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Temporal changes in neurotrophic factors and neurite outgrowth in the major pelvic ganglion following cavernous nerve injury

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Abstract

Despite nerve-sparing radical prostatectomy, nerve damage and erectile dysfunction (ED) prevails and preventing neurodegeneration is of great importance. Neurotrophic factors and neurite outgrowth were characterized in major pelvic ganglia (MPG) following bilateral cavernous nerve injury (BCNI). Young male Sprague-Dawley rats underwent sham or BCNI surgery and intracavernosal pressure to mean arterial pressure ratio (ICP/MAP) was measured 2, 7, 14, 21, 30 and 60 days following injury (n=8/group). MPG gene expression (qPCR) and Western blot was performed for glial cell line-derived neurotrophic factor (GDNF), nerve growth factor (NGF), neurturin (NRTN), neurotrophin-3 (NT3), neurotrophin-4 (NT4), brain-derived neurotrophic factor (BDNF), vascular endothelial growth factor (VEGF) and activating transcription factor 3 (ATF3). Additional rats were injured and MPGs removed 24h, 48h, 3 and 7 days following BCNI (n=3/group). MPGs were cultured in matrigel and neurite outgrowth measured. Erections were impaired early and improved by 60 days in BCNI rats. GDNF, NGF, BDNF and ATF3 gene expression was significantly increased and NT3 decreased in MPGs following BCNI (48h-21d; $p<0.05$). GDNF and NGF proteins levels were elevated in 48h BCNI. MPG neurite outgrowth from 24h and 48h BCNI was higher than sham ($658\pm 19\mu\text{m}$; $607\pm 24\mu\text{m}$; $393\pm 23\mu\text{m}$, respectively, $p<0.05$). Further studies examining the roles of neurotrophic factors in modulating signaling pathways may provide therapeutic avenues for neurogenic-mediated ED.

Keywords

Peripheral nerve injury; erectile dysfunction; neurite outgrowth; activating transcription factor 3

Introduction

The major pelvic ganglia (MPG) are peripheral mixed ganglia consisting of sympathetic and parasympathetic neurons which innervate the urogenital organs and the lower gastrointestinal tract. Due to the location of the ganglia and cavernous nerve (CN), they are susceptible to neuropraxia or neurectomy during pelvic surgeries such as radical prostatectomy (RP) which can result in neurogenic complications including urinary incontinence and erectile dysfunction (ED; Boorjian et al., 2012). To limit this functional consequence of pelvic surgery, so-called nerve-sparing operative techniques have been developed in an attempt to limit nerve damage to neuropraxia, in which spontaneous nerve recovery without surgical nerve realignment is a realistic expectation. To further improve erectile function outcomes of RP surgeries, studies have focused on pharmacological therapies such as phosphodiesterase 5 inhibitors (PDE5i) to limit end organ damage in the penis without any success (Montorsi et al., 2014; Pisansky et al., 2014). In order to develop effective interventions to prevent neurodegeneration and promote neuroregeneration we need a better understanding of the intrinsic processes that take place within the injured MPG and CN.

The peripheral nervous system has demonstrated the ability to regenerate and sprout new axons after injury although many factors contribute to the functional outcome. The severity and type of nerve injury, age or underlying diseases will impact repair and recovery (Scheib and Hoke, 2013). Following injury, distal nerves undergo Wallerian degeneration and a regenerative response is initiated in the neuronal cell bodies (expression of regeneration associated genes) and in Schwann cells (upregulation of neurotrophins, cytokines, chemokines and extracellular matrix proteins; Scheib and Hoke, 2013; Kiryu-Seo and Kiyama, 2011). In sciatic nerve injury, neurotrophic factors such as glial cell line-derived neurotrophic factor (GDNF), brain-derived neurotrophic factor (BDNF), nerve growth factor (NGF), or neurotrophin-3 (NT3) are produced in a temporal fashion to encourage axon growth (Golz et al., 2006). These processes in the MPG are now being studied; however they are not well defined or only assessed at one time point following injury.

To determine the temporal gene expression changes that occur in the MPG following injury, we assessed global gene expression changes in injured MPGs at early (48 hours) and later time points (14 days). Many significant changes were evident at a transcriptional level involving galanin, GDNF, BDNF and NGF signaling pathways (Calenda et al., 2012; Weyne et al., 2014). Additionally, exogenous neurotrophic therapies have been used to promote erectile recovery in diabetic or CN injury rat models of ED. These investigations provide support for the use of neurotrophic factor therapy but have not fully addressed the pathogenesis of CN dysfunction that occurs in the MPG to promote neuritogenesis and regeneration.

In addition to the CN injury rat model of ED, the MPG can be excised and grown on Matrigel to assess its ability to sprout and grow neurites. This is an experimental tool that can be used to assess the ability of different compounds to inhibit or promote MPG neurite growth. Neurite outgrowth in explanted MPG's grown in culture are dependent on neurotrophic factor stimulus (Lin et al., 2003; Bella et al., 2006; Lin et al., 2006). In general,

neurotrophic factors alone or in combination have been shown to enhance MPG neurite outgrowth in young and old MPGs (Lin et al., 2010). MPG Matrigel culture following CN injury has yet to be assessed. In dorsal root ganglia (DRG) culture, *in vivo* sciatic nerve injury lead to twice the length of DRG neurite outgrowth compared to uninjured DRG (Aguis et al., 1998). Injury can prime the nerves for outgrowth by upregulating intrinsic growth factors to allow for repair and regeneration. We sought to determine if the increased gene expression of neurotrophic factors seen *in vivo* following CN injury could impact on the neurite outgrowth of the MPG *in vitro*.

This study examined the time course of intrinsic neurotrophic factors from the GDNF and NGF families in the MPG following a moderate CN crush injury. The degree of injury was assessed by measuring a marker of nerve injury, activating transcription factor 3 (ATF3), and recording erectile function. Additionally, we assessed the time course of neurite outgrowth in cultured control and CN injured MPGs to determine if the intrinsic rise in neurotrophic factors could increase neurite outgrowth in MPGs.

Experimental Procedures

Animals and Experimental Design

Male Sprague Dawley rats (n=119, Charles River, Wilmington, MA) weighing 250–300g were used in this study. Rats had *ad libitum* access to standard rat chow and water. Rats underwent sham (sham, n=16), or bilateral CN injury (BCNI) surgery and tissues were harvested 48 hours (BCNI 48h, n=16), 7 days (BCNI 7d, n=16), 14 days (BCNI 14d, n=16), 21 days (BCNI 21d, n=8), 30 days (BCNI 30d, n=16) or 60 days (BCNI 60d, n=16) following injury to be used for molecular studies. Additional rats underwent sham and BCNI surgeries and MPGs were collected 24, 48, 72 hours or 7 days after injury and cultured in Matrigel (n=3/group). All experiments were conducted in accordance with the Johns Hopkins University School of Medicine Guidelines for Animal Care and Use, the National Institutes of Health Guide for the Care and Use of Laboratory Animals and the Guidelines for the Use of Animals in Neuroscience Research by the Society of Neuroscience.

Bilateral Cavernous Nerve Injury

Under isoflurane anesthesia, the prostate was exposed via a midline laparotomy and the MPG and CN were identified bilaterally. In the BCNI groups, both CNs were injured by crushing with forceps for 3×15 seconds 2–3 mm distal to the MPG (Hannan et al., 2013, 2014). Adequate crush was confirmed by an observable change in nerve color to grey with the neurolemma remaining intact. In sham animals, the CN was identified and the abdomen closed. BCNI surgeries were all performed by the same surgeon.

Measurement of Erectile Responses

Under anesthesia, the right CN was identified. The right cru was cannulated with a 25G needle connected to a pressure transducer to measure intracavernous pressure (ICP). The right carotid artery was cannulated for continuous measurement of mean arterial pressure (MAP). The CN distal to the crush injury was stimulated with a square pulse stimulator (Grass Instruments, Quincy, MA, USA) at a frequency of 20 Hz, 0.5 msec duration, pulse

width of 30 seconds at increasing voltages (2, 4, 6, and 8 volts) for one minute with 3–5 minutes between stimulations. The ratio between the maximal ICP and mean arterial pressure (MAP) obtained at the peak of erectile response was calculated to normalize for variations in systemic blood pressure. Total ICP was measured as the area under the curve (AUC) during the time of stimulation.

Major Pelvic Ganglia Culture and Neurite Outgrowth Assessment

Following sham or BCNI surgeries, MPGs were carefully dissected at 24, 48, 72 hours or 7 days after injury to be cultured in Matrigel (n=3/group). Whole MPGs were carefully separated from the prostatic capsule, excised and kept on ice in serum-free media (RPMI 1640 with 1% Penicillin-Streptomycin, GIBCO) until they were embedded. Reduced growth factor Matrigel was diluted with media and 200µl placed on the bottom of a 24 well plate. Once polymerized, MPGs were placed in the center of the well and covered with 300µl of diluted Matrigel and allowed to harden for 30 minutes at 37°C. MPGs were covered with 1ml of media with VEGF (vascular endothelial growth factor; 25µg/ml, R&D Systems, Minneapolis, MN, USA) which was changed every 24 hours and maintained at 37°C in a humidified atmosphere with 5% CO₂. Photographs of neurite growth at 24, 48, and 72 hours were captured using a Nikon TE200 inverted microscope attached to a CCD Camera and digital images were analyzed with Elements software (Nikon Instruments, Melville, NY, USA). In each area of growth from the MPG the 5 longest neurites were measured. The averages of these measurements defined the neurite length for groups at each time point (20–25 neurites measured per MPG).

Quantitative PCR (qPCR)

Real-time qPCR was used to determine relative expression of neurotrophic factors in MPGs from sham, 48 hour, and 7, 14, 21, 30 and 60 day BCNI rats. Frozen MPGs were homogenized and total RNA purified using the RNeasy system (Qiagen, Hilden, Germany), quantified and then reverse transcribed using Ready-To-Go You-Prime First-Strand Beads (GE Healthcare, Pittsburgh, PA, USA). Real-time qPCR was performed using the StepOnePlus system (Applied Biosystems, Foster City, CA, USA). TaqMan gene expression assays for GDNF (Rn00569510), VEGF (Rn01511601), BDNF (Rn02531967), NTRN (neurturin; Rn01527513), NGF (Rn01533872), NT3 (Rn00579280), NT4 (neurotrophin-4; Rn00566076), ATF3 (Rn00563784) and HPRT1 (hypoxanthine phosphoribosyltransferase 1; Rn01527840) were used. HPRT1 was unchanged between groups and served as an endogenous control in which all values were normalized to HPRT1 transcript levels (Applied Biosystems). All experiments were performed on 8 separate whole MPG cellular fractions from each group with triplicate technical replicate PCR reactions per sample.

Western Blot Analysis

Whole paired MPGs (n=8/group) were excised from sham, 48 hour, and 7, 14, 30 and 60 day BCNI rats and homogenized in cell lysis buffer (Cell Signaling, Beverly, MA, USA). Cellular fractions from homogenized MPGs were isolated for GDNF and NGF western blot analysis. Protein amounts were determined by the BCA kit (Pierce, Rockford, IL, USA), and equal amounts of protein (30 µg) were loaded to 4–20% Tris-HCl gel (Bio-Rad, Hercules, CA, USA). After their separation by SDS-PAGE, the proteins were transferred to

polyvinylidene fluoride membranes and incubated with primary antibodies (GDNF 1:200, NGF 1:200 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH 1:2000); Santa Cruz Biotechnology, Santa Cruz, CA, USA) overnight at 4°C. The membranes were incubated with a horseradish peroxidase-linked secondary antibody and visualized using an enhanced chemiluminescence kit (Amersham Biosciences Corp., Piscataway, NJ, USA). The densitometry results were normalized by GAPDH expression which was unchanged between groups. The intensities of the resulting bands were quantified by using Image J Software (NIH, Bethesda, MD, USA).

Statistical Analyses

Data were expressed as mean $\pm$ SEM. Differences between multiple groups were compared by a one-way analysis of variance (ANOVA) followed by a Tukey's multiple comparisons test (GraphPad Prism 5, San Diego, CA, USA). ATF3, GDNF and NGF expression and ICP/MAP values from sham and all BCNI time points were correlated by calculating Spearman's rank correlation coefficient. P values of less than 0.05 were used as criteria for statistical significance.

Results

Erectile Responses

Erectile function was significantly lower at all time points in a voltage dependent manner following BCNI (Figure 1). Early time points after injury demonstrated a marked decrease in both ICP/MAP and AUC which was lowest at 14 days. Erectile responses began to increase gradually at 30 and 60 days following BCNI and at a higher electrostimulation (6 volts) 60 day injured rats had significantly increased ICP/MAP compared to BCNI 7–30d rats ($p<0.05$). Although erections are improved 60 days following BCNI, they remained significantly lower than sham animals ($p<0.05$). These results are further demonstrated in the representative ICP and MAP tracings for each group (Figure 1C).

Neurotrophic Factor Gene and Protein Expression

To confirm that the decrease in erectile response was due to CN injury, we measured the gene expression of a marker of neuronal injury, ATF3 in the MPG. ATF3 was increased at all time points following BCNI; however only reached significance at 48 hours, 14 and 21 days post-injury (Figure 2A, $p<0.05$).

The gene expression of several neurotrophic factors was assessed in the MPG following injury. BDNF gene expression was significantly increased after 7 days ($p<0.05$) and had returned to sham values by 14 days after BCNI (Figure 2B). VEGF, a known vasculogenic growth factor with neurotrophic properties, and neurturin, a neurotrophic factor related to the GDNF family, was not different following BCNI (Figure 2C & 2D). NT3 and NT4 are members of the NGF family of neurotrophic factors. The gene expression of NT3 was significantly decreased at early time points (48h and 7d) followed by a temporal increase at 14 and 21 days and was lowered again at 30 and 60 days following BCNI (Figure 2E, $p<0.05$). NT4 remained unchanged at all time points following nerve injury (Figure 2F).

The most pronounced change in neurotrophic factor gene expression following BCNI was GDNF. GDNF mRNA increased ~20 fold 48 hours after injury and remained elevated by 5–7 fold up to 21 days post-BCNI (Figure 3A, $p<0.05$). At later time points (30–60d), GDNF gene expression was not different from sham values. NGF gene expression was also significantly elevated 48 hours, 14 and 21 days after crush injury ($p<0.05$) and returned to sham baseline at 30 and 60 days BCNI (Figure 3B). Western blots were performed for these two neurotrophic factors as they had the most pronounced changes in gene expression. Interestingly, GDNF protein amounts were significantly lower 48 hours following injury ($p<0.05$) and at 7 days had returned and were maintained at sham levels (Figure 3C). Similar to GDNF, NGF protein levels were significantly lower 48 hours following injury and were normalized by 14 days BCNI (Figure 3D).

Correlation of Erectile Function and Neurotrophic Factors

Erectile response data (ICP/MAP) recorded after 6 volt electrical stimulation was correlated to the corresponding mRNA ATF3, GDNF or NGF values. Spearman's rank correlation coefficient between ATF3, GDNF and NGF gene expression and erectile responses was -0.8264 , -0.605 and -0.659 , respectively (Figure 4, $p<0.0001$). ATF3 gene expression levels were significantly increased when erectile responses were low and ATF3 mRNA levels were much lower when erections had returned to sham levels (Figure 4B). This strong negative correlation demonstrates that GDNF and NGF gene expression values are high when erectile responses are impaired.

Neurite Outgrowth

The time course of neurite outgrowth was assessed in MPGs cultured following BCNI at 24, 48, 72 hours and 7 days. Neurite outgrowth was measured after the MPGs were incubated for 24, 48 and 72 hours. In all groups neurite outgrowth increased with incubation time (Figure 5). A significant increase in neurite growth was seen in MPGs from rats injured 24 and 48 hours prior to culture compared to sham MPGs (Figure 5E, $p<0.05$). BCNI 72h MPGs grew less than sham after 24 hours of incubation but were no different at 48 or 72 hours of incubation. MPGs that had been injured 7 days prior to culture grew similar to sham MPGs after 24 and 48 hours; however at 72 hours incubation they had significantly increased growth versus sham MPGs ($p<0.05$).

Discussion

This study demonstrates the temporal changes in neurotrophic factors in the injured MPG at early time points when erections are severely diminished, reaching their lowest values after 14 days and beginning to improve by 30–60 days. The gene expression of ATF3, GDNF, NGF, and BDNF were temporally increased and NT3 decreased at times when erectile function was impaired. GDNF and NGF had the greatest increase in MPG gene expression following CN injury. At later time points when erectile function was improved, all neurotrophic factors had returned to sham values. Neurite outgrowth from cultured MPGs was potentiated when the MPG was harvested 24–48 hours after injury, indicating a possible neuroregenerative effect of the observed early ATF3 and neurotrophin upregulation.

We have recently demonstrated a time course of spontaneous recovery of erectile function following BCNI with ICPs stimulated at 8V (Weyne et al., 2014). This study uses the same time course following injury but demonstrates the voltage responses from 2–6V. Similar results to our study were seen in BCNI mice that had a slight increase in erectile function at 4 weeks and significant improvements to erections at 8 and 12 weeks following injury (Jin et al., 2010). Interestingly, mice with CN transection demonstrated no recovery of erections from 3 days to 12 weeks post-injury. These data demonstrate the importance of evaluating early and late time points to ensure that improvements in erectile function following therapeutic interventions are not due to intrinsic recovery of neuronal function.

ATF3 is an early gene upregulated in response to injury (Tsujino et al., 2000) which enhances peripheral nerve regeneration by promoting the growth of injured neurons (Seijffers et al., 2007) and increases neuronal survival (Herdegen et al., 1997). Following axotomy of the sciatic nerve, ATF3 mRNA expression was significantly increased within 12 hours of injury, decreased slightly after 6 weeks and remained elevated in the DRG for 70 days (Tsujino et al., 2000). Explant cultured MPGs expressed increased ATF3 mRNA 8 hours after being cultured and transcript levels were elevated 84-fold following 3 days in culture (Girard et al., 2010). Our results are similar to the crush injured DRG as there was a significant increase in ATF3 gene expression at 48 hours followed by a decrease at 30 and 60 days. There was also a very strong negative correlation between ATF3 transcript levels and erectile function. Further studies are warranted to determine its specific role in CN regeneration and confirm if it may be a marker of CN injury in the MPG.

GDNF, which promotes the survival of central and peripheral neurons, was determined to have the greatest increase in MPG gene expression following injury (Treanor et al., 1996). The expression of GDNF inversely correlated with erectile function. Following CN transection there is a decrease in GDNF mRNA expression in the penis and GDNF protein undergoes retrograde transport from the penis to the MPG (Laurikainen et al., 2000). Our study provides evidence that GDNF is also produced locally in the MPG to promote CN survival in an autocrine fashion. Increasing the expression of GDNF following CN injury via herpes simplex viral (HSV) vector delivery targeted to the MPG has proven successful at recovering erectile function (Kato et al., 2007).

Another member of the GDNF family which promotes neuron growth and survival is neurturin. Although we did not see any significant changes in the gene expression of neurturin in the injured MPG, many others have reported its benefits for the treatment of neurogenic ED. Extended release neurturin placed directly on to the MPG following nerve crush lead to moderate improvement in ICP responses 5 weeks after injury (Bella et al., 2007). Neurturin HSV gene transfer also only mildly increased erectile responses in CN injured rats (Kato et al., 2009). Although neurturin appeared to have had a modest beneficial effect in both rat studies, erections were not recovered to sham levels. Our data support these findings in that neurturin was not activated in response to injury.

NGF was one of the first growth factors to be described and was shown to increase growth of MPG cultured neurons (Tuttle and Steers, 1992; Tuttle et al., 1994). NGF was assessed as an *in vivo* treatment alone or in combination with nerve grafts following CN ablation in rats

(Burger et al., 1991). Treatment with NGF alone minimally increased erectile function while in combination with nerve grafts erections were restored (Burger et al., 1991). Our findings showed a correlation between lower MPG NGF gene expression when neurogenic erectile responses had improved. Additionally, NGF was primarily located in the neuronal cell bodies and fibers in the MPG. In peripheral nerves NGF is transported via a retrograde fashion from the target organ and promotes survival (Zweidel et al., 2005). These data suggest that NGF is an important growth factor in peripheral CN regeneration; however, on its own it may not be a potent enough growth factor to fully recover neuronal function.

BDNF is an important neurotrophin that supports survival of neurons by contributing to the growth, differentiation, and maintenance of neurons. The protein expression of BDNF in the MPG following crush or axotomy has been characterized at early time points (Bella et al., 2007; Bond et al., 2013). Following axotomy, BDNF protein levels and gene expression levels in the MPG were increased after 1 and 5 days (Bella et al., 2007); however, after CN crush BDNF increased after 1 day, decreased to below sham levels by 4 days and normalized after 7 days (Bond et al., 2013). Our results indicate that BDNF gene expression is significantly increased at 7 days and normalized at 14 days. In the rat, overexpression of BDNF delivered intracavernosally via an adeno-associated virus improved erectile responses 4 and 8 weeks following CN injury (Barkicioglu et al., 2001). It is acknowledged that BDNF protein levels were not assessed in the current study and further investigation is warranted to determine BDNF's role in CN regeneration.

NT3, which is responsible for survival and growth of new neurons, was the only neurotrophic factor assessed that demonstrated significantly decreased gene expression in the injured MPG. These results differ from MPG gene expression of NT3 following CN axotomy in which NT3 was unchanged at 1 and 5 days (Bella et al., 2007). We did not see any changes in NT4 or VEGF gene expression following BCNI. Interestingly the majority of studies demonstrating success at increasing MPG neurite growth in vitro or improving erectile function have used these nerve growth factors in combination with each other or BDNF (Lin et al., 2003; Hsieh et al., 2003). Combination treatments in which BDNF and VEGF are overexpressed have been successful at recovering erectile function following CN injury by freezing and/or crush (Chen 2005, Hsieh 2003). It is possible that preservation of nerve function and prevention of neurodegeneration will require a cocktail of nerve growth factors. Furthermore based on our gene expression data the timing of administration of different growth factors may also be critical for treatment.

While we have demonstrated the time course of gene expression of nerve growth factors following CN injury, there is a disconnect between the transcriptional regulation and the protein expression for GDNF and NGF. A week following CN injury, GDNF and NGF protein expression had returned to sham levels; however gene expression remained elevated up to 21 days. It is possible that although protein levels have normalized there may be changes in specific neuron populations that may account for the increased gene expression. Following CN axotomy, there was a greater decrease in the MPG's immunofluorescent staining of GDNF family receptors in parasympathetic versus sympathetic neurons (Palma and Keast 2006). Future studies will serve to assess co-staining of nerve growth factors and

markers of sympathetic, parasympathetic and nNOS positive neurons in sham and injured MPGs.

Neurite outgrowth from MPG neurons can be stimulated by neurotrophic factors. To date the majority of studies have looked at uninjured explanted MPGs. Prior injury to a peripheral nerve is termed 'conditioning' in DRGs and has been shown to increase neurite growth (Smith and Skene 1997). We demonstrated that MPGs from animals injured 24 or 48 hours prior to explant culture had the greatest neurite outgrowth compared to intact MPGs. Additionally, MPGs from later injury time points (3 or 7 days) did not demonstrate as much growth as the early injured MPGs (24 or 48 hours). These results correspond to the neurotrophic factor gene expression data that was highest 48 hours after BCNI and decreased by 7 days after BCNI. Additional studies are required to determine if there are changes in the sympathetic and parasympathetic and nitrergic types of neurites that grow in intact versus injured MPGs and to determine if there is a difference in the response to growth factors.

Some of the limitations to our study are that we do not have protein expression results for all of the nerve growth factors that were assessed. Furthermore, it would be of interest in future studies to assess the distal nerve stump and the target organ to compare the differences in growth factors to those in the MPG. Future studies will also be required to assess the change in growth factor distribution in the MPGs from injured rats by immunofluorescent staining.

Conclusions

Prevention of ED in men undergoing a radical prostatectomy will only become possible when the molecular targets leading to neurodegeneration and neurogenesis following CN injury are fully understood. This study demonstrated that neurotrophic factors such as ATF3, GDNF and NGF following CN injury are significantly elevated. Increased neurite outgrowth is evident in 24 and 48 hour injured MPGs which corresponds with the increased neurotrophic gene expression levels demonstrated after BCNI *in vivo*. The prevention of neurodegeneration and promotion of survival of axons at an early stage after nerve injury are critical. A cocktail of neurotrophic factors administered at critical time points may be required to prevent neurodegeneration. Additional studies examining the roles of neurotrophic factors in modulating neuroregenerative signaling pathways may provide future therapeutic avenues for neurogenic-mediated ED.

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Figure 1. In vivo erectile responses

In vivo erectile responses were assessed in Sham and BCNI rats 48 hours, 7, 14, 30 and 60 days ($n=8/\text{group}$) after BCNI via electrostimulation of the cavernous nerve. Bar graph depicting voltage-dependent erectile responses as measured by the intracavernosal pressure (ICP) to mean arterial pressure (MAP) ratio (A), and total ICP (area under the erectile curve; B) after CNS for 1 min in all groups. Representative tracing of ICP and MAP responses for each group are shown at 6V stimulation for 1 minute as indicated by the black bar along the x-axis (C). Data are represented by mean $\pm$ SEM. BCNI=bilateral cavernous nerve injury; $^*(P<0.05)$ response significantly different compared to sham rats; $^{\delta}(P<0.05)$ response significantly different to BCNI 60d rats.

Figure 2. Gene expression of neurotrophic factors

Bar graphs demonstrating major pelvic ganglion (MPG) gene expression of activated transcription factor 3 (ATF3; A), brain-derived neurotrophic factor (BDNF; B), vascular endothelial growth factor (VEGF; C), neurturin (D), neurotrophin-3 (NT3; E) and neurotrophin-4 (NT4; F) from sham operated rats and various time points from rats following bilateral cavernous nerve injury (BCNI, n=8/group). The gene expression levels were normalized to the gene expression level for glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Data represent mean $\pm$ SEM. *(P<0.05) response significantly different compared to sham rats.

Figure 3. Gene and protein expression of GDNF and NGF

Bar graphs representing major pelvic ganglion (MPG) gene expression of glial cell line-derived neurotrophic factor (GDNF; A), and nerve growth factor (NGF; B) from sham operated rats and various time points from rats following bilateral cavernous nerve injury (BCNI, $n=8/\text{group}$). GDNF and NGF gene expressions were normalized to relative expression of glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Representative Western blots and bar graphs for GDNF (C) and NGF (D) protein expression in MPGs from all groups. Data are normalized to GAPDH protein expression. Data represent mean $\pm$ SEM ($n=8/\text{group}$). *($P<0.05$) response significantly different compared to sham rats.

Figure 4. Relationship between ATF3, GDNF or NGF, and erectile responses

Dot plot depicting the erectile responses as measured by the intracavernous pressure to mean arterial pressure ratio (ICP/MAP) after cavernous nerve stimulation at 6 volts for 1 min in sham and 48 hour, 7, 14, 30 and 60 day bilateral cavernous nerve injury (BCNI) rats (A). Data are respresented by mean $\pm$ SEM (n=8/group). Scatter diagrams demonstrating the relationship between MPG activating transcription factor 3 (ATF3; B), glial cell line-derived neurotrophic factor (GDNF; C) or nerve growth factor (NGF; D) gene expression levels and ICP/MAP to cavernous nerve electrostimulation at 6 V from sham and all injured time points. R=Spearman correlation coefficient. *(P<0.05) ICP/MAP response significantly different compared to sham rats; δ (P<0.05) ICP/MAP response significantly different to BCNI 60d rats.

Figure 5. Time course of MPG neurite outgrowth following injury

Representative images of neurites sprouting from major pelvic ganglion (MPG) from sham animals in the presence of vascular endothelial growth factor (VEGF, B–D) cultured in vitro and the methods of analysis (A). Images were all taken at 100x magnification and white arrow indicates length of neurite. Bar graph depicts neurite length of MPGs from sham, and 24, 48, 72 hours and 7 days bilateral cavernous nerve injured (BCNI) rats after 24, 48 and 72 hours in culture (E). Data represent mean $\pm$ SEM ($n=3$ /group). $^{*}(P<0.05)$ response significantly different compared to sham rats, respective of each time point.