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Neurotrophic effects of brain-derived neurotrophic factor and vascular endothelial growth factor in major pelvic ganglia of young and aged rats

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Abstract

OBJECTIVE—To investigate the neurotrophic effect of brain-derived neurotrophic factor (BDNF) and vascular endothelial growth factor (VEGF) in cultured major pelvic ganglia (MPG) derived from young and aged rats.

MATERIALS AND METHODS—The dorsocaudal region of the MPG was isolated from 12 6-month-old male rats and 12 24-month-old male rats. The MPGs were treated with BDNF, VEGF, or both, at 0, 12.5, 25, 50, 100 and 150 ng/mL to determine the effective concentration for 50% activity (EC₅₀) and optimum dosage for promoting neurite growth. Neurite outgrowth from treated MPGs was measured by microscopy. NADPH diaphorase and tyrosine hydroxylase (TH) staining was used to characterize neurites.

RESULTS—Both BDNF and VEGF promoted neurite sprouting from MPG. Neurite growth was more robust in MPGs derived from young rats (6 months) than from aged rats (24 months). The EC₅₀ for BDNF, VEGF and combined treatment were 10.6, 11.9 and 52 ng/mL in young rats, and 11.3, 12 and 0.75 ng/mL in old rats, respectively. The optimum dosage of both factors for promoting MPG neurite growth in all groups was 25–50 ng/mL. VEGF appeared to favour NADPH diaphorase-positive neurites, whereas BDNF favoured TH-positive neurites.

CONCLUSION—BDNF and VEGF promote neurite growth from cultured MPG; combined treatment produced the most robust neurite outgrowth. Neurite growth from MPGs derived from aged rats was not as robust as it was from MPGs from younger rats. Further studies on the effect of neurotrophins after cavernous nerve injury are warranted.

Keywords

brain-derived neurotrophic factor; vascular endothelial growth factor; major pelvic ganglia; neurite; nerve growth

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CONFLICT OF INTEREST

None declared.

INTRODUCTION

Penile erection is a complex neurovascular event. With sexual stimulation, nitric oxide is released from the cavernous nerves onto smooth muscle cells of the cavernous arteries and corpora cavernosa. Nitric oxide activates guanylyl cyclase, which in turn cleaves GTP into cGMP. Through a series of downstream effectors, cGMP induces decreased intracellular calcium concentration and smooth muscle relaxation, which causes vasodilatation and penile engorgement. An androgenic hormonal milieu and a psychological state conducive to sexual arousal are also critical in the production of normal penile erections [1].

Erectile dysfunction (ED) is defined as the inability to attain and/or maintain an erection sufficient for satisfactory sexual intercourse. ED is a major quality-of-life issue not just for the affected man but for his sexual partner(s). Given the complexity of the erectile process, it is not surprising that insults to the neuronal, vascular, hormonal or psychological milieu can lead to ED.

A particularly troublesome cause of ED is iatrogenic injury to the cavernous nerves, as can occur in many types of urological surgery. Radical prostatectomy, radical cystoprostatectomy and cryoablation of the prostate have all been linked to increased rates of ED, despite the development of cavernous nerve-sparing techniques and minimally invasive methods for treating urological malignancies [2-5]. While nerve regeneration is known to occur after cavernous nerve injury, this process occurs very slowly [6]. It is thought that the prolonged period of cavernous tissue denervation after cavernous neurotomy might lead to atrophy of the corporal structures and subsequent 'venous-leak' ED, even after nerve regeneration [6]. This concept underlies the current interest in 'penile rehabilitation' programmes after radical pelvic surgery, most of which are designed to maintain corporal tissue integrity during the interval between cavernous nerve injury and restoration of nervous enervation [7]. While preservation of corporal tissue integrity is a worthwhile goal in the management of ED after nerve injury, a mechanism by which to enhance the rapid recovery of neuronal innervation to the penis would be preferable to currently available treatments.

Rodent models of cavernous nerve injury have become a mainstay of basic science research on mechanisms and therapies for neurogenic ED related to surgical trauma. Studies have indicated that after cavernous nerve injury in rodents there is significant loss of neuronal nitric oxide synthase (nNOS)-containing nerve fibres and neurones in the corpora cavernosa, and in the major pelvic ganglia (MPG) [8], implying denervation injury similar to what is observed in humans after cavernous nerve injury.

Previous studies have used cultured MPGs *in vitro* to ascertain neurotropic effects of compounds such as vascular endothelial growth factor (VEGF) [9]. This *in vitro* approach permits a relatively rapid assessment of neurotrophic properties of compounds of interest. While the MPG culture method has been used to good effect, to our knowledge there have been no published investigations on the effect of the age of the MPG in determining neurotrophic responses.

In the present study, we used MPG from young and aged rats, and cultured them using techniques established in our laboratory. Cultured MPG were treated with brain-derived neurotrophic factor (BDNF), VEGF, and a combination of both (BDNF+VEGF) at various doses, to assess differences in neurite growth from MPG fragments as a proxy measure for axonal re-growth after stimulation with these trophic factors.

MATERIALS AND METHODS

The study comprised 24 male Sprague-Dawley rats (12 6-month-old 'young' rats and 12 24-month-old 'aged' rats); all animal care, treatments and procedures were approved by the Institutional Animal Care and Use Committee at our university. The rats were killed with an i.p. injection of sodium pentobarbital (200 mg/kg) followed by bilateral thoracotomy.

The dissection and MPG culture were as previously described [9]; briefly, bilateral MPG from each rat were isolated and excised intact. After a rinse in PBS, each isolated MPG was further dissected to isolate the dorsocaudal region (DCR) of the MPG, the portion of the MPG from which the cavernous nerve originates. Each DCR was cut into three pieces of similar size; this yielded a total of 72 pieces of young MPG and 72 of old MPG.

Reduced growth factor Matrigel (RGFM, Becton Dickinson, Mountain View, CA, USA) was diluted three-fold in serum-free RPMI-1640 in a 35-mm culture dish on ice. The diluted RGFM then was spread onto cold sterilized glass cover slips using a sterilized glass slide as a spreader. The coated cover slips were placed in 35-mm culture dishes and incubated at 37°C for 1 h to allow the RGFM to solidify. Subsequently, each MPG fragment was embedded in a 40-μL drop of RGFM that was kept in liquid form in a cold 35-mm plastic culture dish on ice. After a 5-min incubation period at 37 °C to allow the Matrigel to polymerize, 3 mL of serum-free RPMI 1640 medium supplemented with 1× penicillin-streptomycin-fungizone was added. The culture was then maintained at 37 °C in a humidified atmosphere with 5% CO₂.

The cultured MPG fragments were randomly divided into three groups (24 fragments each from both the young and aged groups); a BDNF group, a VEGF group, and a BDNF+VEGF treatment group. Recombinant human BDNF and VEGF (R&D Systems Inc., Minneapolis, MN, USA) was added to the culture medium at concentrations of 0, 12.5, 25, 50, 100 and 150 ng/mL (with the zero concentration group used as the control). In the combined treatment group BDNF and VEGF were dosed at 6.25, 12.5, 25, 50 and 75 ng/mL each. Three MPG fragments from the young and three from the old groups were cultured at each dosage specified. Cultures were incubated for 48–72 h.

At 48 and 72 h, neurite growth from the MPG fragments was photographed at ×20 with a Nikon DXM1200 digital still camera attached to a Zeiss Axiovert microscope, using ACT-1 software (Nikon Instruments Inc., Melville, NY, USA). The digitized images were then analysed with ChemiImager 4000 (Alpha Innotech Corporation, San Leandro, CA, USA) and the longest neurite from each fragment was calculated.

After 72 h of incubation, the culture medium was aspirated and replaced with a fixative solution of 2% formaldehyde, 0.002% picric acid in 0.1 M PBS, at pH 8.0. After 20 min of fixation the MPG fragments were stored in 30% sucrose in PBS at 4°C until use. All MPG fragments were then rinsed three times (10 min each) with PBS and then incubated on a cover slip with 0.1 M NADPH, 0.2 M nitroblue tetrazolium, and 0.2% Triton X-100 in buffer for 30 min at room temperature. Tissues were constantly monitored for colour development. When deep blue staining was detected (indicating the presence of NADPH-diaphorase-positive nerves) the reaction was terminated by washing the tissues with PBS three times for 10 min each. The MPG were then mounted onto glass slides using PBS-buffered glycerine (1:9) as mounting medium. Pictures of stained ganglia were taken with a professional DCS-420 digital camera (Eastman Kodak, Rochester, NY, USA) connected to a microscope and a computer. The digitized images were then analysed with ChemiImager 4000 to determine the length and staining intensity of the neurites.

After NADPH staining was completed cultured MPG and the newly generated neurites were stained immunohistochemically for tyrosine hydroxylase (TH). Tissues were rinsed three times with PBS and endogenous peroxidase activity eliminated by incubating for 10 min in 3% hydrogen peroxide/methanol. The MPG were incubated for 30 min with 3% horse serum in PBS + 0.3% Triton X-100, followed by TH antibody (1:120, Novocastra Laboratories, Newcastle upon Tyne, UK) overnight. MPGs were rinsed three times in PBS and then stained using an avidin-biotin-enzyme complex staining kit with diaminobenzidine as the chromogen (Vectastain, Vector Laboratories, Burlingame, CA, USA). Sections were counterstained with haematoxylin. Cover slips containing the ganglial tissues were then mounted onto glass slides using PBS-buffered glycerine (1:9) as mounting medium. The MPG were then mounted and photographed as described above.

Data were analysed using one-way ANOVA for the difference between groups, with the Student-Newman-Keuls post-ANOVA test; the statistical significance was set at $P < 0.05$.

RESULTS

There was much heterogeneity in the number of neurite sprouts between MPG fragments, and no discernible difference in sprouting between fragments treated with different neurotrophic factors. However, the neurite sprouts in both young and aged MPG in the groups treated with neurotrophic factor were significantly longer than in control MPG. MPG cultured with BDNF and VEGF at >12.5 ng/mL had significantly ($P < 0.05$) longer neurites than controls. The most dramatic enhancement in neurite growth was in MPG treated with 25 ng/mL of both VEGF and BDNF. The combined treatment had the most impressive improvement in neurite growth, suggesting a synergistic effect between these neurotrophic factors. At doses of 100–150 ng/mL, BDNF and VEGF had diminished effects on neurite growth relative to that at lower doses, suggesting that the optimum dose was 25–50 ng/mL. Representative images of neurite growth at 48 h in MPG derived from aged rats and treated with various doses of neurotrophic factors are presented in Fig. 1.

Paired comparisons were made between MPG derived from the young and aged rats at various doses of BDNF, VEGF, or BDNF+ VEGF. Although the aged MPG showed enhancement of neurite growth in response to all treatments, the degree of growth was less than that in MPG from young rats (Fig. 2).

At 48 h all MPG treated with any concentration of BDNF or VEGF had significantly longer neurites than controls, irrespective of MPG age, except for; (i) there was no significant difference in mean neurite length between control MPG and MPG treated with the maximum dose (150 ng/mL) of BDNF or VEGF as monotherapy; and (ii) there was no difference between aged MPG treated with VEGF 12.5 ng/mL vs control aged MPG. All MPG treated with any dose of BDNF+VEGF had significantly longer neurites than the controls, irrespective of age. However, neurites in the young MPG group were on average significantly longer than those in the aged group for BDNF at 12.5, 25 and 50 ng/mL, VEGF at any concentration, and BDNF+VEGF at all but the 150 ng/mL concentration (Fig. 3).

At 72 h all young MPG treated with any dose of either or both neurotrophic factors had longer neurites on average than controls. All old MPG treated with any concentration of BDNF or VEGF had longer neurites on average than controls, except for those MPG treated with BDNF as monotherapy or BDNF+VEGF at the highest dose. Contrary to what was observed at 48 h, the mean length of neurites from aged MPG was not significantly different from that at similar doses in young MPG at most doses of the neurotrophic factors; only at the two lowest doses of VEGF monotherapy were neurites in the young MPG group significantly longer than those in the aged group (Fig. 4).

In the young MPG, the effective concentration for 50% activity (EC_{50}) for BDNF, VEGF and BDNF+VEGF were 10.6, 11.9 and 52 ng/mL, respectively. In the aged MPG, the EC_{50} for BDNF, VEGF and BDNF+VEGF were 11.3, 12 and 0.75 ng/mL, respectively. Notably, even though the response was less in the aged MPG group, a very low dose of VEGF +BDNF promoted neurite growth in these MPG.

Immunocytochemistry showed more TH-positive neurites in MPG treated with BDNF alone or combined than in the VEGF monotherapy group. Conversely, there were more NADPH diaphorase-positive neurones in MPG treated with VEGF alone or combined than in the BDNF-only group. These differences in neurite type were statistically significant (Fig. 5A,B). There were no significant differences in the ratio of TH- to BDNF-positive neurones between young and aged rats. Representative images of neurite staining in MPG derived from rats are presented in Fig. 5C.

DISCUSSION

Neurite differentiation is a critical step in neuronal development and regeneration. While the cellular processes leading to neurite growth are still being ascertained, recent evidence has indicated the importance of the serine/threonine kinase LKB1 and STRAD as critical in this process [10,11]. Downregulation of either LKB1 or STRAD by small-interfering RNAs prevents axon differentiation, and overexpression of these proteins leads to multiple axon formation. Furthermore, interaction of STRAD with LKB1 promotes LKB1 phosphorylation at a protein kinase A (PKA) site S431 and elevates the LKB1 level. Overexpressing LKB1 with a serine-to-alanine mutation at S431 (LKB1(S431A)) prevents axon differentiation. BDNF promotes this kind of differentiation in a manner that depends on PKA-dependent LKB1 phosphorylation [10,11]. Although these studies dealt with central neuronal growth patterns, this pathway might play a role in the peripheral nerve system.

BDNF and VEGF are established neurotrophic factors. BDNF is thought to be particularly important in supporting the survival of neurones after damage while Schwann cells help to guide axonal re-growth [12]. The effect of BDNF appears to be mediated by activation of the JAK/STAT pathway [13,14]. VEGF has been shown to promote neurite outgrowth by interacting with VEGF-receptor-2 and activation of the Rho/ROCK signalling pathway [15].

In vitro studies have established that both BDNF and VEGF stimulate sprouting of neurites from cultured MPG [9]. *In vivo*, intracavernous injection with both VEGF and/or BDNF after cavernous nerve injury has been shown to attenuate the development of ED in rats with cavernous nerve injury, with combined treatment being the most effective [16-18]. Putative mechanisms for this effect have included enhanced neuronal numbers and nNOS positivity in the cavernous nerves of the penis [16].

While existing studies supported the use of BDNF and VEGF as neurotrophins, the most effective dose of the drugs has not been extensively studied. Higher doses (up to 100 ng/mL) have been shown to increase the number, but not the length, of neurites in human retinal explants [19]. We previously reported enhanced neurite growth *in vitro* with BDNF concentrations of 25–50 ng/mL [13,14] but the optimum dosing values for VEGF and VEGF +BDNF was not previously known. This previous observation was confirmed with the current results, showing optimization of neurite length by treatment with neurotrophins at 25–50 ng/mL, and a decline in neurite growth at higher doses.

In addition to a paucity of dosing studies, to our knowledge there has been no investigation of the effect of tissue age and potential senescence on the neurotrophic effect of BDNF and VEGF on cultured ganglia. The question of tissue age as a predictor of response is an important one, as older patients will probably comprise the majority of humans treated with

any sort of neuromodulatory therapy after nerve injury from surgery. Interestingly, while the response in terms of neurite growth differed between the young and aged group, there was no significant difference in EC₅₀ between the young and aged MPG, exception for VEGF +BDNF. In the young MPG, BDNF+VEGF dramatically enhanced neurite growth with a relatively high EC₅₀ of 52 ng/mL. By contrast, small amounts of BDNF+VEGF induced maximum neurite sprouting in the aged group, although the mean length of newly formed neurites was shorter than in the young group. The reason for this difference is not entirely clear from these data. It might be postulated that the reduced EC₅₀ is secondary to enhanced synergistic effects of combined treatment in aged tissues. An alternative explanation would be that the diminished capacity of aged MPG to respond to any sort of neurotrophic treatment means that dose increases might have no effect. Yet another possibility is that toxic side-effects of neurotrophins might be exaggerated in aged tissues, such that only lower doses are safe and effective. Further research is necessary to clarify the reason for these results, which might have relevance for dose optimization studies in translational research of neurotrophic factors involving humans.

In addition to providing data on the efficacy of these neurotrophic factors for potentiation of neurite sprouting, we identified interesting differences in neurite type between MPG treated with BDNF or VEGF as monotherapy. VEGF appears to preferentially favour sprouting of NADPH diaphorase-positive (nitrergic) neurites, whereas BDNF potentiates TH-positive (dopaminergic/adrenergic) neurites. Our findings are in line with previous reports supporting an important role of BDNF in the primary differentiation of dopaminergic neurites [20]. However, this current finding is contrary to our previous report, which suggested that BDNF and VEGF are similarly capable of inducing both TH- and NOS-positive fibres [9]. While adrenergic neurones are generally anti-erectogenic, our previous studies supporting a role for both BDNF and VEGF in restoring erectile function [16,18] indicated that the overall effect of this neurotrophin appears to be positive for erectile function. Further studies are necessary to clarify and confirm our observations, and to more precisely understand these effects.

In the present study we did not address molecular mechanisms for the observed effects. However, numerous investigations have provided a general sense of the mechanisms by which BDNF and VEGF exert their neurotrophic effects. The use of an *in vitro* culture system permits careful and precise modulation of experimental factors but does not necessarily translate to clinical efficacy in the much more complicated *in vivo* system. Further studies of dose optimization *in vivo* are required before consideration can be given to a human translational study

In conclusion, BDNF and VEGF both promote neurite growth from cultured MPG. The consistent optimum dose is 25–50 ng/mL in the *in vitro* system, with combined treatment giving the best effects. Tissue from aged rats did not provide as brisk a neurotrophic response as did tissue from younger rats. The diminished responsiveness of aged tissues to BDNF and VEGF implies that there might be important differences in the responsiveness of aged human tissue to these types of treatments in future clinical trials. There appear to be significant differences in the type of neurite that is stimulated by these two neurotrophins.

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Abbreviations

ED	erectile dysfunction
nNOS	neuronal nitric oxide synthase
MPG	major pelvic ganglia
VEGF	vascular endothelial growth factor
BDNF	brain-derived neurotrophic factor
DCR	dorsocaudal region
RGFM	Reduced growth factor Matrigel
TH	tyrosine hydroxylase
EC₅₀	effective concentration for 50% activity

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FIG. 1.

Neurites sprouting from MPG after stimulation with BDNF, VEGF and VEGF+BDNF at 48 h. The MPG were embedded in RGFM and cultured in vitro. Neurotrophic factors at 0, 12.5, 25, 50, 100 and 150 ng/mL were added to the culture solution. Neurites were longer at all dosages of neurotrophic factors than were control neurites. The number in the upper left hand corner is the number of neurites in the given image, and the arrow indicates the longest neurite in each field. $\times 100$.

FIG. 2.

Difference of neurites from young and old MPG at 48 h; the MPG from 6-month-old 'young' rats (top panel) and 24-month-old 'aged rats' (bottom panel) were treated with 25 ng/mL BDNF (**A,D**), VEGF (**B,E**) and VEGF+BDNF (**C,F**) for 48 h. The combined factors (**C,F**) induced longer neurites than either factor alone. Neurite outgrowth was more robust in the young group. The number in the upper left hand corner is the number of neurites in the given image, and the arrow indicates the longest neurite in each field. $\times 100$.

FIG. 3.

Neurite length from MPG at 48 h; MPGs from young and old rats were treated with BDNF, VEGF, or BDNF+VEGF. The mean ($_{SD}$) length of the neurites (12 for each point) is shown. BDNF- and VEGF-treated MPGs had longer neurites than control MPGs at most concentrations ($\#P < 0.05$ vs control MPG within the same age group). Compared to young MPG, the aged MPG produced significantly shorter neurites at any dose ($*P < 0.05$ for young vs aged MPG when comparing similar concentrations of neurotrophic factors).

FIG. 4.

Neurite length from MPGs at 72 h. MPGs from young and old rats were treated with BDNF, VEGF or BDNF+VEGF. The mean (SD) length of the neurites (12 for each point) is shown. The neurites were longer by $\approx 100 \mu\text{m}$ on average than at 48 h. BDNF- and VEGF-treated neurites tended to be significantly longer than control neurites at most doses ($\#P < 0.05$ vs control MPG within the same age group). Neurites in the aged group tended to be shorter than in the young group at 72 h, but the difference was statistically significant only in the groups treated with the lowest concentrations (12.5 and 25 ng/mL) of VEGF alone ($*P < 0.05$ for young vs aged MPG when comparing similar concentrations of neurotrophic factors).

FIG. 5. NADPH diaphorase and TH staining of neurites at 72 h; NADPH diaphorase (A) and TH (B) immunostaining was used to characterize neurites. Neurites positive for NADPH diaphorase were stained blue and neurites positive for TH were stained brown. (C). VEGF with or without BDNF led to more NADPH diaphorase-positive neurites than with BDNF alone (a, * $P < 0.05$ vs MPG treated with BDNF only). BDNF with or without VEGF led to more TH-positive neurites than MPG treated with VEGF only (b, # $P < 0.05$ vs VEGF only). There was no significant difference in TH and NAPDH diaphorase positivity between young and aged rats in any group ($P > 0.05$).