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ORIGINAL ARTICLE

Local Delivery of Neurotrophin-3 and Anti-NogoA Promotes Repair After Spinal Cord Injury

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Tissue and functional repair after spinal cord injury (SCI) continue to elude researchers. Neurotrophin-3 (NT-3) and anti-NogoA have been shown to promote axonal regeneration in animal models of SCI; however, localized and sustained delivery to the central nervous system (CNS) remains a critical challenge for these and other macromolecular therapeutics. An injectable drug delivery system (DDS) has previously been developed, which can provide safe local delivery to the spinal cord. This DDS, composed of poly(lactic-co-glycolic acid) (PLGA) nanoparticles (nps) dispersed in a hyaluronan methylcellulose hydrogel, was adapted for the tunable bioactive delivery of NT-3 and anti-NogoA. Furthermore, the combined delivery of NT-3 and anti-NogoA from the DDS in an impact/compression model of SCI increases axon density and improves locomotor function. The benefits of this np/hydrogel DDS observed for NT-3 and anti-NogoA demonstrate the utility of the DDS as a local delivery strategy for protein therapeutics to the CNS.

Introduction

The major obstacles to spinal cord injury (SCI) repair are the severe degenerative events that occur following injury,[1] coupled with the low intrinsic regenerative capacity of the central nervous system (CNS).[2] Thus, preclinical and clinical research on treatments for SCI have typically focused on either neuroprotection or tissue repair and regeneration.[3] For the latter, treatments that can induce axonal regeneration are of particular interest for their potential to restore motor and sensory function.

The delivery of small molecules or proteins that induce axonal regeneration has been shown to provide benefit in preclinical models of SCI. The protein neurotrophin-3 (NT-3) is a member of the neurotrophin family, which acts primarily on corticospinal axons and ascending sensory axons through interaction with the TrkC receptor.[4–6] Specifically, NT-3 increases axonal growth and axon sprouting of the corticospinal tract,[7–11] increases growth of dorsal column sensory axons,[12] and prevents neuronal death after axotomy.[13,14] NT-3 has also been shown to promote functional recovery in animal models of SCI.[8–11,15] Yet, to date there have been no human studies of the effect of NT-3 on SCI.[16]

Another promising candidate for the treatment of SCI is the antibody anti-NogoA. NogoA is a myelin-associated inhibitory molecule produced by oligodendrocytes that activates the RhoA and ROCK pathways in neurons to induce growth cone collapse and restrict neurite outgrowth.[17] The antibody 11c7 (anti-NogoA) is an antagonist for NogoA and has been shown to increase axonal sprouting and locomotor function in both rodent[18] and primate[19] models of SCI. A Phase 1 clinical trial of humanized anti-NogoA (ATI355) in spinal cord patients has been completed, with no published results.[20] Previous studies have shown that the combination of NT-3 and other molecules targeting NogoA and related inhibitory molecules, including anti-NogoA, can be beneficial after SCI.[7,11]

A key challenge for protein therapeutics such as NT-3 and anti-NogoA in SCI is achieving local, sustained, and bioactive delivery. The blood–brain barrier and blood–spinal cord barrier limit tissue penetration of systemically delivered macromolecules such as proteins.[21] Previous methods for NT-3 and anti-NogoA delivery have therefore included bolus injection[22] or intrathecal infusion through an osmotic minipump.[12,18] However, these methods require a high dose that is immediately dispersed throughout the CNS and may result in remote off-target effects. Moreover, the implantation of an intrathecal osmotic minipump is invasive and can cause increased scarring and infection.[23,24] Hydrogel-based systems were developed for the local release of NT-3 to the

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injured spinal cord and showed promising results in terms of tissue regeneration[10,25]; however, the hydrogels had to be implanted directly into the partially[10] or completely[25] transected spinal cord, which is a highly invasive strategy.

We designed a minimally invasive, injectable hydrogel system to provide local sustained delivery of therapeutic proteins to the injured spinal cord by injection into the intrathecal space (and not the spinal cord itself). The hydrogel is a physical blend of hyaluronan and methyl cellulose (HAMC) that is shear-thinning and inverse thermal gelling.[26] To better control protein delivery, poly(lactic-co-glycolic acid) (PLGA) nanoparticles (nps) are dispersed within the hydrogel to form an np/hydrogel composite drug delivery system (DDS).[27] This DDS can be injected through a 30G needle into the intrathecal space in proximity to the spinal cord lesion site and is bioresorbable and anti-inflammatory.[28]

We have previously shown that this np/hydrogel DDS can provide sustained release of either NT-3[29] or anti-NogoA[30] *in vitro*. Intrathecal delivery of NT-3 from this DDS was sustained for at least 28 days *in vivo* and induced axon growth after impact/compression SCI.[31] We hypothesized that the combined delivery of two factors, NT-3 and anti-NogoA, would further promote axonal outgrowth in spinal cord tissue and locomotor functional repair with this local controlled delivery strategy in a rat model of impact/compression SCI.

Materials and Methods

Materials

All materials were purchased from Sigma-Aldrich (Oakville, Canada) unless otherwise stated. Artificial cerebrospinal fluid (aCSF) was prepared as previously described.[26] Poly(lactic-co-glycolic acid) (50/50 PLGA, $7–17\times10^3$ g/mol, acid-terminated) was purchased from Sigma-Aldrich. Dichloromethane (DCM) and isopentane were purchased from Caledon Labs (Georgetown, Canada). NT-3 was purchased from Peprotech (Rocky Hill, NJ). The anti-NogoA mAb 11c7 was generously donated by Novartis (Basel, Switzerland). Sodium hyaluronate (HA, $4–1.8\times10^6$ g/mol was purchased from NovaMatrix (Sandvika, Norway) and methyl cellulose (300×10^3 g/mol) was purchased from Shin-Etsu (Tokyo, Japan). Protease inhibitor cocktail (EDTA-free) was purchased from Roche (Mississauga, Canada). The NT-3 ELISA was purchased from R&D Systems (Minneapolis, MN). The anti-NogoA ELISA, which recognizes bioactive anti-NogoA, was performed as described previously[32] with rat NogoA (R&D Systems) as the capture reagent and anti-mouse IgG horse radish peroxidase (HRP; R&D Systems) as the detection reagent. Neurobasal media, B27 serum-free supplement, L-glutamine, and penicillin–streptomycin were purchased from Gibco-Life Technologies (Mississauga, Canada).

The antibodies used for immunohistochemistry (IHC) were 200 kDa neurofilament (NF200, clone NE14, mouse, N5389; Sigma-Aldrich) and glial fibrillary acidic protein (GFAP, rabbit, Z0334; Dako, Burlington, Canada), with anti-mouse IgG 488 (Thermo Fisher Scientific, Burlington, Canada) and anti-rabbit IgG 546 (Thermo Fisher Scientific). The antibodies used for Western blotting were NF200, GFAP, anti-CD68 (ED-1, MCA314R; AbD Serotec, Raleigh, NC), and anti-NogoA (11c7; Novartis), with anti-mouse IgG HRP (R&D Systems) and anti-rabbit IgG HRP (Abcam, Toronto Canada).

All animal experiments were performed following the guidelines set out by the Canadian Council on Animal Care and approved by the University Health Network's Animal Care Committee.

Formulation of composite DDS

PLGA nps were prepared by double-emulsion solvent evaporation.[27,31] aCSF, containing 10 wt% bovine serum albumin (BSA) and 0.4 wt% magnesium carbonate ($MgCO_3$), was added to 120 mg of PLGA dissolved in DCM and sonicated for 10 min on ice (Vibra-Cell, 20% amplitude; Sonics, Newtown, CT) to form the primary emulsion. A secondary emulsion was formed with the addition of 2.5 wt% poly(vinyl alcohol) (PVA) and a further 10 min of sonication (30% amplitude). The emulsion was added to a hardening bath of 2.5 wt% PVA and stirred for 16 h at room temperature. The resultant nps were centrifuged (41,000 *g*; Beckmann-Coulter, Mississauga, Canada) and washed four times with nanopure water and then lyophilized for 3 days. For the formulation containing NT-3, 500 μg of NT-3 was added to the primary aqueous phase in addition to BSA and $MgCO_3$. np size was measured by dynamic light scattering (Malvern, Westborough, MA). The average np diameter was 168 ± 6 nm ($n=3$ batches).

The HAMC hydrogel was prepared by the sequential dissolution of MC and HA in aCSF and then mixed by a dual asymmetric centrifugal mixer (Flacktek, Landrum, SC). The prepared hydrogel was incubated at 4°C with mild agitation overnight to ensure complete polymer dissolution. To prepare the composite np/hydrogel formulation, nps were initially dispersed in aCSF by bath sonication for 5 min, followed by the addition of the hydrogel and speed mixing to completely disperse nps within the gel. Finally, anti-NogoA in phosphate-buffered saline (PBS), or PBS alone, was added to the composite, which in all groups had a final composition of 10 w/v% nps, 1.4 w/v% HA, and 3 w/v% MC.

Evaluation of protein release in vitro

The release of NT-3 and anti-NogoA *in vitro* was studied by incubating 100 μL of the composite DDS in 2 mL centrifuge tubes with 900 μL of aCSF containing 0.1 wt% BSA and protease inhibitor (to reduce anti-NogoA degradation) at 37°C. At each time point, tubes were sampled with complete replacement of supernatant. Samples were stored at −80°C before analysis.

The encapsulation efficiency of NT-3 in the PLGA np was measured by dissolving nps in 0.05 M NaOH for 1 h, followed by dilution in PBS with 0.1% BSA and analysis by ELISA. Release samples of NT-3 and anti-NogoA were also analyzed by ELISA. The protein remaining in the composite at the end of the release period was extracted with 0.05 M NaOH and analyzed by ELISA.

In vitro *bioactivity*

NT-3 bioactivity *in vitro* was assessed by a dorsal root ganglion (DRG) explant assay.[33] Sprague–Dawley rat embryo

DRG (E17) were harvested and pooled in neural basal media with 1 vol% fetal bovine serum, 2 vol% B-27 serum-free supplement, 1 vol% penicillin–streptomycin, and 1 vol% L-glutamine. The undissociated DRG were then placed on 12 mm diameter glass coverslips coated with poly-D-lysine (50 μg/mL in sterile water) and laminin (5 μg/mL in PBS) in a 24-well plate. Control media, media containing soluble (standard) NT-3, or media containing released NT-3 (previously frozen and stored at −80°C) was added to each well. After 48 h of incubation, the DRG were fixed in 4% paraformaldehyde and stained with NF200 and DAPI. DRG were imaged on an Olympus FV1000 confocal microscope. Image analysis was performed with Fiji.[34] For each DRG, the $NF200^+$ area of the multicellular DRG body was subtracted from the total $NF200^+$ area (center+extended neurites) to yield the area of neurite outgrowth. A minimum of five DRG (comprising 1000s of neurons), in a minimum of two wells, were analyzed per group. The anti-NogoA bioactivity was assessed by ELISA, with NogoA as the capture reagent.[32]

The stability of anti-NogoA in the presence or absence of NT-3 was analyzed *in vitro*. Samples of anti-NogoA (500 ng/mL) were incubated in aCSF with or without NT-3 (18 ng/mL) for 1 or 3 days at 37°C. Samples were flash-frozen in liquid nitrogen and stored at −80°C before analysis. The concentration of anti-NogoA was analyzed by ELISA and normalized to a control sample.

Animal surgeries and delivery of composite np/hydrogel DDS

Adult female Sprague–Dawley rats were anesthetized with isofluorane and a T1-2 vertebral level laminectomy was performed. A moderate impact/compression injury was induced by a modified aneurysm clip, exerting 26 g force, compressing the cord for 1 min.[35]

Immediately following the injury, a durotomy 5 mm caudal to the injury site was performed with a 30G angled beveled needle. Ten microliter of the composite DDS containing NT-3 (1.8 μg) and/or anti-NogoA (50 μg), or the DDS alone (Vehicle), was injected through a 30G angled blunt-tipped needle over 30 s into the intrathecal space. The needle was held in place for an additional 1 min to allow gelation to occur. Animals in the SCI control group did not receive a durotomy or injection. Following surgery, the overlying muscles and fascia were sutured closed and animals were placed under a heat lamp to aid recovery. Buprenorphine was administered postsurgery for 3 days to manage pain, and Clavamox was administered orally for 5 days following surgery to prevent infection.

Behavioral analysis

Before surgery, animals were acclimatized to handling for 3 days and then trained on the ladderwalk task[36] for 5 days. Following surgery, Basso–Beattie–Bresnahan (BBB) locomotor scoring[37] was performed weekly for 8 weeks. From weeks 5 to 8, the ladderwalk task was also performed. The BBB motor subscore[38] was also used to assess locomotor function for weeks 5–8. The scoring for each behavioral task was performed by two observers blinded to treatment. Two animals in the SCI group were identified as statistical outliers by the Grubb's outlier test (significance level of alpha = 0.05; GraphPad Prism 5, La Jolla, CA) and removed from the behavioral analysis. The final animal numbers for the behavioral analysis were as follows: $n = 18$ (SCI), $n = 9$ (Vehicle), $n = 10$ (NT-3), $n = 10$ (ANA), and $n = 9$ (NT-3 and ANA).

Immunohistochemistry

Animals were sacrificed 8 weeks after injury and delivery of the composite DDS. For IHC, five animals per group (SCI, Vehicle) or six animals per group (NT-3, ANA, NT-3 and ANA) were injected with a lethal dose of buprenorphine and transcardially perfused with ice-cold 4% paraformaldehyde in 0.1 M PBS. The spinal cord was removed and a 1.5 cm section centered on the lesion site was dissected and cryoprotected in 30% sucrose in PBS.

Tissue was mounted in Cryomatrix (Thermo Fisher Scientific) and sectioned parasagittally into 20 μm sections. Sections were stained with NF200 and GFAP overnight at 4°C, followed by incubation with the secondary antibodies at room temperature for 2 h. Tissue sections were imaged with an Olympus FV1000 confocal microscope. Image analysis was performed with Fiji.[34] For NF200, a region of interest (ROI) was drawn that completely encompassed the tissue at the border of the cavity, as well as two additional ROIs comprising two 250 μm wide segments, 1 mm rostral and caudal to the lesion margin (encompassing the entire dorsoventral area of spinal cord). To quantify axon density, the image was thresholded, converted to a binary image, and the pixel count and total area of each ROI measured. For GFAP, the $GFAP^+$ area surrounding the cavity was used to trace the cavity area. The percentage of spared tissue was then determined based upon a fixed length of spinal cord tissue, accounting for variability in tissue width. In addition, the area of spared tissue was normalized to uninjured spinal cord at the same sectioning level. Three to six sections were analyzed per animal.

Western blotting

Animals were sacrificed 8 weeks after injury and DDS injection. Four animals per group were injected with a lethal dose of buprenorphine and a 1.5 cm section of spinal cord, centered on the lesion site, was harvested and flash-frozen in isopentane. The tissue was then divided into three 0.5 cm segments. The middle segment, corresponding to the lesion epicenter, was suspended in ice-cold RIPA buffer (Cell Signaling Technology, Danvers, MA) containing HALT protease inhibitor (1 mL/100 mg tissue) and homogenized for 30 s with a 3.2 mm steel bead by a minibeadbeater (Biospec Products, Bartlesville, OK). After 30-min incubation on ice, samples were centrifuged at 1000 *g* at 4°C for 15 min. The total protein concentration of the supernatant was assessed after a 10× dilution in water by spectrophotometry (ND-1000 Nanodrop; Thermo Fisher Scientific). Samples were aliquoted and stored at −80°C.

For each Western blot, all samples were simultaneously run on two gels (20 samples in total). Samples were diluted to 1 mg/mL (for GFAP and ED-1) or 1.5 mg/mL (for NogoA) and 10 μL of sample was added to a 10% reducing gel. After separation, protein was transferred to a nitrocellulose membrane using a Transblot Turbo transfer system (BioRad, Mississauga, Canada). Membranes were blocked for 1 h in Tris-buffered saline containing 0.05% Tween-20 (TBS-T) containing 5 wt% skim milk powder (BioRad) and stained

with the primary antibodies overnight at 4°C, followed by 1 h incubation at room temperature for the secondary antibody. Membranes were developed with SuperSignal West Dura Chemiluminescent substrate (Thermo Fisher Scientific) and imaged on a MicroChemi imaging system (DNR Bio-Imaging Systems, Jerusalem, Israel). Band intensity was analyzed with Fiji and normalized to the band intensity of β-tubulin in the same lane (NogoA, ED-1) or a replicate gel (GFAP).

Statistical analysis

All results are reported as mean ± standard deviation. Statistical analysis was performed using GraphPad Prism 5 (GraphPad, La Jolla, CA). Differences between groups for the DRG assay, IHC, and Western blot were assessed by one-way ANOVA with Bonferroni's *post hoc* at 95% confidence intervals. Differences between groups for BBB, BBB motor subscore, and ladderwalk were assessed by two-way ANOVA with Bonferroni's *post hoc* at 95% confidence intervals. Differences between groups for the *in vitro* stability of anti-NogoA were analyzed by student's *t*-test. Grubb's outlier test was used to identify and remove outliers in the behavioral analysis.

Results

Combined release of NT-3 and anti-NogoA in vitro

The combined delivery of NT-3 and anti-NogoA from the composite np/hydrogel DDS was first assessed *in vitro*. Anti-NogoA was dispersed in the np/HAMC composite and released over a period of 10 days, with minimal amounts detected at 21 days and beyond. NT-3 was encapsulated in the nps and a more sustained release, of up to 58 days, was observed. For both NT-3 and anti-NogoA, combined delivery did not significantly alter the release profile in comparison with single-molecule delivery (Fig. 1A).

Importantly, the bioactivity of both molecules was preserved. The anti-NogoA release was measured by an ELISA against rat NogoA, which was previously verified against a cell-based assay for bioactivity.[32] Therefore, the release profile shown in Figure 1A is of anti-NogoA in its bioactive form. The bioactivity of the released NT-3 was assessed by a DRG neurite outgrowth assay (Fig. 1B–E). At all time points, released NT-3 remained bioactive in comparison to the media controls and similar to the positive control (50 ng/mL of fresh NT-3) as determined by the DRG neurite outgrowth assay. Together, these assays demonstrate that the np/hydrogel DDS is able to maintain the bioactivity of both released proteins *in vitro*.

Tissue-level response to sustained delivery of NT-3 and anti-NogoA after SCI

We next examined the effects of combined NT-3 and anti-NogoA (ANA) delivery from the np/hydrogel DDS on the injured rat spinal cord, as well as the effects of each molecule alone. First, we found that the sustained delivery of NT-3 and anti-NogoA was able to promote increased axon density 8 weeks postinjury. In comparison with the injury-only control, all drug treatments increased the axon density in the area surrounding the lesion (Fig. 2A). Caudal

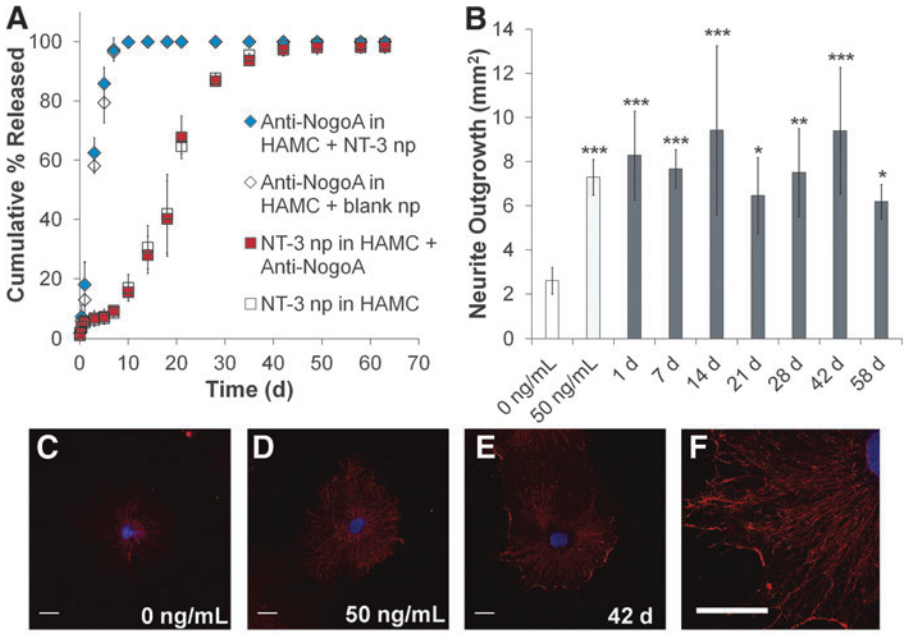


FIG. 1. Bioactive NT-3 and anti-NogoA are released *in vitro* from the composite np/hydrogel DDS. **(A)** Anti-NogoA is released over 10 days, while NT-3 is released over 58 days ($n=4$, mean ± SD). Anti-NogoA remains active over 10–14 days, as determined by ELISA with rat NogoA as the capture reagent. **(B)** NT-3 remains active *in vitro* throughout the release period, as demonstrated by a neurite outgrowth assay (*$p<0.05$, **$p<0.01$, ***$p<0.001$ shows significant difference to 0 ng/mL, $n=5$–6, mean ± SD). Representative images show **(C)** 0 ng/mL and **(D)** 50 ng/mL soluble NT-3 controls, and **(E)** release sample at 42 days, and **(F)** a higher-magnification image of **(E)** (*red* = neurites, NF200; *blue* = aggregated nuclei, DAPI; scale bar: 500 μm). DDS, drug delivery system; np, nanoparticle; NT-3, neurotrophin-3; SD, standard deviation. Color images available online at www.liebertpub.com/tea

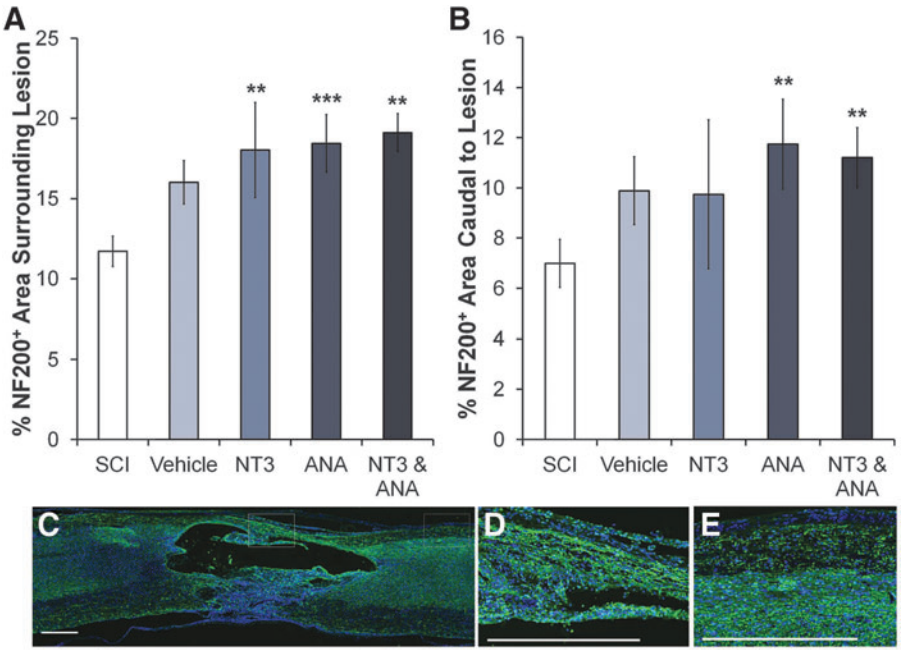


FIG. 2. Delivery of NT-3 and anti-NogoA (ANA) from the composite np/hydrogel DDS promotes axonal regeneration. **(A)** Neurofilament (NF200) density increases in the area adjacent to the cavity with combined NT-3 and anti-NogoA delivery as well as the delivery of each molecule alone (**$p < 0.01$, ***$p < 0.001$ shows significant difference from SCI control, $n = 5$–6, mean ± SD). **(B)** Neurofilament density increases 1 mm caudal to the lesion with combined delivery and anti-NogoA alone (**$p < 0.01$ shows significant difference from SCI control, $n = 5$–6, mean ± SD). **(C)** Immunostained section for combined NT-3 and ANA delivery (*green* = axons, NF200; *blue* = nuclei, DAPI, scale bar: 500 μm). **(D)** Enlarged area adjacent to the cavity, solid outline (*green* = NF200, *blue* = DAPI, scale bar: 500 μm). **(E)** Enlarged area caudal to the lesion, *dashed outline* in **(C)** (*green* = axons, NF200; *blue* = nuclei, DAPI, scale bar: 500 μm). SCI, spinal cord injury. Color images available online at www.liebertpub.com/tea

to the lesion, axon density was higher for combined delivery as well as the delivery of anti-NogoA alone (Fig. 2B). These results indicate that our DDS is able to deliver active NT-3 and anti-NogoA to the injury site, and sustained delivery of these molecules is able to increase axon density.

We then examined the effect of the np/hydrogel DDS on tissue sparing. The cavity area was measured by tracing the $GFAP^+$ border of the lesion, and the amount of tissue sparing was determined relative to a fixed tissue length. No difference in the percentage of spared tissue was observed among all groups (Supplementary Fig. S1A; Supplementary Data are available online at www.liebertpub.com/tea). In addition, no difference was observed among groups for the area of spared tissue normalized to uninjured controls at the same sectioning level (Supplementary Fig. S1B). By Western blot, there was also no difference in the amount of GFAP at the lesion site (Supplementary Fig. S2A). Although there was a high degree of variability within groups, the amount of ED-1 at the lesion site was also not significantly different between groups (Supplementary Fig. S2B). These results suggest that the DDS does not alter the amount of tissue sparing and cavitation after injury.

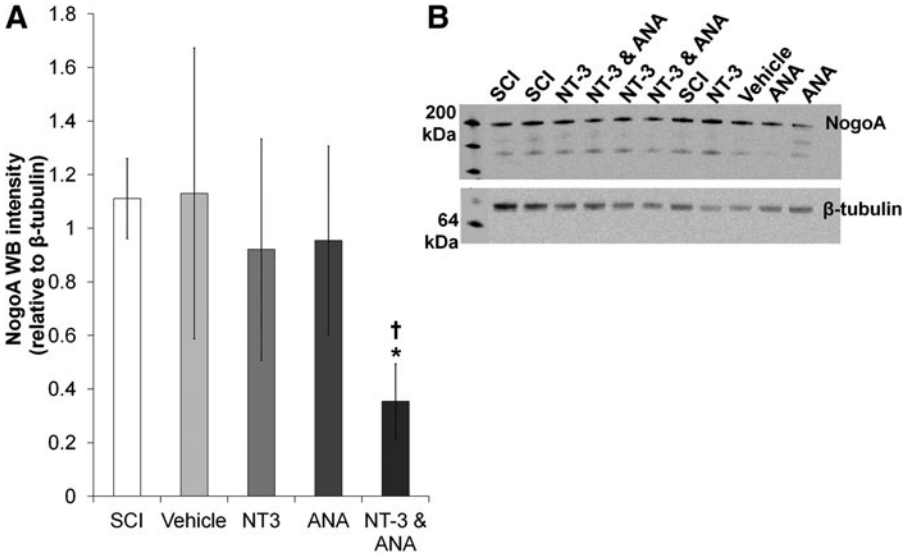


FIG. 3. Delivery of NT-3 and anti-NogoA reduces NogoA at the lesion. **(A)** Quantification of NogoA Western blot intensity at the lesion site, normalized to β-tubulin (*$p < 0.05$ shows significant difference from SCI control, †$p < 0.05$ shows significant difference from DDS control, $n = 4$, mean ± SD). **(B)** Western blot showing NogoA (210 kDa) and β-tubulin (50 kDa).

We investigated the effect of anti-NogoA delivery on the amount of NogoA at the lesion site. By Western blot, there was a significant reduction in NogoA for combined NT-3 and anti-NogoA delivery in comparison with the DDS alone and injury alone; however, delivery of anti-NogoA alone did not show any significant difference (Fig. 3 and Supplementary Fig. S3).

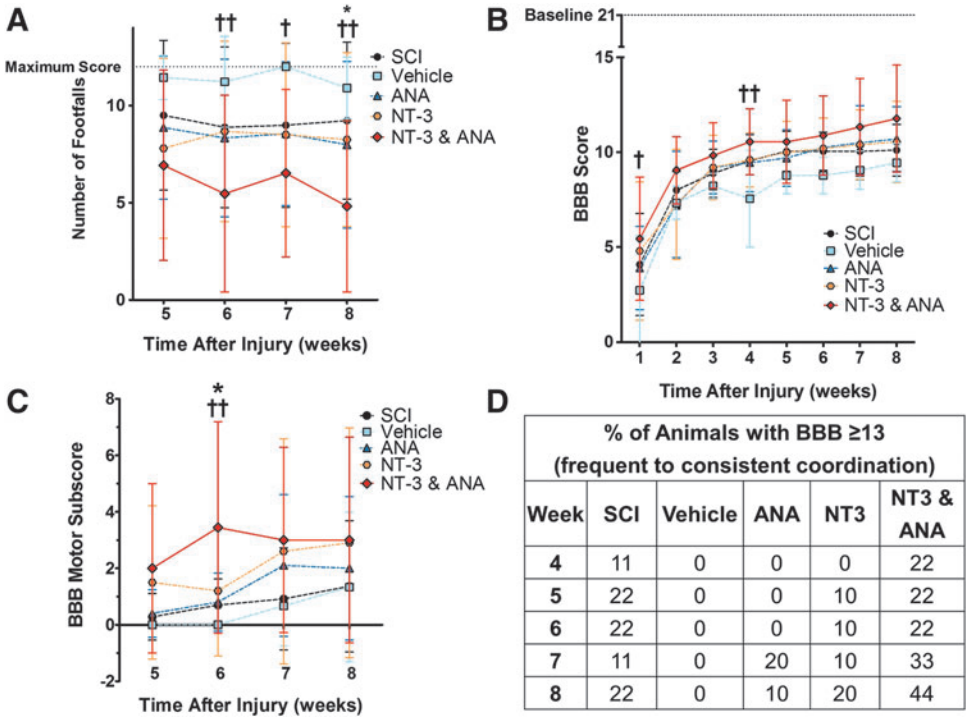
We further investigated the difference observed for combined delivery versus anti-NogoA alone by examining ANA stability in the presence or absence of NT-3 *in vitro*. After incubation for 1 day, no difference in the anti-NogoA bioactivity was observed with or without the addition of NT-3. However, after 3 days at 37°C, a significant increase in the amount of bioactive anti-NogoA was found when coincubated with NT-3 (Supplementary Fig. S4). This suggests that the NT-3 may promote anti-NogoA activity in the combined delivery system.

Effects of combined delivery on behavioral recovery after SCI

The results of the tissue-level analysis suggest that the np/hydrogel DDS can deliver active NT-3 and anti-NogoA to the injured spinal cord to promote repair. We examined this further by investigating the behavioral effects of NT-3 and anti-NogoA delivery with the ladderwalk task, the BBB scoring system, and the BBB motor subscore. Animals treated with the NT-3 and anti-NogoA combined DDS showed significant improvements on the ladderwalk task at 6, 7, and 8 weeks after injury in comparison with the vehicle control and in comparison to the SCI control at week 8 (Fig. 4A). In addition, a significant improvement in the BBB score was seen at 1 and 4 weeks between the dual NT-3 and ANA-treated animals versus those treated with the vehicle alone (Fig. 4B). The BBB motor subscore also revealed a significant difference at week 6 between combined NT-3 and ANA delivery versus vehicle and SCI controls (Fig. 4C). Furthermore, the NT-3 and ANA combined DDS promoted greater recovery of hindlimb–forelimb coordination, as indicated by the greater percentage of animals with a BBB score ≥13 relative to all other groups at weeks 4–8 after injury (Fig. 4D). Therefore, the combined delivery of NT-3 and anti-NogoA from our np/hydrogel DDS improved both tissue regeneration and locomotor function. No functional improvements were seen with single-molecule delivery of either NT-3 or anti-NogoA.

Discussion

We designed the np/hydrogel DDS to provide sustained delivery of both NT-3 and anti-NogoA. As previous studies demonstrated that the delivery of NT-3 through an osmotic minipump for 1 month promoted axon sprouting,[12] we encapsulated NT-3 in PLGA np to prolong its release. In this



Week	SCI	Vehicle	ANA	NT3	NT3 & ANA
4	11	0	0	0	22
5	22	0	0	10	22
6	22	0	0	10	22
7	11	0	20	10	33
8	22	0	10	20	44

FIG. 4. Delivery of NT-3 and anti-NogoA increases functional recovery. **(A)** Combined delivery of NT-3 and anti-NogoA from the np/hydrogel DDS significantly reduces errors on the ladderwalk task from weeks 6 to 8 relative to the vehicle control and at week 8 relative to the SCI control (†$p < 0.05$, ††$p < 0.01$ shows significant difference between NT-3 and ANA and Vehicle; *$p < 0.05$ shows significant difference between NT-3 and ANA and SCI control; $n = 9$–18; mean ± SD). **(B)** Combined delivery of NT-3 and ANA improves the BBB score at weeks 1 and 4 (†$p < 0.05$, ††$p < 0.01$ shows significant difference between NT-3 and ANA and Vehicle; $n = 9$–18; mean ± SD). **(C)** Combined delivery of NT-3 and ANA improves the BBB motor subscore at week 6 relative to the vehicle and SCI controls (††$p < 0.01$ shows significant difference between NT-3 and ANA and Vehicle; *$p < 0.05$ shows significant difference between NT-3 and ANA and SCI control; $n = 9$–18; mean ± SD). **(D)** Combined delivery of NT-3 and ANA increases the percentage of animals with frequent to consistent coordination (BBB score ≥13) relative to all other groups at weeks 4–8. BBB, Basso–Beattie–Bresnahan. Color images available online at www.liebertpub.com/tea

work and in our previous study with a similar PLGA formulation,[31] release was extended to 58 days, with majority of the protein released between days 3 and 28. We also previously found that NT-3 is detectable by ELISA within the spinal cord for at least 28 days following delivery and NT-3 is able to diffuse ventrally throughout the cord.[31]

Anti-NogoA has been shown to be efficacious when delivered through an osmotic minipump for 14 days.[18] Encapsulation of anti-NogoA within PLGA np has been found to substantially reduce its bioactivity both after initial encapsulation and during *in vitro* release, with <20% activity after 7 days.[30] By dispersing soluble anti-NogoA within the np/hydrogel matrix, we observed prolonged bioactive release over 10 days, with bioactive anti-NogoA detected for up to 21 days. Anti-NogoA is an IgG antibody with a molecular weight of 210 kDa,[39] which may account for its slow diffusion through the np/hydrogel DDS. Alternatively, anti-NogoA may interact nonspecifically with the PLGA particles through an adsorption/desorption mechanism to reduce its diffusion. Thus, the np/hydrogel DDS was able to provide sustained release of NT-3 and anti-NogoA over separate time scales of 28 and 10 days, respectively. The ability of this DDS to provide independent control over protein release profile is advantageous and will continue to be probed for future applications with additional therapeutic combinations.

A major obstacle to controlled delivery of proteins is the maintenance of their bioactivity throughout the release period. One factor that affects bioactivity is the reduction in pH inside PLGA np over time as a result of polymer degradation.[40] We previously found that the addition of $MgCO_3$ can improve the bioactivity of released NT-3 for up to 28 days, as determined by a DRG assay.[29] We found in this work that NT-3 released from the np/hydrogel DDS, also containing $MgCO_3$, remained active throughout the entire 58-day release period, even with the higher amounts of encapsulated NT-3 used in this study. In contrast to NT-3, previous studies demonstrated that the encapsulation and release of anti-NogoA result in rapid loss in the bioactivity, with <20% of the protein remaining active after 7 days *in vitro*.[30] The bioactivity of anti-NogoA can be readily assayed by ELISA, in good agreement with cell-based approaches.[32] In this study, we were able to retain anti-NogoA stability during *in vitro* release with the addition of BSA and protease inhibitor to the release medium and by dispersing anti-NogoA in the np/hydrogel DDS as opposed to its encapsulation. We were therefore able to demonstrate sustained and bioactive anti-NogoA release for at least 10 days, with low concentrations of bioactive anti-NogoA detected for up to 21 days. The crucial test of bioactivity is based on the *in vivo* effects. In this study, NT-3 and anti-NogoA delivery induced both tissue and functional repair, indicating that the np/hydrogel DDS is able to preserve the protein activity *in vivo*.

Impact/compression SCI presents a challenging environment to assess axonal sprouting and regeneration. This injury results in a central cavity surrounded by areas of spared tissue,[1] similar to what is observed in humans, but different than hemisection or transection models of SCI where specific axonal tracts are disrupted. We therefore examined the effect of NT-3 and anti-NogoA delivery on axon density by staining for NF200, a general marker for axons. We found that all treatment groups resulted in an increased axon density surrounding and caudal to the lesion, but the combined delivery of NT-3 and anti-NogoA did not result in an additive effect. This is in contrast to a previous study of combined anti-NogoA infusion and cell-based NT-3 delivery, in which only the combined system promoted axonal growth.[11] Combined treatment also resulted in altered electrophysiological responses compared to injury alone or individual treatment.[11] In this study, the combined delivery of NT-3 and anti-NogoA may also have had additional effects, such as long-distance axon growth or improved synaptic connectivity. While not specifically assessed in this study, these tissue effects would be consistent with the improved behavioral outcomes that we observed with combined delivery of NT-3 and anti-NogoA, but not each molecule alone.

Furthermore, NT-3 has previously been shown to promote axonal regeneration,[8,15] increase axon sprouting,[7,9,10] and prevent axonal dieback.[13,14] As we did not distinguish between these mechanisms, the increased axon density that we observed for the delivery of NT-3, anti-NogoA, and the combined system may be ascribed to a combination of regeneration, sprouting, and neuroprotection.

We found no significant differences in tissue sparing among all groups and no differences in GFAP or ED-1 expression at the lesion site. This suggests that the np/hydrogel DDS did not enhance or diminish the inflammatory response following injury, which is consistent with previous findings that the DDS alone is safe for use in the intrathecal space.[28]

The combined delivery of NT-3 and anti-NogoA resulted in a significant reduction in NogoA at the injury site in comparison with the DDS and SCI controls, whereas the delivery of anti-NogoA alone did not. Intrathecal infusion of anti-NogoA alone for 7 days was previously shown to result in the downregulation of NogoA only at a high dose.[41] In this study, combined delivery of NT-3 and anti-NogoA may have either altered the release profile of anti-NogoA *in vivo* to a greater extent than was seen *in vitro* or NT-3 may have increased the stability of anti-NogoA. The latter hypothesis is supported by the *in vitro* stability results, which demonstrate that anti-NogoA stability is improved with the addition of NT-3.

Importantly, the significant differences in axon density observed for the combined delivery of NT-3 and ANA versus controls were reflected in the locomotor functional assays. Combined NT-3 and anti-NogoA delivery improved the BBB score and the BBB motor subscore, as well as reduced the number of footfalls in the ladderwalk task at the end of the testing period in comparison with the vehicle and SCI controls. Furthermore, combined NT-3 and anti-NogoA delivery induced greater recovery of hindlimb–forelimb coordination in comparison with all other groups. A previous study of combined anti-NogoA infusion and cell-based NT-3 delivery in a hemisection model demonstrated that combined treatment improved coordination and fine motor function by establishing a new functional pathway that bypassed the lesion.[11] This was attributed to increased axon growth as well as increased synaptic efficacy. In this study, we did not assess the effects of NT-3 and anti-NogoA delivery on synapse formation or function. Yet, the increased axon density and reduced NogoA, in addition to the improved behavioral outcomes that we observed, suggest that the combined delivery of NT-3 and anti-NogoA is able to

promote functional recovery by inducing tissue repair and regeneration.

Conclusions

SCI results in a degenerative pathological injury that is a challenging target for drug delivery. A minimally invasive np/hydrogel DDS, injected in the intrathecal space, was evaluated for the combined delivery of NT-3 and anti-NogoA directly to the injured spinal cord. Sustained and bioactive codelivery was demonstrated *in vitro* and for the first time *in vivo*. Both combined and individual delivery were able to promote axonal growth after impact/compressive SCI, but only combined delivery showed additional benefits in several functional tasks. This study demonstrates the potential of the np/hydrogel DDS for multifactor drug delivery in tissue regenerative strategies.

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Disclosure Statement

No competing financial interests exist; however, we do hold composition of matter patents on the hydrogel and composite hydrogel.

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