Effect of Electrical Stimulation of Cervical Sympathetic Ganglia on Intraocular Pressure Regulation According to Different Circadian Rhythms in Rats

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Purpose. The purpose of this study was to investigate the relationship between circadian rhythm and intraocular pressure (IOP), and to explore whether electrical stimulation of cervical sympathetic ganglia (SCG) can regulate IOP via neurotransmitter distribution around the Schlemm's canal (SC) in rats.

Methods. Sprague Dawley rats were housed under normal (N-normal), constant dark (N-dark), and constant light (N-light) rhythms ($n = 6$ per group). Electrical stimulation (intermittent wave [20 hertz {Hz}, 2 mA, 10 minutes]) was used to stimulate the SCG. Atropine sulfate eye gel was applied three times a day. DiI was injected into the SCG and anterior chamber. The cross-sectional area and circumference of SC were evaluated using hematoxylin-eosin staining. Immunofluorescence staining was used to evaluate dopamine-β-hydroxylase (DβH) expression in SC endothelial (SCE) cells.

Results. N-Dark increased the IOP, decreased the cross-sectional area of SC, and increased DβH levels in SCE cells. Nerve projection between SC and SCG was detected, and electrical stimulation of SCG upregulated DβH expression in SCE cells. Under normal and constant light rhythms, electrical stimulation of SCG increased DβH and decreased the cross-sectional area and circumference of SC, while simultaneously increasing IOP and decreasing IOP fluctuations. After paralyzing the ciliary muscles, electrical stimulation of SCG decreased the cross-sectional area and circumference of SC under normal and constant light rhythms.

Conclusions. N-Dark increased DβH in SCE cells, reduced the cross-sectional area of SC, and increased IOP. Under the normal and light rhythms, electrical stimulation of SCG increased DβH in SCE cells, reduced the cross-sectional area and circumference of SC, and in turn elevated IOP and decreased IOP fluctuations.

Keywords: cervical sympathetic ganglia, electrical stimulation, intraocular pressure, circadian rhythm, Schlemm's canal, dopamine-β-hydroxylase

Glaucoma is the most common irreversible eye disease causing blindness worldwide,[1] and increased intraocular pressure (IOP) is a key risk factor for glaucoma progression.[2,3] Because IOP fluctuates according to circadian rhythm,[4] an increasing number of investigators believe that devoting attention to the circadian rhythm of IOP is more critical than monitoring IOP in isolation.[4–6]

Rats are nocturnally active mammals. Previous studies have reported that IOP in rats is higher at night than during the daytime, suggesting the presence of an IOP circadian rhythm[7,8]; however, it is unclear whether this IOP rhythm is related to nocturnal activity.

Previous research postulated that increased resistance to the outflow pathway of aqueous humor (AH) could cause IOP elevation.[9] Approximately 75% to 80% of AH is drained via the conventional route, which includes the trabecular meshwork (TM), Schlemm's canal (SC), and the collecting tube. Primary resistance to this route is located between the juxtacanalicular portion of the TM and SC.[9,10] The state of SC (open or closed) is a vital factor in the outflow regulation of AH[11,12]; however, the mechanism of SC regulation remains unclear.

The hypothalamus is an important structure that stabilizes and regulates basic life activities, including the sleep-wake cycle, blood pressure, and heart rate.[13,14] Previous studies have found that the paraventricular hypothalamic nucleus projects to the superior salivatory nucleus and the intermediolateral cell column of the spinal cord.[15,16] The parasympathetic preganglionic fibers from the superior salivatory nucleus project to the pterygopalatine ganglia (PPG), and the sympathetic preganglionic fibers from the intermediolateral cell column innervate the cervical sympathetic ganglia (SCG).[17] Together, the parasympathetic fibers from

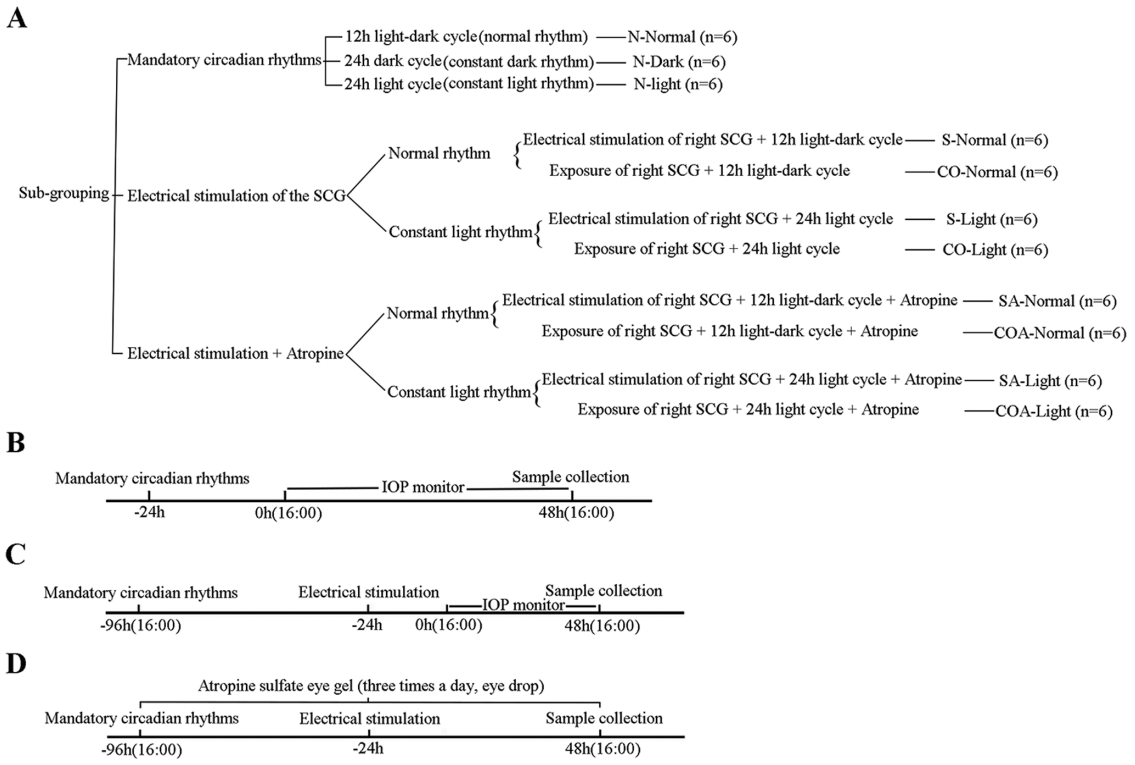

Figure 1. Subgrouping and experiment process plan of this study.

the PPG and sympathetic fibers from the SCG regulate the AH outflow pathway.[17]

Vasoactive intestinal polypeptide (VIP), released by parasympathetic postganglionic fibers in the peripheral nervous system, is expressed by postganglionic neurons in the PPG.[17–19] Our previous studies found that VIP could expand SC and reduce IOP in rat models of chronic IOP hypertension.[18] Electrical stimulation of the sympathetic nerve trunk can increase IOP, whereas excision of SCG can block this effect.[20] These findings suggest that the autonomic nerves may be involved in SC morphology and IOP regulation. However, there is no supportive evidence demonstrating the function of SCG in SC morphology and IOP regulation.

Materials and Methods

Animal Models

Male Sprague-Dawley rats weighing 220 ± 20 g were obtained from the Experiment Animal Center of the Tongji Medical College. Animals were kept at 25°C with sufficient food and water. This study was carried out by the recommendations of the Institutional Animal Care and Use Committee of Huazhong University of Science and Technology and in strict accordance with the guidelines for the ARVO Statement for the Use of Animals in Vision and Ophthalmic Research. After marking the conjunctiva on the midline of the superior, nasal, inferior, and temporal regions in each eyeball, the whole eyeballs of euthanized rats were fixed with 10% formalin for 2 days at 4°C. They were embedded in paraffin to enable SC to be cut perpendicularly to its longitudinal axis. Slices with marker from superior, nasal, inferior, and temporal regions would be chosen for further measurement and analysis.

Rat eyes were divided into three groups (N-normal, N-dark, and N-light, $n = 6$), then supplied them a normal rhythm (lights turned on at 7 AM and off at 7 PM), a constant dark rhythm and a constant light rhythm, respectively (Fig. 1A). After maintaining the corresponding circadian rhythms for 24 hours, we monitored 48-hour IOP starting from 16:00 (0 hour), then rats were euthanized to collect the eyes at 48 hours (Fig. 1B).

DiI, 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate, is one of the most commonly used cell membrane fluorescent probes, and it can be used to trace nerve fibers as a fluorescent tracer.[21,22] Rats under normal rhythm were divided into three groups ($n = 6$). After being anesthetized by intraperitoneal injection of 10% chloral hydrate (4 mL/kg), in the first group, we injected DiI (2.5 mg/mL, 10 µl) into the anterior chamber from cornea where was near Angular margin of the right eyes using 30G needle. In the second group, after being anesthetized, we separated the anterior cervical skin and fascia, then exposed the right SCG, and injected DiI (2.5 mg/mL, 20 µl) into the SCG, finally sutured the incision, and applied amoxicillin on the incision to prevent infection. In the third group, there was no DiI injection. Then, the right SCGs in the first group and the right eyes in the second group were collected after 5 days.

Rats under normal rhythm were divided into two groups ($n = 6$). After being anesthetized, the right SCG was separated and exposed. Then it was treated by electrical stimulation with an Electronic Acupuncture Treatment Instrument (SDZ-II Nerve and Muscle Stimulator, parameters: intermittent wave [20 Hz, 2 mA, 10 minutes], Suzhou Medical

Appliance Factory, Suzhou, China). The right SCGs of rats in the control group were just exposed. The right eyes were immediately collected after electrically stimulating or only exposing SCGs.

Rats were divided into four groups (S-normal, CO-normal, S-light, and CO-light, $n = 6$; see Fig. 1A). After maintaining the corresponding circadian rhythms for 72 hours from 16:00 (–96 hours), different treatments were supplied to rats near the time point of –24 hours (Figs. 1A, 1C). We monitored 48-hour IOP of the right eyes starting from 16:00 (0 hours), then the right eyes were collected at 48 hours (see Fig. 1C).

Rats were divided into four groups (SA-normal, COA-normal, SA-light, and COA-light, $n = 6$; see Fig. 1A). After maintaining the corresponding circadian rhythms for 72 hours from 16:00 (–96 hours), different treatments were supplied to rats near the time point of –24 hours (Figs. 1A, 1D). The right eyes received Atropine sulfate eye gel (2.5 g: 25 mg; Shenyang Xingqi Pharmaceutical Co., Ltd., Shenyang, China) intervention through eye dropping three times a day (08:00, 16:00, and 22:00) during –96 hours to 48 hours, and were collected at 48 hours (see Fig. 1D).

Reagents and Antibodies

The information of reagents and antibodies was shown as following: anti-TH antibody (AB5986p; Millipore, Schwalbach, Germany), anti-DβH antibody (ab19353; Abcam, Cambridge, MA, USA), anti-PECAM1 antibody (A01513-3; Bosterbio, Wuhan, China), donkey anti-rabbit antibody (A21206, Alexa Fluor 488; Thermo, Rockford, IL, USA), donkey anti-goat antibody (A11055, Alexa Fluor 488; Thermo), donkey anti-rabbit antibody (A21207, Alexa Fluor 594; Thermo), and DiI (468495, Alexa Fluor 594; Sigma, Shanghai, China).

IOP Measurement

IOP was measured by using an Icare PRO tonometer (Icare Finland Oy, Vantaa, Finland) in awake rats. Eyes were topically anesthetized with 20% lidocaine hydrochloride before IOP measurements. Measurement of IOP was always performed at 16:00, 20:00, 00:00, 04:00, 08:00, and 12:00 every day. All data were measured within a window of 20 minutes around each clock time. Six measurements were taken from each eye and averaged. All repeated measurements reached the level with a coefficient of variation less than 8%.

SC Measurement

Slices of hydrated and deparaffinized tissues were stained with hematoxylin-eosin. The SC parameters included the cross-sectional area, circumference.[11,18] These parameters were measured by using the ImageJ software (http://imagej.nih.gov/ij/; provided in the public domain by the National Institutes of Health, Bethesda, MD, USA). Optimum image light and contrast were subjectively defined in order to maximize the visualization of the SC. The cross-sectional area and circumference were drawn freehand based on the outline of SC. Each measurement was repeated two times and averaged.

Immunofluorescence Staining

For immunofluorescence (IF), pre-heated to 37°C, SC slices were gently washed three times with phosphate-buffered saline (PBS) and then treated with 10% donkey serum albumin for 1 hour at room temperature to block nonspecific binding. Then, they were incubated with anti-DβH antibody (1:2500) and anti-TH antibody (1:1000) diluted in blocking buffer, respectively, or with anti-DβH antibody (1:2500) and anti-PECAM1 antibody (1:200) simultaneously, at 4°C overnight. Then, they were washed three-times with PBS; the slices were incubated with fluorescently labeled immunoglobulin G secondary antibodies (1:200) for 2 hours at room temperature. In addition, the slices were incubated with DAPI (1:100; Bioss, Beijing, China) for 15 minutes at room temperature. Finally, the slices were observed under a laser scanning confocal microscope (Zeiss LSM 710, Oberkochen, Germany) with excitation wavelengths set to 405 nm for DAPI, 488 nm for Alexa 488, and 594 nm for Alexa 594, respectively.

Immunofluorescence Intensity Measurement

The total area of the SC endothelial (SCE) cells and the total area of DβH-positive expression in SCE cells were measured by using ImageJ. Each measurement was repeated two times and averaged. Then, the percentage of the DβH-positive area (area of DβH-positive expression in SCE cells/ area of SCE cells) was calculated.

Data Analysis

Statistical analyses were performed using two-tailed unpaired *t*-tests by SPSS version 22 (IBM, Armonk, NY, USA). All data for IOP, SC, and immunofluorescence intensity measurement were reported with mean ± standard deviation. The $P < 0.05$ and $P < 0.01$ were both considered statistically significant.

Results

Constant Dark Rhythm (N-Dark) Increased the IOP in Rats

As shown in Figure 2A and Supplementary Table S1, compared with the normal rhythm (N-normal) group, the average IOP in the constant dark rhythm (N-dark) group increased during 0 to 48 hours and 08:00 AM to 08:00 PM (0 hours and 16–28 hours and 40–48 hours, and 28 hours noninclusive) periods; compared with the constant light rhythm (N-light) group, the average IOP in the N-dark group elevated during 0 to 48 hours, 08:00 PM to 08:00 AM (4–16 hours and 28–40 hours, 16 hours, and 40 hours noninclusive) and 08:00 AM to 08:00 PM periods. In the N-dark group, the peak and nadir IOP values were greater than in the N-normal and N-light groups (Figs. 2B, 2C, Supplementary Table S2). The range of IOPs in the N-light group was smaller than that in the N-normal group during the 24 to 48 hour period (7.9 ± 0.5 mm Hg versus 10.8 ± 0.3 mm Hg; $P < 0.01$), whereas there was no significant difference between the N-dark and N-normal groups (Fig. 2D). The results suggested that the N-dark condition increased IOP; however, there was no difference in the fluctuating amplitude of IOP between rats in the N-dark and N-normal groups.

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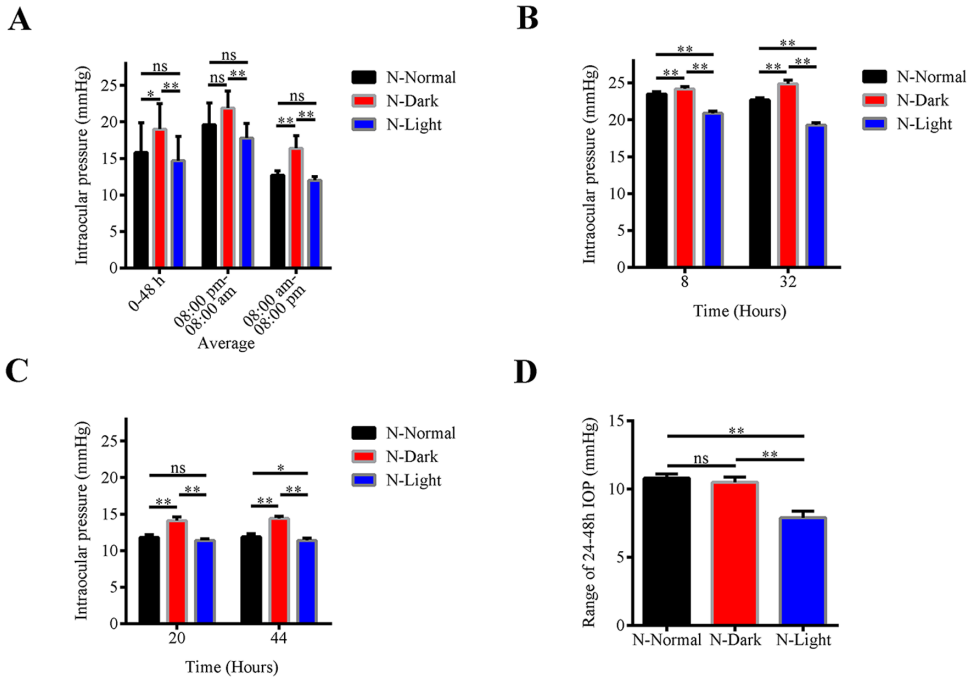

Figure 2. Intraocular pressure (IOP) analyses of rats under the normal, constant dark, and light rhythms ($n = 6$). (**A**) The comparison of average IOP during different periods by using two-tailed unpaired *t*-test; 08:00 PM to 08:00 AM: 4 to 16 hours and 28 to 40 hours (16 hours and 40 hours noninclusive); 08:00 AM to 08:00 PM: 0 hours, 16 to 28 hours, and 40 to 48 hours (28 hours noninclusive). The average IOP increased in the N-dark group compared with the N-normal and N-light groups. (**B, C**) The peak and nadir values of IOP comparison by using two-tailed unpaired *t*-test. The peak and nadir values of IOP were higher in the N-dark group than in the N-normal and N-light groups. (**D**) Range of 24 to 48 hours IOP (24 hours noninclusive) comparison by using two-tailed unpaired *t*-test. There were no statistical differences in the range of 24 to 48 hours IOP between the N-dark and N-normal groups, whereas the range of 24 to 48 hours IOP was decreased in the N-light group. $^{*}P < 0.05$, $^{**}P < 0.01$, ns: no statistical significance.

N-Dark Decreased the Cross-Sectional Area of SC in Rats

To compare the morphological changes in SC under different circadian rhythms, hematoxylin-eosin (HE) staining was used to evaluate the cross-sectional area and circumference of SC (Fig. 3A). As shown in Figure 3B, in the N-dark group, the cross-sectional area of SC decreased compared with SC in the N-normal and N-light groups (939.095 ± 97.130 μm^2 vs. 1513.680 ± 236.439 μm^2 [$P < 0.01$] vs. 1345.798 ± 324.307 μm^2 [$P < 0.01$], respectively). There was no significant difference in the circumference of SC among the three groups (Fig. 3C). The results indicated that the cross-sectional area of SC was decreased in the N-dark group, although the circumference of SC exhibited no statistical differences among the groups.

N-Dark Increased DβH Expression in SC Endothelial Cells In Vivo

To verify the neurotransmitter expression in SCE cells, in vivo expression of tyrosine hydroxylase (TH) and dopamine-β-hydroxylase (DβH) was tested. DβH was expressed along the luminal surface of SC, with no expression of TH (Figs. 4A, 4C). There was multiple co-labeling between DβH and CD31 surrounding the SC lumen, as shown in Supplementary Figure S1. The results implied that DβH was expressed in the SCE cells. The percentage of the DβH-positive area in SCE cells was then compared under different circadian rhythms. As shown in Figure 4B, the percentage of the DβH-positive area in SCE cells in the N-dark group was greater than that in the N-normal and N-light groups (12.596% ± 1.744% vs. 8.734% ± 0.696% [$P < 0.01$] vs. 9.190% ± 0.908% [$P < 0.01$], respectively). The results suggested that DβH expression in SCE cells was upregulated in the N-dark group in vivo.

Nerve Projection Between SC and SCG

The fluorescent tracer dye DiI was used to confirm nerve projection between SC and SCG. Fluorescence was detected in SCG after injection of DiI into the anterior chamber (Fig. 5Ab). Additionally, after DiI injection into SCG, fluorescence was observed around SC (Figs. 5Bf, 5Bh). In addition, DiI fluorescence was partially colocalized with DβH at SC (Fig. 5Cm). The results suggested the presence of nerve projection between SC and SCG, which could be DβH positive.

Electrical Stimulation of SCG Upregulated DβH Expression in SCE Cells

To further explore the relationship between SCG and DβH expression in SCE cells, DβH expression in SCE cells was assessed after electrical stimulation of SCG. As shown in Figures 6A and 6B, in the electrical stimulation (ES) group, the percentage of the DβH-positive area in the SCE cells was greater than that in the control group (12.955% ± 1.780% vs. 7.876% ± 1.306%; $P < 0.01$). This result implied that the ES of SCG increased the expression of DβH in SCE cells in vivo.

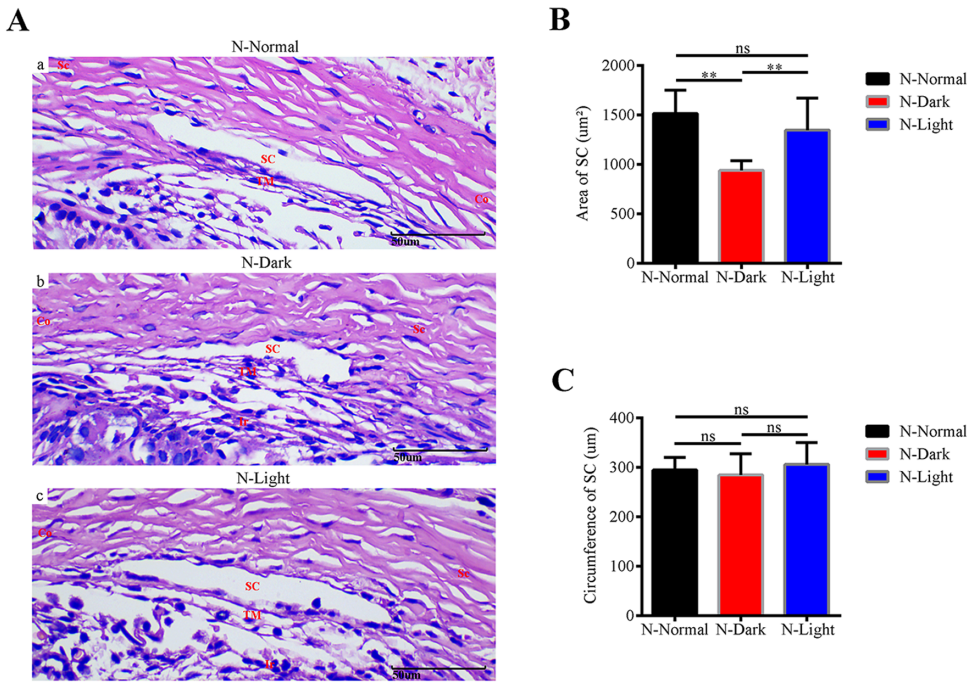

Figure 3. Histologic measurements analyses of SC in rats under the normal, constant dark, and light rhythms ($n = 6$). (**A**) Representative light microscopy images of HE staining showed the location of the SC and TM at the corneoscleral junction of the iridocorneal angle. SC, Schlemm's canal; TM, trabecular meshwork; Ir, iris; Co, cornea; Sc, sclera. (**B, C**) Comparisons of the cross-sectional area and circumference of SC under different rhythms by using two-tailed unpaired *t*-test. In the N-dark group, the area of SC decreased compared with the N-normal and N-light groups, whereas the circumference of SC had no significance among these groups. $^{**}P < 0.01$, ns: no statistical significance. Scale bars: 50 µm.

Electrical Stimulation of SCG Increased DβH Expression of SC Under Normal and Constant Light Rhythms

In the normal rhythm with ES (S-normal) group, the percentage of the DβH-positive area in SCE cells was greater than that in the normal rhythm with SCG exposure (CO-normal) group (13.689% ± 2.270% vs. 8.906% ± 0.919%; $P < 0.01$; Figs. 7A, 7C). Compared with the constant light rhythm with SCG exposure (CO-light) group, the percentage of the DβH-positive area increased in the constant light rhythm with ES (S-light) group (13.637% ± 3.399% vs. 8.263% ± 0.971%; $P < 0.01$; Figs. 7B, 7D). These results suggested that ES of SCG could increase the expression of DβH at SC under normal and constant light rhythms.

Electrical Stimulation of SCG Decreased the Cross-Sectional Area and Circumference of SC Under Normal and Constant Light Rhythms

After electrically stimulating SCG, the cross-sectional area and circumference of SC were evaluated using HE staining (Figs. 8A, 8B). Compared with the CO-normal group, the cross-sectional area of SC in the S-normal group decreased (864.814 ± 275.700 µm^2 vs. 1468.848 ± 294.280 µm^2; $P < 0.01$; Fig. 8C). The circumference of SC in the S-normal group was smaller than that in the CO-normal group (246.375 ± 39.070 µm vs. 311.354 ± 14.603 µm; $P < 0.01$; Fig. 8D). In the S-light group, the cross-sectional area of SC was smaller than that in the CO-light group (845.908 ± 334.229 µm^2 vs. 1559.300 ± 291.384 µm^2; $P < 0.01$; Fig. 8E). The circumference of SC decreased in the S-light group compared with the CO-light group (238.060 ± 57.469 µm vs. 321.608 ± 39.029 µm; $P < 0.01$; Fig. 8F). These results revealed that electrically stimulated SCG downregulated the cross-sectional area and circumference of SC under normal and constant light rhythms.

Electrical Stimulation of SCG Increased IOP and Decreased the Fluctuation Amplitude of IOP Under Normal and Constant Light Rhythms

As shown in Figure 9A and Supplementary Table S1, the average IOP was greater in the S-normal group than in the CO-normal group during 0 to 48 hours and 08:00 AM to 08:00 PM periods. Compared with the CO-normal group, the peak and nadir values of IOP were increased in the S-normal group (Figs. 9B, 9C, Supplementary Table S2). In addition, the range of 24 to 48 hours IOP in the S-normal group was smaller than in the CO-normal group (8.5 ± 0.5 mm Hg vs. 9.6 ± 0.5 mm Hg; $P < 0.01$; Fig. 9D). As shown in Figure 9E and Supplementary Table S1, the average IOP in the S-light group was higher than that in the CO-light group in 0 to 48 hours, 08:00 PM to 08:00 AM and 08:00 AM to 08:00 PM periods. In the S-light group, the peak and nadir values of IOP were elevated compared with the CO-light group (Figs. 9F, 9G, Supplementary Table S2). The range of 24 to 48 hours IOP in the S-light group was decreased compared with the CO-light group (1.2 ± 0.7 mm Hg vs. 5.4 ± 0.2 mm Hg; $P < 0.01$; Fig. 9H). These results demonstrated that ES of SCG increased IOP and decreased the

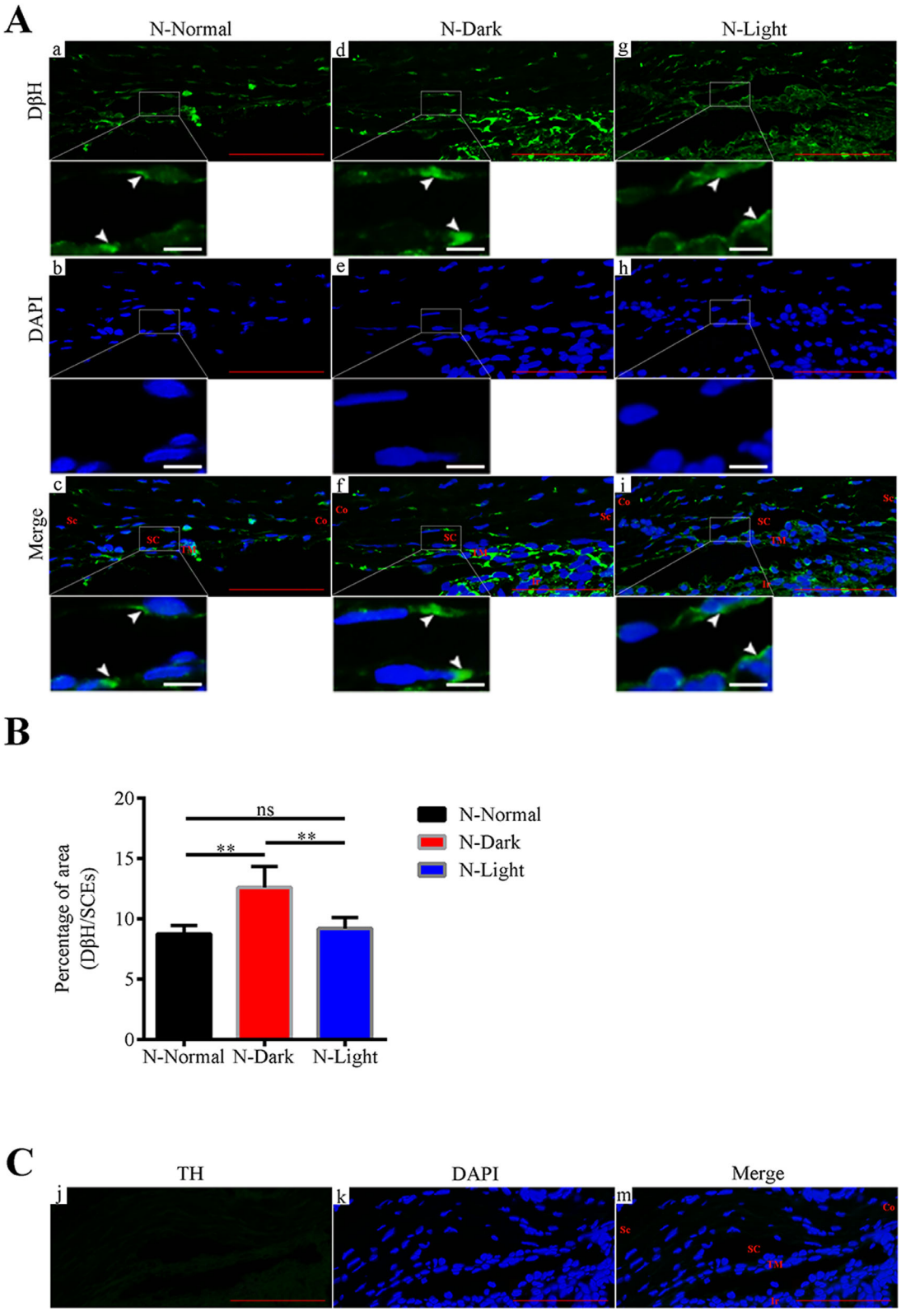

Figure 4. Immunofluorescence staining of DβH and TH in SC endothelial (SCE) cells under different rhythms ($n = 6$). (**A, B**) Representative staining of DβH in SCE cells of rat eyes in different groups. In the N-dark group, the percentage of the DβH-positive area in SCE cells was higher than that in the other groups by using two-tailed unpaired *t*-test. (**C**) Representative staining of TH in SCE cells of rat eyes. There was no significant positive immunofluorescence staining. $^{**}P < 0.01$, ns: no statistical significance. *Red scale bars*: 50 µm, and *white scale bars*: 5 µm. *Arrowheads*: DβH positive expression.

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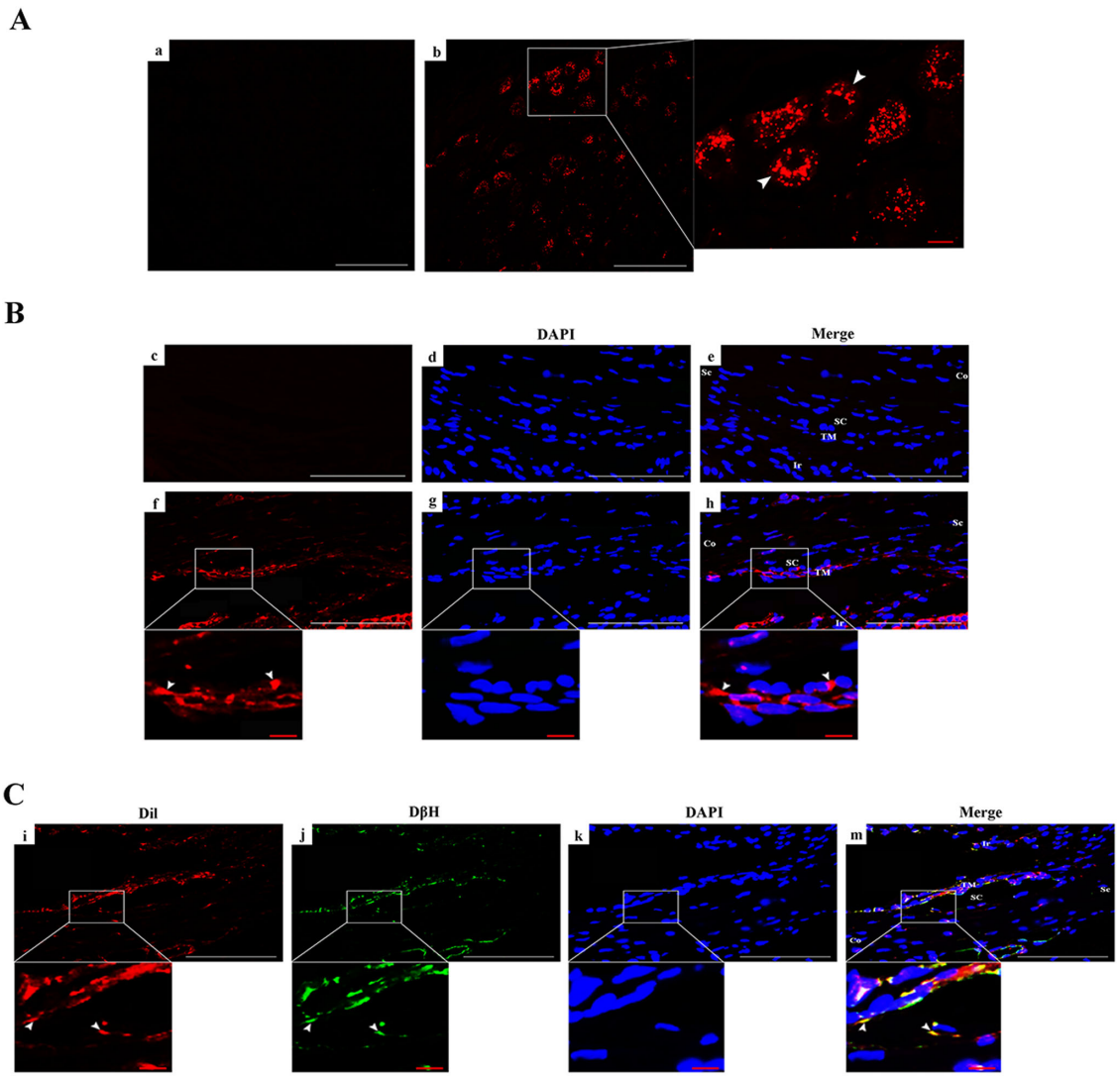

Figure 5. Fluorescence at SCG and SC of rats injecting fluorescent tracer dye DiI ($n = 6$). (**A**) Fluorescence in SCG 5 days after injecting DiI to the anterior chamber of eyes. Compared with the control group, the SCG in DiI injection group showed significant red fluorescence. (**B**) Fluorescence at SC 5 days after injecting DiI to SCG. SC in DiI injection group showed significant red fluorescence. (**C**) Co-label at SC between immunofluorescence staining of DβH and DiI from the SCG. There was partial co-label between DβH and DiI at SC. *White scale bars*: 50 µm, and *red scale bars*: 5 µm. *Arrowheads*: DiI or DβH positive expression, or co-label between DβH and DiI.

fluctuation amplitude of IOP under normal and constant light rhythms.

Electrical Stimulation of SCG Decreased the Cross-Sectional Area and Circumference of SC Under Normal and Constant Light Rhythms With use of Atropine Eye Drops

Atropine has been used to paralyze the ciliary muscles (CMs) of rats. In the normal rhythm with ES (SA-normal) group, the cross-sectional area of SC was smaller than the normal rhythm with SCG exposure (COA-normal) group (854.193 ± 165.367 µm^2 vs. 1129.987 ± 179.939 µm^2; $P < 0.01$; Figs. 10A, 10C). The circumference of SC in the SA-normal group was smaller than that in the COA-normal group (261.481 ± 28.230 µm vs. 293.518 ± 27.406 µm; $P < 0.05$; see Figs. 10A, 10D). In addition, the cross-sectional area of SC was decreased in the constant light rhythm with ES (SA-light) group, compared with the constant light rhythm with SCG exposure (COA-light) group (826.117 ± 139.695 µm^2 vs. 1123.214 ± 167.347 µm^2; $P < 0.01$; Figs. 10B, 10E). The circumference of SC in the SA-light group was decreased compared with the COA-light group (246.213 ± 25.359 µm vs. 270.710 ± 15.767 µm; $P < 0.05$; see Figs. 10B, 10F). These results implied that, except the effect of CM on SC morphology, ES of SCG decreased the cross-sectional area and circumference of SC under normal and constant light rhythms.

Discussion

It is widely recognized that increase and abnormal fluctuations of IOP are significant factors in glaucoma development, especially in primary open-angle glaucoma.[23,24] In this study, we found that SC morphology could be regulated by

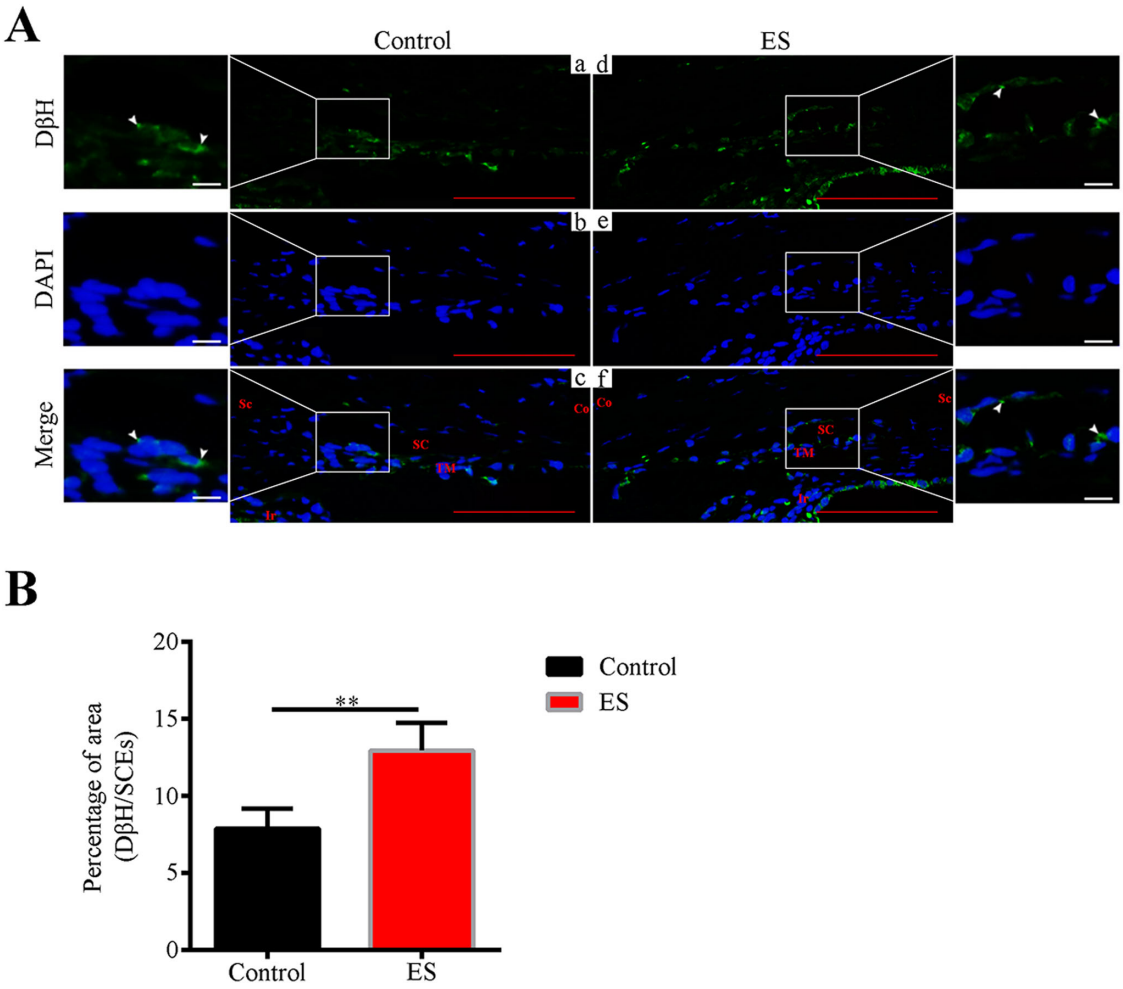

Figure 6. Immunofluorescence staining of DβH in SCE cells of rats treating SCG with electrical stimulation ($n = 6$). (**A**) Representative staining of DβH in SCE cells of rat eyes in electrical stimulation (ES) and control groups. (**B**) Comparison of DβH in SCE cells between ES and control groups by using two-tailed unpaired *t*-test. The percentage of the DβH-positive area in SCE cells was higher in the ES group. $^{**}P < 0.01$. *Red scale bars*: 50 µm, and *white scale bars*: 5 µm. *Arrowheads*: DβH positive expression.

SCG with ES by increasing the expression of DβH around SC. The ES of SCG could increase the expression of DβH at SC, reduce the cross-sectional area and circumference of SC, elevate IOP, and decrease the fluctuation amplitude of IOP under normal and constant light rhythms.

Previous studies have reported that acute constant dark and light exposure did not affect diurnal fluctuation patterns in the 24 hour IOP in rats, rabbits, and mice.[7,25,26] Lozano et al. reported a smaller amplitude, but significant IOP rhythm, after exposure to continuous light for 1 to 7 weeks in rats.[8] In this study, we found that the average IOP increased under a constant dark rhythm compared with a normal rhythm, with analogous fluctuation trends. These results implied that the fluctuation pattern of 24 hours IOP may be altered by constant dark or/and light exposure; however, a short period of stimulation did not affect the fluctuation of IOP.

Abnormal SC morphology is associated with primary open-angle glaucoma, and the coronal diameter and area of SC are negatively correlated with IOP.[27] We found that rats exposed to a constant dark rhythm exhibited increased IOP and decreased area of SC. The reason for this could be that smaller SC increases the outflow resistance of AH, which leads to elevated IOP. However, the circumference of SC demonstrated no differences in the normal, constant dark, and light rhythms. Following ES of SCG, the area and circumference of SC were both smaller under normal and constant light rhythms. We suspect that this could be explained by the following. Under a constant dark rhythm, the sympathetic nerves of rats are excited, and changes in the morphology of SC are regulated by sympathetic nerves from the entire body. However, only local sympathetic nerves are excited after ES of SCG, and, therefore, morphological changes in SC are mainly regulated by local sympathetic nerve projections. Some investigators have found that exercise and acute emotional stress both induce systemic excitement of the sympathetic nervous system.[28,29] Exercise-induced SC expansion and IOP reduction,[28] and acute emotional stress elevated IOP in patients with glaucoma,[29,30] whereas relaxation had the opposite effect.[31] Moreover, ES of SCG could increase IOP.[20] These findings revealed that local excitation of SCG could increase IOP, whereas systemic excitation of sympathetic nerves could increase or decrease IOP. This difference implies that, compared with local sympathetic nerves, the regulation of SC by the excitation of systemic sympathetic nerves was more complicated.

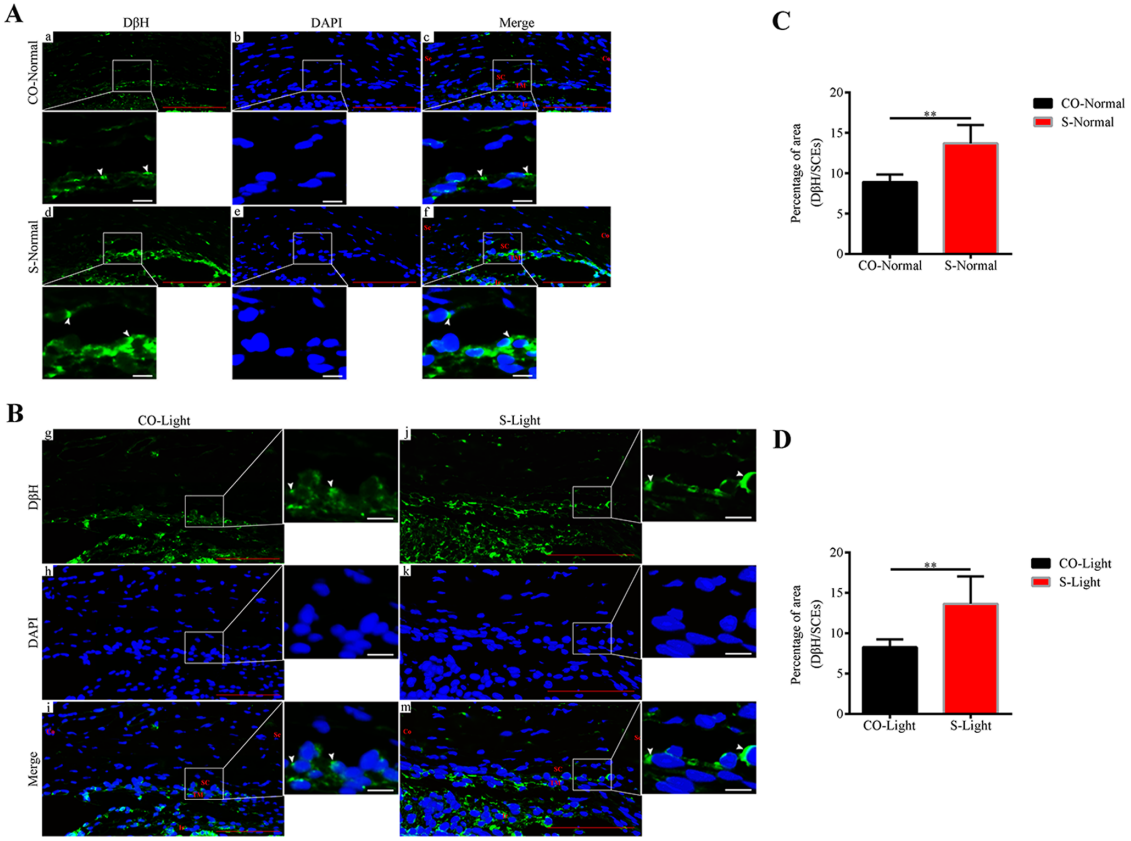

Figure 7. Immunofluorescence staining of DβH in SCE cells treating SCG with electrical stimulation under different rhythms ($n = 6$). (**A, B**) Representative staining of DβH in SCE cells of rats treating SCG with electrical stimulation under the normal and constant light rhythms. (**C, D**) Comparison of DβH in SCE cells between electrical stimulation and control groups under different rhythms by using two-tailed unpaired *t*-test. In the S-normal group, the percentage of the DβH-positive area in SCE cells was higher than that in the CO-normal group. The percentage of the DβH-positive area in SCEs increased in the S-light group compared with the CO-normal group. $^{**}P < 0.01$. *Red scale bars*: 50 µm, and *white scale bars*: 5 µm. *Arrowheads*: DβH positive expression.

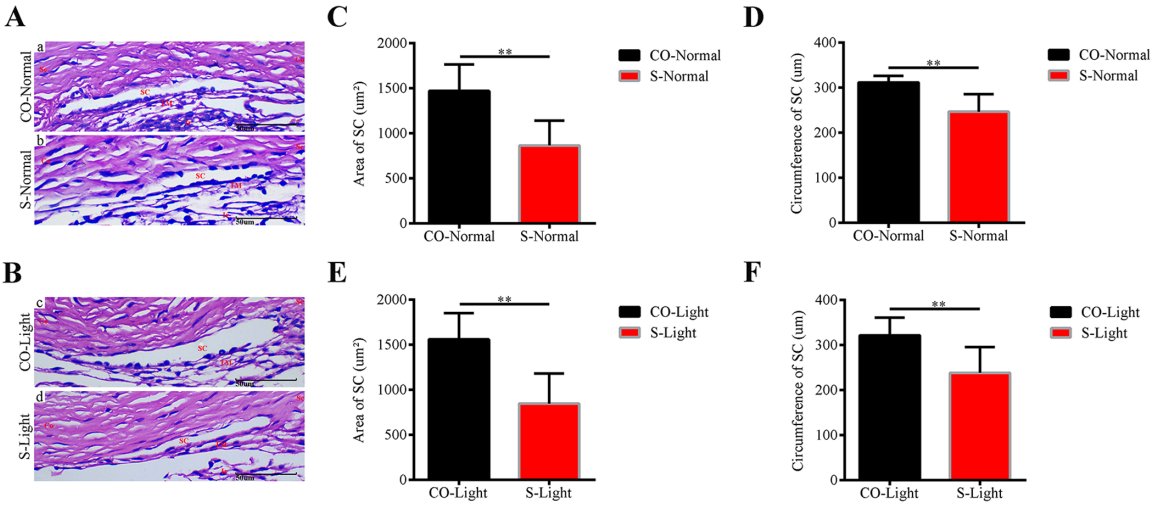

Figure 8. Histologic measurements analyses of SC treating rat SCG with electrical stimulation under different rhythms ($n = 6$). (**A, B**) Representative light microscopy images of HE staining of the SC and TM treating SCG with electrical stimulation under the normal and constant light rhythms respectively. (**C–F**) Comparisons of the cross-sectional area and circumference of SC between electrical stimulation and control groups under the normal and constant light rhythms respectively, using two-tailed unpaired *t*-test. In the S-normal group, the cross-sectional area and circumference of SC decreased compared with the CO-normal group. The cross-sectional area and circumference of SC in the S-light group were smaller than that in the CO-normal group. $^{**}P < 0.01$. Scale bars: 50 µm.

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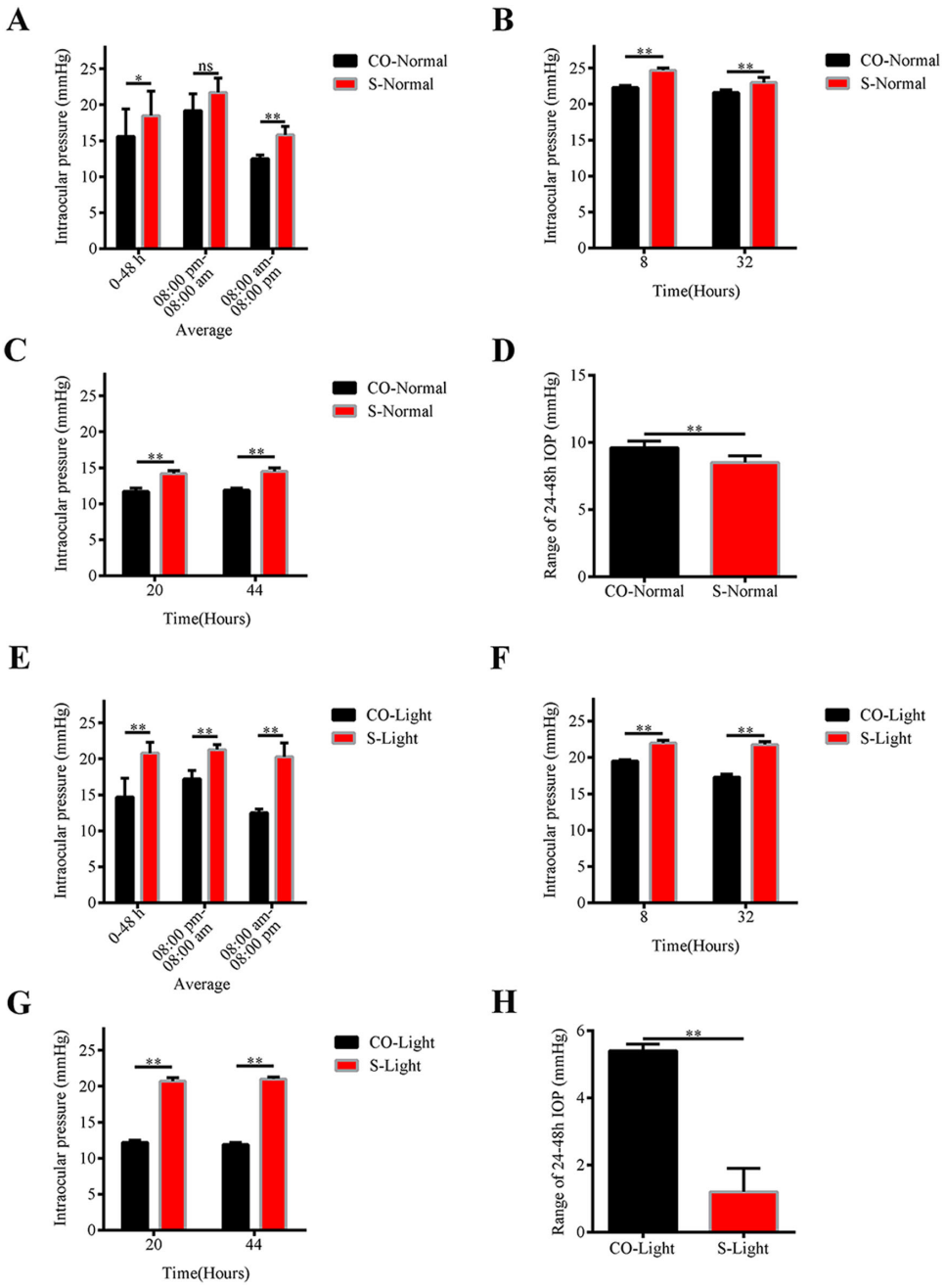

Figure 9. IOP analyses of rats treating SCG with electrical stimulation under different rhythms ($n = 6$). (**A**) The comparison of average IOP during different periods by using two-tailed unpaired *t*-test; 08:00 PM to 08:00 AM: 4 to 16 hours and 28 to 40 hours (16 hours and 40 hours noninclusive); 08:00 AM to 08:00 PM: 0 hours, 16 to 28 hours, and 40 to 48 hours (28 hours noninclusive). The average IOP increased in the S-normal group compared with the CO-normal group. (**B, C**) The peak and nadir values of IOP comparison by using two-tailed unpaired *t*-test. The peak and nadir values of IOP were higher in the S-normal group. (**D**) Range of 24 to 48 hours IOP (24 hours noninclusive) comparison by using two-tailed unpaired *t*-test. The range of 24 to 48 hours IOP in the S-normal group was smaller than that in the CO-normal group. (**E**) The comparison of average IOP during different periods by using two-tailed unpaired *t*-test. The average IOP in the S-light group was higher than that in the CO-light group. (**F, G**) The peak and nadir values of IOP comparison by using two-tailed unpaired *t*-test. The peak and nadir values of IOP increased in the S-light group. (**H**) Range of 24 to 48 hours IOP (24 hours noninclusive) comparison by using two-tailed unpaired *t*-test. The range of 24 to 48 hours IOP in the S-light group significantly decreased. $^{*}P < 0.05$, $^{**}P < 0.01$, ns: no statistical significance.

The TH catalyzes the first and rate-limiting step in the synthesis of catecholamines (CAS), including dopamine (DA), noradrenaline (NA [norepinephrine]), and adrenaline (AD [epinephrine]).[32] DA β-hydroxylase (DβH) is a catalytic enzyme that controls the conversion of DA to NA.[33] The TH and DβH are both sympathetic nerve markers.[34] As neurotransmitters, DA was discovered in the central nervous system,[35,36] whereas NA was discovered in the peripheral sympathetic nerves and brain.[32,36,37] We found that postganglionic nerve fibers from SCG projected to SC, and DβH was expressed around SC, with no TH expression. Therefore, we speculate that the sympathetic neurotransmit-

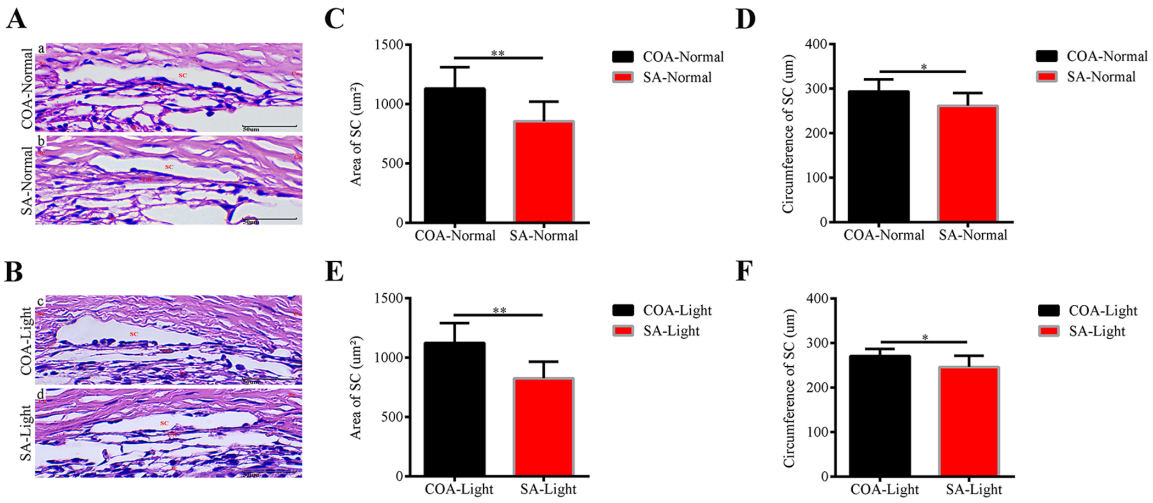

Figure 10. Histologic measurements analyses of SC treating rats with atropine and electrically stimulating SCG under different rhythms ($n = 6$). (**A, B**) Representative light microscopy images of HE staining of SC and TM treating rats with atropine and electrically stimulating SCG, under the normal and constant light rhythms respectively. (**C–F**) Comparisons of the cross-sectional area and circumference of SC between electrical stimulation and control groups under the normal and constant light rhythms respectively, using two-tailed unpaired *t*-test. In the SA-normal group, the cross-sectional area and circumference of SC decreased compared with the COA-normal group. The cross-sectional area and circumference of SC in the SA-light group were smaller than in the COA-normal group. $^{*}P < 0.05$, $^{**}P < 0.01$. Scale bars: 50 µm.

ter at SC is noradrenergic/adrenergic and is released from nerve terminals of the SCG.

We found that variations in IOP fluctuation were different after ES of SCG under different rhythms. After electrically stimulating SCG, IOP fluctuation under a constant light rhythm was smaller than IOP fluctuation under a normal rhythm. Lozano et al. found that the fluctuation of 24 hours IOP (continuous light condition) was smaller after 7 weeks than at 1 and 4 weeks.[8] The ES of SCG may interrupt the current IOP rhythm in rats; when the animals receive new light and dark rhythm stimulations, the IOP fluctuation pattern reflects a newly corresponding alteration.

The pupil diameter is different under dark and light conditions. Pharmacologic or dark-induced mydriasis can increase iridotrabecular contact, bring about a relative pupillary block, and subsequently elevate IOP.[38] Compared with the light condition, the pupil diameter in participants increased under a dark condition, and IOP was elevated in those who experienced a 1.5-hour dark adaptation.[39] Therefore, the elevation of IOP under a dark condition may be relative to not only a change in the SC lumen dimension but also pupil dilation. It is acknowledged that an anterior-segment optical coherence tomography (OCT) and a camera with infrared illumination could be used to measure the pupil size under a constant dark condition.[39,40] Unfortunately, we do not have an anterior-segment OCT for small animals or a camera with infrared illumination and could not measure the pupil diameter under a dark condition. Therefore, in this study, we focused on the regulatory effect of sympathetic excitation on SC morphology, and observed the decrease in the SC cross-sectional area under a dark condition.

Previous researchers observed that CM contraction could expand the potential space within TM and enlarge the SC,[41–44] thus decreasing the AH outflow resistance in this region and leading to a drop in IOP.[45] In this study, to eliminate the effect of CM contraction/relaxation on SC lumen dimension,[42] atropine was used to paralyze CM. Following, we found that, after electrically stimulating the SCG of rats, the cross-sectional area and circumference of SC decreased under normal and constant light rhythms. The results suggested that the SCG might regulate SC morphology by controlling the expression of DβH in the SCEs of rats; however, this mechanism requires further investigation.

SC is a unique and endothelium-lined vascular structure that originates from blood vessels and can be detected with blood vascular marker CD31.[46] In our study, we observed that, after electrically stimulating the SCG, the expression of DβH in SCEs increased, whereas the cross-sectional area and circumference of SC decreased. The mechanism of lumen change in the SC may be analogous to the regulatory mechanism of sympathetic nerves on the vascular endothelium.

DβH was proportionately released along with NA from the vesicles in the sympathetic nerve fibers.[47] NA is released from adrenergic nerves and can induce blood vascular contraction through α1-adrenoceptors.[48] Moreover, α-1D-ARs have been detected in human umbilical vein endothelial cells (HUVECs), α2-ARs have been documented in the uterine artery endothelial cells of ewes.[49–51] In addition, a research reported that VIP, which was released by parasympathetic postganglionic fibers, could regulate the F-actin distribution of SCE cells and SC morphology via the VPAC2 receptor.[18] Thus, we hypothesized that NA might also be able to regulate SC morphology through the α-adrenoceptors of SCE cells.

In this experiment, we used an I-care PRO tonometer to measure rats' IOP. A previous study reported that the IOP readings with an I-care tonometer were consistent with those obtained by the Goldmann applanation tonometer, indicating that the results from I-care should be accurate.[52] Other studies reported that the changes in corneal properties (e.g. central corneal thickness, corneal hysteresis, corneal curvature, and corneal rigidity factor) could influence IOP measurements.[53–55] Additionally, a previous experiment used the retrograde transport method and observed occasionally labeled neurons in the SCG

by applying horseradish peroxidase-wheat germ agglutinin to the central cornea of rats.[56] This implied that SCG electrostimulation might change the biomechanical properties of the cornea, influencing the IOP readings. To investigate the effect of electrostimulation on corneal properties, special instruments (e.g. anterior-segment OCT for small animals, auto keratometer, and Ocular Response Analyzer) are required. However, we currently do not have access to the instruments, and thus could not observe whether SCG electrostimulation changed the biomechanical properties of the cornea. Further studies are required to test the effect of electrostimulation on corneal properties and IOP measurement by I-care.

In conclusion, a constant dark rhythm increased DβH expression in SCE cells, reduced the cross-sectional area of SC, and increased IOP in rats. Moreover, nerve fibers were found to project from the ipsilateral SCG to SC. Under the normal and constant light rhythms, ES of SCG increased DβH expression in SCEs. It also reduced the cross-sectional area and circumference of SC, which, in turn, elevated IOP and decreased the fluctuation amplitude of IOP. The results suggest a novel mechanism for the abnormal fluctuation rhythm of IOP in glaucoma.

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