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Neuronal Nitric Oxide Signaling Regulates Erection Recovery after Cavernous Nerve Injury

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Abstract

Purpose—NO is the major neuronal mediator of penile erection, but its role in EF status after CN injury is uncertain. This study aimed to determine the function of neuronal NO signaling in the pathobiology of EF recovery after partial CN injury using both genetic and pharmacologic mouse experimental paradigms.

Materials and Methods—EF was evaluated in WT and nNOS^{-/-} mice (n=5–7/group) at 1, 3 and 7 days after UCI or sham injury and at day 7 in WT mice treated with the NO synthase inhibitor, L-NAME at baseline and for 6 days following UCI. Apoptosis in the penis was evaluated by Western blot analysis of p-Akt-S473, 3-NT, and caspase-3 expressions after BCI.

Results—ICP was significantly decreased at 1, 3 and 7 days in WT mice but only at day 1 in nNOS^{-/-} mice after UCI compared with sham treatment values (p<0.05). L-NAME-treated WT mice had improved EF compared with the vehicle-treated group response at day 7 following UCI (p<0.05). p-Akt-S473 expression in penes was significantly decreased in vehicle-treated (p<0.05) but not L-NAME-treated WT mice. 3-NT expression in penes was significantly decreased in L-NAME-treated WT and vehicle-treated nNOS^{-/-} mice (p<0.05). Caspase-3 expression in penes was significantly increased in vehicle-treated (p<0.05) but not L-NAME-treated WT mice and vehicle-treated nNOS^{-/-} mice.

Conclusions—Neuronal NO signaling regulates EF recovery early after partial CN injury, exerting an inhibitory role via induction of apoptotic changes in penile tissue. Therapeutic strategies to improve EF recovery after RP may consider targeting pathogenic sites of NO neurobiology.

Keywords

nNOS; penis; apoptosis; neurogenic; erectile dysfunction

INTRODUCTION

ED is a common complication of pelvic surgeries such as RP. The primary etiology of post-pelvic surgery ED is neurogenic, in association with iatrogenic trauma of CNs, which provide autonomic regulation of penile erection. Various investigators have explored interventions to prevent CN degeneration and/or improve CN regeneration in this setting¹. However, this problem persists and prompts an ongoing need for clinical advances to promote EF recovery after pelvic surgery².

Current investigative work in this field acknowledges a complex neuropathic process that accounts for post-pelvic surgery ED³. This process involves derangements in CN regeneration and subsequent cavernous smooth muscle atrophic and fibrotic morphological changes that result from loss of innervation. Molecular mechanisms are at play and include corporal smooth muscle apoptosis, hypoxia, upregulation of profibrotic factors and increased collagen synthesis³.

NO is the main mediator of penile erection⁴. It exerts a neurotransmitter-like role in part based on its synthesis in neurons and its relaxant effect on corporal smooth muscle. The role of neuronal NO and its synthetic enzyme, nNOS, in neurodegeneration/neuroregeneration has been controversial. The beneficial effect of NO in physiologic nerve regeneration is suggested by the findings of a regenerative delay in nNOS^{-/-} mice in a sciatic nerve injury model and also decreased survival of ganglion cells in these mice after axotomy⁵. On the other hand, the possible detriment of neuronal NO in nerve injury contexts has been considered on several grounds. Nerve injury-induced mechanical hypersensitivity is attenuated in mice with genetic knockout and pharmacological inhibition of nNOS⁶. Additionally, inhibition of nNOS prevents neuronal cell loss and promotes axonal regeneration of axotomized facial motor neurons in rats⁷, and nNOS^{-/-} mice exhibit improved functional recovery after spinal cord injury relative to similarly treated WT counterparts⁸.

In this study, we evaluated whether neuronal NO signaling functions in EF recovery after CN injury and, if so, exerts facilitatory or inhibitory effects. We applied a mouse experimental paradigm in which we performed partial CN injury, which mimics CN-preserving RP, and invoked a genetic strategy (use of nNOS^{-/-} mice) in conjunction with a pharmacological approach (use of a NO synthase inhibitor). We also examined changes in protein expressions of p-Akt-S473, 3-NT, and caspase-3, which are involved in cellular apoptosis mechanisms, in penile tissue.

MATERIALS AND METHODS

Animals

Adult (8–10 weeks old) male WT (C57BL6/J strain, from Jackson Laboratories, Bar Harbor, ME) and nNOS^{-/-} (a gift from Dr. Solomon Snyder's Lab, Department of Neuroscience, JHU School of Medicine, Baltimore, MD) mice were used in this study. All experiments were conducted in accordance with the JHU School of Medicine guidelines for animal care and use.

CN Injury

In both WT and nNOS^{-/-} mice, under isoflurane anesthesia, CN crush injury was performed, as described previously⁹. To induce UCI, the right CN was crushed (1–2 mm distal to the major pelvic ganglion) 2 times for 15 seconds by applying tips of an ultra-fine forceps (Dumont #5). CN sham treatment was carried out by exposing the left CNs without crush. BCI was induced similarly by crushing both the right and left CNs. All surgeries were carried out by the same trained investigator.

L-NAME Treatment

WT mice which underwent UCI, BCI or sham treatment were administered the NO synthase inhibitor L-NAME (Sigma-Aldrich, Woburn, MA) 50 mg/kg, ip, immediately after surgical perturbation and 1mg/ml L-NAME (6–7 mg/day) or vehicle in their drinking water for 6 days. After a 24 hour washout period, electrically stimulated EF was studied in the UCI group, and molecular investigation was done using penes collected from the BCI group.

In Vivo Erection Studies

At 1, 3 or 7 days following UCI, EF was measured as CN- electrical stimulation induced changes in ICP in anesthetized mice, as described previously ⁹. To monitor ICP, a 30-gauge needle attached to PE-50 tubing was inserted into the right crus and connected to a pressure transducer. For electrical stimulation of penile erection, a bipolar platinum electrode was placed around the CN and was attached to a Grass Instruments S48 stimulator (Quincy, MA). Stimulation parameters were 4 volts, 16 Hz, with square-wave duration of 5 milliseconds for 1 minute. ICP was recorded (DI-190; Dataq Instruments, Akron, OH) from the start of stimulation to an interval of 1 minute after stimulation ended. Max ICP and ICP area were analyzed using the MATLAB program (Mathworks, Natick, MA). ICP responses to electrical stimulation of the crushed nerve (right CN) were expressed as the percent of the intact nerve (left CN), as described previously ⁹.

Western Blot Analysis

Penes, which were collected at day 7 after BCI, were homogenized, centrifuged, and separated with gel electrophoresis, as described previously ¹⁰. Proteins were then transferred to a polyvinylidene fluoride membrane, and incubated with the following primary antibodies (dilutions indicated) overnight at 4°C: anti-phospho-Akt-S473 (1:1000), anti-Akt (1:2000), anti-caspase-3 (1:15000) (Cell Signaling Technology, Danvers, MA); 3-NT (1:2000; Abcam, Cambridge, MA); anti- β -actin (1:5000; Sigma-Aldrich). Densitometry results were normalized by β -actin and expressed as arbitrary units of % of sham.

Statistical Analysis

The data are expressed as the mean $\pm$ SEM. Statistical analyses were performed using one-way ANOVAs followed by Newman-Keuls multiple comparison tests or by Student's *t* tests when appropriate. A probability of 5% or less was considered significant.

RESULTS

Preserved EF in nNOS^{-/-} Mice after CN Injury

To evaluate the role of neuronal NO signaling in ED following CN injury, we compared the CN-electrical stimulation induced changes in ICP at different time points after UCI in WT and nNOS^{-/-} mice (fig. 1). In WT mice, both max ICP and ICP area were significantly decreased 1 day after UCI and remained decreased up to 7 days compared with sham treatment ($p < 0.05$). In nNOS^{-/-} mice, max ICP was significantly decreased at day 1 ($p < 0.05$) but not at 3 or 7 days after UCI compared with sham treatment. Also in nNOS^{-/-} mice, ICP area was significantly decreased at day 1 after UCI compared with sham

treatment values ($p<0.05$), whereas at days 3 and 7, it was significantly increased compared with that at day 1 ($p<0.05$) although still decreased compared with sham treatment values ($p<0.05$). These results indicate that reduced neuronal NO function under conditions of CN injury correlates with EF preservation.

Preserved EF in L-NAME- treated WT Mice after CN Injury

The role of neuronal NO signaling in CN injury-associated ED was further evaluated in CN injured-WT mice treated with an NO synthase inhibitor. Mice were administered L-NAME as a loading parenteral dose and then orally for 6 days, and EF was evaluated after a 24 hour washout period (fig. 2). Following UCI, L-NAME- treated mice had a significantly elevated ICP response (both max ICP and ICP area) compared with that of vehicle- treated mice ($p<0.05$) further indicating that reduced neuronal NO release after CN injury associates with EF preservation. To confirm the specificity of our dosing protocol using L-NAME, CN electrical stimulation-induced changes were compared in WT mice treated with L-NAME for 7 days continuously or 6 days followed by a 24 hour washout period. In the presence of L-NAME without washout, max ICP was significantly inhibited compared with that of vehicle treatment (15.6 ± 4.5 vs 44.3 ± 6.4 mmHg, respectively, $p<0.05$). ICP response was slightly decreased in the presence of L-NAME with a washout period, but this was not significant (35.2 ± 3.7 vs 44.3 ± 6.4 mmHg, $p>0.05$).

Preserved p-Akt-S473 Protein Expression in L-NAME-treated WT Mouse Penis after CN Injury

To evaluate whether apoptotic mechanisms relate to neuronal NO signaling in CN injury-induced ED, the expression of the antiapoptotic marker, p-Akt-S473, was assessed in our experimental paradigm. p-Akt-S473 expression was significantly decreased in the vehicle-treated WT mouse penis 7 days after BCI compared with the sham treatment value ($p<0.05$) (fig. 3). However, p-Akt-S473 expression was not different in mice treated with L-NAME compared with the sham treatment value ($p>0.05$). These results indicate that reduced neuronal NO release after CN injury correlates with attenuated apoptosis in the penis.

Attenuated 3-NT and Caspase-3 Protein Expressions in L-NAME-treated WT Mouse Penis after CN Injury

The correlation of apoptosis and neuronal NO signaling in CN injury-induced ED was further evaluated by assessing the expressions of the apoptotic markers, 3-NT and caspase-3, in our experimental paradigm. 3-NT expression was significantly decreased in the penis of WT mice treated with L-NAME ($p<0.05$) but not with vehicle 7 days after BCI compared with the sham treatment value ($p>0.05$) (fig. 4A). Caspase-3 expression was significantly increased in the penis of WT mice ($p<0.05$) at 7 days after BCI but did not change in the penis of WT mice treated with L-NAME compared with the sham treatment value ($p>0.05$). To further ascertain whether neuronal NO depletion attenuates apoptosis after CN injury, apoptosis marker protein expressions were evaluated in the penis of CN-injured nNOS^{-/-} mice. In the nNOS^{-/-} mouse penis at 7 days after BCI, 3-NT expression was significantly decreased ($p<0.05$), whereas caspase-3 expression did not change ($p>0.05$) compared with sham treatment values (fig. 4B). These results, taken together, suggest that neuronal NO

release is involved in apoptosis-associated protein tyrosine nitration and induction of apoptosis-associated caspase-3 expression in the penis after CN injury.

DISCUSSION

Neuronal NO is a signaling molecule which functions in a wide variety of physiologic and pathophysiologic processes such as neurotransmission, neuroprotection, and neurotoxicity¹¹. In the urogenital system, nNOS production of NO within CN fibers supplying the penis is well-established as the primary stimulus for neuronally induced penile erection⁴. However, the role of neuronal NO in EF recovery following CN injury has not been clearly elucidated. The present study, using both genetic and pharmacologic approaches, shows that a substantial lack or reduction of neuronal NO production correlates with EF preservation in mouse models of partial CN injury. It also evinces that such reduced neuronal NO signaling correlates with attenuated apoptotic signaling based on our assessment of apoptotic markers in our experimental injury and treatment paradigms. Thus, we infer that nNOS-specific production of NO may play a critical role in the pathobiology of EF recovery after CN injury.

We first charted the time course of CN-stimulated erectile response in WT and nNOS^{-/-} mice following UCI. In our mouse UCI model, ICP measurement is expressed as the percent response of the injured relative to the intact side which has the advantage of comparing injury versus sham treatment in the same animal and permitting each animal to serve as its own control, as described previously^{9, 12}. However, we observed differences in the ICP response to stimulation of R- or L-CNs probably due to response variability in live animal experimentation. Similar to results recently published by Jin et al.¹³, ICP response to electrical stimulation of the CN was decreased about 50% in WT mice compared to sham treatment at day 1 after UCI and remained low at post-injury days 3 and 7. On the other hand, in nNOS^{-/-} mice, which do preserve approximately 10% nNOS activity in the penis¹⁴, erectile response was significantly decreased at 1 day after injury compared with sham treatment, whereas at days 3 and 7 after injury, it was similar to sham treatment responses. The improved EF preservation after CN injury in nNOS^{-/-} mice, relative to that of WT mice, suggests that neuronal NO may act deleteriously on EF recovery after CN injury. This effect was further confirmed in WT mice treated continuously with L-NAME for 6 days with a day of washout. The doses of 50 mg/kg, ip and 1mg/ml in drinking water (6–7 mg/day) were chosen based on mouse in vivo studies, demonstrating that they provide effective acute and chronic inhibition of NOS activity, respectively^{15, 16}. The significant inhibition of CN electrical stimulation-induced ICP response in WT mice treated continuously with L-NAME for 7 days without a washout period is consistent with the established role of neuronal NO in mediating penile erection and the robust efficacy of L-NAME in inhibiting the erectile response. Conceivably, neuronal NO exerts a pathologic role in the intracorporal damage sustained after CN injury (at least early post-injury), distinct from its role in physiologic penile erection. This concept is consistent with known neuropathic actions of NO in the face of neuronal diseases or injury^{8, 17}.

Numerous studies demonstrate that apoptotic changes occur in the penis of rats and mice following CN injury^{18, 19}. Apoptotic changes in the penis have generally been evaluated by

TUNEL assay and/or as changes in caspase-3 expression. In a mouse model of bilateral CN crush injury, apoptosis index was significantly increased after injury as early as 3 days and remained high up to 12 weeks, although the maximum increase was at weeks 1 and 2. In the same study, caspase-3 expression was also significantly elevated at 1 week after injury. Similarly, in our study, we found that caspase-3 expression was increased after injury in WT mice (with and without L-NAME treatment) but not in nNOS^{-/-} mice. Also in WT mice, activity of Akt, an anti-apoptotic signaling molecule, measured as change in the expression of p-Akt-S473, was significantly decreased in the vehicle-treated BCI group but not in the L-NAME- treated BCI group. These findings are also consistent with other studies demonstrating a decrease in Akt phosphorylation following injury¹⁸. In a previous study from our group, we showed that p-Akt-S473 decreased in the rat penis as early as 1 day after UCI²⁰. Although p-Akt-S473 expression returned to baseline levels at 7 days after injury previously, this result is most likely due to differences in the injury model (UCI vs BCI in this study) and the animal strain (rat vs mouse here). Together, our findings suggest the neurodegenerative role of neuronal NO following CN injury leading to apoptotic changes in the penile tissue.

In addition to its well characterized role in physiologic cellular signaling, NO is characterized to mediate pathological conditions¹¹. NO can cause cytotoxicity by reacting with superoxide radicals to produce a potent oxidant peroxynitrite. The propensity to produce superoxide/peroxynitrite differs among individual NOS isoforms, and nNOS is most characteristically associated with the production of superoxide under such conditions as decreased substrate and co-factor availability^{11, 21, 22}. Peroxynitrite has been implicated in secondary oxidative-nitrosative lesions following trauma since it can cause damage to DNA, lipids, carbohydrates and proteins via binding to the amino acids cysteine, methionine, and especially tyrosine. Most of the neurodegenerative disorders are marked by the presence of 3-NT positive proteins, leading to the supposition that increased NO-induced nitrosative stress contributes to degeneration. Therefore, the biomarker for protein nitration 3-NT has extensively been used to evaluate peroxynitrite-induced cellular damage¹⁷. In a mouse model of traumatic brain injury, 3-NT immunoreactivity is attenuated in nNOS^{-/-} mice after injury¹⁷. In our study, 3-NT expression was significantly decreased in nNOS^{-/-} mice after BCI and in L-NAME- treated WT mice following BCI, suggesting that in the context of CN injury neuronal NO provides a source of protein tyrosine nitration in the penis.

Recently, nitrosative stress was suggested to contribute to neurodegeneration through S-nitrosylation (and thus deactivation) of proteins critical for neuronal survival²⁴. In addition to tyrosine nitration, S-nitrosylation is a protein modification with wide-ranging effects on cellular function, especially apoptotic signaling²³. NO can also contribute to apoptosis via nitrosylation of key signaling molecules or via impairment of mitochondrial respiratory chain electron transfer²². Ultimately, multiple factors contribute to how NO affects cell survival, including cell type, redox microenvironment, and the balance of other proapoptotic versus antiapoptotic factors²⁴. It is possible that our findings of increased apoptotic signaling may be mediated not only by NO- induced nitration but also by nitrosylation of several key survival proteins.

NO can be generated not only by nNOS but also by the alternatively characterized NOS enzymes, eNOS and iNOS. The pathophysiologic function of NO is known to be regulated by the expression and activity of these enzymes as well, although their precise roles in neuroprotection/neurotoxicity has been conflicting²⁵. For example, mice lacking iNOS demonstrated a delay in myelinated fiber regeneration following sciatic nerve injury²⁶. Also, in a rat model of bilateral CN resection, iNOS is induced as early as 3 days after injury and is suggested to have an anti-fibrotic role²⁷. On the other hand, in rodent models of spinal cord injury, upregulation of iNOS expression leads to neurotoxicity²⁸. Our study does not rule out roles of iNOS and/or eNOS. However, it is very likely that nNOS is central in the NO-regulated inhibition of EF recovery following CN injury since the effect was observed in nNOS^{-/-} mice.

We recognize that a number of pathophysiologic mechanisms are involved in CN injury-induced ED. The prevailing view is that CN injury deprives the penis of its nerve function at least temporarily and this leads to a loss of corporal smooth muscle cells in the penis and an exaggerated deposition and disorganization of extracellular matrix proteins^{3, 19}. Moreover, decreased neurotrophic factors, increased reactive oxygen species, cavernous hypoxia, and upregulated profibrotic factors such as transforming growth factor- β are involved in subsequent changes in the corpus cavernosum tissue²⁹. Results of our study suggest that neuronal NO operating early after CN injury may act as another molecular factor contributing to cavernous tissue apoptosis and ED.

Despite the proapoptotic actions of NO itself in the context of tissue injury, recent studies demonstrate that NO- independent activation of guanylate cyclase and a downstream target protein, protein kinase G, inhibit neuronal apoptosis and increase cell survival³⁰. Further studies to test the potential protective effects of these effectors distinct from the complications of NO-induced toxicity will contribute significantly to this area of research.

CONCLUSIONS

Our study demonstrates that neuronal NO signaling regulates EF recovery in the early course following partial CN injury, exerting an inhibitory role. Its mechanism of action evidently involves induction of apoptotic changes in penile tissue possibly via nitrosative stress. Modulation of neuronal NO function may provide a new clinical therapeutic strategy for pelvic nerve injuries and related neurogenic ED.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

CN electrical stimulation induced changes in A) max ICP (Δ max ICP) and B) ICP area (Δ ICP area) in WT and nNOS^{-/-} mice at 1, 3 and 7 days after UCI. ICP response is expressed as the percent response of intact nerve stimulation. Values are mean $\pm$ SE, n= 5–7 per group, *P<0.05 vs WT-Sham, †P<0.05 vs nNOS^{-/-}-Sham, §P<0.05 vs nNOS^{-/-}-Crush1.

Figure 2.

Effect of L-NAME treatment on EF following UCI in WT mice. Mice were treated with L-NAME (1mg/ml) in their drinking water for 6 days followed by a day of washout. CN electrical stimulation induced changes in A) max ICP (Δ max ICP) and B) ICP area (Δ ICP area) were evaluated at day 7 after UCI. ICP response is expressed as the percent response of intact nerve stimulation. Values are mean $\pm$ SE, n= 5–7 per group, *P<0.05 vs vehicle treatment.

Figure 3.

Changes in p-Akt-S473 protein expression in WT mouse penis. Following BCI, mice were treated with V or L-NAME (1mg/ml) in their drinking water for 7 days. Values are mean $\pm$ SE, expressed as % of Sham; n= 4–5 per group, *P<0.05 vs Sham.

Figure 4A

Figure 4B

Figure 4. Changes in 3-NT and Caspase-3 protein expression in A) WT and B) nNOS^{-/-} mouse penis. Following BCI, mice were treated with L-NAME (50mg/kg) ip and then (1mg/ml) in their drinking water (WT mice only) or V for 7 days. Values are mean $\pm$ SE, expressed as % of Sham; n= 4–5 per group, *P<0.05 vs Sham.