

ORIGINAL ARTICLE

Herpes simplex virus vector-mediated delivery of neurturin rescues erectile dysfunction of cavernous nerve injury

R Kato^{1,2,5}, D Wolfe^{3,4,5}, CH Coyle¹, JB Wechuck^{3,4}, P Tyagi¹, T Tsukamoto², JB Nelson¹, JC Glorioso³, MB Chancellor¹ and N Yoshimura¹

¹Department of Urology, University of Pittsburgh School of Medicine, Pittsburgh, PA, USA; ²Department of Urology, Sapporo Medical University School of Medicine, Sapporo, Japan; ³Department of Microbiology and Molecular Genetics, University of Pittsburgh School of Medicine, Pittsburgh, PA, USA and ⁴Diamyd Inc., Pittsburgh, PA, USA

Neurturin (NTN), a member of glial cell line-derived neurotrophic factor (GDNF) family, is known as an important neurotrophic factor for penis-projecting neurons. We recently demonstrated significant protection from erectile dysfunction (ED) following a replication-defective herpes simplex virus (HSV) vector-mediated GDNF delivery to the injured cavernous nerve. Herein, we applied HSV vector-mediated delivery of NTN to this ED model. Rat cavernous nerve was injured bilaterally using a clamp and dry ice. For HSV-treated groups, 20 μ l of vector stock was administered directly to the damaged nerve. Delivery of an HSV vector expressing both green fluorescent protein and lacZ (HSV-LacZ) was used as a control. Intracavernous pressure

along with systemic arterial pressure (ICP/AP) was measured 2 and 4 weeks after the nerve injury. Fluorogold (FG) was injected into the penile crus 7 days before being killed to assess neuronal survival. Four weeks after nerve injury, rats treated with HSV-NTN exhibited significantly higher ICP/AP compared with untreated or control vector-treated groups. The HSV-NTN group had more FG-positive major pelvic ganglion neurons than the control group following injury. HSV vector-mediated delivery of NTN could be a viable approach for the improvement of ED following cavernous nerve injury.

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Introduction

Erectile dysfunction (ED) resulting from cavernous nerve injury is a major complication following extirpative surgery of pelvic organs such as the prostate, bladder or rectum. ED is an exceedingly common side effect in men receiving radical prostatectomy.¹ A nerve-sparing procedure has become more widely used to preserve postoperative erectile function but nerve damage remains a frequent complication. Even with nerve-sparing surgery, it takes many months to recover erectile function.^{2,3}

To improve cavernous nerve function after injury, several basic and clinical experiments have recently been reported, including nerve grafting,⁴ nerve reconstruction,⁵ pharmacological neuromodulation using immunophilins,^{6,7} neuroprotection using erythropoietin,⁸ embryonic stem cell injection,⁹ inhibition of neuronal inflammation or neuronal cell death using poly (adenosine diphosphate-ribose) polymerase inhibitor,¹⁰ and gene delivery of neurotrophic and growth factors.^{11,12}

Among these, application of neurotrophic factors to injured nerves presents an ideal option to prevent injury and/or to facilitate nerve regeneration. Although several molecules related to penile erection, such as endothelial NO synthase (eNOS),¹³ neuronal NOS (nNOS)¹⁴ and maxi-K channel,^{15,16} have been used as gene therapy strategies for ED in animals as well as in humans, when considering the mechanism of ED after cavernous nerve injury, neurotrophic factors may be the most appropriate candidates for a gene therapy treatment of nerve injury-related ED.

Neurturin (NTN), a member of glial cell line-derived neurotrophic factor (GDNF) family, has been known as a target-derived survival and/or neurotogenic factor for postganglionic neurons innervating the penis.^{17–20} Laurikainen *et al.*¹⁷ showed that NTN mRNA was expressed in smooth muscle of penile blood vessels and the corpus cavernosum in adult rats, whereas GDNF receptor alpha-2 (GFR α 2) and Ret (common GDNF family receptor) mRNAs were expressed in all cell bodies of the penile postganglionic neurons. Furthermore, mice lacking the GFR α 2 receptor have significantly fewer NOS-containing nerve fibers, which are responsible for penile erection, in the dorsal penile and cavernous nerves.¹⁷ Other groups also demonstrated the role of the NTN–GFR α 2 pathway in the survival and regeneration of sacral parasympathetic neurons, especially for those innervating the penis, by examining their

Correspondence: Dr N Yoshimura, Department of Urology, University of Pittsburgh, Suite 700 Kaufmann Medical Building, 3471 Fifth Avenue, Pittsburgh, PA 15213, USA.

E-mail: nyos@pitt.edu

⁵These authors contributed equally to this work.

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signaling pathways in these neurons.^{18–20} In addition, GFR α 2 and nNOS expression both decreased with age, suggesting the possible involvement of an NTN–GFR α 2 pathway in the age-related alteration of nNOS expression.²¹ On the basis of these data, a recent report showed that treatment with NTN protein at the site of cavernous nerve crush injury facilitated the recovery of erectile function.²²

When considering the method of delivery of these neurotrophic factors to the nerves, one must consider issues such as the short half-life of these factors and unwanted dose-related side effects such as hyperalgesia at higher doses.^{23–26} An ideal delivery system would be safe, requires a single dose and would express the neurotrophic factor(s) in the target neurons for a limited time period. One mechanistically sound paradigm of delivering neurotrophic factors, such as NTN, is through the use of viral vector-mediated gene delivery.

Herpes simplex virus (HSV) has several significant advantages over other viral vectors for the treatment of peripheral nervous system disorders. Replication-defective recombinant vectors, which lack multiple essential gene functions and are non-toxic *in vivo*,^{27–30} have been generated to increase the overall safety of clinical therapeutic applications. These vectors can be prepared to high titer and purity without contamination from wild type.^{31–33} Delivery of the replication-defective vector to neurons innervating the site of vector delivery at the time of surgery would reduce the complications associated with systemic delivery of trophic factors, as the vector would express the neurotrophin solely within the targeted neurons.

Previous studies have demonstrated the utility of replication-defective HSV vectors in the treatment of numerous neurodegenerative disorders of the peripheral nervous system through the expression of trophic factors, such as nerve growth factor, neurotrophin-3 and GDNF,^{34–42} and support their use as therapeutic agents in treating neurological complications of ED. We have demonstrated that replication-defective HSV vectors expressing neurotrophin-3 are effective for improving erectile function in rats with chemically induced diabetes mellitus.³⁶ We have also recently reported that HSV vector-mediated delivery of GDNF (HSV-GDNF) rescued ED following cavernous nerve injury using a rat model of nerve-sparing radical prostatectomy.⁴³

As NTN is reportedly a more selective neurotrophic factor for penis-projecting neurons than GDNF,^{17–20} this study was performed to examine the effects of HSV vector-mediated delivery of NTN (HSV-NTN) in a nerve injury-induced ED model.⁴³ We assessed erectile function by the measurement of intracavernous pressure along with arterial pressure (ICP/AP) and nerve regeneration by a retrograde tracing study using fluorogold (FG) following nerve injury with or without HSV vector administration.

Results

Vector-mediated green fluorescent protein expressions in major pelvic ganglion neurons

To confirm the efficiency of HSV vector transduction into the nerve after pericavernous nerve injections, we investigated the expression profile of the marker gene, green fluorescent protein (GFP), in the major pelvic ganglion (MPG) of HSV-treated rats. In untreated rats, no GFP-positive cells were detected within the MPG (Figure 1a). In HSV-treated rats, approximately 60% of total MPG neurons were GFP-positive (Figures 1b and c). Moreover, GFP expression in MPG was observed up to at least 4 weeks in HSV-treated rats.

Characterization of GFP-positive cells in HSV vector-transduced MPG

We characterized the HSV vector-transduced MPG neuronal cell types following pericavernous nerve vector administration to determine whether the vector preferentially transduced specific neuronal cell types. Jung *et al.*⁴⁴ proposed that the ability to recognize and quantify the nerve fibers releasing NO is the best way to determine the integrity of the NOS-containing nerves and the erectile neural pathway. Another report also revealed that most of the nNOS-positive neurons were penis-projecting neurons.⁴⁵ Therefore, we carried out nNOS immunostaining to identify cavernous nerves, combined with GFP immunostaining. GFP expressions were observed in both nNOS-positive and nNOS-negative neurons in the MPG (Figures 2a and b). Approximately 70% of GFP-positive MPG neurons were positively stained for nNOS.

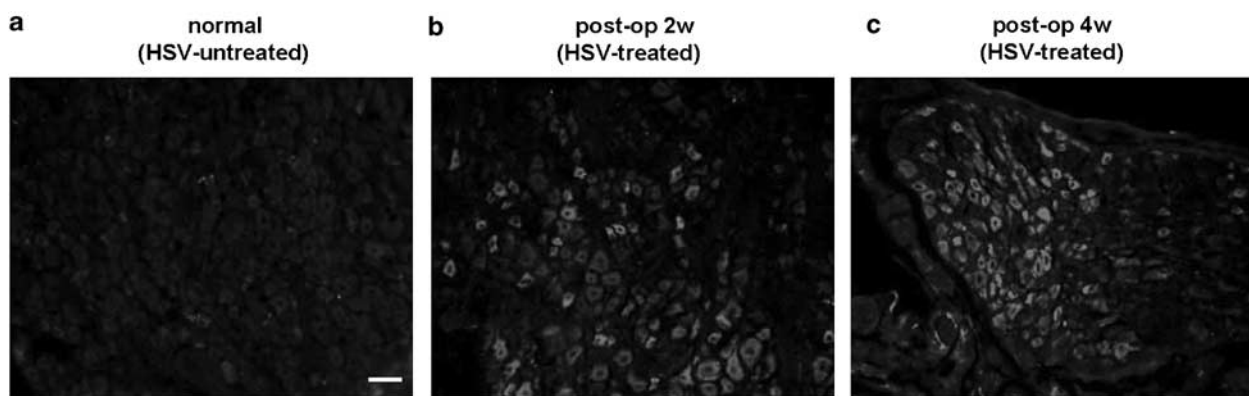


Figure 1 GFP expression in MPG after HSV vector administration. There was no GFP expression in normal uninfected MPG (a). A significant number of GFP-positive cells were seen in MPG from the HSV-treated postoperative 2-week model (b) and postoperative 4-week model (c). Scale bar, 50 μ m. Reduced from $\times 200$. GFP, green fluorescent protein; HSV, herpes simplex virus; MPG, major pelvic ganglion.

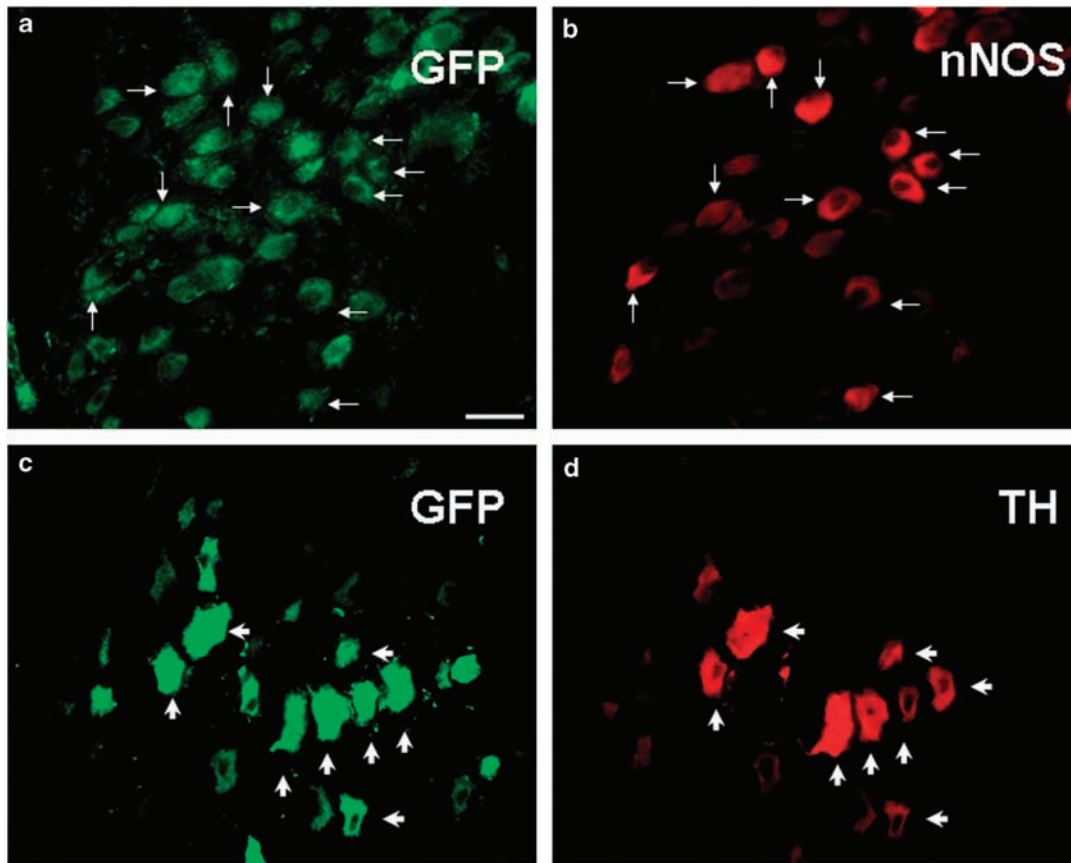


Figure 2 Characterization of GFP-positive cells in MPG by a double-labeling study. GFP immunostaining (a) combined with nNOS immunostaining (b) in the same section and GFP immunostaining (c) combined with TH immunostaining (d) in the same section. Arrows indicate GFP- and nNOS-positive neurons. Arrowheads indicate GFP- and TH-positive neurons. Scale bar, 50 μ m. Reduced from $\times 400$. GFP, green fluorescent protein; MPG, major pelvic ganglion; nNOS, neuronal nitric oxide synthase; TH, tyrosine hydroxylase.

To further characterize the nNOS-negative GFP-positive neurons, we carried out tyrosine hydroxylase (TH) immunostaining, which can identify sympathetic MPG neurons, combined with GFP immunostaining for the presence of the virus (Figures 2c and d). The results of this TH staining demonstrated that approximately 24% of GFP-positive MPG neurons were positively stained for TH.

Changes of ICP/AP after HSV-NTN treatment

We then measured ICP/AP as a means of assessing the functional activity of the HSV neurturin expression vector, HSV-NTN, in this animal model of ED. The mean ICP/AP of normal rats was 0.64 ± 0.04 (mean \pm s.e.m.). ICP/AP of nerve-injured rats decreased with time, and the mean ICP/AP of 2- and 4- week postoperative animals were 0.24 ± 0.05 and 0.17 ± 0.03 , respectively, both significantly lower than those of normal rats ($P < 0.05$). HSV-NTN-treated groups received pericavernous injections of 2×10^7 , 2×10^5 and 2×10^3 total plaque-forming unit (PFU) in a final volume of 20 μ l. Vector was delivered immediately following nerve injury procedure. Control HSV-LacZ-treated group received 2×10^7 total PFU of QOZHG in a total volume of 20 μ l. At 4 weeks after the nerve injury, ICP/AP of the HSV-LacZ group (0.15 ± 0.01) was similar to that of the untreated nerve-injured group (Figure 3). ICP/AP value of the HSV-NTN 2×10^7 group (0.28 ± 0.04) was approximately twice as

high as that of either the HSV-LacZ group ($P = 0.0072$) or the untreated nerve-injured group ($P = 0.0482$). ICP/AP value of the HSV-NTN 2×10^5 or 2×10^3 group (0.26 ± 0.04 and 0.24 ± 0.04) tended to be higher than that of the HSV-LacZ group, but did not reach statistical significance compared with the untreated nerve-injured group ($P = 0.0820$ and 0.1409 , respectively).

FG-positive cells in MPG

To correlate function (ICP/AP) with penile nerve regeneration, a retrograde tracing study using FG was performed as reported earlier.^{5,46,47} We determined the number of FG-positive cells in animals from each treatment group. In the normal MPG, 90–100 MPG neurons per sections were FG-positive 7 days after FG injection in the penile crus (Figure 4a), as similarly reported in earlier studies.^{5,47} The average ratio of FG-positive cells among MPG cells was relatively constant (approximately 30% of total MPG cells) among animals examined although the number of FG-positive cells may be underestimated because of the different uptake efficacies of FG caused by spreading and/or leakage of the injected FG. Two weeks after nerve injury, the number of FG-positive cells significantly decreased and only a few FG-positive neurons remained 4 weeks after nerve injury (Figure 4b). Four weeks after treatment with 2×10^7 HSV-NTN following nerve injury, the number of FG-positive cells was approximately twice as high as that

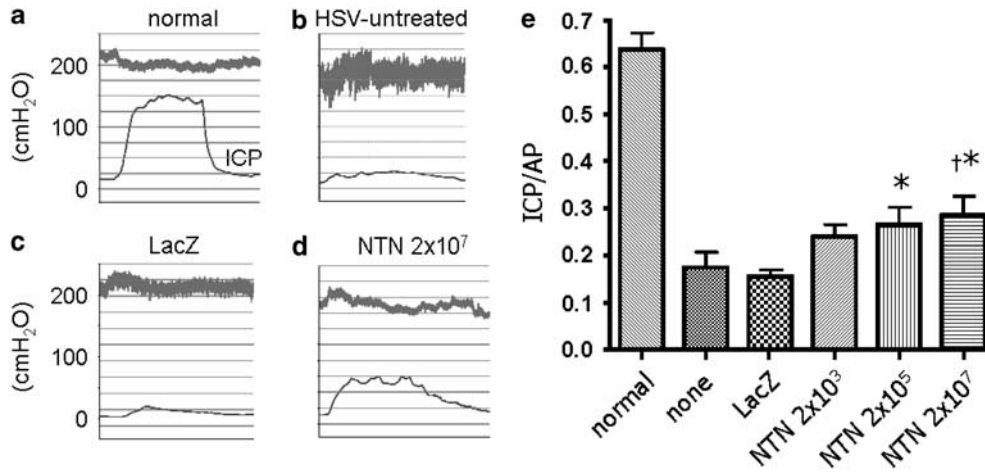


Figure 3 ICP/AP at 4 weeks after the bilateral cavernous nerve injury. Intracavernous pressure (ICP) and simultaneous arterial pressure (AP) of the normal group (a), HSV-untreated nerve-injured group (b), nerve-injured HSV-LacZ control vector group (c) and nerve-injured HSV-NTN 2×10^7 group (d). (e) Rats treated with higher titers (2×10^5 and 2×10^7 PFU) of HSV-NTN exhibited significant recovery of ICP/AP compared with the HSV-LacZ control vector group or HSV-untreated nerve-injured group at 4 weeks after the nerve injury. Crossmark indicates $P < 0.05$ versus 'none' (virus-untreated group) by Student's *t*-test and analysis of variance (ANOVA). Asterisk indicates $P < 0.05$ versus 'LacZ' (HSV-LacZ group) by Student's *t*-test and ANOVA. HSV, herpes simplex virus; NTN, neurturin; PFU, plaque-forming unit.

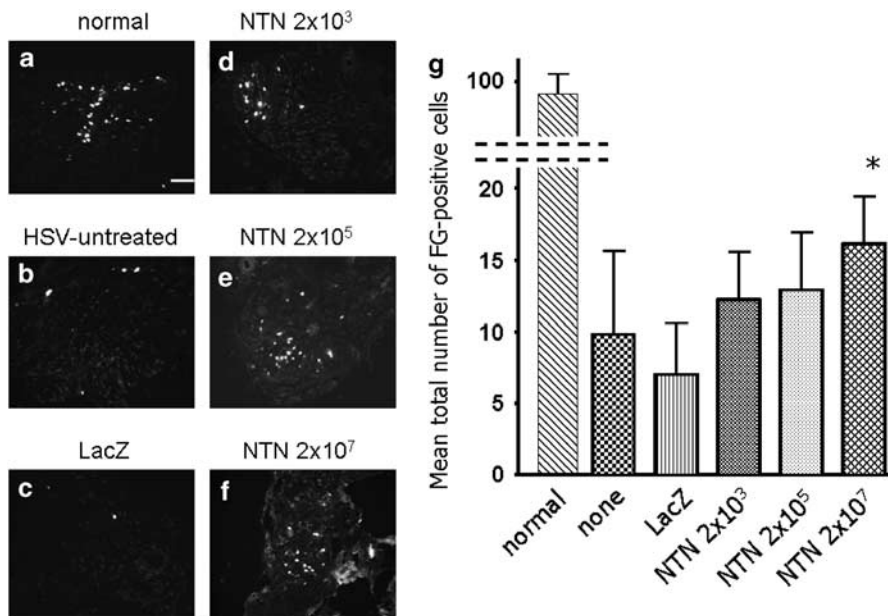


Figure 4 FG-positive cells at 4 weeks after the bilateral cavernous nerve injury. FG-positive cells in MPG of the normal group (a), HSV-untreated nerve-injured group (b), nerve-injured HSV-LacZ control vector group (c), nerve-injured HSV-NTN 2×10^3 group (d), nerve-injured HSV-NTN 2×10^5 group (e) and nerve-injured HSV-NTN 2×10^7 group (f). Scale bar, 100 μ m. Reduced from $\times 100$. (g) The HSV-NTN 2×10^7 group had more FG-positive cells in MPG than the HSV-LacZ group. 'None' means virus-untreated group. Asterisk indicates $P < 0.05$ versus 'LacZ' (HSV-LacZ group) by Student's *t*-test and ANOVA. ANOVA, analysis of variance; FG, fluorogold; HSV, herpes simplex virus; MPG, major pelvic ganglion; NTN, neurturin.

of the control HSV group ($P = 0.0205$) or untreated nerve-injured group ($P = 0.0434$) (Figures 4c–g). The HSV-NTN 2×10^5 or 2×10^3 group did not significantly increase the number of FG-positive cells compared with the control HSV-LacZ group ($P = 0.1011$ and 0.1564 , respectively).

Discussion

Neurturin (NTN) is a member of the GDNF family and has been known as a target-derived survival and/or

neurotrophic factor for postganglionic neurons innervating the penis.^{17–20} Several studies suggested that NTN would be a specific neurotrophic factor for parasympathetic penis-projecting neurons acting through its receptor, GFR α 2, whereas GDNF acts on a broader range of MPG neurons through GFR α 1.^{17–20} We applied a newly constructed HSV vector expressing NTN gene (HSV-NTN) to our nerve injury-related ED model.⁴³

Our functional studies revealed that the ICP/AP values of nerve-injured animals treated with HSV-NTN were higher than those of the control

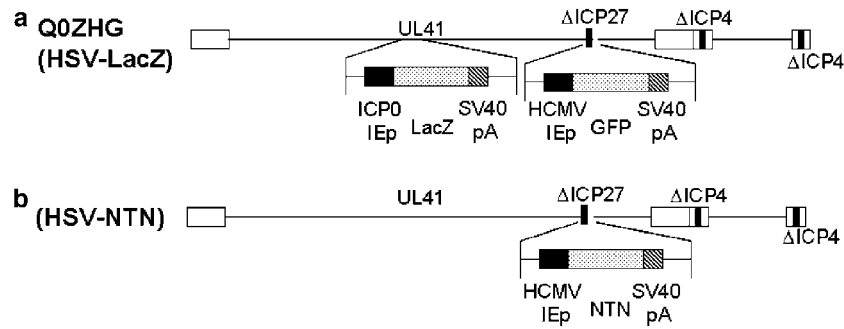


Figure 5 Replication-deficient HSV vectors. QOZHG is a replication-defective HSV vector that expresses lacZ and GFP (a). HSV-NTN contains NTN instead of GFP in the same locus (b). GFP, green fluorescent protein; HSV, herpes simplex virus; NTN, neurturin.

HSV-treated and -untreated nerve-injured groups. In addition, we carried out a retrograde tracing study using FG to assess the degree of nerve preservation/regeneration by examining the number of MPG cells retrogradely labeled by FG injected into the penile crus following cavernous nerve injury. We found a significant improvement in nerve integrity suggesting that delivery of NTN through a replication-defective HSV vector is capable of rescuing nerve damage and ED.

We previously performed similar experiments using HSV-GDNF vectors and found that ICP/AP values of HSV-GDNF (2×10^5 and 2×10^7 PFU)-treated rats were 0.37 ± 0.07 and 0.43 ± 0.04 , respectively.⁴³ These values were higher than those of the HSV-NTN 2×10^5 and 2×10^7 groups, respectively, in this study. Although the GDNF and NTN vectors have not been compared in a single experiment, individual results may indicate that GDNF has stronger effects on cavernous nerve survival or regeneration than NTN. Some previous reports seem to support this assumption. All of MPG neurons expressing vasoactive intestinal polypeptide, a marker for parasympathetic MPG neurons, expressed GFR α 1, which is known to be a GDNF receptor.²⁰ On the other hand, TH-positive sympathetic MPG neurons do not express GFR α 2, an NTN-specific receptor.¹⁸ It is also reported that NTN did not elicit neurite growth in sympathetic neurons or downregulate TH expression.¹⁹ Meanwhile, nerve sprouting is seen in sympathetic neurons as well as in parasympathetic neurons during cavernous nerve regeneration,⁴⁶ suggesting that not only parasympathetic nerve regeneration, but also sympathetic nerve regeneration is important to restore erectile function after cavernous nerve injury although the functional role of penile sympathetic nerves is to induce penile detumescence. Furthermore, GDNF has also been reported to upregulate TH expression.⁴⁸ Taken together, it is assumed that the HSV-GDNF treatment, which can induce sympathetic nerve regeneration, might have an additive effect on cavernous nerve injury-induced ED and that combined therapies using HSV-GDNF and HSV-NTN, which specifically target penile-projecting parasympathetic nerves through GFR α 2 receptors, might act in concert to enhance regenerative responses of injured cavernous nerves.

In conclusion, our results demonstrated that retrograde transport of HSV vector-mediated neurotrophic factor NTN to MPG neurons occurred after the administration of the vector around the cavernous nerves during nerve injury. HSV-NTN vector administration

around the injured cavernous nerve can hasten the process of nerve regeneration and promote the recovery of erectile function (ICP/AP) after cavernous nerve injury. NTN would be one of the possible factors that could be used for this gene therapy application.

Materials and methods

Animals

All animals used in this study were virus-free male Sprague–Dawley rats (250–350 g; Hilltop Laboratory, Pittsburgh, PA, USA). All experiments were performed under the University of Pittsburgh Institutional Animal Care and Use Committee (IACUC)-approved protocols and guidelines.

Viral vectors

The human Neurturin genomic regions were amplified by PCR and cloned into plasmid pCR-8 (Invitrogen, Carlsbad, CA, USA). The nature of the NTN-pCR-8 plasmid was confirmed by restriction analysis and direct sequencing of the plasmid using primers (YY). The NTN locus was recombined into an ICP4, ICP27-deleted vector backbone and the genomic organization confirmed by restriction analysis and sequencing into the NTN gene (Figure 5). NTN expression from the HSV-NTN vector was confirmed by immunohistochemistry on non-complementing Vero cells transduced with either HSV-NTN or control vector QOZHG.⁴⁹ The anti-NTN antibody (X) was used at 1:500 and secondary anti-mouse fluorescein isothiocyanate (Sigma) used at 1:2000 (Data not shown). The control HSV vector, QOZHG,⁴⁹ expresses both GFP and lacZ as gene markers (Figure 5).

The HSV-NTN vector was produced by infecting ICP4- and ICP27-complementing cells, in T-150 flasks in serum-free medium at a multiplicity of infection of 0.1 PFU per cell. The culture was harvested when the infected cells were rounding up and coming off the surface, approximately 3 days postinfection. The harvest was salt-treated, centrifuged to remove cell debris, filtered, and the virus pelleted from the filtrate by centrifugation at 20 000 g for 40 min. The pellet was resuspended in phosphate-buffered saline (PBS), filtered and purified on an ion exchange column. The eluted virus was diluted with 10% glycerol, aliquotted and frozen at -80°C . The stock was titered in complementing cells in duplicate.

Cavernous nerve injury model

Animals were anesthetized with pentobarbital (45 mg kg⁻¹). Through a lower abdominal midline incision, bilateral MPG and cavernous nerves were identified and exposed. The cavernous nerves (approximately 5 mm distal to the MPG) were first crushed using a pair of angled Dumont forceps (Fine Science Tools Inc., Foster City, CA, USA) for 10 s. Then a small solid piece of dry ice was gently applied to the cavernous nerve for at least 30 s. The procedure was performed bilaterally.

In vivo viral administration

HSV vector suspension (20 µl) was applied directly to and around the injured cavernous nerves (10 µl per each side) immediately after the nerve injury using a micropipette. The procedure was performed carefully not to result in further damage to the cavernous nerves. Abdominal wounds were then closed with sutures, and animals were allowed to recover from anesthesia.

Fluorogold injection

Fluorogold injection was performed 7 days before the functional and histological studies. Animals were anesthetized with pentobarbital (45 mg kg⁻¹). The skin beside the penis was narrowly incised and 4 µl of FG (4.0%) was injected into the right penile crus by using a 30 G Hamilton syringe. The needle was left inserted into the corpus cavernosum for 3 min to prevent leakage, and the injected site was immediately closed with instant glue.

Intracavernous and arterial pressure measurement

At 2 or 4 weeks after the nerve injury in animals with or without HSV vector administration, animals were again anesthetized with pentobarbital (45 mg kg⁻¹). The skin of the left neck was opened and a PE50 tube was inserted into the left carotid artery to monitor arterial pressure. Bilateral MPG and pelvic nerves were exposed as described above, and the incision was extended to the skin around the penis. A 23 G needle connected to a PE50 tube filled with heparinized saline (250 IU ml⁻¹) was inserted into the left penile crus. Electrostimulation (20 Hz, pulse width 0.5 ms, 10 V) of the pelvic nerve was applied with a bipolar hook electrode and changes of ICP were measured. Rats were fully anesthetized during the procedure and no body movement or distress was observed during electrical nerve stimulation. The best ICP value was divided by simultaneous mean arterial pressure (ICP/AP), which was measured during the period of electrostimulation of the pelvic nerve, because pelvic nerve stimulation possibly increased AP due to the activation of pelvic nerve afferents to induce pressor responses such as those known as the vesico-vascular reflex.⁵⁰ ICP/AP was measured at 2 or 4 weeks after nerve injury. After ICP measurement, the bilateral MPG was dissected and histological studies were performed.

Experimental groups

We divided the nerve-injured animals into several groups at each time point (2 and 4 weeks after the nerve injury). In the virus-untreated group ($N=10$), bilateral cavernous nerves were injured and no further procedures were given. In the HSV-LacZ group ($N=10$),

HSV-LacZ at a high titer (20 µl of 1×10^9 PFU per ml = 2×10^7 total PFU) was administered around the injured nerves. In the HSV-NTN group, HSV-NTN vectors were administered around the injured nerves and a dose-escalation study was performed using 20 µl of 1×10^9 (2×10^7 total PFU), 1×10^7 (2×10^5 total PFU) and 1×10^5 PFU per ml (2×10^3 total PFU) of HSV-NTN vectors ($N=10$ each). Ten normal rats that received no surgery, were also evaluated as controls.

Immunohistochemistry

After functional experiments, MPG was dissected and immediately fixed in an ice-cold 4% paraformaldehyde solution containing 0.21% picric acid in 0.1 M phosphate buffer (PB) for about 48 h, and then immersed in 0.1 M PB containing 25% sucrose. Serial transverse sections were cut at 12-µm thickness on a cryostat and thaw-mounted sequentially on six gelatin-coated slides (10–12 sections per slide), so that mounted MPG sections in each slide had intervals of 70–80 µm. The sections were air-dried, rinsed in 0.1 M PBS and then pre-incubated for 10 min in 0.1 M PBS containing 1% bovine serum albumin. The sections were then incubated with primary antibodies diluted in 0.1 M PBS containing 1% bovine serum albumin and 0.1% Triton X-100 for 12–24 h in a humidity chamber at 4 °C. The following primary antibodies were used: rabbit anti-GFP (1:200; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and mouse nNOS (1:500; Santa Cruz Biotechnology) or mouse anti-TH (1:1000; Immunostar, Hudson, WI, USA). After rinsing in 0.1 M PBS, the sections were incubated with biotinylated anti-rabbit immunoglobulin G (1:500; Vector Labs, Burlingame, CA, USA) and anti-mouse fluorescent Cy3-conjugated Fragment IgG (1:500; Jackson ImmunoResearch Laboratories, West Grove, PA, USA) diluted in 0.1 M PBS containing 1% bovine serum albumin and 0.1% Triton X-100 for 12–24 h in a humidity chamber at 4 °C. After rinsing in 0.1 M PBS, the sections were incubated with Alexa Fluor 488-conjugated strepto-avidin (1:500; Molecular Probes Invitrogen, Carlsbad, CA, USA) diluted in 0.1 M PBS for 1 h at room temperature. After rinsing in 0.1 M PBS, the sections were coverslipped.

Imaging and quantification

Images of sections were captured directly off the microscope using a digital camera and composed using Adobe Photoshop (R) 7.0J. FG-positive cells were identified through a wide-band ultraviolet filter. GFP-positive cells were identified through a green fluorescent filter and nNOS- or TH-positive cells were identified through a red fluorescent filter. We randomly selected one of six slides and evaluated neuronal profiles of more than 300 cells that had the nucleus in all MPG sections, which had intervals of 70–80 µm between sections, in the selected slides so that we could examine the almost entire MPG and avoid double counting of neurons. We examined and counted apparent positive neurons and disregarded those with weak and ambiguous reactions. The total neurons in each section were also calculated. A total of 10 MPG in each group was evaluated.

Statistical analysis

All values are presented as mean ± s.e.m. Statistical analyses and comparisons among groups were performed using Student's *t*-test where applicable. With

regard to the dose-escalation study, we also used one-way analysis of variance followed by Dunnett's multiple comparison test where applicable. A probability level of less than 0.05 was accepted as significant.

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