gene delivery.

2.6. Histology and immunohistochemistry

Animals were perfused with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.2). Spinal cords were dissected, postfixed overnight at 4 °C, and then transferred to 30% sucrose for 72 h. 10 mm-long horizontal sections of spinal cords centered on the lesion/graft site were sectioned on a cryostat set at 30 μm thickness. In addition, coronal (cross) sections of the C8 spinal cord (3 mm long) were sectioned in the coronal plane at 30 μm thickness, where AAV expressing BDNF or GFP were administered. We blocked the spinal cord 6 mm caudal to the lower edge of the graft/lesion site in all animals for C8 coronal sections. This allowed consistent sampling of values across all animals. For immunolabeling, we performed an antigen retrieval procedure on every 6th free-floating section by incubating sections in 0.01 M Tris-HCl (pH 9.0 at 80°C) for 30 min and post-fixation in 2% paraformaldehyde and 0.2% para-benzoquinone for 5 min (Nagahara et al., 2018). Next, free-floating sections were incubated with primary antibodies against jellyfish RFP (rabbit, Invitrogen at 1:1500 or goat from Chemicon at 1:1500) to label RFP-expressing neural stem cells and their derived axons; BDNF (rabbit anti-BDNF antibody (Chicago Proteintech; made for Tuszynski laboratory at 1:2000 dilution) to label BDNF protein; NeuN (mouse from Chemicon at 1:200) to label mature neurons; ChAT (goat from Chemicon at 1:200) to label spinal cord motor neurons and their processes; human-specific synaptophysin (hSyn) (mouse from Chemicon at 1:1000) to label presynaptic terminals specifically from human axons; hTau (mouse from Chemicon at 1:200) to label human Tau protein; GFP (goat from Invitrogen at 1:750) to label GFP-expressing cells; and TrkB (rabbit from Abcam at 1:500) to label a BDNF receptor tyrosine kinase. Sections were incubated overnight at 4 °C and then incubated in Alexa 488, 594, or 647 conjugated donkey secondary antibodies (1:250, Invitrogen) for 2.5 h at room temperature. For nuclear staining, DAPI (200 ng/ml) was added to the final wash.

2.7. Image analysis and quantification

For examination and quantification of RFP-expressing axon density in C8 spinal cord gray matter where BDNF or GFP was delivered, three representative C8 coronal sections were quantified. BDNF and GFP were
visualized using Alexa 488 conjugated secondary antibodies in the same green channel in an Olympus BX53 fluorescent microscope. The three sections for quantification of axonal density were selected based on their maximal expression of either BDNF or GFP (Moriarty et al., 2022). To measure the RFP-expressing graft-derived axonal density in different regions of the spinal cord, the gray matter was divided into 3 zones: dorsal horn (DH), intermediate zone (IZ), and ventral horn (VH) (Diaz and Morales, 2016) (Fig. 4 E-F). RFP-expressing axon density was quantified in each zone (Fig. 4 E-F). The dorsal horn consisted of spinal laminae I-IV; the intermediate zone consisted of laminae V-VI and the dorsal part of laminae VII; and the ventral horn consisted of laminae VIII-IX and the ventral part of laminae VII. RFP-labeled axon pixel density within each zone was measured using ImageJ software (NIH) on 200× images. The RFP pixel density was then divided by the area of the zone to obtain the average axon density per each zone.

For quantification of human synaptophysin-labeled synapses at RFP-labeled human axonal terminals that directly contacted ChAT-labeled spinal cord motor neurons, five representative coronal sections expressing the maximal RFP along with human-specific synaptophysin and motor neuronal marker ChAT immunolabeling were selected at the C8 spinal cord level. The ventral horn region containing ChAT-labeled motor neurons was imaged using an Olympus FV3000 confocal microscope at 1200× magnification, under high resolution (1024 × 1024 pixel), and in thin (0.5 μm) confocal plane z-stacks. The number of synapses labeled by human synaptophysin that were in direct contact with the motor neuron cell bodies were counted manually on each image that was approximately 110 × 100 μm in size. Twenty images, each containing 2–3 ChAT-labeled motor neurons per image, were quantified in each subject and then averaged for each subject. The density of synapses was expressed as the average number of synapses that were in direct contact with motor neuron bodies per image.

2.8. Statistical analysis

In all quantification procedures, observers were blind to the nature of the experimental manipulation. Comparisons between two groups were tested by two-tailed Student’s t-test (JMP software) at a designated significance level of $P < 0.05$. Data are presented as mean ± standard error mean (SEM).

3. Results

Red fluorescent protein (RFP) immunohistochemistry allowed for unambiguous identification of human NSC grafts and graft-derived axons extending beyond the graft into the caudal host spinal cord. Analysis of horizontal sections revealed that RFP-expressing NSCs completely filled the C5 hemisection lesion cavity when assessed 2.5 months post-grafting (Fig. 1 B). As we reported previously (Lu et al., 2017; Lu et al., 2012b; Lu et al., 2014), densely packed RFP-labeled axons extended out from NSC grafts and traveled rostrally (not showed) and caudally (showed) primarily through host white matter (Fig. 1 C). Double immunolabeling for RFP and the human axonal marker hTau showed co-localization of all RFP-positive processes with human Tau, indicating that all RFP-expressing processes were human axons derived from the NSC graft (Fig. 1 D) (Bell et al., 2021).

To guide appropriate selection of a candidate neurotrophic factor for these studies, we immunolabeled neural stem cell grafts for the BDNF receptor, trkB (Numakawa et al., 2010). Indeed, virtually all graft-derived axons co-localized with trkB (Fig. 1 E), indicating that graft-derived axons expressed the BDNF receptor, trkB. This result is...
consistent with previous descriptions of trkB expression in developing axons (Arimura et al., 2009; Huang et al., 2011). In addition, we performed RNAseq analysis on H9-derived neural stem cells (NSCs) and on their parent ESC line (Kumamaru et al., 2018). The H9 NSCs expressed trkB RNA at levels exceeding the parent ESC line by 724-fold ($2^{9.5}$; Fig. 1F).

To confirm delivery of BDNF into the C8 spinal cord, we performed immunohistochemistry for expression of the control reporter gene, GFP, and the experimental gene, BDNF (Fig. 2). The expression of both exogenous GFP and BDNF was detected mainly in the intermediate zone and ventral horn, indicating that gene delivery was targeted successfully. In control GFP-injected subjects, native BDNF expression was weak except for some fiber labeling in the dorsal horn in lamina 1–3 together with scattered cell body labeling throughout gray matter (Fig. 2C), consistent with a previous study (Conner et al., 1997). In contrast, very robust BDNF expression was detected in the ventral horn and intermediate zone where AAV2-BDNF was delivered (Fig. 2B, D, F). Previous studies report that AAV2 nearly exclusively transduces neurons, including spinal cord neurons (Burger et al., 2004; Taymans et al., 2007); we observed expression of BDNF in gray matter neurons as evidenced by co-localization with neuronal marker, NeuN (Fig. 2B, F). BDNF was detected robustly not only in neuronal cell bodies expressing NeuN, but also in numerous neuronal processes (Fig. 2F).

Analysis of C8 cross sections demonstrated that RFP-expressing, graft-derived human axons were mainly distributed in lateral and ventral white matter ipsilateral to the injury/graft hemiscord in control GFP-treated animals (Fig. 3A, C, E). There was a lower density of axons in host gray matter compared to host white matter (Fig. 3A, C; Fig. 4A, C). In contrast, AAV2-BDNF gene delivery resulted in substantial and significant increases in human axon density in caudal host intermediate and ventral gray matter (Fig. 3B, D, F; Fig. 4B). Graft-derived and RFP-expressing human axonal terminals were often detected in close apposition to BDNF expressing host spinal neurons (Fig. 3D inset), suggesting chemotraction of graft-derived axons to the trophic BDNF sources.

Comparison between (A, C) control and (B, D) BDNF groups demonstrates that robust innervation of the graft-derived and RFP-labeled axonal terminals around the ChAT labeled motor neurons and expression of numerous human synaptophysin (hSyn) at C8 spinal cord at BDNF group, but not in control. (E). Higher magnification and thin plane confocal z-stack images showing co-localization of RFP-labeled human axonal terminals with hSyn in close contact with large ChAT labeled motor neurons. (F) Quantification of hSyn that were in close contact with motor neuron bodies demonstrates significant more synapse number per image than control ($N = 3$; $***p < 0.01$ [p = 0.009]; Two-tailed Student’s t-test). Scale bars, 64 μm (A-B); 11 μm (C—D); 7 μm (E).

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Quantification revealed a striking 5.5-fold increase in RFP-labeled axon terminals in the host ventral gray matter in AAV2-BDNF recipients compared to AAV2-GFP controls ($p < 0.05$, two-tailed Student t-test; Fig. 4G). RFP-expressing axonal density was also increased 2.7-fold in intermediate gray matter compared to GFP-expressing controls ($p < 0.01$, two-tailed Student t-test; Fig. 4G), but not in dorsal gray matter, where BDNF was not expressed (Fig. 4D, G, $p = 0.16$). These results clearly demonstrate that BDNF gene delivery trophically guides and significantly increases innervation of host motor neuron regions by grafted humans axons, regions that exert important roles in forelimb motor function (Bannatyne et al., 2006; Lu et al., 2015; Maxwell and Soteropoulos, 2020; Sinopoulou et al., 2022).
To determine whether these graft-derived axons that innervate host gray matter form synapses with host neurons, particularly with host ventral motor neurons, we performed triple immunolabeling using the human-specific synaptic marker human synaptophysin (hSyn), the motor neuronal marker ChAT, and RFP. Among control subjects that received grafts and GFP-expressing AAV2 injections, human axons were readily detectable in host lateral motor neuron columns at C8 (Fig. 5A). Notably, the density of graft-derived axons in the host lateral motor neuron column was markedly increased by BDNF expression (Fig. 5B). Indeed, many ChAT+ motor neuron axons were densely surrounded by RFP-labeled axon terminals. Higher magnification and high-resolution z-stack confocal microscopic images clearly demonstrated expression of human-specific synaptophysin in RFP+ human axonal terminals in direct apposition to the surface of ChAT+ host motor neurons at C8 (Fig. 5C-D). The co-localization of human-specific synaptophysin and RFP+ axonal terminals was confirmed by 3-dimensional z-stack confocal images (Fig. 5E). Indeed, quantification of the number of synaptophysin+ appositions of graft-derived axon terminals with host ChAT+ motor neuronal bodies revealed a significant, more than 20-fold increase in these contacts (p < 0.01, two-tailed Student t-test; Fig. 5F).

4. Discussion

Neural stem cell and neural progenitor cell grafts have been reported by numerous groups to support host axon growth into spinal cord lesion cavities (Bonner et al., 2011; Ceto et al., 2020; Kadoya et al., 2016; Lu et al., 2012a; Lu et al., 2014; Rosenzweig et al., 2018; Tsuji et al., 2010) and to improve functional outcomes (Cummings et al., 2005; Kadoya et al., 2016; Keirstead et al., 2005; Kobayashi et al., 2012; Kofler et al., 2019; Lu et al., 2012b; Rosenzweig et al., 2018; Salewski et al., 2015; Tsuji et al., 2010). However, functional recovery resulting from stem cell grafting is often incomplete, indicating a need to develop strategies to further improve the effect of stem cell implants. We explored a potential means of enhancing neural stem cell efficacy in this experiment by exploring a trophic hypothesis: would trophic gradients established by BDNF delivery increase stem cell-derived axonal innervation of host neurons caudal to a site of SCI? Indeed, we observed a robust effect, with increases in innervation of host intermediate and ventral gray matter by 5.5-fold and an increase of synaptic contacts onto host neurons by 20-fold. Thus, there is indeed potential that trophic stimulation combined with a neural stem cell graft will improve the number of host-to-graft-to-host connections across a site of injury. It is also possible that trophic simulation could stabilize graft-to-host connections, thereby reducing pruning. Future experiments will explore these possibilities, together with examining functional outcomes.

Previously, others and we reported that trophic gradients increased host axon regeneration into cell grafts placed in spinal cord lesion cavities (Lu et al., 2012a), or when provided as gradients across the lesion, supported host axonal bridging beyond the lesion cavity (Anderson et al., 2018; Bonner et al., 2011; Lu et al., 2012a). However, to the present it had not been explored whether trophic factor gradients would also amplify growth of spinal cord neural stem cell axons. Of course, projections of the developing nervous system are profoundly affected by trophic gradients (Collazo et al., 1992; Klein et al., 1994; Lohof et al., 1993; Ma et al., 2002; von Bartheld et al., 1996). But the environment of the injured adult spinal cord is distinct from development. The present results unequivocally indicate that developing neurons, placed into sites of adult SCI, clearly mount a robust response to BDNF and are guided to its sites of expression to substantially increase synaptic contacts onto host neurons denervated by the injury. While graft-derived axons exhibit substantially increased density and synaptic contacts with alpha motor neurons, it is likely that they also increased contacts onto interneurons, although we did not specifically examine these other cell types because the aim of this work was to increase graft-derived axonal synapses with host alpha motor neurons.

A limitation of this study is the small sample size per group. However, the effect of BDNF delivery on graft-derived axonal density in the ventral horn and intermediate zone was very potent, yielding statistical significance despite the low sample size. Future work will study the effects of over-expressing BDNF in combination to determine potential effects on functional outcomes.

Data availability

No data was used for the research described in the article.

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