Experimental Neurology 223 (2010) 153–165

Contents lists available at ScienceDirect

Experimental Neurology

journal homepage: www.elsevier.com/locate/yexnr

Sensory axon targeting is increased by NGF gene therapy within the lesioned adult femoral nerve

Xinhua Hu, Jie Cai, Jun Yang, George M. Smith *

Department of Physiology, Spinal Cord and Brain Injury Research Center, University of Kentucky, Lexington, KY 40536, USA

ARTICLE INFO

Article history:
Received 16 June 2009
Revised 24 August 2009
Accepted 26 August 2009
Available online 4 September 2009

Keywords:
Femoral transection
Axon reinnervation
Recombinant adenovirus
Nerve growth factor
Axon guidance
Axon targeting
Triple retrograde tracing

ABSTRACT

Even though peripheral nerves regenerate well, axons are often misrouted and reinnervate inappropriate distal pathways post-injury. Misrouting most likely occurs at branch points where regenerating axons make choices. Here, we show that the accuracy of sensory axon reinnervation is enhanced by overexpression of the guidance molecule nerve growth factor (NGF) distal to the bifurcation. We used the femoral nerve as a model, which contains both sensory and motor axons that intermingle in the parent trunk and distally segregate into the saphenous (SB) and motor branches (MB). Transection of the parent trunk resulted in misrouting of axon reinnervation to SB and MB. To enhance sensory axon targeting, recombinant adenovirus encoding NGF was injected along the SB close to the bifurcation 1 week post-injury. The accuracy of axon reinnervation was assessed by retrograde tracing at 3 or 8 weeks after nerve injury. NGF overexpression significantly increased the accuracy of SB axon reinnervation to the appropriate nerve branch, in a manner independent of enhancing axon regeneration. This novel finding provides *in vivo* evidence that gradient expression of neurotrophin can be used to enhance targeting of distal peripheral pathways to increase axon regeneration into the appropriate nerve branch.

Introduction

Incomplete functional recovery has long been observed after surgical repair of transected peripheral nerves (Sunderland, 1978; Vordemvenne et al., 2007). The therapeutic failure often results from misdirection of regenerating axons to target branches which lead to functionally inappropriate end organs (Brushart et al., 2005). Many studies have investigated the underlying mechanism of post-traumatic axon pathway selection (Ahlborn et al., 2007; Brushart, 1988; Madison et al., 1996) to help develop a clinically relevant therapy aiming to increase the specificity of axon regeneration and enhance functional restoration.

The specificity of sensory and motor targets for axon reinnervation was observed by Cajal (1928), who attributed it to "a neurotrophic influence which has an individual and specific character" of end organs. Recent studies demonstrate motor axons preferentially reinnervate muscle pathways as opposed to cutaneous pathways after transection of the parent femoral nerve, even in the absence of end organ contact (Brushart, 1993; Madison et al., 1996). Nerve grafts from denervated cutaneous nerves and ventral roots preferentially supported sensory or motor axon regeneration, respectively (Hoke et al., 2006). These results suggested some specificity of sensory and motor pathways for axon reinnervation. Schwann cells in sensory and motor nerves display differences in growth factor expression, indicating the pathway specificity is due to specific growth factor expression. Nerve growth factor (NGF) is one of the growth factors predominately expressed in denervated and grafted cutaneous nerve (Hoke et al., 2006). This matches its previously reported roles in growth and guidance of sensory axons (Heron et al., 2007; Paves and Saarma, 1997; Zhou et al., 2006). Furthermore, overexpression of NGF within the denervated spinal cord enhanced regeneration of the calcitonin gene-related peptide (CGRP)-positive nociceptive axons across the dorsal root entry zone (Romero et al., 2001), demonstrating a targeting role for lesioned axons.

Femoral transection is a commonly used injury model to assess the specificity of post-traumatic axon reinnervation. The femoral nerve originates from the lumbar plexus II, III, and IV, contains both sensory and motor axons that intermingle in the parent trunk and segregate into separate distal branches: one purely sensory passes subcutaneously along the tibial side of the leg to innervate skin (saphenous branch, SB), while the other leading to quadriceps contains both motor and proprioceptive axons (motor branch, MB). Transection of the parent femoral nerve results in inappropriate axon reinnervation to SB and MB (Brushart, 1988) (Figs. 1A, B). We hypothesized that overexpression of NGF along post-traumatic SB would mediate the accuracy of sensory axon reinnervation.

Abbreviations: Ad, adenoviruses; Ad-NGF, recombinant adenoviruses encoding nerve growth factor; Ad-GFP, recombinant adenoviruses encoding green fluorescent protein; CB, cutaneous branch; CGRP, calcitonin gene-related peptide; DRG, dorsal root ganglia; F488, Dextran AlexaFluor 488; F555, Dextran AlexaFluor 555; FG, FluoroGold; FN, femoral nerve; GFP, green fluorescent protein; MB, motor branch; NGF, nerve growth factor; PN, pectineus nerve; RL, retrograde labeling; SB, saphenous branch; ST, silicon tubing TrkA, tropomyosin-related kinase A.

* Corresponding author. University of Kentucky, Chandler Medical Center, B459 Biomedical and Biological Sciences Research Building, 741 S. Limestone Street, Lexington, KY 40536-0509, USA. Fax: +1 859 257 5737.
E-mail address: gmsmith@uky.edu (G.M. Smith).

0014-4886/$ – see front matter © 2009 Elsevier Inc. All rights reserved.
doi:10.1016/j.expneurol.2009.08.025

To investigate this hypothesis, we administrated recombinant adenovirus encoding NGF (Ad-NGF) along SB in transected femoral nerve. Sequential triple retrograde tracing was performed to assess the accuracy of sensory axon reinnervation.

Material and methods

Experimental animals

Fifty-five female adult (250–300 g) Sprague–Dawley rats (SD rats, from Harlan Sprague–Dawley, Indianapolis, IN) were used in this study. They were organized into 9 experimental groups (Table 1). All surgical procedures and animal maintenance complied with the National Institutes of Health guidelines regarding the care and use of experimental animals and were approved by the Institutional Animal Care and Research Advisory Committee.

Adenoviral vectors

Replication-defective recombinant adenoviruses (Ad) expressing NGF (Ad-NGF) and green fluorescent protein (Ad-GFP, control virus) were constructed as described previously (Romero et al., 2000). In addition to standard deletions in E1 and E3 coding regions, all viruses also encoded the ts125 mutation in the E2a, DNA binding protein, to further reduce potential toxicity after administration in vivo (Romero and Smith, 1998). All plaque-purified adenoviruses were examined for replication-competent adenoviruses via PCR. Viruses were then amplified and purified by double cesium chloride gradient ultracentrifugation. The physical number of viral particles was determined by optical absorbency. The number of infectious particles was estimated using Adeno-X Rapid titer kit (from Clontech, Palo Alto, CA) or directly counting green fluorescent cells 48 h after transfection of 293 cells. The viral particle (vp) to plaque-forming unit (pfu) ratio varied from 10:1 to 100:1.

Femoral transection and adenoviral administration

Deeply anesthetized animals [ketamine (67 mg/kg, i.p.)/xylazine (6.7 mg/kg, i.p.)] underwent transection of the left femoral nerve. Briefly, the femoral nerve was transected 1 mm distal to iliacus nerve, i.e. 6–8 mm proximal to the bifurcated point of SB and MB. The proximal and distal stumps were sutured into a 2-mm-long silicone tubing (1 mm inner diameter, from A-M systems, Carlsborg, WA) with one 10-0 nylon (from Ethicon, Somerville, NJ) stitch. A lesion gap about 0.5–0.8 mm long was maintained between the two stumps.

One week later, some injured animals were treated with Ad-NGF or Ad-GFP. Immediately before virus treatment, animals intraperitoneally received 100 μg of equally combined solution of CD-4 (W3/25) and CD-45 (MRC OX-22) antisera for transient immune suppression, as described previously (Romero and Smith, 1998). Each adenoviral administration consists of injections at three sites (1 mm apart from 2 to 3 mm distal to bifurcated point) along SB. All injections were made with a beveled glass micropipette (30–50 μm external diameter) using a nanoliter injection device (from World Precision Instruments, Sarasota, FL). At each injection, the micropipette was adjusted at an appropriate angle to insert into the nerve vertically at a depth of 0.2 mm with a micromanipulator, and nanoliter (300 nl) quantity of saline solutions containing Ad-GFP ($2.27–3.62\times10^5$ pfu/μl) or Ad-NGF (3.62×10^5 pfu/μl) were slowly infused into the SB at the speed of 1 nl/s. There was 1 min pause between each injection. Our previous studies demonstrated that this procedure resulted in robust transgene expression consistently throughout the injected area with little cell death or tissue damage (Romero and Smith, 1998).

Simultaneous double retrograde tracing

Both SB and MB were transected 2 mm distal to the bifurcated point, and the proximal cut end exposed to 2 μl of 10% Dextran AlexaFluor 555 (F555, 10, 000 mw, from Invitrogen, Eugene, OR) or 10% Dextran AlexaFluor 488 (F488, 10, 000 mw, from Invitrogen, Eugene, OR). F555 and F488 were randomly chosen to apply at SB and MB, respectively, and showed similar efficacy for retrograde labeling. For convenience of description, all SB ends were exposed to F555 whereas all MB ends to F488 during their simultaneous usage in retrograde tracing. Forty-five minutes after tracer application, the cut ends were rinsed robustly with sterile saline and sutured with appropriate distal ends by one 10-0 nylon stitch. L2–L4 DRGs were processed 48 h or 7 days later.

Sequential triple retrograde tracing

Experimental animals underwent sequential triple retrograde tracing in combination with performances of femoral transection and adenoviral administration, as schemed in Fig. 2A. Briefly, SB was cut 4 mm distal to bifurcated point and the proximal end was exposed to 2 μl of 5% hydroxystilbamidine (also known as FluoroGold, FG; from Biotium, Hayward, CA) for 45 min. The cut ends were then rinsed robustly with sterile saline and sutured together by one 10-0 nylon stitch. One week later, femoral transection and adenoviral administration were performed as described above. Three or 8 weeks after transection, 2nd retrograde tracing was performed using the same procedure for simultaneous double retrograde tracing. Seven days were allowed for tracer transportation, and then L2–L4 DRGs were processed for further analysis. In sham control study, 1st and 2nd tracing were performed with an interval of 4 or 9 weeks, in the absence of femoral transection and adenoviral administration to establish baselines.

DRG processing, histology and image analysis

Animals were perfused transcardially with 0.9% NaCl, followed by 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (PB, pH 7.2). L2–L4 DRGs were removed, post-fixed in 4% PFA for 1 h at room temperature, and then move to 30% sucrose in 0.1 M PB at 4 °C. Within 1 week, the DRGs were serially cryostated to 20-μm-thick sections on glass slides and reserved at −80 °C. For double immunostaining of FG and CGRP, air-dried DRG sections were simultaneously incubated with rabbit-anti-FG antibody (1:500, from Fluorochrome, Denver, CO) and mouse-anti-CGRP (1:4000, from Sigma Aldrich, St. Louis, MO) at 4 °C overnight. Next day, the sections were incubated with AMCA-anti-rabbit IgG (1:50, from Jackson ImmunoResearch, West Grove, PA) and Cy5-anti-mouse IgG (1:200, from Jackson ImmunoResearch, West Grove, PA) at room temperature for 45 min. After staining, sections were coverslipped with Fluoromount-G (from SouthernBiotech, San Diego, CA) for fluorescence microscopy. The whole area of each cryosection was photographed with Zeiss Conventional Inverted Microscopy System Axiovert 200 M under 10× objective on a Zeiss LSM510 microscope. All labeled neurons were counted manually from the images.

Femoral crush and adenoviral administration

To evaluate the *in vivo* transgenic expression of NGF and GFP in post-traumatic nerve, adenoviruses were injected into the crushed femoral nerve. The left femoral nerve was tightly held with Dumont forceps no. 5 (from Fine Science Tools, Heidelberg, Germany) for 1 min twice at the site 1 mm distal to iliacus nerve. Previous studies showed that all axons at the crush site would be broken under such a procedure (Irintchev et al., 2005). Immediately after femoral crush, Ad-GFP or Ad-NGF ($\sim5\times10^6$ pfu/μl) was injected along SB with the same injection procedure as described above. One week later, tissue segments from the treated femoral nerve were dissected for either GFP imaging or NGF Elisa.

Fluorescent imaging

Animals were perfused transcardially with 0.9% NaCl 7 days after femoral crush and Ad-GFP injections. Tissue segments of SB and MB