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A multifunctional neurotrophin with reduced affinity to p75^{NTR} enhances transplanted Schwann cell survival and axon growth after spinal cord injury

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ABSTRACT

The lack of regeneration of axonal pathways after SCI is associated with the presence of inhibitory molecules within the glial scar, the loss of the neuron's intrinsic capacity to grow and the absence of growth factors. The NGF family of neurotrophins is a potent growth factor for several types of supraspinal and sensory axons. It is unclear, however, whether the neurotrophin's axon growth-promoting activities after central nervous system (CNS) injuries are mediated through the Trk receptors or p75 neurotrophin receptor (p75^{NTR}) or both. To investigate the role of these receptors in the re-growth of specific fiber tracts after SCI, we created a series of neurotrophins that preferentially bind to either TrkB/C or p75^{NTR} receptors. All the mutations were made on the NT-3/D15A backbone, a multifunctional neurotrophin that can bind TrkB, TrkC and p75^{NTR}. To test the mutants' axon growth-promoting activity after rat contusion SCI, we examined several spinal cord fiber projections after transplanting Schwann cells (SCs) expressing the different multi-neurotrophins. Grafts expressing the NT-3/D15A with reduced binding affinity to p75^{NTR} contained more surviving SCs, and sensory as well as supra-spinal fibers, within the transplant than the NT-3/D15A neurotrophin-SC grafts. These data support the idea that neurotrophins lacking p75 activity can be more effective in promoting axon growth after CNS injury.

Introduction

Grafting rat Schwann cells (SCs) into injured adult rat spinal cord promotes the regeneration of spinal and sensory axons (Xu et al., 1995a, 1997). Even though SC transplantation after SCI provides a favorable environment for axonal regeneration and myelination, it is not enough to facilitate axonal growth beyond the graft and promote robust functional recovery (Fortun et al., 2009; Oudega and Xu, 2006).

To overcome this limitation, several groups have devised combination strategies that combine SC transplantation and delivery of neurotrophins (NTs) by external infusion or transplantation of NT-producing SCs and other cell types (Fortun et al., 2009; Lu and Tuszynski, 2008; Oudega and Xu, 2006; Tetzlaff et al., 2012). This approach has been shown to enhance axonal regeneration of supraspinal as well as spinal and sensory fibers (Bregman et al., 1997; Tuszynski et al., 1994; Xu et al., 1995b). Specific NTs stimulate axonal growth after injury through a variety of cellular mechanisms. Brain derived neurotrophic factor (BDNF) can reverse the atrophy of rubrospinal neurons after axotomy (Kobayashi et al., 1997). NT-3 can prevent the cell death and reduce atrophy of spinal cord projection neurons (Bradbury et al., 1999). BDNF and NT-3 can reduce axonal degeneration and induce sprouting of cortico-spinal axons (Grill et al., 1997; Hiebert et al., 2002; Sayer et al., 2002). These observations suggest that the use of multiple NTs might be necessary for restoring coordinated aspects of locomotion.

Structure-function studies investigating the interaction between the NGF family of NTs and their receptors showed that new NTs could be developed to bind more than one Trk receptor (Ibáñez et al., 1993; Urfer et al., 1994, 1997). One of these multineurotrophins (MNTs), NT-3/D15A, has been found to bind and activate TrkB, TrkC and p75^{NTR} (Urfer et al., 1994). The NT-3/D15A has been used in several models of SCI (Cao et al., 2005; Golden et al., 2007).

Neurotrophins bind Trk receptors and signal through the activation of specific downstream serine threonine kinases to mediate both neurite/axon growth and survival of neurons (Huang and Reichardt, 2003). But the NTs can also bind to p75^{NTR} and thereby trigger apoptosis during development and following injury (Dechant and Barde, 2002; Teng et al., 2010). The proapoptotic effect is thought to be mediated by the proneurotrophin (proNT) form of these molecules (Teng et al., 2010). Since p75^{NTR} and proNTs have been implicated in apoptosis,

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we set out to design MNTs that have reduced affinity for this receptor and therefore transduce mainly growth and survival signals. Here we report the generation of an MNT that does not bind to the $p75^{NTR}$ and testing its effects on the survival of SCs and growth of axons in a SCI model. Where the biological properties of NTs have adapted to specific physiological roles in various animal cells and organs, such attributes often are incompatible with their use as theurapeutic drugs. Therefore, the generation of new NT mutants with modified receptor specificity may be more successful than the naturally occurring proteins for salutary clinical outcomes.

Material and methods

Recombinant DNA techniques and transfections

The mutations were performed on, the human bi-functional neurotrophin, NT-3/D15A, a cDNA previously described (Urfer et al., 1994). For purposes of clarity and to avoid long naming schemes of the various mutants, we will refer to this bi-functional neurotrophin with the general term of multineurotrophin (MNT). All the residues were mutated to amino acid alanine using standard procedures. The cDNAs were cloned into the lentiviral vector pRRLsin.PPT.Th.CMV.MCS.Wpre that carries the CMV promoter (Dull et al., 1998; Follenzi and Naldini, 2002). We constructed the following mutant neurotrophins: 1) NT-3/D15A/R103A, 2) p75-2 (NT-3/D15A/p75-2 denoting the mutations R114A/K115A), 3) p75-22 (NT-3/D15A/p75-22 denoting mutations on Y11A/R68A/R87A/R114A/K115A), 4) p75-23 (NT-3/D15A/p75-23 denoting mutations on R68A/R87A/R114A/K115A), 5) p75-24 (NT-3/D15A/p75-24 denoting mutations on Y11/R87A/R114A/K115A), 6) p75-25 (NT-3/D15A/p75-25 denoting mutations on R87A/R114A/K115A), 7) p75-26 (NT-3/D15A/p75-26 denoting mutations on Y11A/R114A/K115A), 8) NT-3/D15A/R103A,Y51A and 9) NT-3/D15A/R103A/p75-2.

Generation of lentiviral particles

For lentiviral production we used the four plasmid method as described previously (Dull et al., 1998; Follenzi and Naldini, 2002). The virus was concentrated by ultracentrifugation and stored at −80 °C in the presence of PBS and 0.5% bovine albumin. The titers, shown as transducing units (TU) from 10^8 to 10^9 TU/ml, were derived using ELISA for the HIV Gag capsid protein p24.

Western blot and ELISA

To generate neurotrophin supernatants, 293-HEK cells were transfected using Lipofectamine 2000 (Invitrogen, Grand Island, NY). Twenty-four hours post-transfection the medium was replaced with DMEM/F12 containing 10 mg/l insulin and 1 mg/l human transferrin. The supernatants and cells were collected two days later. NT-3 protein was analyzed by the NT-3 Emax ImmunoAssay system (Promega, Madison, WI). Lysates of transfected cells were prepared as described previously (Tsoulfas et al., 1996). Western blotting was performed using a rabbit polyclonal anti-NT-3 antibody (1:500, Peprotech, Oak Park, CA). The enhanced chemiluminescence (ECL) western blotting detection system was used for the visualization of the proteins (GE Healthcare Life Sciences, Piscataway, NJ).

Dorsal root ganglion (DRG) explant neurite outgrowth and survival assays

Cervical and lumbar DRGs were dissected from E15 Sprague Dawley rats. For evaluation of neurite outgrowth, we plated DRG explants on dishes coated with poly L-ornithine (Sigma-Aldrich, St. Louis, MO) and 1 mg/ml fibronectin (Invitrogen) in the presence of purified human NT-3 (Peprotech, Oak Park, CA), human BDNF (Peprotech), conditioned medium with NT-3 mutants (1 ng/ml), and control medium without transfection for two days. Explants were fixed and stained for neuronal tubulin (TuJ1, Covance, Emeryville, CA). We quantified Tuj1 positive areas by capturing pictures using a 4× objective. The intensity was calculated in one-fourth of the area of the explants as in Fig. 2A with image analysis software 3i (Intelligent Imaging, Denver, CO).

DRG dissociated cultures were prepared as described previously (Kleitman et al., 1998). The dissociated cells were plated in neurobasal medium with B-27 supplement and L-Glutamine (Invitrogen). Purified human NT-3, human BDNF, conditioned medium (50 ng/ml), or control medium without transfection was added at days 0 and 3. Cells were maintained for 5 days and then fixed and stained for TuJ1 and DAPI. The quantification was done by capturing pictures of five random areas and counting Tuj1 positive cells using a 4x objective.

Culture of Schwann cells and infection with lentiviruses

The generation and characterization of SC cultures from adult Fischer rat sciatic nerves were described previously (Morrissey et al., 1991). These cells were cultured in the presence of mitogenic factors (20 mg/ml bovine pituitary extract, 2 mM forskolin, and 2.5nM heregulin). The purity of SCs by this method is between 95 and 98%. At passage 1 (P1), cells were infected with the lentiviral particles carrying the different mutants and GFP, grown for 5 days and frozen. For the grafting experiments, cells were thawed (P2) and grown for 4–5 days. The cells were trypsinized to dislodge them from the dishes and collected at the concentration of 2×10^6 cells in 6 μl of DMEM/F12 medium for the injection into the contused spinal cord. Supernatants were collected at P2 and an ELISA assay determined the concentration of secreted neurotrophins. To determine the percentage of infected cells, SCs were fixed and immunostained with an anti-NT-3 antibody (1:200, Peprotech, Oak Park, CA).

Animals and surgical procedures

Adult female Fischer rats (n = total 56, 180–200 g, Harlan, Frederick, MD) were housed according to National Institutes of Health and United States Department of Agriculture guidelines. The Institutional Animal Care and Use Committee of the University of Miami approved all animal experiments. The rats were anesthetized with a mixture of 1.5% isoflurane, 70% nitrous oxide, and 30% oxygen, and then placed on a surgical table on a heating pad (37 ± 0.5 °C). Lacrilube ophthalmic ointment (Allergan Pharmaceuticals, Irvine, CA) was applied to the eyes to prevent drying. All surgeries were performed using aseptic techniques. The spinal column was exposed and a laminectomy performed at T9 and the cord moderately contused using the NYU impactor (10 g, 12.5 mm). Gentamicin (5 mg/kg, intramuscular; Abbott Laboratories, North Chicago, IL) was administered immediately postsurgery once a day for 7 days. The analgesic, Buprenex (0.01 mg/kg of 0.3 mg/ml, subcutaneous; Reckitt Benckiser, Richmond, VA) was delivered postsurgery twice a day for 2 days. The rats were maintained for 7 weeks after injury. One week after injury, the injury site was exposed. A total of 2×10^6 cells in 6 μl of DMEM/F12 medium was injected into the contused area using a 10 ml Hamilton syringe with a pulled glass micropipette (tip diameter 100 μm) held in a micromanipulator at the depth of 1 mm. The glass micropipette was held in place for 3 min and withdrawn slowly. After the injection of SCs, the muscle layers and skin were closed separately. Rats were transplanted with SCs, infected with LV-eGFP (n = 10), LV-NT-3/D15A (n = 12), LV-NT-3/D15A/R103A (n = 11), LV-NT-3/D15A/p75-2 (n = 13) and NT-3/D15A/R103A/p75-2 (n = 10). The rats in each experimental group were randomly divided into subgroups for 1 μm-plastic sections and thin sections for EM (LV-GFP, n = 3; LV-NT-3/D15A, n = 4; LV-NT-3/D15A/p75-2, n = 4). We did not include a group without transplanting SC because previous results have shown multiple times that SC cells alone improve the locomotor behavior (Takami et al., 2002).