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Cyclic AMP-Dependent Post-Translational Modification of Neuronal Nitric Oxide Synthase Neuroprotects Penile Erection in Rats

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Abstract

Objectives—To evaluate nNOS phosphorylation, nNOS uncoupling, and oxidative stress in the penis and major pelvic ganglia (MPG), before and after the administration of the cAMP-dependent protein kinase A (PKA) agonist colforsin in a rat model of bilateral cavernous nerve injury (BCNI) which mimics nerve injury following prostatectomy.

Materials and Methods—Adult male Sprague–Dawley rats were divided into BCNI and sham groups. Each group included 2 subgroups: vehicle and colforsin (0.1 mg/kg/day i.p.). After 3 days, erectile function (intracavernosal pressure) was measured and penes and MPG were collected for molecular analyses of phospho(P)-nNOS (Ser-1412 and Ser-847), total nNOS, nNOS uncoupling, binding of neuronal nitric oxide synthase (PIN) to nNOS, gp91^{phox} subunit of NADPH oxidase, active caspase 3, PKA catalytic subunit alpha (PKA-Cα) (by Western blot) and oxidative stress (hydrogen peroxide [H2O2] and superoxide by Western blot and microdialysis method).

Results—Erectile function was decreased 3 days after BCNI and normalized by colforsin. nNOS phosphorylation on both positive (Ser-1412) and negative (Ser-847) regulatory sites, and nNOS uncoupling, were increased after BCNI in the penis and MPG and normalized by colforsin. Hydrogen peroxide and total ROS productions were increased in the penis after BCNI and normalized by colforsin. Protein expression of gp91^{phox} was increased in the MPG after BCNI and was normalized by colforsin treatment. Binding of PIN to nNOS was increased in the penis after BCNI and was normalized by colforsin treatment. Protein expression of active Caspase 3 was increased in the MPG after BCNI and was normalized by colforsin treatment. Protein expression of PKA-Cα was decreased in the penis after BCNI and normalized by colforsin.

Conclusion—Collectively, BCNI impairs nNOS function in the penis and MPG by mechanisms involving its phosphorylation and uncoupling in association with increased oxidative stress, resulting in erectile dysfunction. PKA activation by colforsin reverses these molecular changes and preserves penile erection in the face of BCNI.

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Competing financial interests

The authors declare no competing financial interests.

Keywords

Phospho-nNOS (Ser-1412 and Ser-847); nNOS uncoupling; neuropathic erectile dysfunction; cavernous nerve injury; oxidative stress; colforsin

Introduction

Erectile dysfunction (ED) is a major clinical complication associated with pelvic surgeries such as radical prostatectomy (RP). Successful sexual intercourse rates at one-year after surgery have been reported to be between 15% – 87% [1]. As intact autonomic nerve supply to the penis promotes the structural and functional health of the cavernosal tissue, cavernous nerve (CN) injury is a predominant cause of RP-associated ED [2]. At the cellular level, CN injury results in apoptosis of corporal smooth muscle and endothelium, upregulation of profibrotic cytokines, hypoxia, increased collagen synthesis, and fibrosis within the corpora cavernosa [3–6]. Molecular conditions underlying these changes involve upregulation of the RhoA-ROCK pathway, dysregulation of growth factor expression, increased oxidative/nitrosative stress, and decreased neuronal nitric oxide (NO) synthase (nNOS) expression in the penile innervation [7–12].

nNOS-containing autonomic nerves are the principal sites in the penis for production of NO, the main mediator of penile erection [13]. In a rat model of CN injury, nNOS protein expression and activity in the penis and major pelvic ganglia (MPG) decrease in parallel with degeneration of nerve terminals [7,14,15]. Furthermore, it is purported that the absence of cavernosal cycling between flaccid and erect state secondary to penile neuropathy results in further structural and functional damage to the corpora cavernosa [16]. Current ED treatments, such as phosphodiesterase type 5 (PDE5) inhibitors, are less effective in men with ED resulting from RP compared to the general ED population [17,18]. Therefore, other therapeutic strategies are needed to preserve the penis and MPG from structural and molecular changes during the period of neural recovery.

Post-translational modification of nNOS on Ser-1412 by protein kinase A (PKA)-mediated phosphorylation constitutes a major regulatory mechanism for NO generation in the penis and MPG [19]. Neuronal stimulation, presumably through the increase in intracellular calcium and adenylate cyclase (AC) activation, causes an increase in intracellular cyclic adenosine monophosphate (cAMP) that activates PKA [20], which phosphorylates nNOS at Ser-1412, stimulating nNOS catalytic activity [19]. This phospho-modification lasts longer than the neuronal calcium transient, thus contributing to both the initiation and maintenance phases of erection [19]. nNOS activity is also regulated by its functional dimerization. Under physiologic conditions, NOSs are homodimeric enzymes that catalyze the conversion of L-arginine to NO [21]. However, under pathologic conditions resulting in a loss of the functional enzyme's dimerization, NOSs can transform into prooxidants, generating predominantly superoxide anion rather than NO ('NOS uncoupling') [22].

The mechanism of ED and, specifically, the molecular basis for the nNOS defect in the neuropathic penis and penile innervation is unclear and requires further investigation. In accordance with the indispensable role of nNOS posttranslational activation in the erectile

process, our hypothesis in the present study was that nNOS posttranslational activation serves as a homeostatic mechanism that is altered in the context of penile neuropathy. We further hypothesized that preserving physiologic nNOS function through cAMP/PKA-mediated nNOS phosphorylation and proper nNOS coupling optimizes nNOS bioactivity that counteracts neuropathic oxidative stress in the penis and penile innervation. Therefore, we studied whether the PKA activator colforsin is capable of improving erectile function in a rat model of bilateral CN injury (BCNI), and if so, whether it is due to preserving optimal nNOS phosphorylation and coupled function, and limiting oxidative stress in the erectile tissue and penile innervation.

Materials and Methods

Animals and Study Design

Adult male Sprague-Dawley rats (325–350 g; Charles River Breeding Laboratories, Wilmington, MA, USA) were used. All experiments were approved by the Johns Hopkins University School of Medicine Institutional Animal Care and Use Committee in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Animals were randomly divided into four groups (n= 8–10/group): Sham + vehicle (Saline); Sham + colforsin (Abcam, Cambridge, MA, USA, 0.1 mg/kg/day); BCNI + vehicle; and BCNI + colforsin (0.1 mg/kg/day). This dose of colforsin was chosen based on our dose-response pilot study, which showed that 0.1 mg colforsin per day is the most effective dose in preserving erectile function (data not shown). Colforsin and vehicle were administered intraperitoneally 2 hours prior to BCNI, 24 hours post BCNI, and 48 hours post BCNI. Twenty four hours after the last treatment, erectile function in response to electrical stimulation of the CN was evaluated, and 10 min after the termination of CN electrical stimulation, the penis and MPG were collected for molecular investigations. We used a one-day washout period in order to exclude the direct calcium-dependent effect of PKA activation on corporal smooth muscle [4–6,23,24]. We chose to perform this study three days following BCNI in order to assess early molecular changes associated with CN injury devoid of penile fibrotic changes, which occur as early as 1 week following BCNI [25].

CN Injury

To perform BCNI on anesthetized rats (2.5% isoflurane with 1 L/min O_2 flow), CNs were identified bilaterally via a midline lower abdominal incision, and crushed 2–3 mm distal to the MPG[26]. BCNI was induced by crushing the CNs with an ultra-fine hemostat (Fine Science Tools, Foster City, CA, USA) at a constant “two-click” pressure for 2 minutes per side. Sham surgeries were completed by exposing the CNs but not manipulating them [27].

Penile Erection Studies

ICP was measured in anesthetized (100 mg/kg ketamine/5 mg/kg xylazine) animals [27]. The electrode was attached to a Grass Instruments S48 stimulator. The stimulation parameters were 16Hz at 1V and 4V with monophasic, square-wave duration of 5ms. The duration of electrical stimulation of CN was 1 min. Response parameters were recorded using data acquisition (DI-190, Dataq Instruments, Akron, OH, USA), and results were

analyzed using Matlab software (Mathworks, Natick, MA, USA). Maximum ICP (maximum pressure that is reached during CN electrical stimulation) and total ICP (ICP area under the curve, indicating the ICP response for the duration of CN electrical stimulation) were expressed per mean arterial pressures (MAP) as a difference from baseline.

Western Blot Analysis

Frozen tissue was homogenized (penis) or sonicated (MPG)[19,28]. nNOS was partially purified from penile homogenates by affinity binding to 2′,5′-ADP Sepharose [28]. Partially purified nNOS samples or penile and MPG homogenates were resolved on 7.5% or 4–20% Tris gels and transferred to polyvinylidene difluoride membrane. Membranes with partially purified nNOS were probed with anti-phospho (P)-nNOS (Ser-1412 and Ser-847) antibodies (polyclonal rabbit, kindly provided by Dr. Solomon Snyder at the Johns Hopkins University, Baltimore, MD, USA, and Abcam, Cambridge, MA, USA, respectively) at 1:6000 and 1:1000 dilutions, respectively (for P-nNOS analyses), anti nNOS antibody (polyclonal rabbit, Cell Signaling Technology, Beverly, MA, USA) at 1:1000 dilution (for nNOS uncoupling analyses), and protein inhibitor of nNOS (PIN) antibody (monoclonal mouse, BD Transduction Laboratories, San Diego, CA, USA) at 1:500 dilution (for PIN binding to nNOS) [29,30]. After probing for P-nNOS (Ser-1412 and Ser-847) or PIN, these membranes were stripped and probed with anti-nNOS antibody (polyclonal rabbit, Solomon Snyder M.D., Johns Hopkins University, Baltimore, MD, USA) at 1:9000 dilution. P-nNOS (Ser-1412 and Ser-847) and PIN densities were normalized relative to those of nNOS in partially purified samples. For immunoblot analyses of dimeric and monomeric forms of nNOS, purified samples (penis) or homogenates (MPG) in Laemmli buffer were not heated, and the temperature of the gel was maintained below 15°C during electrophoresis [31]. For Western Blot analysis of total nNOS (polyclonal rabbit antibody, Cell Signaling Technology, 1:1000 dilution), PKA-Cα (polyclonal rabbit antibody, Cell signaling Technology, Beverly, MA, USA, 1:500 dilution), $gp91^{phox}$ (monoclonal mouse antibody, BD Transduction Laboratories, 1:1000 dilution) and cleaved caspase 3 (polyclonal rabbit antibody, Cell Signaling Technology, 1:1000 dilution), a separate set of homogenates (70–100 μg) was used without purification and standardized to β-actin (monoclonal mouse antibody, Sigma Chemical, St. Louis, MO, USA, 1:4000 dilution). Band densities were quantified using NIH Image J 1.29. The ratio was determined in terms of arbitrary units and expressed relative to the ratio for vehicle-treated sham animals. nNOS uncoupling was represented inversely as a ratio of nNOS dimers to nNOS monomers. To verify that β-actin expression was not affected by BCNI or colforsin treatment, the density of a set of samples was standardized per total proteins (Ponceau S staining). Ratios did not differ from those obtained using β-actin for standardization (data not shown).

In vivo Reactive oxygen species (ROS) Production

In vivo hydrogen peroxide (H_2O_2) and superoxide were measured in the penis via microdialysis on anesthetized rats [32,33]. The penis was freed of skin and fascia, and a linear microdialysis probe (Bioanalytical Systems, Inc., West Lafayette, IN) with a 20 kDa maximal pore size was inserted into the shaft of the penis. Microdialysis probes were perfused with saline containing 100 μM Amplex Ultrared (Molecular Probes, Eugene, OR) and 1.0 U/ml horseradish peroxidase (HRP; Sigma Aldrich, St. Louis, MO) at 1.0 μl/min

with a microdialysis pump (Harvard Apparatus, Holliston, MA). Amplex Ultrared is a fluorogenic substrate, which in the presence of HRP reacts with H_2O_2 to produce the highly fluorescent resorufin [34,35]. Three 15-minute replicate samples were collected, and fluorescence of the dialysate was measured in a fluorometer (BioRad, Hercules, CA) at 510 nm Ex/590 nm Em. 10 U/ml superoxide dismutase (SOD; Sigma Aldrich, St. Louis, MO) was then added to the perfusate, allowing for conversion of superoxide that crosses over the microdialysis membrane into H_2O_2, which then stimulates the conversion of Amplex Ultrared to resorufin by HRP [36]. Three 15-minute replicate samples were collected, from which fluorescence was measured. Following measurements, penile tissue was harvested, and stored at −80°C until analysis.

Statistical Analysis

Data were expressed as means ± SEM. Statistical analyses were performed using one-way analysis of variance (ANOVA), followed by Tukey's multiple comparison test for ICP analyses and in vivo ROS analysis. For Western blots, a modified t-test was used to compare the experimental groups with the normalized control ratio, and to evaluate among the experimental groups. A value of $P < 0.05$ was considered significant.

Results

Effect of BCNI and Colforsin on body weight and blood pressure

There were no group differences in body weight (Sham: 439 gr ± 19.4; Sham + colforsin: 422 gr ± 23.2; BCNI: 447 gr ± 21.7; BCNI + colforsin: 433 gr ± 20.8, $P > 0.05$) or MAP (Sham: 100.2 mmHg ± 1.02; Sham + colforsin: 103.5 mmHg ± 1.94; BCNI: 102.4 mmHg ± 0.06; BCNI + colforsin: 103.8 mmHg ± 3.65, $P > 0.05$) following crush or colforsin treatment.

Effect of BCNI and Colforsin on erectile function

Erectile function was decreased in the BCNI group, as evident by decreased total (ICP)/MAP and max ICP/MAP ($P < 0.05$) at both voltages relative to Sham rats (Fig. 1). Treatment with colforsin augmented ($P < 0.05$) total ICP/MAP and maximum ICP/MAP at both voltages in BCNI rats. Erectile function in Sham animals was not affected by colforsin treatment.

Effect of BCNI and Colforsin on nNOS phosphorylation (Ser-1412 and Ser-847) and total nNOS protein expression in the penis and MPG

Following BCNI, nNOS phosphorylation on Ser-1412 (positive regulatory site) was increased ($P < 0.05$) in the penis (Fig. 2A) and MPG (Fig. 2B). Treatment of BCNI animals with colforsin decreased ($P < 0.05$) nNOS phosphorylation on Ser-1412 in the penis, but not in the MPG. In Sham animals, colforsin treatment had no effect in the penis, although it increased ($P < 0.05$) nNOS phosphorylation on Ser-1412 in the MPG.

Following BCNI, nNOS phosphorylation on Ser-847 (negative regulatory site) was increased ($P < 0.05$) in the penis (Fig. 2C) and MPG (Fig. 2D). Treatment of BCNI animals with colforsin decreased ($P < 0.05$) nNOS phosphorylation on Ser-847 in the penis but not in the

MPG. Colforsin treatment did not affect nNOS phosphorylation on Ser-847 in the penis or MPG in Sham animals.

Protein expression of total nNOS was decreased ($P < 0.05$) in the penis but not in the MPG after BCNI, and was unaffected by colforsin treatment in both tissues (Fig. 3A,B).

Effect of BCNI and Colforsin on nNOS uncoupling in the penis and MPG and on binding of PIN to nNOS in the penis

The ratio of nNOS dimers (functional nNOS)/monomers (nonfunctional nNOS) was decreased ($P < 0.05$) in the penis (Fig. 4A) and MPG (Fig. 4B) after BCNI. Colforsin treatment of BCNI animals increased significantly ($P < 0.05$) the dimer/monomer ratio to Sham levels in both the penis and MPG. Because increased binding of PIN to nNOS has been implicated in nNOS uncoupling, we next measured binding of PIN to nNOS using partially purified penile samples, thus allowing the detection of nNOS and proteins bound to nNOS using specific antibodies [30,31,37]. Binding of PIN to nNOS was increased ($P < 0.05$) in the penis of the BCNI group and normalized ($P < 0.05$) by colforsin (Fig. 4C). We did not evaluate binding of PIN to nNOS in the MPG because this evaluation is not feasible given the limited tissue amount of the MPG.

Effect of BCNI and Colforsin on ROS production in the penis and on protein expression of $gp91^{phox}$ subunit of NADPH oxidase in the MPG

Hydrogen peroxide (H_2O_2) (Fig. 5A) and total ROS (hydrogen peroxide and superoxide) productions (Fig. 5B) were increased ($P < 0.05$) in the penis after BCNI. Colforsin treatment of BCNI animals decreased H_2O_2 and total ROS productions in the penis to levels which were not different from that in Sham animals. Superoxide (O_2^-) production showed a trend ($P = 0.15$) towards increase by BCNI and a trend ($P = 0.13$) towards decrease after colforsin treatment in the penis of the BCNI group (Fig. 5C). ROS production in the penis was not affected by colforsin treatment in Sham animals. We did not evaluate ROS production in the MPG because this evaluation is not feasible given the size of the MPG. However, protein expression of $gp91^{phox}$ was increased ($P < 0.05$) in the MPG of BCNI rats and was normalized ($P < 0.05$) by colforsin treatment (Fig. 5D). Protein expression of $gp91^{phox}$ in the penis was not affected by BCNI (data not shown).

Effect of BCNI on protein expression of active Caspase 3 in the MPG

Protein expression of active caspase 3, an apoptotic marker, was increased ($P < 0.05$) in the MPG after BCNI. Colforsin treatment of BCNI animals resulted in a protein expression level of active caspase 3 which was not different from values in Sham animals (Fig. 6A). Because previous studies demonstrated increased protein expression of caspase 3 and apoptosis in the penis of cavernous injury models as early as 3 days post-injury, we did not evaluate caspase 3 expression in the penis [38–40].

Effect of Colforsin on protein expression of PKA catalytic subunit alpha (PKA-Cα) subunit in the penis

Because cAMP-induced activation of PKA is associated with increased protein expression of PKA-Cα subunit, we measured the expression of this protein in the penis [41,42]. It was

decreased ($P < 0.05$) in BCNI animals and normalized ($P < 0.05$) by colforsin treatment (Fig. 6B). We did not evaluate PKA-Cα expression in the MPG because this evaluation is not feasible given the limited tissue amount of the MPG.

Discussion

This study demonstrates that PKA activation reverses aberrant nNOS signaling and oxidative stress in the neuropathic penis, which is associated with neurogenic ED *in vivo*. The molecular mechanism underlying the reduction in nNOS bioactivity following CN injury involves nNOS phosphorylation, nNOS uncoupling, and elevated free radical production. We found that the PKA activator colforsin protects normal nNOS signaling in the penis from neuropathy and neuroregulated erectile function via preservation of nNOS phosphorylation and nNOS coupled function and abrogation of free radical level production in the penis and its local neural supply. Collectively, we show that nNOS posttranslational regulation is involved in erectile tissue integrity in the face of neuropathy.

nNOS phosphorylation is one of the major mechanisms regulating nNOS bioactivity [19,43]. nNOS activation by phosphorylation can be induced by several protein kinases, including PKA, serine/threonine kinase/protein kinase B (Akt/PKB), AMP-activated protein kinase (AMPK), and Ca2+/calmodulin-dependent protein kinase II (CaM-K II) [43,44]. Phosphorylation of nNOS on Ser-1412 activates the enzyme, while phosphorylation of nNOS on Ser-847 inhibits the enzyme. nNOS phosphorylation on Ser-1412 activates the enzyme's catalytic function by reducing the enzyme's calcium requirement and facilitating electron transfer [45]. Phosphorylation of nNOS at Ser-847 reduces its activity by inhibiting calcium-CaM binding [46]. We have recently demonstrated a major role for PKA-mediated phosphorylation of nNOS (Ser-1412) in mediating penile erection[19]. We now confirm this finding by showing that PKA activator colforsin induced increase in P-nNOS (Ser-1412) in the MPG of healthy, sham-operated rats. Conversely, phosphorylation of nNOS on this site was increased in the penis and penile innervation after CN injury. Increased P-nNOS (Ser-1412) conceivably indicates a deleterious effect of over-activated nNOS in the neuropathic penis and penile innervation. Previous studies demonstrated that over-activation of kinases such as Akt by excessive NO may lead to excessive nNOS phosphorylation on Ser-1412 [46,47]. It is well documented that neuronal insults such as brain ischemia cause overproduction of NO, allowing its conversion into peroxynitrite, which can cause neuronal cell death [48]. While Akt activation in the penis and penile innervation is not associated with physiologic penile erection, it is possible that this pathway becomes activated in pathophysiologic settings such as nerve damage, causing excessive nNOS phosphorylation on Ser-1412 [19]. Colforsin possibly inhibits Akt activation thus preventing excessive nNOS phosphorylation in the penis and associated penile neuropathy. In support of this hypothesis, previous *in vitro* studies demonstrated that cAMP-dependent signaling inhibits Akt activity by blocking the coupling between Akt and its upstream regulators, phosphoinositide-dependent kinase (PDK) and phosphatidylinositol 3-kinase (PI3K), in the plasma membrane [49,50].

Additionally, we showed the involvement of the inhibitory nNOS phosphorylation site (Ser-847) in ED following BCNI. The phosphorylation of nNOS on this inhibitory site was

increased in both the penis and MPG by CN injury, conceivably rendering nNOS less active. Colforsin decreased phosphorylation of this site on nNOS, conceivably recovering physiologic neuronal NO catalysis. While the exact mechanism of this action of colforsin in the penis and penile innervation is not known, it is possible that PKA-dependent activation of protein phosphatase 1/PP2 dephosphorylates nNOS on this site and activates it, as shown previously in hypothalamic neurons [51]. Collectively, our findings demonstrate the physiologic significance of nNOS posttranslational modification by phosphorylation *in vivo* in the preservation of neuroregulated erectile function.

Total nNOS expression in the penis, but not the MPG, was decreased following BCNI, consistent with prior findings by us and others [11,12,14,15]. A previous study described that CN injury decreased nNOS+ nerve fibers in the cavernous nerve distal from the site of nerve crush, but not in the MPG [52]. Decreased expression of nNOS indicates deterioration of nitrergic neurons in the penis and neurogenic ED following CN injury. The lack of effect of colforsin treatment on total nNOS expression here is conceivably due to the short duration of treatment without the influence of regenerated nNOS+ nerve fibers.

An important mechanism of decreased NO bioavailability is functional uncoupling of NOS, characterized by the diversion of electron transfer within the enzyme from L-arginine oxidation. This molecular event reduces molecular oxygen to form superoxide instead of NO[22]. Several mechanisms of nNOS uncoupling have been described, such as limited availability of the cofactor tetrahydrobiopterin (BH_4), the substrate L-arginine, phosphorylation of nNOS at Ser-847, and increased binding of PIN to nNOS [53–56]. Increased nNOS uncoupling in both the penis and MPG was evident following BCNI, as demonstrated by a decreased dimer/monomer ratio. The precise mechanism by which nNOS becomes uncoupled under conditions of penile neuropathy and nerve degeneration is unclear at this time but may be related to increased nNOS phosphorylation on Ser-847 and increased binding of PIN to nNOS. While the mechanism underlying increased nNOS uncoupling by nNOS phosphorylation at Ser-847 is not known, PIN, by occupying the calcium-calmodulin site on nNOS, destabilizes the dimeric form of nNOS and decreases the enzyme's activity [39–40]. Previous studies demonstrated erectile dysfunction in rats with local injection of PIN cDNA, which may be explained by PIN-induced nNOS uncoupling [57,58]. Furthermore, we previously reported that genetic or pharmacologic inhibition of dysfunctional nNOS afforded erectile function preservation after BCNI, suggesting that aberrant nNOS signaling is detrimental for erectile function following penile neuropathy, which is in line with our current findings that overactivated, but uncoupled, dysfunctional nNOS reduces erectile function after BCNI [59]. Recently, nNOS uncoupling has been described in the MPG of rats 14 days following BCNI [7]. We extend this finding and suggest a role for nNOS uncoupling both in the penis and MPG in ED pathophysiology in the CN injury model. Importantly, our findings implicate the binding of PIN to nNOS and nNOS phosphorylation on Ser-847 as potentially important mediators of nNOS uncoupling resulting in neurogenic ED.

Uncoupled nNOS conceivably contributes to decreased NO bioavailability and increased ROS formation by the enzyme, conditions which are both apparent in the neuropathic penis and MPG after BCNI [46,60]. Here, we directly measured superoxide and H_2O_2 produced in

erectile tissue, demonstrating increased levels of these free radicals following BCNI. Superoxide is a highly reactive molecule with well documented adverse effects on neurovascular function, such as quenching of NO, induction of apoptosis, increased endothelial cell permeability, and promotion of neurodegeneration [61,62]. H_2O_2 is critically involved in redox-based signal transduction, which modulates several signaling cascades, including apoptosis, angiogenesis, promotion of endothelial barrier dysfunction, and induction of inflammatory proteins [63]. In addition, we demonstrated an upregulation of the NADPH oxidase subunit $gp91^{phox}$ in the MPG following BCNI, indicating NADPH oxidase as another source of oxidative stress in the MPG. Upregulation of active caspase 3 in the MPG following BCNI suggests that apoptosis takes place in penile innervation, conceivably further contributing to deterioration of nitrergic neurons and ED. Colforsin prevented an increase in free radicals in the penis, and it prevented upregulation of $gp91^{phox}$ and active caspase 3 in the MPG in response to CN injury. PKA stimulation has previously been shown to attenuate superoxide production and NADPH oxidase activity in hypothalamic neuronal and vascular smooth muscle cells [64,65]. Furthermore, PKA stimulation has been found to prevent H_2O_2-induced apoptosis and caspase-3 activation and H_2O_2-induced endothelial cell senescence [66–67]. PKA is also involved in ROS detoxification, as PKA stimulation has been shown to activate several cellular antioxidants, including SOD1, SOD2, SOD3, catalase, peroxyredoxin 2, glutathione peroxidase 1, heme oxygenase-1, and NADPH quinone oxidoreductase (NQO1) [64,66–68]. In this context, colforsin could have suppressed ROS production and/or enhanced ROS detoxification capacity, ultimately resulting in suppression of ROS detected in erectile tissue post-BCNI.

Colforsin is a water-soluble forskolin derivative and AC activator, which increases intracellular cAMP levels with subsequent activation of PKA signaling [69]. The neuroprotective and neurorestorative effects of cAMP have been described in several animal models of peripheral nervous system injury and are thus consistent with our results. In animal models of peripheral nerve injury cAMP, acting through PKA-dependent and PKA-independent mechanisms, transcriptionally activates a series of downstream effectors within injured neurons and Schwann cells to promote neuronal survival and regeneration [70]. Furthermore, cAMP mediated pathways include inhibition of PDE5 and modulation of collagen/smooth muscle synthesis in cultured human corpus cavernosum smooth muscle cells, and relaxation of mouse corpus cavernosum in the absence of functional NO signaling [70–72]. The protective effect of colforsin in our animal model is conceivably due to upregulation of PKA-Cα subunits. Inactive PKA holoenzyme is a heterotetramer composed of two types of subunits: regulatory (R) subunits and catalytic (C) subunits [73]. In the presence of increased cAMP by colforsin, the holoenzyme becomes active by binding two cAMP molecules cooperatively to each R subunit, resulting in a conformational change, thus releasing the two C subunits to phosphorylate downstream targets [44,45]. The summary of BCNI and colforsin on nNOS signaling and oxidative stress in penile innervation is presented in Figure 7. These findings may partially explain previous observations in humans that penile exercise post-RP may improve erectile function possibly due to increase in oxygen dependent cAMP generation during erect state [74,75].

We acknowledge that the use of PKA inhibitors with opposite effects to that of colforsin *in vivo* would substantiate our conclusion that PKA activation preserves nNOS signaling in the

neuropathic penis. Future studies are needed to establish which neuroprotective downstream signaling factors and targets of cAMP, other than nNOS itself, may be induced by colforsin in injured CN nerves and accompanying Schwann cells. Furthermore, for this study we performed colforsin treatment at a single dose just before BCNI to test its acute effect on nNOS signaling. Future studies evaluating dose-response and colforsin treatment given at different time points before and after BCNI may determine the maximum protection on nNOS signaling and possible neurorestorative potential of PKA activation in the penile innervation.

In summary, this study demonstrates that in the context of penile neuropathy nNOS bioactivity is altered via nNOS post translational modification and nNOS uncoupling, resulting in ED, PKA activation by colforsin promotes nNOS bioactivity and counteracts neuropathic oxidative stress in the penis and penile innervation, thereby preserving erectile function.

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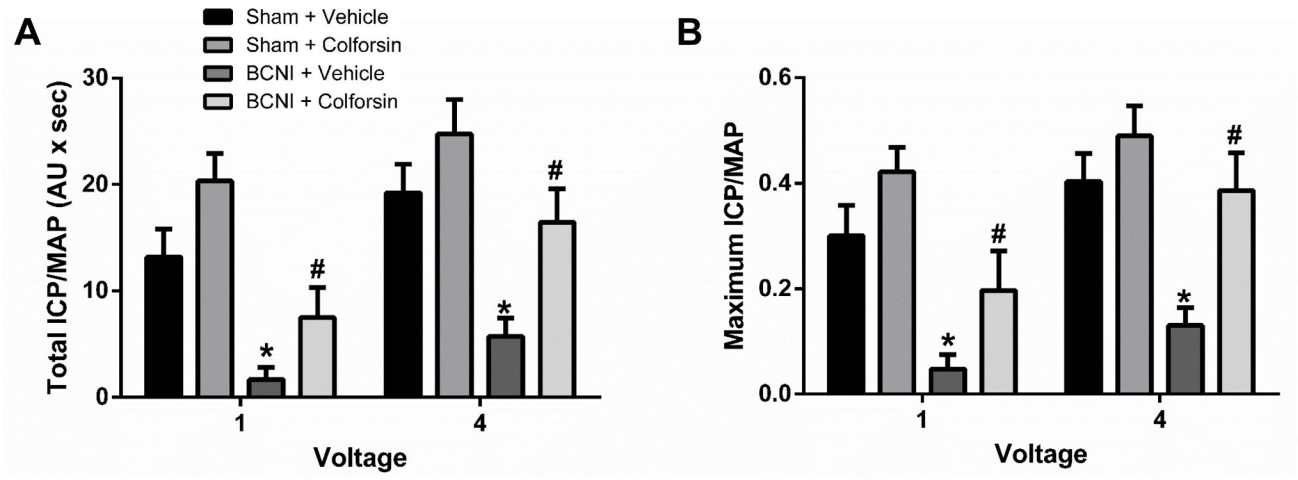

Figure 1.

Effect of BCNI and Colforsin on penile erection. Sham and BCNI rats were treated with Colforsin or vehicle for 3 days. Erectile response to electrical stimulation of the cavernous nerve is indicated by **(A)** total ICP/MAP **(B)** and maximal ICP/MAP corrected for baseline in Sham+vehicle, Sham+Colforsin, BCNI+vehicle, and BCNI+Colforsin treated rats. Each bar represents the mean ± SEM of 8–10. *P < 0.05 vs Sham+vehicle, #P < 0.05 vs BCNI +vehicle. AU = arbitrary units.

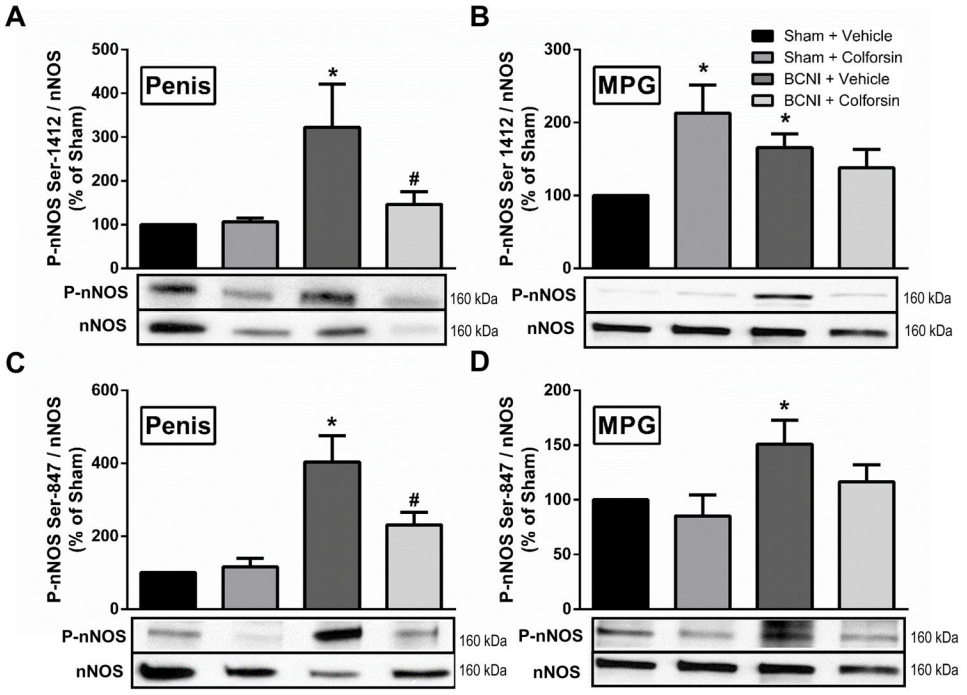

Figure 2.

Effect of BCNI and Colforsin on protein expressions of P-nNOS (Ser-1412 and Ser-847) in the penis **(A and C)** and MPG **(B and D)** of Sham and BCNI rats treated with Colforsin or vehicle for 3 days. Lower panels are representative Western immunoblots, and upper panels are densitometric analyses of P-nNOS (Ser-1412 and Ser-847)/nNOS in the penis and MPG of Sham+vehicle, Sham+Colforsin, BCNI, and BCNI+Colforsin treated rats. Each bar represents the mean ± SEM of n = 8 rats. *P < 0.05 vs Sham+vehicle, #P < 0.05 vs BCNI +vehicle.

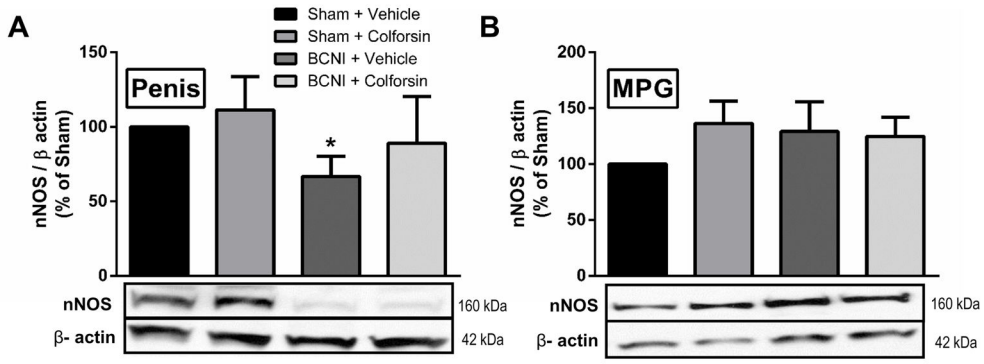

Figure 3.

Effect of BCNI and Colforsin on protein expression of nNOS in the **(A)** penis and **(B)** MPG of Sham and BCNI rats treated with Colforsin or vehicle for 3 days. Lower panels are representative Western immunoblots, and upper panels are densitometric analyses of nNOS/β actin in the penis and MPG of Sham+vehicle, Sham+Colforsin, BCNI+vehicle, and BCNI +Colforsin treated rats. Each bar represents the mean ± SEM of n = 6–8 rats. *P < 0.05 vs Sham+vehicle.

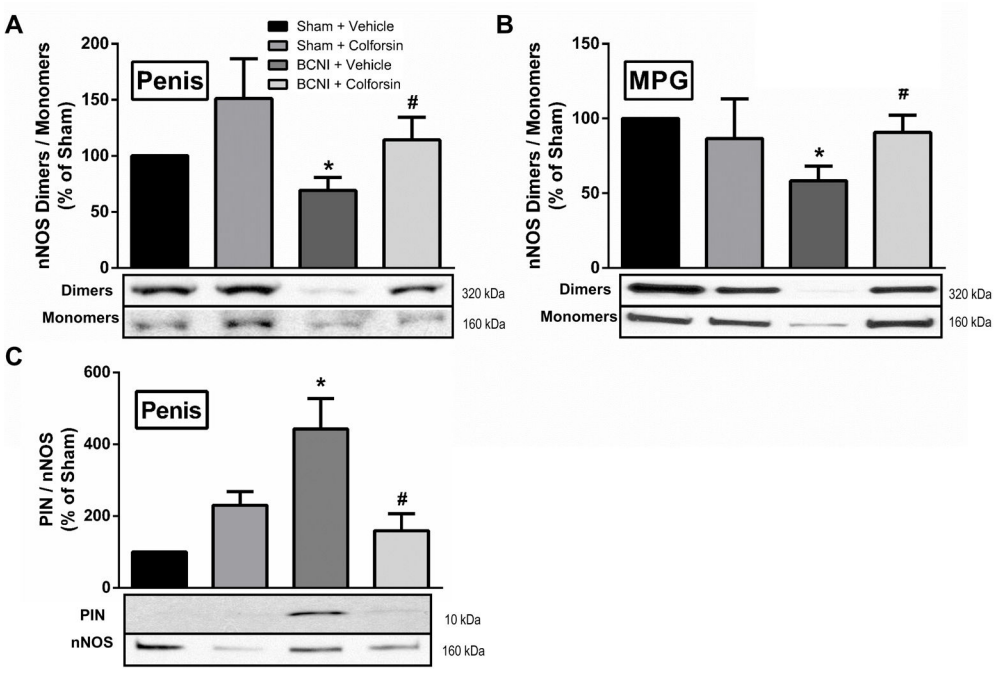

Figure 4.

Effect of BCNI and Colforsin on nNOS uncoupling in the **(A)** penis and **(B)** MPG, and on PIN binding to nNOS in the penis **(C)** of Sham and BCNI rats treated with Colforsin or vehicle for 3 days. Lower panels are representative Western blots and upper panels are densitometric analyses of nNOS dimers and monomers after low-temperature SDS-PAGE in the penis and MPG, and PIN/nNOS in the penis of Sham+vehicle, Sham+Colforsin, BCNI +vehicle, and BCNI+Colforsin treated rats. Each bar represents the mean ± SEM of n = 8 rats. *P < 0.05 vs Sham+vehicle, #P < 0.05 vs BCNI+vehicle.

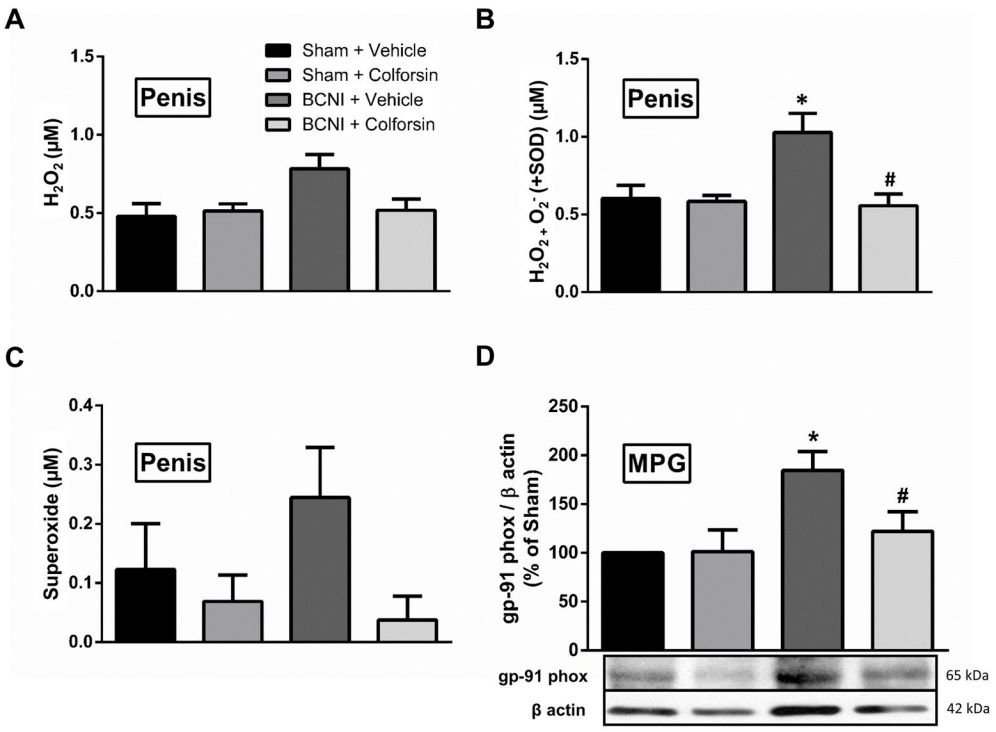

Figure 5.

Effect of BCNI and Colforsin on ROS production in the penis **(A)** H_2O_2, **(B)** H_2O_2+O_2^-, **(C)** O_2^- and on **(D)** protein expression of $gp91^{phox}$ subunit of NADPH oxidase in the MPG of Sham+vehicle, Sham+Colforsin, BCNI+vehicle, and BCNI+Colforsin rats treated for 3 days. Each bar represents the mean ± SEM of n = 7 rats. *P < 0.05 vs Sham+vehicle, #P < 0.05 vs BCNI+vehicle.

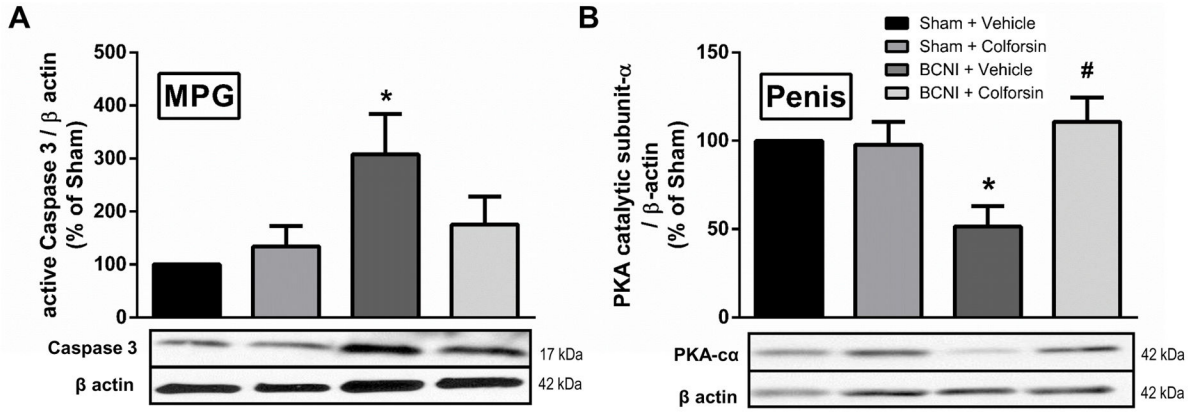

Figure 6.

Effect of BCNI and Colforsin on protein expression of **(A)** active caspase 3 in the MPG and **(B)** PKA catalytic subunit α in the penis of Sham and BCNI rats treated with Colforsin or vehicle for 3 days. Lower panels are representative Western immunoblots, and upper panels are densitometric analyses of active caspase 3/β actin and PKA catalytic subunit α/β actin in the MPG and penis of Sham+vehicle, Sham+Colforsin, BCNI+vehicle, and BCNI+Colforsin treated rats. Each bar represents the mean ± SEM of n = 6–8 rats. *P < 0.05 vs Sham +vehicle, #P < 0.05 vs BCNI+vehicle.

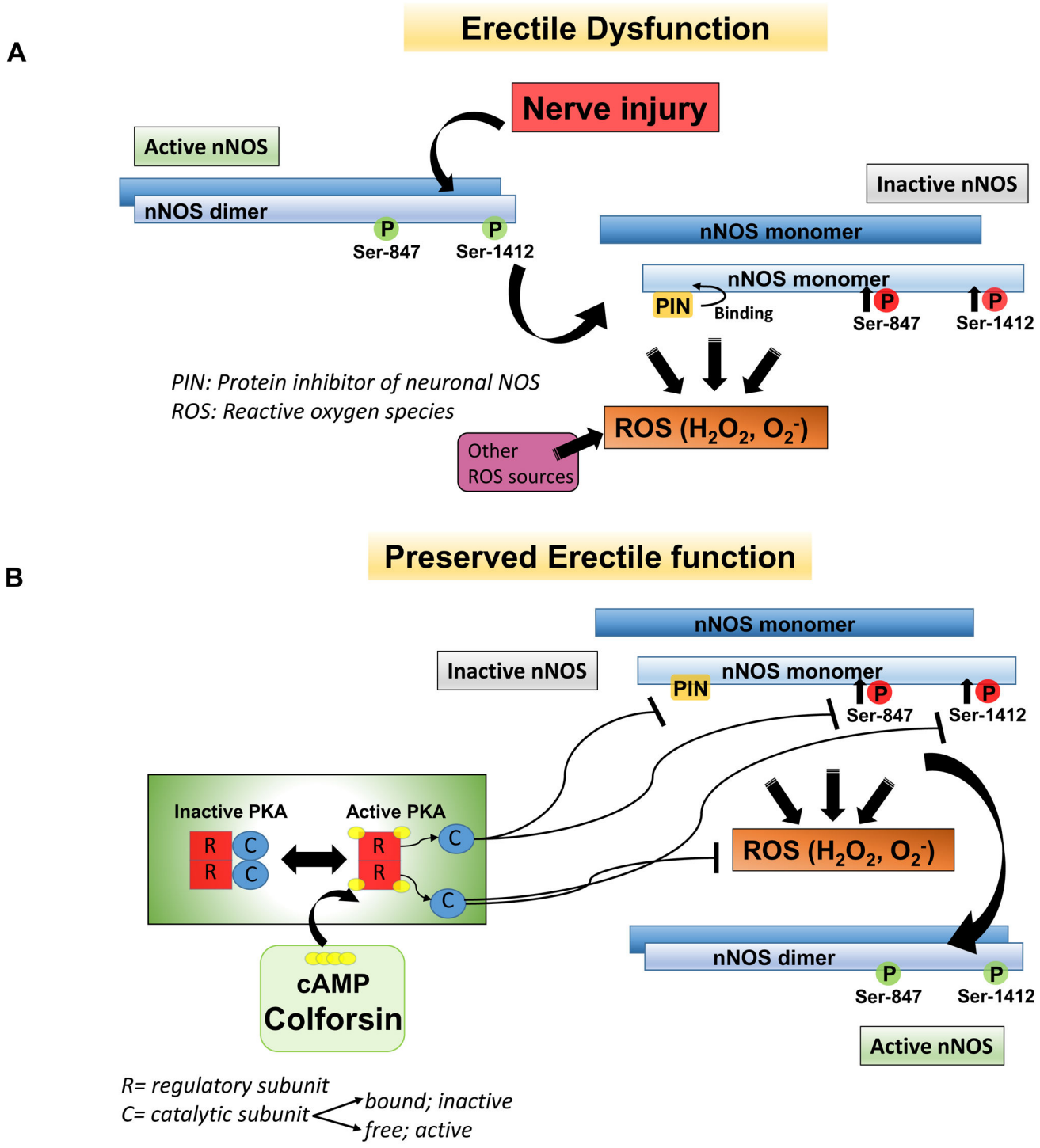

Figure 7. Proposed post-translational modification of nNOS in the penis and penile innervation in cavernous-nerve injury-induced erectile dysfunction and in PKA activator Colforsin-mediated preservation of erectile function

(A) Cavernous nerve injury inactivates nNOS by causing excessive phosphorylation on Ser-1412 and by increasing nNOS uncoupling, the latter due to increased binding of PIN to the enzyme and nNOS phosphorylation on Ser-847. Uncoupled nNOS, together with

activation of other sources of ROS, increases oxidative stress in the penis. All these events result in ED. PIN; Protein inhibitor of neuronal NOS, ROS; Reactive oxygen species. **(B)** PKA activator Colforsin preserves erectile function in the face of cavernous nerve injury by preserving nNOS phosphorylation on Ser-1412 and Ser-847, preventing nNOS uncoupling, and preventing increase in oxidative stress. The protective effect of Colforsin is due to upregulation of PKA-Cα subunits. R; Regulatory subunit, C; Catalytic subunit.