

THE EFFECT OF ADENO-ASSOCIATED VIRUS MEDIATED BRAIN DERIVED NEUROTROPHIC FACTOR IN AN ANIMAL MODEL OF NEUROGENIC IMPOTENCE

MUSTAFA EMRE BAKIRCIOGLU, CHING-SHWUN LIN, PEIDONG FAN, KARL-DIETRICH SIEVERT, YEUT W. KAN AND TOM F. LUE*

From the Departments of Urology and Laboratory Medicine, University of California School of Medicine, San Francisco, California, and Klinik und Poliklinik für Urologie, Westfälische Wilhelms-Universität Münster, Münster, Germany

ABSTRACT

Purpose: We tested the hypothesis that transfecting penile tissue with brain derived neurotrophic factor may facilitate neural recovery and erectile capability after cavernous nerve injury.

Materials and Methods: Of the 34 Sprague-Dawley rats used 10 underwent sham operation and 24 underwent bilateral cavernous nerve freezing and intracavernous injection of adeno-associated virus-LacZ (12) or adeno-associated virus-brain derived neurotrophic factor (12). Erectile function was assessed by cavernous nerve electrostimulation at 4 and 8 weeks, and samples of penile tissue and the major pelvic ganglia were evaluated histologically.

Results: In the brain derived neurotrophic factor group mean maximal intracavernous pressure plus or minus standard deviation was significantly higher than in the LacZ group at 4 and 8 weeks (58.5 ± 11.7 cm. water versus 28.4 ± 5.5 and 61.3 ± 12.5 versus 37.7 ± 7.9 , respectively). In addition, in the brain derived neurotrophic factor group reduced nicotinamide adenine dinucleotide phosphate diaphorase staining and neuronal nitric oxide synthase immunostaining revealed significantly more positive nerve fibers in the dorsal nerves and cavernous tissue than in the LacZ group at each time point and the percent of neuronal nitric oxide synthase positive neurons in the major pelvic ganglia was also significantly greater. Moreover, in the LacZ group most neurons showed a light staining pattern with irregular contours and numerous vacuoles in the cytoplasm.

Conclusions: Intracavernous injection of adeno-associated virus-brain derived neurotrophic factor may prevent the degeneration of neuronal nitric oxide synthase containing neurons in the major pelvic ganglia and facilitate the regeneration of neuronal nitric oxide synthase containing nerve fibers in penile tissue, thus, enhancing the recovery of erectile function after bilateral cavernous nerve injury.

KEY WORDS: penis; impotence; rats, Sprague-Dawley; brain-derived neurotrophic factor; nerve regeneration

Because of the close proximity of the cavernous nerves to the capsule of the prostate, erectile dysfunction is a common complication after radical prostatectomy or cystectomy and prostatic cryosurgery. Although the nerve sparing prostatectomy technique developed by Walsh and Mostwin¹ has significantly reduced the postoperative impotence rate, a large number of patients still have inadequate penile rigidity. Peripheral nerve regeneration is a slow process and the fact that most patients do not recover potency for 6 months to 2 years indicates substantial axonal damage, even with preservation of the neural sheath. The anatomical study of the cavernous nerves of Paick et al revealed a medial and a lateral bundle of cavernous nerves at the level of the prostate, suggesting that in some cases the lateral bundle may be saved, even in nonnerve sparing prostatectomy.²

We have hypothesized that sprouting of the remaining nerves in penile tissue may be more important for regeneration than the regrowth of nerves through the damaged and fibrotic tissues. This concept was confirmed in an animal study that revealed regeneration of the cavernous nerves after unilateral resection.³ In addition, a previous study at our laboratory showed that systemic growth hormone injection

significantly enhanced cavernous nerve regeneration after unilateral injury.⁴ However, our enthusiasm was limited by concerns about the side effects of systemic growth hormone administration and the possibility of enhanced cancer cell growth. Meanwhile we also noted up-regulation of brain derived neurotrophic factor (BDNF) gene expression in rats that had undergone unilateral cavernous neurotomy (unpublished data). This finding prompted us to examine the feasibility of using BDNF to facilitate regeneration of the cavernous nerves.

BDNF was initially characterized as a basic protein present in brain extracts capable of increasing the survival of dorsal root ganglia.⁵ When axonal communication with the cell body is interrupted by injury, Schwann cells produce neurotrophic factors, such as nerve growth factor and BDNF. Neurotrophins are released from the Schwann cells and dispersed diffusely in gradient fashion around regenerating axons, which then extend distally along the density gradient of the neurotrophins.⁶ Local application of BDNF to transected nerves in neonatal rats has been shown to prevent the massive death of motor neurons that follows axotomy.^{7–9} The messenger RNA titer of BDNF increases to several times the normal level 4 days after axotomy and reaches its maximum at 4 weeks.¹⁰ Moreover, BDNF has been reported to enhance the survival of cholinergic neurons in culture.¹¹ In this study we transfected penile tissue by injecting adeno-associated virus (AAV)-BDNF into the corpus cavernosum to effect the

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* Requests for reprints: Department of Urology, University of California, San Francisco, California 94143-0738.

continuous production of BDNF in the penile tissue for several weeks.

MATERIALS AND METHODS

Animals. A total of 34 male Sprague-Dawley rats 3 months old weighing 350 to 400 gm. were divided into a sham operation group of 10 and an experimental group of 24. The rats in the sham operation group underwent periprostatic dissection and identification of bilateral cavernous nerves without other manipulation, and those in the experimental group underwent bilateral cavernous nerve freezing. Several minutes after surgery 12 experimental animals (LacZ group) received intracavernous AAV-LacZ injection and the remaining 12 (BDNF group) received AAV-BDNF. Half of the rats in each group were sacrificed at weeks 4 and 8, respectively, for penile tissue collection. In all animals erectile function was assessed by electrostimulation of the cavernous nerves before sacrifice.

Surgical procedure and transfection technique. Using intraperitoneal anesthesia (50 mg./kg. pentobarbital sodium) each animal was placed on a heating pad to maintain body temperature at 37°C. Through a lower abdominal midline incision the area posterolateral to the prostate was explored. The major pelvic ganglia and cavernous nerve were identified with an operating microscope at 10× to 40×. In the experimental group the cavernous nerve was frozen bilaterally for 1 minute with a thermocouple used to control the temperature (5 mm. diameter handheld microprocessor digital thermometer). Before surgery the thermocouple had been placed in a 15 ml. disposable centrifuge tube filled with ground dry ice and maintained in a thermal flask filled with dry ice. The temperature of the probe at the beginning of the procedure was -80°C, increasing to -50°C at 1 minute. To prevent disruption of the nerve 0.2 ml. saline were used to disengage the tip of the probe from the nerve before removal. After the freezing procedure the right side of the proximal crus was exposed and 0.05 ml. of 10¹⁰ AAV-LacZ or 10¹⁰ AAV-BDNF were injected into 12 rats each through a tuberculin syringe with a 30 gauge needle.

Preparation of AAV-BDNF. Cloning of BDNF Complementary (c)DNA: We used reverse transcriptase-polymerase chain reaction to identify BDNF expression in the human neuroblastoma cell line SK-N-BE(2). We then used the primer pair 5'-CCCTACAGGTCGACCAGGTGA and 5'-CTATAACAATGGATCCACTA to amplify the coding sequence of BDNF from SK-N-BE(2) cDNA. The sequences GTCGAC and GGATCC were designed SalI and BamHI restriction sites, respectively. After digestion with SalI and BamHI the amplified product was cloned into pBluescript (Stratagene, Inc., La Jolla, California) and fully sequenced. BDNF cDNA was then re-cloned into pcDNA4, a modified version of pcDNA3 plasmid (Invitrogen, Inc., Carlsbad, California) that contains the cytomegalovirus promoter for driving the expression of BDNF in mammalian cells.

Construction of rAAV-BDNF: The aforementioned pcDNA4BDNF was digested with SalI to release the expression cassette containing cytomegalovirus immediate-early promoter, BDNF gene and bovine growth hormone poly-A signal. This expression cassette was inserted into the AAV vector pAV53, resulting in the construction of rAAV-BDNF.

Virus Production and Titration: rAAV-BDNF was produced by a 3-plasmid co-transfection method. A total of 20, 15 cm. plates of 293 cells each (50% to 60% confluent) were maintained in Dulbecco's modified Eagle's medium (Gibco BRL, Grand Island, New York) supplemented with 10% fetal bovine serum and 25 mM. HEPES, and co-transfected by the calcium phosphate method with 15 µg. AAV-BDNF vector, and 15 µg. each of pLHP19 (AAV helper plasmid) and pLaden5 (adenovirus helper plasmid). Six hours after transfection the medium was replaced with fresh Dulbecco's modified

Eagle's medium containing 1% fetal bovine serum. Cells were harvested 48 hours after transfection by centrifugation at 1,000 × gravity for 10 minutes. The cell pellets were resuspended in 0.1 M. tris-HCl, 0.15 M. NaCl solution, pH 8.0, and subjected to 4 cycles of freeze-thaw and removal of cell debris. Large-scale rAAV CsCl purification was performed as described previously.¹² AAV-BDNF vector titer was determined by quantitative dot blot hybridization of deoxyribonuclease treated stocks. The AAV vector titer used in the experiment refers to the particle number of AAV vector genomes in the sample, as determined by the quantitative dot blot assay.

Functional evaluation and tissue procurement. At weeks 4 and 8 postoperatively rats in each group underwent repeat exploration for direct electrostimulation of the cavernous nerves before tissue collection. The skin overlying the penis was incised and the ischiocavernous muscle was partly removed to expose the penile crura. A 23 gauge butterfly needle connected to polyethylene-50 tubing was inserted in the right crus for pressure measurement. Electrostimulation was performed with a delicate stainless steel bipolar hook electrode attached to a multi-joint clamp. Each pole was 0.2 mm. in diameter and the 2 poles were separated by 1 mm. Short wave pulses were generated by a Macintosh computer (Apple, Sunnyvale, California) with a custom built constant current amplifier. Stimulus parameters were 1.5 mA., frequency 20 Hz., pulse width 0.2 msec. and duration 50 seconds. Each cavernous nerve was stimulated and intracavernous pressure was measured and recorded with a Macintosh computer programmed with LabVIEW 4.0 software (National Instruments, Austin, Texas). The pressure in each animal was determined as the mean of the 2 sides.

Reduced nicotinamide adenine dinucleotide phosphate (NADPH) diaphorase staining. After sacrifice samples of the major pelvic ganglia and penile tissue were fixed for 3 hours in a cold, freshly prepared solution of 2% formaldehyde and 0.002% picric acid in 0.1 M. phosphate buffer, pH 8.0. Tissues were cryoprotected for 24 hours in cold 30% sucrose in 0.1 M. phosphate buffer, pH 8.0. They were then embedded in OCT compound, frozen in liquid nitrogen and stored at -70°C. Cryostat tissue sections were cut at 10 µm., adhered to charged slides, air dried for 5 minutes and hydrated for 5 minutes with 0.1 M. PO₄, pH 8.0. Sections were incubated with 0.1 mM. NADPH, 0.2 mM. nitroblue tetrazolium and 0.2% Triton X-100 in 0.1 M. PO₄, pH 8.0, for 60 minutes at room temperature. The reaction was terminated by washing in buffer. Coverglasses were added to slides with buffered glycerin as the mounting medium.¹³

The presence of NADPH diaphorase positive nerves was evidenced as a blue stain in the major pelvic ganglia, dorsal nerves and cavernous tissue. The staining pattern was assessed by counting the number of positive neurons in 4 random fields at 400× magnification. The percent of dark and light stained cells in the major pelvic ganglia was calculated by dividing the number of these cells by the total number of positive cells.

Nitric oxide synthase antibody staining. Tissue fixation was the same as for NADPH diaphorase specimens. After freezing 10 µm. cryostat tissue sections were adhered to charged slides, air dried and hydrated for 5 minutes with 0.05 M. phosphate buffered saline (PBS), pH 7.4. Sections were treated with hydrogen peroxide/methanol to quench endogenous peroxidase activity. After rinsing with water sections were washed twice in PBS for 5 minutes, followed by 30 minutes of room temperature incubation with 3% horse serum/PBS/0.3% triton X-100. After draining solution from the sections tissues were incubated for 60 minutes at room temperature with mouse monoclonal anti-neuronal nitric oxide synthase (Transduction Laboratories, Lexington, Kentucky) at 1:500 dilution. After washing for 5 minutes with PBS/TX and then for 5 minutes twice with PBS alone sections were

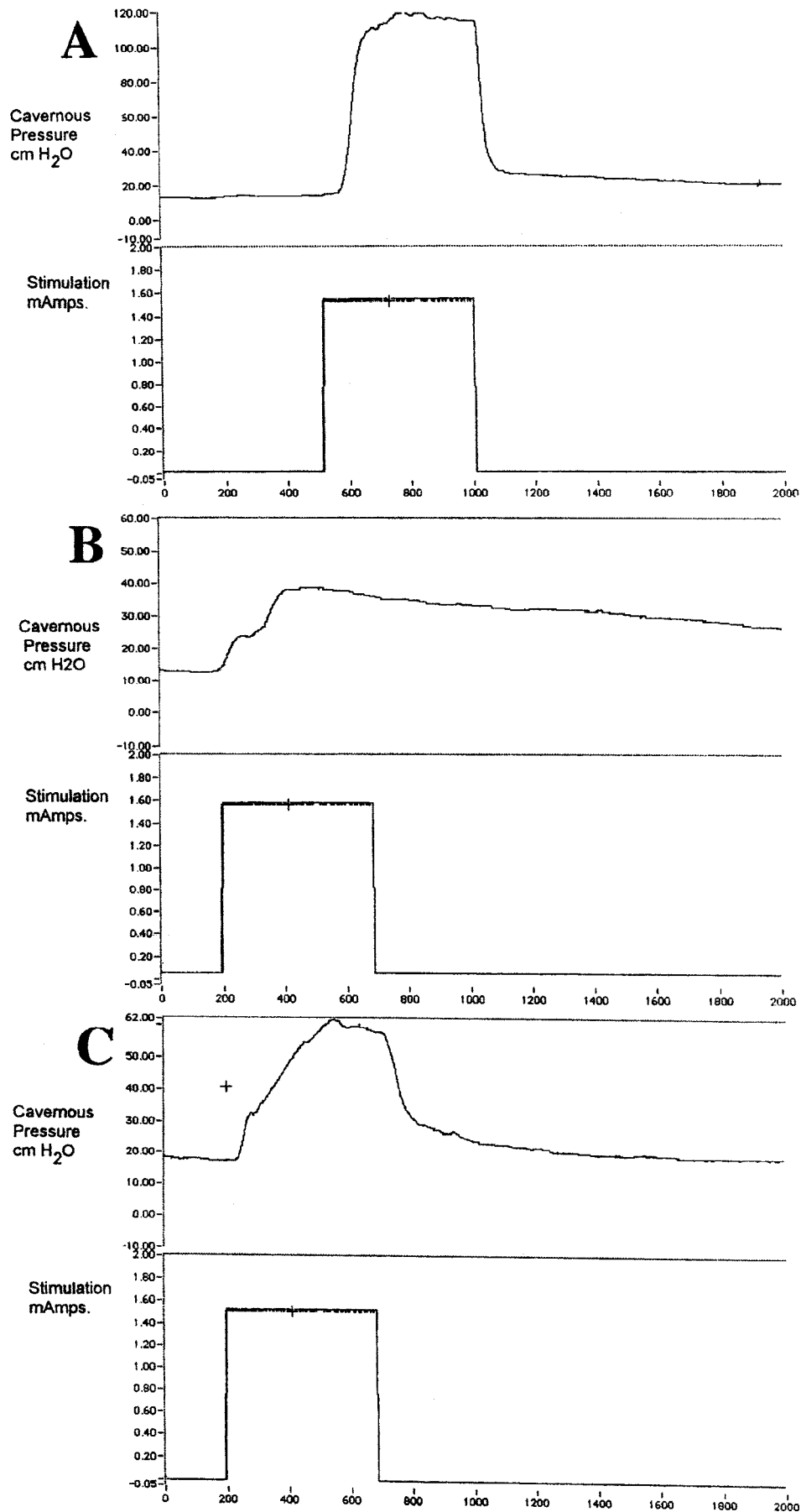


FIG. 1. Electrostimulation of cavernous nerve at 8 weeks at rate of 10 scans per second. A, sham operation group. B, LacZ group. C, BDNF group with higher maximal intracavernous pressure than LacZ group.

immunostained with the avidin-biotin-peroxidase method using an Elite ABC kit (Vector Laboratories, Burlingame, California) with diaminobenzidine as the chromagen, followed by counterstaining with hematoxylin. The nonparametric Mann-Whitney U test and analysis of variance using Statview 4.02 software were done to compare results with $p < 0.05$ considered statistically significant.

RESULTS

Functional studies. In the LacZ and BDNF groups maximal intracavernous pressure in response to bilateral cavernous nerve electrostimulation was less than in the sham operation group. However, the pressure in the BDNF group was significantly higher than in the LacZ group at 4 and 8 weeks (fig. 1 and table 1).

NADPH diaphorase staining. Dorsal Nerve: At week 4 the LacZ group showed significantly fewer NADPH diaphorase positive nerve fibers than the BDNF group. At week 8 the number had increased in each group but there were still significantly fewer in the LacZ group (fig. 2). Compared with the sham operation group the 2 experimental groups showed fewer positive nerve fibers (table 2).

Intracavernous Nerves: Histological evaluation showed significantly fewer NADPH diaphorase positive nerve fibers in the trabecular smooth muscle of the LacZ group than in the BDNF group at 4 and 8 weeks. Compared with the sham

operation group the 2 experimental groups had significantly fewer positive stained nerve fibers (fig. 3 and table 2).

Major Pelvic Ganglia: At week 4 most neurons in the major pelvic ganglia in the LacZ group showed a lighter staining pattern than in the BDNF group (fig. 4). In addition, at 8 weeks most of these LacZ neurons had an irregular cell contour and multiple vacuoles in the cytoplasm (fig. 5). The percent of dark stained cells in the BDNF group at each time point was significantly higher than in the LacZ group and their appearance with a smooth contour and few vacuoles in the cytoplasm was similar to that in the sham operation group. In the 2 experimental groups the percent of dark stained cells was significantly less than in the sham operation group (table 3).

Neuronal nitric oxide synthase immunostaining. Immunostaining of penile tissue and the major pelvic ganglia for neuronal nitric oxide synthase revealed positive staining in the same nerve fibers and neurons as for NADPH diaphorase (fig. 6). The neurons of the major pelvic ganglia in the LacZ group showed lighter staining patterns and many more vacuoles in the cytoplasm than the neurons in the BDNF and sham operation groups (fig. 7).

TABLE 1. Maximal intracavernous pressure in response to electrostimulation 4 and 8 weeks after bilateral cavernous nerve freezing

No. Wks.	10 Sham Operated Subjects (mean cm. water \pm SD)	12 LacZ Subjects (mean cm. water \pm SD)	12 BDNF Subjects (mean cm. water \pm SD)*
4	105 \pm 10.5	28.4 \pm 5.5	58.5 \pm 11.7
8	115 \pm 7.7	37.7 \pm 7.9	61.3 \pm 12.5

* Versus LacZ $p < 0.05$.

TABLE 2. NADPH diaphorase positive nerve fibers in the dorsal nerve and cavernous tissue

No. Wks.	10 Sham Operated Subjects (mean cm. water \pm SD)	12 LacZ Subjects (mean cm. water \pm SD)	12 BDNF Subjects (mean cm. water \pm SD)*
<i>Dorsal nerve</i>			
4	138.5 \pm 10.5	45.7 \pm 5.8	65.5 \pm 15.5
8	140.2 \pm 9.8	55.6 \pm 8.4	86.4 \pm 12.2
<i>Cavernous tissue</i>			
4	103 \pm 9.8	25.5 \pm 3.6	45.5 \pm 10.5
8	110.2 \pm 10.5	35.6 \pm 10.4	66.1 \pm 15.2

* Versus LacZ $p < 0.05$.

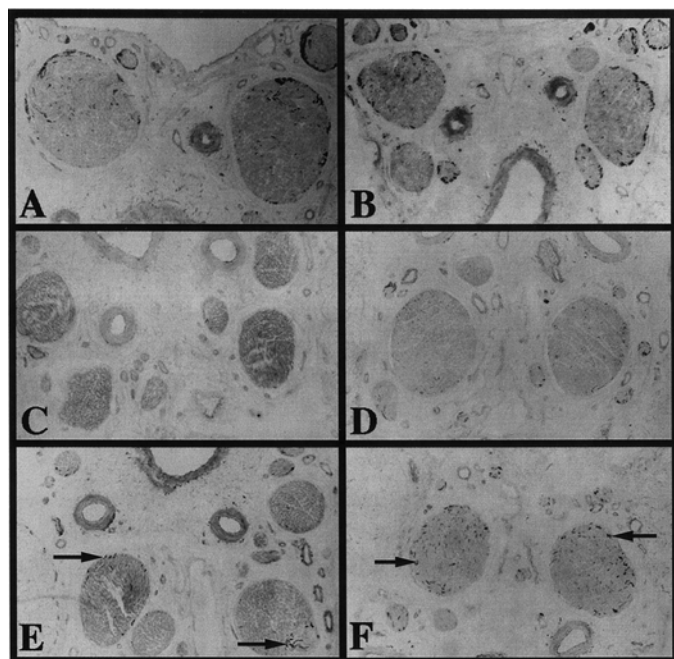


FIG. 2. NADPH diaphorase staining of dorsal nerves. A, sham operation group at 4 weeks. B, sham operation group at 8 weeks. C, LacZ group at 4 weeks. D, LacZ group at 8 weeks. E, BDNF group at 4 weeks with greater number of positive nerve fibers (arrows) than in LacZ group. F, BDNF group at 8 weeks with greater number of positive nerve fibers (arrows) than LacZ group. Reduced from $\times 100$.

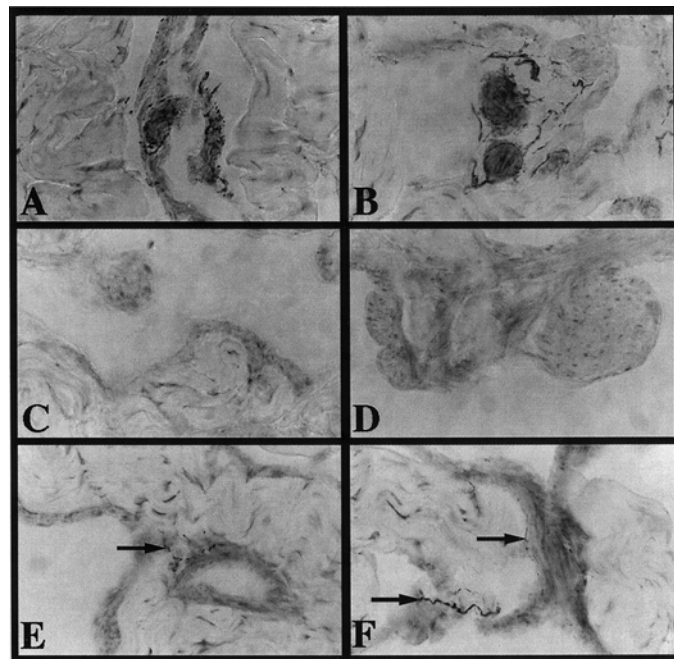


FIG. 3. NADPH diaphorase staining of cavernous tissue. A, sham operation group at 4 weeks. B, sham operation group at 8 weeks. C, LacZ group at 4 weeks. D, LacZ group at 8 weeks. E, BDNF group at 4 weeks with greater number of positive nerve fibers (arrows) than in LacZ group. F, BDNF group at 8 weeks with greater number of positive nerve fibers (arrows) than LacZ group. Reduced from $\times 400$.

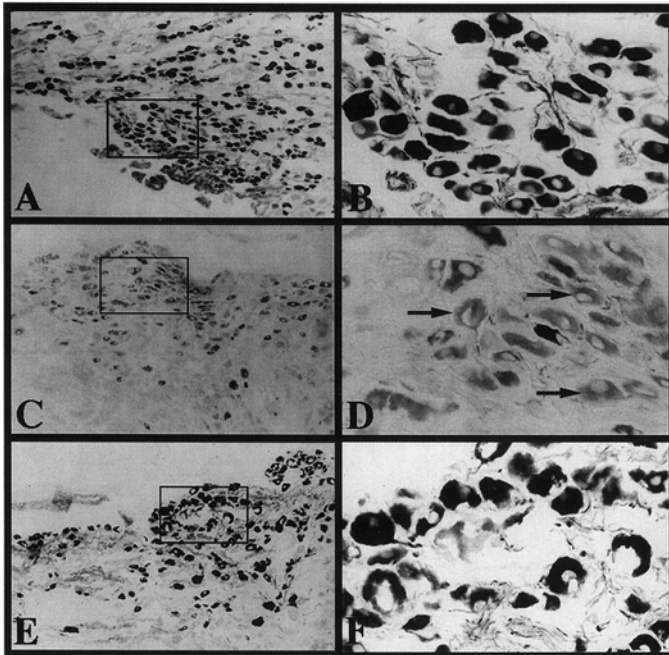


FIG. 4. NADPH diaphorase staining of major pelvic ganglia at 4 weeks. *A*, dark stained neurons in sham operation group. Reduced from $\times 100$. *B*, dark stained neurons in sham operation group. Reduced from $\times 400$. *C*, mostly light stained neurons in LacZ group. Reduced from $\times 100$. *D*, mostly light stained neurons (arrows) in LacZ group. Reduced from $\times 400$. *E*, dark stained neurons in BDNF group, similar to those in sham operation group. Reduced from $\times 100$. *F*, dark stained neurons in BDNF group. Reduced from $\times 400$.

DISCUSSION

Neurotrophins are a class of structurally related growth factors that promote neural survival and differentiation. They stimulate neurite outgrowth, suggesting that they may promote regeneration of injured neurons, and they act as target derived neurotrophic factors to stimulate collateral sprouting in target tissues that produce the neurotrophin.¹⁴ Recently local synthesis and autocrine mechanisms of action have been reported.¹⁵ *In vivo* over expression of a neurotrophic factor through gene transfer would ensure local and continuous neurotrophin production in a manner resembling the physiological response since these proteins are usually produced and secreted by target tissue and glial cells surrounding neurons.

We investigated the use of AAV-BDNF gene transfer to facilitate the recovery of potency after bilateral cavernous nerve injury. Our previous studies have led us to believe that bilateral cavernous nerve freezing in the rat is a suitable model of such injury because the neural sheath is preserved, as in patients who undergo nerve sparing prostatectomy or cryoablation. In addition, the course and extent of functional recovery have been well documented in this model.¹⁶ We used

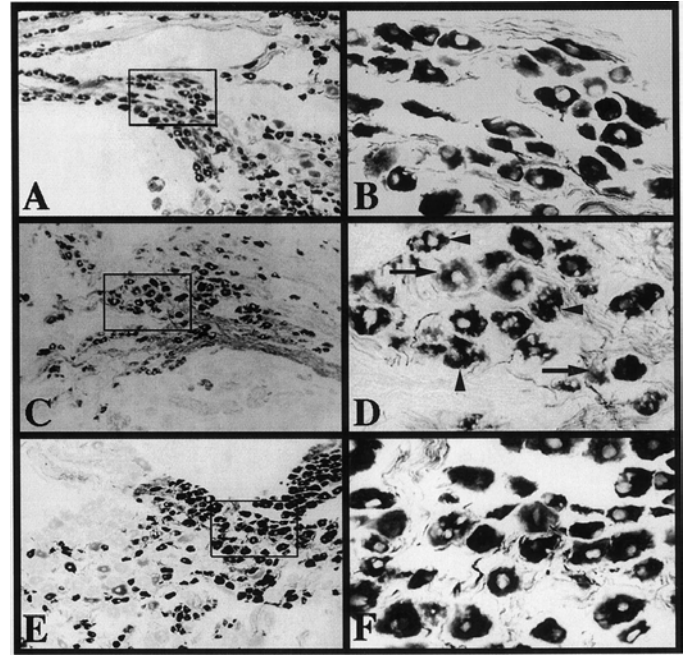


FIG. 5. NADPH diaphorase staining of major pelvic ganglia at 8 weeks. *A*, sham operation group. Reduced from $\times 100$. *B*, sham operation group. Reduced from $\times 400$. *C*, LacZ group. Reduced from $\times 100$. *D*, LacZ group with irregular neuronal contour and cytoplasmic vacuoles (arrowheads) and some neurons with same light staining pattern as at 4 weeks (arrows). Reduced from $\times 400$. *E*, BDNF group. Reduced from $\times 100$. *F*, BDNF group. Reduced from $\times 400$.

maximal intracavernous pressure in response to cavernous nerve electrostimulation to assess the recovery of erectile function. Although apomorphine induced erection may be more physiological, we do not believe that it reliably differentiates partial from full erection in rats with cavernous nerve injury.

In this study the number of neuronal nitric oxide synthase containing neurons in the major pelvic ganglia in the 2 experimental groups was less than in the sham operation group. However, the percent of dark stained neurons was significantly greater in the BDNF group than in the LacZ group at 4 and 8 weeks. Moreover, most neurons in the BDNF and sham operation groups did not show the cytoplasmic vacuoles and irregular cell contour present in the LacZ group. These findings suggest that the production of BDNF protein in penile tissue may be retrogradely transported to the major pelvic ganglia to prevent neuronal damage and preserve neuronal nitric oxide synthase enzymes in the neurons. In turn, it facilitates the recovery of erectile function, as evidenced by the more numerous neuronal nitric oxide synthase positive nerve fibers in the erectile tissue and higher intracavernous pressure in the BDNF group.

A previous study has shown a significantly increased sur-

TABLE 3. NADPH diaphorase positive neurons in the major pelvic ganglia

Group	Mean No. Stained \pm SD		Mean Totals \pm SD	Mean % Stained \pm SD	
	Dark	Light		Dark	Light
<i>4 Wks.</i>					
Sham operation	102 \pm 10.5	21.7 \pm 3.5	123.5 \pm 4.5*	82.2 \pm 5.3	17.3 \pm 6.8
LacZ	32.4 \pm 15.2	52.5 \pm 20.4	85 \pm 20.1	38.5 \pm 17.2	60.7 \pm 17.2
BDNF	61.8 \pm 23.7	33.8 \pm 9	95.7 \pm 22.4	62.1 \pm 13.1†	38.6 \pm 11.8
<i>8 Wks.</i>					
Sham operation	101 \pm 12.3	25.5 \pm 5.5	136.7 \pm 11.3*	80.7 \pm 9.8	19.2 \pm 4.5
LacZ	45 \pm 12.7	53.3 \pm 9.5	98.5 \pm 14.3	45.8 \pm 0.8	54.1 \pm 0.8
BDNF	69.4 \pm 21.1	38.4 \pm 14	107.8 \pm 23.58	64.3 \pm 10.7†	35.6 \pm 10.7

* Versus experimental groups $p < 0.05$.

† Versus LacZ $p < 0.05$.

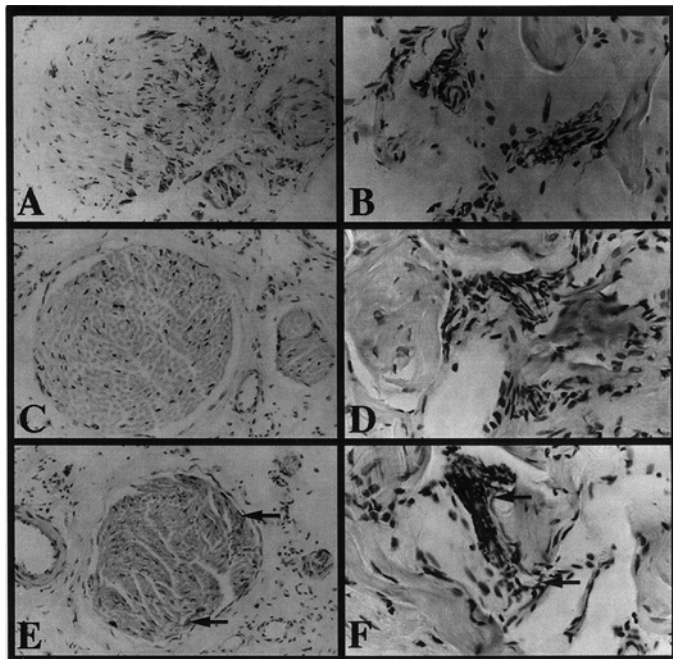


FIG. 6. Neuronal nitric oxide synthase immunostaining of penile tissue at 8 weeks. *A*, dorsal nerve in sham operation group. Reduced from $\times 200$. *B*, cavernous tissue in sham operation group. Reduced from $\times 400$. *C*, dorsal nerve in LacZ group. Reduced from $\times 200$. *D*, cavernous tissue in LacZ group. Reduced from $\times 400$. *E*, dorsal nerve in BDNF group with greater number of positive nerves (arrows) than in LacZ group. Reduced from $\times 200$. *F*, cavernous tissue in BDNF group with greater number of positive nerves (arrows) than in LacZ group. Reduced from $\times 400$.

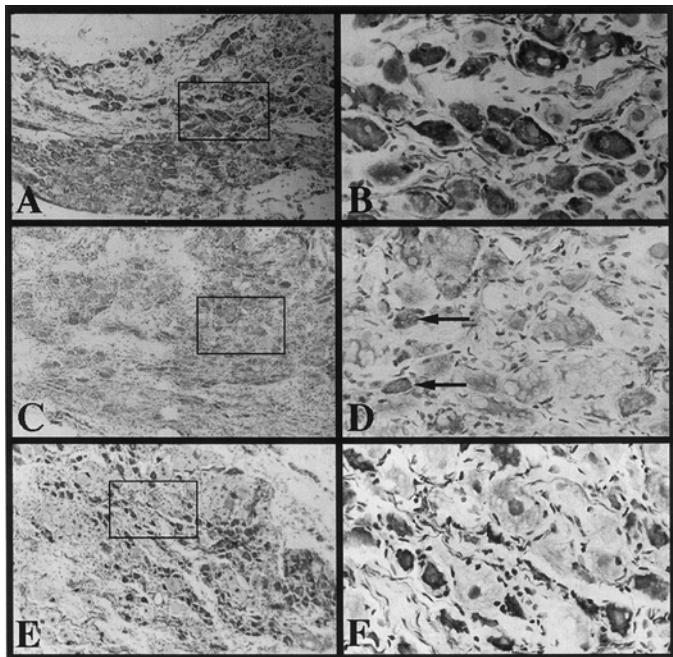


FIG. 7. Neuronal nitric oxide synthase immunostaining of major pelvic ganglia at 8 weeks. *A*, sham operation group. Reduced from $\times 100$. *B*, sham operation group. Reduced from $\times 400$. *C*, LacZ group. Reduced from $\times 100$. *D*, LacZ group with fewer positive stained neurons and lighter staining pattern (arrows). Reduced from $\times 400$. *E*, BDNF group. Reduced from $\times 100$. *F*, BDNF group. Reduced from $\times 400$.

vival of motoneurons 1 week after axotomy in animals pretreated with adenovirus encoding BDNF or glial cell line derived neurotrophic factor.¹⁷ However, because of the dis-

advantages of the adenovirus, we used AAV, a unique member of the nonenveloped, single strand DNA Parvovirus with several properties that distinguish it from other gene transfer vectors. Its advantages include stable and efficient integration of viral DNA into the host genome,¹⁸ lack of associated human disease,¹⁹ broad host range, ability to infect growth arrested cells²⁰ and ability to carry nonviral regulatory sequences without interference from the viral genome.²¹ In addition, no superinfection inhibition is associated with AAV vectors.¹⁸ Infected cells are spread several mm. around the needle tract. AAV infects axon terminals and is retrogradely transported. The injection of AAV vector expressing LacZ into several brain regions has shown transgene expression at as early as 24 hours²² that lasts at significantly decreased levels as long as 6 months.

The penis is a convenient organ for gene therapy because of its external location and slow circulation in the flaccid state. In addition, its sinusoidal structure and the gap junctions between smooth muscles ensure the wide distribution of injected vectors. To our knowledge this is the first demonstration of gene therapy with AAV-BDNF used to facilitate the recovery of neuronal nitric oxide synthase containing nerves and neurons, and consequent erectile function. Because our previous studies have shown that 3 to 6 months may be required for more complete regeneration of cavernous nerves and erectile function in unilateral resection and unilateral freezing models,^{3,16} we are currently performing a further study to examine the effects of higher AAV-BDNF titer and longer followup in this model.

CONCLUSIONS

Our results show that intracavernous injection of AAV-BDNF after freezing bilateral cavernous nerves facilitated the recovery of erectile function, enhanced regeneration of the intracavernous and dorsal nerves, and prevented neuronal degeneration in the major pelvic ganglia. If further studies confirm its effectiveness and safety, intracavernous injection of neurotrophins or other growth factors has the potential to be a curative therapy for neurogenic erectile dysfunction after cryoablation or radical pelvic surgery.

Avigen provided the pLHP19 and pLaden5.

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