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Sonic hedgehog Regulates Brain Derived Neurotrophic Factor in Normal and Regenerating Cavernous Nerves

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Abstract

Introduction—The cavernous nerve (CN) is commonly injured during prostatectomy. Manipulation of the nerve microenvironment is critical to improve regeneration and develop novel erectile dysfunction (ED) therapies. Sonic hedgehog (SHH) treatment promotes CN regeneration. The mechanism of how this occurs is unknown. Brain derived neurotrophic factor (BDNF) facilitates return of erectile function after CN injury and it has been suggested in cortical neurons and the sciatic nerve that BDNF may be a target of SHH.

Aim—To determine if SHH promotes CN regeneration through a BDNF dependent mechanism.

Main Outcome Measures—BDNF and glial fibrillary acidic protein (GFAP) were quantified in PG/CN by Western and a t-test was used to determine differences.

Methods—Sprague Dawley rats underwent: 1. Bilateral CN crush (n=15), 2. SHH treatment of PG/CN (n=10), 3. SHH inhibition in PG/CN (n=14 rats), 4. CN crush with SHH treatment of PG/CN (n=10 rats), 5. CN crush with SHH treatment and BDNF inhibition (n=14 rats), and 6. CN injury and SHH treatment of the penis (n=23).

Results—In normal rats SHH inhibition in the PG/CN decreased BDNF 34% and SHH treatment increased BDNF 36%. BDNF was increased 44% in response to SHH treatment of crushed CNs, and inhibition of BDNF in crushed CNs treated with SHH protein hampers regeneration.

Conclusions—SHH regulates BDNF in the normal and regenerating PG/CN. BDNF is part of the mechanism of how SHH promotes regeneration, thus providing an opportunity to further manipulate the nerve microenvironment with combination therapy to enhance regeneration.

Keywords

Sonic hedgehog; BDNF; cavernous nerve; regeneration; erectile dysfunction

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Introduction

Peripheral nerve regeneration is an important concern to diabetic, aging, and prostate cancer patients who develop erectile dysfunction (ED) as a result of denervation of the penis. Injury occurs through crushing, tension or resection of the cavernous nerve (CN) during prostatectomy or by demyelination and loss of CN fibers with diabetes and aging. ED occurs in 16–82% of men treated by prostatectomy [1] and 20–71% of diabetic patients (Massachusetts Male Aging Study). ED diagnosis has a high impact on men's health since ED has been shown to be an early warning sign for cardiovascular disease [2] and thus may reveal an underlying vascular condition. Loss of innervation causes extensive and irreversible morphological changes in the penis, including smooth muscle apoptosis [3]. PDE5 inhibitors are effective in only 31% of prostatectomy patients [4] and in 41–44% of diabetic men [5]. Thus new treatments are required that can promote CN regeneration and prevent down stream morphological changes in the penis.

Sonic hedgehog (SHH) plays a prominent role in nerve development and guidance and has been implicated as a regeneration factor in peripheral nerves such as the sciatic [6–7] and facial [8] nerve. Our previous studies show that SHH is a critical regulator of both CN homeostasis [9–10] and of smooth muscle apoptosis in the penis [11–13]. When SHH signaling is inhibited in the PG/CN, SHH protein is decreased and apoptosis increased in the penis [10], indicating that SHH signaling in the PG/CN is critical for maintaining SHH signaling in the penis and penile morphology. SHH is abundant in neuronal nitric oxide synthase (NOS1) positive neurons of the PG that innervate the penis and in Schwann cells of the CN [10]. SHH inhibition in the PG causes demyelination and axonal degeneration of CN fibers [9], and SHH treatment of crushed CNs promotes regeneration and return of erectile function [9]. The mechanism of how SHH promotes regeneration is unknown but is important to understand in order to manipulate the nerve microenvironment to enhance regeneration. In this study we examine a new direction of research, to determine the mechanism of how SHH promotes CN regeneration. Based on SHH's role as a central regulator of apoptosis, proliferation and differentiation in other organs, the process is likely multi-factorial, involving induction of several key signaling pathways.

Brain derived neurotrophic factor (BDNF) is important for survival, differentiation, and protection of neural cells and has neuroregenerative effects in the sciatic and facial nerves [14–15]. In the CN, BDNF facilitates recovery of NOS1 fibers, and regeneration of erectile function [16–19]. BDNF also promotes neurite sprouting and enhances myelination of Schwann cells [20]. Studies performed in cortical neurons [21] and in the sciatic nerve [7] suggest that BDNF may be a target of SHH. In this study we examine the hypothesis that the mechanism of how SHH promotes CN regeneration involves BDNF.

Materials and Methods

Animals

Male Sprague Dawley rats (postnatal day, P115–120) were obtained from Charles River. The weight range was 350–425 grams.

Ethics statement

Animals were cared for in accordance with institutional IACUC approval and the National Research Council publication *Guide for Care and Use of Laboratory Animals*.

Peptide amphiphile (PA) delivery vehicle

PAs used in this study had the structure ((C16)-V₂A₂E₂-(NH₂) and (C16)-V₃A₃E₃-COOH and were synthesized at the Northwestern Institute for BioNanotechnology in Medicine Chemistry Core Facility as previously described [22].

Bilateral CN crush

PG/CN were exposed and microforceps (size 0.02 X 0.06mm) were used to crush the CN bilaterally for 30 seconds. This method of CN crush has commonly been used in the literature [23–24] and the extent and reproducibility of crush injury were previously verified [9]. Sham surgery (control) was performed by exposing but not crushing the CN (n=3). Rats were sacrificed 1, 2, 4, and 7 days after injury (n=12) and PG/CN were homogenized for western analysis.

SHH treatment of normal PG/CN

Affi-Gel beads (100–200 mesh) were equilibrated with SHH or bovine serum albumin (BSA, control) protein (0.25µg/µl) overnight at 4°C. Approximately 10–20 beads were injected under the PG bilaterally and rats were sacrificed after 2 days. PG/CN from SHH (n=5 rats) and BSA treated (5 rats) rats were homogenized for western analysis.

SHH inhibition in normal PG/CN

Affi-Gel beads (100–200 mesh, Bio-Rad, Hercules, CA) were equilibrated with 100µl of 5E1 SHH inhibitor (400 µg/ml, Jessel, Hybridoma Bank University of Iowa) or mouse IgG (control) overnight at 4°C. Approximately 10–20 beads were injected under the PG bilaterally and rats were sacrificed after 2 days. PG/CN from 5E1 (7 rats) and IgG treated (7 rats) rats were homogenized for western analysis.

CN crush with SHH treatment of the PG/CN

(C₁₆)-V₂A₂E₂-(NH₂) PA was prepared as previously described [9]. 20mM CaCl₂ was added to a glass slide and 8µl of 100mM PA plus either 2.27µg SHH or BSA (control) proteins were pipetted onto the slide to form the linear PA hydrogel. PG/CN were exposed and bilateral CN crush was performed as described above. PA was transferred with forceps on top of the crushed CNs bilaterally so that each rat received 4.54µg SHH or BSA protein. The release rate of SHH protein from the PA was previously determined to be 90% by 75 hours [9]. Rats were sacrificed after 2 days. PG/CN from SHH (5 rats) and BSA treated (5 rats) rats were homogenized for western.

CN crush with SHH treatment and BDNF inhibition

Bilateral CN crush and SHH PA treatment were performed as described above. Rats were divided into four groups: 1. Bilateral CN crush, 2. Bilateral CN crush/SHH PA/Trk inhibitor K252a (200nM, treatment via Affi-Gel beads injected under the PG), 3. Bilateral CN crush/SHH PA/DMSO (control, treatment via Affi-Gel beads), and 4. Sham. K252a inhibits the kinase activity of Trk receptors at concentrations 200nM [25]. Rats were sacrificed after four days. PG/CN from sham (n=3), CN crush only (n=3 rats), CN crush/SHH PA/K252a (n=4 rats) and CN crush/SHH PA/DMSO treated (4 rats) rats were homogenized for western analysis.

CN injury and SHH treatment of the penis

Rats were randomized into two groups: 1. CN resection/SHH treatment (n=12), and 2. CN resection/BSA treatment (control, n=11). Bilateral CN resection was performed as described previously [9]. CN resection rather than crush injury was performed since previous studies

from our group have extensively examined SHH signaling and apoptosis in the penis of this model [26]. (C₁₆)-V₃A₃E₃-COOH PA was combined with CaCl₂ and SHH or BSA protein and was injected into the corpora cavernosa of the penis where a gel was formed *in vivo* which lined the sinuses to deliver protein in an extended release manner [26]. 100 mM PA (50μl) was added to 5μl of 1.25μg/μl SHH protein in water. 50μl of 200 mM CaCl₂ was added to the SHH-PA solution. The skin covering the penis was retracted and the PA was injected into the corpora cavernosa with a 26-gauge needle. The final amount of SHH protein injected was 6.25μg per rat and rats were sacrificed at 2, and 7 days.

Western

Western was performed on protein samples isolated from PG/CN as previously described [13]. Membranes were blocked for 1 hour in 5% nonfat skim milk in PBS Tween. Membranes were incubated with either 1/200 mouse BDNF (active form, R&D Systems), 1/3000 rabbit GFAP, (DAKO), or 1/50,000 β-ACTIN (Sigma) antibodies overnight at 4°C. Membranes were incubated with chicken anti-rabbit and chicken anti-mouse (Santa Cruz) secondary antibodies for 1.5 hours. Bands were visualized using HRP-conjugated anti-biotin (ECL, Amersham), were exposed to Hyperfilm and were quantified using Kodak 1D software (Rochester, NY), to determine the ratio of the density of BDNF/β-ACTIN and GFAP/β-ACTIN. Samples were run in duplicate and the results were averaged.

Immunohistochemical (IHC) analysis

IHC was performed on normal penis (n=4), CN injured/SHH PA treated and CN injured/BSA PA treated penis tissue assaying for 1/50 rabbit BDNF (Santa Cruz) using the LSAB+ peroxidase kit (DAKO). Negative controls were performed using rabbit IgG in place of primary BDNF antibody (n=4). Nickel was added to the DAB for development of BDNF staining in normal penis, in order to increase resolution.

Statistics

Samples were run in duplicate. The ratio of BDNF/β-ACTIN and GFAP/β-ACTIN were averaged and the results were reported ± the standard error of the mean (SEM). Statistics were performed using the Excel program and a t-test was used to determine significant differences ($\alpha = 0.05$).

Results

BDNF quantification in bilateral CN crushed rats

BDNF protein was quantified by Western in PG/CNs 1, 2, 4, and 7 days after CN crush in comparison to sham controls (Figure 1). A dynamic response was observed with BDNF increasing 38% the first day after injury ($p=0.011$), decreasing 62% below basal levels at 4 days ($p=0.013$) and rebounding to normal levels by 7 days ($p=0.424$).

SHH treatment of normal PG/CN increased BDNF

BDNF protein was quantified by Western in PG/CN from rats that were treated with SHH or BSA (control) for 2 days. BDNF protein increased 36% in the PG/CN in response to SHH treatment ($p=0.007$, Figure 2A).

SHH inhibition in normal PG/CN decreased BDNF

PG/CN were treated with 5E1 SHH inhibitor or mouse IgG (control) and BDNF protein was quantified in the PG/CN by western after two days of treatment. SHH inhibition decreased BDNF protein by 34% (Figure 2B, $p=0.047$).

CN crush with SHH treatment of the PG/CN increased BDNF

Our previous studies performed at 6 weeks after CN injury and SHH treatment of the PG/CN by PA, showed a 58% improvement in erectile function [9], thus showing that SHH promotes CN regeneration. SHH has also been shown to be neuroprotective [27], however the mechanism of how this occurs is unknown. In this study we examined what happens in the PG/CN in response to SHH treatment in the first few days after injury. BDNF protein was quantified by western in PG/CN from rats that underwent CN crush and SHH or BSA treatment by linear PA ((C₁₆)-V₂A₂E₂-NH₂) for 2 days. BDNF protein was significantly increased 44% in SHH treated PG/CN (Figure 2C, p=0.003).

CN crush with SHH treatment and BDNF inhibition decreased SHH induced CN regeneration

The CN was crushed and treated with SHH by linear PA along with either the BDNF signaling inhibitor K252a or DMSO (control). K252a specifically inhibits the kinase activity of Trk receptors at concentrations 200nM [25]. GFAP protein was quantified by western 4 days after crush. GFAP is an intermediate filament protein that maintains structure and function of the cytoskeleton. GFAP increases with nerve injury and decreases to normal levels as regeneration occurs [John Kessler personal communication, 28]. GFAP is useful as a marker of the glial response, with suppressed GFAP after injury being suggestive of decreased injury. Thus quantification of GFAP may be useful as a tool to examine CN status. The intracavernosal pressure was not examined in this study because of the previously documented severity of the crush injury (icp 0.157) [9]. GFAP increased 51% in the crush group relative to the sham (p=0.006). In the CN crush/SHH PA/DMSO group, GFAP decreased 41% relative to the crush only group (p=0.001), indicating a decreased glial response and decreased injury with SHH treatment (Figure 3). In the CN crush/SHH PA/K252a group, GFAP increased 33% relative to CN crush/SHH PA/DMSO group (p=0.033), suggesting increased injury when BDNF is inhibited (Figure 3).

CN injury and SHH treatment of the penis increased BDNF

IHC analysis was performed on normal Sprague Dawley rat penis (Figure 4). BDNF was abundant in longitudinal and circular smooth muscle [29] but was not identified in endothelium. BDNF protein was examined by IHC in penis from rats that underwent CN injury and SHH or BSA treatment by PA injected *in vivo* into the corpora cavernosa. BDNF protein was identified in the penis of control rats however by visual observation BDNF appeared elevated in the penis in response to SHH treatment for 2 and 7 days (Figure 4). We also observed BDNF staining in the nerve bundle that appeared more intense, by visual observation, with SHH treatment (data not shown).

Discussion

Although adult peripheral nerves have an intrinsic ability to regenerate, the endogenous response is limited and does not allow for full recovery of function. Manipulation of the nerve microenvironment to promote neuronal survival and repair is key to improving regeneration strategies. Since SHH is essential for maintenance of CN homeostasis, facilitates CN regeneration [9], and plays an essential role in tissue sculpting in the penis in response to neuronal signals [10], it is a prime candidate for manipulation of its signaling. Acellular nerve grafts [30], use of Schwann cell seeded guidance tubes [31] and alginate supports to bridge the injury gap [32], and treatment with assorted growth factors, including growth hormone [33], vascular endothelial growth factor [17], erythropoietin [34], and neuturin [35], have been used to partially regenerate the CN in animal models. SHH is a regulator of some of these factors in the penis such as VEGF [36]. Since SHH has regenerative effects in both the PG/CN and penis, it has the potential to be a more effective

treatment strategy for ED. In this study we examine a potential mechanism of how SHH promotes CN regeneration.

After CN crush, we observed a dynamic BDNF response in which BDNF was initially elevated in the first 24 hours, decreased below basal levels at 4 days, and returned to normal by 7 days. Bella et al., 2007 [37] previously observed elevated BDNF in the PG at 1 and 5 days after CN transection. This discrepancy may occur because Bella et al., used a resection model, which is a more severe injury, whereas this study utilized a crush model. The Bella et al., study also incorporated only two time points, which would have missed transient changes. Direct comparison of CN resection and crush has not been performed and the microenvironment of the CN in the initial response to injury may not be the same. This is because some degree of regeneration occurs in the crush model whereas in the resection model, return of innervation typically does not occur because axonal sprouts have a difficult time locating and reconnecting to the distal segment of the severed nerve. Increased BDNF in response to axotomy was also noted in rat facial motoneurons [38] and in the sciatic nerve [7]. The biphasic nature of the BDNF curve suggests complex regulation in response to crush injury. A limitation of this study is that BDNF abundance on either side of the CN crush site was not examined because of technical limitations (too little tissue to divide and extract protein). It is possible that there may be differences relative to the site of injury.

The mechanism of how SHH promotes CN regeneration is unknown. We show that BDNF increased 44% in response to two days of SHH treatment, indicating that increased BDNF is part of the regenerative response induced by SHH. The response of BDNF to SHH in the normal CN is very similar to that observed under regenerative conditions, with a 36% increase in BDNF in response to SHH treatment and a 34% decrease in BDNF in response to SHH inhibition. BDNF was observed in smooth muscle of normal rats. Up-regulation of BDNF in response to SHH treatment was also observed in the penis, which is not surprising given the intimate relationship between the CN and corpora cavernosal architecture [10]. Understanding the pathways that regulate regeneration is critical to manipulate the nerve microenvironment to promote regeneration. A potential mechanism of how SHH may impact regeneration through regulation of BDNF is presented below.

In this study we report that SHH is a regulator of BDNF in the CN and penis and propose that BDNF forms part of the mechanism through which SHH impacts regeneration. BDNF is neuroprotective for NOS1 positive neurons and promotes neurite outgrowth through the JAK/STAT pathway after injury [39]. Thus BDNF may prevent degeneration of NOS1 containing neurons in the PG and facilitate the regeneration of NOS1 fibers in penile tissue [18]. SHH is also highly expressed in NOS1 positive neurons of the PG [10] and in Schwann cells of the CN [9]. In response to injury, growth factors are up regulated in Schwann cells and retrograde signaling to neuronal cell bodies is required to maintain them in the absence of axonal signals. While exogenously added SHH protein can be taken up by axons at the crush site [9], our previous work has shown that endogenous SHH does not undergo anterograde or retrograde transport [10]. BDNF is produced not only by PG neurons but also in Schwann cells and by the penile target and thus undergoes both anterograde [40–41] and retrograde transport [37]. Thus it is possible that SHH up-regulates BDNF after injury in order for communication to occur between the crush site and PG neurons. This is an intriguing hypothesis that requires further experimentation beyond the scope of this study to explore.

GFAP was increased after BDNF inhibition in SHH treated rats but not as much as in the CN crush only group. This suggests that either K252a did not completely inhibit BDNF, or that SHH induced regeneration involves factors in addition to BDNF. Since SHH is a key

regulator of multiple pathways during development and in the adult, the BDNF pathway may be one of several regulated by SHH to promote regeneration.

Conclusions

SHH is a regulator of BDNF in normal and regenerating PG/CNs. Part of the mechanism of how SHH promotes CN regeneration involves up-regulation of BDNF. These findings are significant because they suggest that combination therapy with SHH and BDNF may be useful to further enhance CN regeneration and preservation.

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Figure 1.

Graph of BDNF induction from 1–7 days after CN crush in the PG/CN, in comparison to sham controls (n=15). BDNF increased 38% the first day after injury (p=0.011), decreased 62% below basal levels at 4 days after injury (p=0.013) and returned to normal levels by 7 days after injury (p=0.424). Asterisks denote significant differences.

Figure 2.

(A) Western analysis of BDNF protein in normal PG/CN that was treated with SHH or BSA protein for two days (n=10) shows a 36% increase in BDNF in response to SHH treatment ($p=0.007$). (B) Western analysis of BDNF protein in normal PG/CN that was treated with 5E1 SHH inhibitor or mouse IgG (control) for two days (n=14) shows a 34% decrease in BDNF protein with SHH inhibition ($p=0.047$). (C) Western analysis of BDNF in crushed CNs treated with SHH or BSA proteins for two days (n=10) shows a 44% increase in BDNF protein in response to SHH treatment ($p=0.003$). Asterisks denote significant differences.

Figure 3.

(A) Western analysis of GFAP protein in sham, CN crushed, and crushed CNs treated with SHH and either the BDNF inhibitor K252a or DMSO (control, n=14). GFAP increased 51% in the crush group relative to the sham ($p=0.006$). GFAP significantly decreased 41% in the presence of SHH PA by comparison to the crush only group ($p=0.001$) suggesting CN preservation by SHH protein. GFAP significantly increased 33% with BDNF inhibition ($p=0.033$), indicating that SHH induced CN regeneration involves BDNF. Asterisks denote significant differences.

Figure 4.

(A) Immunohistochemical analysis was performed on normal Sprague Dawley rat penis. BDNF was abundant in longitudinal and circular smooth muscle but was not identified in endothelium. 400X magnification. Arrows indicate longitudinal and circular smooth muscle and the unstained endothelium. SM=smooth muscle. (B) Immunohistochemical analysis for BDNF was performed on penis tissue from rats that underwent CN injury and either SHH or BSA treatment by PA injected into the corpora cavernosa. BDNF was increased in the penis in response to SHH treatment of the penis for 2 and 7 days. 100X magnification. Arrows indicate BDNF protein.