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Valproic Acid Prevents Penile Fibrosis and Erectile Dysfunction in Cavernous Nerve Injured Rats

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Abstract

Introduction—Bilateral cavernous nerve injury (BCNI) causes profound penile changes such as apoptosis and fibrosis leading to erectile dysfunction (ED). Histone deacetylase (HDAC) has been implicated in chronic fibrotic diseases.

Aims—This study will characterize the molecular changes in penile HDAC after BCNI and determine if HDAC inhibition can prevent BCNI-induced ED and penile fibrosis.

Methods—Five groups of rats (8–10 wks, n=10/group) were utilized: 1) sham, 2&3) BCNI 14 and 30 days following injury, and 4&5) BCNI treated with HDAC inhibitor valproic acid (VPA 250mg/kg; 14 and 30 days). All groups underwent cavernous nerve stimulation (CNS) to determine intracavernosal pressure (ICP). Penile HDAC3, HDAC4, fibronectin, and transforming growth factor-β1 (TGF-β1) protein expression (Western blot) were assessed. Trichrome staining and the fractional area of fibrosis were determined in penes from each group. Cavernous smooth muscle content was assessed by immunofluorescence to alpha smooth muscle actin (α-SMA) antibodies.

Main Outcome Measures—ICP; HDAC3, HDAC4, fibronectin and TGF-β1 protein expression; penile fibrosis; penile α-SMA content.

Results—There was a voltage-dependent decline ($p<0.05$) in ICP to CNS 14 and 30 days after BCNI. Penile HDAC3, HDAC4, and fibronectin were significantly increased ($P<0.05$) 14 days after BCNI. There was a slight increase in TGF-β1 protein expression after BCNI. Histological analysis showed increased ($P<0.05$) corporal fibrosis after BCNI at both time points. VPA treatment decreased ($P<0.05$) penile HDAC3, HDAC4, and fibronectin protein expression as well

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as corporal fibrosis. There was no change in penile α-SMA between all groups. Furthermore, VPA-treated BCNI rats had improved erectile responses to CNS ($P<0.05$).

Conclusion—HDAC-induced pathological signaling in response to BCNI contributes to penile vascular dysfunction after BCNI. Pharmacological inhibition of HDAC prevents penile fibrosis, normalizes fibronectin expression, and preserves erectile function. The HDAC pathway may represent a suitable target in preventing the progression of ED occurring post-RP.

Keywords

Cavernous nerve; Erectile dysfunction; Histone deacetylase; Transforming growth factor beta; Fibrosis; Autonomic nerve injury

Introduction

Men undergoing radical prostatectomy (RP) to treat localized prostate cancer commonly experience erectile dysfunction (ED) following surgery [1]. At the time of nerve-sparing RP, profound effects including traction injury and local inflammatory changes in the neuronal microenvironment of the autonomic fibers innervating the penis cause pathophysiological alterations in the end organ. The pathological changes that occur in the penis include decreased smooth muscle and endothelial cell content and increased fibrosis of the corporal tissue [2,3]. Transforming growth factor beta (TGF-β) as well as other fibrotic cytokines has been shown to induce fibrogenesis in the corpora cavernosa [4]. Elevated TGF-β expression and fibronectin has been noted in animal models of cavernous nerve (CN) injury-induced penile fibrosis [5,6]. Moreover, penile biopsies of the corporal tissue following RP show increased collagen deposition and fibrosis thus supporting the pre-clinical findings [7,8]. Current research strategies to prevent ED following RP have focused on pharmacological intervention that prevents penile corporal fibrosis in an effort to preserve the hemodynamic mechanisms of penile erection.

Recent attention has focused on novel signaling pathways which regulate collagen deposition, fibrogenic cytokines, and smooth muscle cell proliferation [9]. The role of increased histone deacetylase (HDAC) enzymatic activity in the development and progression of fibrosis in chronic disease has been well established. The balance between histone acetyltransferase (HAT) and HDAC regulates gene transcriptions of a wide variety of mediators for cellular functions including protein-protein interactions, localization, and binding activity in vascular smooth muscle cells [10,11]. HDAC activation controls inflammation, cell proliferation, and fibrosis by catalyzing the removal of acetyl groups from lysine residues in various organs, including the heart, lung, and kidney [12]. Several studies have demonstrated that increases in TGF-β can abrogate HDAC deacetylation which leads to vascular remodeling and fibrosis in a number of disease states including pulmonary and renal fibrosis as well as cardiac hypertrophy [12–14]. Valproic acid (VPA) is a non-specific HDAC inhibitor commonly used as an anticonvulsant or mood stabilizing drug. Recently in hypertensive and myocardial ischemic animal models, VPA-mediated HDAC inhibition prevented inflammation, smooth muscle hypertrophy, and TGF-β induced fibrosis [15,16].

In the present study we evaluated the pharmacological effects of HDAC inhibition using VPA in a rat model of CN injury induced ED. We hypothesize that VPA mediated inhibition of HDAC will prevent penile fibrosis and preserve penile hemodynamics to neurogenic-stimulus.

Materials and Methods

Animals and Drug Treatment

Male Sprague-Dawley Rats (Charles River, Wilmington, MA, USA; n=50) aged 8–10 weeks were used in this study. All experiments were conducted in accordance with the Johns Hopkins University School of Medicine guidelines for animal care and use. Animals were randomly separated into the following groups (n=10/group): i) Sham (exposure of CN); ii) BCNI (bilateral cavernous nerve injury) 14 days; iii) BCNI 30 days; iv) BCNI 14 days + valproic acid (VPA); v) BCNI 30 days + VPA. Following BCNI, treated rats (BCNI 14d +VPA, BCNI 30d+VPA) received VPA daily (250 mg/kg via intraperitoneal (i.p.) injection) and sham, BCNI 14d and BCNI 30d received vehicle injections (saline). This dose was selected based two studies performed in rats that demonstrated VPA to inhibit apoptosis and inflammation in ischemic retinas and spinal cord injury [17,18].

Bilateral Cavernous Nerve Injury

Animals were anesthetized with ketamine/xylazine (100 +10 mg/kg, i.p. injection). The prostate was exposed via a midline abdominal incision. The cavernous nerve and MPG were identified posterolateral to the prostate. Bilateral crush injury was induced by applying Dumont #5 forceps to the CN 2–3 mm distal to the MPG on both sides. The forceps were held to complete closure three times for 15s each, causing a moderate crush injury [19]. In sham-operated rats, abdomens were closed following identification of the MPG and CN without crush injury. The same surgeon performed all crush surgeries. Erectile responses were measured and tissues collected at 14 and 30 days following BCNI.

Measurement of Erectile Responses in vivo

Rats were anesthetized with ketamine/xylazine (100/10 mg/kg) and erectile responses were assessed as previously described [18]. Briefly, the carotid artery was cannulated to measure mean arterial pressure (MAP). The right crura was exposed and a 25G needle connected to PE-50 tubing with 250 U/mL heparin was inserted to measure intracavernosal pressure (ICP). The major pelvic ganglion and CN were identified and an electrical stimulator was placed around the CN proximal to the crush injury. MAP and ICP were measured with a pressure transducer connected to a data acquisition system (ADInstruments, Colorado Springs, CA, USA). The CN was stimulated with a square-pulse stimulator (Grass Instruments, Quincy, MA, USA) at a frequency of 20 Hz with a pulse width of 30 seconds and a 0.5 millisecond duration. The length of stimulation was for 1 minute, at increasing voltages (2, 4, 6, 8 volts) with rest periods of 2 to 3 minutes between subsequent stimulations. The ratio between the maximum ICP and MAP obtained at the peak of the erectile response was calculated to normalize for variations in systemic blood pressure. Total ICP was determined by the area under the erectile curve (AUC; mmHg*sec) from the beginning of CN stimulation until the ICP returned to baseline pressures. Erectile responses

were measured 14d and 30d after nerve crush injury. After functional testing, animals were euthanized and penes were harvested for Western blot and histological analysis.

Western Blot Analysis

Whole penes were excised and homogenized in a buffer containing 50 mM Tris-HCl (pH 7.5), 2 mM EDTA, 2 mM EGTA, 150 mM NaCl, 50 mM NaF, 10% glycerol, 10 mg/ml leupeptin, 2 mg/ml aprotinin,10 mg/ml trypsin inhibitor, 1 mM phenylmethylsulfornyl fluoride and 1 mM Na3VO4. Cellular fractions from homogenized penes were isolated for histone deacetylase-3 (HDAC3), histone deacetylase-4 (HDAC4), transforming growth factor beta 1 (TGF-β1), and fibronectin (FN) 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 (HDAC-3 1:1000, HDAC-4 1:1000, TGF-β1 1:2000, FN 1:500 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) 1:2500) 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. The intensities of the resulting bands were quantified by using Image J Software (NIH, Bethesda, MD, USA).

Histology

Rat penes were harvested (n=5/group), fixed in 10% formalin, paraffin embedded and cut in 6 μm sections. Sections were deparaffinized, rehydrated and endogenous peroxidases were quenched with 3% H_2O_2. Nonspecific binding of IgGs was blocked using normal goat serum 1:50 in 0.1% bovine serum albumin in phosphate-buffered saline. Sections were incubated overnight at 4°C with primary antibodies for alpha-smooth muscle actin (α-SMA 1:1000; Abcam, Cambridge, MA, USA) and appropriate species-directed secondary antibodies (Fluorescein (FITC) 1:600; Life Technologies, Grand Island, NY, USA) were applied to the sections (60 min at room temperature). Control staining in the absence of primary antibodies was performed to evaluate unspecific staining by the secondary antibodies (data not shown). Additional slides were stained by Masson's trichrome method to assess collagen content [20]. Fluorescent images were taken with E800 Nikon microscope and CCD camera (Nikon Instruments Inc, Melville, NY, USA) and light microscope images of trichrome staining with E-600 Nikon and DXM camera. Image J Software was used to quantify the area of α-SMA fluorescence stain and the ratio of collagen (blue color) to smooth muscle (red) in the trichrome stained penes (4 high power images of the cavernosum per rat penis). The ratio of collagen to smooth muscle was expressed as a percentage and termed %Fibrosis.

Statistical Analysis

Data were expressed as mean ± standard error of the mean (SEM). Differences between multiple groups were compared by one–way analysis of variance followed by a Tukey's multiple comparisons test using Prism 5 (GraphPad Software Inc, La Jolla, CA, USA). P-value of < 0.05 was used as criteria for statistical significance.

Results

Treatment with VPA preserves erectile function in BCNI rats

Following treatment with VPA, all animals were in good health and there was no difference in body weights (Sham: 417g±8.2; BCNI 14d: 423g±6.5; BCNI 30d: 440g±9.3; BCNI 14dg +VPA: 430g±5.8; BCNI 30d+VPA: 458g±11.5, $p>0.05$) or baseline MAP (Sham: 100.0mmHg±6.16; BCNI 14d: 93.1mmHg±3.51; BCNI 30d: 101.8mmHg±4.43; BCNI 14d +VPA: 92.8mmHg±3.73; BCNI 30d+VPA: 90.4mmHg±4.24, $p>0.05$) between the groups. There was a significant decrease in erectile function in both BCNI 14d and BCNI 30d rats compared to sham rats (Figure 1). Both ICP/MAP and total ICP across all voltages for BCNI 14d and BCNI 30d were lower in contrast to sham rats ($p<0.05$). Treatment with VPA improved ICP/MAP to sham levels at 4 volts and greater than sham levels at 6 and 8 volts for BCNI 14d+VPA and BCNI 30d+VPA rats (Figure 1A). Total ICP was significantly increased at 4, 6, and 8 volts for BCNI 14d+VPA and BCNI 30d + VPA versus BCNI 14d rats; however total ICP in both treated groups remained lower than in sham rats across all voltages (Figure 1B).

HDAC3, HDAC4, TGF-β1 and fibronectin protein expression

At 14 days following BCNI, there was a significant increase in HDAC3, HDAC4 and fibronectin protein expression compared to sham-operated rats (Figures 2, 3). In contrast, BCNI 30d penes did not demonstrate any significant increases in HDAC3, HDAC4 or fibronectin compared to sham penes (Figures 2, 3). Following treatment with VPA for 14 days, BCNI 14d+VPA penes had decreased protein expression of HDAC4 (↓27% vs BCNI 14d) and fibronectin (↓14% vs BCNI 14d) although not significantly different. There was no change in protein expression of HDAC3 in BCNI 14d+VPA penes compared to BCNI 14d penes. Additional treatment with VPA for 30 days, lowered HDAC3, HDAC4 and fibronectin protein levels to sham expression levels. There was a slight increase in TGF-β1 protein expression in BCNI 14d and BCNI 30d injured penes and VPA treatment in BCNI 30d+VPA decreased TGF-β1 compared to sham penes; however these data were not significantly different (Figure 3).

BCNI-induced penile fibrosis was prevented with VPA treatment

Masson's trichrome staining for collagen and smooth muscle within the corpora cavernosum was evaluated in penile cross sections. The ratio of collagen to smooth muscle was depicted as the percentage of fibrosis (Figure 4). Following BCNI at both 14 and 30 days, there was a significant increase in fibrosis compared to sham penes ($p<0.05$). This enhanced fibrosis was reversed with VPA treatment in both 14 and 30 days injured penes (Figure 4). To confirm that these changes were due to increased collagen deposition, smooth muscle content was also assessed by immunofluorescence staining to α-SMA (Figure 5). The area of cavernosal smooth muscle was unchanged with injury and subsequent treatment with VPA (Figure 5).

Discussion

This study is the first to examine the ability of HDAC inhibitors to prevent a decline in erectile function in a rat model of CN injury. Following BCNI in rats, there was an increase in penile HDAC3, HDAC4, TGF-β1, and fibronectin protein expression at 14 days, in addition to enhanced penile fibrosis and decreased erectile function. Treatment with VPA prevented a decrease in ICP/MAP at 14 and 30 days following BCNI. Furthermore, VPA treatment lowered penile HDAC3 and HDAC4 protein expression and preserved penile morphology by decreasing TGF-β1 and fibronectin expression in the penis. These finding suggest that HDAC inhibition can preserve erectile function following CN injury by maintaining penile morphology and inhibiting changes in extracellular matrix.

Penile fibrosis as a result of CN injury has been well established in experimental models (mice, rats, rabbits) and has been demonstrated in two studies examining men who had undergone radical prostatectomy [3,7,8]. In the present study, we found significant increases in penile HDAC3 and 4 protein expressions 14 days following CN injury which were associated with penile fibrosis. The role of HDAC in the fibrotic process has been assessed in multiple diseased states. In a mouse model of unilateral ureteral obstruction, HDAC is involved in the regulation of signal transducer and activator of transcription 3 (STAT3) and fibroblast proliferation in renal fibrosis [21]. Treatment with an HDAC inhibitor, trichostatin A (TSA), inhibited fibroblast activation and diminished the expression of renal α-SMA and fibronectin [21,22]. The role of HDAC in a rat model of cardiac infarct demonstrated that the addition of an HDAC agonist (theophylline) further exacerbated cardiomyocyte hypertrophy and collagen formation [23]. In the penes from CN injured rats, we observed a temporal increase in HDAC3 and HDAC4 at 14 days following BCNI that was lowered by 30days and completely normalized to sham levels with VPA treatment. Additionally, VPA treatment decreased fibronectin protein expression and fibrosis thereby improving erectile function. These data suggest that HDAC inhibition can prevent the pathological alterations that occur in the penis after neuropraxia.

Many ED animal models of CN injury have demonstrated a decrease in corporal smooth muscle content [24–26]. These data are typically assessed by measuring the smooth muscle content in penes stained with Masson’s trichrome and comparing it to the amount of collagen stained. In an animal model of a more severe crush injury in which needle drivers were used to crush for 2 minutes, there was a significant decrease in α-SMA and lower ICP/MAP (0.1) compared to the current study (0.25) [25]. Furthermore, transection and excision of the CN disrupts axonal communication to the penis and results in greater loss of corporal α-SMA and no erectile response is evident upon CN stimulation [27,28,29]. We assessed penile smooth muscle content by immunofluorescence staining with antibodies specific to α-SMA and found that there were no differences in smooth muscle content in sham and BCNI rats injured by forceps which were held to complete closure 3 times for 15 seconds.

In both RP-induced ED and pulmonary hypertension, phosphodiesterase (PDE) 5 inhibitors are commonly prescribed to promote vasodilation and decrease fibrosis. However, in hypoxia-induced pulmonary hypertension in rats, HDAC inhibition caused a greater

reduction in pulmonary arterial pressure compared to tadalafil treated rats [12]. Futhermore, in diabetic nephropathy, HDAC inhibition decreased glomerular volume and collagen, nitrotyrosine concentration, and urine albumin excretion [30]. In our current study, HDAC inhibition recovered erections in CN injured rats 14 and 30 days following injury. The improvement in neurogenic induced erections correlated with preserved penile morphology and reduction in penile fibrosis and TGF-β expression. We have not assessed PDE5 inhibition therapy in our CN crush model though others have demonstrated chronic sildenafil or tadalafil treatment decreased corporal fibrosis, apoptosis and smooth muscle content in CN resection rat and mouse models [31–34]. However, these studies did not assess erectile function via cavernous nerve stimulation. A direct comparison of these two treatments alone or in combination may be interesting to pursue in future investigations.

In the current study, TGF-β1 was only slightly increased following CN injury. The CN crush injury is a moderate injury that may not significantly increase penile TGF-β1 to the higher levels documented following CN resection in animal models or spinal cord injuries in men [28,35,36]. Elevated TGF-β1 in the penis has been shown to activate the mothers against decapentaplegic (SMAD) proteins which bind the SMAD binding element on the gene of interest and increase matrix synthesis [6]. The role of HDAC in TGF-β1 induced collagen synthesis was assessed in human hepatic cells treated with VPA. HDAC inhibition suppressed the transcription of TGF-β1 via SMAD2/3 and maintained acetylation of histone H3 and H4 leading to attenuated collagen synthesis [37]. In renal fibrosis from unilateral ureteral obstruction or diabetes in rats, HDAC inhibition suppressed TGF-β1 production, decreased phosphorylation of SMAD3, and differentiation and proliferation of fibroblasts to myofibroblasts [38,39]. Further studies are required to elucidate if the mechanism of improved erections and fibrosis is via HDAC mediated decreases in TGF-β1 and SMAD pathways.

Considerable research efforts have also demonstrated the neuroprotective effects of HDAC inhibition in neurodegenerative diseases and CNS injuries. Following injury to the optic nerve in mice, HDAC3 nuclear expression was increased in the dying retinal ganglion cells and inhibition of HDAC prevented cell loss [40]. Similarly in optic nerve crushed rats, treatment with VPA prevented retinal ganglion cell death [17]. In rat primary neuronal cultures, nonspecific inhibition of HDAC protected neurons from oxidative-stress death and increased neurite outgrowth [41]. VPA also induces proliferation and myelination of rat Schwann cells and sciatic nerve regeneration [42,43]. Although the current study did not assess any markers of neurodegeneration or neuroprotection, it is possible that HDAC inhibition prevented a decline in erectile function by protecting the CN from undergoing apoptosis and degenerating. Furthermore, by preventing neurodegeneration and preserving neuronal input to the penis, there would be less oxidative stress and initiation of fibrosis in the penile end organ.

The purpose of this study was to assess the ability of HDAC inhibition to prevent the occurrence of erectile dysfunction following CN injury. Further studies must be undertaken to elucidate the exact mechanisms concerned with the improvement of erectile function following the administration of VPA including measuring gene expression and HDAC activity as decreased protein expression does not necessarily translate into lowered

functional activity. The ability of HDAC inhibition to prevent apoptosis in both the corporal tissue and the CN requires further understanding. Another limitation is that HDAC protein expression was not assessed in the MPG and CN. Assessing earlier time points following CN injury may also help reveal if HDAC inhibition is neuroprotective in the CN or if it regulates TGF-β synthesis via a SMAD dependent mechanism. Additionally, exploring the effect of other doses of VPA or other HDAC inhibitors may help to further prevent the decrease in the total ICP (AUC) that was evident in CN injured rats.

In conclusion, the results of our study demonstrate that early treatment with VPA can be used as a potential therapeutic strategy to combat RP-induced ED by preserving the penile morphology and erectile function in a rat model. VPA is a safe, widely used FDA approved drug with minimal common dose-dependent side effects such as dyspepsia and weight gain. HDAC inhibition is also a promising drug target for cancer therapy and is currently undergoing several clinical trials [44]. In an animal xenograft model of prostate cancer VPA decreased tumor size and HDAC inhibition decreased the transformation of tumor precursor cells in the prostate in patient's thereby reducing their risk of developing prostate cancer [45,46]. Thus, VPA could be used to treat men undergoing RP to prevent the incidence of ED without concern about its effects on prostate cancer recurrence. Further studies are required to fully understand the mechanisms of HDAC inhibition in the penis and the CN; however this is a promising new clinical application for RP-induced ED which currently lacks a treatment that cures the underlying disease process.

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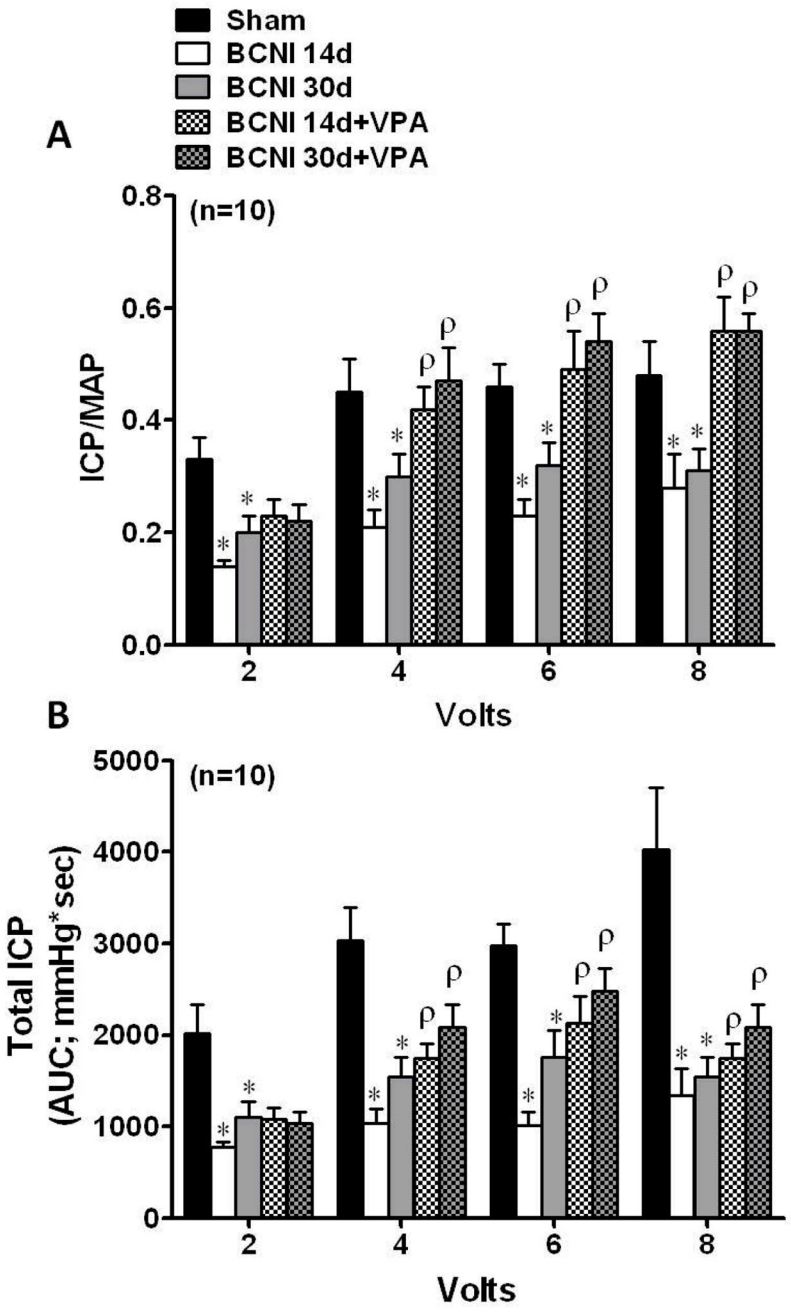

Figure 1.

In vivo erectile responses were assessed 14 and 30 days after CN injury via electrostimulation of the CN. 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 4V stimulation for 1 minute as indicated by the black bar along the x-axis (C). *n*=number of animals in each group; BCNI= bilateral cavernous nerve injury; VPA=valproic acid;

*(P<0.05) response significantly different compared to sham rats; ρ(P<0.05) response significantly different to BCNI14d rats without treatment.

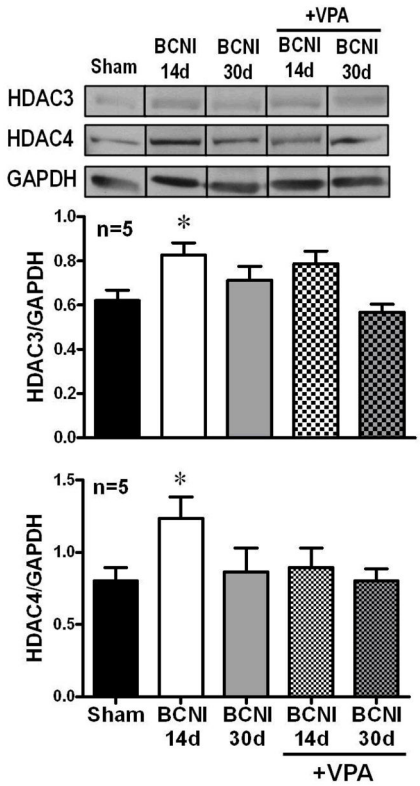

Figure 2.

Western blot analyses demonstrate the expression of HDAC3 and HDAC4 proteins in penile tissue of all groups. Data are normalized to GAPDH protein expression. *n*=number of tissue samples in each group; BCNI=bilateral cavernous nerve injury; VPA=valproic acid; HDAC=histone deacetylase; GAPDH=glyceraldehyde 3-phosphate dehydrogenase; *($P<0.05$) response significantly different compared to sham rats.

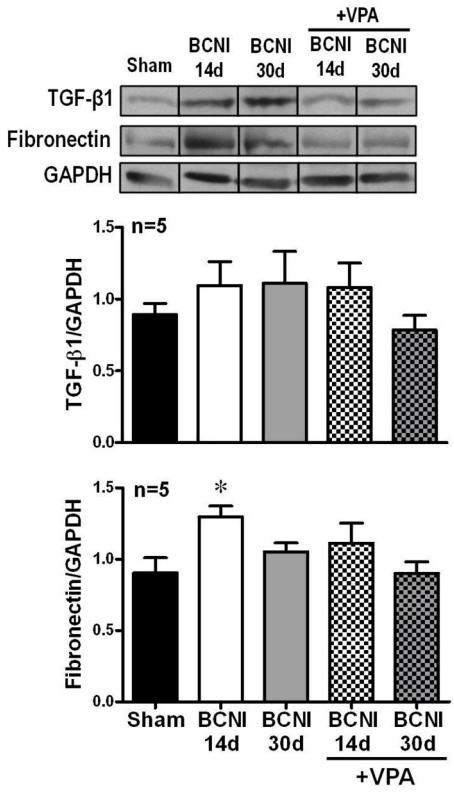

Figure 3.

Representative Western blots for TGF-β1 and fibronectin protein expression in penile tissue of all groups. Data are normalized to GAPDH protein expression. *n*=number of tissue samples in each group; BCNI=bilateral cavernous nerve injury; VPA=valproic acid; TGF-β1=transforming growth factor beta 1; GAPDH=glyceraldehyde 3-phosphate dehydrogenase; *($P<0.05$) response significantly different compared to sham rats.

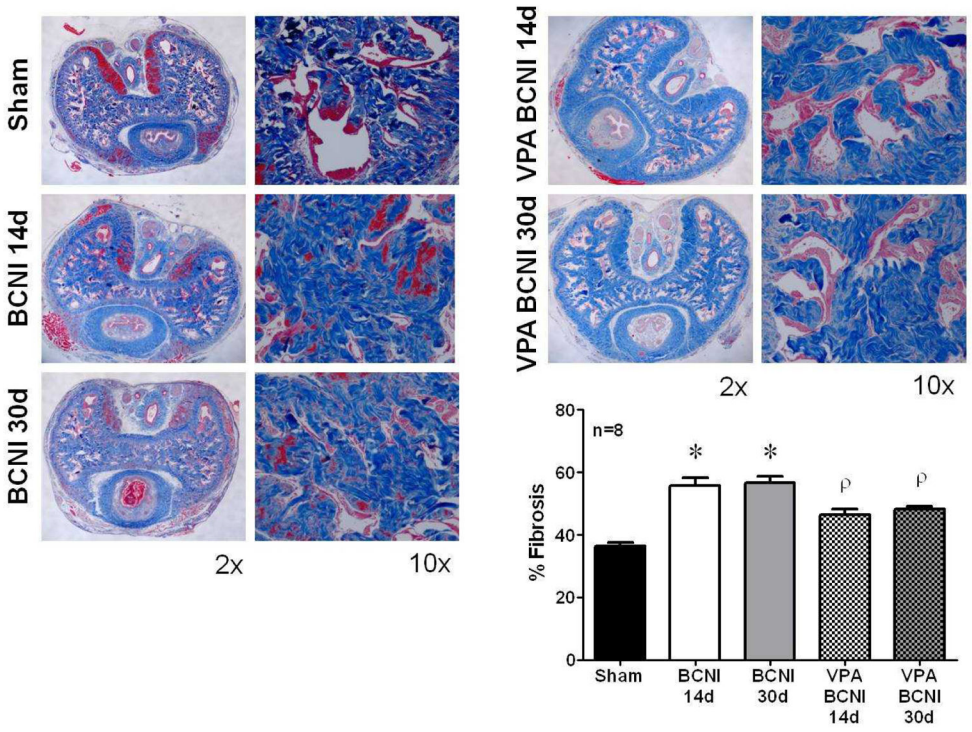

Figure 4.

Representative images of the corpus cavernosum stained with Masson's trichrome in all groups. In all images, the smooth muscle stains red, collagen and connective tissue stain blue and the cytoplasm stains a light pink. The % fibrosis was assessed as the ratio of collagen to smooth muscle. *n*=number of tissue samples in each group; BCNI=bilateral cavernous nerve injury; VPA=valproic acid; *($P<0.05$) response significantly different compared to sham rats; ρ ($P<0.05$) compared to BCNI 14d and 30d without treatment. Magnification 2x and 10x.

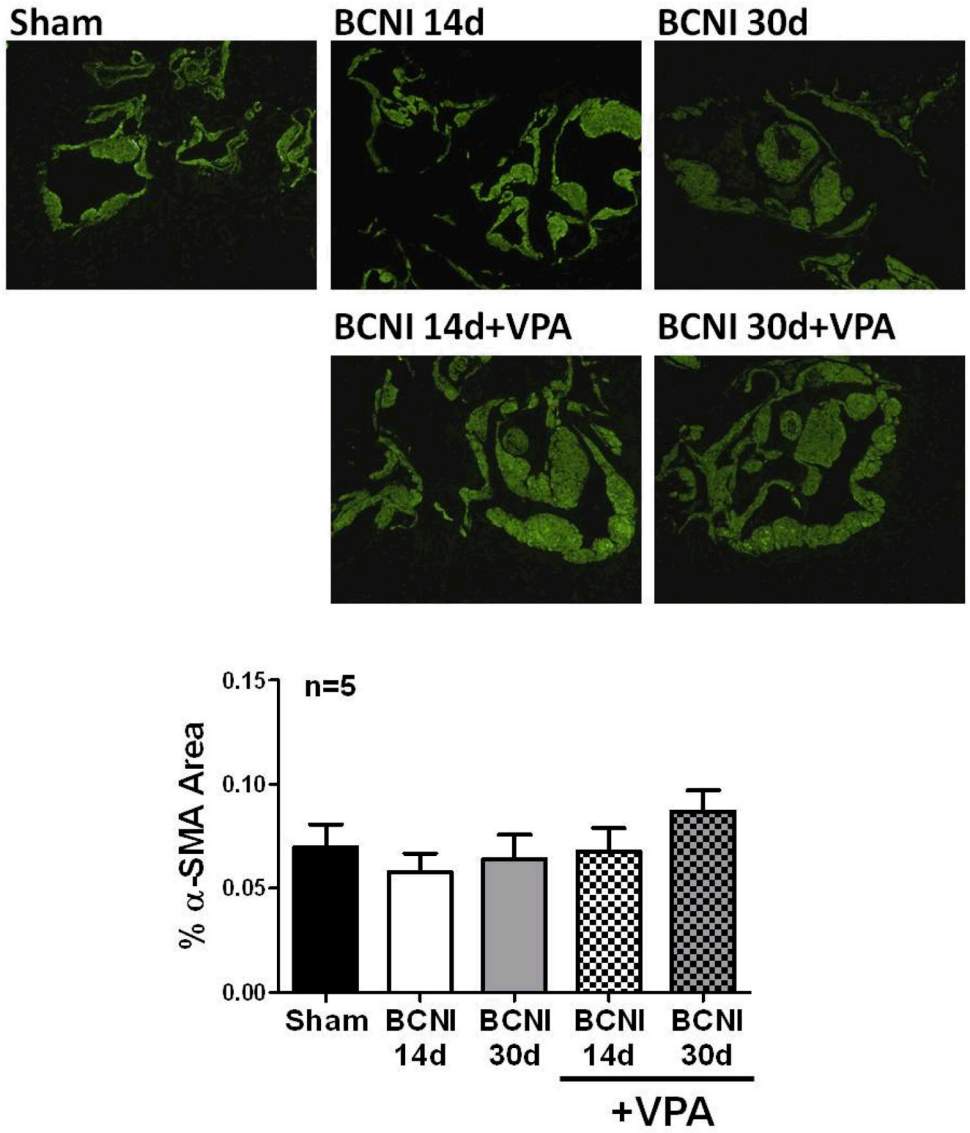

Figure 5.

Immunofluorescent staining of rat penes with antibodies for α-smooth muscle actin (α-SMA, green stain) in all groups. Four quadrants of the corporal tissue were imaged and the percent area of α-SMA was measured and averaged. *n*=number of tissue samples in each group; BCNI=bilateral cavernous nerve injury; VPA=valproic acid. Magnification 20x.