Neutralizing antibody to proNGF rescues erectile function by regulating the expression of neurotrophic and angiogenic factors in a mouse model of cavernous nerve injury

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Directed by Professor Chang Hee Hong

The Doctoral Dissertation submitted to the Department of Medicine the Graduate School of Yonsei University in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Medical Science

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Doo Yong Chung

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ABSTRACT

Despite advances in surgical techniques and robotic procedures, radical prostatectomy induces some degree of cavernous nerve injury (CNI) and causes denervation-induced pathologic changes in cavernous vasculature. The precursor for nerve growth factor (proNGF) is known to be involved in neuronal cell apoptosis and microvascular dysfunction through its receptor $p75^{NTR}$. Here, we determined the differential expression of proNGF/$p75^{NTR}$ and examined the effectiveness of proNGF neutralizing antibody (anti-proNGF-Ab) on erectile function in mice with CNI. Twelve-week-old C57BL/6 mice were used and distributed into 3 groups: sham operation group and bilateral CNI group treated with intracavernous injections of PBS (days -3 and 0; 20 μL) or of anti-proNGF-Ab (days -3 and 0; 20 μg in 20 μL of PBS). Erectile function was measured in response to electrical stimulation of the cavernous nerve at 2 weeks after treatment, and the penis tissues were then harvested for histological and biochemical studies. We also determined the effect of anti-proNGF-Ab on neural preservation in *ex vivo* cultured major pelvic ganglion (MPG). We observed increased

cavernous expression of proNGF and $p75^{NTR}$ after CNI. Intracavernous administration of anti-proNGF-Ab increased penile nNOS and neurofilament content by enhancing the expression of neurotrophic factors (NGF, brain-derived neurotrophic factor, and neurotrophin-3). Anti-proNGF-Ab preserved the integrity of cavernous sinusoids, such as pericytes, endothelial cells, and endothelial cell-cell junctions, by regulating the expression of angiogenic factors (angiopoietin-1, angiopoietin-2, and vascular endothelial growth factor); and induced endogenous eNOS phosphorylation in CNI mice. These changes significantly rescued erectile function in CNI mice. Anti-proNGF-Ab also enhanced neurite sprouting from MPG exposed to lipopolysaccharide. The preservation of damaged cavernous neurovasculature through inhibition of proNGF/$p75^{NTR}$ pathway may be a new avenue to treat radical prostatectomy-induced erectile dysfunction.

Key Words: erectile dysfunction; radical prostatectomy; cavernous nerve injury; proNGF, $p75^{NTR}$

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I. INTRODUCTION

Radical prostatectomy (RP) is one of the most commonly used therapeutic approaches for patients with localized prostatic cancer. Despite the good long-term oncologic outcomes, the rate of postoperative erectile dysfunction (ED) must not be neglected, depending on the surgical approaches and on the surgical experiences [1-3]. Although more advanced surgical techniques and instruments, such as nerve-sparing techniques and robotic procedures, were used, the various degrees of cavernous nerve injury (CNI) or neurapraxia will not be avoidable [2,4] . The prevalence of ED following RP was reported to be very broad from 14 to 90% [3]. Moreover, the response rate to the currently available oral phosphodiesterase type 5 inhibitors (PDE5Is) is much lower in men with ED following RP than in the general ED population [5].

Various strategies including neuromodulation have shown beneficial effect on recovery of erectile function at the preclinical level [6,7] . However, a clinical trial

with GPI 1485 [8], a non-immunosuppressive analogue of FK506, failed to show improvement of erectile function in men undergoing nerve-sparing RP. This finding suggests that the restoration of cavernous nerve function alone may not be enough to restore erectile function, because denervation-induced pathophysiologic changes cavernous vasculature, such as cavernous endothelial dysfunction and smooth muscle apoptosis, have already begun [9,10]. Therefore, the functional and structural rearrangements of the damaged penile neurovascuature are necessary to overcome RP-induced ED.

Nerve growth factor (NGF) is one of the neurotrophic factor family and is produced from its precursor proNGF by proteolytic cleavage [11,12]. Accumulating evidences suggest that proNGF is not just a precursor of NGF, but has distinct biological roles [11-13]. NGF and proNGF act as ligands of tropomyosin receptor kidnase A (TrkA), p75 neurotrophin receptor ($p75^{NTR}$), and sortilin [14,15]. NGF has a high affinity to TrkA and enhances neuronal cell survival and proliferation, whereas proNGF is known to induce neuronal cell apoptosis mainly binding to $p75^{NTR}$ in pathological conditions, such as Parkinson's disease, Alzheimer's disease, and spinal cord injury [14-19]. Moreover, the proNGF and $p75^{NTR}$ pathway is also known to be involved in microvascular complications, such as endothelial cell and pericyte dysfunction, and neurodegeneration in animal models of diabetic retinopathy [17,20]. These findings suggest that inhibition of proNGF/$p75^{NTR}$ pathway is a promising therapeutic strategy to treat various neurological and vascular diseases.

We recently reported in diabetic mice that inhibition of the proNGF and $p75^{NTR}$ pathway restored erectile function by enhancing cavernous angiogenesis and neural regeneration [21]. In the present study, therefore, we examined differential expression of proNGF/$p75^{NTR}$ in the corpus cavernosum after CNI. Next, we also determined the effectiveness of proNGF-neutralizing antibody (anti-proNGF-Ab) in restoring erectile function in a mouse model of CNI.

II. MATERIALS AND METHODS

1. Animals and treatment

C57BL/6 pathogen-free male mice were purchased from Orient Bio (Gyeonggi, South Korea) and maintained under specific-pathogen-free (SPF) facility. The experimental protocol was approved by Institutional Animal Care and Use Subcommittee of Inha University. Ten-week-old male mice were used in this study. The mice from each group were anesthetized with intraperitoneal injection of ketamine (100 mg/kg) and xylazine (5 mg/kg). The mice were distributed into three groups: sham operation group and bilateral CNI group treated with repeated intracavernous injections of PBS (20 μL) or of anti-proNGF-Ab (Biosensis, Thebarton, Australia; 20 μg/20 μL) at days -3 and 0. Animal models were established as previously described [10] (Jin, *et al.*, 2010). Briefly, sham operation group underwent exposure of the prostate to enable visualization of the cavernous nerves bilaterally without any direct cavernous nerve manipulation. Cavernous nerve injury was induced by crushing the cavernous nerve with a non-serrated hemostat applied with full tip closure to each side of cavernous nerve 1 mm distal to the ganglion for 2 minutes. PBS or anti-proNGF-Ab was injected into the midportion of the corpus cavernosum by a 30-gauge syringe. Electrical stimulation of the cavernous nerve was done 2 weeks after treatment for evaluation of erectile function. Penile tissue from a separate group of animals were collected for histological and biochemical analysis.

2. Measurement of erectile function

Erectile function was measured as previously described [10]. Briefly, bipolar platinum wire electrodes were placed around the cavernous nerve and electrical stimulation was done at 5 volts, 12 Hz frequency, 1 msec pulse width, and 1 minute duration. Maximal intracavernous pressure (ICP) was recorded during tumescence. Area from the beginning of cavernous nerve stimulation until 20 seconds after stimulus termination

(area under the curve) was determined as the total ICP value. Noninvasive tail-cuff system (Visitech Systems, Apex, NC, USA) was used to measure systemic blood pressure. The ratios of the maximal ICP (cm H_2O) or total ICP (area under curve) to the mean systolic blood pressure (MSBP, cmH_2O) were calculated to normalize variations in systemic blood pressure.

3. *Ex vivo* neurite sprouting assay

The mouse major pelvic ganglion (MPG) tissues were prepared and maintained as described previously [22] with minor modifications. The MPG tissues were dissected from male mice under a microscope and suspended into sterile Hank's balanced salt solution (Gibco, NY, USA). After washing twice in PBS, the tissues were cut into small pieces and plated on poly-D-lysine hydrobromide-coated (Sigma-Aldrich, St. Louis, MO, USA) 12-well plate. Matrigel was added to cover the whole MPG tissue and placed on ice for 5 minutes prior to incubation at 37^{o}C for 10-15 minutes in a 5% CO_2 atmosphere. Tissues were suspended in 1 mL of complete Neurobasal medium (Gibco) supplemented with 2% serum-free B-27 (Gibco) and 0.5 nM GlutaMAX™-I (Gibco). Lipopolysaccharide (LPS; Sigma-Aldrich, St. Louis, MO, USA) was added to MPG tissue culture media for 24 hours to mimic neuroinflammatory condition. The dishes were then incubated at 37℃ in a 5% CO_2 atmosphere. Neurite outgrowth was observed seven days after seeding. The area of neurite sprouting was quantitatively analyzed with an image analyzer system (National Institutes of Health [NIH] Image J).

4. Histological examinations

The penis tissues and cultured MPGs were fixed in 4% paraformaldehyde at 4℃ for 24 hours. Tissues were washed several times with PBS and then immersed in antibody diluent solution (Invitrogen, Carlsbad, CA, USA) at room temperature for 1 hour to minimize nonspecific binding of antibodies. The samples were incubated with antibodies to proNGF (Sigma-Aldrich, St. Louis, MO, USA; 1:100), $p75^{NTR}$ (Merck

KGaA, Darmstadt, Germany; 1:100), PECAM-1 (Millipore, Temecula, CA, USA; 1:50), platelet-derived growth factor receptor-β (PDGFR-β; Abcam, Cambridge, MA, USA; 1;50), phospho-histone H3 (PH3, Millipore; 1;50), endothelial nitric oxide synthase (eNOS; BD Bioscience, San Jose, CA, USA; 1:50), NG2 (Millipore; 1:50), claudin-5 (Invitrogen; 1:50), occludin (Invitrogen; 1:50), phospho-eNOS (Cell signaling Technology, Danvers, MA, USA; 1:50) βIII-tubulin (Abcam; 1;100), and neuronal NOS (nNOS; Santa Cruz Biotechnology, Santa Cruz, CA, USA; 1:50). Sections were washed several times and then incubated with tetramethyl rhodamine isothiocyanate- or fluorescein isothiocyanate-conjugated secondary antibodies (Zymed Laboratories, South San Francisco, CA, USA) at room temperature for 2 hours. For nuclei staining, 4,6-diamidino-2-phenylindole (DAPI, Vector Laboratories Inc., Burlingame, CA, USA) contained mounting solution was used. Optical signals and digital images were collected with confocal microscope (FV1000, Olympus, Tokyo, Japan). Quantitative analysis of histological sections was measured with an image analyzer system (NIH Image J)

5. TUNEL assay

ApopTag in situ Apoptosis Fluorescein Detection Kit (S7111; Chemicon) was used for Tunnel Assay. The TUNEL assay was performed using an. DAPI contained (Vector Laboratories Inc.) mounting solution was applied to the chamber to visualized cell nuclei. The numbers of apoptotic cells in the corpus cavernosum were counted at a screen magnification of 400×.

6. Western blot

Equal amounts of protein (30 μg per lane) were electrophoresed on sodium dodecyl sulfate-polyacrylamide gels (10% to 15%), transferred to polyvinylidene difluoride membrane, then probed with antibodies to proNGF (Sigma-Aldrich; 1:500), $p75^{NTR}$ (Millipore; 1:1000), occludin (Invitrogen; 1:1000), claudin-5 (Life Technologies,

Carlsbad, CA, USA), zonula occludens-1 (ZO-1; Invitrogen; 1:500), angiopoietin-1 (Ang1; Novus Biologicals, Centennial, CO, USA; 1:500), angiopoietin-2 (Ang2; Abcam; 1:500), vascular endothelial growth factor (VEGF; Novus; 1:500), phospho-eNOS (Cell Signaling Technology; 1:500), eNOS (BD Bioscience; 1:500), TrkA (Millipore; 1:500), TrkB (Sigma Aldrich; 1:500), NGF (Santa Cruz Biotechnology, Dallas, TX, USA; 1:500), neurotrophin-3 (NT-3; Santa Cruz Biotechnology; 1:500), brain-derived neurotrophic factor (BDNF; Santa Cruz Biotechnology; 1:500), or β-actin (Abcam; 1:5000). The results were quantified by densitometry. The results were quantified by NIH Image J.

7. Statistical analysis

The results are expressed as mean ± SE. Statistical analyses were performed using SigmaStat 3.5 software (Systat Software Inc., Richmond, CA, USA). For parametric data, one-way ANOVA test was applied for multiple intergroup comparisons followed by Newman-Keuls posthoc tests. Mann-Whitney U test or Kruskal-Wallis test was used to compare nonparametric data. Probability values less than 5% were considered significant.

III. Results

1. Increase in proNGF and $p75^{NTR}$ expression in the penis of CNI mice

Immunofluorescent staining and Western blot analysis revealed that the expression of proNGF and $p75^{NTR}$ protein was increased significantly in cavernous tissue from CNI mice compared with that in the sham group (**Fig 1**). Immunofluorescent staining of mouse erectile tissue showed that some proportion of proNGF and a majority of $p75^{NTR}$ expression were overlapped with cavernous endothelial cells (**Fig. 1A**). Intracavernous administration of anti-proNGF-Ab decreased the expression of proNGF and $p75^{NTR}$ in the cavernous tissue of CNI mice (**Fig 1**).

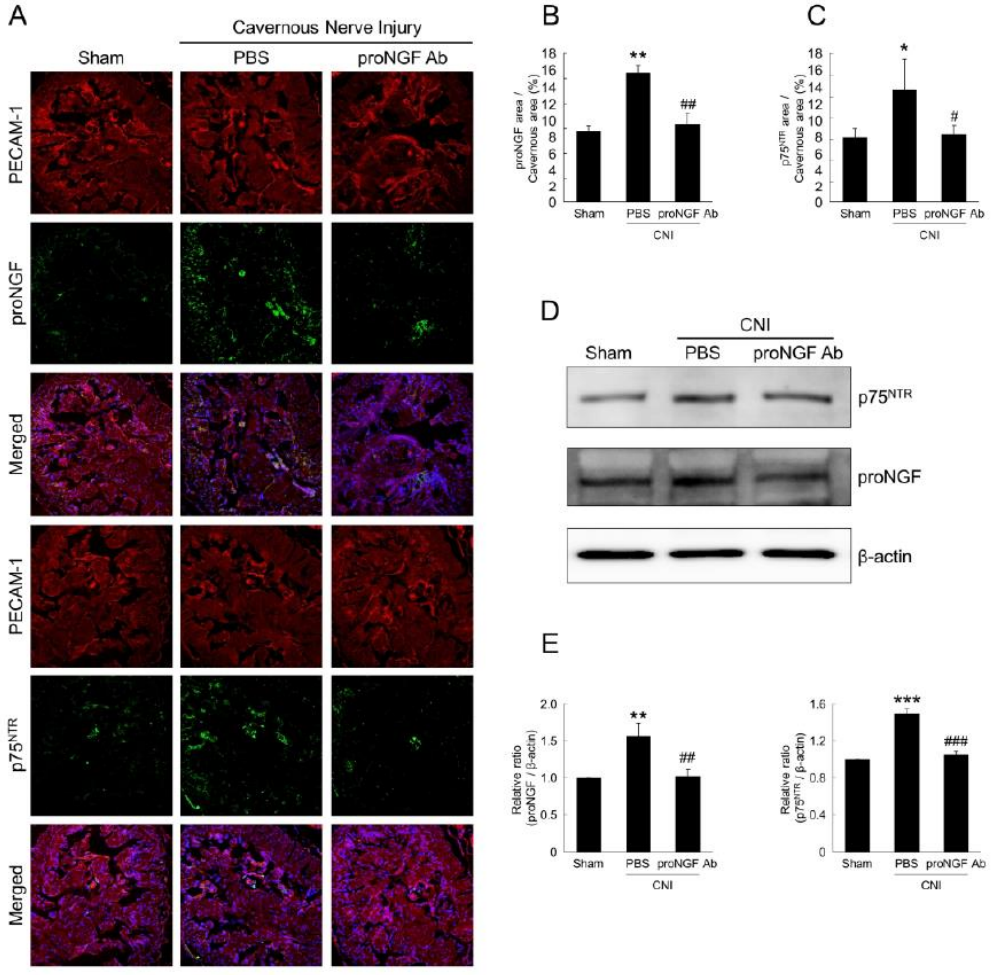

Figure 1. The cavernous expression of proNGF and p75^{NTR} is up-regulated in CNI mice.

(A) PECAM-1 (red) and proNGF or p75^{NTR} (green) staining in the corpus cavernosum of sham control and cavernous nerve injury (CNI) mice 2 weeks after repeated intracavernous injections of PBS (days -3 and 0; 20 μL) or anti-proNGF-Ab (days -3 and 0; 20 μg/20 μL). **(B, C)** Quantitative analysis of cavernous proNGF and p75^{NTR} immunopositive area in cavernous tissue was performed by image analyzer (N = 4). *P < 0.05, **P < 0.01 vs. the sham group. #P < 0.05, ##P < 0.01 vs. PBS-treated CNI

group. **(D)** Representative Western blots for proNGF and $p75^{NTR}$ in mouse cavernous tissue. **(E)** Normalized band intensity values for proNGF and $p75^{NTR}$ (N = 4). $^{**}P < 0.01$, $^{***}P < 0.001$ vs. the sham group. $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ vs. PBS-treated CNI group. Data in graphs are presented as mean ± SE.

2. Anti-proNGF-Ab preserves penile nerve content by enhancing the secretion of neurotrophic factors in CNI mice

The expression of nNOS and βIII-tubulin-positive nerve fiber was significantly decreased in the dorsal nerve bundle and the corpus cavernosum tissue of the PBS-treated CNI group compared with the sham control group. Local delivery of anti-proNGF-Ab into the corpus cavernousum preserved nNOS-contained fiber and axonal contents (βIII-tubulin) in the CNI mice (**Fig. 2A, 2C, and 2D**). We further examined neuroprotective effect of anti-proNGF-Ab (1 μg/mL) in *ex vivo* cultured MPG tissues exposed to LPS. At 7 days after incubation, immunofluorescent staining of MPG tissue with antibody to βIII-tubulin revealed a significant decrease in neurite sprouting from MPG tissue exposed to LPS, which was preserved by treatment with anti-proNGF-Ab (**Fig. 2B and 2E**).

In addition, we also asked whether the effects of proNGF neutralizing antibody were mediated by the production of neurotrophic factors. The cavernous expression of neurotrophic factors (NGF, BDNF, and NT-3) and their receptors (TrkA and TrkB) determined by Western blot analysis was significantly higher in CNI mice treated with anti-proNGF-Ab than in PBS-treated CNI mice, which was comparable to the level found in sham controls (**Fig. 2F-2K**).

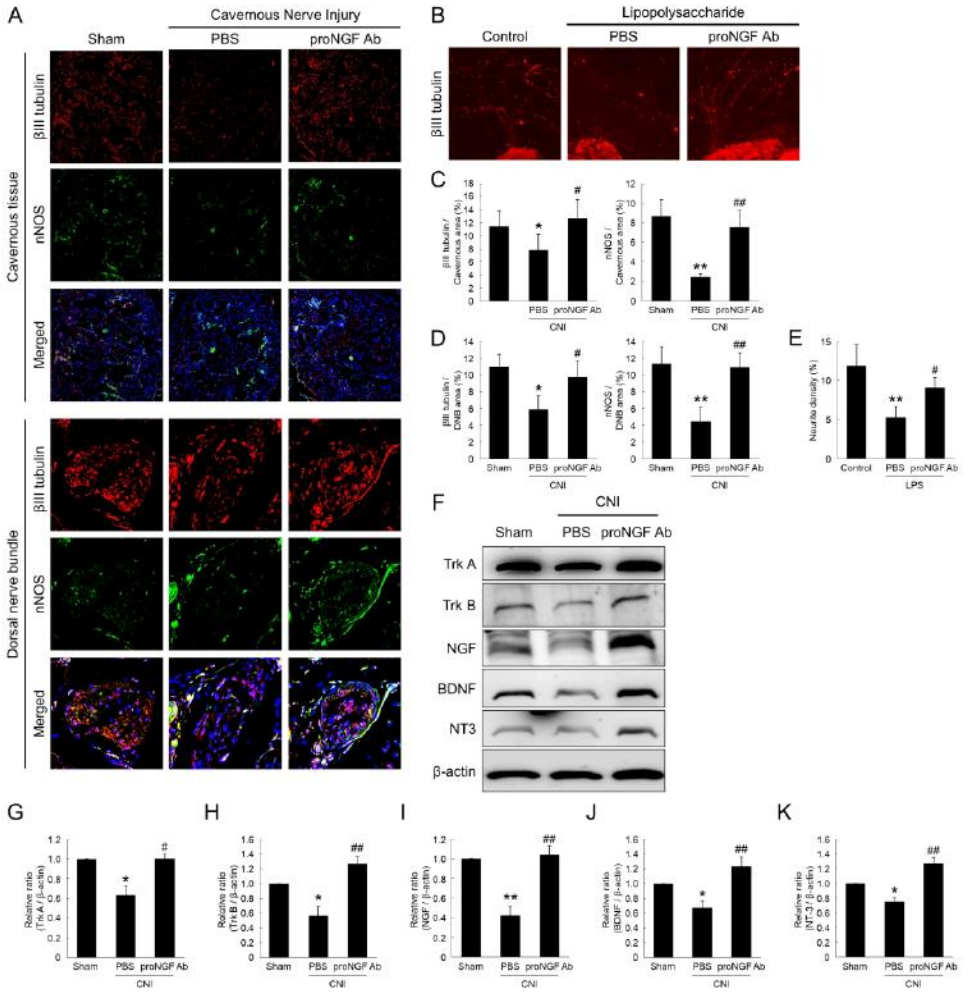

Figure 2. Anti-proNGF-Ab induces neural regeneration by enhancing the secretion of neurotrophic factors in CNI mice.

(A) Beta III tubulin (red) and nNOS (green) staining in the corpus cavernosum of sham control and cavernous nerve injury (CNI) mice 2 weeks after repeated intracavernous injections of PBS (days -3 and 0; 20 μL) or anti-proNGF-Ab (days -3 and 0; 20 μg/20 μL). **(B)** Beta III tubulin (red) staining of MPG tissue exposed to lipopolysaccharide (LPS), which were treated with anti-proNGF-Ab. **(C, D)** βIII-

tubulin and nNOS immunopositive area in corpus cavernosum or dorsal nerve bundle by ImageJ (N = 6). *P < 0.05, **P < 0.01 vs. the sham group. #P < 0.05, ##P < 0.01 vs. PBS-treated CNI group. **(E)** Quantitative analysis of βIII-tubulin immunopositive neurite length by ImageJ (N = 4). **P < 0.01 vs. the control group. #P < 0.05 vs. PBS-treated group. **(F)** Representative Western blots for neurotrophic factors and their receptors. **(G-K)** Normalized band intensity values (N = 4). *P < 0.05, **P < 0.01 vs. the sham group. #P < 0.05, ##P < 0.01 vs. PBS-treated CNI group. Data in graphs are presented as mean ± SE. NGF = nerve growth factor; BDNF = brain-derived growth factor; NT-3 = neurotrophin-3.

3. Anti-proNGF-Ab preserves regenerative potential of endothelial cells and pericytes in CNI mice

Cavernous tissues from sham and CNI groups were stained with antibodies to PECAM-1 and NG2 at 2 weeks after treatment. The cavernous endothelial cell and pericyte contents were significantly lower in the PBS-treated CNI mice than those in the sham control mice. Intracavernous administration of anti-proNGF-Ab preserved PECAM-1-positive endothelial cell and NG2-positive pericyte contents in the CNI mice 2 weeks after treatment (**Fig. 3**). To determine whether the preservation in cavernous endothelial cell and pericyte content was result of cell proliferation, we assessed the number of phosphohistone H3 (a nuclear protein indicative of cell proliferation) expressed in endothelial cells and pericytes. We observed significant increases in phosphohistone H3-positive endothelial cells and pericytes in CNI mice 2 weeks after treatment with anti-proNGF-Ab (**Fig. 4A, 4B, 4E, and 4F**).

4. Anti-proNGF-Ab decreases apoptosis of cavernous endothelial cells and pericytes in CNI mice

TUNEL assay revealed that the number of apoptotic cells was higher in the cavernous endothelial cells and pericytes of PBS-treated CNI mice than those

in the sham controls. Intracavernous administration of anti-proNGF-Ab significantly reduced the number of apoptotic cells in cavernous endothelial cells and pericytes of CNI mice (**Fig. 4C, 4D, 4G, and 4H**).

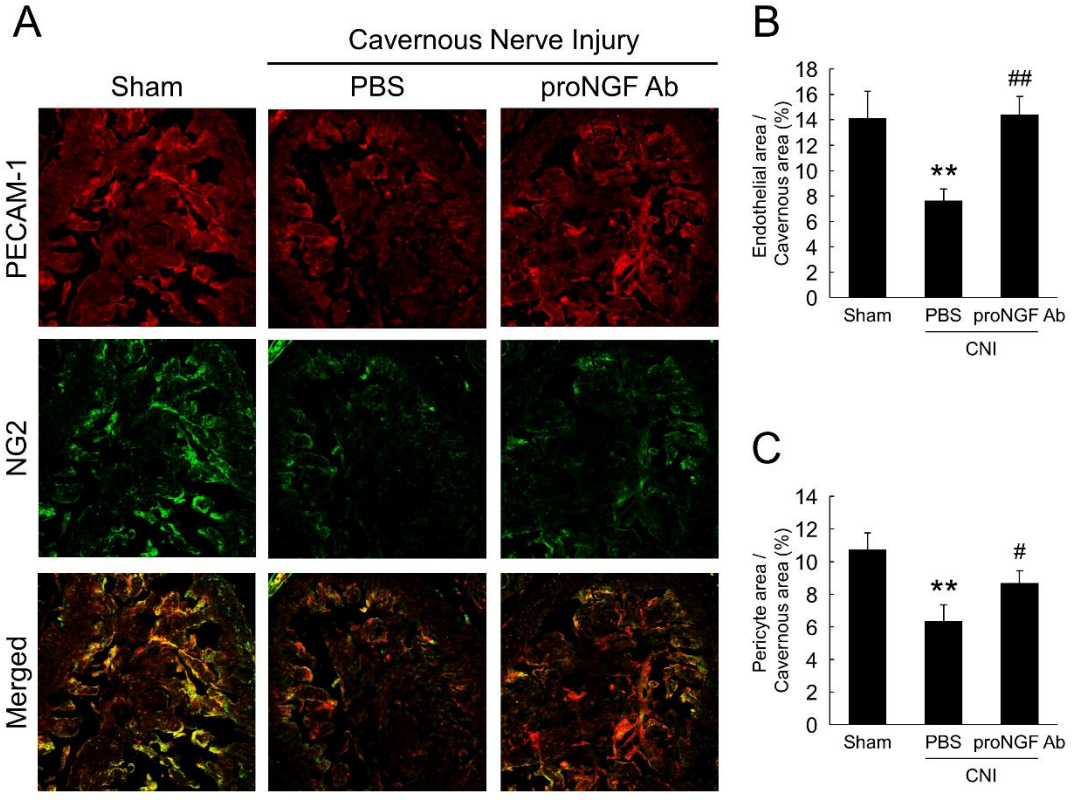

Figure 3. Anti-proNGF-Ab restores cavernous endothelial cell and pericyte contents in CNI mice.

(A) PECAM-1 (red) and NG2 (green) staining in the corpus cavernosum of sham control and cavernous nerve injury (CNI) mice 2 weeks after repeated intracavernous injections of PBS (days -3 and 0; 20 μL) or anti-proNGF-Ab (days -3 and 0; 20 μg/20 μL). **(B, C)** Endothlial cell- and pericyte-immunopositive area were quantified by image analyzer (N = 6). ***P* < 0.01 vs. the sham group. #*P* < 0.05, ##*P* < 0.01 vs. PBS-treated CNI group. Data in graphs are presented as mean ± SE.

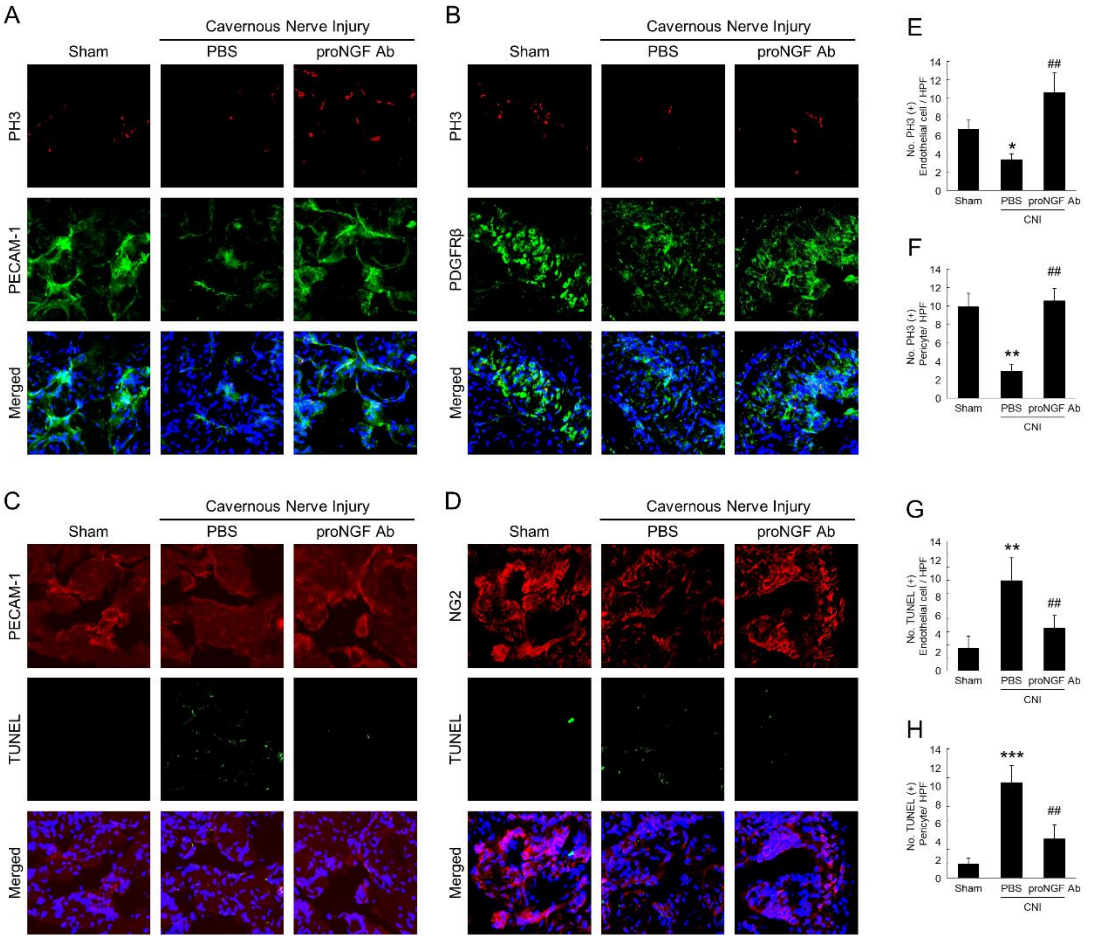

Figure 4. Anti-proNGF-Ab promotes proliferation and decreases apoptosis of cavernous endothelial cells and pericytes in CNI mice.

(A, B) PH3 (red) and PECAM-1 (green) or PH3 (red) and PDGFR-β (green) staining in the corpus cavernosum of sham control and cavernous nerve injury (CNI) mice 2 weeks after repeated intracavernous injections of PBS (days -3 and 0; 20 μL) or anti-proNGF-Ab (days -3 and 0; 20 μg/20 μL). Nuclei were laveled with DAPI (blue). **(C, D)** PECAM-1 (red) and TUNEL (green) or NG2 (red) and TUNEL (green) staining in each group of mice. **(E-H)** Number of PH3- or TUNEL-positive endothelial cells or pericytes per high-power field (N = 6). **P* < 0.05, ***P* < 0.01, ****P* < 0.001 vs. the

sham group. *##P* < 0.01 vs. PBS-treated CNI group. Data in graphs are presented as mean ± SE. PH3 = phosphohistone H3; PDGFR-β = platelet-derived growth factor receptor-β; TUNEL = Terminal deoxynucleotidyl transferase dUTP nick end labeling.

5. Anti-proNGF-Ab preserves cavernous endothelial cell-cell junction proteins and induces eNOS phosphorylation in CNI mice

We performed immunohistochemical staining and Western blot to evaluate the cavernous expression of endothelial cell-cell junction proteins (claudin, occludin, and/or ZO-1) in sham control and CNI mice 2 week after treatment. The cavernous expression of endothelial cell-cell junction proteins was significantly lower in the PBS-treated CNI mice in the sham control. Intracavernous administration of anti-proNGF-Ab significantly preserved cavernous endothelial cell-cell junction proteins in the CNI mice (**Fig. 5**).

Immunofluorescent staining and Western blot analysis revealed that the cavernous phospho-eNOS and eNOS expression were significantly lower in the PBS-treated CNI mice than in sham controls. Anti-proNGF-Ab preserved the expression of cavernous phospho-eNOS and eNOS in the CNI mice (**Fig. 6**).

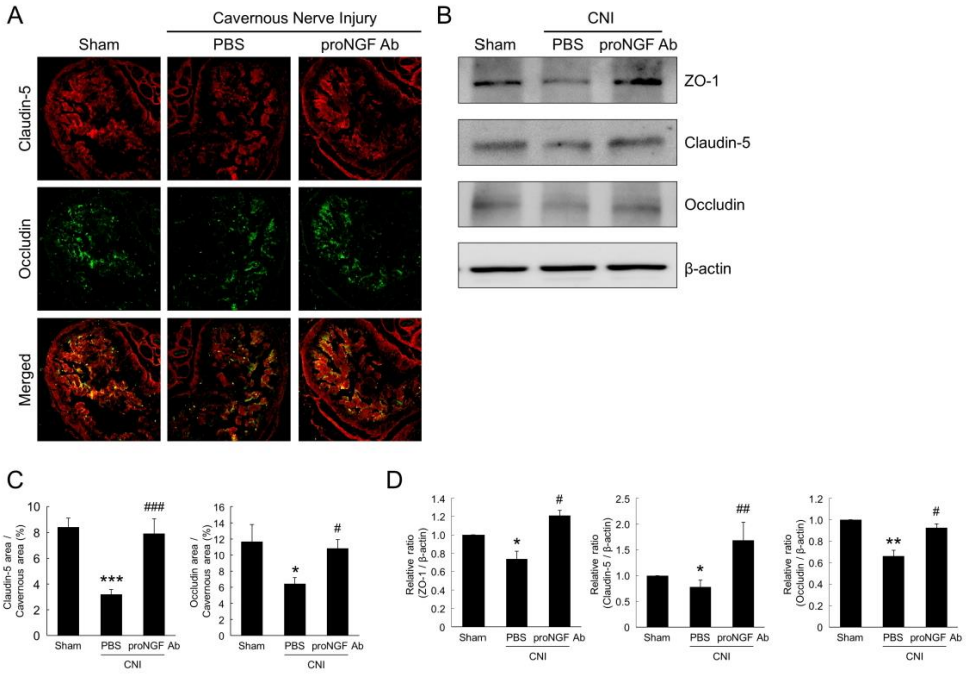

Figure 5. Anti-proNGF-Ab restores cavernous endothelial cell-to-cell junction in CNI mice.

(A) Claudin-5 (red) and occludin (green) staining in the corpus cavernosum of sham control and cavernous nerve injury (CNI) mice 2 weeks after repeated intracavernous injections of PBS (days -3 and 0; 20 μL) or anti-proNGF-Ab (days -3 and 0; 20 μg/20 μL). **(B)** Representative Western blots for claudin-5, occludin, and zonula occludens (ZO-1) in mouse cavernous tissue. **(C)** Quantitative analysis of claudin-5- and occludin-immunopositive area by image analyzer (N = 6). **P* < 0.05, ****P* < 0.001 vs. the sham group. #*P* < 0.05, ###*P* < 0.001 vs. PBS-treated CNI group. **(D)** Normalized Western blots band intensity values for claudin-5, occluding, and ZO-1 (N = 4). **P* < 0.05, ***P* < 0.01 vs. the sham group. #*P* < 0.05, ##*P* < 0.01 vs. PBS-treated group. Data in graphs are presented as mean ± SE.

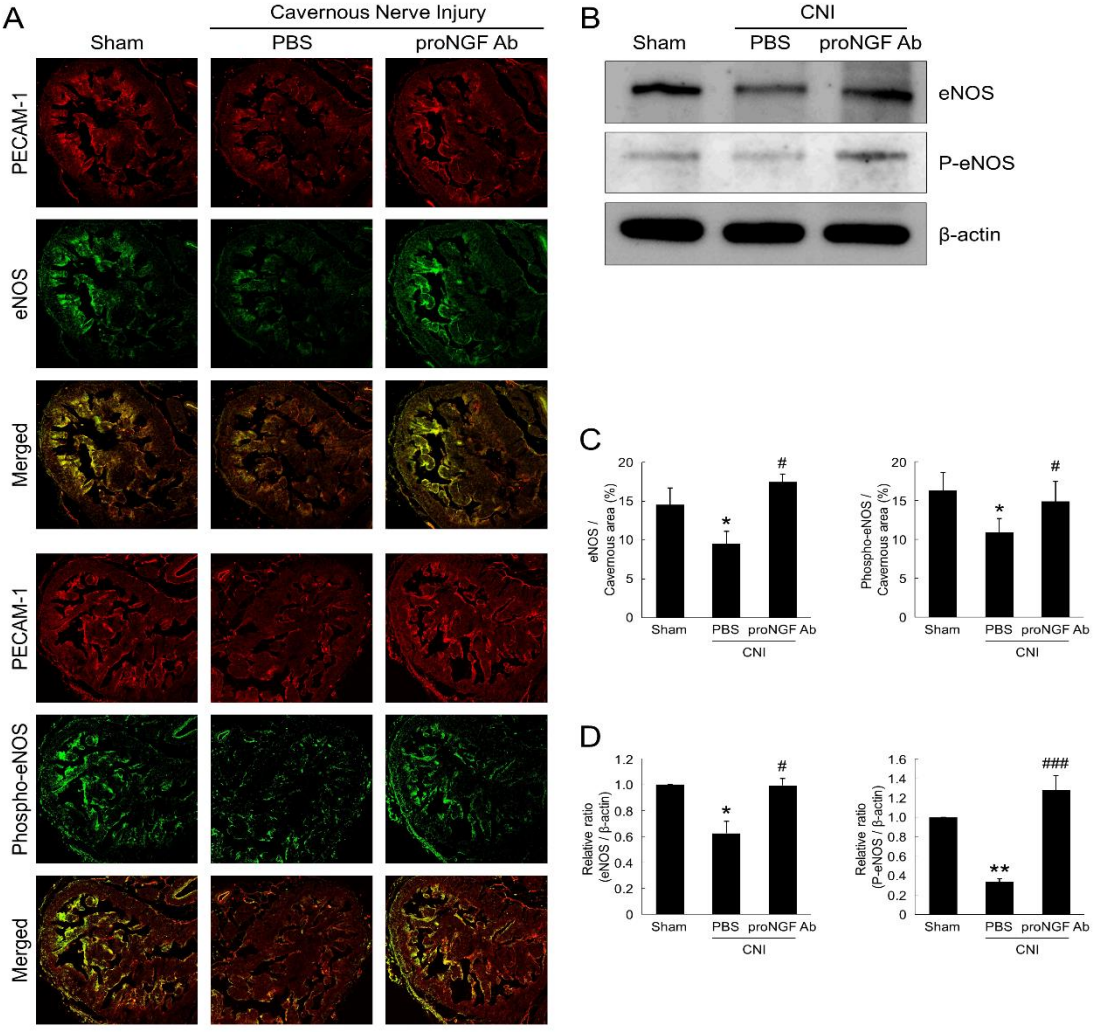

Figure 6. Anti-proNGF-Ab induces cavernous eNOS, eNOS phosphorylation in CNI mice.

(A) PECAM-1 (red) and eNOS (green) or PECAM-1 (red) and phospho-eNOS (green) staining in the corpus cavernosum of sham control and cavernous nerve injury (CNI) mice 2 weeks after repeated intracavernous injections of PBS (days -3 and 0; 20 μL) or anti-proNGF-Ab (days -3 and 0; 20 μg/20 μL). **(B)** Representative Western blots for eNOS and phospho-eNOS in mouse cavernous tissue. **(C)** Quantitative analysis of

eNOS- and phospho-eNOS-immunopositive area by image analyzer (N = 6). *P < 0.05 vs. the sham group. #P < 0.05 vs. PBS-treated CNI group. **(D)** Normalized Western blots band intensity values for eNOS and phospho-eNOS (N = 4). *P < 0.05, **P < 0.01 vs. the sham group. #P < 0.05, ###P < 0.001 vs. PBS-treated CNI group. Data in graphs are presented as mean ± SE.

6. Anti-proNGF-Ab regulates the expression of angiogenic factors in CNI mice

To determine whether the angiogenic potential of anti-proNGF-Ab is mediated by regulation of the angiogenic factors, we performed immunohistochemical staining and Western blot analysis. The cavernous expression of Ang1 and VEGF was down-regulated, and of Ang2 was up-regulated in the CNI mice, which was returned to the level of the sham controls after treatment with anti-proNGF-Ab (**Fig. 7**).

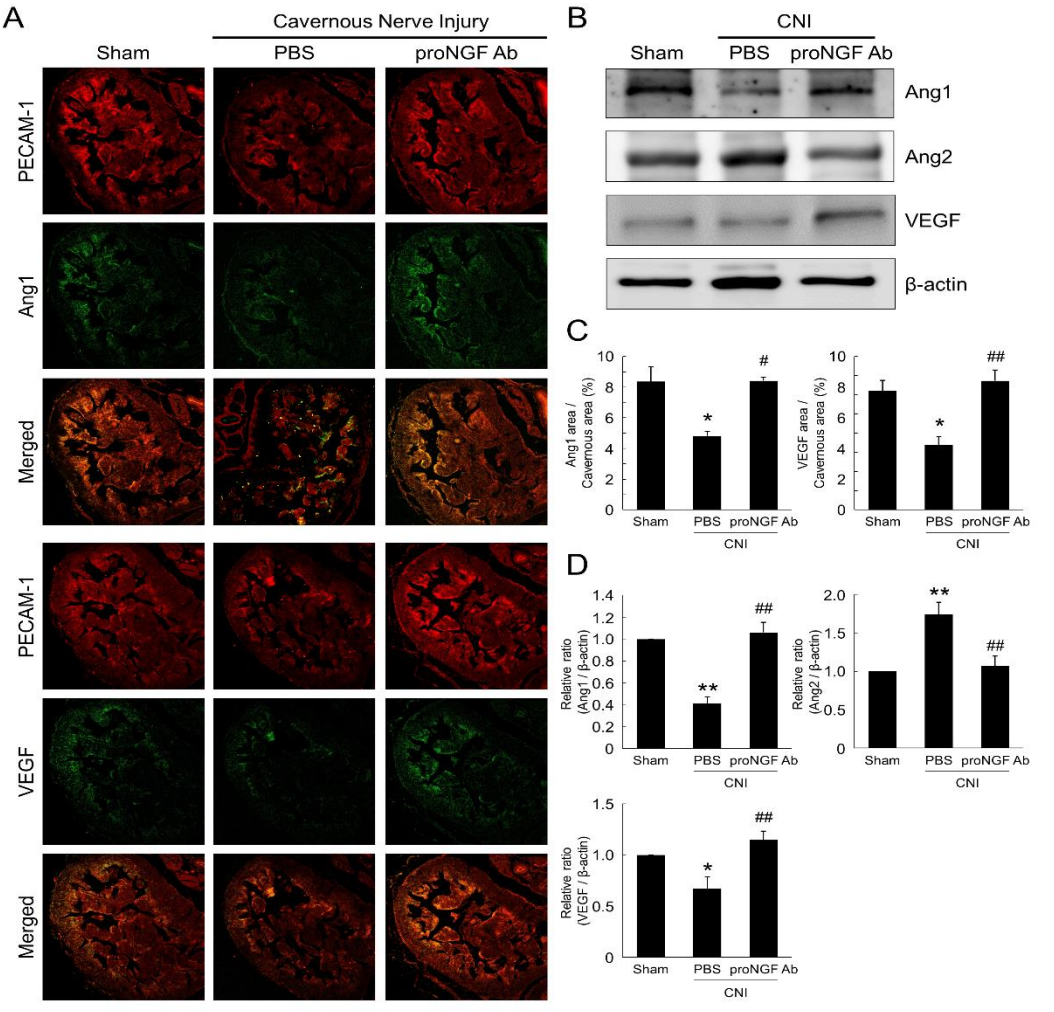

Figure 7. Anti-proNGF-Ab regulates the expression of angiogenic factors in CNI mice.

(A) PECAM-1 (red) and angiopoietin-1 (Ang1, green) or PECAM-1 (red) and vascular endothelial growth factor (VEGF, green) staining in the corpus cavernosum of sham control and cavernous nerve injury (CNI) mice 2 weeks after repeated intracavernous injections of PBS (days -3 and 0; 20 μL) or anti-proNGF-Ab (days -3 and 0; 20 μg/20 μL). **(B)** Representative Western blots for Ang1, angiopoietin-2

(Ang2), and VEGF in each group of mice. **(C)** Quantitative analysis of Ang1- and VEGF- immuno positive area by image analyzer (N = 6). *P < 0.05 vs. the sham group. #P < 0.05, ##P < 0.01 vs. PBS-treated CNI group. **(D)** Normalized Western blots band intensity values for Ang1, Ang2, and VEGF (N = 4). *P < 0.05, **P < 0.01 vs. the sham group. ##P < 0.01 vs. PBS-treated CNI group. Data in graphs are presented as mean ± SE.

7. Anti-proNGF-Ab rescues erectile function in CNI mice

To determine physiologic relevance of proNGF-Ab, we measured ICP after electrical stimulation of the cavernous nerve in sham control and CNI mice 2 weeks after treatment. The ratios of maximal ICP and total ICP to MSBP were significantly lower in PBS-treated CNI mice than in sham controls. In agreement with anti-proNGF-Ab-mediated neural regeneration and angiogenesis, intracavernous administration of anti-proNGF-Ab significantly rescued all erection parameters in CNI mice, which reached up to 75% (maximal ICP at 5V) or 80% (total ICP at 5V) of sham control values (**Fig. 8**).

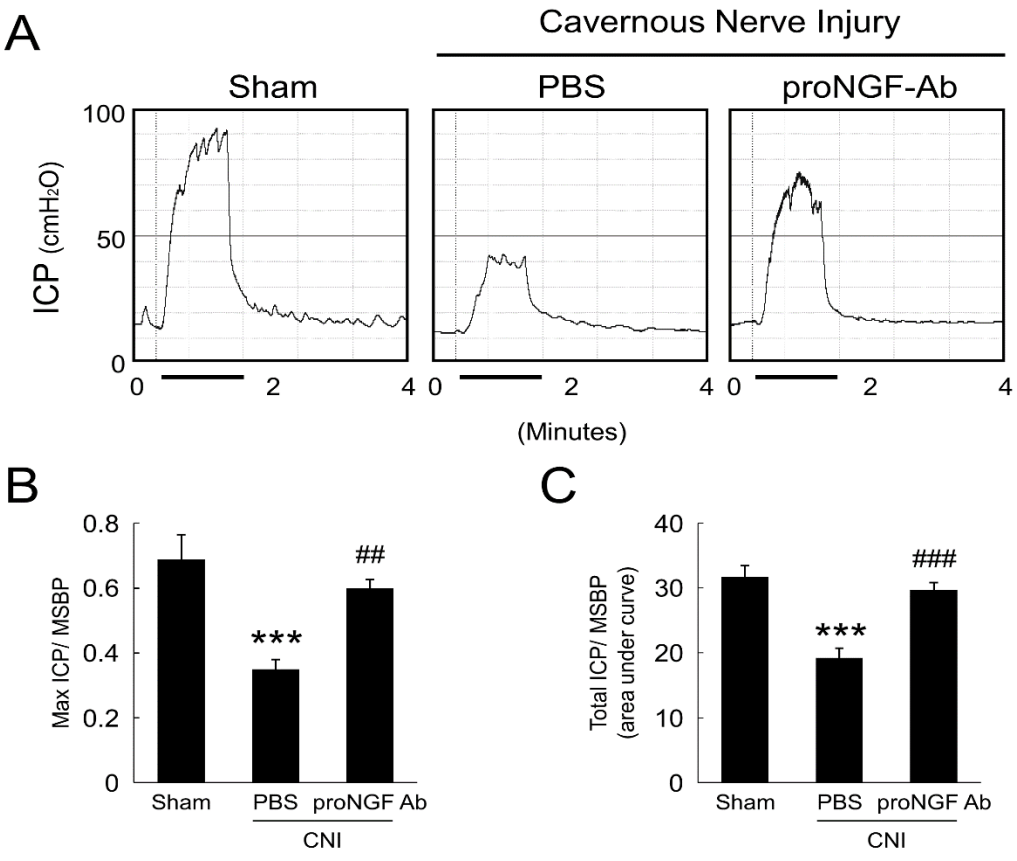

Figure 8. Anti-proNGF-Ab restores erectile function in CNI mice.

(A) Representative intracavernous pressure (ICP) responses in sham group and cavernous nerve injury (CNI) group. Electric stimulation of the cavernous nerve was done 2 weeks after intracavernous injections of PBS (days -3 and 0; 20 μL) or anti-proNGF-Ab (days -3 and 0; 20 μg/20 μL). The stimulus interval is indicated by the solid bar. **(B, C)** Ratio of the mean maximal intracavernous pressure (ICP) and total ICP (area under the curve) to mean systolic blood pressure (MSBP) were calculated for each group (N = 6). ****P* < 0.001 vs. the sham group. *##P* < 0.01, *###P* < 0.001 vs. PBS-treated CNI group.

IV. DISCUSSION

Here, we determined the differential expression of proNGF/p75^{NTR} in the mouse corpus cavernosum and examined the mechanisms by which the inhibition of this signal pathway with anti-proNGF-Ab rescue erectile function in the mice with CNI. The cavernous expression of proNGF and p75^{NTR} was up-regulated after CNI. Local delivery of anti-proNGF-Ab into the corpus cavernosum of CNI mice increased penile nNOS and neurofilament contents by enhancing the expression of neurotrophic factors. Anti-proNGF-Ab successfully preserved endothelial cell and pericyte contents as well as the integrity of endothelial cell-cell junction proteins by regulating the expression of angiogenic factors (Ang1, Ang2, and VEGF), and induced endogenous eNOS phosphorylation in CNI mice.

Anti-proNGF-Ab also enhanced neurite sprouting in *ex vivo* cultured MPG exposed to LPS.

In the present study, we observed an up-regulation of proNGF/p75^{NTR} in the corpus cavernousum of CNI mice compared with that in the sham control group. Previous studies also reported the overexpression of proNGF pathway in a variety of neurodegenerative diseases and diabetic retina [16,17,20,23-27]. We recently reported the overexpression of proNGF/p75^{NTR} in the corpus cavernousum of diabetic mice. These findings give us a rationale to use the blockade of proNGF/p75^{NTR} pathway in various disease conditions including ED.

The proper synthesis of nitric oxide from nNOS in the intact cavernous nerve and diffusion into the adjacent smooth muscle cells are critical for the initiation of penile erection [28]. It has been reported that oxidative stress breakdowns the balance of NGF and proNGF and stimulates the expression of p75^{NTR} [24]. The upregulation of proNGF/p75^{NTR} pathway induces apoptosis of retinal ganglion cells [29]. In the present study, intracavernous administration of anti-proNGF-Ab preserved nNOS- and βIII-tubulin-positive neuronal contents in the dorsal nerve bundle and cavernous tissue of

CNI mice. We further determined the neurotrophic effect of anti-proNGF-Ab in *ex vivo* cultured MPG tissue. To mimic *in vivo* neuroinflammatory condition, the MPG tissue was cultivated under LPS-treated condition. The neurite outgrowth was profoundly impaired in LPS-treated MPG tissue, whereas treatment of MPG with anti-proNGF-Ab enhanced neurite sprouting. And finally, we examined the mechanisms by with proNGF neutralizing antibody induces neural protection. The cavernous expression of neurotrophic factors (BDNF, NGF, and NT3) and their receptors (TrkA and TrkB) was significantly lower in the CNI mice than in the sham control mice. Intracavernous administration of anti-proNGF-Ab significantly increased the expression of neurotrophic factors and their receptors in CNI mice.

The integrity of cavernous sinusoids also plays a crucial role in maintaining penile erection through the production of NO from eNOS. Similar to the result from a recent study by ours in a mouse model of diabetic ED [21], anti-proNGF-Ab preserved cavernous endothelial cell contents in CNI mice by promoting endothelial cell proliferation and by inhibiting endothelial cells apoptosis. It was also reported in diabetic retina that siRNA-mediated silencing of $p75^{NTR}$ successfully blocked proNGF-induced endothelial cell apoptosis [30]. In accordance with these findings, intracavernous administration of anti-proNGF-Ab enhanced eNOS phosphorylation in CNI mice.

Endothelial cell-cell junction plays a critical role to protect organ from noxious stimuli, especially in brain (blood-brain barrier) and retina (blood-retina barrier) [31]. We also reported the importance of cavernous endothelial cell-cell junction in the pathogenesis of diabetic ED [32]. Inhibition of proNGF/$p75^{NTR}$ pathway prevented the breakdown of blood-retina barrier [24]. Similar to the previous results from diabetic corpus cavernosum [32], the cavernous expressions of endothelial cell-cell junction proteins, such as claudin-5, occludin, and ZO-1, were profoundly decreased in CNI mice compared with that in sham controls. Intracavernous administration of anti-proNGF-Ab preserved endothelial cell-cell junction proteins in the corpus

cavernousum of CNI mice.

The interaction between endothelial cells and pericytes play a crucial role in vascular homeostasis [33]. We recently documented a cross-talk between cavernous endothelial cells and pericytes in a mouse model of diabetic ED [34]. It was reported that inhibition of proNGF/$p75^{NTR}$ pathway restored pericyte content in the retinal blood vessels [33] or corpus caverinnousm of diabetic mice [21]. In agreement with these findings, anti-proNGF-Ab profoundly preserved cavernous pericyte content in CNI mice.

In the present study, the expression of Ang1 and VEGF was down-regulated, and that of Ang2 was up-regulated in the corpus cavernosum of CNI mice. ProNGF neutralizing antibody restored the expression of Ang1, Ang2, and VEGF to the levels of sham controls. Ang1 generates non-leaky and stable vasculature by binding it receptor Tie2, whereas Ang2 antagonize Ang1 upon binding to Tie2 receptor and is known to destabilize blood vessels [35]. Although VEGF is a potent angiogenic factor, but by itself induces inflammation, which causes the formation of unstable blood vessel with increased vascular leakiness [36]. In contrast, Ang1 counteracts VEGF-induced inflammation and synergistically induces angiogenesis when co-administration with VEGF [37]. Therefore, the regulation of angiogenic factors may contribute to the mechanism responsible for anti-proNGF-Ab-mediated preservation of cavernous vascular integrity in CNI mice.

This study has some limitations. The mechanism by which the regulation of neurotrophic and angiogenic factors by anti-proNGF-Ab were not clarified. The erectile function preservation induced by anti-proNGF-Ab was relatively short-term and partial. Additional studies are necessary to determine whether the different dosing or frequency of anti-proNGF-Ab would induce long-lasting and complete preservation of erectile function. Despite these limitations, this study will give us valuable information to overcome the limitations of current oral PDE5Is in men with RP-induced ED.

V. CONCLUSIONS

Our results suggest that inhibition of proNGF/$p75^{NTR}$ pathway preserves damaged penile neurovascular structure and restores erectile function in CNI mice probably by regulating the expression of both neurotrophic and angiogenic factors. With ongoing further research, we believe that the development of new therapeutics by using proNGF neutralizing antibody may be a way to overcome RP-induced ED.

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ABSTRACT (IN KOREAN)

해면체 신경 손상의 마우스 모델에서 proNGF 중화 항체를 통한 신경 영양 및 혈관 신생 인자의 발현 조절에 따른 발기 부전 치료

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정 두 용

전립선 암의 치료인 근치적 전립선 절제술은 로봇 수술을 비롯한 수술적 기술의 발전에도 불구하고 일부의 해면체 신경 손상 (CNI)을 유발 하며 또한 이에 따른 해면체 혈관 구조의 병리학적 변화를 유발하여 발기 부전을 일으킨다. 이전 연구들에서 proNGF는 수용체 $p75^{NTR}$ 을 통하여 신경 세포의 미세혈관 기능 장애 및 세포자연사와 관여 하는 것으로 알려져 있다. 따라서 우리는 CNI를 유발한 마우스모델에서 proNGF/ $p75^{NTR}$ 발현 변화를 조사하고, proNGF 중화 항체 (anti-proNGF-Ab)의 효과에 대하여 조사하였다.

12주령의 C57BL/6 마우스를 사용하여 3그룹으로 분류 하였다. 1. Sham operation 그룹, 2. 양측 CNI를 시행 후 2회 PBS (days -3 and 0; 20 μL)를 처치한 그룹 3. 양측 CNI를 시행 후 2회 anti-proNGF-Ab (days -3 and 0; 20 μL).

발기 기능은 처치 후 2주뒤 해면체 신경의 전기 자극을 통하여 측정 하였으며, 페니스 조직을 통하여 조직학 및 생화학적 조사를 시행하였다. 우리는 추가로 ex vivo 실험으로 주요 골반 신경 절 (MPG)을 배양하여 anti-proNGF-Ab에 신경 재생 효과를 알아보았다.

실험 결과에서 CNI를 유발 시킨 마우스에서 proNGF/ $p75^{NTR}$ 의 발현이 증가됨을 관찰 하였다. 그리고 CNI 마우스의 페니스에 anti-proNGF-Ab를 주입 하였을 때 신경 영양 인자(NGF, brain-derived neurotrophic factor, and neurotrophin-3) 및 nNOS 가 회복 되는 것을 관찰 하였다. 또한 anti-proNGF-Ab 은 혈관 생성 인자들(angiopoietin-1, angiopoietin-2, and vascular endothelial growth factor) 및 endothelial cell-to-cell junction 단백질 발현을 조절 함으로써 해편체 혈관 주피세포 와 내피세포 및 해면정맥굴의 보존성을 회복 하고 eNOS 인산화를 유발 하였다. 이러한 변화는 CNI 마우스에서 발기 기능을 현저히 회복 시키는 변화를 보여주었다. 또한 anti-proNGF-Ab 는 lipopolysaccharide 에 노출 시킨 MPG에 신경 돌기 발아에 향상을 야기 시켰다.

따라서 우리는 proNGF / $p75^{NTR}$ 경로의 억제를 통하여 손상된 해면체 신경과 혈관의 재생은 근치적 전립선 절제술로 발생한 발기 부전 치료에 새로운 길을 열 수 있다고 생각한다.

Key Words: 발기 장애; 근치적 전립선 절제술; 해면체 신경 손상; proNGF, $p75^{NTR}$