MATERIALS AND METHODS

1. Cavernous nerve injury

Cavernous nerve injury (CNI) model was made conferring to protocol [25]. Eight-week-old age-matched male mice were divided into five groups: three treatment groups received bilateral crush injury to the cavernous nerve whereas the control group underwent sham surgery to expose the cavernous nerve, but without manipulation. Preoperatively, the mice were anesthetized with a xylazine (5 mg/kg) and ketamine (100 mg/kg) combination that was administered by intraperitoneal injection. The surgical area was shaved and disinfected with 2% chlorhexidine and 70% isopropanol alcohol, and the surgery was done under a dissecting microscope with the subject placed on a 37 °C heating pad. A lower midline abdominal laparotomy (1 cm) incision of the skin and peritoneal layer was made and extended until the pelvic organs and cavernous nerve plexus were exposed. The cavernous plexus was visualized for the CNI induced by applying maximal mechanical pressure to the pre-exposed cavernous nerve 1 mm distal to the ganglion with a non-serrated hemostat (Karl Stortz Co.) for 2 minutes on each side. Two separate closures of the peritoneum and skin were undertaken with 6-0 Vicryl (Poliglactin 910; Ethicon). Mice were placed on a heating pad upon recovery.

2. Erectile function evaluation

Erectile capacity was assessed by intracavernous pressure (ICP) according to protocol [24]. Sedated animals received intraperitoneal xylazine (5 mg/kg) and ketamine (100 mg/kg). Systemic blood pressure was measured with a tail-cuff blood pressure monitoring system (Visitech Systems). A lower midline (2–3 cm) abdominal incision was made to the abdominal skin and peritoneum. The bladder and prostate were retracted laterally to expose the cavernous nerve. The cavernous nerve was stimulated with a 26 g bipolar platinum electrical wire stimulator (Biopac System Inc.) that was positioned under the cavernous nerve. A 25G needle connected to a pressure transducer (Biopac System Inc.) was inserted into the degloved penile crus. Approximately 30 μL heparinized solution (250 U/mL) was injected into the cavernous to prevent the clogging of the needle tip by a blood clot. The study's stimulation settings were 5 volts, 12 hertz, 1 millisecond pulse width, and 1 minute duration. ICP was measured with Biopac Student Lab (BSL 3.7.7, Biopac System Inc.). During tumescence, the maximal intracavernous pressure (maxICP) and total ICP reflected by the area under the curve were recorded. Variations of systemic blood pressure were normalized by calculated ratios of the area under the curve (total ICP) or maximum ICP (cm H_2O) to the mean systolic blood pressure (MSBP).

3. Histologic examinations

Penile tissues intended for immunofluorescence were preserved in 4% paraformaldehyde overnight. The tissue was frozen quickly in optimal cutting temperature (OCT) gel and cut into 10-μm-thick sections. Prior to the analysis, the slide was washed with PBS (Gibco) several times to remove the OCT. Tissue sections were incubated overnight with primary antibodies against; neurofilaments (NF) (1:100; Sigma-Aldrich), neuronal nitric oxide synthase (nNOS) (1:100; Santa Cruz Biotechnology Inc.), PECAM-1 antibody (1:100; Millipore), neuron-glial antigen-2 (NG-2) (1:100; Millipore), phospho-endothelial-nitric-oxide synthase (p-eNOS) (1:100; Cell Signaling), myelin basic protein (MBP) (1:100; Invitrogen), anti-S100 (1:100; Abcam), and smooth muscle α-actin (α-SMA) (1:100; Sigma-Aldrich) at 4 °C. After 10 minutes of rinsing with PBS, the slides were treated with secondary antibodies specific to the species of interest: tetramethyl-rhodamine-isothiocyanate (TRITC) or fluorescein-isothiocyanate (FITC)-conjugated (1:300; Zymed Laboratories) for 2 hours at room temperature and mounted with DAPI-containing (Vector Laboratories, Inc.) nuclear dye. Fluorescence signals were observed using a confocal microscope (K1-Fluo; Nanoscope Systems, Inc.). Image J was used for quantitative analysis (NIH 1.34, http://rsbweb.nih.gov/ij/).

Quantification of immunofluorescent images involved adjusting threshold parameters using the "Image–Adjust–Threshold" function for precise selection of areas of interest, with a dark background setting for enhanced contrast in fluorescence images. After setting the threshold, the "Analyze–Set Measurements" and "Limit to Threshold" features, then we manually corrected the desired region of interest (ROI) using a tool like the freehand or ellipse,

and then uses the "ROI Manager" to define and confine the parameters for quantification to the selected areas only. The process concluded with the "Analyze–Measure" command within the ROI Manager to obtain results, and "Analyze Particles" for cell analysis and intensity assessments within regions of interest. For accurate comparisons across multiple specimens, consistent staining, image acquisition, processing techniques, and settings are crucial, along with using a homogeneous backdrop for quantifying staining intensity.

4. BrdU-incorporation assay

Neuroinflammatory-like conditions were simulated by treating PC-12 cells with lipopolysaccharide (LPS) continue with 15 minutes LED irradiation for each session for 5 days, followed by treatment at 37 ℃ for 1 hour with 10 M BrdU (5'-bromo-2'-deoxyuridine; Sigma-Aldrich). Using anti-BrdU antibody (1:200; Bio-Rad), the fixed cells were stained. BrdU+ cells were enumerated in 400x high-power fields using a confocal microscope (K1-Fluo).

5. TUNEL assay

Cell death in PC-12 was measured by TUNEL with an ApopTag Fluoresce in Situ Apoptosis Detection Kit (Ca# S7111; Chemicon) following the instructions provided by the manufacturer. Using confocal fluorescence microscopes, we counted the number of apoptotic cells in each high-magnification field at 200x. Fluorescence signals were observed using a confocal microscope (K1-Fluo). Image J was used for quantitative analysis (NIH 1.34).

6. Neurite outgrowth analysis

Major pelvic ganglion (MPG) and dorsal root ganglion (DRG) tissues were obtained and preserved using a published protocol [25]. Tissue was removed under dissecting microscope, deposited in Hank's balanced salt solution (Gibco), rinsed, and washed twice with PBS. Tissues cut into 1- to 2-mm pieces were plated on poly-D-lysine hydrobromide-coated 6-well glass-bottom plates, covered with Matrigel (BD Biosciences) and incubated at 37 ℃ for 10 minutes in a 5% CO_2 environment with 1 ml of Gibco complete neurobasal medium (0.5 nM), GlutaMAX™-I 2%, and serum-free B-27 (Gibco). In order to replicate an *in vivo* neuroinflammatory condition, LPS (Sigma-Aldrich) was introduced into the culture medium and allowed to incubate for a duration of 24 hours. Neurite outgrowth was assessed after five days of photobiomodulation (PBM) treatment. Tissue was fixed in 4% paraformaldehyde for 1 hour, washed several times with PBS and immunofluorescence-labeling to anti-βIII tubulin antibody (1:100; Abcam).

The quantification of neurite outgrowth area was performed using Image J (NIH 1.34), by measuring βIII tubulin immune-positive area. Images underwent a transformation process to convert them into 8-bit graphics, which facilitated the selection of the ROI. The optimal neurite sprouting region was determined by altering the threshold and selection tool. Any residual noise that was not eliminated by the thresholding process was manually deleted.

For PC-12 neurite outgrowth experiments, the rat pheochromocytoma PC-12 cell lines were acquired from the American Type Culture Collection (ATCC) and cultivated in Dulbecco's modified Eagle Medium (DMEM, Gibco), supplemented with 10% horse serum (Gibco), 5% fetal bovine serum (Gibco), and 1% penicillin/streptomycin (Gibco). The cells were seeded in growth media at a density of 2×10^4 cells per well on collagen type I-coated 6-well culture plates (BD Biosciences). Subsequently, LPS 10 μL/mL (Sigma-Aldrich) was added 24 hours post seeding and the cells were subjected to heat, RED, NIR, or a combination of these treatments for a duration of 15 minutes each day, for five consecutive days. The medium was changed every two days. Before conducting the study, the slide underwent a washing process using PBS and was then treated with a 4% paraformaldehyde solution for one hour. After fixation, the slide underwent immunofluorescence labeling with NF (1:100, Sigma-Aldrich). The data collected consisted of counts derived from three separate wells, with each well containing 10 randomly selected images. We only evaluated cells that had neurite lengths 1.5 times the width of cell bodies. Fluorescence signals were observed using a confocal microscope (K1-Fluo). Image J (NIH 1.34) was used for measuring neurite length and number of branching.

7. Western blotting

After the assessment of erectile function, the penile tissue was immediately placed in a container containing liquid nitrogen for preservation. A small amount of liquid nitrogen was used to grind the tissue. The protease and

phosphatase inhibitors (1:100; Sigma-Aldrich) were mixed with RIPA buffer (Sigma-Aldrich) to lyse the tissues. The ELx800G Universal Microplate Reader was used to determine the protein content (BioTek Instruments Inc.). Equal amounts of protein (30 μg per lane) were electrophoresed on 8% to 12% sodium dodecyl sulfate-polyacrylamide gels and transferred to polyvinylidene fluoride (PVDF) membranes. After blocking with 5% non-fat dried milk for 1.5 hours at room temperature, the membrane was incubated at 4 ℃ overnight with primary antibodies, p-eNOS (9571, 1:3,000; Cell Signaling), neurotrophin-3 (NT-3) (1:3,000; Santa Cruz), phospho-phosphoinositide-3-kinase (p-PI3K) (1:3,000; Cell Signaling), vascular endothelial growth factor (VEGF) (1:3,000; Santa Cruz), brain-derived neurotrophic factor (BDNF) (1:3,000; Santa Cruz), nerve growth factor (NGF) (1:3,000; Santa Cruz), angiopoietin-1 (ab8451, 1:3,000; Abcam), and glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) (1:5,000; ABclonal), then washed five times with PBS-T. Thereafter, signals were visualized using the ECL detection system (Amersham Pharmacia Biotech, Piscataway).

Supplement Table 1. Physiologic and metabolic parameters: 2 weeks after PBM treatment

	Sham	CNI			
		Heat	RED	NIR	RED+NIR
Body weight (g)	26.03±0.51	27.10±0.94	26.59±1.79	27.62±0.96	25.82± 0.64
MSBP (cm H_2O)	79.21±3.46	82.81±2.14	76.14±2.06	80.41±2.16	75.76±2.14

Values are the mean±standard error of the mean for n=6 animals per group.
Body weight and MSBP measured two weeks post-PBM irradiation therapy with heat, RED, NIR, or RED+NIR for five consecutive days.
PBM: photobiomodulation, CNI: cavernous nerve injury, NIR: near-infrared, MSBP: mean systolic blood pressure.